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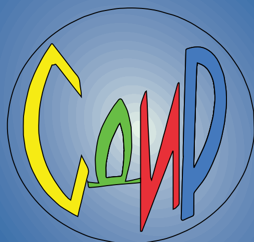

# THE SECOND CONGRESS OF THE SERBIAN ASSOCIATION FOR CANCER RESEARCH

with international participation

"CANCER RESEARCH: PERSPECTIVES AND APPLICATION"

# PROCEEDINGS BOOK

October 2015, Belgrade, Serbia

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**THE SECOND CONGRESS OF THE SERBIAN ASSOCIATION FOR CANCER RESEARCH with international participation "Cancer research: perspectives and application"**

**SDIR-2**

2-3 October 2015, AeroClub, Uzun Mirkova 4/II, Belgrade, Serbia

Serbian Association for Cancer Research (SDIR) is a member of the European Association for Cancer Research (EACR).

**President of SDIR2 Congress**

dr sc. Zorica Juranić, Principal Research Fellow

**Co-president of SDIR2 Congress**

dr sc. med. Siniša Radulović, Professor of Clinical Pharmacology


THE SECOND CONGRESS OF THE SERBIAN ASSOCIATION FOR CANCER RESEARCH
with international participation "Cancer research: perspectives and application"
Belgrade, 2-3 October 2015

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**THE SECOND CONGRESS OF THE SERBIAN ASSOCIATION FOR CANCER RESEARCH with international participation "Cancer research: perspectives and application"**

2-3 October 2015, AeroClub, Uzun Mirkova 4/II, Belgrade, Serbia

*Dear colleagues,*

*It is a great pleasure to announce the Second Congress of the Serbian Association for Cancer Research with international participation which will take place in Belgrade on October 2-3, 2015. Our national society SDIR is the member of the European Association for Cancer Research (EACR).*

*The main goal of SDIR-2 "Cancer research: perspectives and application" is to enable scientists and clinicians from Serbia and foreign countries to learn about the latest achievements in the field of basic and translational cancer research, to exchange their ideas and research experience and to establish scientific collaborations.*

*The main topics of the congress will be the following:*

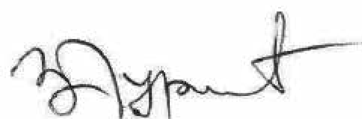
1. *Immune response and its modulation in cancer*
2. *Molecular changes in cancer development and progression*
3. *Anticancer drugs investigation*
4. *Clinical oncology: Era of molecular markers.*

*Thirty distinguished invited lecturers from Serbia and foreign countries already confirmed participation.*

*We cordially invite cancer researchers and clinicians to attend the Second Congress of the Serbian Association for Cancer Research.*

*We look forward to seeing you in Belgrade!*

*Kind regards,*



Principal research fellow,
dr Zorica Juranić
SDIR president

Principal research fellow,
dr Siniša Radulović
Co-president of SDIR2

## ACCREDITATION

The congress SDIR2 is accredited by the Serbian Health Council for continuing medical education (No A-1-1758/15). Invited speakers will be awarded with 15 CME credits, oral presentations with 13 CME credits, poster presentations with 11 CME credits and passive attendances with 9 CME credits.

## SPONSORS

This meeting was financially supported by the Ministry of Science, Education and Technological Development of the Republic of Serbia, the European Association for Cancer Research, the Institute of Oncology and Radiology of Serbia, the Oncology Section of the Serbian Medical Society (SLD), Uni-chem, ProMedia, Alfa genetics d.o.o., Analysis d.o.o, SUPERLAB and Roche d.o.o.

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16. Gordana Bogdanović, Academy of Medical Sciences of the Serbian Medical Society
17. Gordana Konjević, Institute of Oncology and Radiology of Serbia, Belgrade, Serbia

## PROGRAMME

***Friday, 2nd October 2015***

8.00 – 9.00 Registration

9.00 - 9.30 **Opening Ceremony**

Welcome speech by dr Zorica Juranić, president of SDIR

Welcome speech by dr Siniša Radulović, co-president of SDIR2 congress

Welcome speech by prof. dr Radan Džodić, director of the Institute of Oncology and Radiology of Serbia, president of Oncology Section of the SLD

9.30 - 11.15 **Plenary lectures**

***Chairmen: Zorica Juranić, Siniša Radulović, Radan Džodić***

9.30 -10.00 ***Lecture sponsored by EACR**

**Keisari Y[1], Confino H.[1], Schmidt M.[2], Umansky V.[3] Kelson I[1,2]*

*[1]Sackler Faculty of Exact Sciences, Tel Aviv University, Israel, [2] German Cancer Research Center, Heidelberg, [3]Ruprecht-Karl University of Heidelberg, Mannheim, Germany*

**Cure of metastatic tumors by intratumoral alpha radiation in combination with immunostimulants and inhibitors of immune suppressor cells**

10.00 – 10.30 *Novaković S.*

*Institute of Oncology, Ljubljana, Slovenia*

**Tumor vaccines and their role in oncology: Our experience with the creation of different tumor vaccines**

10.30 – 11.00 ***Lecture sponsored by EACR**

*Solary E.*

*Gustave Roussy, Paris, France*

**Chronic myelomonocytic leukemia, pathophysiological insights**

11.00 – 11.15 *Discussion*

11.15 – 11.45 *Coffee break*

11.45 – 13.30 **Session: Immune response and its modulation in cancer**

***Chairmen: Zorica Juranić, Gordana Konjević, Yona Keisari***

11.45 -12.00 *Popović L.*

*Oncology Institute of Vojvodina, Sremska Kamenica, Medical School Novi Sad, University of Novi Sad, Novi Sad Serbia*

**Anti-PD1/PDL1 checkpont inhibitors: a new frontier in cancer immunotherapy**

12.00 -12.15 *Cikota Aleksić B.*

*Institute of Medical Research, MMA, Belgrade, Serbia*

**The association of TNF-alpha, FCGR2A, FCGR3a and FN genes polymorphism with characteristics, course and outcome of diffuse large B-cell lymphoma**

12.15– 12.30 *Besu-Žižak I.*

*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*

**The role of immunoreactivity to food proteins in the etiopathogenesis of different diseases**

12.30 – 12.45 *Mirjačić Martinović K.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Functional and immunophenotypic characteristics of NK cells and their subsets in metastatic melanoma patients**

12.45 – 13.00 *Vuletić A.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
***In vitro* activation of natural killer cells from regional lymph nodes of melanoma patients with interleukin-2 and interleukin-15**

13.00 – 13.15 *Matić I.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**CD26 in various malignancies**

13.15 – 13.30 *Discussion*

13.30 – 15.00 *Lunch*

15.00 – 16.30 **Session: Molecular changes in cancer development and progression I**
***Chairmen: Mirjana Branković Magić, Srđan Novaković***

15.00 – 15.15 *Tanić M.*
*UCL Cancer Institute, University College London, London, UK*
**Tracking (epi)genomic landscape during cancer progression in circulating tumor DNA**

15.15 – 15.30 *Krivokuća A.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Hereditary breast cancer genetics: looking for BRCA3?**

15.30 – 15.45 *Šupić G.*
*MMA Medical School, Institute of Medical Research, MMA, Belgrade, Serbia*
**Cancer epigenetics: from mechanisms to clinical applications**

15.45 – 16.00 *Gužvić M.*
*Faculty of Medicine, University of Regensburg, Germany*
**Identification and molecular characterization of metastasis founder cells**

16.00 – 16.15 *Ozretić P.*
*Ruđer Bošković Institute, Zagreb, Croatia*
**Expression of PTCH1b Tumor Suppressor Gene Is Controlled by Different 5' Untranslated Region Cis-Regulatory Elements**

16.15 – 16.30 *Discussion*

16.30 – 17.00 *Coffee break*

17.00 – 18.30 **Session: Clinical oncology: Era of molecular markers**
***Chairmen: Džodić Radan, Radmila Janković***

17.00 – 17.15 *Džodić R.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Lymphonodal metastases in clinically N0 patients with incidental thyroid microcarcinomas**

17.15 – 17.30 *Pešić A.*
*Pfizer Serbia*
**Academic clinical trials**

17.30 – 17.45 *Jezeršek Novaković B.*
*Institute of Oncology, Ljubljana, Slovenia*
**The diffuse large B cell lymphoma - can we at least approach study results in everyday clinical practice**

17.45 – 18.00 *Tomašević Z.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Breast cancer and brain metastases: clinical, pathological and molecular characteristics**

18.00 – 18.15 Radosavljević D.
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Metastatic lung and colorectal cancer: Impact of molecular markers on systemic treatment – Serbian experience**

18.15 – 18.30 *Discussion*

***Saturday, 3rd October 2015.***

9.00 – 10.00 **Plenary lectures**
***Chairmen: Zorica Juranić, Siniša Radulović***

9.00 - 9.30 ***Lecture sponsored by EACR**
*Ulukaya E.*
*Medical School of Uludag University, Bursa, Turkey*
**Palladium (II)-based anticancer compounds: A new hope or an illusion?**

10.30 – 10.00 *Dimas K.*
*School of Health Sciences, University of Thessaly, Larissa, Greece*
**Sigma receptors as targets for the development of novel targeted anticancer therapies**

