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ONCOLOGY INSIGHTS

Aims and Scope

Oncology Insights is a yearly oncological open-access peer-reviewed journal that publishes new research from different areas of oncology. It strives to provide a platform for the exchange of cutting-edge research and knowledge in the field of oncology. This journal aims to advance the understanding, prevention, diagnosis and treatment through the dissemination of high-quality scientific discoveries.
The journal applies a fair and accurate peer review process, employing double-blind review methodologies. Acceptance of manuscripts is based on their scientific merit, originality, clarity, and contribution to the field.

Topics

Oncology Insights covers a wide spectrum of topics within the field of oncology, including but not limited to:

- Basic and Translational Research
- Clinical Oncology
- Radiation Oncology
- Surgical Oncology
- Pediatric Oncology
- Hematologic Oncology
- Palliative Care
- Epidemiology and Public Health
- Cancer Genetics
- Immunotherapy and Targeted Therapies
- Experimental Therapeutics
- Computational Biology and Artificial Intelligence

About/Information

Oncology Insights welcomes various types of contributions including original research articles, review articles, case reports, case studies, clinical trials, registered reports, comments, brief communications, editorials, letters to the editor, perspectives, and conference papers from a wide range of disciplines related to cancer research.
Through encouraging interdisciplinary collaborations, the journal welcomes contributions that integrate oncology with related fields such as immunology, genetics, biochemistry, radiology, and other relevant disciplines. The journal places a special emphasis on publishing research that highlights emerging trends, novel technologies, and innovative approaches in cancer research and clinical practice.
Oncology Insights is intended for a diverse readership, including oncologists, researchers, clinicians, nurses, allied healthcare professionals, patients, patient advocates, policymakers, and all stakeholders involved in the prevention, diagnosis, and treatment of cancer. It adopts a global perspective, encompassing research from diverse regions addressing oncological challenges that may vary across different populations.
The journal is committed to upholding the highest ethical standards in research and publication provided by established international guidelines.
Periodically, Oncology Insights may publish special issues focusing on specific topics to highlight particular areas of interest or emerging needs.
Authors are provided with clear and comprehensive guidelines for manuscript preparation, including structure, formatting, and other specific requirements.

Esteemed colleagues,

It is a rare honor and privilege in a scientist's career to shape joint efforts and dedication of a group of scientific enthusiasts into a tangible outcome - ***Oncology Insights, the Official Journal of the Serbian Association for Cancer Research*** (srp. Srpsko društvo istraživača raka, SDIR).

The first volume of Oncology Insights has been derived from years of scientific contributions of many individuals and institutions who have selflessly devoted their expertise, ideas and time to establish the SDIR society that today resonates with integrity and charm. In the future, we will strive to maintain those standards, always aiming higher. Thus, we encourage researchers, physicians, nurses, laboratory technicians, as well as patients, survivors, caregivers, and patient advocates to offer their valuable expert insights that will stimulate future progress of oncology in Serbia and worldwide.

Over the last 20 years, we have witnessed remarkable progress in the field of cancer research. Oncology Insights aims to play an integral role in supporting that progress by providing a platform for sharing cutting-edge research, creating a space for new collaborations, partnering established researchers with young investigators, and serving as a home for oncology professionals of various specialties dedicating their careers to this challenging research field.

Oncology Insights pledges to evolve, adapt, reinvent, redefine, and reshape its content to serve its members and inevitable advances in the field. We hope you will be a part of its success story by providing evidence-based, unbiased multidisciplinary content, feeling both an honor and a duty to treat cancer research with the same care, passion, and dedication which individuals with cancer deserve and expect.

Please tune all your senses to enjoy the intellectual feast spread through the pages of this inaugural journal volume. The future of Oncology Insights will be shaped by you.

With kind regards,

Milena Čavić, SDIR President
Editor-in-Chief
Oncology Insights
Official Journal of the Serbian Association for Cancer Research

The first number of Oncology Insights includes
PROCEEDINGS BOOK of
THE SIXTH CONGRESS OF THE SERBIAN ASSOCIATION FOR CANCER RESEARCH
with international participation

From Collaboration to Innovation in Cancer Research

2nd – 4th October 2023
Royal Inn Hotel, Belgrade

SDIR-6 ORGANIZER
Srpsko društvo istraživača raka (SDIR)
Serbian Association for Cancer Research (SACR)
www.sdir.ac.rs

Dear colleagues,

We are very pleased to welcome you to the 6th Congress of the Serbian Association for Cancer Research (SDIR) with international participation "From Collaboration to Innovation in Cancer Research" which will be held on October 2-4 2023, at the Royal Inn Hotel, Kralja Petra 56, Belgrade, Serbia.
During the three-day congress, lectures will be given by distinguished Serbian and international researchers, covering the following topics:

- Tumour metabolism and biology
- Epigenetics and gene regulation in cancer
- Bioinformatics and artificial intelligence in cancer research
- Omics approaches in cancer research
- Therapy response and resistance
- Clinical and translational oncology
- Immunooncology
- New and challenging drug targets
- Pathways to innovation in cancer research

We are pleased to announce that our sixth congress is actively supported by the European Association for Cancer Research (EACR). National and regional cooperation is also important, and so representatives from our friend societies will be attending our congress.
The timing of the organisation of SDIR-6 is important for the establishment of our national society's journal Oncology Insights. The abstracts of the sixth congress will be published in the very first issue of the journal.
Advances and innovations in cancer research are based on growing scientific knowledge and collaboration. We believe you will enjoy the lively atmosphere of the congress and that fruitful scientific discussions will help you build new collaborations and develop new ideas.

We look forward to welcoming you in Belgrade!

Kind regrads,

on behalf of the SDIR-6 Organizing Committee

Prof. dr Katarina Zeljić
Faculty of Biology, University of Belgrade
President of the SDIR-6 Organizing Committee

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LEGACY INSIGHTS

The foundation of the Serbian Association for Cancer Research

(personal point of view)

Dr Zorica Juranić, Principal Research Fellow (retired)
Institute for Oncology and Radiology of Serbia
First SDIR President


Dr Zorica Juranić

Exchange of knowledge is of the utmost importance for the progress of science. This is especially of value for rapidly developing sciences such as medicine, where many people´s lives depend on the expertise and skills of doctors. Therefore, there is always a need for organisations aiming to exchange ideas and news, especially in the field in oncology. One of the organisations which is actively working in the field of oncology is The European Association for Cancer Research (EACR).

In a letter in 2010, Mr Andrew Binns (at that time the treasurer of EACR) informed me, as I was already a member of EACR, of the possibility for application of our national society to become a member of EACR, whose members already were at that time national societies of United Kingdom, Spain, Hungary, Croatia, Netherlands, Sweden, Israel and many other countries. This made me remember my previous participation in EACR meetings. One of them was held in Genova, Italy in 1991, where I was with my esteemed colleages Professor Ivan Spužić and Professor Gordana Konjević, and where we heard the very interesting plenary lecture from Professor Olav Hilmar Iversen and had the honour of presenting our own results. Then I thought of the EACR meeting held in Granada, Spain in 2006, which was characterized by ground-breaking results in cancer research and therapy. Of special importance was the session chaired by the famous scientist from the Roswell Park Memorial Institute, Professor Enrico Mihich (1928-2016), who, a long time ago in 1985, had published a review paper with completely new insights in cancer research and therapy; from proper diagnosis of cancer as a set of different biological, hormonal and immunological entities, to corresponding specific therapeutical modalities. Professor Mihich was one of the first scientists who emphasized the importance of personalized approach in therapy of cancer patients. Very appropriate text describing Professor's Mihich (Henry) research was writen many years later by his colleagues Graham Pawelec and Ostrand-Rosenberg, describing his approach in learning about cancer which was published in Cancer Immunol Immunother in 2017:

„...At this time, Henry introduced and provided experimental evidence for the counterintuitive idea that lasting therapeutic effects of certain anticancer drugs depend on the drug-induced modulation of immunity against the tumor. This idea met with a degree of skepticism, because it was "obvious" that chemotherapeutics were immunosuppressive—a clear example of a scientific paradigm proving very resistant to change, but thereafter following the classic path of new ideas in science and becoming fully accepted as equally as obvious now as it was ridiculous then. Henry's tenacity in promulgating this idea against the resistance of the research community played a major part in its current acceptance…"

I was happy that I had had the opportunity to meet and speak with this distinguished, eminent scientist. It was crucial for me and my future work.

At that moment I thought that it would be important for cancer researchers from Serbia to form our own Serbian society for cancer research, and aim to become a member of EACR. Then we could apply for funds for younger researchers for their participations in scientific meetings, apply for grants for research Laboratories abroad in order to learn new methods, or to get funds for the organization of national society meetings.

At this time, in Serbia, the nucleus for cancer research was in the Departement of Experimental and Clinical Oncology, at the Institute of Oncology and Radiology of Serbia (IORS). With the help and ethusiasm of my fellow physicians and cancer researchers, we founded our Serbian Society for Cancer Research (serbian SDIR: Srpsko Društvo Istraživača Raka) on the 16th of November 2010. Founders of SDIR were: Zorica Juranić, Zora Nešković-Konstantinović, Tatjana Stanojković, Željko Žižak, Irina Besu Žižak, Sandra Aranđelović, Ivana Matić, Emina Mališić, Aleksandra Erić, Nevenka Gligorijević, Lana Filipović, Tijana Vujasinović, Zaki Abu Rabi, Ksenija Kanjer, Radmila Janković, Tatjana Srdić-Rajić, Katarina Mirjaćić-Martinović, Ana Vuletić, Milena Čavić, Siniša Radulović, Milan Markićević, Gordana Konjević, Nataša Todorović-Raković, Dragica Nikolić-Vukosavljević and Suzana Vasović. And

later on, we made the successful application for membership in EACR.

The one rule to become a member of SDIR was to work in the field of cancer research. I remember the names and scientific work of our colleagues who, by their excellent research and education of young scientists, created the basis for modern cancer research in Serbia. One of the most famous (successor of the eminent Ksenofon Šahović who participated in the formation of the Institute of Radiology as the cancer research center, and succesor of Professor Emillija Višnjić) was the distinguished scientist Professor Blagoje Nešković MD, PhD (1907-1984). It must be emphasized that the organization in the Laboratory for Experimental Oncology as the research unit of the Faculty of Medicine, University of Belgrade made by dr Nešković was enthusiastically created not only for that time, but also for future research. Even now, 60 years later, the basic teamwork organization, and the basic scientific methods (mainly cell cultures in vitro, histo- and citology, the use of inverted, fluorescent and scaning microscopy) within scientific projects are preserved and are used together with new technologies. Under the leadeship of Blagoje Nešković, a multiprofessional team consisting of chemist Bulka Babin, biologist Zoran Ajdarić, physicist Đoka Polić, pharmacist Ljubinka Đurić, physical chemists Dragica Nikolić-Vukosavljević and me, Zorica Juranić and technician Delimir Marković, studied processes of *in vitro* cell growth and development, the mechanism of action of various biological response modifiers like vinca alkaloids and also the effect of various fractions of viper venom, or of formamide on malignant cells *in vitro*. Dr Nešković was passionately interested in the determination of the energy of malignant cell metabolism by isothermal microcalorimetry, working mainly in the field of experimentral oncology.

The results from his most known study, his PhD thesis, dealing with investigations on the developmental phases in intermitosis and the preparation for mitosis of mammalian cells *in vitro* were published in International Review of Cytology in 1968. Findings from his studies of the effect of various agents on the developmental phases of the *L* strain cells *in vitro* were published in Nature 1965. As it was known that metabolites of polycyclic aromatic hydrocarbons benzo(a) pyren are strong carcinogens, Blagoje Nešković with Vesna Šoškić presented their analysis on the detection of small quantities of 3,4-benzpyrene directly from chromatographic paper in Nature in 1961. Dr Nešković was actively intrested not only in the processes of cell morphology and growth, but also in learning the secondary DNA structure. Under his leadership, thermal analysis of the disruption of secondary DNA structure, (the determination of the DNA melting point, and of DNA melting profile) was introduced as a method in the Laboratory for Experimental Oncology. Thermal denaturation of the secondary DNA structure and hibridization of single strand DNA into double helix are among the main procedures used even nowadays in the methods of the determination of primary DNA structure, of DNA sequencing. They are included in the methods for detection of (non) specific mutations in DNA, in the tests which are the basis for patient selection for modern, targeted cancer therapies. Interests of Prof. Nešković also included hormone receptor sensitive tumors. Nowadays, in clinics, the determination of the presence of steroid receptor positive tumors (by immunohistochemistry) is of the main importance for patients, who can be treated with hormonal therapy. There are many of us who are grateful to Professor Blagoje Nešković for his amazing scientific legacy.

IORS, Department of Experimental Oncology, 1987. First row: (left to right) Radovan Vukićević, Đoka Polić, dr Ivan Spužić, dr Nikola Vujanović and Ljubinka Đurić. Second row: (left to right) dr Ljiljana Vučković-Dekić, Dragica Nikolić-Vukosavljević, Zorica Juranić, Ivanka Šami, dr Nevenka Stanojević-Bakić, dr Dušanka Milošević, Slavica Kutanoski and dr Milica Marinković

Many others have contributed to the scientific work of SDIR at the Institute for Oncology and Radiology of Serbia. The scientific work of Nikola Vujanović (now Professor Emeritus at the Pittsburg Cancer Institute) and of Academician Ivan Spužić with the team of their colleagues, professors Ljiljana Vučković-Dekić, Nevenka Stanojević Bakić, Dušanka Milošević, Milica Marinković, Ivanka Šami, Dragica Nikolić-Vukosavljević, Ljiljana Dimitrijević, Gordana Konjević, Mirjana Branković-Magić and Zorica Juranić, is the cornerstone of the basic research in tumor biology and immunology at IORS. Their work was performed in collaboration with medical doctors known for their passionate work in the introduction of novelty in patient therapy, professors Labuda Mitrović, Svetislav Jelić, Zora Nešković-Konstatinović, Davorin Radosavljević, Nada Babović, Suzana Vasović, Ivan Popov, Siniša Radulović, Snežana Šušnjar, Vladimir Kovčin, Radmilo Tomin, Slobodan Nikolić, Radan Džodić, Ivan Marković, Miomir Šašić, Zorica Tomašević, Nenad Milanović, Suzana Matković, Zoran Tomašević, radiologists Vera Šobić, Slobodan Čikarić, Ljiljana Radošević-Jelić, Mirjana Durbaba, Vesna Plešinac-Karapanžić, Nenad Borojević, Miodrag Đorđević epidemiologist, along with Professor Nikola Mitrović and Ljuba Marković involved in the functional organization of IORS as the National Cancer Research Center. Researchers from the Institute for Oncology in Sremska Kamenica, Novi Sad, professors Vladimir Baltić, Aleksandar Kerenji and Gordana Bogdanović, as well as Professor Slađana Filipović from Niš, with their work in medical oncology also contributed to the development of the basis for modern cancer research in Serbia.

The First Scientific Meeting of the Serbian Association for Cancer Research was held in the Sava Centar in Belgrade on 29th and 30th of November 2012. It was an EACR sponsored event, with the main topic: "The Novelties in Cancer Research" and was related to new data in the field of basic and translational cancer research. In order to strengthen the precious collaboration between cancer researchers and clinicians, crucial for true significant advance in oncology, this meeting was held together with the "49th Cancerology Week" which was devoted to the study of infectious agents as a cause of neoplastic diseases; 450 participants (medical doctors, cancer researchers and nurses) attended the meeting. Invited speakers were Prof. Yehuda Shoenfeld and Prof. Sonja Levanat, the president of Croatian Association for Cancer Research (HDIR). Prof. Shoenfeld from Israel gave a lecture on the prospective role of vitamin D in the prevention of malignant and autoimmune diseases, emphasizing the important connection between immunity and cancer. Invited speaker from Croatia, prof. Sonja Levanat presented new data on the involvement of the Hedgehog signaling pathway in the pathogenesis of ovarian carcinoma and colon cancer and suggested the potential for developing better therapies based on the inhibitors of Hh-Gli pathway. Dr Zorica Juranić, SDIR president, highlighted the significance of personalized nutrition for cancer prevention by giving a comprehensive overview of the results from her own laboratory as well as new literature data in this field, pointing that in some patients with hematological malignancies there is specific enhanced immunity to some food antigens.

These were beginnings of SDIR…

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SDIR evolution: important steps from 2016 to 2022

Dr Mirjana Branković Magić, Principal Research Fellow (retired)
Institute for Oncology and Radiology of Serbia

In the time period between 2016 and the beginning of the 2022, I was honored to be the second president of the Serbian Association for Cancer Research (SDIR).

SDIR was formed at the Department of Experimental Oncology, Scientific Division of the Institute for Oncology and Radiology, in 2010. In 2011 SDIR became an affiliated National Society of the European Association for Cancer Research (EACR). In these first days of our young association, many good things were done – an international two-year SDIR Congress was established. Each new management has its own vision of development. Our vision was directed towards translational research and a better collaboration between experimental and clinical oncology in Serbia. Besides basic research, we were interested in the development of new techniques, and new research options that may be transferred into clinical diagnostics and treatment of cancer patients.

The successful growth of our society has been confirmed by the expanding members' network - starting from 25 at our founding session, through 68 when we were awarded the status of EACR National affiliated society, to the current SDIR member count of 242.

Between 2016 and 2022, we held 3 SDIR Congresses under the auspices of the EACR. All were with international participation:

SDIR-3 "Challenges in anticancer research: translation of knowledge to improve diagnosis and treatment" 3rd. SDIR Congress, 2017. We were pleased to host distinguished international cancer researchers: dr Nicola Normanno, prof. dr Yannoukakos Drakoulis, prof. dr Srđan Novaković (sponsored by EACR), prof. dr Esat Mahmut Özsahin (sponsored by EACR), dr Ignacio Ochoa Garrido, prof. dr Engin Ulukaya and prof. dr Konstantinos Dimas.

SDIR-4 "Bringing science to oncology practice: Where is Serbia? 4th SDIR Congress, 2019 with 5 international eminent speakers: Arkaitz Carracedo, Engin Ulukaya, Joanna Kopecka, Ana Šipak Gašparović, Vita Šetrajčić Dragoš. Dr Arkaitz Carracedo, the EACR Board member and representative for Early Career Researchers gave an inspiring plenary lecture. We organized for the first time a very successful E-poster session. To enrich the scientific programme and enable young talented researchers to present their results to the broader international scientific community we have selected 12 participants with abstracts for a short oral presentation. This became our tradition for every future SDIR congress.

SDIR-5 "Translational potential of cancer research in Serbia" 5th SDIR Congress, 2021 was held as a virtual event due to the Covid 19 pandemic. The excellent plenary lecture was delivered by EACR president Caroline Dive. SDIR-5 congress was the most successful congress in the history of SDIR, with 206 registered participants including speakers, from 15 countries worldwide. The fact that the congress was virtual enabled us to create the high-quality scientific programme and to invite distinguished international speakers in the field of oncology.

During that period we tried to spread our activities to different institutions involved in cancer research. The new SDIR board successfully continued with these activities.

FUTURE HORIZONS

IN CANCER

SDIRSACR

Review/Perspective

The importance of sex as a biological variable in cancer research

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Abstract
Sex is an important biological variable that has an impact on all aspects of human health and disease. Yet, it is greatly unappreciated in both basic and translational cancer research, and most concerningly in cancer clinical trials. In this review we summarize how patients' biological sex influences cancer risk, the biology of cancer and its response to anticancer therapy. We present data from the past decade on the genetic, genome-wide, metabolic and immune differences between sexes and how they relate to cancer development and progression. Ultimately, we highlight the importance of considering sex as a variable in all aspects of cancer research and recommend guidelines for implementation.

Introduction

There is growing evidence that non-reproductive cancers are initiated earlier, associated with higher incidence and greater mortality in males than in females (1). Hormones play a role in observed sex differencesin cancer incidence and outcome, however in recent years it has become apparent that genetic and epigenetic foundations are equally important (2, 3). Factors that affect metabolism, immune response and response to therapy also differ by sex. Females generally have better response to treatment, yet it is associated with higher toxicity (4).Despite the evidence from both basic and translational research that implies that sex bias in cancer exists, it is most commonly overlooked. In this review we will highlight the significance of including sex as a variable in all stages of cancer research from early cell and animal testing to clinical trials; and underline the importance of sex segregated analysis that will lead to novel discoveries and improved personalized treatment for cancer patients.

Sex and gender defined

Many researchers are still unfamiliar with the distinction between sex and gender. In humans, sex refers to the biological and physiological attributes that distinguish male, female and/or intersex (5). Sex chromosomes, hormones and reproductive organs serve as biological determinants of sex. Genetic sex refers to XX and XY chromosomes that are present in every cell in our bodies, thereforeall cells have sex (6). The impact of sex on human health is dynamic and changes throughout life. In biomedical research, the factor of sex deserves an integrative approach (7) as important differences between males and females exist in body composition (percent of fat and muscle), hormonal status, metabolism, immunity and pharmacokinetics and pharmacodynamics of drugs. Gender refers to sociocultural attitudes, behaviors and identities. Gender attitudes and behaviors can change with time and place, vary from society to society, and can intersect with sex, age, socioeconomic status, sexual orientation and ethnicity (5). Gender differences in cancer can only be studied in humans and cannot be modeled in preclinical models. Important gender differences in cancer are, for example, exposure to risk factors, care seeking and therapeutic choices. In this review terms male and female will refer to the biological sex.
In most cancer types, males have higher incidence and higher mortality rate compared to females (2) (Figures 1 and 2). Despite having higher incidence rates in some types of cancer like breast, thyroid and gallbladder for example, females have better survival than males. This implies that fundamental biology of sex differences affects cancers of all types (8).

Genetics of sex disparity in cancer

The X chromosome

Molecular mechanisms driving sex differences in disease are poorly understood and most approaches in precision medicine assign therapy without considering sex as a variable. Genetic and genome-wide sex differences influence both cancer biology and outcome. As X chromosome inactivation is incomplete, some genes can be expressed from both alleles in females, resulting in important sex bias based on sex chromosomes (2). A portionof the reduced cancer incidence in females as compared to males across a variety of tumor types has been attributed in part to the male-biased mutations in genes that escape X-inactivation. In more than 20 cancer types higher mutation rate in males was identified for 6 tumor suppressor genes termed EXITS – escape from the X-inactivation tumor suppressors (9). Two X-linked genes that can escape X-inactivation have a role in immune response – FOXP3 (10) and CD40L(11) – are associated with increased susceptibility to autoimmune disease in females, but may present an advantage in anti-tumor response. Numerous genes that engage in p53 networks are also located on the X chromosome. This is of importance as four key processes that link to cancer sexdisparity: immune response, regulation of apoptosis and cell cycle, metabolism and DNA repair are linked to the tumor suppressor p53. In males, there are also higher frequencies in p53 mutations (12).Chromatin

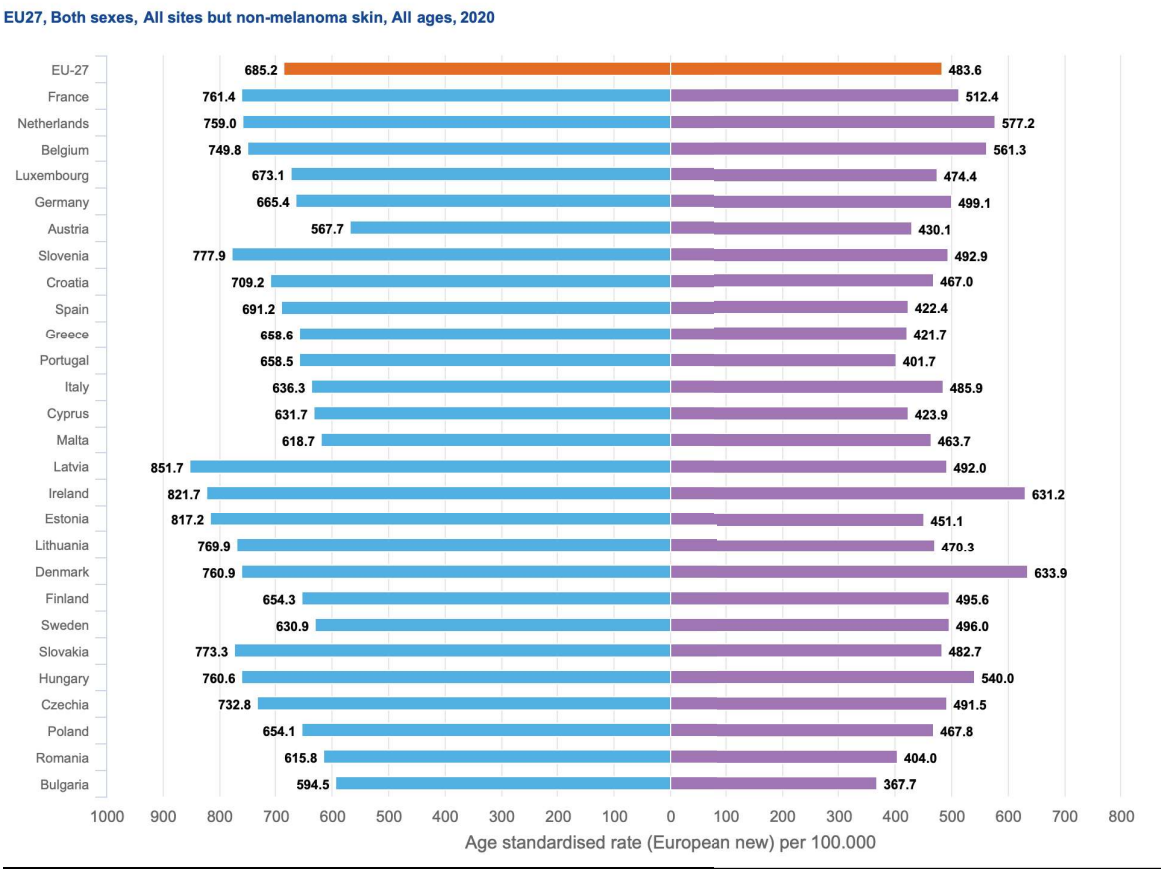

Figure 1. Estimates for cancer incidence per country from the European Cancer Information System (ECIS) From https://ecis.jrc.ec.europa.eu, accessed on 15/09/2023 © European Union, 2023.

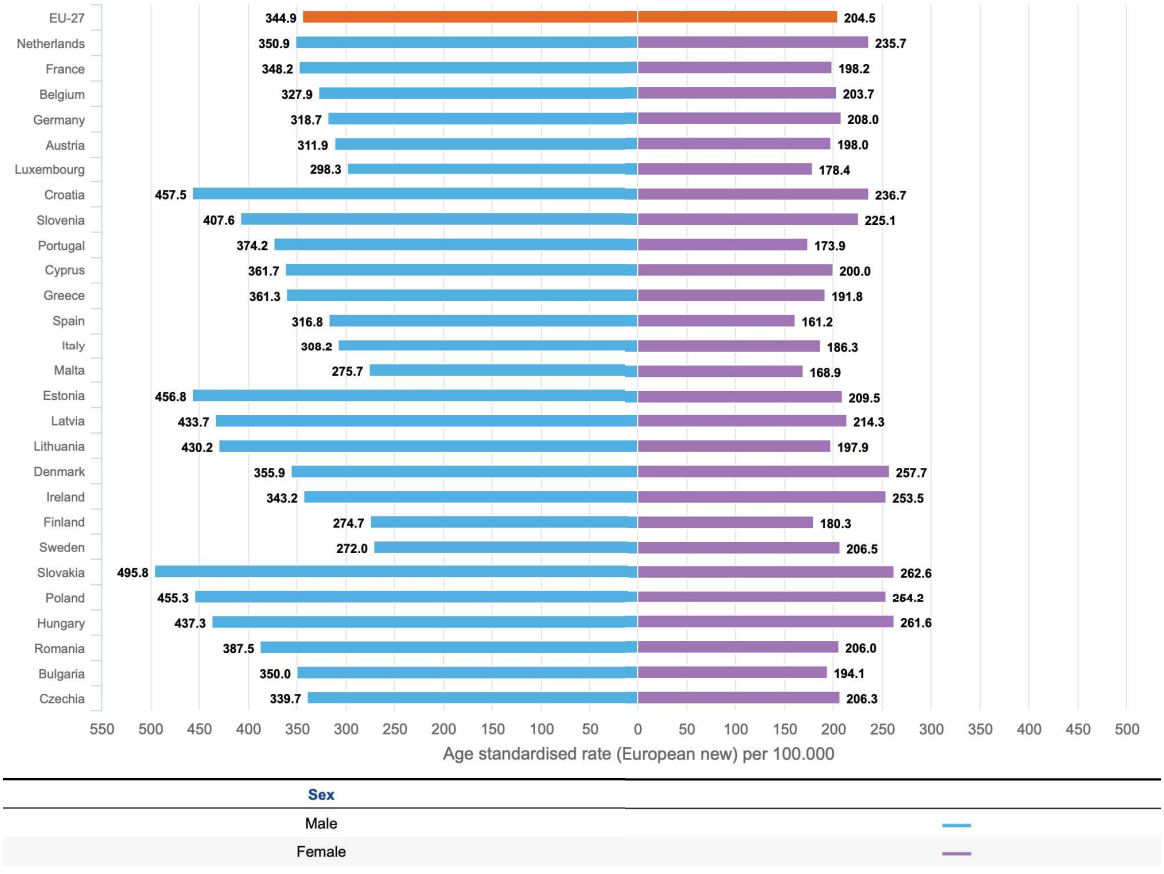

Figure 2. Estimates for cancer mortality per country from the European Cancer Information System (ECIS) From https://ecis.jrc.ec.europa.eu, accessed on 15/09/2023 © European Union, 2023.

accessibility is as well strongly dependent on patient sex, especially on the X chromosome (13, 14). Further support for the effect of biallelic X chromosome gene expression on cancer development comes from a study showing that women with Turner's syndrome (X chromosome monosomy) have increased risk for solid tumor development compared to XX women; and men with Klinefelter syndrome (with two or more X chromosomes) have lower risk for solid tumor development compared to XY men (15).

Sex bias in gene expression and mutational burden

Comprehensive analysis of molecular-level differences between male and female cancer patients in 13 cancer types revealed both sex-biased gene expression patterns for more than 60 individual genes and sex-biased molecular signatures comprising of more than a thousand of genes per signature (16). Importantly, more than 50 percent of clinically actionable genes showed sex-biased expression. For example, a major therapeutic drug target in lung adenocarcinoma, EGFR, showed female-biased mRNA expression (16), that may have contributed to the better response to the EGFR inhibitor erlotinib in female patients (17).

Large differences between the two sexes in mutation density and in the frequency of mutation of specific genes in cancer were also reported; these were suggested to be associated with sex biases in DNA mismatch repair genes and microsatellite instability (18). The first study that investigated genomic differences underlying sex bias was on metastatic melanoma, where authors found that male patients had significantly more missense mutations than female patients (19). Interestingly, somatic mutations have accumulated earlier in the life span in males than in females, implying that the differences in ageing rates account for at least a part of the observed bias (20). The differences in mutational sex bias, however, were not common across tumor types. Some tumor types showed sex-biased differences in SNV mutation profiles, others in CNA mutational profiles, and some in both. Sex-biases existed in both coding and non-coding cancer drivers (21). The mechanisms behind these differences remain to be elucidated.

It is often hard to reach statistical power in sex-stratified analysis of genetic variants (22), nevertheless they can reveal important disparities in cancer. A single nucleotide polymorphism in a negative regulator of p53, MDM2, has been shown to increase cancer risk in several cancer types in females but not in males (23). Many genes related to drug metabolism have genetic variants that impact males and females to a different extent. These can be of influence in the clearance of chemotherapy and associated toxicity.

Sex-biased methylation patterns have been observed in many human tissues, influenced by the presence of the sex chromosomes and sex hormones. Genome wide analysis in 13 cancer types also reported sex-dependent methylation patterns, where most of the genes that had sex-biased methylation had sex-biased expression (16). It is important to note though, that very few methylation studies have included X and Y chromosomes in the analysis.

Taken together, sex biases in activity, repair and folding of the human genome are associated with differences in cancer incidence and outcome between men and women (24). Including the equal number of male and female patients in studies, and segregating the analysis by sex is of utmost importance in the era of high-throughput sequencing and genome wide association studies. Although higher incidences in males dictate the bias, one can design to prospectively collect or retrospectively analyze equal number of patients per sex.

Sex-related differences in cancer metabolism

Many metabolic processes differ between healthy females and males. Both gonadal hormones and X-linked genes contribute to glucose and lipid metabolism and obesity (25). Consequently, associated disease risks differ between sexes (26). In the 13 non-reproductive cancers from the TCGA database significant sex differences in glycolysis, bile acid and fatty acid metabolism were observed (16). High blood glucose levels are associated with higher prevalence and mortality in cancer, as high glucose promotes cell proliferation, invasion and migration, induces the apoptotic resistance and can enhance the drug resistance in tumor cells (27, 28). Fasting hepatic glucose uptake is generally higher in males than in females (29), and increased blood glucose concentration is associated with higher cancer risk in liver and colorectal cancer in males but not in females (30, 31). Using transcriptome analysis, male-specific decreased survival related to glycolytic gene overexpression was found in patients with glioma (32). Further evidence that glucose metabolism has sex-biased impact in cancer outcomes comes from the meta-analysis of 8 cohort studies on colorectal cancer (CRC). This study revealed that while metformin (glucose levels reducing drug) decreased overall mortality of CRC patients with Type 2 diabetes mellitus, females with T2DM using metformin had a lower CRC-specific mortality than males (33). There are 435 registered clinical studies on metformin for repurposing in cancer treatment, with 82 currently recruiting (clinicaltrilas.gov accessed on 15.09.2023) and one can only assume that analysing treatment outcome segregated by sex would lead to novel insights.

Both diabetes and obesity are associated with increased cancer risk (34). Visceral adiposity is higher in men, while subcutaneous fat accumulates more in females (35). Visceral fat is a source of pro-oncogenic adipokines that especially contribute to the development of hepatocellular cancer (36). While it is recognized that lipid metabolism and obesity are associated with inflammation that disproportionately increases cancer risk in males (37), sex-segregated studies that will elucidate the exact mechanisms behind this disparity are still lacking.

Sex disparity in cancer immune response

Sex is a biological variable that strongly affects the immune system, in both innate and adaptive response. Sex chromosomes and sex hormones (as well as nutrition and microbiota) regulate differential response between females and males (38).

In adults, differences in lymphocyte subsets including B cells (higher in females), $CD4^+$ T cells (higher in females), $CD8^+$ T cells (higher in males) and CD4/CD8 ratios (higher in females) are well documented(38). Furthermore, activity of CD4 subsets also differs between sexes. The difference in mounting an inflammatory immune response between the sexes translates into difference in immune defense against cancer (37). It was recently shown that male $CD8^+$ T cells exhibited impaired effector and stem cell-like properties compared with female $CD8^+$ T cells, where androgen receptor inhibited the tumor-infiltrating $CD8^+$ T cell activity by regulating transcriptional programs epigenetically (39).
Around fifty genes that are involved in innate and adaptive immunity are X-linked (40). More robust and heterogeneous immune response in females gives an advantage in anti-tumor response during cancer development. On the contrary, sex dependent mutational burden and sex dependent inherent immune surveillance differences give males an advantage in response to immune checkpoint blockade therapy. Immune response in tumors in males and females should be studied separately, from ***in vitro*** assays using both female- and male-derived model systems to informed patient selection strategies that will better hone immunotherapy approaches.

Sex differences in response to cancer treatment

Drug development has historically followed one size fits all model. However, there are significant differences in therapeutic response and toxicity in male and female cancer patients. Given the body composition, women often receive greater relative dose, that may translate into greater toxicity, but also better response. In the phase II and III clinical trials for chemotherapy, immunotherapy and targeted therapy, spanning four decades and including more than 23,000 cancer patients (excluding sex-specific cancers), women had 34% increased risk of severe adverse effects from therapy (4). Particularly large differences were observed for patients receiving immunotherapy. It is important to note that in these combined 202 analyzed trials women were presented with 37.9% of patients. Indeed, systemic review of randomized immunotherapy clinical trials in the last 10 years showed that women were strongly under-represented (41). Patient response to the immune checkpoint blockade therapy (with inhibitors of PD1, PDL1 and CTLA4) showed divergent patterns for sex bias (42). Women had better response rates in non-small cell lung carcinoma compared to males, but males had better response in colorectal cancer and in six out of seven clinical trials in melanoma (42). Elucidating the opposing sex disparities in response to immune checkpoint blockade demands adjustment for further confounding factors that differ between these cancer types, such are smoking, tumor purity, and age at diagnosis. Inclusion of sufficient number of females in these trials is also fundamental, as mentioned above.
Very few studies investigated response to radiotherapy treatment by sex. Radiotherapy offered advantage in females at the expense of toxicity in oesophageal squamous cell cancer, while underlying biological mechanism was not investigated (43). In this study cardiac toxicity occurred at significantly lower doses in females than in males. Toxicity of chemotherapy is also higher in females. This is a consequence of sex- related differences in pharmacogenomics, pharmacokinetics and pharmacodynamics of drugs in males and females (44). Women have consistently worse safety profile with slower processing of most drugs, higher accumulation of lipophilic drugs, decreased gastric motility, stomach acidity and kidney excretion, which result in slower excretion and elimination of therapeutics. In spite of these known differences, most treatment strategies do not account for sex. However, despite the toxicity, females survive longer than males in response to most of the chemotherapeutics: DNA alkylating agents, antimetabolites, antimitotics and anticancer antibiotics (37).

Existing policies and recommendations

In 1993 National Institutes of Health (NIH) in the United States mandated the enrollment of women in human clinical trials and twenty years later demanded the same in preclinical investigation – to be performed in both male and female animals (45), as several surveys showed that in many biological disciplines researchers used disproportionately higher number of male animals. European commission adopted similar policies in 2014. Mandated policies raised concerns that including both sexes in research will waste resources and slow down research (46). Others have pointed out that the costs of not taking sex into account are even higher as they result in failed clinical trials, misdiagnosis and inappropriate therapies for women and omission of fundamental biological principles (7). Unfortunately, even today not all researchers are fully aware nor they adhere to these recommendations in cancer research. Zucker and Beary analyzed almost 2000 animal studies and found a male bias in 8 out of 10 biological disciplines (47). It was often assumed that results from male animals applied to females, and studies where both sexes were included frequently failed to analyze results by sex. Lack of interest in sex differences is harmful but also presents a missed opportunity for innovation (48). How taking sex as a biological variable has led to novel discoveries can be seen in the recent years. For example, untargeted metabolomic analysis of colon tumors segregated by sex revealed sex differences in energy production and amino acid metabolism and helped define a novel subphenotype in women with right-sided colon cancer with implications for stratifying patient outcome (49). In a murine model of colon cancer with inducible KRASG12D mutation and conditional null alleles of APC and p53 tumor suppressors, male animals had higher metastatic rate and worse outcome (50). This finding led to a discovery that a Y-chromosome coded histone demethylase down-stream of mutated KRAS decreases tight junction integrity in cancer cells making them more prone to migration. By analyzing data segregated

by sex, our group has reported that in melanoma tumor suppressor nischarin had positive prognostic value only in female patients, while in males it was associated with tumor B cell infiltration and negative patient outcome (51). This will have implications on the potential for repurposing of nischarin agonists for treatment of melanoma.

Many a time failure to translate research findings from basic to translational research to therapeutic benefit were blamed on issues of subjective bias, inappropriate experimental design and statistical analysis as causal factors. But more recently, it was acknowledged that studying predominantly one sex also contributes to failure to translate. Some journals have recognized this importance and have changed the guidelines to demand that sex is no longer ignored as a biological variable (52). This editorial noted that while the reporting of sex is encouraging, it is not enough to state the sex of the cells, animals and human specimens analyzed, but to improve practices further, they encouraged researchers to analytically study both sexes. European Union policy review on how inclusive analysis can improve research and innovation (5) suggests that all studies in humans and animals should consider whether sex is a covariate, confounder, or explanatory variable and report sex even at the level of cells and tissues used in research. Namely, female and male cells can exhibit sex differences in transcriptional profile in culture, as well as differences in growth rate, metabolism and response to stimuli (53).

Conclusions

Parameter of sex is largely omitted in both basic and translational cancer research spanning from cell line testing, validation inin vivo models in mice and zebrafish, as well as in patient biopsies. Most researchers do not consider sex specificity in study design and interpretation and molecular mechanisms underlying sex bias in cancer remain largely unknown. Females are largely underrepresented in both clinical trials and animal studies, and results are often generalized to both sexes based on research performed on males. It is essential to consider sex in every stage of preclinical and clinical research to improve prevention, diagnosis and treatment for all cancer patients. In both research and clinical practice sex should be used as a stratifying factor andsex-specific analyses should be performed. These considerations could lead to novel findings and development of treatments that increase efficacy while limiting toxicity.

In the last decade, a new concept has emerged that tumor types with the same histopathological phenotype may have distinct molecular etiologies in men and women (24). Identifying differences between the sexes will be of utmost importance in improving cancer diagnosis and treatment in the era of personalized medicine. Including sex into experimental design helps achieve responsible, rigorous and reproducible science (54). If incorporating both sexes in the research design is not possible, this should be indicated in article titlesand trial reports (47).