10.00– 10.15 *Discussion*

10.15– 11.15 **Session: Anticancer drugs investigation I**
***Chairmen: Siniša Radulović, Engin Ulukaya***

10.15 – 10.30 *Pešić M.*
*Institute for Biological Research, "Sinisa Stankovic", Belgrade, Serbia*
**New approaches in overcoming multidrug resistance in cancer**

10.30 – 10.45 *Bogdanović G.*
*Academy of Medical Sciences of the Serbian Medical Society, Belgrade*
**Fullerenes: biological activity and potential biomedical application**

10.45 – 11.00 *Aranđelović S.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Platinum complexes as anticancer-drugs: properties and perspectives**

11.00 – 11.15 *Discussion*

11.15 – 12.00 *Coffee break*

12.00 – 13.00 **Session: Anticancer drug investigation II**
***Chairmen: Konstantinos Dimas, Gordana Bogdanović***

12.00 – 12.15 *Gligorijević N.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Development of Ru(II)-arene complexes with pyridine derivatives as anticancer agents**

12.15-12.30 *Žižak Ž.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Antitumor activities of macrofungi extracts**

12.30 – 12.45 *Čavić M.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**G protein-coupled receptor heteromers as new pharmacological targets in lung cancer**

12.45 – 13.00 *Discussion*

13.00 – 14.00 **Session: Molecular changes in cancer development and progression II**
***Chairmen: Karmen Stankov, Sonja Levanat***

13.00 – 13.15 *Slade N.*
*Ruđer Bošković Insitute, Zagreb, Croatia*
**p53/p63/p73 protein network**

13.15 – 13.30 *Radulović M.*
*Institute of Oncology and Radiology of Serbia, Belgrade, Serbia*
**Image Analysis of Breast Tumour Histology Images in Prognosis of Distant Metastasis Risk**

13.30– 13.45 *Stankov K.*
*Clinical Center of Vojvodina, Medical School Novi Sad, University of Novi Sad, Novi Sad, Serbia*
**C-KIT signaling in cancer**

13.45– 14.00 *Discussion*

14.00 – 15.30 *Lunch*

15.30 – 17.00 **Poster Session & Short talks**

17.00-17.30 Coffee break

17.30 – 18.30 **Best Poster Award & Closing remarks**

## OP01: Cure of metastatic tumors by intratumoral alpha radiation in combination with immunostimulants and inhibitors of immune suppressor cells

*Keisari Y.*[1], *Confino H.*[1], *Schmidt M.*[2], *Umansky V.*[3], *Kelson I.*[2]
[1]*Department of Clinical Microbiology and Immunology;* [2]*School of Physics and Astronomy, Sackler Faculty of Exact sciences, Tel Aviv University, Israel;* [3]*Skin Cancer Unit, German Cancer Research Center, Heidelberg and Department of Dermatology, Venereology and Allergology, Ruprecht-Karl University of Heidelberg, Mannheim, Germany*

Ablation strategies are non-surgical debulking procedures that can eradicate small tumors and release tumor antigens and damage associated molecular pattern molecules (DAMPs) for the induction of anti-tumor immunity. Such anti-tumor immune responses can destroy residual malignant cells in primary tumors and distant metastases. In this way, the tumor can serve as its own antigenic vaccine after ablation. In situ tumor destruction by radiotherapy, mainly by external beam gamma-irradiation, was reported to facilitate anti-tumor immune reactivity. We aimed to develop an intratumoral alpha radiation based tumor ablation system, which can eliminate the primary tumor and enforce systemic anti-tumor immunity.

Radiation by heavy particles (neutrons, protons, alpha particles and heavy ions), is defined as high-Linear Energy Transfer (LET) radiation that deposit more energy along the path they take through tissue than do x-rays or gamma rays and thus cause more damage to the cells they hit. High LET radiation interacts directly with the critical target in the cell, leading to the chain of physical and chemical events that eventually produce the biological damage.

High LET radiations such as alpha particles have a high relative biological effectiveness (RBE), and only a few hits are required to ensure cell lethality. Although being highly cytotoxic, alpha based radiotherapy has a very limited medical applicability for the treatment of solid tumors due to the short way of alpha particles in tissue (40-90 µm). We developed a potent tumor ablation brachytherapy, which is the only modality currently available that provides an efficient method for prolonged treatment of the entire volume of solid tumors by alpha radiation. Our approach termed Diffusing Alpha emitters Radiation Therapy (DaRT) is based on the intra-tumoral insertion of radium-224 loaded wires (3.66 d half-life), which release by recoil short-lived alpha-emitting atoms into the tumor. These atoms disperse in the tumor, and spray it with highly destructive alpha radiation particles.

*In vivo* experiments, which tested the ablative performance of DaRT against murine and human derived solid tumors, from various histological origins, indicated that the responses to the interstitial radiation varied between tumors. DaRT achieved substantial tumor growth retardation, extended survival, reduced lung metastases and even complete cure of animals bearing murine squamous cell carcinoma (SCC), lung, pancreatic, colon, prostate and breast mouse derived tumors, and human derived tumors.

Applied as a monotherapy, tumor ablation by DaRT enhanced the anti-tumor immune responses in both high and low immunogenic experimental tumor models as evident by resistance to a re-inoculated tumor compared to the control. The treatment also reduced the lung metastatic load. While most (93%) of the mice in the control group developed lung metastases only 56% in the DaRT treated group carried lung metastases. Moreover, DaRT in combination with CpG retarded the growth of DA3 derived tumors more effectively than each treatment alone.

We also explored new approaches to facilitate systemic anti-tumor immunity by combining DaRT tumor ablation with enhancement of anti-tumor reactivity by neutralization of immunosuppressive cells such as regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSC).

Mice bearing DA3 mammary adenocarcinoma with metastases treated with DaRT wires in combination with an MDSC inhibitor (sildenafil) or a Treg inhibitor (cyclophosphamide at low dose) achieved a higher degree of primary tumor ablation and a better control of lung metastases. Combination of all four therapies, i.e. DaRT, Treg and MDSC inhibitors and the immunostimulant CpG, led to a complete rejection of primary tumors, to the elimination of lung metastases, and extended survival compared to the corresponding controls.

Therapy with DaRT combined with the inhibition of immunosuppressive cells and immunostimulation with CpG enforced both local and systemic anti-tumor immune responses and displayed a significant anti-tumor effect in tumor-bearing mice.

DaRT offers a technique to eliminate local and distant malignant cells, regardless of their replication status, by stimulating specific anti-tumor immunity through the supply of tumor antigens from the destroyed tumor. This treatment can be applied in patients when surgery is not an option or before surgical removal of tumors.

## OP02: Tumor vaccines and their role in oncology: Our experience with the creation of different tumor vaccines

*Srdjan Novaković*

*Institute of Oncology Ljubljana, Zaloška 2, 1000 Ljubljana, Slovenia*

**Background:** In the last few years, great progress has been made in the fields of immunology, tumor physiology and molecular biology(1,2). On the basis of that progresswe were able to acquire extended knowledge on tumor biologyand the role of the immune system during tumor development, which helped us develop different biological strategies against cancer(3). According to the principles applied for their creation, current biological therapies for cancer can be divided into two major groups. The first group comprises approaches based on the principles of classical tumor immunology. Therefore, the classical biological therapeutics against cancer include different non-specific immunomodulators, cytotoxic or immuno-modulatory cytokines, growth factors, immuno-modulatory monoclonal antibodies, host defense cells (i.e. tumor infiltrating lymphocytes, lymphokine activated killer cells, antigen presenting cells), as well as different tumor vaccines. The second group comprises approaches based on principles of molecular tumor immunology, such as vaccination with genetically-modified tumor cells or specific tumor antigens, the adoptive transfer of *in vitro* activated genetically-modified effector cells, the in vivo transfer of prodrug-activating enzyme genes into the tumor cell, the introduction of multidrug resistance genes into the bone marrow or stem cells, the in vivo transfer of wild-type tumor suppressor genes into tumors, etc.At the Institute of Oncology Ljubljana, during the last 10 years, the research in the field of tumor vaccines has been oriented to the creation of classical as well as genetically modified tumor vaccines. Classical tumor vaccines were created using different immunostimulators and irradiated tumor cells, while the genetically modified tumor vaccines were created by generating and transferring expression cassettes containing different cytokine genes into tumor cells(4-6). The general aims of those studies were: 1) to achieve the assembly of a potent tumor vaccine that would be able to trigger both immediate and long lasting antitumor immune response, while being easy to produce, highly controllable and having no undesired side effects on normal tissue; 2) additionally clarify thefunction of particular immunological cells in vaccination, and select the most important targets

for new tumor vaccines or approaches; 3) to assess different transfection methods and perform fine tuning of the selected method for transient expression of genes of interest in the mammalian cells; 4) to generate our own expression cassettes with chosen genes and test their usefulness in forcing anti-tumor immunity; 5) to determine the optimal conditions for creating hybridomas.

**Material and Methods**

*Cell line*. Murine B16F1 melanoma cells.

*Animal tumor model.* The experiments were performed on 8-10 week-old syngeneic female C57Bl/6 mice. Tumors were induced by i.p. or s.c. inoculation of $5x10^5$ viable B-16 tumor cells. Tumor vaccine preparation. B16F1 tumor cells were irradiated sublethally with 60 Gy on Darpac 2000X X-ray unit. The irradiated tumor cells were mixed with MVE-2(1,2-co-polymer of divinyl ether and maleic anhydride) or CpG ODN 2395 class C.

*Isolation of mouse mononuclear cells (mMNC) and dendritic cells (mDC) from spleens*. Spleens were cut and MNC were isolated by gradient centrifugation on Ficoll. DC (CD11c+) were analyzed by flow-cytometry.

*Control plasmid.* Theplasmid pSVβ-galactosidase that directs the synthesis of β-galactosidase enzyme has been used (Promega, Madison, Wisconsin).

*Gene transfer and cell hybridization*. Receptor mediated gene transfer (RMGT), Particle delivery gene transfer by helios gene gun, Lypofectionand Gene transfer by electroporation were used. The cell hybridization was made using electroporator).

**Results**

*Tumor vaccine composed of irradiated B16 melanoma cells and MVE-2*

The experiments included mice bearing subcutaneous (s.c.) or intraperitoneal (i.p.) tumors. Regardless of route of vaccination (subcutaneously or intraperitoneally) the tumor vaccine was capable of causing a delayin the development of s.c. tumors but not prevent it completely. Intraperitoneal pre-vaccinationprevented the development of i.p. tumors in at least 40% of experimental mice. Repeated vaccination resulted in a long-term protection against development of i.p. tumors. The crucial mechanism of action of the vaccine was through the activation of phagocytic cells and through an increase in portions of CD25+, CD69+ as well as of IFN-γ+ or IL4+ cells among CD3+ T lymphocytes.

*Tumor vaccine composed of irradiated B16 melanoma cells andCpGODN class C*

The next approach forgenerating a tumor vaccine was to includeanimmunostimulator that would be capable of direct activation of dendritic cells. Since the Toll-like receptor 9 (TLR9) is significantly involved in the differentiation and maturation of APCs,we decided to create the vaccine composed of sublethally irradiated tumor cells and CpG ODNs as ligands for TLR9. In this way we expected anaugmentationin the presentation of antigens to B and T effector cells. Indeed, one injection of the tumor vaccine significantly prolonged the survival of animals compared to the control group. In a fraction of mice that had been treated with a single injection of the tumor vaccine containing different quantities of CpG ODN, a full antitumor protection was achieved. The most convincing tumor prevention effect was achieved with a single injection of the tumor vaccine followed by two additional injections of CpG ODN. In more than 80% of mice that had survived the first tumor challenge a long lasting immunity was induced.The percentage of CD86+CD11c+ cells among CD11c+ cells was the highest in mice pre-vaccinated with tumor vaccine containing 30 μg/mouse of CpG ODN followed by two additional doses of CpG ODN.

A set of 7 genes of interest (CD11c, MyD88, CD86, CD27, CD62L, CD44, IL-15) and 2 housekeeping genes (GUSB, HPRT1) were investigated using the TaqMan Gene Expression Assays. Based on detected expression profile we have demonstrated that tumor vaccine composed of CpG ODN and irradiated tumor cell targets the APCs (including the DCs). This kind of vaccine primarily triggers the differentia-

tion and maturation of APCs in the spleen, which then migrate to the bone marrow. Once in the bone marrow, these APCs (especially the DCs) play a crucial role in the development and maintenance of long-lived memory T cells capable of preventing a relapse of malignant disease.

***Genetically modified tumor vaccines***

Using a biotechnological approach, we generated expression cassettes containing hIL2, hIFNγ, hTNFα, hGMCSF, hGCSF or combinations of two of the aforementioned genes. The expression cassettes were used for transient transfection of tumor cells producing an example of genetically modified tumor vaccines. The confirmation of successful transfection as well as of proper functioning of transfected genes,was done by detecting specific proteins (encoded by transfected genes) in the cells' growth media.

Another approach to the generation of genetically modified vaccines included the creation of tumor-dendritic cell hybridomas. The fusion was accomplished by an Eppendorf electroporator. Unfortunately, the results with these vaccines in in vivo tumor models have not come even close to the ones achieved with classical tumor vaccines.

**Conclusion:** The initial research in the field of tumor vaccines at the Institute of Oncology Ljubljana was headed towards the creation of different types of vaccines and exploration of their mechanisms of action. Classical tumor vaccines exerted tremendous potential for inducing preventive antitumor immunity. They have a wide range of influences on different types of immune cells,provoking immediate andlong lasting immunity. The resulting tumor-specific memory cellspredominantly reside in bone marrow and peritoneal tissue.However, the impact of these vaccines for the treatment of already existing tumors is negligible. On the other hand, genetically modified tumor vaccines have more specific effects, yet they are far more demanding for preparation and adequate control of their effect. Through perplexingexperimental work on creation of tumor vaccines, tremendous amounts of knowledge have accumulated and disseminated among the students and medical professionals. Based on this knowledge many different immunologic and molecular techniqueswere adopted and used for routine diagnostic procedures.

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**Key words:** cancer vaccines, immune system, neoplasms, transfection

## OP03: Chronic myelomonocytic leukemia: strategies to improve diagnosis and treatment

*Eric Solary*
*Gustave Roussy, Paris-Sud University, Inserm U1170, F94805 Villejuif, France.*

The response Chronic myelomonocytic leukemia (CMML) is the most frequent myelodysplastic syndrome / myeloproliferative neoplasm in the classification of myeloid malignancies by the World Health Organisation. This clonal hematopoietic malignancy usually occurs in the elderly. The median overall survival of CMML patients is about 30 months, one third evolving to acute myeloid leukemia while the others die from the consequences of cytopenias. Allogeneic stem cell transplantation, which is the only curative therapy, is rarely feasible because of age. In patients ineligible for transplantation, intensive chemotherapy results in low response rates and short response duration. The cytidine analogs azacytidine and decitabine (5-aza-2'-deoxycytidine) were approved for the treatment of chronic myelomonocytic leukemia. These drugs were originally described as cytotoxic drugs, but low doses also cause DNA demethylation by inactivation of DNA methyltransferases. It remains unclear whether the response to these drugs, which is always transient, results from a cytotoxic or an epigenetic effect.

The lecture will be dedicated first to disease diagnosis, introducing a flow cytometry approach that facilitates CMML recognition and patient follow-up. Secondly, a comprehensive analysis of genetic alterations identified in CMML cells by combining whole exome and whole genome sequencing will be presented. Third, sequential whole exome and RNA sequencing together with DNA methylation analyses in untreated patients and patients treated with a hypomethylating drug will demonstrate that clinical response to cytidine analogs is associated with a dramatic decrease in DNA demethylation but the size of the mutated clone remains unchanged, arguing for a predominantly epigenetic effect of these drugs. Lastly, we will introduce current hypothesis for CMML pathogenesis.

## Session: Immune response and its modulation in cancer

# OP04: Anti-PD-1/PD-L1 checkpoint inhibitors: a new frontier in cancer immunotherapy

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Human immune system plays a key role in recognizing and removing the malignant cells. However, cancer cell presents with a number of personal mechanisms helping it escape the host immune response and thus enabling it to multiply and metastasize. One of the principal mechanisms is presentation of so called immune-regulators (checkpoints) which warrant cancer cell to be missed by immune system as a foreign cell (1). For the last few years checkpoint inhibitors grew out to start a whole new revolution in the treatment of cancer and seem promising to become one of the most important treatment modalities in the future.

**Checkpoint inhibitors mechanism of action and clinical trials rationale**

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is the first receptor to be investigated for the cause of checkpoint blockade. Its function is to decrease T lymphocyte activity. T-cells attack tumor cells who present tumor specific antigens in the form of peptides produced by the tumor itself, banded to the major histocompatibility complex (MHC) molecules. These antigens can be produced by oncogenic viruses, dedifferentiated genes, can be epigenetically regulated, but what is common to all of them is that they are not familiar with human immune system and are recognized by host immune cells (2). Antigen-MHC complex recognition by T-lymphocytes is complex and requires more than antigen-MHC complex, but also costimulatory signals activation, that is CD28 receptor binding on the surface of T-cells with B-7 molecules (CD80 i CD86) at the surface of antigen presenting cells (Fig 1.) (3,4) Apart from certain lymphoma subtypes, malignant cells do not express B7 molecules on their surface which makes them invisible for the immune system. Binding CD28 to B7 enables T-cells to recognize and remove tumor cell especially if the T cells are already present in greater number in tumor infiltrate(3,5).

CTLA-4 is a molecule very similar to CD28 receptor, presented on T-lymphocytes and bonded to B7 molecules, however with much higher affinity in comparison to CD28 (3). After being discovered CTLA-4 was considered to be one of the stimulators (6) of T-cell response, however CTLA-4 has inverse role and decreases T-cell activation, so that its expression prevents T-lymphocyte from recognizing foreign cell (7).

Programmed Cell Death-1 (PD-1) is negative regulator of T-lymphocyte activation and expresses its function only after it is binds to its' ligands PD-L1 and PD-L2. When it binds to one of these ligands PD-1 inhibits T-cell signaling pathways that lead to their activation (Fig. 2) Different types of cancer cells express PD-L1 receptor: melanoma, hepatocellualar carcinoma, lung cancer, kidney, breast, ovary, esophagus, glioblastoma, pancreas and certain lymphoma subtypes. In some of them PD-L1 positivity carries out worse prognosis. In kidney cancer PD-L1 expression is associated to 4.5 greater risk of death and shorter survival.