Taken together, there is a strong rationale and growing evidence that patient sex is an important variable and it is time for sex bias in basic research and clinical medicine to end, as it will improve therapy for both sexes. For guidance on how to include sex as a biological variable in your research we recommend the SAGER guidelines (48) and the Gendered Innovations Annex A from the European Commission Directorate (5). We will conclude with The European Society for Medical Oncology recommendation that Men and women with nonsex-related cancers should be considered as biologically distinct groups of patients, for whom specific treatment approaches merit consideration (55).

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Review/Perspective

The role of microbiota in cancer patients

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Abstract
The human microbiota, a diverse community of microorganisms inhabiting various regions of the body, has emerged as a crucial player in maintaining health and influencing disease development. This complex ecosystem, known as the microbiome, comprises bacteria, archaea, fungi, protozoa, and viruses, with its composition influenced by factors such as birth mode, lifestyle, diet, genetics, and antibiotic use. Notably, the gut microbiota, constituting over 90% of the human microbiota, plays a pivotal role in preventing pathogen colonization, preserving intestinal mucosal barriers, and shaping the immune system.
This article explores the multifaceted relationship between microbiota and cancer. Differences in microbiota composition are observed in patients with various cancer types, raising questions about their roles in cancer development. Certain microorganisms are present in the tumor microenvironment, potentially influencing cancer progression through interactions with cancer cells. Moreover, the microbiota can affect the effectiveness of cancer therapies, introducing the possibility of personalized interventions.

Human microbiota

The human microbiota represents a complex community of all microorganisms that inhabit various regions of the human body (skin, mucous membranes of the digestive, respiratory, and urogenital tracts, and the conjunctiva of the eye). This diverse aggregate of microorganisms has significant importance both in maintaining human health and in the development of certain diseases. The symbiosis between the human body and the microbiota represents a “superorganism” called the holobiont (1). The term “microbiome” was first introduced by Nobel laureate Joshua Lederberg in 2001 to emphasize the importance of this complex ecological community (2). The term “human microbiome” refers to the collection of all microorganisms that constitute the microbiota, their genes, gene functional products, and metabolites (3,4).
The microbiota of healthy individuals comprises saprophytic and opportunistic pathogenic bacteria, archaea, fungi, protozoa, and viruses (mostly bacterial viruses – bacteriophages) (5).
Contemporary research on the microbiota involves analysing the prevalence of specific genera and species (including both quantitative and qualitative analysis), as well as their gene expression at the RNA level. However, research was hindered by the fact that conventional microbiological methods for culturing samples were not applicable, because certain microbiota bacteria cannot be cultivated *in vitro* conditions. New research technologies (meta-transcriptomics, metagenomics, metabolomics, and bioinformatics tools) have significantly contributed to shedding light on the importance of the microbiota (6).
Today, it is known that microorganisms forming the microbiota form organised communities within the region they inhabit, and that they communicate among themselves by producing various signalling molecules. These interactions contribute to the stability of the ecological community and better defence against competitive (pathogenic) microorganisms (1,7).
Numerous factors influence the composition of the microbiota, including the mode of delivery (natural childbirth or C-section), lifestyle, geographic location, dietary habits, genetic factors, and the use of antibiotics (8, 9).

The formation of microbiota

When does the formation of the microbiota begin? The long-held belief was that colonisation of the human body begins immediately after birth. However, in recent years, research results have become available indicating that microorganism colonisation starts *in utero* (the prenatal colonisation phase). Some studies suggest the existence of microbial communities in the placenta and amniotic fluid, with their origins traced back to the mother’s oral cavity (10). The next phase of microbiota development occurs in the birth stage. The newborn’s organism becomes colonised during the delivery itself. The mode of delivery is one of the factors influencing the composition and prevalence of microbiota species in the newborn.
The oral mucosa, conjunctiva, and skin of newborns delivered vaginally will exhibit a higher prevalence of lactobacilli compared to infants born via Cesarean section, who will be colonised by streptococci living on the mother’s skin. Diet plays a significant role in shaping the gut microbial communities during the first year of life. The gut microbiota of formula-fed infants tends to be enriched in species predominant in adults, such as *Roseburia*, *Clostridium*, and *Anaerostipes*. In contrast, *Bifidobacterium* and *Lactobacillus* dominate the gut microbiota of breastfed infants during their first year (11,12). The first two years of life are considered the most crucial for microbiota formation. In childhood and adolescence, the microbiota changes to a lesser extent. In adulthood, the microbiota is characterized by significantly lower plasticity (13).

The impact of microbiota in health and disease

It is clear that the interaction between microorganisms that make up the microbiota and the human organism persists throughout an individual's entire life. This interaction is an important component in maintaining human health. Researchers worldwide have paid the greatest attention to the microbiota of the gastrointestinal tract (GIT), as it constitutes more than 90% of the human microbiota.

The GIT microbiota plays a significant role in preventing the colonization of pathogenic microorganisms, preserving the mucosal barrier of the intestines, and participating in the maturation of the immune system. In the GIT, immune tolerance is established towards a large number of commensal microorganisms that constitute the microbiota, whose composition undergoes significant changes during the first three years of life. At the same time, the GIT maintains an immune response against pathogenic microorganisms and also responds to opportunistic microorganisms should they enter sterile regions of the body. Here a dense mucus layer plays an important role in the maturation of the immune system by separating microorganisms from the microbiota and the intestinal epithelium. This mucosal barrier is not only mechanical but also induces the development of a tolerogenic phenotype in immune cells (14,15,16).

Generally, the diversity of the GIT microbiota is considered a good indicator of 'healthy' intestines. Reduced diversity of GIT microbiota (dysbiosis characterized by a lower diversity) has been observed in various conditions, such as psoriatic arthritis (17), celiac disease (18), inflammatory bowel disease (19), andeczema (20), among others.

Today, there is significant interest among research groups worldwide in understanding the role of altered gut microbiota. In fact, the question arises as to how significant dysbiosis is in the pathogenesis of various diseases (carcinomas, diabetes, inflammatory bowel diseases, cardiovascular diseases, mental illnesses, allergies, etc.) and whether the composition of the microbiota can have predictive significance.

The microbiota and cancer

The association between microorganisms and cancer can be viewed from different perspectives: 1) which microorganisms can lead to malignant transformations of human cells, 2) whether dysbiosis plays a role in carcinogenesis, 3) whether human microbiota can have a protective function in cancer, 4) how human microbiota affects the success of therapy in oncology patients, and 5) what is the significance of the tumour microbiome.

While the development of cancer cells is generally associated with genetic predisposition and environmental factors, it is important not to overlook the fact that microorganisms can be linked to changes in cell biology and the onset of malignancy (21). Of a multitude of microorganisms inhabiting the Earth, ten have been shown to be associated with alterations in cell biology and are linked to the malignant transformation of human cells (*Helicobacter pylori*, *Clonorchis sinensis*, *Opisthorchis viverrini*, *Schistosoma haematobium*, hepatitis B virus, hepatitis C virus, human papillomavirus, Epstein-Barr virus, Merkel cell virus, and Kaposi sarcoma-associated herpesvirus). This group of oncogenic microorganisms is associated with the development of epithelial cancers (gastric cancer, liver cancer, urinary bladder cancer, cholangiocarcinoma, cervical cancer, as well as other anogenital cancers, lymphoma, skin cancer and Kaposi sarcoma) (5, 22).

Microbial metabolitescan also be associated with carcinogenesis. One of the more extensively studied microbial oncometabolitesis colibactin, which is produced by *Escherichia coli*, found in the human colon. Colibactin is a cytotoxin known to be directly associated with the development of colorectal cancer (23). The mechanism through which colibactin damages DNA involves the alkylation of adenine residues on various DNA strands and the formation of DNA interstrand links (24).

In animal models, it has been demonstrated that enterotoxigenic *Bacteroides fragilis* can induce carcinogenesis of colonic epithelial cells through reactive oxygen radicals that damage DNA (25).

When we examine the composition of the microbiota in oncological patients, we can observe differences compared to healthy controls. One clinical study demonstrated variations in the composition of the gut microbiota between a group of patients with benign breast tumours and a group of patients with malignant breast tumours. In the group with benign tumours, a higher percentage of the genera *Clostridium*, *Faecalibacterium*, *Lachnospira*, *Erysipelotrichaceae*, *Romboutsia*, *Fusicatenibacter*, *Xylophilus*, and *Arcanobacterium* was observed. Differences in the prevalence of certain genera were also noticed among different types of breast cancer (Table 1). It was found that the percentage of the genus *Citrobacter* was statistically significantly higher in the group with malignant breast tumours (26). This could be significant, as precursor studies in animal models (27) have shown that *Citrobacter rodentium* promotes colon tumour growth. In one clinical study, Goedert et al. (28)demonstrated that the genera *Dorea* and *Lachnospiraceae* were present in a lower percentage in the gut microbiota of the postmenopausal breast cancer patients compared to healthy controls.

Clinical studies that have monitored changes in the GIT microbiota in patients with colorectal cancer show that there is a lower percentage of commensal microorganisms in the intestines of these patients, which can affect the gut mucosal immune response. The most frequently isolated pathogen in these patients is enterotoxigenic *Bacteroides fragilis*. It is still not entirely clear whether dysbiosis results from the development of cancer or if it predates carcinogenesis in the colon (29, 5).

In this group of patients, it has been shown that there is a polymicrobial biofilm in the intestines dominated by the genera *Sporobacter*, *Peptostreptococcaceae*, and *Ceilonellaceae*. Hence, research is underway to determine whether such organization of microorganisms could potentially affect the proliferation of epithelial cells and cancer progression via polyamine metabolism (30).

Tumormicrobiome

Research by Kovacs et al. (31) underscores the significance of the fact that members of the human microbiota are often in direct contact with cancer cells, forming part of the tumour microenvironment. Recently, the concept of the tumour microbiome has been introduced: although it is not yet precisely defined, it encompasses all microorganisms within the tumour tissue, on the surface of cancer cells, or inside them. Currently, it is not known whether these microorganisms constitute a permanent niche or represent a transient colonization of the tumour (32,33). The most extensively researched direct interactions between microorganisms from the tumour microenvironment and cancer cells include autophagy mediated by intracellular microorganisms and the inflammation caused by extracellular microorganisms. It has been demonstrated that *Fusobacteriumnucleatum* alters the process of autophagy in colon cancer cells, leading to their chemoresistance and migration (34). It is well-known that chronic inflammation increases the risk of malignancy. The presence of inflammatory cells in the tumour tissue microenvironment promotes the proliferation and migration of cancer cells (35).

Table 1. Genera of microorganismshighly prevalent in the gut microbiota of patients with different forms of breast cancer

PR positive vs. PR negative breast cancer	PR negative vs. PR positive breast cancer	ER positive vs. ER negative breast cancer	ER negative vs. ER positive breast cancer
Prevotellaceae *Tyzzerella*	*Barnesiellaceae* *Lactobacilliaceae* *Lactobacillus* *Prevotellaceae* *Cloacibacillus* *Acinetobacter* *Hydrogenophilus* *Rhodobacteriae* *Hydrogenophilaceae*	*Megasphaera* *Roseburia* *Prevotellaceae*	*Bacteroides* *Bacteroidaceae* *Puniceicocceae* *Opitutales* *Hydrogenophilus* *Hydrogenophilaceae*
Ki-67 low vs. Ki-67 high expression	**Ki-67 high vs. Ki-67 low expression**	**Her2-positive vs. Her2-negative breast cancer**	**Her2-negative vs. Her2-positive breast cancer**
Lactobacillus *Clostridium* *Clostridiaceae* *Megasphaera* *Proteus* *Burkholderiaceae*	*Ruminiclostridium* *Tenericetes* *Mollicutes* *Ruminococcaceae_UCG* *Izimaplasmatales* *Sporobacter* *Syntrophomonadaceae*	*Megasphaera* *Barnesiellacea* *Alloprevotella* *Lachnospiraceae* *Moraxellaceae* *Acinetobacter* *Enorma* *Flavonifractor* *Burkholderiaceae*	*Enterococcus* *Peptostreptococcus*

PR- progesterone receptor, ER- oestrogen receptor, Her2 – human epiderma growth factor receptor 2
Yang P, Wang Z, Peng Q, Lian W, Chen D. Comparison of the Gut Microbiota in Patients with Benign and Malignant Breast Tumors: A Pilot Study

The microbiota and anti-cancer therapy

Perhaps one of the greatest potentials of the GIT microbiota lies in the fact that microorganisms can metabolize not only various host products but also drugs (36, 37). Several studies have indicated a correlation between the effectiveness of anti-programmed cell death protein-1 (PD-1) treatment for malignant melanoma and the makeup of the GIT microbiota. Based on meta and bioinformatic analyses, it has been determined that a positive response to therapy is associated with the presence of the bacteria from the *Lachnospiraceae* and *Ruminococcaceae* families (phylum *Firmicutes*) and the *Actinobacteria phylum*. The presence of Gram-negative bacteria is linked to unfavourable treatment outcomes (38, 39, 40).

Conclusion

In conclusion, while the exact mechanisms underlying the microbiota-cancer connection are still evolving, it is clear that microbiota composition holds predictive and therapeutic potential in oncology. It is also worth noting that cancer therapy itself, as well as frequent antibiotic usage, can also lead to dysbiosis, which may further impact treatment efficacy in specific cases. The fact that the composition of the microbiota can influence a favourable treatment outcome opens the possibility of the application of faecal transplantation, or of selectively enriching the GIT microbiota with specific microorganisms. Understanding this intricate relationship may pave the way for innovative approaches in cancer prevention, treatment, and management.

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PROCEEDINGS BOOK

ABSTRACTS

Plenary Lectures
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LECTURES

PL1

Unconventional approaches to the treatment of cancer

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There is increasing evidence that further activation of signaling pathways that are already activated by oncogenic mutations can be as lethal to cancer cells as their inhibition. This lethality is thought to result from overloaded stress response pathways that are unable to compensate for the increased mitogenic activity. We used small molecule inhibition of protein phosphatase 2A(PP2A) to hyperactivate both WNT and MAPK signaling in colon cancer cells and used genome scale CRISPR screens and compound screens to find the vulnerabilities of cells that experience hyperactivation of oncogenic signaling. Our data indicate that the inhibition of PP2A combined with the inhibition of the WEE1 kinase to perturb the mitotic stress response results in DNA replication stress followed by mitotic catastrophe. Cells that have developed resistance to this drug combination have downregulated oncogenic signaling and consequently fail to form tumors in vivo. Our data suggest that paradoxical activation of oncogenic signaling forces cancer cells to evolve towards a less malignant phenotype.

PL2

Targeting KRAS: achievements and drawbacks

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Lung adenocarcinoma (LUAD) is among the leading causes of cancer-related death worldwide, with approximately one-third of patients harboring KRAS mutations. In particular, G12C is the most prevalent alteration. Direct KRAS inhibitors sotorasib and adagrasib binding the target in its inactive conformation and locking the GDP state (RAS-OFF inhibitors) have shown early signs of clinical activity in patients with KRASG12C-mutant LUAD, but responses are short-lived and the development of resistance is inevitable. We developed in vitro and in vivo models of resistance to sotorasib and adagrasib to validate and characterize both acquired and adaptive mechanisms of therapeutic escape, as well as to test new treatment options as a single agent or in combination. Strategies to overcome resistance with the new generation of RAS-ON inhibitors targeting the active GTP-bound form of RAS were also investigated in patient-derived models. We propose that acquired genetic drivers rewire KRASG12C tumor cells signaling and that this rewiring is therapeutically actionable. Moreover, RAS-ON inhibitors targeting GTP-bound KRAS may be an effective alternative to treat disease relapse due to adaptive mechanisms of signal reactivation by wild-type RAS isoforms.
Keywords: KRAS, MAPK pathway, drug resistance

INVITED LECTURES

L01

Discovery of novel HDAC inhibitors for therapy of triple-negative breast cancer – preclinical study

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Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer that has poor survival rates due to the absence of specific molecular markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). In the era of precision oncology, it is recognized that an imbalance in post-translational modifications of histones, such as histone lysine acetylation and deacetylation, is closely linked to tumor initiation and progression. Two groups of enzymes control the reversible nature of histone post-translational acetylation: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Isoform-specific targeting of HDACs is considered a rational strategy to develop safe anticancer therapeutics compared to non-selective HDAC inhibitors. However, non-selective HDAC inhibitors have been more extensively studied in clinical trials. This work presents the design and discovery of potent HDAC inhibitors that selectively target HDAC6 isozyme, using 1-benzhydryl piperazine as a surface recognition group with different hydrocarbon linkers. Through in vitro screening, two HDAC6-selective inhibitors with nanomolar IC50 values and two non-selective HDAC inhibitors were identified. Structure-based molecular modelling was utilized to investigate the impact of linker chemistry on the potency of synthesized inhibitors against HDAC6. The anti-cancer, anti-migratory, and anti-invasive activities of these compounds were evaluated using breast cancer cell lines (MDA-MB-231 and MCF-7). Experiments on a zebrafish MDA-MB-231 xenograft model demonstrated that a novel non-selective HDAC inhibitor (compound 8b) with a seven-carbon-atom linker exhibited potent effects against tumor growth, metastasis, and angiogenesis at low micromolar concentrations.
Keywords: anticancer drug, breast cancer, histone deacetylases,hydroxamic acid

L02

Estrogen Receptor Beta promoter methylation as a possible biomarker in breast cancer

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Since the estrogen receptor alpha (ERα), together with the progesterone receptor (PR) and the herceptin receptor 2 (HER-2), are the dominant factors determining the groups of breast cancer (BC) patients, breast cancer treatment depends on the presence or absence of these three molecules. Approximately 70% of patients receive hormone treatment targeting the estrogen receptor alfa, with tamoxifen (selective oestrogen receptor modulator) being the first choice as it inhibits further proliferation of cancer cells. However, 30% of patients do not respond to existing hormone therapy, raising the question of new targets and treatment options. Non-responders include patients who have acquired resistance to standard treatment and triple-negative breast cancer patients (TNBC), characterized by the absence of ERα, PR and HER-2. One of the unexplored potentials for treatment is a protein homologue of ERα, estrogen receptor beta (ERβ), as many studies show ERβ expression in ERα-negative patients. The estrogen receptors alpha and beta belong to the superfamily of nuclear receptors, and their dominant ligand is estrogen. When estrogen binds to estrogen receptors, they form dimers (homo or heterodimers) and bind ERE sequences of target genes (estrogen receptor elements). In a heterodimeric state, ERβ can inhibit ERα transactivation and thus influence the signalling pathways. ERα and ERβ are encoded by highly homologous genes (ESR1 and ESR2), resulting in two highly homologous

protein structures. The human ESR2 gene contains eight exons. The last two coding exons of the ESR2 gene are alternatively spliced encoding ERβ transcriptional variants (ERβ1-5), resulting in altered C-terminal domains of the ERβ protein. These transcriptional variants can have dominant positive or negative functions or no function at all. While ERα is crucial for the growth and proliferation of breast tissue, ERβ plays a role in the normal development of breast tissue, ovaries, testes, brain and adrenal glands. Study reports show that ERβ has an antiproliferative, pro-apoptotic and tumour-suppressive function. Its function in breast development also implies its function in tumourigenesis. However, the expression of ERβ mRNA and protein expression is unclear. Various studies on ERα-positive tumours show that ERβ is a tumour suppressor. The studies on ERα-negative tumours show controversy, whereby ERβ could be proliferative or suppressive. ERβ expression is often associated with smaller tumour size, lower grade and the absence of metastases. In TNBC patients, the association between clinical outcomes and ERβ is unclear. Some studies associate ERβ with prolonged survival, others with shortened survival, while in others, no association has been demonstrated. There are many reasons for these contradictions. The first reason is unprecise methods of measuring ERβ levels, with differences in baseline material. In some studies, the amount of ERβ is estimated by quantitative PCR, while in others, by antibodies. Secondly, the researchers prevalently use non-specific antibodies that cannot detect the existence of specific ERβ isoforms. ERβ expression changes during BC progression. In the early stages of BC, ERβ levels decrease, while more advanced stages show a complete loss of ERβ. However, some studies report increased ERβ expression in metastatic tissues. Researchers should pay particular attention to the molecular mechanisms that alter ERβ expression, with epigenetic mechanisms being the most crucial. One of the most important mechanisms for tumour initiation and development is gene promoter methylation. DNA methylation is an inheritable epigenetic modification in which DNA methyltransferases (DNMTs) promote the transfer of the methyl group from S-adenosyl L-methionine (SAM) to 5'-cytosine of the CpG dinucleotide. CpG methylation is a crucial regulatory mechanism that begins early in embryogenesis. In the promoters of genes central to development, such as housekeeping genes and some tissue-specific genes, there are unmethylated regions called CpG islands. CpG islands encompass about 500 to several thousands of base pairs, and the CpGdinucleotides within them are more abundant than in the other genome locations. CpG islands in coding genes' promoter regions of cancer cells are regularly hypermethylated, causing gene silencing. The silenced genes are commonly tumour suppressor genes, such as ERβ. ERβ gene promoter region contains two exons, exon OK and exon ON. Most studies have been done on ON exon, linking hypermethylation of ON exon with decreased ERβ expression. Initially, the researchers noticed ON exon hypermethylation in prostate cancer, and prostate cancer cell treatment with a demethylation agent, 5'-AZAC, led to ERβ expression activation. Also, during the progression of prostate cancer, a hypermethylation level increased. These results were consistent with some studies on breast cancer patients and cell lines. There is scant data on the association between ERβ hypermethylation and survival. Usually, studies show correlations between ERβ1 expression and survival. The clinical potential of ERβ promoter methylation is yet to be examined. Additional research on this molecule and its expression mechanisms should determine its predictive, diagnostic, and treatment potential.

Keywords: Estrogen Receptor beta, DNA Methylation, Tumor Suppressor

L03

A new approach to the design of metal-based antineoplastic drugs

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High potential of cisplatin in oncology has raised the interest in metal-based drugs as potential antineoplastic drugs. Transition metals offer diverse chemistries for the design of anticancer agents. Ligand affinity and possible coordination geometries of the metal center are important bioinorganic principles for design. Metal-ligand bonds are closely related to the HSAB nature of metals and their preferred ligands. Many factors could affect metal–ligand complex formation including the formation of competing equilibria–solubility products, complexation, and/or acid–base equilibrium constants–sometimes referred to as „metal ion speciation". Bearing in mind all the effects, new approach is based on heteronuclear complexes which contain two reactive centers differ differing in Lewis acidity, geometry and kinetic characteristics, connected with π-acceptor bridging ligands. The incorporation of two metals that differ in Lewis acidity, can affect the kinetic properties in the same complex and may improve selectivity for cancerous cells increasing the antitumor activity and decreasing toxicity to normal cells. Design of novel hetero-nuclear platinum(II)-zinc(II) complexes could be highly effective. The complexes of these two metal ions have different coordination geometries, kinetic properties, affinity and reactivity towards biologically relevant nucleophiles. According to the hard-soft acid-base principle, dissimilar reactivity of metal centers will results in different coordination modes of biomolecules and

in increment of cytotoxicity. The nature of the linker between the different metal centers may have a significant impact on the reactivity. The set of heteronuclear complexes with general formula cis/trans-Pt-L-Zn(terpy-X) (where L= pyrazine or 4,4′-bipyridyl, terpy-X = 2,2′:6′,2′′-terpyridine or 4′-chloro-2,2′:6′,2′′-terpyridine) has been synthesized and characterized. The antiproliferative action of the heterometallic complexes and CDDP as a referent compound was determined in a panel of human tumor cell lines (A549, HCT116, LS-174, SW-480 and MDA-MB-231) and one non-tumor fibroblast cell line (MRC-5) using the MTT assay. The results have shown that heteronuclear complexes with zinc center coordinated with terpy-Cl less cytotoxic effect, and haven't showed significant reduction of cell viability. The influence of negative inductive effect of chloride could be main reason for the decrease of the electrophilicity of both Zn(II) and Pt(II) centers and less reactivity.

Keywords: Heterometallic Complexes, Structure-Reactivity Correlation, Antineoplastic Agents

L04

Approaches to targeting cancer cell resistances in preclinical research

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Background: Considering that cancer is one of the leading causes of death worldwide there are more and more attempts and available strategies for cancer treatment. Preclinical and clinical research aims to discover anticancer drugs that are efficient enough, and a number of ongoing studies explore potentially effective anticancer drugs. Although many of them exhibit remarkable efficacy during preclinical investigations and primary treatment, various side effects and drug resistance often develop in many cancer patients. Cancer cell resistance is an important problem in cancer treatment and the main cause of chemotherapy failure. Drug resistance can occur due to the activation of intrinsic (pre-existing) or acquired (induced by drugs) mechanisms. Both can lead to multidrug resistance (MDR), which occurs when cancer cells develop resistance to multiple chemotherapeutic drugs, including anticancer drugs with different chemical structures and mechanisms of action. Multidrug resistance is caused by various microenvironment, genetic and epigenetic factors, including changes and commonly mutations in oncogenes and tumor suppressor genes, hypoxia, inflammation, etc. Whatever the cause, it altered the expression of MDR genes responsible for the development of resistance against anticancer agents and significantly limited the effectiveness of chemotherapy. MDR phenotype includes changes in many target proteins, decreased drug entry, detoxification and altered drug metabolism (metabolic enzymes), increased DNA repair capacity, avoidance of apoptosis, increased ejection of drugs from the cancer cells, the presence of cancer stem cells, and many other mechanisms.[1] The most common drug resistance mechanism is the increased efflux of drugs, mediated by ATP-dependent ABC transporters, such as the MDR1 gene that encodes P-glycoprotein (P-gp), which is mainly overexpressed in various cancers and has been associated with the development of resistance against numerous chemotherapeutics. Other drug-resistant transporters may also contribute to resistance development, such as MRP1 and 2 (MDR-associated protein-1 and 2), BCRP (Breast Cancer Resistance Protein), and many others which have not been extensively investigated. Additionally, molecules and enzymes responsible for drug metabolism are also involved in the reduction of the cytotoxic effect of chemotherapeutics and play a role in cancer cell resistance. These enzymes are a part of phase I and II of drug metabolism which are related to the detoxification of xenobiotics. Cytochrome (CYP) isoforms are mostly included in phase I of drug metabolism and detoxification, whose overexpression has been observed in various cancers and associated with their enhanced resistance. Altered expression of enzymes involved in phase II of drug metabolism, including glutathione-S-transferases (GSTs) which conjugate xenobiotics with glutathione (GSH) in cancer cells may also enhance their MDR. Even the increased GSH and enhanced detoxifying ability of GSH in cancer cells are associated with decreased activity of the chemotherapeutic agents, as well as dysregulation of Bcl-2 expression, as a pro-survival and antiapoptotic regulator which upregulates target genes such as P-gp, NF-kB and many other transcriptional factors.[2] Understanding the molecular mechanisms and identification of MDR genes that result in the development of drug resistance is increasingly important. Numerous attempts have been devised to overcome it. Many compounds, called MDR inhibitors or modulators have been tried and tested in the development of effective treatment modalities, including develop of ABC transporter inhibitors, as the most important strategy. Chemical substances have been tested in numerous clinical attempts for their MDR modulatory activity, however not successful. Thus, discovering of the new, safer ones is necessary. Generally, there is research progress in the overcoming of drug resistance via natural products such as the MDR modulators, because of their multiple targets, little systemic toxicity, and other beneficial effects.[3] These compounds have the potential to become more successful than many of the previously developed modulators in three generations of inhibitors. The most investigated are plants and their metabolites, however in recent years animal products occupy an important place. The focus of research in this study is the underlying mechanism of cancer cell resistance development, identification of the main molecules

related to colon cancer cell resistance, as well an attempt for its modulation. Our research includes basic/preclinical research, about natural compounds that aim to inhibit or overcome MDR development in colon cancer cells. According to the natural role of intestine epithelium and exposure to the xenobiotics these cells often have innate resistance, high expression of member transporters before chemotherapy and a high potential to develop MDR. **Material and Methods:** Identification the biomarkers which may underline the mechanism of colon cancer cell resistance to 5FU on parental and resistant cell line model systems. Cancer drug resistance indicators include the inhibitory concentration that inhibits 50% of cell growth (IC50 value), cell resistance index (RI), the cell growth curve (cell viability in real time- RTCA), apoptotic index, as well as biomarkers of resistance on gene and protein level. In our experimental conditions, the large number of natural compounds have been tested in an attempt to overcome these resistance mechanisms or minimize the resistance to chemotherapy. Their potential to modulate resistance-related gene and proteins were monitored by the qPCR method, immunocytochemistry, and colorimetric methods. Another strategy was the combined treatment of anticancer drugs together with an MDR inhibitor (natural compounds as potential MDR inhibitors). **Results:** Our results indicate that many natural derived compounds, including plant extracts and their individual metabolites[4], animal products, including animal venoms and their isolated compounds, like as melittin from bee venom and L-amino acid oxidase from snake venom show potential to modulate MDR.[5,6] Our results indicate that natural compounds are suitable and promising in overcoming the resistant phenotype of cancer cells, including high cytotoxic and proapoptotic activity, reduced necrotic activity, inhibitory activity to MDR efflux pump expression on gene and protein level, inhibition of enzymes of phase I and II biotransformation, modulation of apoptosis via Bcl-2 proteins and reversing the recovering of the cell after longer time exposure (growth curve in real time indicate cell recovering even in aggressive treatment). Our results indicate that natural products can modulate the several most implicated transporters in cancer drug resistance (P-gp, MRP1, 2, 5 and 8, BCRP). Their expression was downregulated in many investigated substances from natural origin. Also, they interact with biomolecules and altered expression of specific genes responsible for colon cancer cell resistance on 5-fluorouracil, where they are efficient in sensitising MDR cells to 5-fu. This offers the possibility of combined use with chemotherapeutic agents, where our research also showed great potential, through synergistic effects due to inhibition of transporters which reduce the effective intracellular concentration of the drug. **Conclusion:** Better understanding of the mechanisms of MDR and potential targets/biomarkers of resistance should improve the ability to reverse it and provide guidance for future research of new effective strategies in cancer treatment. Downregulation and inhibition of genes and proteins involved in the development of resistance should improve efficacy of the anticancer treatment, where natural products have great potential. Also, data has shown that their combination with other drugs significantly increased the efficiency of cancer treatment and deserves future detailed investigation.

Keywords: Colon Cancer Cells, Multi Drug Resistance, Natural Product

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L05

Small hydrophobic molecules in multi-targeted cancer therapy: disruption of plasma membrane and mitochondrial functions

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Background: One of the most promising approaches in cancer therapy involves drugs with heterogeneous anticancer activity that simultaneously hit multiple targets within the cell. Small hydrophobic molecules, as antimicrobial drugs with ionophoric properties as well as amphiphilic drugs with potential to locally perturb composition of lipid bilayers throughout the cell, have been successfully repurposed to target cancer. Multiple targets include various membrane-bounded organelles, contributors of multidrug resistance (MDR), ion channels, membrane receptors and lipidomic homeostasis. Adamantane-containing crown ethers represent small hydrophobic molecules with good potential to hit multiple targets in breast cancer. As we previously demonstrated, crown ethers exhibit potential for ion transport, while hydrophobicity of the attached adamantane substituents prominently contributes to their bioactivity. Moreover, crown ethers inhibit a major MDR contributor, P-glycoprotein (MDR1). Breast cancer is heterogeneous disease comprised of different subtypes, the most hazardous being characterised by poor prognosis and inefficient chemotherapy. Breast cancer chemoresistance is often related to a high percentage of cancer stem cells (CSCs) within the tumour bulk, especially in triple negative breast cancer (TNBC). CSCs subpopulation within a tumour overlaps with a subpopulation of cells that have undergone epithelial to mesenchymal transition (EMT), developmental program activated during cancer invasion and metastasis. EMT is characterised by changes in plasma membrane composition and modifications of cellular organelles, remodelling of cytoskeleton, metabolic reprogramming and other adopted traits that increase the survival of invasive cell. Since breast CSCs represent vital, but extremely heterogeneous and plastic subpopulation within breast cancer, approach involving drugs that hit multiple targets is crucial. Thus, we investigated the potential of representative adamantyl crown ether compound to target breast cancer cells and breast EMT-model cells. **Material and methods**: We performed molecular dynamics (MD) simulations and next generation sequencing, followed by in vitro study of cell death, membrane perturbations and ionophoric ability upon treatment. Cell lines used were EMT-model cells consisting of cells induced to undergo EMT (HMLE cells transduced with lenti-Twist and HMLE cells transduced with an empty control vector), SUM 159 TNBC cells, MCF7 breast adenocarcinoma cell line and MJ90 (HCA2) fibroblasts. **Results**: Molecular dynamics simulation indicates that the adamantyl crown ether compound readily diffuses deeply into the centre of the lipid bilayer, but is not able to transport cations through the membrane. In addition, the compound forms large aggregates that are orientated towards the water phase of the membrane. The compound affects expression of genes involved in membrane lipid composition, vesicular transport and cytoskeletal remodelling, all of which is crucial for the maintenance of membrane integrity. All treated cell lines exhibit growth inhibition followed by necrotic cell death. We measured an increase in membrane permeability, the effect being most prominent in EMT-model cells. The compound depolarises mitochondrial membrane potential and decreases cellular ATP levels. In addition, the treatment changed mitochondrial morphology in EMT-model cells. Measurements of ion fluxes confirmed inability to transport ions, as predicted by MD simulation. However, we measured changes in the membrane potential of cells rapidly after the treatment, which correlated with intracellular Ca2+ level increase. **Conclusion**: Our proprietary adamantyl crown ether targets breast cancer cells and breast EMT-model cells by physical disruption of plasma membrane and impairment of mitochondrial function. Thus, we propose application of small hydrophobic compounds as adamantyl crown ethers to target membrane integrity and mitochondrial function in EMT, and potentially CSCs, among tumours.
Keywords: Breast cancer, Mitochondrial Function, Plasma Membrane, Small Hydrophobic Molecules

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L06

Good cop-bad cop: different roles of hsa-miR-93-5p in colorectal cancer

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Background: Colorectal cancer (CRC) is a heterogeneous disease that ranks third and second globally in terms of incidence and mortality rates, respectively. Five-year survival of patients with CRC is approximately 90% if diagnosed in the early stages, and only 13% if the advanced disease is present. About 25% of patients already have CRC metastases (mCRC) with the primary CRC diagnosis, while half of the patients will develop metastases with further disease progression. The most common organ in which CRC metastasizes is the liver (colorectal cancer liver metastasis, CRLM). Almost half of CRC patients will die due to complications caused by the presence of metastases, so it is extremely important to discover new therapeutic approaches, as well as prognostic and predictive biomarkers, in order to reduce such a high mortality rate. In the era of personalized medicine, various treatment modalities are available to CRC and mCRC patients, including resective surgery, systemic chemotherapy, and novel targeted biologics, which significantly improve the outcome of CRC patients. The main goal of neoadjuvant systemic chemotherapy is to render currently unresectable disease amenable to resection. The standard cytotoxic drugs used in systemic chemotherapy for the treatment of CRC are: 5-fluorouracil (5-FU), oxaliplatin, and irinotecan applied as single agents or combined. It has been shown that the combination of systemic chemotherapy with targeted biological agents (e.g., bevacizumab which targets vascular endothelial growth factor) leads to a better therapy response compared to the use of systemic chemotherapy alone. MicroRNA (miRNA) molecules belong to a large class of small regulatory non-coding single-stranded RNA molecules that exert negative post-transcriptional regulation of gene expression. MiRNAs are involved in the regulation of fundamental cellular processes such as cell proliferation, differentiation and death, thus these molecules have been proposed as one of the regulators of oncogenesis, considering that they can have an oncogenic or tumor-suppressive role, which can be tumor-specific. The miRNA expression pattern is consistently and reproducibly altered in CRC compared with normal intestinal mucosa, and this expression pattern changes during the progression from normal colon, through adenoma to colorectal cancer. Not surprisingly, microRNAs have been implicated in the CRC growth, progression, metastasis, and response to therapy. MiRNAs have also been studied as potential diagnostic, prognostic and predictive biomarkers, and therapeutic agents or targets. To date, a small number of molecular biomarkers have been identified that can predict a patient's response to therapy and thus help doctors in decision making to select the right therapy for a given patient. Identification of new validated predictive and prognostic biomarkers will be necessary to improve the quality of life and outcome of CRC patients. Hsa-miR-93-5p, together with hsa-miR-106b and hsa-miR-25, belongs to the miR-106b-25 cluster located on the 515 bp long region of chromosome 7q22, within intron 13 of the MCM7 gene. Interestingly, hsa-miR-93-5p has been reported to have oncogenic and tumor-suppressive roles in different tumor types. This systematic review aims to present the current knowledge on the

role of hsa-miR-93-5p in the processes related to colorectal carcinogenesis, metastasis, and response to chemotherapy in patients with primary and metastatic colorectal cancer. Also, the role of hsa-miR-93-5p as a potential prognostic and predictive biomarker is described. **Material and Methods:** PubMed database was searched using keywords hsa-miR-93-5p, colorectal cancer, metastatic colorectal cancer, response to therapy, 5-fluorouracil, oxaliplatin, irinotecan, bevacizumab, predictive biomarker, and prognostic biomarker for available relevant literature data. **Results:** Literature data shows no consensus regarding the direction of hsa-miR-93-5p expression in CRC compared to normal mucosa, with most studies indicating elevated hsa-miR-93-5p expression, while others emphasize decreased hsa-miR-93-5p expression. In various tumors, the oncogenic or tumor-suppressive role of hsa-miR-93-5p has been demonstrated. As for CRC, several studies have shown that hsa-miR-93-5p inhibits proliferation, invasion, migration, autophagy and tumor formation in vivo, reduces viability, induces apoptosis, and may suppress the immune evasion of CRC cells, indicating a tumor-suppressive role. Some of the molecular mechanisms by which hsa-miR-93-5p regulates these processes include Wnt/β-catenin and PI3K/AKT signaling pathways, also downregulation of several genes including ERBB2, p21 and VEGF, and reduction of MMP-1, MMP-2, and MMP-9 proteins. Results of our still unpublished work are in contrast to the aforementioned studies where the anti-tumor effect of hsa-miR-93-5p in CRC was proven. We have shown that hsa-miR-93-5p stimulates in vitro tube formation thereby participate in promoting CRC angiogenesis, but does not affect viability, cell cycle, anoikis and migration in vitro, as well as tumor growth in the in vivo chick embryo model. MiRNA expression is often altered by anti-tumor drugs. A number of studies associate hsa-miR-93-5p with response to standard and targeted chemotherapeutic drugs used for the treatment of CRC and mCRC. One study showed downregulation of hsa-miR-93-5p after treatment with 5-FU and oxaliplatin for 24 h, while other showed increase of hsa-miR-93-5p after treatment with 5-FU for 24 h in a different CRC cell line. Results on the metastatic CRC cell line SW620 showed that the hsa-miR-93-5p expression 72 h after treatment with individual chemotherapeutic drugs (5-FU, oxaliplatin, irinotecan) and their combinations (5-FU + oxaliplatin and 5-FU + irinotecan) was reduced compared to the control treatment. When comparing these studies, one should definitely pay attention to the used CRC cell line, as well as the used concentration and the treatment duration. Besides standard chemotherapeutic drugs, the effect of targeted molecular agent bevacizumab on hsa-miR-93-5p expression was also analyzed in vitro, applied alone or in combination with 5-FU + oxaliplatin. However, neither bevacizumab nor bevacizumab in combination with 5-FU + oxaliplatin for 72 h did not change the hsa-miR-93-5p expression in SW620 metastatic CRC cell line. miRNAs present in the tissue or as freely circulating molecules in the blood have been proposed as promising biomarkers for predicting response to systemic and targeted therapy in CRC patients. One study investigated whether the hsa-miR-93-5p expression was altered in patients who received neoadjuvant chemotherapy based on the combination of 5-FU + oxaliplatin, compared to those who did not receive neoadjuvant chemotherapy using samples of metastatic tissue and serum from patients with CRLM. Also, in order to examine the translational potential of hsa-miR-93-5p as a predictive biomarker for response to neoadjuvant chemotherapy in patients with CRLM, the association of high and low expression of hsa-miR-93-5p in metastatic tumor tissue and serum was analyzed in patients who showed a chemotherapy response compared to those who did not. These results showed that neoadjuvant chemotherapy based on the combination of 5-FU + oxaliplatin did not affect the expression of hsa-miR-93-5p in metastatic tumor tissue or serum in CRLM patients, and that hsa-miR-93-5p has no predictive potential in patients with CRLM. Numerous studies indicate that miRNAs expressed in the tumor or in various body fluids could be potential prognostic biomarkers for CRC and mCRC and thus predict the disease outcome. It was suggested that miRNAs in combination with carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) tumor markers could improve the discrimination of patients in relation to the disease outcome. One study investigated whether hsa-miR-93-5p expressed in CRLM or serum of these patients correlates with the levels of CEA and CA 19-9 tumor markers. The results showed that hsa-miR-93-5p expressed in CRLM shows a moderately negative correlation with CEA level. So far, four studies investigated the potential prognostic utility of hsa-miR-93-5p in CRC patients. All studies found that decreased hsa-miR-93-5p expression was associated with early disease recurrence and worse overall and disease-free survival. Regarding the role of hsa-miR-93-5p in mCRC, hsa-miR-93-5p expression is known to be higher in CRLM compared to normal colon tissue and compared to primary CRC. One study investigated the prognostic significance of hsa-miR-93-5p for one-year disease-free survival and early disease recurrence in CRLM patients. The results showed that there was no difference in one-year disease-free survival in patients with increased or decreased hsa-miR-93-5p expression in CRLM or serum. However, it was shown that the elevated hsa-miR-93-5p expression in the serum was significantly associated with early disease recurrence during one-year follow-up. **Conclusion:** This systematic review summarizes the role of hsa-miR-93-5p in colorectal carcinogenesis, response to therapy and disease prognosis. Tumor-suppressive role of hsa-miR-93-5p has been demonstrated in CRC. Standard chemotherapeutics for the treatment of CRC affect the expression of hsa-miR-93-5p, but different in vitro studies have shown conflicting results. Numerous studies confirm the prognostic utility of hsa-miR-93-5p in CRC patients, as it has been shown that reduced hsa-miR-93-5p expression is associated with early disease recurrence and worse overall and disease-free survival. In mCRC, hsa-miR-93-5p has been shown to participate in promotion of angiogenesis in CRC and mCRC by stimulating in vitro tube formation. It has also been shown that standard

chemotherapeutical drugs 5-FU, oxaliplatin, irinotecan and their combined regimens, but not the targeted agent bevacizumab, affect the hsa-miR-93-5p expression in SW620 cells in vitro, but the effect of neoadjuvant chemotherapy on hsa-miR-93-5p expression was not observed in CRLM patients. Hsa-miR-93-5p has no predictive potential in CRLM patients treated with 5-FU + oxaliplatin. Interestingly, hsa-miR-93-5p in CRLM showed a moderately negative correlation with the level of the tumor marker CEA, but further research should confirm the usefulness of their combination for the disease prognosis. Finally, elevated hsa-miR-93-5p expression in the serum of CRLM patients was significantly associated with the early disease recurrence, which is why it was proposed as a potential prognostic biomarker for the early disease recurrence.

Keywords: colorectal cancer, hsa-miR-93-5p, metastasis, predictive biomarker, prognostic biomarker, response to therapy

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L07

Network based approaches in cancer research - chances and challenges

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Cancer, a complex and multifaceted disease, presents a significant challenge to researchers and clinicians alike. Traditional reductionist approaches have provided valuable insights into the biology of cancer, but they often fall short in capturing the intricate interplay of molecular components that drive tumorigenesis and progression. Network-based analysis approaches have emerged as powerful tools in cancer research, offering a holistic perspective on the intricate molecular networks governing cancer initiation, progression, and treatment response. This talk explores the key elements of network-based analysis in cancer research, highlighting their importance and impact. It will present two examples, namely co-expression networks and directed acyclic graphs (DAGs) to identify potential therapeutic targets and reverse engineer gene regulatory networks. Both examples show the intricate interaction of AXL with cancer promoting pathways, and the impact of gender on AXL expression and pathway regulation. Further, the talk addresses several challenges in network based analysis approaches, such as network optimization and automatic deduction of structured causal models. Finally, the talk touches the integration of multiomics data using methods such as MINT. This talk underscores the significance of network-based analysis in advancing our understanding of cancer biology and its translation into clinical applications. The integration of big data, computational methodologies, and biological networks holds promise for more effective cancer treatments and improved patient outcomes.

L08

Tackling omics research in pathology in a low-budget setting

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In the past decade, both research and diagnostics in pathology have moved from single change and single molecule analysis to multiplex analysis ranging from comprehensive panels of genesandwide epigenome profiling towhole genome and exome analysis and metabolome profiling.[1] Although the price per sample for such wide analyses has become lower in developed countries, the access to appropriate instrumentation is still very limited in developing countries. To overcome this problem, researchers can use omics data from publicly available cohorts. In this lecture, the potential of such resources and the pitfalls of their use will be discussed through examples together with the problem of validation of acquired results. Questions related to the dynamics of normal human tissues can be evaluated using data from Genotype-Tissue Expression (GTEx) project or Human Protein Atlas (HPA).[2,3] GTEx is a valuable resource for the understanding of the role of genetic and gene expression variation in biological changes in human tissues and cells. It provides data acquired from tissue RNA sequencing of a high number of samples and data acquired from single nuclei sequencing from a smaller number of frozen tissues.[2,4] In recent versions of the HPA, tissue and single cell transcriptomics were integrated with immunohistochemistry based proteomics of various tissues.[5] In such way, other researchers have the opportunity to evaluate temporal and spatial dynamics of normal tissues and cells on the RNA and protein level. Different bioinformatical approaches can be applied on tissue transcriptome to gain the information about cellular heterogeneity and cell-level temporal dynamics.[6–8] Comparative analysis of single cell transcriptome and proteome based on the manual annotation of immunohistochemically stained tissues proved to be a good strategy for detection of missing proteins.[9] Here, some examples will be shown to illustrate how HPA can be used in the analysis of spatial dynamics of protein expression in human skin (data not published). Moreover, computational methods have found its place in cellular profiling of cancer tissues to better characterize invasive capacity and aggressiveness of tumors.[10] Still, in many of such research efforts validation of the results on the protein level is missing. Moreover, many of them utilize datasets available through the Cancer Genome Atlas (TCGA)[11] but don't replicate results in other publicly available cohorts. Researchers should be critical when using TCGA data, especially in relation to histological classification and grading of tumors. These are important aspects in cancer research as they are the most prone to change over the years.[12] As such, pathologists should be a part of a research to advise on these changes and to reevaluate the diagnosis beforehand.

Keywords: Open Data, Transcriptomics, Proteomics, Computational Analysis

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L09

Sex as a biological variable in preclinical melanoma research

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Background: In humans, biological sex refers to the attributes that distinguish male, female and intersex (chromosomes, gonads, genitals and hormones). Patient's biological sex influences cancer risk, the biology of cancer, response to treatment and the metabolism of anticancer drugs. In melanoma sex differences in mutational burden, immune response at baseline and response to several therapeutic modalities are pronounced; yet in preclinical research they are largely disregarded. **Material and methods:** Sex differences in melanoma biology, patient response to treatment and survival will be overviewed. Preclinical drug repurposing study from our group with melanoma patient material and *in vivo* study in mice will be presented as an example how considering patient and animal sex as a variables leads to novel discoveries. **Results and conclusions:** We found that druggable target nischarin had opposite prognostic role in male and female melanoma patients. Differences stemmed from the differentially activated signaling pathways and B cell infiltrate distribution in tumors of male and female patients. Ultimately, *in vivo* response to nischarin agonist differed in male and female melanoma bearing mice. A patient's sex may affect the biology of non-sex related cancers significantly

and impact both prognosis and response to treatment. Men and women should be considered as biologically distinct groups of patients for whom specific treatment options should be tailored. The influence of sex should always be considered in preclinical study design.
Keywords: biological sex, melanoma, preclinical research

L10

The importance of adequate molecular diagnostics in the era of precision oncology – focus on lung cancer

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By definition, precision oncology entails delivering the right cancer treatment to the right patient at the right time. More precisely, precision medicine aims to *deliver treatments targeted to the needs of individual patients on the basis of genetic, biomarker, phenotypic or psychosocial characteristics that distinguish a given patient from other patients with similar clinical presentations*, with the goal of improving patient outcomes. Precision oncology is not a new concept, its beginnings can be traced back to the 1970s with tamoxifen as the first precision treatment being used with much success for hormone-receptor positive breast cancer. It was the development of technology in the form of complex and comprehensive molecular and genetic diagnostic modalities however, that enabled it's use to its full potential in diagnostics and therapy of malignant disease. Beginning with the discovery of the Philadelphia chromosome positive chronic myeloid leukaemia and the use of imatinib-mesilate in its treatment, multiple molecular targets have been discovered and multiple targeted agents approved for therapy of various cancers. However, this promise of precision oncology is also its challenge because of the number of steps that need to be taken in a precise and correct way in order to implement it in clinical practice. That has led to the invention of a Molecular Tumour Board as a derivative of the well-established Multidisciplinary Tumour Board. A Molecular Tumour Board is not only multidisciplinary in nature, but also multiprofessional, requiring the collaboration of physicians, molecular biologists, geneticists, bioinformatics experts, ethicists and many more, all in order to interpret the multitude of data obtained by comprehensive genomic testing and enabling the most appropriate treatment for each individual patient.
Why is lung cancer the paradigm of precision oncology? First of all, its incidence and mortality make it still one of the most important public health problems worldwide. Up until 20-30 years ago, the outcomes for these patients were hugely unsatisfactory, with 5-year survival rates less than 10% in metastatic disease. The first major breakthrough came in 2004 with the discovery of driver mutations in the gene for the epidermal growth factor receptor (EGFR) and the first approved targeted therapy with EGFR tyrosine-kinase inhibitor (TKI) gefitinib. This therapy provided a huge improvement of patient outcomes, with response rates of up to 80% and progression-free survival of 10-12 months, and overall survival of about 24 months in metastatic lung adenocarcinoma, which was until that time, an unheard outcome. Since then, many other driver mutations have been identified in non-small cell lung cancer (NSCLC) such as ALK translocations, ROS1, KRAS, BRAF, RET, which in turn has led to over 30 drugs being approved for treatment, and this list is constantly growing. Current guidelines of all major oncology societies list as many as 10 genetic alterations which need to be tested prior to starting treatment. This translates to almost 50% of all NSCLC patients who can be treated using precision oncology, and these are patients whose overall survival has been extended to an incredible time period of over 5 years in metastatic disease. It is recommended that molecular testing should be reflex at diagnosis, i.e. done at once using a comprehensive genomic testing platform such as Next Generation Sequencing (NGS), since tumour sampling can be a major issue in lung cancer treatment. Very often, due to the poor general condition of lung cancer patients, biopsy cannot be repeated and tumour samples are scarce, so being frugal with molecular testing is a must. This is where other type of samples play an important role. Plasma, pleural effusion, cerebrospinal fluid, even urine are an excellent source of tumour DNA, and can be used for molecular testing.
Precision oncology also has an important role in the sequential and real-time monitoring of patients and responses. Treatment with targeted therapy in NSCLC eventually and inevitably leads to the development of resistance through various mechanisms. Identifying the driving mechanism of resistance can lead again to the prescribing of adequate targeted treatment if available. In EGFR-driven disease, the first resistance mechanism discovered is the development of T790M mutation, which makes the disease resistant to first and second generation EGFR TKIs. Since then, many, some simple, some very complex, mechanisms of resistance have been discovered, many of which can only be discovered using NGS. This therefore emphasises the need for ensuring availability of not only adequate molecular diagnostic tools, but also expert interpretation of obtained data in the clinical concept, and of course, availability of appropriate drugs. All this has lead to the formation of the Precision Medicine Working Group by the European Society of Medical Oncology (ESMO), whose task is to give guidance for the appropriate use of precision oncology tools in everyday clinical practice. In their position paper on the use of NGS, it is stated that multigene NGS panels should be used to diagnose

level I alterations in all patients presenting with advanced non-squamous NSCLC.
It has been shown in several clinical studies, that if patients whose lung cancers harbour driver mutation are not treated with adequate target therapy, their outcomes are worse than patients whose tumours do not harbour driver mutations. It is, therefore, necessary to utilise all molecular diagnostic tools of precision oncology at our disposal to diagnose, monitor and follow all our patients with NSCLC, in order to ensure the best possible outcomes.
Keywords: Precision oncology, Molecular testing, Non-small cell lung cancer, Targeted therapy

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L11

High-throughput screening of multidrug-resistance markers in non-small cell lung carcinoma patient-derived cells – contribution to personalized treatment

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Introduction: Cancer remains one of the leading causes of death globally, despite significant advancements in cancer treatment over the past decades. A major challenge in cancer therapy is multidrug resistance (MDR), which is responsible for over 90% of deaths in cancer patients receiving both traditional chemotherapeutics and novel targeted

drugs. MDR arises from various mechanisms, including elevated metabolism of foreign substances (xenobiotics), enhanced drug efflux from cells, increased DNA repair capacity, and genetic factors such as gene mutations, amplifications, and epigenetic alterations. It is categorized into two types: primary resistance, which exists before initiating therapy, and acquired resistance, which develops after the initial treatment. The incidence of primary resistance to cancer treatment can be remarkably high (up to 60%) in certain cancer types. Furthermore, the majority of cancer patients are likely to develop resistance at some point during treatment. Although, the various underlying mechanism for drug resistance development in tumors have been highlighted in the past years, enhanced drug efflux, caused by increased expression of ATP-binding cassette (ABC) membrane transporters, is one of the major contributors to MDR. Among the known ABC transporters, three members, P-glycoprotein (P-gp, encoded by the MDR1 gene), Multidrug Resistance-Associated Protein 1 (MRP1), and Breast Cancer Resistance Protein – BCRP or Placenta ABC Protein – ABC-P), have been linked to chemoresistance to various drugs. P-gp and BCRP regulate various chemical compounds' distribution, absorption, and excretion. However, their overexpression can interfere with drug administration, reducing drug bioavailability and intracellular concentration. There is a significant correlation between increased expression of P-gp in cancer cells and enhanced resistance to drugs like paclitaxel, etoposide, DOX, and vinblastine. Overexpression of P-gp has been observed in approximately 50% of all human cancers. While in some tumor types, such as lung, liver, kidney, rectum, and colon, increased P-gp expression has been observed before chemotherapy treatment, in others, such as acute lymphoblastic leukemia and acute myeloid leukemia, it has been noticed after exposure to anticancer agents. Overexpression of P-gp and BCRP has been associated with poor clinical response and MDR in patients. Therefore, the pharmacological inhibition of the efflux function of these transporters was pursued as a strategy to overcome resistance to anticancer drugs in the clinic. However, despite showing high efficacy in preclinical studies, none of the P-gp inhibitors have been approved yet by the U.S. Food and Drug Administration (FDA) for clinical use in cancer treatment. Taking into account all the above-mentioned it is clear that screening and assessment of MDR markers in patient's cancer cells could play an important role in personalized treatment approaches. Expressing MDR markers in cancer cells could predict a patient's response to specific drugs or drug classes, allowing the selection of the most effective treatment regimen and avoiding using drugs that are likely ineffective due to resistance. Moreover, the presence of MDR markers associated with resistance to multiple drugs could guide the design of personalized treatment regimens with a combination of drugs that have a higher chance of overcoming the patient's specific drug resistance profile. Monitoring the expression level of MDR markers during the course of treatment could provide valuable insights into the development of drug resistance, and would allow healthcare professionals to adjust the treatment plan if drug resistance emerges, ensuring that the patient receives the most effective therapy. Our team established a promising method for high-throughput screening for MDR markers in non-small cell lung carcinoma (NSCLC) patient-derived cells, which implies pharmacological screening and an ex vivo experimental setting. It enables gaining valuable insights into patient characteristics and drug responses that may not be apparent through conventional sequencing or clinical trials. This strategy has the potential to improve personalized cancer treatment approaches, offering patients more effective and tailored therapies based on their individual characteristics and drug responses. **Methodology: Patient-derived NSCLC cell cultures.** Samples from NSCLC patients are collected from the Thoracic Surgery Clinic at the Clinical Center of Serbia. The histological grade is determined by histopathological analysis of the surgical specimen. Collected NSCLC samples are used to establish patient-derived NSCLC cell cultures comprising cancer and stromal cells (mainly fibroblasts). It is well known that the sensitivity of cancer cells depends on their interaction with the microenvironment including neighboring cells. The primary cultures obtained from the samples are grown for 1-2 weeks prior to drug testing. **Fluorescence immunoassay for high-throughput identification of cancers cells and MDR markers in NSCLC patient-derived cell cultures.** The fluorescence immunoassay utilizes antibodies against CK8 and CK18, which are expressed in nearly all carcinomas of epithelial origin, to identify epithelial cancer cells. Co-staining of CK8/18 with Hoechst 33342 allows the identification and quantification of two types of cells: CK8/18-negative (non-cancer cells) and CK8/18-positive (cancer cells). This immunoassay is also used to identify and quantify changes in the expression of MDR markers ABCB1, ABCC1, and ABCG2 both in cancer and non-cancer cells in primary NSCLC cultures that may occur during chemotherapy and tyrosine kinase inhibitors (TKIs) treatment. Co-staining of ABCB1, ABCC1, and ABCG2 with CK8/18 and Hoechst 33342 enables the identification of four types of cells in NSCLC primary cell cultures: drug-sensitive non-cancer cells, MDR non-cancer cells, drug-sensitive cancer cells, and MDR cancer cells. For validation of the immunoassay patient-derived cells are seeded in 384 well-plates and treated with 5 different concentrations of 8 chemotherapeutics known to induce overexpression of MDR markers (cisplatin, carboplatin, paclitaxel, docetaxel, gemcitabine, vinorelbine, etoposide, and pemetrexed), allowing the ex vivo evaluation of NSCLC MDR profile. Validated immunoassay is further used to evaluate the expression of MDR markers ABCB1, ABCC1, and ABCG2 (MDR profile) in patient-derived cell cultures after treatment with a panel of 10 TKIs (erlotinib, gefitinib, afatinib, osimertinibcrizotinib, alectinib, ceritinib, nintedanib, dabrafenib, and trametinib), allowing the evaluation of MDR profile in both cancer and stromal cells. The sensitivity of cancer and stromal cells for each individual NSCLC patient to a particular TKI is assessed using a discriminative immunoassay employing CK8/18 antibodies cocktail. **Whole Exome Sequencing (WES)**. Paired patient samples (normal and tumor)

were subjected to a DNA isolation procedure using Qiagen genomic DNA extraction kit, recommended for NGS applications. Isolated DNA samples underwent WES analyses by Novogene Company. Bioinformatics and statistics tools were employed to define clinically relevant gene alterations in MDR markers ABCB1, ABCC1, and ABCG2. **Results:** In order to understand how NSCLC patient cells respond to chemotherapy and targeted therapy, ex vivo testing was performed. The maximum concentration of drugs in human plasma that the patient is exposed to during therapy (Cmax) was used as an upper limit for drug concentration during testing, with four lower concentrations also used. The results showed that patient-derived cells display individual differences in sensitivity to both chemo and targeted therapeutics. IC50 values, which indicate sensitivity, fell within the concentration range for most chemotherapeutics. Only some chemotherapeutics (cisplatin, etoposide, docetaxel, gemcitabine, and pemetrexed) showed selectivity towards cancer cells with lower IC50 values in cancer than in stromal cells. Among TKIs, only erlotinib was efficient with IC50 below Cmax, showing selectivity towards cancer cells in all investigated patient-derived cell cultures. A number of chemotherapeutics increased the expression of ABCB1, ABCC1, and ABCG2, while TKIs afatinib, alectinib, ceritinib, osimertinib, and trametinib did not affect these transporters. Some TKIs increased the expression of ABC transporters, with nintedanib showing the potential to select cancer cells with higher MDR marker expression. WES showed significant ABCC1 gene instability, while ABCB1 had many SNPs with clinical relevance for drug response. ABCG2 had the lowest number of SNPs, but intron deletions were still identified. However, the clinical significance of these changes is currently unknown. **Conclusion:** Screening for multidrug-resistance markers through a high-throughput process provides valuable information about how a patient will respond to therapy. This process can identify if the MDR phenotype is already present or if it can be induced with targeted or chemotherapy. Based on this information, it can provide recommendations for a patient's mono- and combined therapy. This methodology has the potential to greatly impact cancer treatment strategies and improve patient outcomes by tailoring therapies to individual patient profiles. Ultimately, this will benefit a wider range of patients with non-small cell lung carcinoma and other cancers, as it leads to more precise and targeted treatment selections.

Keywords: Multidrug-Resistance Markers, NSCLC, Patient-Derived Cell Cultures, Personalized Therapy,

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L12

Circulating cytokines as potential biomarkers of disease progression in BRAFwt metastatic melanoma patients receiving anti-PD-1 therapy

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Background: Melanoma represents the most lethal type of skin tumor with a 5-year survival rate of 29% in metastatic melanoma (MM) patients. The clinical application of immune checkpoint inhibitors (ICIs), monoclonal antibodies that block inhibitory molecules primarily CTLA-4 (Cytotoxic T-lymphocyte Antigen-4), PD-1 (Programmed Cell Death-1), PD-L1 (Programmed Cell Death Ligand-1), has dramatically improved the outcome of MM patients by increasing their antitumor immune response. Despite efficacy of ICIs, clinical outcomes remain highly variable and over 50% of treated patients do not obtain any clinical benefit. Therefore, predictive biomarkers for response to this therapy still represent an important clinical need. However, none of the investigated parameters has demonstrated a clinically-useful role in distinguishing between responder and non-responder patients. Current biomarkers, primarily PD-L1 expression, are mainly measured in tumor tissue and their clinical use is still limited. Therefore, the analysis of circulating biomarkers obtained from peripheral blood may represent an alternative solution[1]. The measurement of cytokines in plasma and serum is minimally invasive and repeatable and it gives the opportunity for the analysis of the systemic immunity of the patients before and during the treatment[2]. Cytokines are small soluble proteins that are secreted by activated immune or tumor cells and they have the role in the regulation of tumor development and progression, and in the regulation and modulation of host immune response. Contrary to interferon-gamma (IFN-γ), a cytokine that increases tumor immunogenicity and enhances the cytotoxic function of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), transforming growth factor beta (TGF-β), interleukin (IL)-6, IL-8 and IL-10 inhibit anti-tumor immune response by recruitment and activation of various immunosuppressive cells in tumor microenvironment (TME)[3]. Even if the role of these cytokines in the regulation of the immune response is well known, their implication as potential biomarkers of response to ICIs is still controversial. In this study, we analyzed the levels of TGF-β, IFN-γ, IL-6, IL-8 and IL-10 in serum and plasma of BRAF wild type (wt) MM patients prior to anti-PD-1 therapy, Pembrolizumab to determine whether these cytokines could be biomarkers of disease progression (DP). **Patients and Methods:** This study involved 32 BRAFwt MM patients (stage IV according to 8th modified American Joint Committee on Cancer staging system)[4] of the Institute of Oncology and Radiology of Serbia (IORS) treated only with Pembrolizumab and 30 healthy controls (HCs). The patients and HCs were age and sex matched with no evidence of infection, autoimmune or any other disease. The study was approved by the Ethical Committee of IORS and all investigated subjects signed the informed consent before inclusion in the study. In patients, the tumor assessment was performed at baseline and every 12 weeks of therapy and clinical response was classified according to immune-response evaluation criteria in solid tumors (iRECIST)[5]. Therefore, patients were divided into two groups: 14 patients were with disease control, i.e. without DP (Non-DP) (patients who achieved complete response (CR), partial response (PR), or stable disease (SD)) and 18 patients achieved DP. Based on the localization of distant metastases, patients were divided into 3 groups. Patients that had metastases in distant skin, the subcutaneous layer or in distant lymph nodes (M1a) and patients with metastases in the lungs (M1b) were included in M1a+M1b group, while the patients with distant metastases in non-central nervous system (CNS) viscera were included in M1c group. The patients in group M1d metastasized to the CNS. Also, according to the serum values of enzyme lactate dehydrogenase (LDH) the patients were divided in the two groups: LDH- group included patients with normal LDH serum values (230-460 IU/L), whereas LDH+ patients had elevated LDH (≥460 IU/L). Pretherapeutic LDH values were obtained from the patients' database. Peripheral blood samples obtained from patients were collected prior to the first administration of therapy. The concentrations of TGF-β, IFN-γ, IL-8 and IL-10 in sera and IL-6 in plasma of MM patients and HCs were determined by commercial uncoated ELISA kits (Invitrogen, Massachusetts, United States), according to manufacturer's instructions. For comparison of patients and HCs, disease and treatment characteristics among different groups of MM patients, Mann-Whitney exact test, the Kruskal-Wallis, Wilcoxon rank sum, Pearson chisquare and Fisher exact tests were used. The Spearman's rank correlation was used for linear correlation investigation. The statistical significance level was set at $p \leq 0.05$. The Receiver Operating Characteristics curve (ROC) methods were applied to investigate these cytokines' discriminative potential for DP. **Results:** Pretherapy values of the investigated cytokines in MM patients were compared to age-matched HCs. The obtained data showed significantly higher baseline levels of TGF-β, IFN-γ, IL-6, and IL-10 in MM patients but similar level of IL-8 ($p \leq 0.05$, $p < 0.01$, Mann–Whitney exact test). Furthermore, the analysis of the differences in the values of investigated cytokines

between MM patients with different localisation of distant metastases has shown that patients in M1d group had higher values of plasma IL-6 compared to M1a + M1b group of patients ($p \leq 0.05$, Kruskal Wallis test). We have also shown that LDH+ patients had statistically significant higher values of IL-6, as well as IL-8 compared to LDH- patients ($p \leq 0.05$, $p < 0.01$, Wilcoxon rank sum test). Also, we have shown in MM patients the statistically significant positive correlation between values of LDH and IL-6 ($\rho=0.3716$), as well as LDH and IL-8 values ($\rho=0.4047$), and also between values of IL-6 and IL-8 ($\rho=0.5567$) ($p \leq 0.05$, Spearman's rank correlation). Furthermore, regarding their response to Pembrolizumab, investigated MM patients were divided in two groups: Non-DP and DP patients. By analysing the cytokine values between these two groups of patients, we have shown that DP patients had significantly higher values of IL-6 ($p<0.01$, Wilcoxon rank sum test). By applying ROC analysis, we confirmed the discriminative potential of IL-6 for the occurrence of DP in investigated patients with the new ROC cut-off value of 3.021302 pg/ml. However, the analysis for the other cytokines did not show significant discriminative potential for the occurrence of DP. Furthermore, we examined if there was any difference in various parameters between subgroups of patients based on ROC cut-off value for IL-6. This analysis revealed that the two groups of patients did not significantly differ in gender and age. However, the patients with IL-6 level above the cut-off were predominantly composed of patients with elevated LDH and metastasis in CNS (M1d group) and also these patients received fewer therapy cycles, had significantly lower progression-free survival (PFS) and showed higher pretherapy level of IL-8 in sera. There were no differences between these two groups of patients when TGF-β, IFN-γ and IL-10 were evaluated ($p>0.05$, Wilcoxon rank sum test). **Discussion:** In this work we aim to identify cytokines as new biomarkers of DP to Pembrolizumab in BRAFwt MM patients. We show that IL-6 is significantly higher in patients with DP compared to non-DP patients. Contrary to these results, TGF-β, IFN-γ, IL-8 and IL-10 are similar between investigated groups of patients. IL-6 is a key pleiotropic immunomodulatory cytokine secreted by both normal and tumor cells. By supporting a chronic aberrant inflammation in TME it induces immunosuppression not only by accumulation of immunosuppressive myeloid cells, myeloid derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), but also by the prevention of apoptosis of these cells, that directly or indirectly inhibit antitumor lymphocytes, NK cells and CTLs[3]. Since the onset of application of ICIs, several studies have investigated the association between baseline IL-6 level and response to therapy. In this sense, elevated pre-treatment IL-6 levels have been associated with reduced ICI response rate in MM patients[6], as well as in non-small-cell lung cancer patients[7]. However, Yamazaki et al.[8]have shown that responders (CR/PR) treated with anti-PD-1 drug, Nivolumab had remarkably higher level of pretreatment IL-6 than non-responders in advanced BRAFwt, but also BRAF mutated MM patients. Overall, baseline IL-6 level is a strong prognostic marker of ICI therapy and the majority of findings suggest that increased level of IL-6 is an indicator of progression and poor prognosis of this treatment. By applying ROC analysis, we confirmed the discriminative potential of IL-6 for the occurence of DP in investigated patients with the new cut-off value of 3.021302 pg/ml. Wang et al.[9] suggested cut-off of 4 pg/ml for plasma IL-6 in melanoma patients treated with targeted therapy and/or ICIs. Furthermore, in our work patients with IL-6 equal or higher than 3.02 pg/ml had significantly lower PFS, fewer therapy cycles, as well as higher level of serum LDH and IL-8 compared to patients with low IL-6 levels. Tsukamoto et al.[10] have reported that MM patients treated with Nivolumab with on-/pretreatment IL-6 values equal or higher than 1.516 pg/ml exhibited a shorter PFS, whereas changes in LDH level were not associated with IL-6 level. Existing studies on the association of cytokine profile with patient response to anti-PD-1 therapy were mostly preformed on heterogeneous groups of melanoma patients that comprise patients treated with various PD-1 inhibitors, or even patients treated with anti-PD-1 and anti-CTLA-4 agents in combination. Our study was conducted on a homogeneous group of patients treated with Pembrolizumab, solely. In this study, baseline IL-6 was found to be a biomarker of DP and poor prognosis in BRAFwt MM patients treated with Pembrolizumab. Moreover, we proposed the new cut-off value of this cytokine and the possibility that higher values of baseline circulating IL-6 could help to estimate whether the patients are at high risk of DP. Therefore, the evidence available from this study implies that IL-6 could be a possible immunosuppressive target and that therapeutic blockade of IL-6 in combination with ICI may enhance survival in MM patients.

Keywords: BRAF wild type, Disease progression, IL-6, Metastatic melanoma patients, Pembrolizumab

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L13

Targeting chitinase 3-like 1 for the treatment of pancreatic cancer

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies worldwide, with a 5-year survival rate of approximately 12%[1]. Owing to the dense desmoplasticstroma, PDAC remains associated with an extremely poor prognosis, with drug resistance and the lack of alternative therapeutic strategies hampering the effectiveness of treatment[2-4]. The standard treatment in PDAC is still gemcitabine, which is administered alone or in combination with other chemotherapeutic agents, namely paclitaxel[5]. Another treatment option for PDAC patients is FOLFIRINOX, a combination regimen of 5-fluorouracil/leucovorin with irinotecan and oxaliplatin; however, its use remains limited due to its toxicological and adverse health effects[6]. Importantly, our previous work demonstrated the involvement of Chitinase 3-like 1 (CHI3L1) in PDAC cellular resistance to gemcitabine[7]. The Cancer Genome Atlas (TCGA) analysis revealed that high expression levels of CHI3L1 are associated with low overall survival of PDAC patients and with no improvement in patient's response to gemcitabine. Therefore, our work identified CHI3L1 as a promising therapeutic target for PDAC[7]. Furthermore, we found pentoxifylline, which is an antifibrotic drug clinically approved for other diseases, as an inhibitor of CHI3L1 able to counteract such resistance, suggesting a potential for this drug as an adjuvant therapy[4,7]. In agreement with our results, other studies have reported an association between high expression levels

of CHI3L1 and low survival, poor prognosis and advanced tumor stage in patients with pancreatic cancer[8-9]. Therefore, our work opened new perspectives on the possibility of exploring drugs that target CHI3L1 in combination with conventional chemotherapy, to overcome drug resistance and improve treatment protocols for PDAC patients [10]. In the present work we identified, by in silico molecular docking, putative CHI3L1 inhibitors. In addition, we assessed the chemosensitizing effect of one of the identified CHI3L1 inhibitors presenting good docking scores, on PDAC treatment. First, we performed the in silico analysis (AutoDockVina) on the CHI3L1 protein to identify potential CHI3L1 inhibitors, using 11,741 molecules from the DrugBank database. Our molecular docking screening revealed that 568 molecules presented higher affinity towards CHI3L1 than a well-known ligand, chitotetraose. A score value of -11.8 kcal/mol was obtained for darifenacin, a muscarinic receptor antagonist, suggesting the ability of this drug to target CHI3L1 with high efficiency. Thus, further studies were conducted to validate the potential of darifenacin in PDAC treatment. For that, using 2 PDAC cell lines (BxPC-3 and PANC-1), and also primary PDAC cells obtained from a resected patient, we evaluated the effects of darifenacin (alone or in combination with gemcitabine, or with gemcitabine plus paclitaxel) on the % of cell growth, by the sulforhodamine B assay (SRB). The cytotoxic effect of darifenacin against normal immortalized pancreatic ductal cells (HPNE) was also assessed. Moreover, recombinant CHI3L1 protein was used to confirm the impact of darifenacin on CHI3L1-induced PDAC cellular resistance to therapy (by the SRB assay). The effect of darifenacin on Akt activation was also analysed, by ELISA. Finally, the association between the expression levels of a known molecular target of darifenacin, cholinergic receptor muscarinic 3 (CHRM3), and therapeutic response was evaluated by immunohistochemistry of paraffin-embedded tissues from surgical resections of a 68 patients' cohort. Our work demonstrated that darifenacin inhibited 50% of the growth of BxPC-3 and PANC-1 PDAC cell lines, at 26 µM and 13.6 µM respectively. A similar effect was observed in primary PDAC cells (with a GI50 of 30 µM). Interestingly, at concentrations above 50 µM, darifenacin did not cause significant cytotoxic effect against the normal HPNE cells. Importantly, darifenacin sensitized human PDAC cells to standard chemotherapies, such as gemcitabine and gemcitabine plus paclitaxel. Using molecular approaches, we verified that darifenacin reverted CHI3L1-induced PDAC cellular resistance to therapy, and decreased Akt phosphorylation. Furthermore, an association between high CHMR3 expression and reduced therapeutic response to gemcitabine was observed in a cohort of 68 PDAC patients, further suggesting the potential of darifenacin for PDAC treatment. Overall, this work expects to propose novel therapeutic strategies combining CHI3L1 pharmacological inhibitors with conventional chemotherapy, to overcome PDAC drug resistance. Notably, our work highlights the potential of using darifenacin as a novel chemosensitizer for PDAC treatment. Future work will explore other CHI3L1 inhibitors identified *in silico*.

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L14

Establishment of a first cancer Biobank at the Institute for Oncology and Radiology of Serbia – advantages, challenges and future perspectives

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Background: Procurement and storage of different biospecimens from cancer patients has been a standard practice over decades at the Institute for Oncology and Radiology of Serbia (IORS). However, such organized repositories were exclusively project-based. The type and amount of collected samples was defined by research needs, or by limited capacity of the storage units, while collecting of biospecimens relied on hands-on involvement of researchers and medical professionals. Although necessary ethical guidelines were respected and the whole process was well controlled by individual researchers, there were several disadvantages that needed to be addressed and resolved. Information on the stored samples was not collected systematically and tracking was difficult. Most biospecimen were disposed after project end due to storage space shortage rendering the previous collection efforts non-economic. Within the framework of the Horizon Europe STEPUPIORS (101079217) project, the goal was to solve the identified drawbacks of project-based repositories and to create a highly organized and well controlled biobanking system. The biobank was envisioned to function in accordance with internationally accepted technical, scientific and logistical standards and guidelines while respecting the national legislative, with the aim to contribute to elucidation of pathophysiology, diagnosis, and treatment of cancer. Organized biobanks provide assurance that patients' biospecimens and derived data are collected, stored, and managed in a way that enables optimal sharing and usage with minimal risk of misuse. Here we present the road towards the establishment of a proper procedural workflow for setting up a first rectal cancer biobank at IORS, and the identified advantages, challenges and future perspectives. **Material and Methods:** Future biobanking personnel attended intensive online and in person training and performed expert visits to each partner institution with insight into biobanking practices. Biobanking procedures were developed according to recommendations of the International Society of Biological and Environmental Repositories (ISBER), the Biobanking and BioMolecular resource Research Infrastructure (BBMRI), European Research Infrastructure Consortium (ERIC), and EU regulations followed by partner institutions. A rigorous evaluation of ethical and legal regulations was performed, respecting national and European legislation. Essential biobank equipment and laboratory information software were procured to ensure maximum accordance with infrastructural, storage and data protection requirements. Decisions were made during regular weekly meeting of IORS biobanking staff and consensus consortium approvals reached on all aspects. Management and internal and external oversight committees were formed of members of all four partner institutions. **Results:** Fifteen professionals of different disciplines (5 physicians, 3 biochemists, 4 molecular biologists, 3 pharmacists),

all IORS employees, have been gathered sharing a mutual intention to define a sequence of action necessary for the process of setting up a biobank. Within the first year, the STEPUPIORS team has undertaken landmark steps to improve both our knowledge and technical aspects as those involved to establish high-quality criteria for routine biobanking practice. At the very beginning, we were pursuing ways to reconcile some diversities in legal regulations between Serbia and the European Union, which is of crucial importance for future biospecimens and data exchange. Two expert visits of IORS professionals to STEPUPIORS partner biobanks provided on site education regarding different management strategies, funding models, and biosafety requirements that are mandatory for running a biobank. Attending a Basic biobanking course at the Medical University of Graz notably expanded our understanding of minimal requirements for building a biobank, sample handling and logistics, biobank sustainability and risk assessment. We implemented the acquired knowledge to develop a first set of 13 Standard operating procedure (SOP) protocols with appropriate annexes covering patient recruitment, documentation and biospecimens management. We evaluated different models of biobanking Informed consent documents and adapted ours to serve both the patients and the medical community, while respecting all bioethical regulations and data protection legislative. We performed a thorough market and quality analysis of available biobanking laboratory information systems to be implemented which would best support data collection, analysis and management while providing strong security and protection of patients' privacy. We further defined equipment and space requirements to maintain a safe environment for the biospecimen. Special attention has been dedicated to potential biosafety hazards and measures that should be implemented to minimize potential risks while handling biohazardous materials. In August 2023, biobanking software procurement was finalized and the biobank is planned to be fully operational in October 2023. A procedural basis for the collection of a planned project cohort of around 100 locally advanced rectal cancer (LARC) patients was successfully introduced. Scientific and management oversight committees including members of partner institutions were formed from the initiation of the biobank to ensure high-quality biobank-related research and innovation that will advance the treatment of LARC patients. Although the biobank has primarily been established within the framework of the STEPUPIORS project as a project deliverable, the collected rectal cancer cohort is planned to be used for a broad spectrum of future research projects. The developed SOPs and IT infrastructure will allow further advancement in cancer research at our Institute, as it has been envisioned to be expanded to other cancer types and the organized collection of diagnostic samples. The first IORS biobank might be useful as a pilot project for other biobanks planned to be formed in Serbia in the future. **Conclusion:** Building a biobank is a challenging project even in countries with appropriate scientific and health-related funds. Aside from legislative issues and the need for improvement of current infrastructure and performance, it requires a highly dedicated and well-orchestrated team of professionals with on-call duty to ensure maximum safety. Within the framework of the STEPUPIORS Horizon Europe project, the first rectal cancer Biobank has been successfully established at the Institute for Oncology and Radiology of Serbia and is expected to become fully functional by the end of 2023. Plans for its future expanding to accommodate other cancer subtypes are under way.

Keywords: Biobank, bioethics, data protection, rectal cancer

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L15

Advancing reversible immunocapture toward scalable purification of extracellular vesicles

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Background: Extracellular vesicles (EVs) are supramolecular structures secreted by majority of cells[1]. Based on their mechanism of release and size, EVs are categorized as exosomes (EXO), microvesicles/shedding particles, and apoptotic bodies. Their content differs according to the characteristics of the cells from which they originated[2]. EVs are involved in a myriad of physological and pathological proceses and correct elucidation of their role in these processes is highly dependent on the reliability of the method used for their purification[3]. Currently available chemical/physical protocols for sample fractionation are time-consuming, often scarcely reproducible and their yields are low. Immune-capture based approaches could represent an effective purification alternative to obtain homogeneous EV samples[4]. **Methodology:** We have set-up an easy-to-operate chromatography system for the purification of intact EVs based on a single domain (VHH) antibodies-copolymer matrix suitable for biological samples as different as conditioned

cell culture medium, human plasma (healthy volunteers and patients with prostate cancer) and urine. Methacrylate based copolymer is a porous solid support the chemical versatility of which enables its efficient functionalization with VHHs. Isolated EVS have been characterized using biochemical and physicochemical methods. **Results:** The system allowed isolation of EVs in a single step chromatographic approach, using diluted starting material. Combined analyses of morphological features and biomarker (CD9, CD63 and CD81) presence indicated that the recovered EVs were exosomes. The lipoprotein markers APO-A1 and APO-B were both negative in tested samples. The system was applied to pathology of prostate cancer where it demonstrated the potential to isolate biomarker-class EVs[5]. We are the first report demonstrating the successful application of spherical porous methacrylate based copolymer coupled with VHHs for the exosome isolation from biological fluids. This inexpensive immunoaffinity method has the potential to be applied for the isolation of EVs belonging to different morphological and physiological classes.
Keywords: Exosomes, Single-Domain Antibodies, Biomarkers

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L16

Dying of cancer cells feeds the others to create more aggressive tumor

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Cancer is a deadly disease where the cancer cells are resistant to cell death. Normally, billions of body cells die every day just as a physiological cell death, in other words, programmed cell death / apoptosis. However, cancer cells are programmed to be immortal with some mechanisms. Thereby, one of the hallmarks of cancer is to be unable to die. Interestingly, if the cancer cells are killed by a chemotherapeutic drug, then neighboring cells become more proliferative and maybe even more malignant. First observation comes from our group's initial study that was performed many years ago. Since then, this phenomenon has not taken a big attention. In our study, we discovered that if the patients basal (before chemotherapy) apoptotic rate is higher, then their overall survival gets worse than the others with normal or lower apoptotic rate. This finding reasons us that dying of cancer cells actually makes the others more aggressive. In this task, we discuss the reasons for this interesting phenomenon.