PD-L1 expression and worse prognosis is perceived in melanoma patients. Triple negative breast cancer is the most aggressive alternative of breast cancer. PD-L1 expression is noted in about 60% of these tumors and correlates to high grade and larger tumor size at diagnosis. All of this was rationale for introduction of PD-1/PD-L1 inhibitors in numerous clinical trials (1,10).

**Clinical efficacy of PD-1/PD-L1 inhibition**

A great number of antibodies which prevent PD-1 receptor binding are currently investigated in different phases of trials in diverse cancers. Although having different structures they can be divided in two distinctive groups: PD-1 blocking group, and PD-L1 blocking group. AMP-224 is a fusion protein that binds PD-L2 and most probably reduces the number PD-1 positive T-cells (1,11). Their mechanism is similar however it is believed that PD-L1 binding antibodies decrease T-cell activity by interaction with B7 as well (12). On the other hand anti-PD-L1 antibodies do not block the PD-L2 influence, whose role is still not completely resolved (1).

PD-1/PD-L1 blocking has showed promising results in large numbers of tumors and has made an improvement in treating some types of cancer that did not have enough efficient systemic therapies available so far, such as squamous cell lung cancer and urothelial carcinoma (10,13). In previous years it was considered that immune therapy is an ineffective strategy in lung cancer treatment. With PD-L1 receptor discovery and it's expression in different subtypes of lung cancer, rationale was set up for investigation of immune therapy in this frequent malignancy.

Nivolumab, anti-PD1 IgG4 monoclonal antibody was investigated in Ib phase in previously treated, advanced lung cancer patients. One year overall survival (OS) was 50%, while two- year OS was 45%. The response was seen even in squamous cell cancer, in which none of the so far investigated target therapies has shown. The efficacy was shown in PD-L1 positive as well as in PD-L1 negative patients, somewhat higher in PD-L1 positive group. Similar efficacy was shown in another anti-PD1 antibody, pembrolizumab, as well as with anti-PD-L1 antibody atezolizumab (10,14,15). Melanoma is another malignancy whose baseline treatment even before has been immune therapy with interferon alpha and interleukin-2. Clinical studies involving pemrolizumab, nivolumab and pidilizumab (16-18) have shown significant efficacy of this medications in metastatic melanoma. What make it more significant are responses even after progression to ipilimumab, CTLA-4 inhibitor. Like melanoma, urological malignancies have shown response to immunotherapy. Trials involving anti-PD1/PD-L1 antibodies have shown significant response in kidney cancer (19), and maybe even more important in metastatic urothelial cancer that had only modest therapeutic options so far. In patients with metastatic urothelial carcinoma treatment response was 52% in patients with IHC 2/3 PD-L1 positivity, and there are also responses noted in patients with PD-L1 0/1 status where overall response is 11%. In 16 out of 17 patients who responded to the treatment none had registered progression even after third month of the treatment (13). Except the efficacy in solid tumors, nivolumab has demonstrated 87% response in heavily pretreated patients with Hodgkin lymphoma (20).

**Immune-related response criteria and immune-related adverse events**

Early studies with CTLA-4 antibodies have noted that patients having progression according to the RECIST (21) criteria, had response in later phases if they have continued treatment with the same agent (22). Apart from that, 25% of the patients treated with ipililumab (anti-CTLA-4 antibody) and who survived more than 4 years, had never response better than progression according to RECIST (23). Because of that so called immune-related response criteria have been developed (24). The basic of these criteria is the fact that checkpoint inhibitors do not produce early response to the treatment, so progressions according to RECIST are not announced as progressions in the early stages of the treatment.

Immune-related adverse events (irAEs) are also specific and are based on autoimmune phenomena originating in blocking the receptors inhibiting the immune response. The most common irAEs are

dermatological in form of different rashes. Diarrhea as a consequence of immune colitis is relatively common irAE. The rise in transaminases (transaminitis) is observed in most of the patients treated with CTLA-4 i PD-1 antibodies, however grade 3 and 4 transaminitis are relatively rare, while up to 10% in cases with ipilimumab treatment and even less often after treatment with PD-1/PD-L1 antibodies. The treatment of irAE is conduced in accordance to the protocols for autoimmune phenomena, corticosteroids, mycophenolate-mofetil, and in more severe cases ifliximab (30,31).

## Future directions

Although PD-1/PD-L1 antibodies accomplished revolutionary effect in treatment of malignant diseases, certain number of patients does not respond to the treatment with these drugs, while others accomplish long disease progression free periods. Because of that the investigators are trying to apprehend certain biomarkers that might help anticipate the patients with the highest benefit of the treatment. IHH expression PD-L1 2/3 is connected to the better treatment response, however patients with low or no expression of PD-L1 gain benefit as well. Absolute lymphocytic count, greater nuber of tumor-infiltrating lymphocytes or ICOS (inducible costimulator) expression and like vise, can lead to better response to immune therapy treatment (32-34). We are left to see whether combination of anti-PD-1/PD-L1 antibodies with chemotherapy, other target or immune therapy is secure and efficient and whether combined approach can gain benefit to the patients. Combination with radiotherapy is investigated as well.

Immune therapy is the future of cancer treatment happening right here before our eyes. Many trials in process will lead us to the answers which of the possible treatment options of this promising method is optimal for our patients.

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# OP05: The association of TNF-alpha, FCGR2A, FCGR3a and FN genes polymorphism with characteristics, course and outcome of diffuse large B-cell lymphoma

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**Background:**. Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma (NHL), accounting for about one-third of all newly diagnosed lymphoma cases. With respect to morphology, clinical features, response to treatment and outcome, DLBCL is highly heterogeneous disease (1). This heterogeneity is largely defined at the (immuno) genetic level.

For many years, cyclophosphamide/doxorubicin/vincristine/prednisone (CHOP) chemotherapy has been the standard therapy for advanced DLBCL providing a long-term overall survival rate (OS) of about 40%. Introduction of rituximab (R) in the treatment of DLBCL has improved OS and relapse free survival (RFS) and established R-CHOP therapy as the standard care in DLBCL (1). Rituximab is a monoclonal IgG1 antibody against CD20, but its exact mechanism of activity is not completely understood. Posible mechanisms of action include antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and induction of apoptosis. Recent studies demonstrated that rituximab induces polarization of B-cells, involving a reorganization of CD20; such polarized cells are preferentially killed by NK cells (2). ADCC is modulated by FCGRs that are expressed on leucocytes. (Auto)antibodies bind to FCGRs and activate immune cell functions, including fagocytosis and the release of inflammatory mediators. Binding affinity of FCGR may be determined by gene polymorphisms. In FCGR2A, the presence of histidine (H) or arginine (R) at amino acid position 131 is encoded by A519G SNP. It is known that the H isoform (encoded by FCGR2A 519G allele) has a higher affinity to IgG2 than the R isoform (encoded by 519A allele). Considering FCGR3A, 559C allele encodes the high-binding isoform to IgG1 and IgG3 with valine (V) at amino acid position 158, while 559A allele encodes the low-binding phenylalanine (F) isoform (3).

Tumor necrosis factor alpha (TNFα) is a cytokine involved in systemic inflammation. The primary role of TNF is in the regulation of immune cells; dysregulation of TNF production has been implicated in a variety of human diseases including cancer. TNF-α is thought to influence lymphoma genesis through up-regulation of inflammatory and anti-apoptotic signals, possibly via the nuclear NF-kB pathway. In addition, TNF-α was also found to be elevated in patients with NHL. High plasma levels of TNF represented valuable prognostic markers in lymphoma patients. *In vitro* studies have suggested that high-levels of TNF-α can reduce cellular sensitivity to apoptosis-inducing chemotherapeutic agents and contribute to the emergence of drug-resistant disease (4). The production of TNF-α is influenced by the promoter polymorphism at position 308 (-308A/G). The presence of -308 A allele is associated with higher constitutional and inducible expression of TNF-α, (5).

Fibronectin (FN1) is one of the basic elements of the extracellular matrix, which mediates the processes of cell adhesion, cell growth, migration, and differentiation. It has been shown that the adhesion of FN1 to malignant cells increases their resistance to apoptosis induced by chemotherapy and rituximab, while the introduction of monoclonal antibodies (e.g., natalizumab), which block contact of B-lymphocytes with fibronectin, enhances the cytotoxic effects of chemotherapy and rituximab. Several studies have demonstrated that single nucleotide polymorphisms may influence gene expression or modify the biological activities of the functional protein (6,7).

In the present study, we analyzed the association of $FCGR2A_{519}$, $FCGR3A_{559}$, TNF-$\alpha_{308}$, FN1 *Msp*I and *Hae*III polymorphisms with clinical characteristics [age (> 60 ≥), sex, Ann Arbor stage (I/II versus III/IV),

B symptoms, bulky disease and IPI risk score (low/low-intermediate versus intermediate-high/high], response to therapy and survival of DLBCL patients.