L17

The role of Hedgehog signaling pathway in plasticity, stemness and resistance of melanoma

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The Hedgehog signaling pathway is a signal transduction pathway crucial for embryonic development: it is involved in tissue patterning; it is very active in the neural crest and during limb differentiation and contributes to patterning of many organs. In the adult organism, the pathway is quiescent in differentiated cells, and active in somatic stem cells, where it is responsible for stem cell maintenance and quickly activated during tissue repair after injury. The pathway is activated by binding of the Hedgehog (HH) ligand (either Sonic HH, Indian HH of Desert HH, depending on the tissue) to the transmembrane receptor Patched (PTCH1), which then dimerizes and acts as a channel for an unidentified molecule, which then releases its inhibitory effect on the protein Smoothened (SMO). The most likely candidate for this molecule is cholesterol, although the exact mechanism of this interaction is still being investigated. SMO then translocates from the intracellular membranes to the tip of the primary cilia, where it accumulates and triggers the activation of the GLI transcription factors (GLI1, GLI2 and GLI3). These transcription factors are activated by their release from the protein Suppressor of Fused (SUFU) by phosphorylation events involving protein kinase A (PKA) and gylcogen synthase 3 kinase beta (GSK3B). GLI proteins then translocate to the nucleus and trigger expression of genes involved in proliferation, cell cycle progression and activation of other signaling pathways. The three GLI proteins differ in their structure, with GLI1 containing only the transactivation domain, while GLI2 and GLI3 additionally contain a repressor domain. In the absence of the HH signal, GLI2 and GLI3 proteins are phosphorylated, ubiquitinated and cleaved, resulting in the truncated repressor form of the proteins (GLI2R and GLI3R) which contains only the N-terminal repressor domain while the C-terminal transactivation domain is cleaved off. This cleavage has not been reported for GLI1, so GLI1 is considered to be the activator of the pathway, while GLI2 and GLI3 can be in either activator or repressor form depending on the cell context. This interrelation between the three GLI proteins is called "the GLI code", and it is still not completely elucidated. It depends on a variety of internal and external signals in the cell which can activate the Hedgehog pathway non-canonically, resulting in GLI activation regardless of the presence of the ligand. The Hedgehog-GLI signaling pathway is often activated in many tumor types, where it contributes to their proliferative properties and stemness. Activation of the pathway can be ligand-independent or ligand-dependent. The ligand-independent activation is usually the result of mutations in the pathway components: inactivating mutations in PTCH1 or activating mutations in SMO can render the signal cascade constitutively active, and the binding of the HH ligand does not affect its activity. Ligand-dependent activation, as the name suggests, relies on the binding of the HH ligand to the receptor and activation of the signaling cascade. Based on the source of the ligand we can differentiate three types of ligand-dependent activation: (1) autocrine signaling, where the cell overproduces the HH ligand and receives it, thus amplifying the signaling cascade, (2) paracrine signaling, where the tumor cell produces the ligand, the stromal cell receives the signal, and in response produces growth factors and proteins that generate a favorable microenvironment for the tumor cell, or (3) the reverse paracrine cascade, where stromal cells produce the HH ligands, and the tumor cells receive it and activate the signaling cascade, resulting in transcription of genes associated with cell cycle progression, proliferation, survival and invasion. Additionally, the Hedgehog-GLI signaling pathway can also be activated non-canonically through other signaling pathways. In non-canonical activation, GLI transcription factors can be regulated by signals from other pathway, such as the MAPK pathway, and trigger transcription of target genes independently of the membrane components of the pathway. Several inhibitors of the Hedgehog-GLI signaling pathway have been developed, and some are even used in a clinical setting for treatment of basal cell carcinoma of the skin, such as vismodegib and sonidegib. Basal cell carcinomas often contain mutations in *PTCH1* or *SMO* genes, and therefore inhibitors targeting the SMO protein can effectively block the downstream signaling cascade and bring benefit to the patients. However, for most other solid tumors inhibition at the membrane level is not as effective, most likely due to the non-canonical contribution to GLI activation. Inhibitors targeting GLI proteins, such as GANT61, are available in a research setting, where they show very good results, but due to their poor bioavailability are not suitable for clinical use. Several research groups are trying to develop and test other GLI inhibitors, and this is a promising future research direction. We decided to investigate the GLI code in melanoma, as the Hedgehog signaling pathway has been associated with this disease. It is known that melanoma cells originate from the neural crest, which is regulated by Hedgehog expression during embryogenesis. It is also known that skin stem cells express Hedgehog signaling components, and that this pathway is activated after injury and during wound healing in the skin. Hedgehog signaling activity is required for melanocyte proliferation in vitro, and inhibition of Hedgehog signaling prevents development of melanomaspheres in vitro and melanoma xenografts in mice. This is especially true for RAS-driven melanoma. GLI1 and GLI2 overexpression has been associated with metastatic melanoma, worse survival in patients and resistance to therapy. The expression of GLI3,

however, has not been investigated at all in the context of melanoma. GLI3 has long been considered the repressor of the pathway, and its role in general has been poorly investigated. However, recent evidence suggests that it can also be detected in its full-length active form and act as a transcriptional activator and respond to Hedgehog pathway inhibitors. Therefore, our strategy was to perform RNA-seq on three melanoma cell lines overexpressing GLI1, GLI2 or GLI3 in order to identify unique and overlapping targets of these three transcription factors. The cell lines chosen for the screening are representative of the three most common mutational backgrounds in melanoma: (1) *BRAF*-mutated, (2) *NRAS*-mutated, and (3) wild type for both of these genes. We identified 814 differentially expressed genes (DEG) in the GLI1-transfected cells, 941 DEG in the GLI2-transfected cells, and 58 DEG for the GLI3-transfected cells. Gene set enrichment analysis revealed that GLI1 and GLI2 had highly overlapping targets, associated with signaling pathways WNT, MAPK and HIPPO and with several cancers, including basal cell carcinoma and melanoma. Additionally, we performed ChIP-seq analysis on the same three melanoma cell lines to increase the stringency of the method and to identify direct transcriptional targets. The ChIP-seq analysis was done on the endogenous GLI proteins present in the cell lines, to avoid any possible effects of overexpression, which might lead to off-target binding of GLI proteins to their target genes. We identified 183 GLI1 target genes, 399 GLI2 target genes, and 131 GLI3 target genes, including mRNA (92%), miRNA (3%) and lncRNA (5%). The gene lists from both analyses were compared, and overlapping genes were identified: three for GLI1 and 10 for GLI2, while there was no overlapping genes for GLI3 protein. Gene lists were additionally analyzed with the GeneAnalytics software on the Gene Cards platform for their function, role in tumors and role in MAPK signaling pathway, reducing the number of genes of interest to 21 target gene candidates. These 21 target genes were additionally analyzed in GEPIA, The Human Protein Atlas and UCSC databases, and finally experimentally validated by qPCR on 11 melanoma cell lines (the original 3 cell lines plus 8 additional melanoma cell lines). Out of 21 selected targets, 15 were validated as novel targets of GLI proteins, considering their expression in melanoma cell lines and possession of GLI binding motifs in their promoter regions. Out of these 15, four targets with the most consistent expression patterns in all tested cell lines were chosen for functional analysis using other functional models: cell lines stably overexpressing SHH protein (model of pathway hyperactivation), cell lines resistant to Hedgehog pathway inhibitor GANT-61 (model of pathway downregulation), and 3D spheroid models. Our studies confirm that in melanoma, the HH-GLI signaling pathway is in crosstalk with other signaling pathways and that many of the identified target genes are members of other cancer-associated pathways, such as WNT, MAPK and HIPPO. Many of the identified targets were identified previously by other researchers, but often in different models and diseases, not necessarily melanoma. This includes *PTCH1* and *GLI1* genes, which are members of the Hedgehog pathway but also regulated by the pathway itself by a feedback autoregulatory loop. In our study they were identified by both RNA-seq and ChIP-seq methods, confirming the validity of both methods. The cross-talk with the MAPK signaling was also an expected finding, as other researchers have identified non-canonical activation of the Hedgehog pathway by the MAPK. MAPK pathway is the most often activated pathway in melanoma, with almost 50% of all melanoma harboring a mutation in the *BRAF* gene, and additional 15-20% in the *NRAS* gene, both of which are members of the MAPK signaling cascade. However, we have also identified that Hedgehog signaling affects the transcription of genes involved in the MAPK signaling pathway, so the cross-talk is two-directional. To investigate the cross-talk between Hedgehog and MAPK signaling pathways, we developed melanoma cell lines resistant to Hedgehog pathway inhibitor GANT-61. This inhibitor was the most effective out of all tested Hedgehog pathway inhibitors on a panel of 13 melanoma cell lines we have tested in our laboratory, and this corresponds to available literature data on the effectiveness of Hedgehog inhibitors on melanoma. This inhibitor acts directly on GLI proteins, blocking their transcriptional activity. By continuous propagation of cell lines in increasing concentrations of GANT-61, we have developed two resistant cell lines. In the resistant cell lines, a signaling switch between Hedgehog signaling and MAPK signaling occurs: the Hedgehog pathway components are downregulated, while MAPK is activated. This is reflected on the newly identified Hedgehog signaling target genes, which are also downregulated in this model. The resistant cells demonstrate the characteristics of epithelial-mesenchymal transition, such as changes in morphology (elongation of cell bodies and spindle-like phenotype), upregulation of vimentin (a mesenchymal marker) and downregulation of E-cadherin (an epithelial marker). They also demonstrate increased features of stemness, such as upregulation of stemness markers (OCT4, SOX2 and NANOG, depending on the cell line), and increase in markers of invasion such as matrix metalloproteinases (MMP2, MMP9). The cells demonstrate increased migratory properties by the scratch assay, and increased colony forming potential in vitro compared to their non-resistant counterparts. Interestingly, there was generally no cross-resistance on other Hedgehog pathway inhibitors that act at the membrane (cyclopamine, vismodegib, sonidegib) or cytoplasmatic components (lithium chloride) of the pathway, and only transient cross-resistance to another inhibitor acting at the level of GLI proteins, arsenic trioxide. There was also no cross-resistance to a RAS inhibitor salirasib, suggesting that other treatment options remain open in cells resistant to GANT-61.

Keywords: Hedgehog-GLI Signaling, GLI Proteins, MAPK, RNA-seq, ChIP-seq, Drug Resistance, Stemness

L18

What is new in care of adolescents and young adults, AYA with cancer

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Cancer in Adolescents and Young Adults (AYA) population is uncommon and it counts about 2-4% of all malignancies[1]. The incidence of cancer increases with age and consequently cancer is 2.7 times more frequent between the ages of 15 and 30 years, than in the under 15 age group. Recent data retrived from Global Cancer Observatory, crude and age-standardised (World Standard Population) incidence and mortality rates, have shown cancer burden in Europe. Incidence and mortality varied widely between countries with the highest mortality observed in Eastern EU countries. Cancers of the female breast, thyroid and male testis were the most common cancers across countries followed by melanoma of skin and cancers of the cervix. Variations in cancer incidence rates across different populations may reflect different distribution of risk factors, variations in the implementation or uptake of screening as well as overdiagnosis. AYA cancer mortality disparities may be due to variation in early-stage diagnoses, different public education and awareness of cancer symptoms, different degrees of access or availability of treatment[2].

The optimal AYA age range remains an elusive topic on which to agree. This age group is usually characterised as a subpopulation that is in transition between childhood and older adulthood. Therefore, perhaps it is inevitable it will vary by culture, geography, economics and time. It is generally accepted that the definition of childhood encompasses 0 to 14 years of age, definition of adolescence encompasses 15 to 19 years of age and agreement that adulthood starts at approximately 20 years of age, but lack of consensus still remains regarding the upper age limit of 'young adulthood', which has been inconsistently reported as 24, 35 and 39 years.

It is well documented that traditional health care models do not meet the specific needs of AYA and do not provide expert cancer care focussed on this age group. Subsequently, survival gains in AYA patient population have improved modestly compared with older adults and children with cancer[6]. Poorer outcomes in AYAs could also be attributed to the specific biology of the cancer. AYA patients also tend to be diagnosed at later stages and their access to clinical trials is lower than older patients, but information is limited as this population has not historically been a focus of cancer control and research.

The Cancer in AYA Working Group (WG) is a joint venture between European Society for Medical Oncologists (ESMO) and the European Society for Paediatric Oncology (SIOPE) created in 2016 with the aim to increase awareness amongst the medical and paediatric oncology communities and enhance knowledge on specific cancer issues in adolescents and young adults. The group advocate for an increase in research capacities in tumour types affecting these patients and cultivate collaborative relations between medical and paediatric oncologists, as well as with other healthcare professionals involved in AYA care.

In 2017 and 2021 the ESMO-SIOP AYA WG conducted a survey among health-care professionals, which characterised patterns of under-provision and inequity in AYA cancer care across Europe, urging priority training for health care workers along AYA cancer patients' journey, and focused research on AYA cancer care[3,4]. This surveys suggests the need to expand provision of excellent care for the particular needs of AYA patients not only among health professionals but also among health care authorities who are in charge for strategic planning, future national programs for AYA and curriculum regarding AYA cancer care. It also identifies geographical areas where deeper involvement and improvement is needed. This surveys should also encourage provision of specialised rehabilitation services, focused to improve AYA lives after cancer.

ESMO/SIOPE WG has published position paper in 2021, which reflects the position of this working group regarding current AYA cancer care, the challenges to be addressed and possible solutions[5]. Key challenges include the lack of specific biological understanding of AYA cancers, the lack of access to specialised centres with age-appropriate multidisciplinary care and the lack of available clinical trials with novel therapeutics. Key recommendations include diversifying interprofessional cooperation in AYA care and specific measures to improve trial accrual, including centralising care where that is the best means to achieve trial accrual. This defines a common vision that can lead to improved outcomes for AYA with cancer in Europe.

There is considerable opportunity for many international or national organisations to work together in raising the profile of AYA cancer related issues, in providing education, in encouraging research and collaboration.

Keywords: adolescents and young adults, cancer, interdisciplinary, clinical trials, education

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L19

Control of IFN-γ Responsiveness and Metastatic Potential in Melanoma by GSTA4

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Background: Interferon-gamma (IFNγ) is a crucial effector molecule of antitumor immunity. This cytokine promotes the excessive production of reactive oxygen species (ROS) in tumor cells, which leads to DNA damage and senescence [1]. Recently it has been discovered that IFNγ can also trigger cancer cell ferroptosis by fostering lipid peroxidation [2]. Cancers often evade antitumor immunity by losing their responsiveness to IFNγ. Consequently, IFNγ becomes a critical player in the immunoediting process, selecting tumor cells with immunoevasive properties [3]. Defects in responsiveness to IFNγ in cancer cells significantly contribute to the limited success of cancer immunotherapy in clinics [4], emphasizing the importance of understanding the mechanism behind the IFNγ-mediated immunoediting process. To address this issue, we investigated how tumor cells escape IFNγ-dependent immune response through immunoediting by analyzing originally established immune-escape variants of melanoma cells. **Material and Methods**: We used a previously established in vivo model in which antitumor immunity was IFNγ dependent [5]. Mouse B16 melanoma cells expressing ovalbumin as a tumor-specific antigen (B16OVA) were subcutaneously inoculated in OVA-immunized B6 mice. In this model, tumor growth suppression by host IFN-γ lasts for a limited time, after which all tumors progress. Next, we established cancer cell lines with different in vivo immunological experiences. Tumor cells were isolated from same-sized tumors from wild-type (WT) untreated mice (established cell lines were named "NIMM"), from WT OVA-immunized mice after the cessation of immune control of tumor growth (established cell lines were named "IMM"), or from IFNγ knockout (IFNγ KO) OVA-immunized mice (established cell lines were named "GKO-IMM"). IMM, NIMM, and GKO-IMM cells were re-challenged in OVA-immunized mice to test their ability to provoke antitumor immunity. Instead of immunization with OVA antigen, in some experiments, the anti-PD-1 antibody was administered intraperitoneally to initiate tumor-specific immunity in vivo. To examine changes in phenotype resulting from the IFNγ immunoediting process, total RNA was extracted from parental B16OVA cells and immune-escaped IMM cells. Gene expression was analyzed using a GeneChip system with GeneChip Mouse Gene 2.0 ST Array. mRNA and protein expression of selected genes was quantitatively determined by real-time PCR and western blotting, respectively. GSTA4 overexpression or knockdown was performed to determine its functional role in the immunoevasive phenotype of IMM cells. Cell sensitivity to IFNγ and 4–hydroxynonenal (4–HNE), a lipid peroxidation product, was estimated by WST–8 cell viability assay. CellROX Deep Red reagent was used to detect IFNγ-induced intracellular ROS accumulation. Transwell invasion assay was used to assess melanoma cells' in vitro metastatic potential. In the in vivo experimental lung metastasis model, cells were injected into the tail vein and metastasized tumor colonies on the surface of the lungs were counted. The correlation of GSTA4 expression in human melanoma patients with tumor-free survival rates, and response to anti–PD1 treatment

in correlation with GSTA4 expression and survival rates were obtained from publicly available databases. **Results:** Upon re-challenging into OVA-immunized mice, IMM cells showed unrestrained progression, while the growth of NIMM and GKO-IMM tumors was suppressed. In addition, only IMM cells specifically lost OVA antigen expression, indicating that these cells gained the ability to evade the OVA-specific antitumor immune response. In line with in vivo data, IFNγ treatment in vitro reduced the viability of parental B16OVA, NIMM, and GKO-IMM cells, while the viability of IMM cells was intact. Interestingly, IFNγ upregulated the expression of MHC class I (H-2Kd) and PD-L1in IMM cells, suggesting that these cells did not have the defect in IFNγ signaling. We found that the lack of IMM cell responsiveness to the IFNγ-induced cytostatic effect was due to the acquisition of resistance to the IFNγ-induced oxidative stress response. Gene expression analysis using DNA microarray revealed that the most upregulated gene in immunoevasive IMM cells was glutathione-S-transferase-4 (GSTA4). GSTA4 is a member of a family of detoxification enzymes that play an essential protective role in cellular oxidative stress responses [6]. GSTA4 overexpression in parental B16OVA cells reduced ROS production and increased their resistance to the IFNγ-induced cytostatic effect in vitro. Consequently, the growth of B16OVA cells overexpressing GSTA4 was more aggressive in OVA-immunized mice than that of parental B16OVA cells. In parallel, the knockdown of GSTA4 in IMM cells led to increased intracellular ROS levels and decreased viability upon in vitro IFNγ treatment. IMM tumors were resistant to anti-PD1 treatment in vivo, and the knockdown of GSTA4 reinvigorated their responsiveness. In addition to the role in acquired resistance to IFNγ, we found that the upregulation of GSTA4 was also responsible for the higher metastatic potential of IMM tumors. Next, we confirmed the results from the mouse model in human melanoma. GSTA4 expression levels in Malme3M, UACC 62, and MeWo melanoma cell lines inversely correlated with their sensitivity to in vitro IFNγ treatment. Database analysis revealed a significant correlation between the expression of GSTA4 and the metastasis-free survival rate of human melanoma patients. Melanoma patients with low GSTA4 expression were better responders and showed a better progression-free survival rate to anti-PD1 therapy, further supporting the clinical relevance of our findings. **Conclusion**: In this study, we uncovered a new mechanism through which cancer cells evade immune surveillance and enhance their ability to metastasize by developing resistance to oxidative stress responses through GSTA4 upregulation. Our results suggest that targeting the oxidative stress response in cancer cells emerges as a promising therapeutic strategy to overcome immune resistance and regulate the progression of metastasis [7].

Keywords: Immunotherapy, Interferon-gamma, Melanoma, Neoplasm Metastasis, Oxidative Stress

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L20

MicroRNAs – biomarker properties in prostate cancer

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Background: Prostate cancer (PCa) is the second most commonly diagnosed malignancy in males and one of the leading causes of cancer-related deaths worldwide. Together with these striking statistics, overdiagnosis and overtreatment, as causes of unnecessary morbidity and the major problems in clinical management of PCa, qualify this malignancy for one of the leading global health issues. Therefore, among the prevailing aims in modern scientific research on PCa is to identify novel molecular markers useful for improving risk stratification, diagnostics and decision making. Due to their functional significance in PCa and their diverse regulatory properties, microRNAs emerged as candidates for biomarker research on PCa. Variants in microRNA-encoding genes that could influence microRNA biogenesis, stability of mature microRNAs, the efficiency of target gene regulation, as well as target specificity, were recognized as potential PCa-related loci. Furthermore, their chemical stability, the minimal invasiveness of sample acquisition and their known involvement in the molecular mechanisms underlying PCa pathogenesis, are the key features of microRNAs that make them plausible candidates for novel circulatory biomarkers of prostatic neoplasia. The main aim of our research group – PROSTATSERBIA, a member of The Prostate Cancer Association Group to Investigate Cancer Associated Alterations in the Genome (PRACTICAL) consortium, was to evaluate microRNA-related genetic variants and microRNA molecules from plasma and circulatory exosomes as novel biomarkers of PCa risk and disease progression. In an attempt to overcome the limitations of single-locus main effect evaluations to detect joint effects of multiple genetic variants, we conducted the analysis of potential epistatic interactions between variants located in microRNA genes and in genes encoding the components of RNA-induced silencing complex (RISC) in relation with PCa risk. Furthermore, we aimed to conduct quantitative data synthesis of our novel and previously published findings in order to elucidate the involvement of these genetic variants in predisposition for PCa development and for aggressive cancer phenotype. **Patients and methods:** PROSTATSERBIA team collected blood samples of patients diagnosed with PCa, benign prostatic hyperplasia (BPH) and healthy male volunteers (around n=400 each), which were used in genetic association studies and circulatory biomarker research. For individuals diagnosed with PCa, clinicopathological characteristics were determined, including initial serum prostate-specific antigen (PSA) level, Gleason score (GS) and the clinical stage of localized cancer, and patients were stratified according to PCa aggressiveness. Single nucleotide variants were selected based on previous results from genome-wide association studies or by candidate gene-approach and were genotyped by using adequate techniques, including PCR-RFLP, allele-specific PCR, Taqman SNP Genotyping Assays and other real-time PCR-based genotyping assays using specific probes, as well as high resolution melting analysis. Genotyping results were included in the Multifactor dimensionality reduction (MDR) analysis, aimed at evaluating the presence of statistical epistatic interactions. Results from our experiments, together with other previously published findings, were included in PRISMA-compliant meta-analyses. MicroRNAs from plasma and plasma-derived exosomes were quantified by qPCR. Exosomes were extracted from plasma by affinity-based isolation method and characterized by bead-based flow cytometry and transmission electron microscopy. **Results:** For our initial genetic association study on microRNA-related variants and PCa in Serbian population, we selected rs2910164 within hsa-miR-146a gene and did not demonstrate its association with PCa susceptibility. Similar findings, suggesting the lack of effect of rs2910164 on PCa risk, were found in our subsequent meta-analysis. Still, this genetic variant showed association with an increased GS (P=0.045; OR=1.70, 95%CI 1.01–2.87), higher PCa aggressiveness (P=0.0033; OR=2.32; 95%CI 1.30–4.13) and the presence of distant metastases (P=0.049; OR=1.95, 95%CI 1.01–3.79), when assuming over-dominant genetic model. Among the three other analyzed genetic variants which affect the structure of microRNA precursors, rs895818 located within a gene encoding miR-27a was the only one that showed association with PCa risk (P=0.035; OR=1.38, 95%CI 1.02–1.86). Still, we found that the minor allele of rs3746444 within hsa-miR-499 associated with a reduced risk of PCa progression, while carriers of rs895818 allele C also had an increased susceptibility to the development of distant PCa metastases. As for the genetic variants located in regulatory regions of microRNA genes, allele C of rs4705342 within hsa-miR-143/145 gene was found to increase the risk of PCa occurrence (P=0.031 for codominant model, P=0.0088 for recessive model). Minor allele G of rs1076064 located in a regulatory region of hsa-miR-378 was found to associate with various prognostic parameters of PCa, as well as with disease aggressiveness. For hsa-miR-34b/c variant rs4938723 association with the clinical stage of PCa was established (Pdom=0.0046; OR=0.36, 95 % CI 0.17-0.76). In our attempt to evaluate the effects of microRNA-related variant on PCa risk and aggressiveness, we also conducted a case-control study which included variants within microRNA-binding sites of KLK3, VAMP8 and MDM4 and yielded evidence of the association between all three tested genetic variants and the values of standard parameters of PCa progression. For KLK3 variant rs1058205, minor allele C demonstrated association with the lower serum PSA score in PCa patients (PSA

> 20 ng/ml vs. PSA < 10 ng/ml comparison, Prec = 0.038; ORrec = 0.20, 95%CI 0.04–1.05). Also, rs4245739 within MDM4 and rs1010 in VAMP8 were found to associate with GS and tumor stage. We also evaluated the biomarker properties of genetic variants located within genes encoding the components of RISC and found no evidence to support the association of rs3742330 in DICER1, rs4961280 in AGO2, rs784567 in TARBP2, rs7813 in GEMIN4 and rs197414 in GEMIN3 with PCa susceptibility. However, rs4961280, rs3742330 and rs7813 showed association with GS and/or the stage of localized PCa. Rs3742330 minor allele G also demonstrated association with lower PCa aggressiveness (P=0.036; OR=0.14, 95%CI 0.023–1.22, for recessive model). These genetic variants were further included in MDR tests of the effects of epistatic interaction on the risk of developing PCa which yielded statistically insignificant results. However, MDR results for the comparison of PCa patients with high and low PCa aggressiveness were statistically significant for the analysis that included rs11614913 in hsa-miR-196a2, with the 3-locus best model comprising this genetic variant, rs7813 and rs784567 (P=0.027). When it comes to circulatory microRNAs, our study aimed at clarifying the significance of miR-21 and miR-375 as PCa biomarkers, as well as at comparing different types of specimens as a source of relevant microRNAs, yielded no evidence of differences in the expression of the analyzed microRNAs between PCa patients and patients with BPH. For miR-375, results remained insignificant in tests of association with the values of standard prognostic parameters of PCa and disease aggressiveness. However, the level of exosomal miR-21 was elevated in PCa patients with high serum PSA values, as well as in patients with more aggressive PCa. At the same time, correlation tests showed the lack of statistical significance when comparing the relative expression of miR-375 and miR-21 in matched plasma and exosome samples. **Conclusions:** According to our data, rs2910164 in hsa-miR-146a and rs3746444 in hsa-miR-499 qualify for genetic variants potentially associated with PCa aggressiveness in Serbian population. Our study provided the first evidence of association between rs895819 and PCa risk, as well as of its genetic association with the presence of distant metastases among PCa patients. Similarly, we identified variant rs4705342 located in the regulatory region of microRNA-encoding gene as PCa susceptibility locus in Serbian population, while for rs1076064 and rs4938723 we demonstrated the association with PCa progression parameters. As for variants in protein-coding genes, analyzed polymorphisms which disrupt the microRNA-binding sites or affect the function of RISC components qualify for PCa progression-associated loci. Our results further support the potential prognostic role of exosomal miR-21 expression levels in PCa, while diagnostic significance of miR-21 and miR-375 derived from plasma and plasma exosomes was not confirmed.
Keywords: Exosomes, MicroRNA, Polymorphism, Prostate Cancer, RNA-Induced Silencing Complex

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L21

Significance of molecular diagnostics in therapy of chronic lymphocytic leukemia

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Abstract in extenso:
Chronic lymphocytic leukemia (CLL) is a malignancy of mature CD5+ B lymphocytes that is characterized by exceptional clinical and biological heterogeneity. The Rai and Binet staging systems, developed in the late 1970s to early 1980s, are used in clinical practice to stratify CLL patients into risk categories and to help guide clinical follow-up options: to treat or to watch and wait. However, in early-stage disease, these systems are unable to predict what patients will face the progression to a more aggressive disease. That means, a number of molecular markers with prognostic and/or predictive impact exist and their assessment is strongly recommended in all patients prior to treatment initiation. One of the first recognized prognostic genomic aberrations in CLL include those detected by fluorescence in situ hybridization (FISH): del(17p), del(11q), trisomy 12 and del(13q), and the immunoglobulin heavy variable (IGHV) gene somatic hypermutation (SHM) status. Moreover, the rapid development of genomics techniques greatly expanded the understanding of CLL at the molecular level in the past decade. This resulted in the discovery of many newer prognostic markers based on chromosomal aberrations or gene mutations. For instance, next-generation sequencing (NGS) studies have led to the discovery of recurrently mutated genes in CLL, such as *NOTCH1*, *SF3B1*, *BIRC3*, *XPO1*, *POT1*, *NFKBIE* and *EGR2*, that are associated with poor clinical outcome. Among all of these biomarkers, the distinction between markers of prognostic and predictive values should be made. Prognostic markers refer to biomarkers that can provide information regarding the patient's outcome regardless of treatment. They are often assessed before treatment to help guide decisions on to treat or not. Markers associated with overall survival (OS) or time to first treatment (TTFT) represent such examples. On the other hand, predictive markers are related to therapeutic interventions with the ability to predict treatment response to a drug. These markers are normally assessed when patients receive the particular therapy. Some markers can be both prognostic and predictive. The National Comprehensive Cancer Network guideline recommends testing of *TP53* genetic alterations, *IGHV* mutation status, and several well-established cytogenetic markers for CLL prognostication. Of these, *TP53* mutations, *IGHV* unmutated status, del(17p), and del(11q), as well as complex karyotype (the presence of three or more unrelated clonal chromosomal abnormalities in a sample), are associated with poor prognosis. Normal karyotype and trisomy 12 are considered as intermediate prognostic factors, whereas del(13q) is associated with a favorable prognosis. The higher frequencies of the previously mentioned unfavorable markers (except for IGHV) found in the treated population usually imply the clonal evolution during disease progression or change in clonal dynamics induced by therapies, especially chemotherapies. Different molecular and genomic techniques are employed for detecting molecular biomarkers in CLL. For IGHV mutation status, the preferred method is Sanger sequencing to detect mutations in genomic DNA or cDNA following PCR, and align the resulting sequences to the germline IGHV using the IMGT/V-QUEST analytic tool, where ≥ 98% homology to the germ line is interpreted as unmutated, >2% nonhomology as mutated, and 97.0% to 97.9% is interpreted as borderline. Prognostically significant chromosomal abnormalities are frequently detected using fluorescence in situ hybridization, array comparative genomic hybridization or conventional karyotyping. Fluorescence in situ hybridization, although offers a high sensitivity and specificity, requires prior knowledge of chromosomal lesions for the probe designs and are limited to the chosen panel genes. The technique has limitations in detecting possible complex cytogenetic abnormalities, as well. On the other hand, karyotyping and array comparative genomic hybridization provide genome-wide coverage. Despite the fact that array comparative genomic hybridization does not effectively detect balanced chromosomal rearrangements, it uncovers more genomic abnormalities than karyotyping as the probe-based technology examines the chromosomal structure at a much higher resolution. Development in NGS technology in the past two decades, made the technique, especially targeted sequencing of gene panels, much less costly and accessible. Currently, in Serbia, genetic techniques such as FISH, conventional karyotyping, Sanger sequencing and NGS are available for detection of CLL biomarkers. Advances in the understanding of CLL pathogenesis have consequently led to the development of several highly effective targeted therapies, including Bruton tyrosine kinase (BTK), phosphatidylinositol 3-kinase, and BCL2 apoptosis regulator (BCL2) directed inhibitors. B-cell survival and proliferation is regulated by the BCR signaling pathway. In normal B cells, BCR is triggered by antigen ligation, leading to activation of a cascade of tyrosine kinases, including BTK. BCR signaling is aberrantly activated in many B-cell malignancies, including CLL. Ibrutinib has demonstrated high clinical efficacy acting as an irreversible potent inhibitor of Bruton's tyrosine kinase and targets several key components of the BCR pathway. However, despite having 80% to 90%

response rate, 10% to 15% of CLL patients, who respond initially, develop ibrutinib resistance and disease relapse in 2 to 3 years on ibrutinib treatment, mainly because of the acquisition of a BTK C481S mutation. The mutation prevents the drug from forming a covalent bond with the C481 residue that weakened the drug-BTK binding by 500-fold. As a result, BCR signaling and cell proliferation were restored in the tumor cells. BTK mutations may be found in approximately 70% of CLL patients who progressed on ibrutinib treatment. Another resistance mechanism is through acquired activating mutations in PLCG2, which is found in approximately 10% of the cases. Given these evidences, the current National Comprehensive Cancer Network guideline recommends testing for BTK and PLCG2 mutations for CLL patients receiving ibrutinib who are suspected of having disease progression. NGS has become the optimal method for detecting BTK or PLCG2 mutations in the setting of ibrutinib treatment, as multiple mutations in both genes may occur in the same specimen. Currently, approximately 20% of CLL patients who progressed on ibrutinib do not have either BTK or PLCG2 mutations; thus, with NGS, it is possible to uncover other less common but yet undefined drug-resistance mutations. In addition to BTK and PLCG2 mutations known to confer ibrutinib resistance, other molecular markers have been associated with an upfront high risk of relapse on ibrutinib treatment. It has been reported that complex karyotype, del(17p)/TP53 mutation, and del(18p) at baseline before ibrutinib treatment are strongly associated with disease relapse. Other approved targeted agents for CLL treatment include the phosphatidylinositol 3-kinase inhibitors idelalisib and duvelisib and BCL2 inhibitor venetoclax. For venetoclax, a novel BCL2-G101V mutation was identified to prevent drug activity through drug-protein interaction. Each patient with CLL may have several clinical and molecular markers of conflicting prognostic significance simultaneously, making the precise prognostication challenging. Today is of the greatest importance to apply ultrasensitive techniques to reveal molecular relapses after therapy initiation and to detect minimal residual disease after patients achieve complete responses. Keywords: Chronic Lymphocytic Leukemia, Molecular Biomarkers, Molecular Techniques, Target Therapy

POSTER SESSION

ORAL AND POSTER PRESENTATIONS

ORAL PRESENTATIONS

O01

The PDK-1 inhibitor GSK2334470 induces cell death and G1 cell cycle arrest in human pancreatic cancer cells

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Background: The aim of this work was to investigate the *in vitro* anticancer activity of the small molecule GSK2334470, a reported PDK-1 inhibitor, against human pancreatic ductal adenocarcinoma (PDAC) cell lines. PDK1 (phosphoinositide-dependent kinase-1) is a constitutive serine/threonine kinase that acts as a master kinase, phosphorylating and activating a subset of the AGC family of protein kinases, such as the RSKs, key downstream kinases of the MAPK/ERK pathway and being involved in the regulation of the PI3K/AKT/mTOR pathway. **Material and methods:** PANC-1, MIA, PaCa-2, BxPC3 and AsPC-1 were used to study the in vitro activity of this small molecule. An SRB cytotoxicity assay was first performed to determine the *in vitro* anticancer properties of this compound. A clonogenic assay and a wound healing (scratch) assay were then performed to test the anti-proliferative and anti-migratory capacity of the inhibitor, both in terms of the ability of individual cells to form colonies and their ability to migrate. Flow cytometry was used to identify the cell cycle phase at which GSK2334470 acts in both synchronised and non-synchronised cells in the G1 phase. WB was then used to elucidate the mechanisms by which this molecule induces cell cycle arrest. Finally, the effect of the inhibitor was also compared with silencing of the kinase by specific siRNA. **Results:** The data obtained from the above experiments suggest that GSK2334470 has significant anti-proliferative effects against all PDAC cell lines tested, with PANC-1 being the most sensitive as the GI50 was found to be around 10μM. In addition, GSK2334470 was able to inhibit both colony formation at a concentration of 0.1μM and pancreatic cancer migration at concentrations close to 1μM. Finally, GSK2334470 was observed to act via a cell cycle phase-specific mechanism, as it was found to arrest the cell cycle at the G0/1/S transition. WB showed that GSK2334470 was able to downregulate the activation of PDK1 with subsequent activation of GSK3a/β and finally the downregulation of cyclin D1, consistent with the G1 arrest observed. **Conclusions:** GSK2334470 PDK-1 inhibitor was found, for the first time worldwide, to exhibit promising *in vitro* anticancer activity against PDAC cell lines, which was further found to be time and dose dependent. Further studies are underway to understand the mechanism of action of this small molecule.
Keywords: Pancreatic cancer, PDK1, GSK2334470, Cell cycle arrest,

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O02

Suppressor effects of the mixed ligand platinum (II) saccharinate complexes (*trans*-[Pt(sac)$_2$(PPh$_3$)$_2$] and *trans*-[Pt(sac)$_2$ (PPh$_2$Cy)$_2$])on *in vitro* and *in vivo* angiogenesis

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Background: Metal-based chemotherapeutics, including platinum complexes are promising agents for the treatment

of cancer with greater efficacy than chemotherapeutic agents. The aim of the present study is to investigate the anti-angiogenic effects of the new platinum(II) complex with phosphine and saccharinate ligand, platinum (II) saccharinate complex *trans*-[Pt (sac)$_2$(PPh$_3$)$_2$] (MP2 complex),and *trans*-[Pt (sac)$_2$ (PPh$_2$Cy)$_2$)] (MP6 complex), known to exhibit anti-cancer activity. **Materials and methods:** The cytotoxic effect of platinum (II) saccharinate MP2 and MP6 complexon human umbilical vein endothelial cells (HUVECs) was investigated using the SRB and ATP assays. Molecular markers of angiogenesis and survival pathways were also examined using Western blotting. *In vitro* tube formation assay and *in vivo* chick embryo chorioallantoic membrane (CAM) were used to evaluate the anti-angiogenic activity of MP2 and MP6 complex. **Results:** Based on the SRB and ATP assay, cell viability was inhibited in a dose-dependent manner at all tested concentrations (1.56-100µM) of MP2 and MP6 complex after 24-48 h. Especially, 12.5 µM and higher concentrations of MP2 and MP6 complexes significantly decreased cell viability. In addition, MP2 and Mp6 treatment resulted in a dose- and time-dependent decrease in the expression of phospho-SRC, phosphor-p38, phospho-VEGFR-2, phospho-FAK, phosphor-ERK1/2 and phospho-Akt. MP2 and MP6 (6.25-12.5 µM) also strongly reduced tube structure compared to vehicle and positive control at all times tested The MP2 complex at a concentration of 40 µg/pellet showed a very strong anti-angiogenic effect on CAM compared to the positive control (±)-thalidomide. The 20 µg/pellet concentration of MP2also showed a strong antiangiogenic effect, but a weak effect was observed with the low concentration of MP2(10 µg/pellet). For the MP6 complex a strong anti-angiogenic effect was again shown at the concentration of 40 µg/pellet. The 20 µg/pellet concentration of MP6 exhibited a weak antiangiogenic effect, while no effect was observed at the low concentration of MP6 (10 µg/pellet). **Conclusions:** The results of our study suggest that the platinum(II) saccharinate complexes have significant anti-angiogenic activity *in vitro* and *in vivo*. However, further *in vivo* experiments are needed to prove that the anti-angiogenic effects of novel platinum(II) saccharinate complexes are worthy of clinical use.

Keywords: Angiogenesis, CAM assay, Platinum (II) saccharinate complex

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O03

All-trans retinoic acid activities in Merkel cell carcinoma: implication of the retinoic gene signature

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Background: Merkel cell carcinoma (MCC) is a rare, but aggressive skin cancer. About 80% of MCCs is linked to oncogenic Merkel cell polyomavirus (MCPyV). Since the currently available MCC therapies are remarkably limited, novel approaches are required. The biological activity of all-trans retinoic acid (ATRA) is mediated by retinoic acid (RAR) and retinoid X (RXR) receptors, which are ligand-dependent transcription factors that activate genes crucial for cell differentiation. Dysregulations of RAR/RXR receptors lead to carcinogenesis. Although a strong in vitro/in vivo antitumor activity of ATRA has been proved in different carcinoma types, the effect of this drug in MCC is still unknown. **Material and Methods:** Herein, we investigated in vitro the ATRA activities against MCPyV-positive MCC (MCCP), PeTa and WaGa, and -negative (MCCN) cells, MCC13 and MCC26, MCC cell lines and normal human lung fibroblasts MRC5, as control. The ATRA effect was evaluated testing MCC cell (i) proliferation, (ii) migration, (iii) colony formation abilities and (iv) apoptosis. Proliferation, migration and clonogenicity were evaluated by WST-1, wound healing and colony formation assays, respectively. Apoptosis/cell death were evaluated via Annexin-V and propidium iodide assays. Apoptosis was molecularly evaluated by RT2 Profiler PCR mRNA array and by western blot analysis. Retinoic pathway was evaluated by RT2 Profiler PCR mRNA array. **Results:** ATRA treatment led to a significant reduction in MCC cell proliferation, migration and clonogenicity, while increasing apoptosis/cell death in MCC cell lines compared to untreated cells. MCCP cells were slightly more ATRA-sensitive compared to MCCN cells. No significant effects have been found in the ATRA-treated control cell line. Gene expression array indicated a significant overexpression of several pro-apoptotic genes in MCC cells. High levels of pro-apoptotic proteins have been found following ATRA treatments in MCC cells, while being almost undetectable in untreated cells. Pro-apoptotic markers were almost undetectable in ATRA-treated MRC5. Numerous retinoic signaling genes, including BMP2, FOXA1 and MAFB, were differentially expressed in ATRA-treated MCC compared to untreated cells. **Conclusions:** Overall, our in vitro data indicate that ATRA is effective in reducing MCC cell growth, while presenting strong pro-apoptotic effects and favoring cell death, by modulating the retinoic receptor pathway. These results point to ATRA as a potential novel effective drug for the MCC therapy.

Keywords: ATRA, All-trans retinoic acid, MCC, Merkel cell carcinoma, therapy, retinoic signaling, MCPyV

O04

Predicting response to chemoradiotherapy in locally advanced rectal cancer using MRI-based radiomics features

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Background: Locally advanced rectal carcinoma (LARC) is typically treated with neoadjuvant chemoradiotherapy (nCRT) followed by surgery. Identifying predictive biomarkers is crucial for selecting patients who will benefit most from neoadjuvant treatment. This study aimed to develop a predictive model using radiomics features extracted from MRI scans to predict the response of LARC patients to nCRT. **Materials and methods:** Between June 2020 and January 2022, we prospectively enrolled 75 LARC patients who underwent long-course nCRT. Radiation therapy was administered using volumetric modulated arc therapy-simultaneous integrated boost technique, along with concomitant chemotherapy (5FU, Leucovorin) during the first and fifth week of treatment. Treatment response (TR) was evaluated in week 8 after completing nCRT. For patients with complete clinical response (cCR) and initially distant located tumor no immediate radical surgery was suggested and they were enrolled in a strict follow-up program ("watch and wait" approach). Responders were defined as those with cCR and postoperative TRG1 and TRG2 categories, as per the Mandard classification. Non-responders were classified as TRG3-5. Initial pelvic MRI imaging was available for 71 out of 75 patients, and 3D T2-weighted (T2W) contrast sequences were utilized for tumor segmentation. **Results:** Among the patients, 46.6% were responders. Tumor morphology was assessed through the calculation of 2092 shape, first-order, and second-order radiomic features. TR was considered the outcome of interest. The least absolute shrinkage and selection operator (LASSO) technique was employed to identify the most predictive and non-redundant features associated with the outcome. Out of the 2092 radiomic features, LASSO selected eight features for the model. The final model, further selected through multivariate regression, included two features (maximum 2D diameter and complexity) with an area under the curve (AUC) of 0.76. **Conclusions:** The application of radiomics in LARC holds potential for assisting clinicians in tailoring treatment plans and making informed decisions for individual patients. Further prospective studies with larger cohorts are needed to validate these preliminary findings.

Keywords: chemoradiotherapy, response to treatment, MRI, radiomics

O05

Transcriptomic profiling of the early stage squamous cell lung cancer

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Abstract in extenso. **Background:** Lung cancer is a leading cause of death, and squamous cell lung cancer (SqCLC), a frequently diagnosed histological subtype of lung malignancy, is represented with high mortality and limited treatment options. Identification of potential targets suitable for drug development using high-throughput methods is still lacking. Therefore, the aim of this study was to analyze expression profiles of mRNA in SqCLC aiming to identify

the key molecules associated with tumorigenesis and prognosis. **Materials and Methods:** Samples of primary SqCLC (N=23) were collected during surgical resection. Healthy control tissues (N=4) were obtained during pathological sections from healthy individuals who died from accidental deaths and were not having any lung changes. Study inclusion criteria for FFPE lung tumor specimens were: tumor should be of squamous origin, there should be 60% or more tumor cells confirmed in specimen and not more than 30% of necrotic tissue. Validation cohort (SqCLC=225; Healthy=288) was downloaded from uniformly realigned expression datasets from TCGA, TARGET and GTEx. We performed differential gene expression analysis, pathway enrichment analysis and gene ontology analysis on RNA-seq data obtained from experimental and validation cohorts. Differential expression analysis was performed in R using DESeq2 package. Differentially expressed genes were functionally annotated by Gene Ontology analysis performed by using web-based tool available at http://geneontology.org/, and PANTHER complete annotation sets. The influence of differentially expressed genes on overall survival analysis was performed using Xena browser. Kaplan-Meier and log-rank test was used to evaluate the difference in survival rates between the two groups (high or low expression). We have also estimated levels of tumor infiltrated immune cells in experimental cohort, followed by immune subtypes identification. For the purpose of this analysis we used sample-level enrichment method gene set variation analysis (GSVA) to estimate relative abundance of sixteen immune populations in tumor microenvironment. **Results:** We identified 1381 up-regulated and 1085 down-regulated genes, common for both cohorts. The most significant up-regulated genes were involved in cell-cycle regulation pathways, while down-regulated genes predominately belonged to immune-related pathways. Survival analysis performed on selected genes, commonly dysregulated in both cohorts, performed only on nonmetastatic patients, identified novel prognostic biomarkers associated with OS in early-stage SqCLC patients (HOXC4 is associated with worse OS, p=0.0001, while LILRA5 with better OS, p=0.0029). Our results on immune cell profile show that there is a difference in tumor infiltrating immune-cells levels among tumor samples. Based on calculated GSVA enrichment scores, we identified four immune-subtypes, characterized as “less” (subtypes 1 and 2) or “more immunogenic” (subtypes 3 and 4). In general, based on immune cell infiltration profiles, “more immunogenic” subtypes exhibit higher levels of infiltration with NK bright cells and CD8+ T cells, in comparison to “less immunogenic”. Also, higher levels of infiltration with antigen-experienced cells, like central memory T cells, effector memory T cells and follicular helper T cells, were associated with more immunogenic subtypes. **Conclusions:** Results of the current study identified differentialy expressed genes associated with the development of SqCLC. These genes were shown to be involved in cell-cycle regulation pathways (up-regulated genes) and immune-related pathways (down-regulated genes). This study also identified individual genes, like HOXC4 and LILRA5, associated with OS. In general, results of our study provide a spectra of the transcriptome profile of SqCLC, giving a new information on carcinogenesis mechanisms and prognosis. Future functional research is necessary, in order to investigate in more details the potential application of detected genes in diagnosis and treatment of the SqCLC.
Keywords: lung cancer, NSCLC, squamous cell carcinoma, profiling, mRNA

O06

The role of p53 family in melanoma development and therapy resistance

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Metastatic melanoma is one of the most aggressive tumors, with frequent mutations of protein kinase BRAF. Despite the significant improvements in treatment with BRAF/MEK inhibitors, the majority of patients develop resistance whose mechanisms are still not completely understood. The TP53 gene, the guardian of the genome, which is altered in majority of human cancers, is rarely mutated in melanoma. Notably, the TP53 gene possesses two distinct promoters, alternative translation initiation sites, and undergoes alternative splicing, giving rise to 12 diverse isoforms with distinct functions. Additionally, p53 family members, TP73 and TP63, generate multiple isoforms with different functions. We assume that shorter isoforms of the p53 family may act as modifiers of p53-dependent responses, including its tumor suppressor functions. Using quantitative qPCR and western blot analysis, we have analyzed the p53/p73 expression profiles in a panel of human melanoma cell lines, in normal conditions or after the treatment with common DNA-damaging agents or targeted therapy, as well as in clinical specimens. Our results show that human melanoma cells express a wide array of p53/p73 isoforms, with Δ160p53α being the most variable. Noteworthy, higher Δ133p53β and p53α mRNA had a negative impact on the overall patients' survival. Additionally, we generated two melanoma cell lines with acquired resistance to BRAF inhibitor vemurafenib after prolonged exposure to the drug. The resistant cells showed an altered expression of p53 and p73 isoforms, specifically an increased expression of potentially pro-

oncogenic Δ40p53β and a decrease in tumor-suppressive TAp73β. Finally, we have studied the expression profile of the p53/p73 isoforms in a panel of five patient-derived melanoma cell lines that harbor mutations in BRAF and show different sensitivity to BRAFi and/or MEKi. We have found that increased levels of p53 isoforms (p53α, p53β, and Δ40p53β) and lower levels of tumor-suppressive TAp73β isoform could correlate with acquired resistance to BRAFi/MEKi and/or BRAFi targeted therapy. We, therefore, propose that p53 family isoforms can play a role in melanoma cells' aggressiveness and could be a potential marker and target for melanoma therapy.
Keywords: isoforms, melanoma, p53, p73, resistance, targeted therapy

O07

The anticancer effects of triterpene saponin deglucocyclamine isolated from *Cyclamen hederifolium*

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Background: Following the traditional Serbian use of cyclamen tubers in the treatment of the most aggressive forms of lung cancer, we performed methanolic extraction of fresh tubers of Cyclamen hederifolium to isolate and identify bioactive constituents. The triterpene saponin deglucocyclamine (SDGC) was identified as a major constituent of cyclamen extract, and its anticancer effects were studied using a panel of NCI-60 cell lines and primary cell cultures obtained from patients with non-small cell lung cancer (NSCLC). **Material and Methods:** The cyclamen tubers were ground, lyophilized, and extracted with methanol at room temperature with the use of an ultrasonic bath. The part of the methanol extract was further fractionated by dissolving in H2O and then washed with CH2Cl2. The water layer was extracted with n-BuOH. The butanol extract was fractionated by isocratic CC on silica gel with CHCl3−MeOH−H2O eluent. This resulted in the isolation of triterpene saponin deglucocyclamine (SDGC, C52H84O22) which was identified using 1D and 2D NMR spectra. SDGC was tested at 10 μM against a panel of NCI-60 cancer cell lines and then over a concentration range of 0.01-100 μM using the sulforhodamine B (SRB) assay. SDGC was also tested in the 0.01-10 μM concentration range against 5 primary patient-derived NSCLC cell cultures (2 stage IB, 2 stage IIA, and 1 stage IIB) using the MTT assay. Cell death analysis was performed in patient-derived NSCLC cells using annexin/propidium iodide staining and flow cytometry. **Results:** SDGC at 10 μM after 72 h significantly inhibited cell growth of all tested cancer cell lines in the NCI-60 panel. Therefore, SDGC IC50 values were evaluated across the entire NCI-60 panel, ranging from 600 nM to 1 μM. In patient-derived NSCLC cells, SDGC IC50 values were between 1.3 μM and 4.6 μM after 72 h of treatment. SDGC at 10 μM induced late apoptosis and necrosis, significantly reducing the percentage of viable cells to 40% after 48 h. At the same concentration, cisplatin was ineffective against patient-derived NSCLC cells. **Conclusion:** The triterpene saponin deglucocyclamine (SDGC), whose anticancer effects have not been studied before, showed promising results against NSCLC, melanoma, colon, breast, ovarian, kidney, prostate, and CNS cancer cell lines, as well as patient-derived NSCLC cells. Further more detailed studies of SDGC at the cellular and molecular level are planned.
Keywords: anticancer, cyclamen, NCI-60, non-small cell lung carcinoma, patient-derived cell culture

O08

The effect of diiron thiocarbyne complex on tumor cells of different grade

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Background: Iron is an important trace element with a broad range of functions in diverse physiological processes and a tightly regulated metabolism. Over the years, numerous studies have indicated that cancer cells exhibit an iron-seeking phenotype, meaning they have higher demands for iron than healthy cells. This feature may serve as a foundation for a new approach to cancer therapy. In order to develop an anticancer drug with improved efficacy,

higher selectivity and reduced toxicity, a new organo-diiron complex with a bridging thiocarbyne ligand (FeSDAP) was synthesized. **Material and Methods:** The cytotoxic effect of FeSDAP was investigated on mouse cancer cell lines (B16-F1 low-invasive melanoma, B16-F10 high-invasive melanoma and 4T1 breast cancer), as well as on mouse embryonic fibroblasts (NIH-3T3). For investigation of its mechanism of action, flow cytometry and light microscopy were used. To investigate how 72h long exposure to DMAP *in vitro* affects the potential of B16-F1 and B16-F10 cells to form tumor in vivo, respective subcutaneous synegenic models in C57BL/6 mice were used. **Results and Conclusions:** Treatment with FeSDAP decreased viability of all cells after 72 hours, with significantly less potent effect on embryionic fibroblasts compared to cancer cells, suggesting FeSDAP may possess selectivity towards a malignant phenotype. Melanoma cells were almost equally sensitive to the treatment, but more sensitive than breast cancer cells, so both B16-F1 and B16-F10 were selected for further comparative investigation. Treatment with FeSDAP inhibited proliferation of melanoma cells and caused substantial change in their morphology, which was even more pronounced when it comes to B16-F10 cells. After microscopic evaluation, it was shown that melanoma cells went into senescence. Prominent morphological change of B16-F10 cells was caused by transdifferentiation into Schwann Cell-Like Cells. Further investigation of tumorigenic potential of treated melanoma cells in mice showed that the average tumor size in the groups that received treated cells was significantly smaller, suggesting that melanoma cells have persistently reduced potential to form tumor after single in vitro treatment with FeSDAP. Ultimately, these results strongly indicate that investigated diiron thiocarbyne complexes may display a promising antitumor potential that will be investigated in more detail.
Keywords: cell transdifferentiation, cellular senescence, iron compounds, melanoma

O09

The effects of cisplatin-ibuprofen conjugate free and immobilized in mesoporous nanostructured silica on the change of morphology of mouse melanoma cells, and antitumor potential *in vivo*

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Background: Active contribution of cyclooxygenase enzymes (COX) and their products, in particular prostaglandin E2, to tumor progression makesthis enzyme an attractive target for molecular therapy in cancer. The combination of conventional chemotherapeutic drugs with COX1/2 inhibitors, and further enhancement of their delivery into target tissue can be a highly prospective approachin cancer therapy, especially in advanced stages. Accordingly, a cytostatic and anti-inflammatory drug conjugate was synthesised, as well as its immobilization in mesoporous nanostructured silica SBA-15. Detailed evaluation of the cytotoxic potential and the mechanism of action of this conjugate and the appropriate materialon B16 cells was further performed*in vitro*and *in vivo*. **Material and Methods:** Cell viability of B16 melanoma cells was determined by MTT and CV assays. Cell morphologywas estimated byhematoxylin–eosin andOil Red O staining using light microscopy, whilechanges in thenucleiwere validatedby PI staining using fluorescent microscopy. Differentiation of melanoma cells was determined by measurement of tyrosinase activityand the presence of melanin. Syngeneic C57BL/6 mice modelwas used for *in vivo*assessment of the tumorigenic potential of B16 cellsexposed tofree and SBA-15 loaded conjugate*in vitro*, as well as for the evaluation of the antitumor potential of the experimental substances given in the therapeutic regimen. **Results and Conclusion:** Exposure to free or immobilized cisplatin-ibuprofen conjugatedecreasedthe viability of the B16 cell culturewhile morphology of survived cells was changed. Cytoplasm of enlarged and elongated cells showed intensive granularity with enhanced lipid contentand huge irregularly shaped nuclei with prominent heterochromatin foci, all of which indicated senescent state. Increased activity of tyrosinase and the presence of melanin compared to the control, referred to the differentiation of melanoma cells toward primary phenotype.Further inoculation of pretreated B16 cells into C57BL/6 mice showed decreased potential to form tumor incomparison to tumorigenic potential of untreated cells.Additionally, *in vivo* application of free and SBA-15 immobilized conjugatein therapeutic regimentled to statistically significant reduction of tumor volume, with only fewer signs of toxicity compared to cisplatin as positive control.New knowledge about this compound and corresponding materialis reflected in their antitumor potential on mouse melanoma cells, which opens numerous possibilities for further research.
Keywords:cell differentiation, cisplatin, ibuprofen, melanoma, nanoparticles, senescence

O10

Role of claudins 3, 4 and 7 in triple negative breast cancer progression

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Background: Breast cancer is the most commonly occurring malignancy and the leading cause of cancer-related death in women. Triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype and is associated with high recurrence rates, high incidence of distant metastases and poor overall survival. The aim of this study was to investigate the role of cludin 3, claudin 4 and claudin 7 in TNBC promotion and progression. Claudins are tight junction (TJ) integral membrane proteins that are key regulators of the paracellular pathway. **Materials and methods:** This is a retrospective analysis of 125 patients with triple-negative breast cancer operated at the Institute of Oncology and Radiology of Serbia in the period from 2009 to 2014. The expression of claudin 3, 4 and 7 was observed using the immunohistochemical staining method. The Allred scoring system was used with cut-off values: ≤4 and >4 (low/ high expression). **Results:** Our results showed that the expression of claudins 3 and 4 correlate with higher nuclear gradus and low desease free interval (DFI). More over, the expression of claudin 3 and claudin 4 correlates (Spearman test $p<0.0001$). In addition, high expression of claudin 7 is significantly related to low DFI of patients ($p<0.005$) and distant metastases. **Conclusions:** We concluded that claudin 3, claudin 4 and claudin 7 have significant impact on TNBC progression. Namely, elevated expression of these proteins significantly correlates with low DFI and distant metastases. In other words, elevated expression of claudins is a bad news for TNBC patients. Therefore, the expression of claudins could be a good prognostic marker for TNBC patients and potential target for future therapy protocols.
Keywords: Triple-negative breast cancer (TNBC),claudin 3, claudin 4, claudin 7

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O11

Impairment of cystatin F activation can increase the cytotoxicity of NK cells

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Background: Natural Killer (NK) cells utilize granule mediated cytotoxicity as the predominant pathway in cancer cells killing. The main effector molecules required for this process, granzymes and perforin are synthetized and stored in their inactive forms and require proteolytic processing by cathepsins C, H, and L for their activation. We have identified a protease inhibitor cystatin F as a potent regulator of these cathepsins and consequently cytotoxic efficacy in NK cells. Cystatin F activity is regulated by various factors, including expression levels, N-glycosylation, and proteolytic activation. In lysosomes, cathepsin V cleaves 15 N-terminal amino acids from cystatin F, thereby activating it from an inactive dimeric form to an active monomer. Cystatin F was found expressed in glioblastoma tumor tissue. The aim of this study was to assess NK cell function in glioblastoma patients and to enhance NK cell cytotoxicity by inhibiting cystatin F activation. **Material and Methods:** To counter the cystatin F effects of on NK cell cytotoxicity, a new small molecular inhibitor of cathepsin V was developed. After molecular docking of small molecular compounds from commercial libraries with cathepsin V, selected compounds were evaluated by enzyme kinetics for enzyme inhibition, selectivity, and reversibility of binding. The effect of the most potent, selective, and reversible-acting cathepsin V inhibitor on cystatin F activation was tested by western blot. NK cells were isolated from peripheral blood mononuclear cells of healthy donors and glioblastoma patients and analysed by flow cytometry. **Results and Conclusions:** We found that patient NK cells had significantly reduced cytotoxic efficacy and increased content of cystatin F compared to healthy donor NK cells. However, even healthy NK cells were susceptible to the effects of cystatin F. Recombinant cystatin F reduced cytotoxicity, increased IFN-γ secretion, and decreased cathepsin C and granzyme B activity. Treatment of NK

cells with cathepsin V inhibitor decreased the monomerization of cystatin F and increased their cytotoxicity.
Keywords: Cytotoxicity, NK cells, Protease inhibitor

O12

Cisplatin-Killed Cells as a Preferable Method for Generating Tumor Cell-Based Vaccines

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Background: Anticancer vaccines based on whole tumor cells are a promising strategy for cancer therapy, as cell lysates potentially carry all tumor antigens, eliminating the need to search for better antigen candidates, which is necessary in the case of mRNA, protein, or peptide-based anti-tumor vaccines. However, it is debatable how to generate whole tumor cells lysates, leading to the best immune response and antitumor effect. In the present study, we prepared different variants of whole tumor cell-based vaccines using CT26 murine colon adenocarcinoma model and evaluated their efficiency. **Material and Methods:** We prepared tumor CT26 cell-based vaccines for Balb/c mice immunization (5×10^5 cells in 100 µl of PBS per animal, subcutaneously, 3 animals in each group) as follows: Pathogenesis group – CT26 cells without exposure; F/T group – cells after freezing-thawing (2 hours before vaccination); PDT group – cells after photodynamic treatment (5 µg/ml Chlorine E6 photosensitizer, 6 J/cm2 irradiation 2 hours before vaccination); Cisplatin group –cells treated cisplatin (24 hours before vaccination). The doses of photodynamic treatment and cisplatin were chosen in vitro to provide a 99.9% cell death rate within 24 hours after vaccination time. No tumor development was observed in F/T, PDT, and Cisplatin groups and on the 14th day after vaccination, all animals were euthanized, spleens were taken, and splenocytes were isolated. In each group, 2×10^5 splenocytes were co-cultured with 1×10^4 CT26 cells in the presence of 1000 U/ml interleukin-2 for 72 hours. The supernatants were analyzed for interferon-gamma levels according to the standard ELISA protocol. **Results:** The mean values (±SD) of interferon-gamma concentrations measured were: 2123 (52), 4150 (79), 4126 (73), and 9065 (143) pg/ml in Pathogenesis, F/T, PDT, and Cisplatin groups, respectively. The significant difference in the immune response to tumor cell-based vaccination compared to tumor process ($p < 0.005$) was shown. Mice immunization with cisplatin-killed cells caused the strongest interferon-gamma response of isolated splenocytes after their co-cultivation with tumor cells. **Conclusions:** The study results suggest that the use of cisplatin for the generation of tumor cell-based vaccines is preferable compared to the classical method of preparing cell lysates or using photodynamic exposure.
Keywords: cancer, vaccine, whole tumor cells, cisplatin

O13

Modes of Activity and Prognostic Significance of the Hedgehog-GLI Signaling Pathway in Prostate Cancer

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Background: Prostate cancer (PC) is the second most frequent cancer in males. Localized disease can be treated successfully, but resistance to androgen deprivation (AD) therapy quickly occurs. The Hedgehog-GLI (HH-GLI) signaling pathway is involved in many aspects of embryonic development as well as stem cell maintenance and tissue homeostasis in adult organisms. However, its aberrant activation in adult cells has been linked with the development of various tumors, including prostate cancer. Therefore, we aimed to investigate the role of HH-GLI pathway in maintaining androgen-independent (AI) PC growth and assessed its prognostic significance.**Material and Methods:** We used PC cell line LNCaP, which was made AI. Expression of Hedgehog-GLI members and androgen receptor (AR) targets was

determined by qPCR and western blot (WB), in parental cells and after pathway inhibition by cyclopamine or GANT-61 treatment. Co-immunoprecipitation (co-IP) and proximity ligation assay (PLA) were used to investigate the physical interaction between the sonic hedgehog ligand (SHH-N) and AR. Immunohistochemistry (IHC) was used to determine GLI1, GLI2, GLI3 and PTCH1 expression in 159 PC and 65 healthy prostate tissue samples. Overall survival was calculated using the Kaplan-Meier method.**Results:** After cyclopamine or GANT-61 treatment, we found various modes of HH-GLI signaling, depending on androgen availability. For instance, a short-term AD led to canonical signaling, whereas in AI cells, SHH-N could non-canonically bind to AR through its cholesterol modification. Inhibition of this interaction, after cholesterol depletion by MβCD, led to AR signaling down-regulation, which implies that SHH-N activated AR and sustained AI. Results on tissue samples showed statistically significantly increased expression of all four studied proteins in PC tissues compared to healthy prostate. Decreased GLI1, increased GLI2 and PTCH1, and nuclear GLI3 localization were associated with a shorter overall survival of PC patients.**Conclusions:** Our study showed existence of various modes of HH-GLI signaling in PC, and that targeting SHH:AR interaction might be used for advanced PC treatment, while some of the main members of the HH-GLI pathway, which are commonly used as markers of its activity, showed potential as prognostic biomarkers for PC.

Keywords: Androgen, Biomarkers, Hedgehog Proteins, Prostatic Neoplasms, Receptors, Zinc Finger Protein GLI1

O14

Platelet-released factors boost proliferation of multiple myeloma cells and changes in bone marrow stroma with implications of NFκB pathway involvement

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Background: Multiple myeloma (MM) is a hematological malignancy characterized by bone marrow (BM) infiltration with plasma cells, a high occurrence of thrombin generation, and thrombosis. However, the role of platelets in MM has not been unexplored. Additionally, BM mesenchymal stem/stromal cells (BMSCs) participate in MM pathophysiology through a complex interplay with MM cells in BM niche. Since recent studies showed strong positive association between platelet activation states and MM progression, here we investigated effects of platelet-released factors in MM and neighboring BMSCs. **Material and Methods:** Fresh platelet-rich plasma (PRP) was collected from whole blood of healthy donors. Non-malignant and MM-affected BM specimens from sex and age-matched patients undergoing orthopedic surgery were processed and used for isolation of mononuclear cells and BMSC expansion. Cell-free conditioned media were prepared by subsequent 24h incubation of thrombin-activated PRP (aPRP) hydrogels. Cells were exposed to PRP- or aPRP-released factors. Cellular and molecular assays were performed to determine cell functions, protein and gene expression. Statistical significance was estimated by non-parametric tests. **Results:** Platelet-released factors from both PRP and thrombin-activated (aPRP) significantly increased metabolic activity and proliferation of MM cell lines (AMO1, U266, OPM2). In addition, PRP and particularly aPRP increased presence of CD138+ cells within MM-BM mononuclear cells in hypoxic conditions (3% O2). Although BMSCs supported proliferation of MM cells, presence of aPRP did not change it. However, we found upregulated HSP60, PDGFRα and α-SMA protein levels in BMSCs, followed by increased inflammatory (Il-6, Il-8), and decreased osteogenic (Alp) gene expression in aPRP-exposed BMSCs when compared to control. This suggested that platelet-released factors induced inflammatory "cancer-educated" phenotype in BMSCs. When applied selective NF-κB inhibitor (PDTC), PRP and aPRP effects on MM cell growth were abolished, while it appeared that NF-κB signaling was only partly involved in aPRP-regulated profile of BMSCs. **Conclusions:** Our findings for the first time suggest that activated platelet-released factors contribute to myeloma cell growth and possible induction of inflammatory phenotype in BMSCs. Obtained results can contribute to a better understanding of platelets involvement in MM and BM stroma crosstalk and establishing novel anti-cancer strategies.

Keywords: platelets, multiple myeloma, mesenchymal stem/stromal cell, NFκB signaling, platelet-rich plasma

O15

In vitro anticancer activity of kaempferol-derived flavonoids against pancreatic adenocarcinoma

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Background: Pancreatic cancer is one of the most lethal cancers worldwide. The aim of this study was to investigate the *in vitro* anticancer activity of kaempferol-derived flavonoids in pancreatic ductal adenocarcinoma. Specifically, kaempferol and its glycosylated derivatives tiliroside and its semi-synthetic derivative peracetylated tiliroside (Tac) were tested for their in *vitro* anticancer activity against pancreatic cancer cells. **Material and methods:** The cytotoxic activity of the congeners and some RSK inhibitors was tested against the human pancreatic adenocarcinoma cell lines (PANC-1, MIA PaCa-2 and BxPC3) using the SRB method. The most active compound, Tac, was further evaluated for its ability to inhibit colony formation using a colony forming assay and to inhibit PANC-1 migration using a scratch assay. In addition, after exposing the cells to specific concentrations (10μM and 20μM) and time intervals (6h / 12h / 24h), we examined its effect on the viability of PANC-1 cells using the trypan blue method. The ability of Tac to enhance the activity of the clinical drugs gemcitabine (GEM) and 5-fluorouracil (5FU) against pancreatic cancer cells was also tested in combinatorial experiments. Finally, we investigated whether Tac is involved in the MAPK pathway by inhibiting the function of p90RSK kinases by Western blot analysis to study the expression levels and phosphorylation status of p90RSKs. **Results:** Based on the results of the study described above, it was observed that the peracetylated derivative of tiliroside, Tac, exhibited the strongest antiproliferative and cytotoxic effect against the human pancreatic cancer cell line PANC-1. This effect was found to be both dose- and time-dependent. Strong synergism was documented when cells were treated with various combination ratios of TAC with GEM and 5FU. Finally, immunoblotting revealed that Tac affects the function of p90RSK by inhibiting some of the most important phosphorylations for the activation of these kinases. **Conclusions:** The results indicate significant in vitro anticancer activity against the PANC-1 cancer cell line via a dose- and time-dependent mechanism involving inhibition of p90RSKs. The mechanism of action of this compound appears to be related to RSKs but is still under investigation in our laboratory.
Keywords: Kaempferol-derived flavonoids, Tiliroside, RSK, Pancreatic cancer

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O16

Amassing a treasure trove for drug repurposing using chemoproteomics

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Most of the human proteome remains pharmacologically unaddressed. Hence, in the context of personalised medicine, following-up on discoveries of dysregulated cellular pathways with suitable drugs can be arduous. Because small molecule drugs hit off-targets more often than not, drug repurposing constitutes a fast drug discovery strategy for unaddressed proteins.We have developed chemoproteomics assays that allow us to systematically identify the off-targets of classes of drugs. For such assays, affinity matrices for particular families of proteins (e.g. the kinome) need to be engineered. These are made of selections of bead-immobilised analogues of promiscuous drugs. The proteins, which they specifically bind to, constitute the native protein “panel” of the assay. They remain bound to the beads after washing and can be identified and quantified by LC-MS/MS after digestion (i.e. using a bottom-up proteomics readout). When a lysate is incubated with a drug of interest, the drug binds its targets in accordance with its affinity for each and every of them, and henceforth reduce the enrichment of the targets by the affinity matrix. The dose-dependent reduction of the quantity of a protein remaining on the affinity matrix across the lysate aliquots incubated with increasing doses of a drug, designates this particular protein as a target of the drug and provides an affinity measure. Henceforth, for each drug, a selectivity profile is obtained.With such a chemoproteomics technology, we have notably revealed the target landscapes of clinical HDAC and kinase drugs that include the affinities for all (off-)targets and selectivity metrics. Exploring this treasure trove allows to e.g. propose chemical probes to validate biological targets, or explore HDAC inhibitors as MBLAC2 leads in medicinal chemistry programmes or repurpose kinase inhibitors for

protein kinases such as EPHA2 that lack dedicated drugs.
Keywords: drug discovery, kinase, HDAC, proteomics

O17

Characterization of heterogeneity of cancer-associated fibroblasts isolated from PDAC patients

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Background: Pancreatic cancer is one of the most lethal cancers with a five-year survival rate of 5% to 12%. Pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of all pancreatic cancers and is characterized by a dense stroma that can occupy up to 80% of the total tumor volume. Most of the stroma consists of activated pancreatic stellate cells (PSCs), which are the major source of cancer-associated fibroblasts (CAFs) in PDAC. CAFs are crucial for the formation of desmoplastic stroma and participate in tumor progression and metastasis, and are responsible for chemoresistance. There are at least two phenotypes of CAFs in PDAC – myofibroblasts and inflammatory fibroblasts – which differ in localization, function and expression levels of biomarkers. As CAFs contribute to the pathology of the disease it is of utmost importance in PDAC research to have adequate models that will reflect the heterogeneity of CAFs. The aim of this study was to isolate, characterise and propagate CAFs from PDAC tumors for *in vitro* research. **Patients and methods:** We isolated fibroblasts from PDAC tissue of three patients by the outgrowth method. Fibroblasts were characterized by determining the expression of myCAF and iCAF markers (αSMA, FAP, COL1, FN, IL-6) by immunocytochemistry, western blot and qRT-PCR. To model cancer-stroma interactions we used transwell co-cultures of CAFs with PANC-1 cancer cells and performed dot blot quantification of growth factor and cytokine expression. **Results and conclusions:** We isolated and analysed CAF populations from three patients and propagated them up to 10 passages. The fraction of myCAFs and iCAFs varied between established cell lines, as well as cytokine/growth factor profile in CAF-PANC1 co-culture. Given the patient-to-patient variability, it is very important that in the further course of research we have CAFs models that will properly reflect the heterogeneity of patients and that will give clearer answers to how the tumor microenvironment affects chemoresistance in PDAC.
Keywords: PDAC, cancer-associated fibroblasts, heterogeneity

O18

Exploring the anticancer activity of essential oil of *Satureja montana L.* from Montenegro

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Background: It has been demonstrated that aromatic plant essential oils (EO) contain phytochemicals with anti-inflammatory, antioxidant, and anticancer activities. The goal of this study was to examine the cytotoxicity and the anticancer action mechanisms of an EO extracted from *Satureja montana* plant species, originating from Montenegro. **Material and methods:** The cytotoxic activity was assessed against human cancer cell lines: cervical adenocarcinoma HeLa, malignant melanoma A375, colorectal adenocarcinoma LS 174T, lung carcinoma A549, as well as against normal human lung fibroblasts MRC-5 by MTT assay. Using flow cytometry, it was examined how HeLa cells were distributed throughout the cell cycle after treatment with EO and whether caspase-3, caspase-8, and caspase-9 were activated. RT-qPCR was used to assess the expression levels of genes and miRNA in HeLa cells. **Results:** *Satureja montana* EO exerted strong cytotoxicity against human cancer cell lines, with IC50 values ranging from 0,12 to 0,18 µL/mL, The strongest cytotoxic activity of EO was observed against lung carcinoma A549 cells with an IC50 value of 0.12 µL/mL and LS 174T colorectal adenocarcinoma cells with IC50 value of 0.13 µL/mL. The lowest cytotoxicity was determined against normal lung fibroblasts MRC-5 (IC50 value of 0.19 µL/mL). After 24 hours of treatment with *Satureja montana* EO, a striking increase in the proportion of HeLa cells in the subG1 phase was seen in comparison with control cells. Pretreatment of HeLa cells with caspase inhibitors showed that *Satureja montana* EO activated all three investigated caspases to trigger apoptosis. In comparison to control cells, *Satureja montana* EO treatment of HeLa cells decreased

MMP2 gene expression levels, and elevated *MMP9* and *VEGFA* gene expression levels. *Satureja montana* EO treatment increased the expression levels of the tumor supressive miR-16 and miR-34ain HeLa cells, and increased levels of miR-21, with oncogenic role in cervical cancer. **Conclusion:** *Satureja montana* EO showed strong cytotoxic effects on tested cancer cells. EO exerted prominent apoptotic activity in HeLa cells via intrinsic and extrinsic pathways. Decrease in the expression levels of *MMP2* gene, involved in invasion and metastasis, and cancer suppressive miRNAs, further support its potential to be utilized as a therapeutic agent in cancer treatment.
Keywords: cytotoxicity, *Satureja montana*, essential oil, apoptosis

POSTER PRESENTATIONS

P01

A pilot study of the association between variants rs25487 of *XRCC1* gene, rs1801320 of *RAD51* gene, and rs13181 of *ERCC2* gene and acute toxicity of radiation therapy after radical prostatectomy in patients with prostate cancer

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Background: Prostate cancer is one of the most common malignant diseases in men older than fifty. Radiotherapy is a golden standard for the treatment of prostate cancer, but the occurrence of toxicity in some patients affects the schedule and the outcome. The most common types of toxicity are gastrointestinal toxicity (GI) and genitourinary toxicity (GU). Genetic variants of DNA repair genes *XRCC1*, *RAD51,*and *ERCC2* introduce changes in the expression and function of proteins and might be associated with acute toxicity of radiation therapy. The aim of this pilot study was to explore the association between variants rs25487 of *XRCC1*, rs1801320 of *RAD51,* and rs13181 of *ERCC2* and acute toxicity of radiation therapy after radical prostatectomy in patients with prostate cancer. **Material and methods:** The study included 40 patients (age range 68.4, median 69) treated with radiation therapy after radical prostatectomy. Genomic DNA was isolated from blood leukocytes. Restriction fragment length polymorphism analysis (RFLP-PCR) was used for *XRCC1*, *RAD51,* and *ERCC2* genotyping. Statistical analysis included Fisher's exact test. **Results:** Out of 40 examined patients, 18 (45%) showed acute toxicity. Among patients with toxicity, 9 manifested GU toxicity, 6 GI and 3 patients manifested both types of acute toxicity. In the group with GI toxicity, 1 recessive homozygote, 2 heterozygotes and 3 dominant homozygotes for *XRCC1* were detected. One recessive homozygote, 1 heterozygote and 4 dominant homozygotes were detected for *RAD51*. For *ERCC2* was detected one dominant homozygote and 5 heterozygotes. In the group with GU toxicity, 1 patient was recessive homozygote for *XRCC1*, 2 were heterozygotes and 5 were dominant homozygotes for *XRCC1*. Seven dominant homozygotes and 2 heterozygotes were detected for *RAD51* and *ERCC2*. In the group of patients with both types of toxicity, all of them were dominant homozygotes for *XRCC1* while two patients were dominant homozygotes and one was heterozygote for *RAD51*. One patient was dominant homozygote and two were heterozygotes for *ERCC2*. **Conclusion:** The association between analyzed polymorphisms and acute toxicity was not detected, which may be a consequence of the small number of enrolled patients and low statistical power. A prospective study involving more patients is currently ongoing in an effort to profile patients which would require closer monitoring for the occurrence of acute toxicity by a minimally invasive and low-cost method.
Keywords: acute toxicity, *ERCC2*, polymorphism, prostate cancer, *RAD51*, *XRCC1*.

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P02

Overview and data management of gastropancreatic oncology biobank sample and data collection

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Background: In the last three years, the involvementof Biobanks intranslational research has escalated.Research in COVID-19 has been globally prioritized, with apublication rate in the field of biobanking of8.5%. Remarkably, the impact of Biobanks' collaboration in another cutting-edge area, oncology, is even higher, accounting for 20.3%

ofpublished papers in the biobanking setting. TheGastropancreaticOncology Biobank collection was born in2008 to provide oncological research groups with high quality human biological samples, as well as its associated demographic and pre-analytical data.Our Biobankcoordinates and ensures the quality of the collection, processing, storage and procurement of this cohort to assure optimal sample quality and traceability. Of note, a critical step is the importance of monitoring the pre-analytical and demographic data availability and their correct annotation. Likewise, although the biobank workflow includes well documented checkpoints to avoid any issues that can compromise the quality of the samples or their derivatives or further implications in downstream applications, incidental events can occur. Therefore, it is very important to control them in order to improve the Biobank's well-functioning.In this project we aimed to: (i) To promote and enhance sample and data utilization ratesby researchers of the Gastropancreatic Oncology cohort in order to foster biomedical research; and(ii)To detail the data managementpipeline and to analyze the features of incidental eventsof the Gastropancreatic Oncology Biobank collection of the Hospital Clínic de Barcelona - FundacióRecercaClínic Barcelona – Institutd'InvestigacionsBiomèdiques August Pi iSunyer Biobank (HCB-FRCB-IDIBAPS Biobank).

Material and methods:

Gastropancreatic oncology repositorygeneral workflow

Blood samples are collected from donors of the Gastroenterology Clinical Service of HCBto obtain plasma, serum, DNA, and peripheral blood mononuclear cells (PBMCs). Fresh blood samples are sent to the HCB-FRCB-IDIBAPS Biobank for registering, processing, and storage.Samples are coded and linked to the clinical registries for subsequent traceability and clinical characterization in the biobank's standardized Laboratory Information Management System (LIMS) named NorayBanks.This softwareenables the traceability of samples and their associated data, and the codification/ pseudonymization of samples (according to Spanish legal-ethical frameworkand European Union regulations).

Data management and incidental events

The HCB-FRCB-IDIBAPS Biobank catalogue includes more than 700,000 samples and it is categorized in different biobank sample collections according to their etiology. For this reason, the control of available data is crucial for the proper functioning of the facility. In this sense, a pipeline and a workflow for data management was designed and established in the HCB-FRCB-IDIBAPS Biobank.After a pilot study to assess the viability of the workflow, we implemented the pipeline and the data management workflow was extended to all the biobank catalogue collection, including the Gastropancreatic Oncology collection The data management workflow is based on a Python-coded computational pipeline, with 7 quality checkpoints, which has been designed to ensure: i) data annotation compliance; ii) clean-up of mismatches and duplicates; iii) filling of empty variables and missing data; and iv) assessment of sample incidental events, which allows the determination of the most prevalent incidental events and their cause.

Results:

Gastropancreatic oncology repository

The GastropancreaticOncology repository includes 164,670 aliquots of samples from 10,563 donors from the HCB (Last update 29/06/2023). Samples from different timepoints (follow-up samples) have been collected from 287 donors. The sample types available are plasma (55,556 aliquots), serum (57,764), PBMCs (4,921), EDTA blood (3,397), isolated DNA (10,237) and normalized DNA (32,795) and comprise the following features or diagnostics: colorectal cancer (207 cases), in situ carcinoma (34), invasive carcinoma (58), medium (8,147) and moderate risk of colorectal cancer (168), high risk of colorectal cancer (polyposis syndrome or non-polyposis syndrome) (950), pancreatic cancer (35), intraductal papillary mucinous neoplasm or mucinous cystic neoplasm (64), relative of patient with pancreatic cancer (72), chronic pancreatitis (51), gastric cancer (82), relative of patient with gastric cancer (13), patient at low risk of gastric cancer (8) and patient with gastric cancer precursor lesion (1).Until now, 1,557 aliquots have been procured, and there is the challenge to increase sample utilization rate to guarantee biobank sustainability paired to contribution to science.