**Patients and methods:** The study included a total of 84 patients with DLBCL, aged 18 to 74 years (median 47.5), who were diagnosed, treated and followed-up at the Military Medical Academy, Belgrade. Patients with previous history of hematological malignancy, cancer or HIV-related DLBCL were not included in this study. All patients received R-CHOP therapy (6-8 cycles). As a consolidation therapy for high-risk patients, autologous stem cell transplantation (ASCT) was performed in 27 cases.

Genotyping was performed after PCR amplification and digestion with restriction endonuclease *Bst*UI (FCGR2A), *Nla*III (FCGR3A), *Nco*I (TNF-α), *Msp*I and *Hae*III (FN1). The differences in genotype frequencies between DLBCL patients with different variables of disease were assessed using the $\chi^2$ test and, if required, Fisher's exact test. Survival curves were generated using the method of Kaplan and Meier and compared by log-rank test. A p value < 0.05 was considered as statisticaly significant.

**Results:** The observed genotype frequencies for $FCGR2A_{519}$, $FCGR3A_{559,}$ $TNF\text{-}\alpha_{308}$, FN1 *Msp*I and *Hae*III were in Hardy-Weinberg equilibrium.

Considering FCGR2A, HH genotype was found in 49% of patients, HR in 42%, while RR genotype was found in 9% of patients. Patients with HR genotype the most often had DLBCL in CS III or IV (OR 6.1 95% CI 1.18-31.54; RR 1.8519 95 CI % 1.21-2.8; p=0.02). Comparing other clinical characteristics, we didn't observe significant statistical difference in the frequencies of FCGR2A genotypes. Furthermore, there was no significant statistical difference in the CR rate, incidence of relapse, duration of RFS, (event free survival) EFS and OS in patients with different FCGR2A genotypes.

Considering FCGR3A, VV genotype was found in 30% of patients, VF in 50%, while FF genotype was found in 20% of patients. The trend of higher incidence of genotypes containing F allele (VF and FF) was observed in patients aged 60 or older ($\chi^2$ test, p=0.07). Considering characteristics of DLBCL, CR rate and incidence of relapse, we didn't observe significant impact of FCGR3A genotypes. Analyzing the duration of EFS and OS, there was no significant statistical difference between patients with different FCGR3A genotypes. However, we observed the trend that patients with FF genotype had better RFS than patients with VV and VF genotypes (log rank test, p=0.05).

TNF-α genotyping revealed that 52% of DLBCL patients had GG genotype while 48% of them were carriers of variant A allele. There was no significant statistical association between TNF genotypes and clinical characteristics. However, we observed an association between the *TNF-α 308* G/A polymorphism and outcome. The carriers of GG genotype showed higher sensitivity to R-CHOP therapy. Patients with GA/AA genotypes had significantly decreased OS compared to homozygous G-allele carriers (3-years OS 61.3% for AG/GG versus 82.5% for GG genotype; p=0.048). In respect to EFS, there was a trend toward a less favorable outcome for GA/AA genotypes (3-years EFS 48.1 %) in relation to GG genotype (3-years 70%), but, statistical significance was not reached (p=0.07).

FN1 genotyping included 60 patients; *Hind*III EE genotype was present in 8% of patients, EF in 72% and FF in 20% of patients. Considering FN1 *Msp*I, CC genotype was found in 26% of patients, CD in 52%, while DD genotype was found in 22% of patients. Preliminary data didn't indicate clinical impact of FN1 genotypes.

**Conclusion:** The present study included DLBCL patients who were diagnosed, uniformly treated and controlled in one institution. In this ethnically matched group of patients, frequencies of analyzed genotypes were similar to those previously reported for other European populations. We reported the association of FCGR2A HR genotype and adverse clinical stages III or IV. A higher frequency of FCGR3A F allele in DLBCL patients older than 60 years and impact of FCGR3A FF genotype on DFS were noticed only as a trend. This study and previous reports suggest the association of FCGR2A and FCGR3A genotypes with DLBCL characteristics, rather than the course and outcome of DLBCL. However, the possible approval of new anti-CD20 antibodies with better affinity for the FCGR3A F allele in clinical usage may

impose the need for FCGR3A genotyping as an integral part of the routine diagnostic practice. In addition, we report that the TNF-a -308 A allele is associated with adverse response to R-CHOP therapy. This finding has possible clinical implications regarding treatment. In a selected group of patients, the use of immunomodulatory agents or TNF inhibitors (especially lenalidomide) may be an attractive approach.

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**Key words**: diffuse large B-cell lymphoma; gene polymorphism; FCGR2A; FCGR3A; TNF-α; FN1

## OP06: The role of immunoreactivity to food proteins in the etiopathogenesis of different diseases

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**Background**: Food is one of the major antigenic challenges that the human immune system may encounter. The impact of food intolerance on human health has been very extensively studied, especially the intolerance to antigens such as gliadin (a wheat protein fraction) and cow's milk proteins. Immunoreactivity to food proteins can be dealt with as part of some diseases and can play a role in the etiopathogenesis of the others.

Non-Hodgkin lymphoma (NHL) represents heterogeneous group of diseases either B or T cell origin. The association of persistent activation of immune system through the chronic infection with various agents or the association with autoimmune diseases such as lupus, psoriasis, Sjögren's syndrome is also well documented in recent papers (1). The association of celiac disease is also reported in diffuse large B cell NHL as well as in enteropathy-associated T cell NHL. Celiac disease is a genetically determined, chronic, inflammatory bowel disease, gluten-induced. The high degree of coincidence in the presence of enhanced levels of immune complexes in circulation (CIC) and of immunoreactivity with gliadin in immunofixation (after the serum protein electrophoresis in agarose gel in veronal buffer, at pH 8.6) especially in NHL patients points that some antigliadin immunoreactivity unrevealed in ELISA tests could be hidden in CIC. The enhanced immunity to cow's milk proteins (CMP) in some patients with NHL could be developed too.

The enhanced immunoreactivity to some of the food antigens which is found in some patients with NHL, but also in some people with non-malignant disorders points that the intolerance of food antigens is not exclusively related to malignant NHL disease (2).

Recurrent aphthous ulcers (RAU) are currently one of the most common oral disorders, with a worldwide distribution. Ulcers are inflammatory processes of unknown etiology, characterized by the presence of painful, recurrent, single or multiple ulcerations of the mucosa. It was found the strong association between high levels of serum anti-CMP IgA, IgG and IgE antibodies and clinical manifestations of RAU (3). Cow's milk contains several proteins; they can be generally classified in two major groups: caseins and whey proteins.

**Patients and Methods:** Sera from 58 patients with various form of NHL of B cell lymphoma and with T cell NHL, 50 patients with RAU and 50 control persons, for screening for immunity to gliadin, and to CMP were analyzed. The determination of serum IgA, IgG and IgE immunoreactivity to gliadin or to CMP was done by home-made ELISA tests, using sheep antihuman IgA, IgG and IgE secondary antibodies. As a measure of the level of immune complexes in circulation, the absorbance at 450 nm of serum with 3.75% PEG 6000 was determined. After the patient's serum separation on agarose gel by electrophoresis, solution of crude gliadin in 1%, or 0.5% SDS was put over the slides for immunoprecipitation. After immunofixation and washings, gels were stained with Amidoblack, or Coomassie Brilliant Blue.

**Results:** The enhanced immunoreactivity of serum IgA with gliadin was found in 10/58 patients with lymphoma and the enhanced immunoreactivity of serum IgG in only 2/58 patients. The determination of CIC levels reveals that enhanced levels of CIC are found in 18/55 analyzed patients with NHL and just in one healthy control person (2).

The high degree of coincidence in the presence of enhanced levels of immune complexes in circulation and of immunoreactivity with gliadin in immunofixation especially in NHL patients, points that some antigliadin immunoreactivity unrevealed in ELISA tests could be hidden in immune complexes. It was shown that the 41.18% of patients with the enhanced levels of CIC had immunoreactivities in immunofixation with gliadin (2).

The enhanced immunity to cow's milk proteins in some patients with NHL could be developed too. Presented data also showed the enhanced immunoreactivity of serum IgA with CMP higher than cutt off value, in 7/58 examined patients and enhanced immunoreactivity of serum IgG with CMP in 8/58 patients with lymphoma (2).

The enhanced immunoreactivity to some of the food antigens which is found in some patients with NHL, but also in some people with non-malignant disorders points that the intolerance to food antigens is not exclusively related to malignant NHL disease. Results from study showed that the levels of anti CMP IgA, IgG, and IgE antibodies were significantly higher and more frequently found in subjects with RAU in comparison with healthy controls (3).

The increased humoral (IgA or IgG) immunity to CMP has been found in 32 out of 50 patients with RAU, while 17 of them showed the enhanced both IgA and IgG immunoreactivity to CMP. The increased humoral (IgA, IgG and IgE) immunity to CMP has been found in 16 out of 50 patients with RAU (3).

It was found the strong association between high levels of serum anti-specific cow's milk proteins (anti-SCMP) IgA, IgG, and IgE antibodies, especially to caseins: α-, β-, and κ-casein from cow's milk and clinical manifestations of RAU. The increased humoral (IgA and/or IgG and/or IgE) immunity to α-casein has been found in 36 of 50 subjects, to β-casein in 31 of 50, and to κ-casein in 26 of 50 subjects with RAU. Serum immunity to the whey proteins in subjects with RAU was not in so high percentage expressed (4).

The cross-reactivity between milk proteins from different animal species was evaluated by Restani et al. (5). Authors observed that cow's and buffalo's milk present a similar protein composition. Ewe's and goat's milk are similar to cow's milk but with evident reduction in α-casein fraction (5) and this

findings are in accordance with our results - the levels of serum antifresh cow's milk IgA, IgG, and IgE antibodies were significantly higher than the levels of serum antifresh goat's milk, in subjects with RAU with proven increased immunoreactivity to CMP ($P = 0.0003$; $P < 0.0001$; $P < 0.0001$) (6).

**Conclusions:** In individuals with increased immunoreactivity to some food proteins, it should be consider applying of appropriate diet. Our data showed that besides the enhanced humoral anti-gliadin immunity (which sometimes could be hidden in CIC), the immunity to CMP in some patients with NHL could be developed too. Regarding NHL, it means that if malignant genotype in immunocompetent cells exists, the presence of persistent activation of immune system by some of food antigens might create good immunological milieu of cytokines for its support.

Detection of the enhanced levels of the immune reactions in patients with RAU, to protein(s) present in milk and milk products, is from this point of view, of great importance, because appropriate restriction diet can induce remission of RAU. These patients with RAU with increased immunity to CMP could consider the use of goat's milk as the alternative protein source, because of the different protein composition.

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**Key words:** cow's milk proteins, gliadin, non-Hodgkin lymphoma, recurrent aphthous ulcers

## OP07: Functional and immunophenotypic characteristics of NK cells and their subsets in metastatic melanoma patients

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**Background:** Natural killer (NK) cells are a subset of lymphocytes that play a central role in the innate immune response toward tumors without prior sensitization. They directly kill target cells by releasing perforin and granzymes from their preformed granules and have an immunomodulatory role by producing various cytokines, primarily IFN-γ [1]. In humans, NK cells are defined as $CD3^-CD56^+$ and they are divided in two *developmentally,* functionally and phenotipically different subsets, mature, cytotoxic, $CD3^-CD56^{dim+}$ and less mature, immunoregulatory, $CD3^-CD56^{bright+}$ [2]. NK cell function is re-

gulated by a large panel of germline-encoded activating and inhibitory receptors [3]. The most important activating NK receptors are NKG2D, natural cytotoxicity receptors (NCR) such as NKp46, NKp30, NKp44, and DNAX accessory molecule-1 (DNAM-1). The ligands for these receptors, stress inducible molecules, are commonly absent on normal cells, whereas, they become highly expressed on tumor cells [4]. Killer immunoglobulin-like receptors (KIRs) belong to the immunoglobulin superfamily and they are responsible for the inhibition of NK cell-mediated lysis of normal cells that express MHC-I molecules. In this sense, according to the "missing-self" hypothesis, the activation of NK cells occurs in contact with malignantly transformed cells that have lost MHC-I molecules, and have therefore become susceptible to lysis [5]. Melanoma, a potentially fatal form of skin cancer that arises from pigment cells, melanocytes, is characterized by a rapid progression to distant organs, as well as by a limited efficiency of currently applied therapeutics [6]. Aside from that, it has been demonstrated, in both murine and human models, that melanoma cells are susceptible to NK cell-mediated cytotoxicity [7]. However, it is well known that tumor cells and numerous immunosuppressive cells in tumor microenvironment such as myeloid-derived suppressor cells (MDSC) by producing cytokines, growth factors and enzymes evolve various mechanisms to create immunosuppression [8]. In this study, it was of interest to analyze, the expression of various activating and inhibitory receptors, as well as intracellular effector molecules in NK cells and their dim and bright subsets in metastatic melanoma (MM) patients in comparison to healthy controls (HC). The results obtained in this study could be of importance as various NK cell parameters may be good biomarkers of impaired NK cell activity that is associated with increased susceptibility to melanoma progression.

**Patients and methods:** Peripheral venous blood was obtained from 40 metastatic melanoma (MM) patients (stage IV according to 7$^{th}$ modified AJCC/UICC staging system) [9] and 30 healthy controls (HC), age and gender matched, with no evidence of any disease or infection. Blood was drawn at the time of diagnosis prior to chemotherapy. Before inclusion in the study, informed consent was signed by each patient and healthy volunteer and approved by Ethical committee of Institute of Oncology and Radiology of Serbia. Furthermore, MM patients were divided in 2 groups based on the localization of distant metastases according to AJCC/UICC staging system. Patients that have metastases in distant skin, the subcutaneous layer or in distant lymph nodes and normal LDH serum level (M1a) and patients with metastases in the lungs (M1b) were included in M1a+M1b group, while the patients with metastases in vital organs other than the lungs with normal serum LDH level or the patients that have any distant metastasis with elevated LDH were included in M1c group. Peripheral blood mononuclear cells (PBMC) were isolated from obtained peripheral venous blood samples. NK cell cytotoxic activity was evaluated from freshly isolated PBMC, against K562, NK cell sensitive tumor cell line, using radioactive $^{51}$Cr test. Flow cytometry method was used for the analysis of the expression of CD107a degranulation marker, IFN-γ production, the expression of STAT-1, perforin, IL-12Rβ1 and IL-12Rβ2, as well as the expression of activating NKG2D, NKp46, DNAM-1 and inhibitory CD158a and CD158b KIR receptors on $CD3^{-}CD56^{+}$ NK cells and their $CD3^{-}CD56^{dim+}$ and $CD3^{-}CD56^{bright+}$ subsets. The percentage of $CD14^{+}HLA\text{-}DR^{-}$ MDSC cells in PBMC was also estimated by Flow cytometry. The transcription level of DAP10 and SHP-1 signaling molecules from native PBMC was analysed by RT-PCR. The investigated parameters between MM patients and HC were compared by Mann Whitney U test, while correlation analysis was evaluated by Spearman's correlation.

**Results:** MM patients compared to HC had significantly decreased NK cell activity, lower expression of CD107a degranulation marker on $CD3^{-}CD56^{+}$ NK cells and their cytotoxic $CD3^{-}CD56^{dim+}$ subset and impaired IFN-γ production in all investigated NK cell subsets. These results were followed by lower expression of various molecules that regulate NK cell effector functions, perforin, STAT-1, IL-12Rβ1, IL-12Rβ2 on NK cells and both their subsets. Analysing the expression of NK cell receptors and their signaling molecules we show that the expression of NKG2D activating receptor and its DAP10 signaling molecule was decreased in MM patients compared to HC contrary to the expression of activating

NKp46 and DNAM-1 receptors, as well as inhibitory CD158a and CD158b KIR receptors and their SHP-1 signaling molecule that were similar in MM and HC. Furthermore, analyzing the expression of NKG2D receptor on NK cells and their subsets after *in vitro 4 h co-culture of PBL with* K562 or FemX human melanoma tumor cell line we showed a significant decrease in the expression of this receptor on NK cells and cytotoxic $CD3^{-}CD56^{dim+}$ subset in HC only after co-culture of PBL with FemX tumor cell line. Contrary to this, in MM patients we show a significant decrease in the expression of NKG2D receptor on NK cells and both their subsets after co-culture of PBL with K562 and especially with FemX cell line. In MM patients only, there was positive correlation between decreased NKG2D expression and impaired cytotoxicity, as well as lower CD107a degranulation marker expression of NK cells. The lowest expression of NKG2D and NKp46 receptors was obtained in MM patients with unfavourable clinical prognosis and outcome that belong to M1c subclass. Additionally, we showed for the first time increased percentage of immunosuppressive MDSC, $CD14^{+}HLA\text{-}DR^{-}$ cells, in PBMC of MM patients.

**Conclusion:** The alterations in various parameters of NK cells and their subsets, as well as an increase in the percentage of immunosuppressive MDSC in MM patients shown in this study may give new insight into the mechanisms of impaired cytotoxic and immunoregulatory function of NK cells in these patients. Furthermore, these molecules and cells could represent the biomarkers of impaired NK cell effector functions indicating cancer associated immunosuppression that may facilitate tumor progression. Furthermore, they can be used as parameters for the evaluation of standard or developing immunotherapy in advanced melanoma patients.

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**Key words**: metastatic melanoma patients, NK cells, NKG2D, NKp46, perforin, STAT-1

## OP08: *In vitro* activation of natural killer cells from regional lymph nodes of melanoma patients with interleukin-2 and interleukin-15

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**Introduction:** Cutaneous melanoma is a type of skin cancer with the highest mortality rate. Melanoma is an immunogenic tumor poorly sensitive to chemotherapy and insensitive to irradiation. Regional lymph nodes (LN)s represent the first barrier in lymphogenic dissemination in melanoma. In antitumor immunity an important role is played by natural killer (NK) cells, the effector cell subpopulation of innate immune system that is able to immediately recognize malignantly transformed cells and lyse them by cytolytic enzymes (perforin and granzymes). In melanoma patients NK cells have been studied mostly in peripheral blood and in tumor tissue, while NK cells in regional LNs of these patients have been scarcely investigated [1].