Data management and incidental events

The use of a LIMS allowseasy access to samples information, filtering options and future sample procurements registry. Recently, a new data manager was incorporated to handle the huge amount of data exchanged and to ensure the availability and consistency of the minimum essential data and other specific associated data (annotations achieved for almost 90% sample donors).These tools are currently being used to evaluate the whole Biobank data catalogue.Of 10,563 donors of the GastropancreaticOncology repository, 8,867 cases have a complete available dataset (83.94%), 25 donors have no associated data (0.2%) and the other 15.86% are incomplete but in process of being obtained. The incidental event rate in the biobank is 0.84% of the total processed aliquots (852,642). Of the 7,167 incidental events, 36.75% affected the OGP collection. The incidental event rate in the OGP collection is 11.84% of the 22,239total processed samples. The analysis determines that, of the 2,634 OGP incidental events,41.23% of compiled incidental events were due to the blood condition before its reception (e.g., hemolyzed samples, cloudy samples, >24h post-extraction processing). Therefore, the occurrence of incidental events has been monitored by the workflow to establish corrective actions that avoid any possibility to compromise the quality of the sample or its derivatives or further implications in downstream applications.

Conclusions:
Gastropancreatic oncology collection is the result of a well-established synergy of multidisciplinary collaborations between clinical / research centers and platforms with a high strategic value.
Sample and data procurement to researchers should be promoted and enhanced to foster biomedical research.
The implementation of the data management pipeline and the monitoring of incidental events enables the continuous improvement of the collection, enhancing oncology research.
Keywords: Gastropancreatic oncology, biobank, data management, colorectal cancer, pancreatic cancer, gastric cancer, sample procurements, quality checkpoints.

P03

Detection of resistant *EGFR* T790M mutation from liquid biopsy samples of patients with advanced non-small cell lung cancer: comparison of qPCR and dPCR detection methods

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Background: In Serbia, at the Institute for Oncology and Radiology of Serbia, testing for the presence of resistant *EGFR* T790M mutation from March 2023 is performed from liquid biopsy samples of patients with advanced non-small cell lung cancer who progressed on therapy with first and second generation tyrosine kinase inhibitors using qPCR and dPCR methods. Our aim was to compare the sensitivity of *EGFR* T790M mutation detection between these two methods. **Materials and methods:** Liquid biopsy samples consisted of 25 blood plasma samples and 4 pleural effusion samples. Testing for the presence of the *EGFR* T790M mutation was performed using the Applied Biosystems QuantStudio Absolute Q Digital PCR System (Thermo Fisher Scientific) and the TaqMan probe Hs000000029_rm for *EGFR* p. T790M mutation and Absolute Q™ DNA Digital PCR Master Mix (5X). **Results:** In the period March-June 2023, 29 samples of liquid biopsies from patients with advanced non-small cell lung cancer were tested for the presence of the resistant *EGFR* T790M mutation, including 25 samples of blood plasma and 4 samples of pleural effusion. The total detection percentage of the resistant T790M mutation by qPCR was 17.24%, while this percentage was higher when testing was done by dPCR, which was 31.03%. 4 of 25 (16%) blood plasma samples were T790M positive when tested by qPCR, while 6 of 25 (24%) samples were dPCR positive for the same mutation. 1 of 4 (25%) pleural effusion samples were positive for T790M by qPCR, while 3 of 4 (75%) pleural effusion samples were positive by dPCR. On the total sample, the % agreement in the results between these two methods was 86.21%, while the value of the kappa coefficient was 0.633, which indicates a “moderate” agreement of the results. All mutations detected by qPCR were also detected by dPCR, but not vice versa. **Conclusion:** With the introduction of the dPCR method for testing the presence of the resistant *EGFR* T790M mutation, the percentage of its detection has increased, and therefore the number of patients who might benefit from osimertinib therapy has increased. Once again, pleural effusion has been shown to be an excellent source for the detection of the resistant T790M mutation.
Keywords: *EGFR* T790M, liquid biopsy, non-small cell lung cancer.

P04

Histomics: Bridging Radiomics and Histopathology Towards Advancing Prognostication of Breast Cancer Metastasis

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Abstract in extenso:

Background: Breast carcinoma has become the most prevalent cancer, surpassing lung cancer and isthe leading cause of cancer-related fatalities in women [1].

Histopathology has a major role in cancer diagnosis and prognosis by providing insights into cellular-level tumor characteristics. It is the basis for determination of pathological grade (1-3, histological assessment of the tumor cell differentiation) and tumor type (mainly based on growth types: invasive ductal, lobular, medullary, mucinous, papillary carcinoma). Traditional diagnostic and prognostic tests rely on the microscopic visual examination of tumor histopathology specimens. However, subjectivity poses a concern within this method. To address this, computer-assisted image analysis applications have emerged to enhance objectivity, focusing on quantifying specific cellular structures of diagnostic and prognostic significance, such as nuclear morphology, mitotic activity, and tubular formation. Often referred to as "pathomics", these approaches aim to overcome subjectivity in assessment [2-4]. In addition to such targeted approach, the unselective analysis of texture information is based on the underlying hypothesis that histopathology contains distributed prognostic information that remains untapped by visual or computational evaluations targeting specific structures. Previous studies have investigated this unselective approach for prognostic purposes [5-7].

Our objective was to more deeply exploit prognostic information from histopathology images by exploring the potential of unselective radiomics analysis, which is a widely used method for analyzing 3D scans but has not yet been explored in histopathologic images.

Methods: The analysis workflow included the preparationof tumor tissue sections, H&E staining, selectionof tissue sections, image acquisition, image normalization, conversion from color to grayscale, extraction of radiomics features, feature selection by of LASSO and multivariable backward stepwise binary regression, prognostic evaluation and validation.

Results and Discussion: Our earlier study demonstrated modest prognostic performance when applying the basic five GLCM features to the analysis of routinely H&E-stained histopathology slides, with AUCs ranging from 0.57 to 0.63 [10]. In contrast, our current 2D radiomics study, conducted on the same group of breast carcinoma patients, yielded significantly improved prognostic performance, with individual features reaching AUCs of 0.75. The superior performance of the current radiomics study, which utilized up to 1208 radiomic features compared to the basic GLCM analysis with only five features may be explained by the fact that radiomics analysis enabled a more comprehensive and detailed characterization of tumor heterogeneity by extracting a larger number of features. This expanded feature set encompasses a wider range of quantitative information, including grayscale intensity relationships, several co-occurrence feature types such as GLCM, GLRLM, GLSZM, GLDM, NGTDM and several image transformations obtained by wavelet, square, square root, logarithm and gradient filters. Such diversity of features allowed for a more comprehensive assessment of tumor characteristics, potentially capturing increased amount of relevant information related to metastasis risk.

The use of medical images in clinical oncology has been expanding from its primary role as a diagnostic tool to a role in precision medicine. To our knowledge, there have been no previous reports of using radiomics methodology to analyze histopathology structure. One report described radiomics-based features for pattern recognition of lung cancer histopathology and metastases, but these features were calculated from computed tomography images, not from histopathology slides [8]. Standard pathology diagnostics relies on the visual microscopic assessment of cellular and tissue morphology by trained experts, which remains subjective and qualitative [9]. A field of comprehensive quantitative pathology data mining called “pathomics” has emerged in the last decade, which also implements computational analysis to histopathology images. However, unlike our current application of exhaustive radiomics texture analysis, pathomics is focused on histomorphometry, with specific visual targets such as the spatial characterization of tumor and stromal regions, the examination of nucleus shapes and textures, the classification of cell types, the quantitative characterization of lymphocytic infiltration, and the estimation of cell counts labeled with various biomarkers by immunohistochemical staining [2]. We propose the term “historadiomics” to describe the exhaustive computational analysis comprising the first-order features together with non visual texture of histological sections.

Radiomics aims to extract high-dimensional imaging features from medical images to improve the reliability of cancer diagnosis, prognosis and prediction. However, such comprehensiveness of the radiomics analysis is its major strength but also its main weakness, due to increase in likelihood of false discoveries, where by statistically significant results are actually due to chance[12]. Therefore, we compared the prognostic performance of the moderately dimensional radiomics analysis including 93 features (AUC=0.83) and the very highly dimensional analysis including 1208 features (AUC=0.92). As expected, the model with more features exhibited better prognostic performance. This suggests that the additional features captured more information about prognosis and metastasis, leading to improved prognostic performance. However, the moderately dimensional analysis with 93 features still delivered very good performance, with an AUC of 0.83.

The radiomic scores were calculated using the feature coefficients presented in Table 1, utilizing the formula: score = value of variable1 * coefficient1 + value of variable2 * coefficient2 + value of variable3 * coefficient3. Because of z-score normalization of feature values, coefficients in Table 1 do reflect the relative importance of features. Table 1 shows the expected prognostic performance of scores, with the score including only CP parameters exhibited the

weakest performance, whereas the score combining CP with 1208 radiomic features demonstrated the most robust prognostic performance.

Table 1. Composition of models for prognostication of metastasis occurrence

Feature	Lambda	Coefficient	P-value
Model[a]: Only CP features; AUC=0.66 (0.53-0.79); P=0.02			
pT	-	0.531	0.03
ER	-	0.262	0.20
Model[b]:CP and 93 radiomic features; l = 0.091; AUC=0.83 (0.72-0.95); P=0.00			
ER	0.086	0.648	0.01
Original FirstorderMean	0.114	2.731	0.02
Original GLCM Imc2	0.200	-0.999	0.00
Original GLDM DependenceVariance	0.099	-0.566	0.15
Model[b]:CP and 1208 radiomic features; l = 0.111; AUC=0.92 (0.84-0.99); P=0.00			
ER	-	0.663	0.01
Original FirstorderMean	0.116	3.230	0.00
Wavelet HHH FirstorderRmad	0.234	-0.773	0.03
Wavelet HLL GLCM Idmn	0.223	1.348	0.01

[a]Feature selection was performed by use of the multivariate stepwise binary regression based on probability of P=0.20 for entry and P=0.20 for removal.

[b]Feature selection performed by use of the logistic LASSO regression, followed by multivariate stepwise binary regression based on probability of P=0.20 for entry and P=0.20 for removal.

Abbreviations: CP, clinicopathological; pT, pathological tumor size; ER, estrogen receptor; LASSO, least absolute shrinkage and selection operator; GLCM, Gray-Level Co-occurrence Matrix; Imc2, Informational Measure of Correlation; Rmad, Robust Mean Absolute Deviation; Idmn, Inverse Difference Moment Normalized.

The performance of the calculated models is visually demonstrated without statistical arbitration in Figure 1, illustrating how increasing numerical values of each feature stratify tumors with and without metastasis. The stratification performance of the CP score and CP+93 radiomic features score was less pronounced on the low-risk side, unlike previous reports that demonstrated better stratification of the low-risk group [11]. On the other hand, the score composed of CP+1208 radiomic features showed a nearly symmetrical performance on both risk sides, likely due to its exceptionally strong overall prognostic capacity (Figure 1).

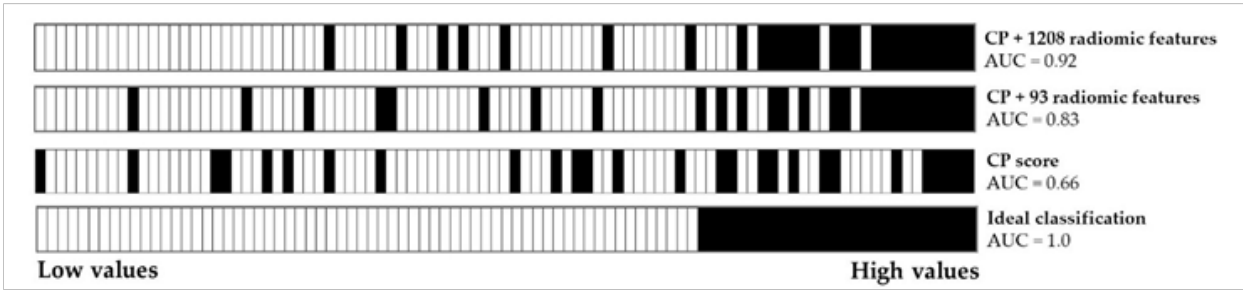

Figure 1. The prognostic performance of the computed models. A simplified visual representation of the classification between patients with and without metastasis occurrence,illustrated as the alignment of continuous values with the actual metastasis outcome. In this representation, black and white fields correspond to patients with and without actual metastasis occurrence, respectively. The continuous values of the indicated scores are ordered from lowest (left) to highest (right) to show how increasing values enrich patients with higher metastasis risk. An ideal discrimination with AUC=1.0 is included for comparison.

In conclusion, we propose a deep textural analysis of histopathological slides using an unselective radiomic analysis methodology. We showed that such Historadiomics analysis can achieve excellent prognostic performance in moderate-dimensionality format and outstanding prognostic performance using very high-dimensionality. This type of analysis has the potential to enable precision medicine in two ways: on its own, and by developing multimodal prognostic and predictive approaches that incorporate historadiomics features with other data, such as visual histopathological examination, radiomics, pathomics, genomics, clinicopathological and laboratory data.

Keywords: breast cancer, histopathology, radiomics, prognosis, texture analysis, tumor, histology, metastasis, biomarker, dimensionality

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P05

Effects of promoter methylation and mutation on *BRCA1/2* expression in ovarian cancer

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Background: Ovarian cancer is a highly lethal disease, and Serbia has the second-highest incidence of the disease worldwide. A significant proportion of ovarian cancer cases are attributed to germline and somatic alterations in the *BRCA1/2* genes. *BRCA1* and *BRCA2* are tumor suppressor genes coding for proteins involved in DNA double strand break repair by homologous recombination. DNA methylation at CpG sites in the promoter region can change gene expression. In this study we investigated the relationship between gene expression and promoter hypermethylation of *BRCA1/2* genes in ovarian cancer patients previously tested for somatic mutations as part of standard diagnostic

procedure. **Materials and Methods:** We employed a retrospective cohort design for the investigation, which was carried out at the Institute for Oncology and Radiology of Serbia. Starting material for this study was FFPE tissue of 47 ovarian cancer patients who were previously tested for somatic *BRCA1/2* mutations, as well as promoter methylation. FFPE blocks were cut by hand considering percentage of tumor cells and the position of the tumor tissue in the block. RNA was extracted by RNeasy FFPE Kit (Qiagen, Hilden, Germany) according to manufacturer recommendation. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Vilnius, Lithuania). Expression measuring was performed by real time PCR (Light cycler 480, Roche) using SYBR Green chemistry (Applied Biosystems, Vilnius, Lithvania). For every sample there were 3 tested genes, one housekeeping (*GAPDH*) and two target (*BRCA1/2*) genes. Statistical analysis was performed using non parametric test (Mann Whitney t-test). **Results:** We examined *BRCA1/2* expression in two groups, *BRCA1/2* promoter non-methylated and methylated samples, as well as *BRCA1/2* mutated and wild type. With a p value of 0.0042, we discovered a significant difference in *BRCA1* expression between non-methylated and fully/intermediary methylated *BRCA1* patients, but no statistically significant difference was observed for *BRCA2* gene. **Conclusion**: Several cellular biological mechanisms regulate the expression of the BRCA1and BRCA2 proteins. While we observed a silencing effect of *BRCA1* promoter methylation, no such effect was found for *BRCA2,* indicating that other mechanisms may be in play to regulate its expression.
Keywords: *BRCA1/2* genes, MSP, NGS, ovarian cancer, promoter hypermethylation, somatic mutation

P06

Ultra-short cfDNA fragment detection during systemic therapy of advanced-stage colorectal cancer

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Background: Circulating cell-free DNA (cfDNA) has gained significant attention as a biomarker of response to systemic therapy. Distinct mutational signatures, DNA methylation patterns and fragmentation profiles of tumor-derived cfDNA have been reported in cancer patients. The aim of the study was to evaluate longitudinally the changes in cfDNA fragmentation profile and correlate it with disease progression in colorectal cancer (CRC) receiving anticancer therapy. **Material and methods:** We recruited 14 patients with advanced stage colorectal cancer (CRC) and performance status 0-2 at diagnosis undergoing standard-of-care therapy at the Institute for Oncology and Radiology of Serbia. All patients were initially tested on PD-L1 expression as well as on mutations in *EGFR* and *KRAS* genes. Consecutive plasma samples were collected at diagnosis, during treatment and after termination of the therapy. Magnetic based cfDNA extraction kit (MagMax) was used for isolation of cfDNA from 0.5 mL of plasma, and quantified using Qubit HS dsDNA assay and ddPCR. Contamination with genomic DNA was determined using a B-cell-specific ddPCR assay. Fragment size distribution of cfDNA was determined by Agilent Bioanalyzer HS dsDNA assay. Custom single-stranded DNA library prep method was used to generate libraries for genome-wide DNA methylation analysis and targeted sequencing of actionable mutations (QIASeq solid Tumor hotspot panel). **Results:** While absolute concentrations measured by ddPCR (~17.51 ng/mL of plasma) were generally lower than those estimated by Qubit (~23.88 ng/mL of plasma) there was a strong correlation (Pearson correlation, R=0.97). In a 41% of samples there was small contamination by peripheral blood cells gDNA (<6% of total cfDNA), and one sample was discarded with 19% contamination. Beside the expected mononucleosomal 168bp cfDNA fraction was observed in all samples, we observed an ultra-short fraction of cfDNA (50bp mode) in 79% of samples. **Conclusions:** Application of magnetic-based cfDNA extraction and use of single-stranded DNA library preparation method allowed us to detect a previously unrecognized ultra-short cfDNA fraction in colorectal cancer patients. The analysis of DNA methylation and mutational profiles of cfDNA fractions is ongoing.
Keywords: colorectal cancer, cfDNA, ddPCR

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P07

Comparison of variant calling tools for mutation analysis of *BRCA1* and *BRCA2* genes in patients with epithelial ovarian cancer

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Background: Serbia is the world's leading country in incidence and mortality rate of breast and ovary cancer. Mutations such as single nucleotide variants (SNVs), indels, insertions, deletions, in damage repair genes, most often in *BRCA1* and *BRCA2* genes, can lead to forming breast and ovarian cancer. Current therapy is based on PARP inhibitors which cause synthetic lethality in *BRCA*-mutated cells and it is dependable on accurate variant detection. **Matherial and methods:** In this study, specimens of 11 patients with serous epithelial ovarian cancer from Institute for Oncology and Radiology were sequenced on MiSeq Illumina sequencer, after which raw data were analyzed bioinformatically. Bioinformatic analysis included checking quality control of raw FASTQ sequences with fastqc tool, trimming with trim galore tool, mapping them on reference genome (hg19) with bwa mem tool and variant calling. We used six variant calling tools including FreeBayes, Mutect2, GATK HaplotypeCaller, Samtools, VarScan and VarDict callers and evaluated the similarities and differences between the callers for detection SNV/indels in *BRCA1* and *BRCA2* genes, as well as their concordance rate, unique variant rates and clinical relevance. **Results:** Similarity between the callers was searching for germlines and somatic mutations in tumor only samples. The differences were reflected in the target specificities which implied that HaplotypeCaller and Vardict were calling variants only in chromosome 13 and 17 and the rest of the callers searched the variants all over the genome. Results showed the highest concordance rate between VarScan and HaplotypeCaller and high concordance rate between Samtools, FreeBayes and Mutect2. VarDict had the highest unique variant rate, where as VarScan and HaplotypeCaller had the lowest. Also, the VarScan showed highest clinical relevance, that is, it showed the highest percentage of variants with VAF>5% and coverage>500 on chromosome 13 and 17. **Conclusion:** Variant caller VarDict has the lowest concordance rate, while the VarScan and HaplotypeCaller have the highest concordance rate in comparison to other variant calling tools. Variant callers VarScan and HaplotypeCaller havethe lowest unique variants rate, while the VarDict has the highest unique variants rate. Variant callers VarDict and HaplotypeCaller show only target-specific variants, while the other variant calling tools also show target-nonspecific variants. Variant calling tool VarScan is most clinical relevant tool.
Keywords: breast and ovary cancer, *BRCA1* gene, *BRCA2* gene, data analysis, variant calling tools, sequencing
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P08

Expression and heteromerization of adenosine A2A and dopamine D2 G protein-coupled receptors in neuroendocrine tumors of the lung

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Background: Dopamine and adenosine receptors have been recognized as new pharmacological targets for lung neuroendocrine tumors and their expression linked with NET aggressiveness. The aim of this study was to evaluate the differences in genetic and protein expression and potential heteromerization of A2AR and D2R receptors in NETs and to correlate it with patient and tumor characteristics, therapy response and disease recurrence. **Patients and methods:** In silico analyses using Human Protein Atlas and String were performed to evaluate the significance of A2AR and D2R receptors in NETs. The carcinoid patient groups consisted of 26 typical (TC, 15 females and 11 males, age range 17-75, median 50) and 26 atypical (AC, 12 females and 14 males, age range 29-72, median 53) samples. Evaluation of genetic and protein expression were performed by RT-PCR and Western blot. A2AR-D2R heteromerization was evaluated by Proximity ligation assay. For large cell NET, the NCI-H460 cell line and the resistant NCI-H460/R cell line, obtained by

gradual doxorubicin treatment were used. The functional properties were analyzed by studying the MAPK pathway, Dynamic Mass Redistribution (DMR) and the production of cAMP. Statistical significance was set at $p<0.05$. **Results:** No significant differences in expression of A2AR and D2R were detected between TC and AC at the genetic level. At the protein level, AC had statistically significant higher expression of A2AR than TC ($p= 0.0458$). No A2AR-D2R heteromers were detected in TC samples, while 80% of AC samples showed heteromerization. The existence of the A2AR-D2R heteromers was confirmed in both large cell NET cell lines, with 80% and 85% of cells containing heteromers, and a ratio of 13 and 16 heteromers per heteromer-containing cell, respectively. Co-stimulation with both agonists did not produce an additive effect (negative cross-talk). Antagonist binding to one of the receptors blocked signaling of the other receptor (bidirectional cross-antagonism) in both cell lines (MAPK and DMR results). By studying cAMP production, negative cross-talk was detected in both cell lines and cross-antagonism only in NCI-H460. **Conclusions:** Functional differences were observed in the expression and heteromerization of A2AR and D2R between typical and atypical neuroendocrine lung tumors and the sensitive and resistant neuroendocrine large cell tumor cells. This data will be validated in a prospective cohort of lung cancer patients.

Keywords: Adenosine, dopamine, GPCR, heteromerization, lung cancer, neuroendocrine tumors

P09

Detection of viral proteins in locally advanced rectal cancer patient samples by mass spectrometry – predictive potential for response to neoadjuvant chemoradiotherapy

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Background: Phenotypic profile of patients with locally advanced rectal cancer (LARC) is still unexplored and represents a field of great potential. Standard treatment for LARC involves neoadjuvant chemoradiotherapy (nCRT) followed by total mesorectal excision. Response to therapy varies and there is an unmet clinical need for new predictive markers. The aim of this study was to detect viral proteins in locally advanced rectal cancer patient biopsies by mass spectrometry and to examine their predictive potential for response to nCRT. **Materials and methods:** Data-independent acquisition mass spectrometry (DIA-MS) was performed on a carefully selected LARC patient cohort treated with nCRT. Patients were assessed for tumor response in week 8 post-nCRT (pelvic MRI scan and rigid proctoscopy). No immediate surgery was suggested for patients with clinical complete response (cCR) and initially distant-located tumors ("watch and wait" approach). Response after surgery was assessed using histopathological tumor regression grading(TRG) categories from postoperative specimens using the Mandard scale. Responders(R) were defined as patients with cCR without operative treatment and TRG 1/2. Non-responders(NR) were patients classified as TRG 3-5. Twenty patient samples with the most distinctly different responses to therapy were chosen for comparison – 11 NR and 9 R. DIA-NN was used for the deconvolution of the mass spectra and the Perseus software was used for the statistical analysis of data. **Results:** In total 12 non-human proteins were identified in 20 rectal cancer FFPE samples. After initial processing 7 proteins originating from viral particles were detected, including proteins encoded by the following genes: L4 from Human adenovirus 2,5 and 12, U17/U16 from Human herpesvirus 6A, U44 from Human herpesvirus 7, UL26 from Human herpesvirus 1, UL47 and UL82 from Human cytomegalovirus and L1 from HPV28, HPV30 and HPV53. Differential expression analysis suggested that protein encoded by L4 gene was differentially over-expressed in R/NR ($p= 0.046$). **Conclusion:** Locally advanced rectal cancer (LARC) patient samples contain proteins derived from viral genomes. The detection of these viral proteins suggests the significance of infections in the development of rectal cancer and its response to therapy.

Many of these viruses have been associated with the development of other types of cancer, indicating a promising avenue for further validation and research in this area.

Keywords: data-independent acquisition mass spectrometry, neoadjuvant chemoradiotherapy, proteomics, rectal cancer, viral infection, viral proteins

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P10

Prognostic value of combined hematological/biochemical indexes and tumor clinicopathologic features in colorectal cancer patients — a pilot single center study

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Background: Colorectal cancer (CRC) is a significant public health problem. There is increasing evidence that the host's immune response and nutritional status play a role in the development and progression of cancer. The aim of our study was to examine the prognostic value of clinical markers/indexes of inflammation, nutritional and pathohistological status in relation to overall survival and disease free survival in CRC. **Patients and Methods:** The total number of CRC patients included in the study was 111 and they underwent laboratory analyses within a week before surgery. Detailed pathohistological analysis and laboratory parameters were part of the standard hospital pre-operative procedure. Medical data were collected from archived hospital data. Data on the exact date of death were obtained by inspecting the death registers for the territory of the Republic of Serbia. All parameters were analyzed in relation to the overall survival and survival period without disease relapse. **Results:** The follow-up median was 42 (24–48) months. The patients with the III, IV and V degrees of the Clavien–Dindo classification had 2.609 (HR: 2.609; 95% CI: 1.437–4.737; p = 0.002) times higher risk of death. The modified Glasgow prognostic score (mGPS) 2 and higher lymph node ratio carried a 2.188 (HR: 2.188; 95% CI: 1.413–3.387; p < 0.001) and 6.862 (HR: 6.862; 95% CI: 1.635–28.808; p = 0.009) times higher risk of death in the postoperative period, respectively; the risk was 3.089 times higher (HR: 3.089; 95% CI: 1.447–6.593; p = 0.004) in patients with verified tumor deposits. The patients with stage III/IV and tumor deposits tumor deposits had 1.888 (HR: 1.888; 95% CI: 1024–3481; p = 0.042) and 3.049 (HR: 3.049; 95% CI: 1.206–7.706; p = 0.018) times higher risk of disease recurrence, respectively. The emphasized peritumoral lymphocyte response reduced the risk of recurrence by 61% (HR: 0.391; 95% CI: 0.196–0.780; p = 0.005). **Conclusions:** Our study presents evidence that standard laboratory parameters, which do not present any additional cost for the health system, may provide additional information on the CRC patient outcome and lay the groundwork for a larger prospective examination. In our patient cohort, Clavien–Dindo classification of postoperative complications, modified Glasgow prognostic score, lymph node ratio, tumor deposits and peritumoral lymphocyte response were factors that were significantly associated with survival of operated patients.

Keywords: biochemical indexes, colorectal cancer, prognostic value, tumor clinicopathologic features

P11

The polymorphisms of genes encoding antioxidant enzymes modulate the risk for testicular germ cell tumor

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Background: The simultaneous analysis of redox biomarkers and polymorphisms encoding for regulatory and catalytic antioxidant proteins was performed in order to evaluate their potential role in the development of testicular germ cell tumor (tGCT). **Patients and Methods:** NRF2 (rs6721961), GSTM3 (rs1332018), GSTO1 (rs4925), GSTO2 (rs156697), GSTO2 (rs2297235), SOD2 (rs4880) and GPX3 (rs8177412) polymorphisms were assessed in 88 patients with tGCT (52 seminomas) and matched controls, through various logistic regression models. The plasma levels of DNA oxidative damage (8-hydroxy-2'-deoxyguanosine, 8-OHdG), protein oxidative damage (thiol groups, TG) and lipid oxidative damage (malondialdehyde , MDA) were measured in the group of tGCT patients. **Results:** A significant association between variant GPX3rs8177412*TC+CC genotype and risk of overall tGCT, as well as seminoma development, was found. Moreover, carriers of variant SOD2rs4880*TT genotype were at almost 3-fold increased risk of seminoma development. Interestingly, combined SOD2rs4880*TT/GPX3rs8177412*TC+CC genotype conferred a 7-fold higher risk for tGCT development. In addition, the carriers of the GSTO1rs4925*C/A*C/C genotype exhibited an increased risk for tGCT development. Significant association with increased risk of tGCT was observed in carriers of GSTO2rs2297235*A/G*G/G genotype, and in carriers of combined GSTO2rs156697*A/G*G/G and GSTO2rs2297235*A/G*G/G genotypes. Moreover, the carriers of the GSTO2rs156697*G allele were in particular at higher risk of developing seminoma compared to the carriers of GSTO2rs156697*AA genotype. Haplotype H7 (GSTO1rs4925*C/GSTO2rs2297235*G/GSTO2rs156697*G) carriers exhibited higher risk of tGCT, however, without statistical significance. Still, the presence of assessed genetic variants was not associated with significantly higher levels of redox biomarkers in both tGCT patients, as well as in those diagnosed with seminoma. **Conclusions:** In conclusion, the polymorphic expression of certain antioxidant enzymes might affect their protective antioxidant activity, therefore predisposing susceptible individuals toward higher risk for testicular GCT development. Keywords:testicular GCT, oxidative stress, polymorphism, antioxidant proteins, redox biomarkers

P12

Complementarity of miR-203a-3p and ETS-1 sequences may influence agressiveness of papillary thyroid carcinoma

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Background: Papillary thyroid carcinoma (PTC) is the most frequent endocrine tumor, with large variability in histological features and clinical outcomes. Therefore, novel indicators for identifying high-risk PTC patients are constantly required. In order to determine the prognostic value of ETS-1 in PTC patients, ETS-1 protein levels in divergent cell compartments were associated with known clinicopathological parameters of PTC patients. Furthermore, cytoplasmic ETS-1 levels, paired with miR-203a-3p levels, were linked to the incidence of unfavorable clinical characteristics of the patients. **Patients and Methods:** 74 cases of fresh frozen PTC tissue samples and matched archival PTC tissue samples. ETS-1 was tested immunohistochemically. Quantitative RT-PCR was used to determine the levels of miR-203a-3p. The results were related to the clinicalopathological characteristics of the patients. Bioinformatic analysis was performed using freely available online tools for the prediction of biological interactions. **Results:** ETS-1 was found in the cytoplasm of 91.9% PTC cases and the nucleus of 78.4% PTC cases, at variable levels. It was also found in some areas of the adjacent

non-malignant thyroid tissue, with predominately nuclear localization. Cytoplasmic levels of ETS-1 protein correlated with the pT status of PTC patients ($p = 0.045$, $r = -0.236$). Nuclear levels of ETS-1 correlated with the lymph node metastasis of the patients ($p = 0.034$, $r = -0.248$). Bioinformatic analysis and model prediction revealed that miR-203a-3p shares a seed sequence with the 3'-untranslated region of ETS-1 mRNA. The mutual distribution of ETS-1 and miR-203a-3p average levels differs between aggressive and non-aggressive PTCs ($p < 0.05$). In a group of PTC patients with aggressive clinical features (higher grade, invasion or higher levels of tumor infiltration), ETS-1 levels were proportional to miR-203a-3p levels, whereas in non-aggressive PTCs, average ETS-1 expression was lower in the miR-203a-3p highly expressed group than in the low expresing one. **Conclusion:** ETS-1 could be used to identify high-risk PTC patients, however its cell localization should be considered. MiR-203a-3p may bind to ETS-1 mRNA in non-aggressive PTC and so downregulate its protein expression and influence PTC progression. In contrast, it appears that such regulation is disrupted in aggressive PTCs, since average ETS-1 protein levels follow miR-203a-3p level of expression.
Keywords: cancer biomarker, prognostic factor, microRNA, subcellular localization

P13

Characterization of nischarin expression in pancreatic ductal adenocarcinoma

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Background: Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal, aggressive malignancy with a high metastatic rate. PDAC is characterized by a lack of early symptoms, leading to late-stage diagnosis and poor clinical outcomes. Recent research is focusing on identifying novel biomarkers that may also serve as drug targets both in the epithelial cancer compartment and in the cancer stroma. Nischarin (NISCH), a multifunctional scaffolding protein, has been shown to have a tumor suppressive role in several cancer types. Its expression and role have not been reported for PDAC. The purpose of this study was to examine the level and localization of NISCH in tumor tissue form the PDAC patients. **Materials and Methods:** To gain preliminary insights, we utilized online databases (ProteinAtlas and TIMER) to analyze NISCH expression in PDAC and its association with tumor purity and selected markers of the tumor epithelium and the stroma (ACTA2, FAP, VIM, CDH1, CDH2, COL1A1, FN1). Next, NISCH expression was examined in total of fifty PDAC tissues representing different tumor regions derived from 10 patients (5 male and 5 female) by qRT-PCR. All the tumor regions were also examined by qRT-PCR for co-expression of NISCH and stromal and epithelial markers. In select patient samples, NISCH protein expression and co-localization with E-cadherin or α-smooth muscle actin was examined by immunohistochemistry. **Results:** Public database analysis revealed a significant downregulation of NISCH in PDAC tissues compared to the normal pancreatic tissue. Based on the correlation with the tumor purity, and partial correlation with both selected mesenchymal and epithelial markers (VIM, CDH2, CDH1, FN1), nischarin may be present in both the epithelial and the stromal compartment of PDAC. RT-qPCR analysis of distinct tumor regions of PDAC patients revealed great heterogeneity of NISCH expression within the tumors. Immunohistochemistry staining confirmed that nischarin could be observed in both the tumor epithelial and in stromal compartment.**Conclusions:** Based on the data from the public databases, NISCH expression was significantly downregulated in PDAC, suggesting its potential tumor suppressor role. The correlations observed between nischarin expression, tumor purity, and select epithelial and mesenchymal markers both in the public databases and in our patient cohort imply that nishcarin is expressed in both the cancer and the stromal compartment of the tumor. This is of importance, as there are several approved nischarin agonists that may be repurposed. Our findings imply that effects of these agonists have to be examined not only on cancer cells, but also on the cells of the tumor stroma, such are cancer-associated fibroblasts, tumor vessels and the immune infiltrate.
Keywords: biomarkers, PDAC, nischarin

P14

Expression profile of CD81 gene transcripts in colorectal cancer

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Background: The activity profile of alternative promoters may be an indicator of tumor characteristics. Alternative promoters of CD81 gene were shown to be differentially active in colon and rectal cancer tissue. The promoter active in colon and rectal cancer gives rise to transcripts CD81-205 and CD81-215, while the promoter active in normal gut mucosa gives rise to transcripts CD81-203 and CD81-213. This study aimed to analyze the relative abundance of the CD81 gene transcripts in colorectal cancer. **Material and Methods:** Transcripts generated from two alternative promoters of CD81 gene were analyzed by qPCR in the following sets of samples: human colon cell lines grown as 3D spheroids (non-malignant HCEC-1CT and malignant DLD1, HCT116 and SW620); ten pairs of tumor and non-tumor tissue samples from patients with colon cancer; five pairs of tumor and non-tumor tissue samples from patients with rectal cancer. The total expression of CD81 gene was analyzed as well. **Results:** Analyzed transcripts were represented with low frequency in the total amount of CD81 gene transcripts (0.2-3.3% for cell lines; 0.2-15.3% for colon tissue; 0.3-19.1% for rectal tissue). In non-malignant cell line HCEC-1CT, an approximately equal level of the transcripts of both promoters was shown. In the malignant cell lines and all analyzed tissue samples, the relative abundance of transcripts CD81-205 and CD81-215 was higher than transcripts CD81-203 and CD81-213. **Conclusions:** These findings showed the biomarker potential of the CD81 gene alternative transcripts and indicate their potential role in colorectal cancer. Increased transcript abundance in both tumor and non-tumor tissue samples in comparison to the cell lines indicates their stromalorigin.
Keywords: biomarker, cancer, colon, rectum

P15

Genetic polymorphisms of enzymes involved in redox homeostasis can influence survival in smokers and overweight patients with prostate cancer

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Background: Prostate cancer (PC) represents a major cause of mortality in developed countries. Nevertheless, due to disease heterogeneity, in certain cases, prognosis can be difficult to anticipate. It is believed that oxidative damage can also affect prostate carcinogenesis. Genetic single nucleotide polymorphisms (SNPs) of enzymes involved in redox homeostasis can lead to oxidative damage and contribute to patients' shorter overall survival. The aim of this study was to investigate whether SNPs of glutathione peroxidase 1 (GPX1 rs1050450), superoxide dismutase 2 (SOD2 rs4880) and regulatory antioxidant protein nuclear factor erythroid 2-related factor 2 (Nrf2 rs6721961) stratified by different age groups and acquired risk factors (obesity, hypertension, and smoking status) can affect overall survival. **Material and Methods:** Total of 235 patients with histologically confirmed prostate cancer treated at the Institute forOncology and Radiology of Serbia and the Urology Clinic of the Clinical Center of Serbia in Belgrade were included in the study. The epidemiological data was collected from standard questionnaires and patients' medical records. Isolated DNA from whole blood was used for quantitative polymerase chain reaction (qPCR) to detect SOD2 and GPX1 gene polymorphisms and polymerase chain reaction with confronting two-pair primers (PCR-CTTP) to detect Nrf2 gene polymorphism. The follow-up time was up to 98 months or death, whichever came first. At the end of the follow-up, 76 patients died, 146 were alive and 13 were lost. Patients were stratified in groups by obesity observed by body mass index (BMI), smoking status, presence/absence of hypertension and age. **Results:** In the overweight group of patients (BMI 25-29.9) shorter survival was observed for carriers of Nrf2*C/C genotype compared to carriers of at least one variant Nrf2*T allele (78 vs 91 months; p=0.022). In smokers, patients with variant GPX1*T/T genotype

had shorter survival when compared to patients with GPX1*C/C or *C/T genotype (71 vs 90 months; p=0.021). SOD2 gene polymorphism showed no difference in survival across all investigated groups. **Conclusions:** Nrf2 (rs6721961) and GPX1 (rs1050450) gene polymorphisms can be potential predictor factors in certain PC patients' subgroups. Keywords:glutathione peroxidase 1, prostate cancer, regulatory antioxidant protein nuclear factor erythroid 2-related factor 2, single nucleotide polymorphism, superoxide dismutase 2

P16

Expression of long non-coding RNA HOTAIR in rectal cancer as a potential predictor of response to chemoradiotherapy

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Background: Patients with locally advanced rectal cancer are mainly treated with chemoradiotherapy (CRT) before surgery. Less than 20% of patients respond completely to neoadjuvant CRT. To avoid unnecessary treatment, biomarkers are being sought to identify patients with rectal cancer who do not respond to therapy. The HOX Transcript Antisense Intergenic RNA (HOTAIR) is a long non-coding trans-acting RNA molecule that is frequently deregulated in cancers of the digestive tract and plays a role in chemoresistance. The aim of this study was to investigate HOTAIR as a potential biomarker for predicting treatment response in patients with rectal cancer. **Methods:** The study group consisted of 14 patients with rectal cancer treated with neoadjuvant CRT. RNA was isolated with TRIzol reagent from samples of rectal cancer and non-tumour tissue before and after therapy. The relative expression of HOTAIR, normalized to GAPDH, was analyzed by qRT-PCR. **Results:** There was no difference in HOTAIR expression level between rectal cancer samples before (0.0017±0.0052) and after CRT (0.0019±0.0059), p>0.05. HOTAIR was significanty upregulated in non-tumour tissue before (0.0039±0.0119) compared to non-tumour tissue after therapy (0.0002±0.0002), p=0.0085. No differences in HOTAIR expression were detected between responders and non-responders in rectal cancer tissue before and after therapy, or in non-tumor tissue before and after CRT, p>0.05. **Conclusion:** Long non-coding HOTAIR cannot be used as a biomarker of response to therapy in patients with rectal cancer.
Keywords: long non-coding RNA, HOTAIR, rectal cancer, therapy reponse

P17

Prognostic potential of redox status, SLFN11, and PD-L1 in colorectal cancer patients

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Background: Previous studies revealed that oxidative stress, implicated in various diseases, may be an essential progenitor in carcinogenesis, including colorectal cancer (CRC). Excessive generation of free radicals, redox imbalance, and consequential DNA damage can affect intestinal cell homeostasis and lead to neoplastic transformation. Schlafen 11 (SLFN11) protein recently emerged as pivotal in DNA damage conditions, with predictive potential for tumor response to cytotoxic chemotherapies, particularly DNA-damaging agents. Additionally, recent discoveries showed that the Programmed death ligand 1 (PD-L1) protein can be found on malignant cells, providing an immune evasion mechanism exploited by different tumors. Therefore, our study aimed to investigate the significance of redox status parameters, SLFN11, and PD-L1 proteins as prognostic biomarkers in patients with CRC. **Patients and Methods:** In this study, we included 130 CRC patients and compared all measured and calculated parameters between patients who died and those who survived during the one-year follow-up. We investigated the following redox status parameters:

spectrophotometrically measured in the serum superoxide dismutase (SOD), sulfhydryl (SH) groups, advanced oxidation protein products (AOPP), malondialdehyde (MDA), pro-oxidant–antioxidant balance (PAB), and superoxide anion (O2−) and calculated Prooxidative Score, Antioxidative Score, and Oxy Score as a comprehensive index of oxidative stress status. Serum protein levels of SLFN11 and PD-L1 were determined using the ELISA method. **Results:** The SLFN11 protein levels were significantly higher in the serum of patients who died during the first year of follow-up (p=0.041). On the other hand, measured redox status parameters, calculated scores, and PD-L1 protein levels did not differ significantly among living patients and those who died during the first year of follow-up. **Conclusion:** The SLFN11 protein levels may harbor prognostic potential in patients with CRC. Since this is, to our best knowledge, the first study to evaluate SLFN11 concentrations in the serum of CRC patients by the ELISA method, further studies need to validate this result in an independent patient cohort.