NK cell cytotoxic activity against tumor cells can be modulated by various cytokines. The important role in activation of NK cell cytotoxic function is mediated by: interleukin (IL)-2 and IL-15, the two cytokines that belong to the same cytokine family. Based on its biological activity, IL-2 has been used for more than two decades in therapy of metastatic melanoma and IL-2 therapy represents the proof of principle that activation of immune cells may result in tumor rejection and represents a milestone in cancer immunotherapy [2]. However, this treatment has shown also adverse effects and the effectiveness of this cytokine in antitumor immunity is limited by its ability to induce differentiation and maintenance of T regulatory cells (Treg) that are suppressive in antitumor immune response. IL-15, although, less cytotoxic and less effective in inducing Treg cell activity compared to IL-2, provides similar immune-enhancing effects on tumor reactive T and NK cells. Therefore, IL-15 generated tremendous interest in bringing this agent to clinical testing in cancer patients including the metastatic melanoma patients [3].

The aim of this study was to investigate the effect of IL-2, a cytokine that is used in metastatic melanoma treatment and IL-15, a cytokine from the novel generation, on antitumor cytotoxic function of scarcely investigated NK cell population from regional LNs of melanoma patients. The effect of these two cytokines on NK cells was also analyzed in the light of the expression of several activating and inhibitory NK cell receptors that regulate NK cell antitumor cytotoxicity.

**Patients and Methods:** In this study were included fifty melanoma patients in clinical stage II- IV, according to modified AJCC/UICC staging system that underwent regional LN dissection. Excised LNs were subjected to standard pathohistological examination performed in the Department of pathology in the Institute of Oncology and Radiology of Serbia. Tumor infiltration was evaluated by at least two independent examinations of hematoxylin/eosin stained sections per LN. For the purpose of this research, LN tissue samples were taken immediately after surgical excision and placed in large volume of RPMI 1649 tissue culture medium (CM) supplemented with 10% of fetal calf serum. LN tissue was subsequently dissociated to single cell suspension and mononuclear cells (MNC)s were purified. Isolated MNCs were *in vitro* treated for 72 h and 7 days with IL-2 (200 IU/ml) and IL-15 (25ng/ml) at 37ºC in atmosphere with 5% $CO_2$.

NK cell cytotoxic activity was estimated by two methods. By standard radioactive $^{51}$Chromium release assay, NK cell cytotoxicity was determined against tumor K562 erythromyeloid cell line for 80:1 effector to target cell (E:T) ratio. The other method used for measuring NK cell cytotoxicty activity was analysis of CD107a degranulation marker expression on gated $CD3^-CD56^+$ NK cells by flow cytometry after MNC stimulation with target K562 and melanoma (FemX and A375) tumor cell lines in 3:2 E:T ratio.

The expression of CD69 activation antigen, activating receptors (NKG2D and CD16) and inhibitory killer cell immunoglobulin-like receptors (KIR)s (CD158a and CD158b) and was analyzed on $CD3^{-}CD56^{+}$ NK cells by flow cytometry. The transcription of perforin gene (*PRF1*) was analyzed by rt-PCR, while the expression of synthesized perforin was analyzed by Western blot method.

Significance of differences between investigated parameters after cytokine treatments and control CM treatments was estimated by nonparametric Wilcoxon signed- rank test.

**Results:** After 72 hours of *in vitro* cultivation the standard chromium release assay showed that initial low values of NK cell antitumor cytotoxicity in non-metastatic and in metastatic regional LNs of melanoma patients substantially and significantly increased after treatment with IL-2, as well as with IL-15. Moreover, both cytokine treatments induced the expression of CD107a marker which represents the hallmark of cytotoxic granule content release, after contact with erythromyeloid and melanoma target tumor cell lines, on NK cells from both LN groups.

Concomitantly with NK cell cytotoxicity, after 72 hours of cultivation, IL-2 and IL-15 cytokine treatments increased the transcription of gene encoding cytotoxic molecule preforin as well as the level of this protein with more abundance of the fraction of the mature perforin form in both non-metastatic and in metastatic regional LNs.

After longer, 7 day *in vitro* treatments with IL-2 and IL-15 the enhancement of NK cell cytotoxicity persisted as well as the increased percentage of NK cell degranulation in both LN groups after co-culture with K562, as well as with FemX melanoma cell line in both non-metastatic and metastatic regional LNs. While both cytokine treatments increased NK cell cytotoxicity, only the treatment with IL-2 increased the prevalence of $CD3^{-}CD56^{+}$ NK cells in investigated LNs, whereas IL-15 treatment did not affect the percentage of NK cells.

After 7 days of *in vitro* cultivation the expression of CD69 early activating antigen, increased significantly after IL-2, as well as after IL-15 treatment on NK cells from both non-metastatic and metastatic regional LNs. Furthermore, IL-2 and IL-15 cytokine treatments on NK cells from both non-metastatic LNs and metastatic regional LNs increased the expression of NKG2D, the most prominent activating NK cell receptor that on binding to stress-induced ligands on malignantly transformed cells induces NK cytotoxicity. Moreover, both cytokines increased the expresson of CD16 activating receptor and CD158b KIR that are inherent to the mature [4] and cytotoxic NK cell phenotype.

**Conclusion:** In summary, our data show that both IL-2 and IL-15 cytokine treatments substantially increase antitumor cytotoxicity of NK cells from both non-metastatic and metastatic regional LNs of melanoma patients by increasing perforin level and inducing more mature $CD16^{+}KIR^{+}$ NK cell phenotype. This study gives an insight into the effects of these two cytokines on NK cell population in regional LNs that due to its localization represents the first line of antitumor immune defense in melanoma. The ability of IL-2 and IL-15 to activate NK cell from regional LNs regardless of LN tumor infiltration may provide relevant implication for novel immunotherapeutic interventions involving NK cell population from regional LNs of patients with malignant tumors either in adoptive cellular immunotherapy or as immunotherapeutic targets.

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**Key words**: cytokines, lymph nodes, melanoma, NK cells

## OP09: CD26 in various malignancies

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Dipeptidyl peptidase IV (DPPIV/CD26) is a transmembrane glycoprotein expressed on the surface of B and T lymphocytes, NK cells and macrophages, as well as on the surface of epithelial cells of various tissues and endothelial cells of blood vessels. The soluble form of DPPIV/CD26 is found in biological fluids, such as serum, plasma, semen, urine, synovial and cerebrospinal fluids. Lymphocytes are considered as one of the major sources of soluble DPPIV/CD26 in serum, shed from the membrane by proteolytic cleavage. This multifunctional protein is implicated in regulation of immune and inflammatory processes, as well as in the control of neoplastic transformation [1]. By cleaving dipeptides from N- terminal end of peptides and polypeptides that have proline or alanine in the second position, DPPIV regulates the activity of cytokines, chemokines, growth factors and hormones involved in the development and progression of malignant tumors. Binding of DPPIV/CD26 to components of extracellular matrix, such as collagen and fibronectin, points to its role in adhesion, migration and invasion of cancer cells.

Alterations in DPPIV/CD26 cell surface expression in addition to changes in serum DPPIV enzymatic activity or soluble CD26 levels are associated with various types of cancer [1]. Literature data point out tumor-promoting or even opposite tumor-suppressing properties of DPPIV, which seem to be dependent of its expression levels and interaction with specific molecules in the tumor microenvironment [1]. Decreased serum DPPIV enzymatic activity, related with impaired immune functions, is found in patients with various types of hematological malignancies and different solid malignant tumors [1]. While, increased serum DPPIV enzymatic activity, which is associated with enhanced immunity, occurs in hepatic cancer and in some hematological malignancies [1].

Robust investigations have proposed DPPIV/CD26 as a potential useful biomarker for early stage diagnosis and prognosis for several solid malignant tumors and hematological malignancies.

Our initial research dealing with the CD26 in cancer was the first to demonstrate the significantly decreased percentage of CD26+ overall white blood cells and significantly decreased expression of CD26 on the surface of lymphocytes in patients with breast cancer in comparison to healthy control group [2]. Although there were no significant changes in serum DPPIV enzymatic activity in the group of patients with breast cancer when compared to healthy control group, the increased frequency of these patients had higher serum DPPIV activity, pointing to possible role of this molecule in the breast tumor development. However, it must be noted that the same number of molecules of DPPIV can exert different activity depending on the specific microenvironment [1]. We did not find correlations between the levels of DPPIV enzymatic activity or its expression levels on lymphocytes and metastatic status or other histopathological properties of malignant breast tumors.

The next study of our group focused on changes in DPPIV/CD26 in melanoma showed for the first time a significantly lower DPPIV enzymatic activity in addition to decrease in the percentage of CD26+ overall white blood cells in patients with melanoma in relation to healthy individuals [3]. The observed changes could be explained by significant decrease in the percentage of lymphocytes in patients with melanoma, caused by tumor-specific immunosuppression or by alterations in lymphocyte homing

induced by modified chemokine gradients. Since cell surface CD26 associates with extracellular adenosine deaminase (ADA), changes in CD26 expression on lymphocytes might, at least to some extent, influence their overall number as a result of fact that ecto-ADA free lymphocytes are not capable to catalyze and inactivate potentially toxic adenosine to non-toxic inosine [1]. There were no differences in mean fluorescence intensity of CD26 expression on lymphocytes between the group of patients with melanoma and the group of healthy persons. Decrease of the mentioned parameters observed in the group of patients with melanoma was not dependent on the presence of metastatic disease, since differences between the subgroup of melanoma patients without metastatic disease and the subgroup of patients with metastatic disease were not statistically significant. Obtained data support the relationship between circulating DPPIV and the immune system and might indicate its possible role in the complex molecular mechanisms of melanoma pathogenesis.