Keywords: colorectal cancer, oxidative stress, SLFN11, PD-L1

P18

Interleukin-6, a potential plasma biomarker for diagnosis and prognosis of thyroid neoplasms

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Background: Thyroid neoplasms include benign tumors – thyroid adenoma (TA), and malignant tumors of various histological types: papillary thyroid carcinoma (PTC) – the most common and usually indolent, anaplastic thyroid carcinoma (ATC) – the most aggressive, and several other types such as follicular, medullary and poorly differentiated. Despite the progress in understanding the epidemiology and genetic landscape of thyroid tumors, the diagnosis, prognosis and treatment approach require further improvement. Interleukin-6 (IL-6) is a pro-inflammatory cytokine with a central role in the regulation of immune and inflammatory responses including autoimmune thyroid diseases. Studies have revealed a potential impact of IL-6 in the development, progression and control of thyroid cancer. The aim of this study was to provide novel aspects for the preoperative differential diagnosis and/or prognosis of thyroid cancer. To achieve this, we assessed the circulating levels of IL-6 in patients with benign and malignant thyroid tumors of various histotypes, compared them with healthy volunteers, and correlated the results with clinicopathological parameters. **Patients and Methods:** The study included 43 patients with benign or malignant thyroid tumors, surgically treated at the Center for Endocrine Surgery, Clinical Center of Serbia. IL-6 protein levels were determined in plasma samples by quantitative ELISA. Parametric and nonparametric statistical tests were used for data analysis. **Results:** IL-6 concentrations in patients with either TA or carcinoma (PTC, ATC) were significantly higher compared to the healthy volunteers (Mann Whitney test). The highest concentrations were detected in ATC patients (Median±SD 15.97±0.71 pg/mL), being significantly higher compared to TA and PTC (2.14±1.34 pg/mL and 1.96±2.12 pg/mL, respectively). In PTC microcarcinoma, IL-6 was higher compared to controls, but there was no significant difference compared to other PTC or TA (Mann Whitney test). The correlation analysis with clinicopathological parameters in PTC patients revealed a trend towards the association of increased IL-6 plasma levels with the presence of nodal and distant metastases. No other significant associations were found. **Conclusion:** Patients with thyroid adenoma or carcinoma have increased plasma IL-6 levels that are in proportion with the aggressiveness of the thyroid tumor, suggesting that IL-6 might be a candidate biomarker for diagnosis and prognosis of thyroid neoplasms.

Keywords:biomarker, blood plasma, interleukin-6, thyroid neoplasms

P19

The effect of tyrosine kinase inhibitors in high-grade glioma patient-derived cells

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Background: High-grade gliomas are the most frequently diagnosed malignant brain tumors in adults, with a very unfavorable prognosis. Although various strategies have been applied in the clinical setting, no significant progress has been made in the treatment of high-grade glioma. Clinical trials continue to expand into new approaches such as targeted agents and immunotherapy. Here, we performed pharmacological screening of tyrosine kinase inhibitors (TKIs) on patient-derived glioma cells *ex vivo* and assessed the expression of multidrug resistance (MDR) marker in glioma and stromal (non-glioma) cells. The effects of TKIs have been compared with chemotherapeutic agents approved for the treatment of high-grade glioma. **Material and Methods:** Primary patient-derived cell cultures were established from resections of high-grade gliomas. After short-term culturing (2-3 weeks), a mixed population of glioma and non-glioma cells was treated with 4 TKIs (alectinib, dabrafenib, trametinib, and nintedanib), as well as temozolomide (TMZ) and carmustine (BCNU). The maximum achieved concentration in human plasma during therapy (Cmax) was set as the upper limit and 4 lower concentrations were also used during the study. An immunofluorescence assay allowing discrimination of glial fibrillary acidic protein antibody-positive glioma cells versus negative non-glioma cells was performed using an ImageXpress Pico high-content imager (Molecular Devices) with CellReporterXpress 2.9 software. The MDR marker (ABCB1) was analyzed with the corresponding antibody in the same immunoassay. **Results:** Among the compounds tested, alectinib and TMZ did not affect cell growth and did not change the number of ABCB1-positive cells. Other compounds significantly inhibited the growth of glioma cells. However, they were not selective towards glioma cells, on the contrary, they showed greater cytotoxicity in non-glioma cells. The number of glioma cells positive for the ABCB1 marker increased significantly after treatment with dabrafenib, nintedanib, and BCNU, while trametinib and did not change ABCB1 expression in glioma cells. Stromal (non-glioma) cells generally followed the pattern of ABCB1 observed in glioma cells. **Conclusions:** Novel functional immunoassay may provide valuable information on the sensitivity of high-grade gliomas to different TKIs and possible treatment outcomes based on the expression of MDR marker.
Keywords: ABCB1, high-grade glioma, immunoassay, patient-derived cell culture, tyrosine kinase inhibitors

P20

The significance of interleukin-8 in hormonally dependent early breast cancer – association with the established parameters ER/PR and HER2

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Background: Interleukin-8 (IL-8) is a multifunctional cytokine linked to cancer progression. Studies have confirmed high IL-8 levels in HER2-enriched and basal-like (ER–) primary breast tumors. The aim of this study was to evaluate the relationship between intratumoral IL-8 protein levels and clinical outcome in hormone dependent (ER+) primary breast cancer patients. **Patients and methods:** The study included 65 early-stage breast cancer patients with detectable levels of hormone receptors (ER>0, PR>0), all of whom had not received any prior hormonal or chemotherapeutic systemic therapy. The median follow-up was 144 months. Steroid hormone receptor status was determined by ligand-binding assay. HER2 status (absence or presence of gene amplification) was determined by chromogenic in situ hybridization (CISH). IL-8 protein levels were determined in cytosol tumor extracts by quantitative ELISA. ER level of 10 fmol/mg, PR level of 20 fmol/mg and the median IL-8 concentration level of 88.8 pg/mg, were used as cut-off values. **Results:** There was a significant difference in relapse free survival (RFS) between IL8low and IL8high subgroups of patients (Log rank test, p=0.002). Considering subgroups of patients stratified in different phenotypes according to receptor status and the median IL-8 value, if IL-8 is highly expressed, the influence of ER is weaker and there was no significant difference in RFS between subgroups with ERlowIL8high and ERhighIL8high phenotypes. The same is true for PR and HER2 and there was no significant difference in RFS between subgroups with PRlowIL8high and PRhighIL8high phenotypes, neither between subgroups with HER2−IL8high and HER2+IL8high phenotypes. On the other hand, subgroup with ERhighIL8low

phenotype had significantly longer RFS compared to those with ERlowIL8high and ERhighIL8high phenotypes (p=0.02, p=0.04, respectively); subgroup with PRlowIL8low phenotype had significantly longer RFS compared to those with PRlowIL8high and PRhighIL8high phenotypes (p=0.003, p=0.02, respectively); and subgroup with HER2–IL8low phenotype had significantly longer RFS compared to those with HER2–IL8high and HER2+IL8high phenotypes (p=0.01, p=0.02, respectively). **Conclusions:** IL-8 is a potential biomarker of unfavorable prognosis in hormone dependent breast cancer that is associated with the established parameters ER/PR and HER2. Receptor-mediated signaling could act additively with IL-8 signaling in progression of hormone dependent breast cancer.
Keywords: biomarker, breast cancer, HER2, hormone receptor, interleukin-8.

P21

Variant rs745430558 in the *SMAD4* gene promoter as a biomarker for adenocarcinoma of the pancreas

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Background: Our previous study has identified variant rs745430558 in the *SMAD4* gene promoter as potential biomarker for adenocarcinoma of the pancreas. The allele delTT (10T instead of 12T) was present in malignant pancreatic tissue with a prevalence of 88%. As analysis of cfDNA in liquid biopsy represents a noninvasive approach for the diagnosis and monitoring of malignancies, the aim of this study was to determine the presence of 12T and 10T alleles in the peripheral blood of patients with suspected pancreatic malignancy. **Material and Methods:** The study was performed using cell-free DNA (cfDNA) isolated from the serum of 15 patients with morphological alterations of the pancreas. The presence of 12T and 10T alleles was assessed by allele specific quantitative real-time PCR. **Results:** Of 15 analyzed samples, 13 were diagnosed with adenocarcinoma of the pancreas (AcP), 1 with neuroendocrine tumor (NET), and 1 with pancreatitis. The 10T allele was present in 84.7% of cases with AcP and also in the sample from the patient with NET. In patient with pancreatitis only the 12T allele was detected. **Conclusion:** Our research has shown that the results of liquid biopsy of patients with AcP are in agreement with tissue specimens analysis. Targeted detection of the rs745430558 10T variant in patients with suspected pancreatic malignancies could be a potential biomarker for diagnosis of AcP inthefuture.
Keywords: cfDNA, liquid biopsy, pancreatic cancer

P22

Effect of BET inhibitors on cancer stem cells sorted from primary oral cancer cell culture

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Background: Oral cancer is the most common malignant tumor in the oral and maxillofacial region, and squamous cell carcinoma (OSCC) accounts for 80% of tumors of the oral cavity. Despite improvements in OSCC management, survival rates remain relatively low and the discovery of novel anti-neoplastic agents are urgently needed. The study investigated the cytotoxic effect of three BET inhibitors (JQ1, iBET-151, iBET-762), and one antitumor plant alkaloid (paclitaxel) on cancer stem cells (CSCs), sorted from primary oral cancer cell culture. **Material and methods:** Magnetic sorting was used to gain CD44 and CD133 positive cells. Double negative cells served as a control. Cells were seeded in 96 well plates, and 10 µM dose of drugs were added to the wells. After 24, 72 hours, and 7 days MTT was performed. **Results:** Real-time PCR analysis confirmed adequate sorting of the double positive (CD44+ and CD133+) cells, with negligible to none of the marker's expression in double negative cells. After 24h of treatment no significant cytotoxicity of the drugs was observed, in comparison to untreated cells. On 48h of treatment there was significant reduction of the cells in the presence of the drugs, but no difference was observed between CSCs and control cells. In longer treatment period (7 days), there was significant difference in cell survival between CSCs and control, in presence of the drugs, for JQ1 ($p<0.05$), paclitaxel ($p<0.01$), iBET 151 and iBET 762 ($p<0.001$). **Conclusions:** The investigated drugs were relative efficient in treatment of tumor cells, but CSCs remain more resistant to the therapy in comparison to the control.New investigations should be aimed at the successfulreduction of CSCs.
Keywords: cancer stem cells, iBET, magnetic sorting, oral cancer cell line, qPCR

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P23

Precision medicine in gastrointestinal oncology – gemcitabine-based systemic chemotherapy in patients with advanced/metastatic pancreatic carcinoma

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Background: Therapeutic options in patients with advanced/metastatic pancreatic carcinoma are very limited. The results of clinical studies in this group of patients have shown the activity of nab-paclitaxel plus gemcitabine combination. We analyzed the efficacy and safety of gemcitabine plus nab-paclitaxel combination in patients with advanced/metastatic pancreatic carcinoma. **Material and methods:** We included patients with advanced/metastatic pancreatic carcinoma, ECOG PS 0-2, treated in our hospital. The patients received systemic chemotherapy nab-paclitaxel 125 mg/m2 and gemcitabine 1000 mg./m2 D1, D8, and D15 q4wks until disease progression or unaccepted toxicity. We analyzed: overall survival (OS), time to progression (TTP), overall response rate (ORR) and side effects. **Results:** We analyzed a total of seven patients. The median overall survival (mOS) was 8.0 months, 1-yr survival was 42.8%. The median time to progression (mTTP) was 4.7 months and the response rate (RR) was 28.5%. The most common grade 3 side effects were: neutropenia in 42.8% of patients, fatigue in 28.5% and neuropathy in 14.2%. There were no cases of febrile neutropenia nor of treatment-related deaths. **Conclusion:** The results confirmed the activity of nab-paclitaxel plus gemcitabine combination in patients with advanced/metastatic pancreatic carcinoma with acceptable tolerability.
Keywords: pancreatic carcinoma, chemotherapy

P24

Precision medicine in gastrointestinal oncology – therapeutic approach in patients with braf mutant metastatic colorectal cancer: a retrospective analysis

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Background: The *BRAF* mutation (mt) is present in 8% – 15% of patients with metastatic colorectal carcinoma (mCRC) and has a very poor prognosis. In our work, we presented the results of treatment and survival in this group of patients in a retrospective analysis as one of the tertiary centers. **Material and Methods:** We analyzed patients with mCRC tested for *KRAS, NRAS* and *BRAF* mutation, left-sided primary tumor, treated in our hospital between November 2019 and February 2023. Patients with *BRAF*mt mCRC were correlated with *KRASwt/BRAFwt* patients. **Results:** Out of a total of 218 patients, analyzed for *KRAS, NRAS* and *BRAF* mutation, 73 patients had a primary tumor of the right colon, while 145 patients had a KRASwt primary tumor of the left side of the colon. In this group, 12 (8.2%) patients had the *BRAFmt* tumor. Patients were treated with systemic chemotherapy based on fluoropyrimidine in combination with the VEGF-inhibitor – bevacizumab (*KRASwt/BRAFmt* tumor), while patients with the *KRASwt/BRAFwt* tumor were treated with systemic chemotherapy in combination with EGFR inhibitors. After 31.0 months of follow-up we analyzed 39 (53.4%) patients. The median overall survival (mOS) was 10.7 months in patients with the BRAFmt tumor and 17.4 months in patients with the KRASwt/*BRAFwt* tumor. Time to progression (PFS) for *BRAFmt* and *KRASwt/BRAFwt* patients was 5.5 and 9.7 months respectively. The response rate (ORR) was achieved in 5 (41.6%) patients with BRAFmt mCRC. The most common grade 3 toxicity side effects were: neutropenia in 20.6%, hypertension in 6.8%, diarrhea in 16.5% and neurotoxicity in 5.5% of the patients. **Conclusion:** The results showed a poor median overall survival (mOS) in patients with *BRAFmt* mCRC. A two-drug chemotherapy is a reasonable therapeutic option in this group of patients. We suggested that patients with *BRAFmt* mCRC should be included in clinical studies for better outcome.
Keywords: metastatic colorectal carcinoma, *BRAF*, chemotherapy

P25

Iron metabolism in the prognosis of epithelial ovarian cancer

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Background: Hepcidin, the central iron hormone, and its receptor ferroportin are critical for the regulation of systemic iron homeostasis. Under high circulatory iron condition hepcidin is secreted by the liver and triggers the degradation of the iron-exporter protein ferroportin, thereby sequestering iron in the cell. Dysregulation of iron homeostasis has been observed in many cancers. We aimed to investigate correlation of the expression of hepcidin (*HAMP*), ferroportin (*FPN*) and seven other iron metabolism genes (transferrin-iron receptors (*HFE, TFR1*), divalent metal transporter 1 (*DMT1*), heme oxigenase (*HMOX*), hypoxia inducible factor 1A (*HIF1A*), *IL6* as inflammatory cytokine and stimulator of hepcidin expression and *IL10* as anti-inflammatory cytokine) with clinicopathological characteristics as well as survival of epithelial ovarian cancer (EOC) patients. **Patients and Methods:** Sixty two fresh frozen EOC were included in the study. SYBR Green qPCR was used to determine gene expressions. Fold changes were calculated using ddCt method with Ribosomal protein L4 (*RPL4*) as reference gene. To test the differences in gene expressions between FIGO stages, tumor grades, histological subtypes, presence of residual tumor, and distal metastasis Wilcoxon rank sum test were used. The log-rank test was used for progression free survival (PFS) and overall survival (OS) analysis. P-values ≤0.05 were considered statistically significant, while p-values between 0.05 and 0.1 were pointed out as a statistical trend. **Results and Conclusions:** The higher expression of *HAMP*, *HIF1* and *HMOX1* ($p<0.001$, $p=0.006$ and $p<0.001$, respectively) as well as lower expression of *HFE* ($p=0.001$) are related to advance FIGO stages 3/4. Higher expression of *HAMP* and *HMOX1* are observed in higher tumors grades ($p=0.044$; $p=0.055$). No significant differences were observed between expression of other examined genes and clinicopathological characteristics. We followed-up 46 patients who received taxane-carboplatine chemotherapy for 1-63 months (median 10.5) for PFS and 1-106 months (median 28.5) for OS. Based on median fold change value, the patients were divided into low and high expression group for each investigated gene. The expressions of investigated genes are not related to PFS. However, the lower expression of *HFE* and *DMT1* were associated with poor OS ($p=0.064$; $p=0.039$). In conclusion, the obtained data indicate that iron metabolism gene signature could be potential prognostic biomarker for EOC. Keywords: gene expression, hepcidin, iron metabolism, ovarian cancer

P26

Can serum HER2 testing add prognostic value to routine tissue HER2 analysis for primary breast cancer patients?

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Background: Tissue expressed human epidermal growth factor receptor 2 (HER2) is an established, but not completely reliable, parameter of breast cancer; and it is determined in routine clinical practice by histology. The aim of this study was to evaluate the association of serum HER2 levels with tissue HER2 expression to investigate if this added prognostic value for primary breast cancer patients. **Patients and methods:** The study included 66 primary breast cancer patients with a median age of 45 years. All patients received adjuvant hormonal therapy, with 20% (13/66) of these patients also receiving trastuzumab. The median follow-up period was 103 months (41 – 172 months) for patients without a recurrence; the median period until the recurrence was 45 months (20 – 89 months). Serum HER2 protein levels were measured by ELISA. Prognostic performance was evaluated by the receiver operating characteristic (ROC), Cox proportional hazards regression and Kaplan-Meier analyses. Classification of patients into tissue HER2low and HER2high subgroups was performed by a pathologist based on: (i) immunohistochemistry (IHC) score determination (0, 1+, 2+, 3+) and (ii) HER2 2+ IHC score verification by chromogenic in situ hybridization (CISH). Classification of patients into serum HER2low and HER2high subgroups was performed using the outcome-oriented cut-off point categorization approach. **Results:** During the follow-up 20% of patients developed recurrence. There was a significant difference between serum HER2 levels of patients with and without recurrence (Mann-Whitney rank sum test). The cut-off point for the serum HER2 levels was 6.0 ng/mL. A significant prognostic association was observed for the age, grade, nodal status, FSH, estradiol and serum HER2 by the Cox regression. The recurrence incidence was 29% for the serum HER2high subgroup, but only 10% for the serum HER2low subgroup. Serum HER2 levels did not correlate with tissue expression of ER, PR or HER2, neither with measured serum hormones, but did correlate significantly with the unfavorable feature of nodal spread. **Conclusion:** Serum HER2 testing by ELISA has potential as an additional test to

routine tissue HER2 analysis for primary breast cancer patients. Our conclusion is based on three main findings: (i) there was no correlation between tissue and serum HER2 expression; (ii) there was no difference in patient outcomes with respect to targeted therapy; and
(iii) serum HER2 values had prognostic significance.
Keywords: biomarker, breast cancer, ELISA, HER2, targeted therapy

P27

Prognostic significance of the localization of the primary tumor and HER2-receptor expression in KRAS wild-type metastatic colorectal cancer treated with anti-EGFR therapy

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Background: Treatment of metastatic colorectal cancer (mCRC) remains a clinical challenge since a certain percentage of patients with RAS/RAF wild type (wt) tumor do not respond to anti-EGFR therapy. The reasons may be a consequence of primary or secondary resistance or the excessive expression of HER2 receptors in patients with mCRC. The localization of the primary tumor (LPT) can also contribute to variable treatment response and disease outcomes since segments of the large bowl represent distinctive molecular entities. The aim of this study was to examine the prognostic value of LPT, as well as to investigate the role of HER2-receptor expression in patients with mCRC. **Patients and Methods:** This study included 181 patients (101 left LPT and 80 right LPT) with KRASwt mCRC, who received anti-EGFR therapy at the Oncology Institute of Vojvodina. KRAS mutation status was determined using Real-Time PCR methodology, while HER2-receptor expression was detected immunohistochemically. The effect of anti-EGFR antibody therapy was analyzed using progression-free survival (PFS) and overall survival (OS) in relation to the LPT and the HER2-receptor expression. **Results:** The median OS in patients with left-side tumors was significantly better than in patients with right-side localized tumors (43 vs. 33 months, Mantel-Cox p=0.005; Breslow p=0.001), as well as median PFS (6 vs. 3 months, Mantel-Cox p <0.001; Breslow p<0.001). According to the multivariate Cox regression analysis of OS and PFS, right tumor localization also represents an independent prognostic factor (p=0.022; HR 1.46; 95% CI 1.06-2.01; p=0.004; HR 1.60; 95% CI 1.16-2.21, respectively). The OS of HER2 positive patients is worse than in HER2 negative patients (p = 0.339), while the PFS of HER2 positive patients was significantly worse (p<0.001) compared to HER2 negative patients (despite a relatively small number of HER2 positive patients). **Conclusion:** Localization of the primary tumor is an important prognostic marker in the KRASwt mCRC patients since results demonstrated that patients with right-sided primary tumors have a statistically significantly shorter PSF and OS. The role of HER2 receptor expression requires further examination, although we noted a low expression level of HER2 receptor in KRASwt mCRC; these patients also had a shorter PFS and OS.
Keywords: Colorectal Neoplasms, humanOncogene Protein HER-2,KRAS protein, Precision Medicine

P28

Expression profile of sex hormone receptors in head and neck cancer: unraveling gender disparities

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Background: Sex hormone receptors (SHRs), including those for androgen, estrogen, and progesterone, play a complex role in various types of cancers, including head and neck squamous cell carcinoma (HNSCC). HNSCC is a diverse group of cancers that originates in the squamous cells lining the mucosal surfaces of the head and neck region. Interestingly,

the relative risk for HNSCC is up to five times higher in males compared to females. Therefore, it is considered that the endocrine microenvironment could be one of the risk factors linked to HNSCC. A gender-specific risk suggests either the existence of specific risk factors that affect only males or that females have defensive hormonal and metabolic features. Therefore, we have started to study in more detail the role of nuclear (AR, ER and PR), as well as membrane SHRs in HNSCC. In addition to the traditional nuclear SHRs, which are established biomarkers for hormone-dependent cancers, a detailed molecular characterization of membrane SHRs in HNSCC can open the door to understanding the different signaling pathways that drive cancer progression. **Material and Methods:** Using quantitative real-time PCR (qPCR) we analyzed mRNA expression of nuclear and membrane androgen, estrogen, and progesterone receptors, in 93 primary HNSCC tumors, 26 positive lymph nodes, and 40 healthy oral mucosa fresh tissue samples. The difference in relative gene expression levels of SHRs in HNSCC tissue samples was compared with the patient's age, gender, HPV status and primary tumor site. **Results:** Our results have shown that the median age at the time of diagnosis was 64 years. Patients older than the median age have higher expression of the CACNA1C, SCN2A, PAQR5, and PAQR6 genes. Additionally, 79.8% of patients were men, and 20.2% were women. Interestingly, expression levels of sex hormone receptors PGRiB, OXER1, CACNA1C, GPER1, SCN2A, PAQR9, and PGRMC2 were significantly higher in women. Furthermore, HPV DNA was detectable in 18 (15.1%) of 119 tumor tissue samples. Only OXER1 showed a difference in the expression level between primary tumor sites and metastases. **Conclusions:** To sum up, our results indicate the existence of an association between the differential gene expression of SHRs, primarily membrane ones, and demographic parameters, pointing out the potential of membrane SHRs to influence critical cellular processes and impact HNSCC progression.
Keywords: Cytoplasmic and Nuclear,Gene Expression, Receptors, Squamous Cell Carcinoma of Head and Neck

P29

Circulating cytokine changes in BRAFwt MM patients during anti-PD-1 therapy

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Background: Despite the promising results of immune checkpoint inhibitors (ICIs) in therapy of metastatic melanoma (MM) patients, clinical benefits still remain variable and only a subset of patients show long-term response. Therefore, biomarkers that can predict efficacy, as well as the safety of this immunotherapy are urgently needed. Cytokines are small soluble proteins that regulate immune system. They are easily detected and monitored in peripheral blood and therefore they could be used as potential biomarkers of response and immune-related adverse events (irAEs) to ICIs therapy. **Patients and Methods:** In this study we measured the levels of transforming growth factor beta (TGF-β), interferon-gamma (IFN-γ), interleukin (IL)-6, IL-8, IL-10 in the sera and plasma of 32 BRAF wild type (wt) MM patients by ELISA method. The blood samples were collected before and after the 4th, 8th, 12th, and 16th cycle of Pembrolizumab, PD-1 inhibitor therapy, until one year or the disease progression (DP). A cycle was defined as 3 weeks of treatment. **Results:** In patients with disease control, i.e. without disease progression (non-DP), TGF-β and IL-6 first significantly decreased during the therapy, while after that they started to successively increase reaching the level similar to the initial value by the end of the follow up. Furthermore, in this group of patients the level of IFN-γ increased while the levels of IL-8 and IL-10 decreased at final points of the clinical follow up. In patients with DP the level of IL-6 increased at the time of progression compared to the baseline level, while IL-8 decreased when the best response was achieved. Also, in patients with pseudoprogression of disease the level of IL-6 and IL-10 significantly increased compared to the pretreatment values. However, no significant changes of the investigated cytokines levels were noted in patients with appearance of irAEs. **Conclusions:** Changes in circulating cytokine level found in this study could be used to monitor and predict clinical benefit fromPembrolizumab therapy in BRAFwt MM patients. Dynamic of changes in these potential biomarkers might be a promising tool to characterize long-term responders.
Keywords: BRAF wild type, Cytokines, Metastatic melanoma patients, Monitoring, Outcome predictors, Pembrolizumab

P30

Prognostic significance of pathologically detected extramural venous invasion (EMVI) in rectal carcinoma

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Background: Rectal carcinoma (RC), a common malignancy of the gastrointestinal tract, remains a great clinical challenge due to the increased risk of local and/or systemic recurrence. The mechanism of primary tumor progression and dissemination may be the crucial prognostic factor. Direct vascular spread, especially venous invasion, has been previously recognized and validated as an important predictor of adverse prognosis. Extramural venous invasion (EMVI) is characterized by the presence of tumor cells within veins outside the bowel wall and is strongly associated with poor survival, increased risk of local recurrence, systemic recurrence, and death. The aim of this study is to examine the prognostic value of pathologically detected EMVI and its relationship with other available clinicopathological parameters of patients with RC. **Patients and Methods:** This retrospective study included 100 untreated and non-metastatic RC patients (50 EMVI+ and 50 EMVI-) who underwent curative resection between January 2016 and June 2018 and were followed for the next five years (median follow-up of 71.1 months). The presence of EMVI was assessed on standard hematoxylin and eosin-stained histolological sections of postoperative tumor specimens samples, confirmed by a consultant pathologist in arbitrary cases, and in accordance with validated College of American Pathologist (CAP) guidelines. **Results:** The presence of EMVI within a selected cohort of RC patients significantly associated with female gender (p=0.039), T4 stage (p=<0.001), N2 stage (p=<0.001), less number (n≤3) of involved lymph nodes (p=<0.001), excessive lymphatic infiltration (p=0.044), presence of perineural invasion (p=0.002), positive circumferential margin (CRM) (p=0.003), and TNMIII stage (p=<0.001). In addition, within EMVI+ patients, metastases, dominantly in the liver (13/19, 68%), and death outcomes were more frequent events (p=0.013 and p=0.032, respectively), while survival analyses revealed that EMVI+ patients had significantly shorter overall survival (OS, p=0.035) and disease-free survival (DFS, p=0.030). **Conclusion**: Obtained results strongly suggest that the EMVI type of vascular invasion, considered independently of classical stage parameters and separately from lymphatic invasion, has the potential to be a reliable predictor of the course and outcome of the disease, which should be confirmed on a larger cohort of patients with RC.
Keywords: Extramural Venous Invasion (EMVI), Predictive Medicine, Rectal Cancer

P31

Genomic instability as a prognostic marker in malignant brain cancer

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Introduction: Glioblastoma and Astrocytoma are diffuse malignant brain tumors and characterized as the most aggressive and invasive brain cancers. Glioblastoma IDH wild-type is a primary brain tumour that develops de novo, and Astrocytoma IDH mutant is a secondary tumour which arises by progression from lower tumour grades. They are characterized by poor survival, resistance to therapy and poor prognosis which develops as a consequence of genomic instability. Genomic instability also contributes to tumour heterogeneity and provides the genomic diversity necessary for selection. **Materials and methods:** 31 patients with Glioblastoma IDH wild-type and Astrocytoma IDH mutant, grade 3 and 4, were analysed for the presence of genomic instability using AP-PCR, DNA profiling method. Comparing DNA profiles between tumour tissue and normal tissue (blood) of the same patient, we detected qualitative and quantitative changes. Qualitative changes are detected as the presence and absence of bands and are the manifestation of microsatellite instability (MIN). Quantitative changes are the representation of chromosomal instability (CIN) and are detected as differences in the intensity of bands. Survival analyses were performed using Kaplan & Maier test for survival data in relation to different histological tumour type and genomic instability. Statistical differences were considered significant for p≤ 0,05. **Results:** Patients with Glioblastoma IDH wild-type have significantly shorter survival compared to other histological types (p=0,025). For each histological type that we analysed and each type of instability,

MIN, CIN and total genomic instability, two groups of patients were made – those with high and low instability. Patients with Glioblastoma IDH wild-type that have low total genomic instability have significantly shorter survival (p=0,045) compared to other analysed types of brain cancer. Patients with Astrocytoma IDH mutant grade 4 who have high total genomic instability and high CIN have significantly shorter survival (p=0,018, p=0,007 respectfully). **Conclusion:** Patients with Glioblastoma IDH wild-type have shorter survival which makes this tumour the most aggressive and malignat of all analysed tumours. Our results show that low genomic instability in Glioblastoma IDH wild-type and high genomic instability lead by high CIN in Astrocytoma IDH mutant, gradus 4 contribute to shorter survival, which makes genomic instability a potential good prognostic marker.
Keywords: Astrocytoma,DNA profiling, genomic instability, Glioblastoma, survival

P32

Head and neck cancer: single- and two-stage reconstruction

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Background: In head and neck oncology, surgical treatment frequently results in microvascular reconstruction. Oncologic resection followed by immediate reconstruction is often associated with prolonged working and surgical duration, challenging a surgeon's concentration level and potentially worsening patient outcome. To improve the surgeon's performance and to reduce risk of potential complications, we implemented a two-stage procedure in patients with head and neck cancer. This study critically analyzed the surgical outcomes, organizational benefits, and investigated job satisfaction among affected health care professionals. **Patients and methods:** A retrospective data analysis of patients who had undergone microvascular reconstruction after oncologic head and neck surgery between 2010 and 2021 included 33 patients (n = 33). Twenty patients underwent single-stage reconstruction (group 1, n = 20) and 13 patients underwent two-stage reconstruction (group 2, n = 13) with 12.2 (± 7.4) days between surgeries. **Results:** The mean surgical duration, and mean start and end time of the reconstructive surgery component differed significantly (p = 0.002). The mean total complication rate (p = 0.58) did not differ significantly, although a trend toward higher demands for blood products was observed in group 1. There was no significant difference in five-year survival (p = 0.28). A questionnaire on subjective work performance was answered by the affected health care professionals (n = 34) and it revealed that 88% preferred long surgeries to be scheduled first and that 97% work most efficiently in the morning. **Conclusions:** Two-stage reconstruction is a suitable option in selected head and neck cancer patients offering the possibility of optimizing preoperative planning and organization. This may result in regular working hours, reduced surgeon fatigue, and improved job satisfaction without compromising patient outcomes or survival.
Keywords: head and neck cancer, head and neck reconstruction, mitigation strategies, patient safety, staged reconstruction, surgeon fatigue

P33

Simultaneous *EGFR* L858R and T790M mutations in treatment-naïve metastatic lung adenocarcinoma: a case study and therapeutic implications

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Background: The use of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) is now standard of care in the first-line treatment of patients with advanced adenocarcinoma of the lung who harbor *EGFR* mutations. Patients with the L858R mutation are candidates for first-generation (gefitinib, erlotinib) and second-generation (afatinib) EGFR-TKIs. While the introduction of EGFR-TKIs undoubtedly improves treatment outcomes for patients with *EGFR*-mutated lung adenocarcinoma, a large proportion of patients eventually develop resistance. The most common mechanism of acquired resistance is the occurrence of the T790M mutation in exon 20 of the *EGFR* gene. It has been shown that the T790M mutation can also occur as a primary mutation in patients who have not received EGFR-TKI therapy. This case study presents a rare case in which a patient was diagnosed with concurrent L858R and T790M mutation at the time of diagnosis. **Material and Methods:** This study presents a case of a non-smoking female patient diagnosed with stage IV lung adenocarcinoma at the age of 71 years. DNA isolation was performed from formalin-

fixed, paraffin-embedded (FFPE) tissue blocks using the Cobas® DNA Sample Preparation Kit. Molecular assays were performed using the Cobas® EGFR Mutation Test v2 on the Cobas® 4800 platform (Roche Diagnostics) and TaqMan probes for EGFR p.T790M and p.L858R mutations and the Absolute QTM DNA Digital PCR Mix (5X) on the QuantStudio Absolute Q Digital PCR platform (ThermoFisher). **Results:** Real-time PCR detected L858R and T790M mutations in this patient's tumor sample. These mutations were confirmed by dPCR. In this particular case, as in any case where the T790M mutation is detected, the patient is a candidate for the treatment with third-generation TKI (osimertinib), regardless of the stage of the disease. Considering that osimertinib in Serbia is only approved for patients who developed T790M after therapy with 1st and 2nd generation TKIs, and had disease progression, this case is a good argument for expanding the current indication. **Conclusions:** Several studies have shown that the use of first line osimertinib in these cases leads to a prolongation of progression-free survival (PFS) and overall survival (OS). Although de novo (pre-treatment) T790M mutations are very rare, the use of dPCR as a more sensitive method could increase the number of newly detected T790M mutations, potentially expanding the pool of patients eligible for osimertinib therapy.
Keywords: de novo T790M mutation, EGFR, L858R mutation, lung adenocarcinoma, osimertinib

P34

Tracing the connection between trace metals and oxidative stress in malignant brain tumors and hydrocephalus

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Background: The ability of metals to traverse the blood-brain barrier has led to the hypothesis that chronic metal exposure could elevate the risk of certain brain diseases, including tumor development. Since the role of reactive oxygen species (ROS) and trace metals in disease onset remains incompletely elucidated, this study aims to explore their potential interplay in the bloodstream of patients with malignant brain tumors (MBTs) and hydrocephalus. **Patients and methods:** Blood samples were obtained from 45 patients with MBTs and patients with hydrocephalus. We included 45 age- and gender-matched control blood samples for comparative analysis. Blood plasma and red blood cells (RBCs) were utilized to assess various parameters. A total of ten metals were analyzed, including essential Mn, Co, Zn, Cu, Fe, and Se, as well as toxic Ni, As, Cd, and Hg using ICP-MS. Additionally, we examined the activities of CAT, CuZn SOD, GSH-Px, GST, GR, AchE, GSH, TBARS, and SH. **Results:** Controls exhibited significantly lower levels of As, Se, and Cd but higher levels of Ni, Co, Zn, and Fe compared to patients with MBTs and hydrocephalus. Intriguingly, Cd levels were significantly higher in the hydrocephalus group than in the MBT group, whereas Mn levels were significantly lower in the latter. In terms of oxidative stress parameters, both hydrocephalus and MBT patients exhibited higher CuZn SOD activity and lower SH levels than the control group, indicating elevated oxidative stress. Notably, AchE activity was increased in hydrocephalus patients when compared to the other two groups. **Conclusions:** Our findings strongly suggest a compelling relationship between trace metals and biomarkers of oxidative stress in MBTs and hydrocephalus. We encourage extensive investigations in the future, involving a larger cohort of clinical samples.
Keywords: malignant brain tumors, hydrocephalus, trace metals, essentially/toxicity, oxidative stress

P35

Anti-cancer activity of newly synthesized derivatives of nicotinic acid on several monolayer and three-dimensional solid tumor models

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Background: Six newly synthesized derivatives of nicotinic acid were assessed for anticancer properties on different solid tumor models. **Materials and Methods:** Compounds 1-6 were investigated on four human cell lines (LoVo –

colorectal adenocarcinoma, SkoV3 – ovary adenocarcinoma, A549 – lung non-small cell carcinoma, and Mcf7 – mammary adenocarcinoma) for the ability to induce cell death by means of Calcein AM/propidium iodide (PI) staining, type of cell death (Annexin V/PI), cell cycle changes, activation of caspase-8/-9, generation of mitochondrial superoxide radicals (MtSR), anti-migratory activity, and growth inhibition of three-dimensional (3D) malignant cultures. Incubation periods on monolayer cells included 24 hrs for types of cell death (1-100 µM), cell cycle changes, and migration assay (1 and 10 µM), while 6 hrs (75 µM) for all other assays. 3D cultures were treated over period of 6 days. **Results:** LoVo cell line has revealed the highest sensitivity toward the applied treatments, where 1, 3, and 5 achieved death in more than 50 % of treated cells. However, Annexin V/PI staining disclosed all LoVo cells ended up in necrosis, which is unfavorable outcome. On the contrary, 3 was the only compound that attained notable incidence of apoptosis in A549, SkoV3 and Mcf7 cell lines. Compound 3 displayed interesting patterns in its activity, such as bell-shaped dose-response curves in A549 and Mcf7 cells within range of 30-100 µM, whereas that was not recorded in Skov3 up to 100 µM. The accumulation of cells at the G0/G1 phase was seen at lower concentrations of 3 in each of treated cell lines, but instead of progressing to cell cycle arrest it was decreasing concurrently with the rise in applied concentration of 3. The increase in MtSR generation was found only in Skov3 cells that were least affected compared to A549 and Mcf7 lines. Independent activations of caspases -8 and -9 were detected in all three cell lines treated with 3, which strongly indicates that this compound has the ability to trigger at least two different mechanisms of death in malignant cells. Additionally, 3 scored statistically significant inhibition of cell migration and successfully restrained growth of 3D tumor masses comparing to non-treated control. **Conclusion:** According to our results, compound 3 is a small molecule which triggers apoptotic death in malignant cells via mechanisms that deserve to be investigated in further studies.
Keywords: derivatives of nicotinic acid, apoptosis, inhibition of cell migration, 3D cultures

P36

The effect of *Lactobacillus salivarius* on AKT-mTOR signaling pathway in normal, dysplastic, and oral cancer cell co-cultures

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Background: In oral cancer, aberrant activation of AKT-mTOR signaling contributes to uncontrolled cell growth, metastasis, and therapeutic resistance. Targeting this pathway holds promise for developing novel therapeutic approaches against oral cancer.The impact of oral probiotics, particularly *Lactobacillus salivarius* (LS), in oral cancer is an area of growing research interest.It has been suggested that LS may modulate the immune response, influence the oral microbiome, and inhibit the growth of oral cancer cells through various mechanisms, such as the modulation of host immune signaling pathways. Hence, the aim of the study was to assess the different effects of LS presence on AKT-mTOR signaling pathway in normal, dysplastic, and oral cancer cells. **Materials and Methods:** The co-cultures of LS and normal (stem cells from apical papilla), dysplastic (DOK cell line) and oral cancer (SCC-25 cell line) cells were made in ratio 10:1 respectively. The co-cultures were kept in complete growth medium without antibiotics (DMEM/F12 and 10% Fetal Bovine Serum) at standard conditions (5% CO_2, 100% humidity, 37 °C).After 2, 4, 6, and 12h of co-culturing, total RNA was extracted using TRIzolreagent. Complementary DNA was created following the manufacturer's instructions with the Revert Aid First Strand cDNA Synthesis kit. Following that, qPCR analysis of PI3K (Phosphoinositide 3-Kinase), AKT (Protein Kinase B) andmTOR (Mammalian Target of Rapamycin)was carried out on the Line Gene-K Fluorescence Real-time PCR Detection System with the Maxima SYBR Green/ROX qPCR Master Mix. **Results:** During the first 2 to 4h of incubation with LS, significant downregulation of all markers was noted in dysplastic and cancerous cell lines, while upregulation was observed in normal cells. Opposite to that, all genes were upregulated in the three cell cultures after 6 to12h of co-culturing with LS. **Conclusions:** These findings suggest that (1) LS has a marked modulatory effect on AKT-mTOR signaling pathway, potentially influencing cell growth and oral cancer progression; (2) a significant impact of co-culturing time was noted on AKT-mTOR gene expression: shorter (2-4h) and longer (6-12 h) culture timesexerted opposite effects. Further research is needed to fully understand the mechanisms and therapeutic implications of LS in oral cancer.
Keywords: AKT-mTOR, co-cultures, dysplastic cell line, oral cancer cell line, qPCR

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P37

Violacein enhances the cytotoxic effect of commonly used chemotherapeutics on rhabdomyosarcoma cells

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Background: Investigation of natural compounds showing specific toxicity to tumor cells aims to improve the efficacy of available therapies. Our previous research demonstrated the cytotoxic activity of the bacterial pigment violacein against rhabdomyosarcoma (RMS) cell lines. RMS is the most common soft tissue malignancy in children. In this study, we evaluated the cytotoxicity of violacein on RMS cells in combination with conventional chemotherapeutics doxorubicin, irinotecan, and vinflunine. **Material and Methods:** Toxicity of doxorubicin, irinotecan, and vinflunine was assessed on three cell lines representing different RMS subtypes (SJRH30, RD, and HS-729). IC25 concentrations for each of them, causing a 25% reduction in cell viability, were calculated using the results of MTT viability tests. Cells were then treated with the IC25 concentrations of doxorubicin, irinotecan, or vinflunine in combination with violacein at its IC25 concentration. The effects of the combined treatments were evaluated by MTT assays. The coefficients of drug interaction were calculated using Foucquier and Guedj method, indicating synergy, additivity, or antagonism. **Results:** Sensitivity to chemotherapeutic agents varied among RMS cell lines. The SJRH30 was most sensitive to irinotecan and vinflunine but most resistant to doxorubicin. RD was most sensitive to doxorubicin but highly resistant to vinflunine. HS-729 exhibited the highest resistance to irinotecan. When violacein was combined with doxorubicin, irinotecan, or vinflunine, we observed either additive or synergistic effects, depending on the cell line. The combination of violacein and doxorubicin showed the highest degree of synergy, particularly in RD cells. **Conclusions:** Combining violacein with commonly used chemotherapeutic agents has the potential to enhance RMS treatment efficacy. The next steps would be to understand underlying mechanisms and evaluate safety and effectiveness in preclinical and clinical settings.
Keywords: antineoplastic agents, cytotoxicity, doxorubicin, rhabdomyosarcoma, violacein

P38

Anticancer effects of non-toxic repurposed drugs on hamster fibrosarcoma – fast applicable in oncology

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Background: Some drugs developed for other illnesses might be repurposed for cancer treatment, to kill cancer cells by hitting previously recognized or unrecognized molecular target or unknown target. This study investigated such drugs, with proven *in vitro* anticancer effects, *in vivo* on fibrosarcoma in hamsters. **Material and Methods:** Anticancer efficacy of selected repurposed drugs: mebendazole, metformin, diclofenac, 2-Deoxy-D-glucose, deoxycholic acid, caffeine, itraconazole, nitroglycerin, disulfiram and selected two-component combinations were tested on fibrosarcoma experimentally induced by BHK21/C13 cells in Syrian golden hamsters. Tumor biophysical characteristics, histology and immunohistochemistry were assessed. Blood samples were collected for hematological and biochemical analyses and the main organs were toxicologically analyzed. **Results:** This study showed that two-drug combinations: metformin with 2-Deoxy-D-glucose, metformin with deoxycholic acid, metformin with caffeine, metformin with itraconazole, metformin with nitroglycerin and metformin with disulfiram can significantly ($P < 0.05$) suppress fibrosarcoma in hamsters with doses equivalent to achievable oncological human doses, without toxicity and influence on biochemical and hematological tests. **Conclusion:** All efficacious repurposed drug combinations recorded in our study on hamster

fibrosarcoma can be recommended for further clinical trials.
Keywords: BHK-21/C13, cell culture, drug effects, fibrosarcoma, hamsters, NF-kB
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P39

Potential of Tamoxifen-based Copper(II) Dichloride in Breast Cancer Therapy

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Background: Estrogen receptor-positive (ER+) breast cancer accounts for approximately 70% of all cases and, concordantly, anti-estrogen therapies present a leading therapeutic choice. Interestingly, tamoxifen, which is the most commonly used drug, has also been proven effective in hormone-independent forms of breast cancer, suggesting the existence of intracellular off-targets. Frequent acquisition of therapy resistance presents a platform for the design of tamoxifen derivatives with a 2,2'-bipyridine unit enabling the coordination of transition metal moieties, such as copper(II) dichloride. Copper (Cu) is an essential element involved in the regulation of cellular growth and development. Disruption of its delicate homeostasis results in severe toxicity and hard medical conditions. Increased demand of cancer cells for this micronutrient makes it a valuable candidate for drug design in cancer treatment. The mechanism of action of Cu complexes is typically based on their ability to induce deadly oxidative stress. This study evaluated the efficacy of a copper–tamoxifen hybrid drug on a panel of breast cancer cell lines with varying receptor expression status. **Material and Methods:** The viability of breast adenocarcinoma cell lines MCF-7, MDA-MB-361, MDA-MB-231, 4T1 and glioma U251 was estimated by MTT and CV assays. Flow cytometric analysis of cells stained with annexin V-FITC/propidium iodide, ApoStat, acridine orange, dihydrorhodamine 123 (DHR), dihydroethidium (DHE) or 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF) was used to evaluate cell death, caspase activity, autophagy, production of reactive oxygen and nitrogen species (ROS/RNS), respectively. **Results:** The Cu-tamoxifen hybrid drug displayed substantially higher hormone-receptor (HR) independent cytotoxic activity compared to previously reported metal complexes with a similar tamoxifen vector. Massive caspase-dependent apoptotic cell death is partially attenuated by an autophagic process that counteracts death signals. In contrast to the platinum analogue, the copper-based tamoxifen derivative reduces ROS/RNS that may be associated with the intracellular accumulation of the reduced form of CuI which is important for cuproptosis. **Conclusion:** This study demonstrates the potential of the copper–tamoxifen hybrid drug as an intriguing alternative to commonly used platinum complexes in treatment of cancer. Its safety and efficiency will be further estimated *in vivo*.
Keywords: Breast neoplasms, copper, tamoxifen, therapeutics

P40

The mechanism of action of ruthenium compounds on ovarian tumor cells OVCAR-3

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Abstract in extenso:
Background: From its discovery to the present day, cisplatin and structurally related platinum-based drugs represent an important class of compounds used in cancer therapy (1). The main problem in treatment and antitumor therapy is the occurrence of resistance to platinum-containing compounds and toxicity to healthy tissues. Researchers are for decades working on the development of new antitumor drugs that will successfully replace cisplatin and overcome

the unwanted effects (2-5). Ruthenium complexes have also found their way into this type of research(2-5).In the present study we investigated the cytotoxic activity of four newly synthesized ruthenium(II)-arene complexes with acetylpyridine-type ligands: C1, C2, C3, and C4. Cisplatin (CDDP) was used as the reference compound. **Material and Methods:** The *in vitro* antitumor activity of complexes was examined in epithelial tumor cells derived from ovarian adenocarcinoma (OVCAR-3) after 72 hours of continuous drug exposure using the MTT assay in 2D and 3D cell culture models. Toxicity was evaluated in normal lung fibroblasts (MRC-5).Morphological characteristics of treated cells in 2D and 3D models were examined and imaged every 24 h during treatment. Further, mechanism of action of two selected complexes C2 and C3 was analyzed using flow cytometry for determination of cell cycle perturbations (by propidium iodide staining) or apoptosis inducing potential(by Annexin V-FITC and propidium iodide staining). **Results:** Complexes exhibited very low cytotoxicity and only complexes C2 and C3 reached IC_{50} values up to 300 µM, being 296.13±3.64 and 297.97±8.98, respectively on OVCAR-3 cells. Hence, they were selected for further analysis of the molecular mechanisms underlying their activity towards OVCAR-3 cells.

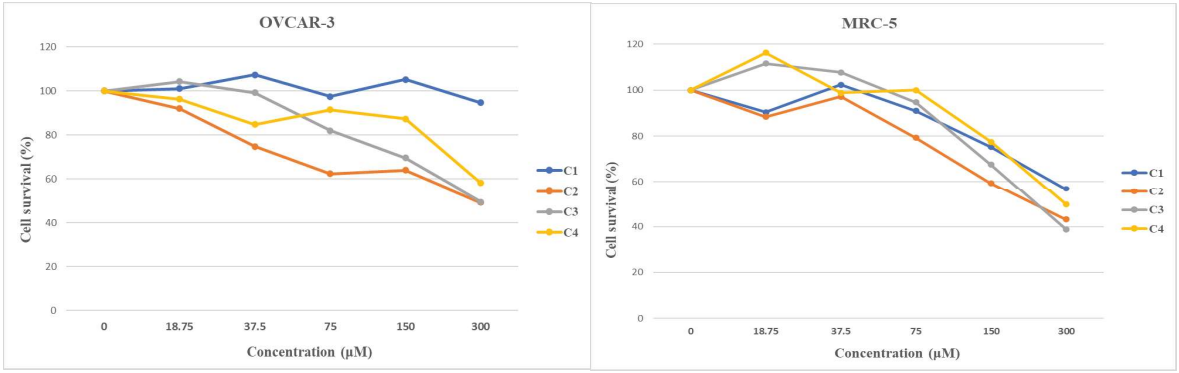

Figure 1. Cell survival diagrams after 72 h of treatment of OVCAR-3 and MRC-5 cells with complexes C1–C4.

Flow cytometry was used to determine whether complexes C2 and C3, compared to cisplatin, have the ability to induce changes in the cell cycle of OVCAR-3 cells after 48 and 72 hours of continuous drug treatment and PI staining. The results are presented in Figure 2.

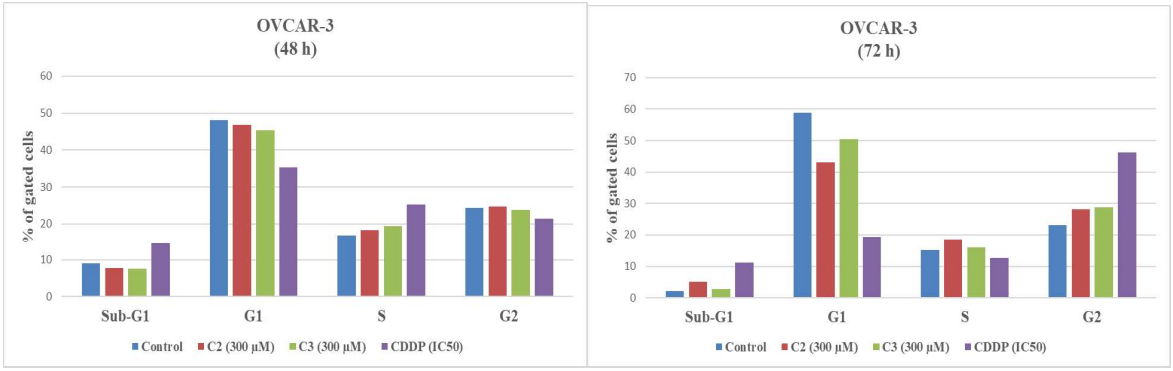

Figure 2. Cell cycle distribution diagrams of OVCAR-3 cells 48 and 72 h after treatment with complexes C2, C3 and CDDP.

Flow cytometry was used to analyze apoptosisin OVCAR-3 cells induced by complexes C2, C3, and CDDP, using double staining with Annexin V-FITC and PI after 72 hours of continuous drug treatment at concentrations corresponding to IC_{50} values (Table 1). Based on the values presented in the table, it can be concluded that the number of viable cells was significantly reduced, and approximately 50% of cells (55.82% for C2 and 53.10% for C3) entered the apoptotic or late apoptotic phase, similar to the values obtained after treatment with CDDP (64.83%).

Table 1. Results of apoptosis analyzed by flow cytometry for complexes C2, C3, and CDDP presented as percentages (%), obtained after 72 hours of treatment.

	Deadcells (%)	Cells in apoptosis and late apoptosis (%)	Living cells (%)	Cells in early apoptosis (%)
Control	1.39	0.71	97.74	0.16
C2	1.16	55.82	27.74	15.28
C3	0.03	53.10	21.92	24.95
CDDP	2.56	64.83	20.26	11.74

Investigated complex C2 exhibited dose dependent growth inhibition in 3D models of OVCAR-3 cells after 72 h treatmentwith IC_{50} being 212.30±1.03 μM (Figure 3).

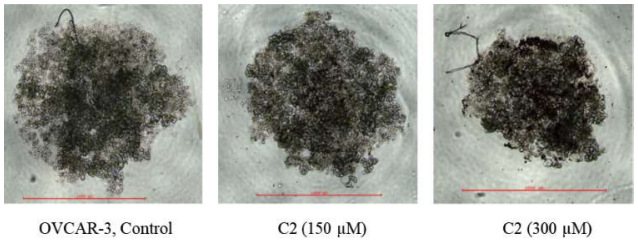

Figure 3. Growth inhibition of OVCAR-3 multicellular tumor spheroidstreated with C2 complex.

Conclusion: Based on the results of the MTT assay in 2D and 3D models of OVCAR-3 cells we determined that complex C2 exhibited dose dependent growth inhibition in 2D model and preserved its activity in 3D model. Complex C2 show some potential of interfering with cell cycle in OVCAR-3 cells and inducing apoptosis. These results can be considered promising for further investigation of the mechanisms of action of thisruthenium(II) complex and further chemical modification.

Keywords: Cytotoxicity, Ruthenium(II) complex, 3D cell culture, ovarian cancer cells
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P41

Multidrug resistant non-small cell lung cancer cells present collateral sensitivity to platinum-based drugs

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Non-small cell lung cancer (NSCLC) is one of the most frequent cancers worldwide, with a 5-year survival rate of 15%. Conventional chemotherapy with taxanes and platinum (or alkylating) agents remains the standard treatment option for patients with NSCLC. However, multidrug resistance (MDR) remains a major obstacle, limiting the effectiveness of available treatments. Surprisingly, some drugs have been found to exert a stronger antitumor effect on MDR cells than on their counterpart sensitive cells. This effect is known as collateral sensitivity. Our work aimed to: i) select MDR NSCLC cells and establish MDR cell lines from a parental sensitive cell line; ii) employ the selected MDR cell lines to identify collateral sensitizer drugs. First, we have successfully generated two drug resistant cell lines, A549-CDR1 and A549-CDR2, by treating sensitive A549 cells with increasing concentrations of paclitaxel. These two selected cell lines were resistant to paclitaxel for at least 31 days without drug treatment, which was verified by the Sulforhodamine B (SRB) assay. Moreover, overexpression of drug efflux pumps (verified by Western Blotting – WB) and increased drug efflux (verified with the Rhodamine-123 accumulation assay) were detected in both resistant cell lines, compared to the sensitive A549 cells. In addition, our results showed that both A549-CDR1 and A549-CDR2 cells were resistant to several other anticancer drugs including docetaxel, vinorelbine, doxorubicin, etoposide and gemcitabine (evaluated by the SRB assay), confirming their MDR profile. However, and most interestingly, both MDR cell lines were more sensitive to platinum-based drugs (cisplatin, carboplatin and cyclophosphamide) than their parental cells, indicating that these drugs caused a collateral sensitivity effect in these two MDR cell lines. Since platinum-based drugs cause DNA damage, ongoing work aims to verify whether the two MDR cell lines have increased susceptibility to DNA damage, which could explain the observed collateral sensitivity effect of these drugs in the MDR cells. Overall, we established two MDR NSCLC cell lines presenting a collateral sensitivity effect to platinum-based drugs. These cell models could be a valuable tool to better understand mechanisms underlying the collateral sensitivity phenomenon to overcome chemotherapy resistance in NSCLC.
Keywords: chemotherapy, collateral sensitivity, multidrug resistance, NSCLC, platinum agents, taxanes

P42

Anoikis as a novel mode of shikonin derivatives anticancer action on C6 glioma cells

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Background: Shikonins are naturally occurring naphthoquinones found in the roots of several genera of the Boraginaceae family, widely known for their antiinflammatory, antioxidant, antimicrobial, and anticancer properties. This study aimed to investigate the antitumor potential of six shikonins isolated from the roots of Onosmavisianii against highly aggressive rat glioma cell line C6 and to explore the mechanisms involved. **Material and Methods:** Cell viability was estimated by MTT and CV assays. Cell death, proliferation rate, and caspase activity were assessed using

flow cytometric analysis of annexin V-FITC/propidium iodide, CFSE, and ApoStat staining, respectively. Fluorescent microscopy of propidium iodide-stained cells was employed for the detection of nuclear morphology. To evaluate the viability of detached cells, an acidic phosphatase assay was used. The cells' property to adhere was assessed by cell adhesion assay while western blot was engaged to measure the expression of relevant proteins responsible for the observed phenomenon. **Results and Conclusions:** All examined shikonins dose-dependently decreased the viability of C6 cells, with compounds 5 and 6 being the most potent ones. Compound 5 had a more profound effect on the proliferation rate of C6 cells than compound 6, resulting in almost 70% of inhibition of cell division. Additionally, compound 5, but not compound 6 generated a significant number of early and late apoptotic cells in treated cultures as detected by flow cytometry. In collision with this, typical morphological signs of apoptotic cells were not observed, and fluorescent microscopy revealed only the presence of enlarged nuclei. This paradox was resolved by the discovery of massive detached cell presence, indicating that glioma cells underwent anoikis, a cell attachment-dependent programmed cell death, in response to treatment with both agents. Decreased ability of C6 cells to adhere to the extracellular matrix and compromised integrin signaling was further confirmed by adhesion assay and western blot, respectively. Interestingly, while compound 5 triggered caspase-mediated anoikis, compound 6 realized anoikis in a caspase-independent manner and under sustained ERK1/2 activation, indicating the deviation from standard proanoikis signaling. This study represents the first proof of shikonin derivatives' strong anticancer potential realized through the inductionof anoikis of highly proliferative and invasive malignant glioma cells.
Keywords: anoikis, integrin signaling, glioma

P43

Different mitochondrial response in A549 KRASG12S cells and Mcf7 KRAS wild type cells to the treatment with mitochondrial superoxide radicals triggering agent 2-(1-Benzyl-4-piperidinylamino)-4-(4-chlorophenyl)-4-oxo-N-phenylbutyramide (BPCPh)

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Background: We previously published that derivatives of aroylacrylic acid phenylamides induce mitochondrial superoxide radicals (MtSR) generation and apoptosis in cancer cells. As KRAS mutant cell lines have dysfunctional mitochondria with increased basal cellular reactive oxygen species (ROS) levels, they could respond differently to MtSR triggering agents. In this study we treated A549 cells (harboring KRASG12S mutation) and Mcf7 cells (KRAS wt) to observe and compare alterations at the mitochondrial (Mt) level upon treatment with BPCPh that acts as a powerful generator of MtSR. Both cell lines are p53 wt. **Material and Methods:** Apoptosis was determined by Annexin V/propidium iodide (PI) staining and Sub-G0/G1 analysis, MtSR were detected by MitoSOX Red, total ROS by DCFDA, Mt potential was defined with MitoTracker CMX Ros and Mitochondrial potential kit/PI staining, MitoTracker Green FM was employed for Mt mass observation. Analyses were performed on FACS Calibur cytometer and Carl Zeiss fluorescent microscope**. Results:** In both A549 and Mcf7 cells apoptosis was evident after 24 hrs of BPCPh treatment. This outcome was completely reversed by N-acetylcysteine co-incubation, implying ROS generation as responsible for cell death in both cell lines. Nevertheless, at 6 hrs of treatment with 50 μM of BPCPh, the response at Mt level was drastically different in the two cell lines. Contrary to A549 cells, MtSR production in Mcf7 cells was vigorous followed by momentous boost of cellular ROS. There were no notable changes of Mt potential in A549-treated cells compared to non-treated control, but decrease in Mt mass was seen in a modest percentage of those that underwent BPCPh treatment. On the contrary, significant Mt hyperpolarization and gain in Mt mass were recorded in Mcf7 cells. Microscopic examinations showed that BPCPh treatment led to interruption of Mt networking in both cell lines. While Mt in A549 seemed to have preserved size and integrity, they have been relocated toward the plasma membrane. In Mcf7 cells, the remaining Mt were massive and repositioned near the nuclei. **Conclusion:** A549 and Mcf7 cells displayed different strategies to overcome treatment with BPCPh. While Mcf7 cells have evidently undergone mitochondrial swelling and fission, in A549 cells mitophagy may be underlying process that should be further confirmed. Our results could contribute to better understanding of Mt plasticity in cancer cells in response to strong pro-oxidant agents.
Keywords: KRASG12S mutation, mitochondrial superoxide generation, cellular ROS, apoptosis

P44

Anticancer activity of diphenyltin(IV) compounds bearing carboxylato N-functionalized 2-quinolones

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Background: The limited efficacy of conventional metal-based chemotherapeutic drugs is attributed to resistance, high toxicity, and numerous side effects, thus providing a platform for the design of new metal-based drugs with enhanced properties. Organotin(IV) compounds have already been recognized as promising agents due to their ability to inhibit tumor growth both *in vitro* and *in vivo*. Following this concept, new diphenyltin(IV) complexes incorporating carboxylato N-functionalized 2-quinolones ligands were assessed on different cancer cell lines. **Material and Methods:** Evaluation of anticancer activity *in vitro* of the newly synthesized diphenyltin(IV)complexes bis (3-(4-methyl-2-oxoquinolin-1(2H)-I)propanoato)diphenyltin(IV), and bis (2-(4-hydroxy-2-oxoquinolin-1(2H)-yl)ethanoato)diphenyltin(IV) (1−3, respectively) as well as ligand precursors (HL1, HL2, and HL3) was determined after 72 h on a panel of cancer cell lines of human and mouse origin (MCF-7, A375, HCT116, 4T1, B16, CT26) using MTT and CV assays.Complex 1 and HCT116 cells were selected for further analysis of the potential mechanism, Flow cytometry for the assessment of cell death, proliferation, caspase activation and production of active oxygen/nitrogen species as well as fluorescent microscopy for detection of nuclei morphology were employed. **Results:** Obtained results showed a dose-dependent viability decrease in all cell lines exposed to complexes 1−3 with IC50 values in the low micromolar range. Ligand precursors, HL1−HL3 showed no activity up to 200 μM. Complex 1 inhibited cell proliferation and provoked caspase-dependent apoptosis in HCT116 cells. The enhanced presence of autophagosomes determined after the treatment with complex 1 was found to be protective, opposing apoptosis. The scavenging potential of tested complex 1 on ROS/RNS production can be connected with abolished viability and suppressed proliferation, since HCT116 cells are potent producers of ROS. **Conclusion:** Taking all together, novel diphenyltin(IV) complexes present promising anticancer agents and should be further tested *in vivo*.
Keywords: apoptosis, cancer, cytotoxicity, melanoma

P45

Bismuth ferrite nanoparticles increase ROS production and p62 expression in A375 melanoma and HeLa cells

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Background: Cancer nanomedicine is a rapidly developing field that uses nanoparticles (NPs) for the diagnosis and treatment of cancer. Currently, many nanomaterials with different shapes, sizes, structures, and compositions have been investigated to produce effective anticancer NPs. The interest in the biomedical applications of bismuth-containing nanoparticles, such as bismuth ferrite (BFO-NP) is a result of their promising properties such as cost-effectiveness, chemical inertness, high stability, and simplicity of functionalization. **Material and Methods:** A375 human melanoma and HeLa cervical carcinoma cells were used to study the antitumor activity of BFO-NP. Clonogenicity of treated cells was analyzed by colony forming assay, while cell death was examined using flow cytometry. DCF-DA fluorescent assay was applied to measure ROS production. Protein expression of p62 and TfR1 was detected by Western blot. Cell migration was analyzed using a wound scratch assay, while an SRB assay was used to assess cell adhesion. **Results:** BFO-NP (200 ng/μL) significantly reduced the clonogenicity of A375 and HeLa cells by 46 and 60%, respectively. Detected ROS production was increased considerably, especially for A375 melanoma cells, and amounted to 400%. The number of late apoptotic and/or necrotic cells increased by 10-12%, compared to the control. Significantly increased expression of autophagy-related protein p62 was observed in both cell lines after BFO-NP treatment. Ferroptosis-related transferrin

receptor (TfR1) expression was slightly increased in treated A375 end HeLa cells (~14%). The noticed increase in cell adhesion ranged from 20-30% followed by a decrease in cell migration. **Conclusion:**BFO-NP is a promising antitumor agent with a significant inhibitory effect on A375 and HeLa cell growth and metastatic potential. Molecular mechanisms involved in these processes include ROS production and increased p62 expression. Reduced metastatic potential resulted from the inductionof cell adhesion and decreased cell migration.
Keywords: bismuth ferrite nanoparticles, cell death, cell migration, ROS.

P46

Stimulation and inhibition of NF-kB by repurposed drugs – effects on hamster fibrosarcoma

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Background: NF-kB transcription factors are key regulators of apoptosis, autophagy, necroptosis and turns up everywhere in cancer life and death. This study investigated how the regulation of NF-kB by repurposed drugs in oncology affect experimental fibrosarcoma development and progression in hamsters. **Material and Methods:** Anticancer efficacy of certain drugs was tested on fibrosarcoma experimentally induced by BHK21/C13 cells in Syrian golden hamsters. Used repurposed drugs with in vitro verified NF-kB inhibitory effect were: metformin, caffeine, itraconazole, nitroglycerin. Used drug with known NF-kB stimulatory effect was mebendazole. Tumor biophysical characteristics, histology and immunohistochemistry were assessed. Blood samples were collected for hematological and biochemical analyses and the main organs were toxicologically analyzed. **Results:** Our study showed that combinations of NF-kB inhibitors: metformin with caffeine, metformin with itraconazole and metformin with nitroglycerin, in human equivalent doses could be efficacious ($p < 0.05$) against fibrosarcoma growth, which can rescued by mebendazole, without toxicity and influence on biochemical and hematological tests. **Conclusion:** Combinations of repurposed drugs with NF-kB inhibitory effect: metformin with caffeine, metformin with itraconazole and metformin with nitroglycerin could be an important therapeutic option in oncology.
Keywords: BHK-21/C13 cell culture, drug effects, fibrosarcoma, hamsters, NF-kB

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P47

Targeting Tumor pH: The Role of Sodium Bicarbonate in Cancer Treatment

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Background: Tumor acidity is a hallmark of cancer that promotes cancer progression and treatment resistance. It is associated with metabolic reprogramming and the use of glycolysis, which results in high lactic acid production. Sodium bicarbonate (SB) potentially can alkalize the tumor microenvironment, and SB per oral administration has shown promising anticancer effects in numerous preclinical studies and some clinical reports. However, the question of local or systemic use of SB in cancer therapy is unclear. Buffering therapy does not counteract standard treatment and can be used in combination to increase effectiveness. For some manifestations of the tumor process, like malignant ascites (MA), clinicians would have been able to transfer the local use of SB from preclinical studies to the clinic quickly. In this study, we evaluated the effect of MA local treatment with SB. **Material and Methods:** We performed the intraperitoneal perfusion procedure with SB/sodium chloride (SC) solution in ICR (CD-1) mice with Erlich ascites carcinoma one week after tumor cells injection. The perfusion procedure consisted of tumor ascites evacuation,

double intraperitoneal administration of 10 ml 0.9 % SC solution (pH~5.5, pH- group, n=14) or 10 ml 1 % SB – 0.675 % SC solution (pH~8.2, pH+ group, n=14) with 10 minutes incubation followed by evacuation and intraperitoneal washing with 0.9 % SC solution. Mice in the control group (n=14) were intact. **Results:** The measured ascites pH values were 6.9±0.1 in all groups. Flow cytometry revealed that the multiplication capacity of Erlich carcinoma decreased in the pH+ group on the 11th day after treatment. The percentage of apoptotic and dead cells was equal in all groups. However, the difference was in the percentage of cells in G2/M, G0/G1, and S-phases: 24.1±3.1 %, 30.6±3.5 %, 45.3±5.4 % in the pH+ group vs. 43.6±7.1 %, 20.9±3.4 %, 35.5±9.5 % in the pH- group, respectively. The survival analysis showed that in the pH+ group, mice had a median survival of 30 days after tumor cells injection, which was significantly different from the median survival of 18 days in the pH- group and 14 days in the control group ($p<0.05$). **Conclusions:** Sodium bicarbonate has an antitumor effect, probably due to its alkalizing properties. Intraperitoneal perfusion with SB solution could be an effective alternative to paracentesis or addition to chemotherapy in palliative care of malignant ascites.
Keywords: cancer, ascites, sodium bicarbonate, treatment

P48

Antitumor potential and impact on redox homeostasis of the essential oil of Black pepper (*Piper nigrum L.)*

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Background: Cancer represents one of the biggest challenges that must be handled as a multifaceted global health issue. Colorectal carcinoma is the third most commonly diagnosed cancer worldwide despite the advanced therapeutic and surgical interventions. Breast cancer is the leading cause of death among female cancers. Free radicals are known to be involved cancer progression, but overproduction may lead to a negative impact on therapy by causing chemoresistance. The active compounds isolated from Black pepper have antitumor effects, but the bioactivity of Black pepper essential oil (BPEO) is rarely studied. The aim of this study was to investigate antitumor capacity and mechanisms of redox potential of BPEO on HCT-116 and MDA-MB-231 cells. **Material and methods:** The increasing concentrations of BPEO (from 1 µg/mL to 200 µg/mL) were applied to cells during 24 h and 72 h after which the evaluation of proliferation, oxidative/antioxidative status and nitrite production of treated cells was performed. **Results:** In this study, we found that BPEO significantly inhibited the proliferation of HCT-116 and MDA-MB-231 cells in dose-dependent manner. The stronger antiproliferative effect has been shown in MDA-MB-231 cells, especially after long-term treatment with the highest applied concentration, where the percentage of viability was reduced by over 46%. The results also showed decreased concentrations of superoxide anion radicals in treated cells, which indicate their significant antioxidative role. Elevated levels of nitrites indicate high levels of nitric oxide (NO) production and suggest its higher bioavailability due to antioxidative environment. **Conclusions:** These results indicate a significant antitumor activity of BPEO, and further evaluation of these compounds in potential therapeutic application is suggested.
Keywords: BPEO, breast cancer cell line, colon cancer cell line, nitric oxide production, superoxide anion radicals concentrations

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Antiparasitic drug Ivermectin, a potential anticancer drug

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Background: Ivermectin (IVM) is a macrolide antiparasitic drug with a 16-membered ring that is widely used for the treatment of many parasitic diseases IVM not only has strong effects on parasites but also has potential antiviral effects (inhibit the replication of flavivirus, inhibitory effect on the SARS-CoV-2 virus). In addition, IVM shows potential for clinical application in asthma and neurological diseases. Recently scientists have discovered that IVM has a strong anticancer effect. This suggests that ivermectin may be an anticancer drug with great potential. **Materials and methods:** IVM was tested for its anticancer activity against human cervical adenocarcinoma (HeLa) and human colorectal adenocarcinoma (LS-174T) cell lines. Cells were seeded into 96-well plates. After the adhesion, on the next day, cells were treated with five different concentrations of the IVM. Stock solution of IVM, were prepared in dimethyl sulfoxide, diluted with a complete nutrient medium, and applied to target cells. The treated cells were incubated for 72h and the cytotoxic effects were determined by the MTT assay. **Results:** IVM exerted a dose-dependent antiproliferative action at micromolar concentrations toward investigated tumor cell lines, detected by MTT test. The obtained results indicate that HeLa cells showed a slightly higher sensitivity (IC50 value 0.62 µmol ± 0.049) than LS- 174T (IC50 value 0.88 µmol ± 0.049). **Conclusions:** Our experimental results show that IVM might be a new potential anticancer drug for therapy of human cancer.
Keywords: Ivermectin, MTT assay, anticancer drug

P50

Role of the SALL2 transcription factor in epithelial-mesenchymal transition and its implication in tumor malignancy in colorectal cancer

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Background: Colorectal cancer (CRC) is the second leading cause of global cancer-related deaths. Its primary mortality arises from metastasis, particularly in the advanced stages of the disease, which is when it is commonly diagnosed. An essential process inducing metastasis is the epithelial-mesenchymal transition (EMT), a dynamic phenomenon where epithelial cells acquire mesenchymal traits. Phenotypic traits include increased cellular migration and invasion, higher proliferation, cytoskeletal reorganization, and loss of cellular polarity. At the molecular level, transcription factors linked to EMT (such as Snail, Slug, Zeb1, among others) are responsible for the expression status of crucial genes that uphold epithelial integrity. Within this framework, it is imperative to identify novel factors governing tumor progression and the EMT. Here, we investigated the involvement of the SALL2, a transcription factor significantly downregulated during CRC progression. **Material and Methods:** To this aim, we generated CRC cell lines and organoid models, with gain and loss of SALL2 function, and evaluated phenotypic traits (proliferation, migration, invasion, cytoskeletal reorganization) and molecular changes. **Results:** We found that loss of SALL2 leads to increased EMT-induced characteristics and the upregulation of mesenchymal markers such as Snail, Slug, Twist1, Zeb1, and N-cadherin. **Conclusions:** Ours findings underscore the significant impact of SALL2 loss on CRC progression by positively regulating the EMT. Future studies will evaluate in vivo how Sall2 loss impacts CRC progression and metastasis.
Keywords: Colorectal cancer, EMT, SALL2, Transcription Factor

P51

Cytotoxic activity of extract of *Helichrysum plicatum* DC. on human cancer cells *in vitro*

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Background: Everlasting flowers (*Helichrysum plicatum*) represent a significant source of pharmacologically active secondary metabolites (flavonoids naringenin, kaempferol, apigenin) related to proven spasmolytic, antioxidant, antimicrobial and cytotoxic activity. A critical point in development of polyphenol rich extracts of *H. plicatum* is their limited stability, which can be solved using microencapsulation technique spray drying. The aim of this study was to determine the cytotoxic potential of *H. plicatum* extract *in vitro* on human cervical carcinoma cell line – HeLa, colon cancer cell line – LS-174, prostate cancer cell line PC-3 and normal lung fibroblast cells MRC-5. **Material and methods:** The dried flowers of H. *plicatum* were purchased from the Institute for Medicinal Plant Research "Dr Josif Pančić" (Belgrade, Serbia). The plant material was subjected to a percolation process with an ethanol-water mixture (50:50) for 12 hours, the ratio of solid to solvent being 1:5. After the percolation process, the ethanol was removed using a rotary evaporator (Buchi rotavapor R-114). Then, the obtained extract from the *helichrysum* flowers was spray dried in a Labtex ESDTi spray dryer, and the dried extract was stored in amber glass tubes. The extract of *H. plicatum* was measured and then dilutions were prepared in RPMI-1640 medium. Cytotoxic activity was determined by the colorimetric MTT assay. **Results:** The results of cytotoxic activity of extract of *H. plicatum* against tumor and normal cells are expresed as IC_{50} (half-maximal inhibitory concentration – average ± standard deviation from three independent experiments).

IC_{50} [µg/mL] Av±SD*			
HeLa	LS-174	PC-3	MRC-5
196±16	273±48	297±5	502±44

In all cancer cell lines the extract of *H. plicatum* showed significant cytotoxic activity, with the highest activity in the cervical cancer cell line – HeLa, with good selectivity in activity against all cancer cell lines in comparision to normal MRC-5 cells. **Conclusions:** This study demonstrated the potent anticancer activity of the extract of *H. plicatum*. Future research could show the activity of the extract in other cancers, and identify the main compound that gives it its anticancer activity.
Keywords: cancer cell line, cytotoxicity, extract, *H. plicatum*,

P52

The role of ROS in MAPK-dependent autophagy involved in phorbol myristate acetate-induced macrophage differentiation of HL-60 leukemia cells

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Background: Reactive oxygen species (ROS) have been implicated in autophagy induction and mitogen activated protein kinases (MAPK) activation which both participate in the differentiation of hematopoietic and leukemic cells. We assessed the role of ROS in MAPK activation and autophagy induction in phorbol myristate acetate-(PMA) induced macrophage differentiation of HL-60 leukemia cells. **Material and methods:** The macrophage markers CD11b, EGR1, CSF1R, and IL-8 were assessed by RT-qPCR and flow cytometry. The activation of MAPK was assessed by ERK and JNK immunoblotting, while autophagy was monitored by LC3-II and p62 immunoblotting. Pharmacological inhibition was used to determine the role of MAPK and autophagy in HL60 cell differentiation. Intracellular ROS production was determined by flow cytometric analysis of the green fluorescence emitted by non-selective redox-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate. Antioxidant N-acetylcysteine (NAC) was used to determine the role of ROS in MAPK activation, induction of autophagy and HL-60 macrophage differentiation. **Results:** PMA-triggered

differentiation of HL-60 cells into macrophage-like cells was confirmed by elevated expression of macrophage markers CD11b, EGR1, CSF1R, and IL-8. The induction of autophagy was demonstrated by the increase of autophagic flux. Pharmacological inhibition of ERK or JNK suppressed PMA-triggered autophagy induction and differentiation of HL-60 cells into macrophage-like cells. PMA increased the intracellular ROS generation and the antioxidant NAC reduced the expression of macrophage markers EGR-1, CSF1R, IL-8 and CD11b in PMA-treated HL-60 cells. NAC also blocked PMA-induced LC3-II and ERK phosphorylation, but only slightly reduced the phosphorylation of JNK and did not affect the levels of p62. **Conclusion:** Our study revealed the partial involvement of ROS in MAPK-dependent autophagy in the differentiation of HL60 cells, indicating ROS/MAPK-mediated autophagy for further investigation in differentiation therapy of AML.

Keywords: ROS, leukemia, differentiation, autophagy, ERK, JNK

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Monitoring of the presence of EGFR-mutated DNA during EGFR-targeted therapy may assist in the prediction of treatment outcome

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Background: The aim of our trial was to evaluate the prognostic significance of qualitative ctDNA analysis on different stages of EGFR mutated non-small cell lung cancer (NSCLC) treatment. **Materials and Methods:** We included 99 patients amendable for the first line treatment with either gefitinib/erlotinib (n = 87), afatinib (n = 10) or osimertinib (n = 2). Sequential qualitative analysis of ctDNA with cobas® EGFR Mutation Test v2 were performed before first dose, after 2 and 4 months of treatment, and on progression. **Results:** Our analysis showed clinically significant heterogeneity of EGFR-mutated NSCLC treated with 1st line tyrosine kinase inhibitors (TKIs) in terms of progression-free and overall survival. When treated with conventional approach, i.e. monotherapy with TKIs, the patients falls into three subgroups based on ctDNA analysis before and after 2 months of treatment. Patients without detectable ctDNA at baseline (N = 32) possess the best prognosis on duration of treatment (PFS: 24.07 [16.8-31.3] and OS: 56.2 [21.8-90.7] months). Those who achieve clearance after two months of TKI (N = 42) have indistinguishably good PFS (19.0 [13.7 – 24.2]). Individuals who retain ctDNA after 2 months (N = 25) have the worst prognosis (PFS: 10.3 [7.0 – 13.5], p = 0.000). 9/25 patients did not develop ctDNA clearance at 4 months with no statistical difference in PFS from those without clearance at 2 months. **Conclusion:**Prognostic heterogeneity of EGFR-mutated NSCLC should be taken into consideration in planning further clinical trials and optimizing the outcome of patients.

Keywords: EGFR, lung cancer, tyrosine kinase inhibitors.

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Benefit of immunotherapy administration on overall survival of patients with NSCLC according to real world data analysis

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Background: Recently, there are lots of prospective randomized trials that showed significant benefit of immunotherapy addition to the 1st line treatment of NSCLC. Administration of immunotherapy is preferable for almost all NSCLC patients in first line setting. However, inaccessibility of immunotherapy for healthcare of some countries and variation of benefit for patient with different prognosis made essential real world data analysis and investigation of different predictive biomarkers. This trial focus on overall survival analysis of NSCLC patients treated with or without immunotherapy in real world setting, selection of significant prognostic factors and testing of immunotherapy impact on survival in prognostically different groups. **Materials and Methods:** A cohort of 415 patients with advanced stage NSCLC who received first line systemic therapy was retrospectively analysed. Propensity score matching was performed to calculate overall survival and confirm the benefit of immunotherapy on real world data. We defined prognostic significance of several clinical and laboratory characteristics using Cox proportional hazards models. **Results:** The median follow-up was 9,8 months. According to propensity score matching median OS was 11,1 months [95% CI 8,8–13,3] in chemotherapy group versus 15,5 months [95% CI 13,1 – 17,9] in immunotherapy group which confirmed the benefit of immunotherapy addition on real world data. Several demographic and clinical characteristics including sex (p=0,007), lymph nodes involvement (p<0,001), chemotherapy usage in first line treatment (p=0,054), low neutrophil count (p=0,040) and neutrophil/lymphocyte ratio (p=0,002) in peripheral blood sample demonstrated statistically significant influence on univariate and multivariate Cox regression model. **Conclusion:** The benefit of immunotherapy addition in 1st line treatment of NSCLC is confirmed by real world data analysis. Further investigation of prognostic biomarkers can make possible selection of patients with the biggest magnitude of benefit from immunotherapy.
Keywords: immunotherapy, lung cancer, prognostic biomarkers

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