In addition, our study about CD26 alterations in hematological malignancies showed significantly decreased serum DPPIV enzymatic activity in patients with Non-Hodgkin lymphoma, patients with leukemia and patients with multiple myeloma when compared to healthy individuals [4]. The significantly diminished DPPIV activity was associated with significantly reduced percentage of CD26+ lymphocytes in patients with Non-Hodgkin lymphoma and patients with leukemia. It is noteworthy that CD26 is proposed as a potential prognostic marker for B-cell chronic lymphocytic leukemia, in addition to molecular markers CD38 and ZAP-70 [5]. Furthermore, cell surface antigen CD26/DPPIV is considered as a novel promising diagnostic marker of leukaemic stem cells in chronic myeloid leukaemia [6].

CD26 has been suggested as a potential useful diagnostic and prognostic marker for colorectal cancer [7-9]. The levels of soluble CD26 in serum were found to be diminished in patients with colorectal cancer in comparison to healthy persons. A prospective, controlled, double-blinded study showed that measurement of sCD26 serum levels in addition to faecal blood immunochemical test may represent an important strategy for colorectal cancer screening in family risk-individuals [8]. What is more, a subpopulation of CD26+ cancer stem cells connected with increased invasiveness and chemoresistance was identified in patients with colorectal cancer [9]. Investigation of CD26+ cells in the primary colorectal cancer has been indicated as possibly useful for prediction of development of distant metastases [9].

We decided to evaluate further the possible clinical utility of assessment of the levels of DPPIV serum activity and the levels of CD26 expression on lymphocytes in patients with metastatic colorectal cancer. Our preliminary results did not show significant changes in serum DPPIV activity in the group of patients with metastatic colorectal cancer before oncological therapy in comparison to group of healthy control persons. Among 45 patients included in the study up to now, 20% of patients had increased DPPIV activity, while 18% of patients had decreased DPPIV activity. Our finding is in line with results of other groups that some of the patients with metastatic colorectal cancer had elevated soluble CD26 concentrations. In addition, we observed significantly decreased percentage of CD26+ lymphocytes and decreased percentage of lymphocytes in this group of patients in relation to healthy control individuals. In contrast to reduced percentage of CD26+ lymphocytes, patients with metastatic colorectal cancer had increased expression of CD26 on the surface of lymphocytes when compared to healthy controls, although observed difference was not statistically significant. Assessment of the clinical outcome of investigated patients will help to clarify the clinical significance of observed CD26 alterations in colorectal cancer.

The different levels of DPPIV expression on the surface of malignant cells in addition to high substrate specificity of this multifunctional protein, suggested that DPPIV might be used for selective enzymatic activation of anticancer prodrugs [10]. *In vitro* investigation of the functional activity of L-glycyl-L-prolyl dipeptide prodrug on a panel of malignant cell lines showed that activation and antiproliferative action of tested prodrug were dependent on the cell surface expression levels of DPPIV and indicate DPPIV as an useful potential target for specific delivery of chemotherapeutics [10].

A large and constantly growing body of literature data point out the important role of DPPIV/CD26 in the complex interplay between immune system and malignant tumors. The underlying mechanisms and possible clinical implications of alterations in DPPIV enzymatic activity accompanied with reduced percentage of CD26+ lymphocytes, that we observed in patients with different malignancies require further examination.

**Key words:** cancer, dipeptidyl peptidase IV activity, DPPIV/CD26 expression, immune system cells

## Session: Molecular changes in cancer development and progression I

### OP10: Tracking the evolution of epigenetic landscape during cancer progression by targeted bisulfite sequencing of circulating tumour DNA (ctDNA)

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**Background**: It has been long recognized that at the time of diagnosis most of human tumors are composed of subpopulations of cells with distinct phenotypes, and invasive and metastatic capacities. Recent cancer genomics studies provided deeper insight into intra- and inter-tumoral heterogeneity [1] demonstrating that most cancers are composed of several genetic subpopulations, supporting clonal evolution model in cancer progression [2]. The success of systemic cancer therapy is often halted by the emergence of secondary drug resistance due to cancer cell evolution and phenotypic heterogeneity [3]. Selection pressures exerted by therapeutic agents operate on the phenotypic variability acquired by the accumulation of genetic and epigenetic alterations, allowing new dominant subclones to emerge with a growth advantage manifested by increasing malignancy.

Genomic DNA methylation is one of the most important epigenetic modifications in eukaryotes necessary for cellular differentiation and plays a crucial role in gene regulation of many oncogenes and tumor suppressor genes. Aberrant DNA methylation has been heavily implicated in cancer initiation and progression, both through epigenetic silencing of individual genes through promoter CpG island hypermethylation, and on a genome-wide scale by global hypomethylation leading to activation of oncogenes, promotion of genomic instability and CpG mutator phenotype [4]. Pronounced intra-tumor heterogeneity of promoter DNA methylation has been documented in a variety of tumorsand increased global intratumor DNA methylation heterogeneity has been correlated with adverse patient outcome [5]. Furthermore, it has been documented that specific epigenetic changes such as MGMT promoter hypermethylation in glioblastoma, or global hypermethylation in bladder cancer, play a key role in development of chemoresistance [6]. In contrast to increasing knowledge regarding genetic events associated with drug resistance, we lack a detailed understanding of the particular epigenetic modifications and alterations involved in the acquisition of resistance phenotypes and the evolution of the epigenetic landscape during cancer progression, especially concerning the extent to which cancer cells undergo reversible phenotypic changes.

Understanding the selective effects of therapeutic interventions requires longitudinal tumour sampling, during disease progression and throughout treatment. However, owing to their invasive nature, repeated tumour biopsies cannot always be performed routinely in the clinical setting, and are also liable to sampling bias. Liquid biopsies in the form of cell-free circulating tumour DNA (ctDNA) isolated from blood plasma or serum have shown great promise to be used as non-invasive markers in cancer patients providing an insight into the composition of both primary tumour and metastases,

and an opportunity to assess clonal dynamics throughout the course of disease. Presence of detectable ctDNA was demonstrated even in early stage tumours across 14 cancer types, and several studies demonstrated the utility of ctDNA for monitoring tumour dynamics during treatment in patients with advanced disease by both targeted and whole exome sequencing of ctDNA. ctDNA carries not only tumour- specific changes in its sequence but also distinctive epigenetic marks, namely DNA methylation patterns similar to the patterns in the primary tumour. However, even though methods for targeted detection of genetic alterations in ctDNA are already established (e.g. Fluidigm, digital PCR, RainDance), there are no such methods available for targeted bisulfite sequencing.

The aim of this project is to develop a novel pipeline for high-throughput targeted bisulfite sequencing of ctDNA that will allow us to test for the presence of variant DNA methylation marks at single base resolution in minute quantities of ctDNA. Secondly, under the umbrella of the Cancer to chronic disease (C2c) initiative [7] we will explore the dynamics of DNA methylation changes during cancer progression in circulating tumor DNA (ctDNA), whether specific methylation changes are associated with differential patient outcome, and what is their underlying biology. This will be achieved through large-scale targeted bisulfite sequencing of longitudinally collected plasma ctDNA samples from patients undergoing systemic therapy acquired through ongoing prospective studies for colon cancer (Oncotrack, and NOCRC) and non-small cell lung cancer (TRAICERx).

**Material (Patients) and Methods**: Blood plasma samples were isolated from 10ml whole blood of healthy consenting volunteers. To determine the efficiency of the extraction procedure synthetic dsDNA fragments were spiked in the sample prior to extraction and detected by qPCR,Several different commercial methods for ctDNA extraction (Qiagen'sQIAamp Circulating nucleic acids kit, Norgen's Plasma/Serum Cell-Free Circulating DNA Purification, Macherey-Nagel'sNucleoSpin® Plasma XS) were compared. Genomic DNA contamination was estimated by an assay targeting the B-cell specific VDJ-rearranged IGH locus. Average DNA yield was estimated by PicoGreen assay (Invitrogen), and single gene copy number assay (RnasePamd BDP1). DNA integrity was determined by Agilent Bioalanyzer High Sensitivity DNA Analysis Kits.Mock ctDNA to be used for the optimization of the targeted protocol was prepared by sonicating known concentrations of Reference genomic DNA, and DNA methylation standards with different ratios of fully methylated (M.SSSI treated) to fully umethylated (Whole genome amplified) DNA, to 50-300bp range.

For the optimization of the protocol for targeted bisulphite sequencing of ctDNA three commercial and custom methods for ultra-low input NGS library preparation, in combination with target enrichment are being benchmarked: 1) Microfluidics-based picodroplet PCR enrichment and library prep on the ThunderStrom system (Raindance); 2) Post-bisulfite adaptor tagging (PBAT) protocol for library preparation in conjuction with hybridization-based target enrichment (Agilent SureSelect); 3) in-house one-step library prep and target enrichment method based on the MMLV-RT enzyme template switching and tailing (SMART). Starting from nanogram quantities of mock ctDNA (5-100ng/ml) we will generate sequencing libraries and perform target enrichment, coupled with Illumina MiSeq sequencing. Candidate targets were selected based on literature search and from own and publically available data at The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC). Currently, 450k DNA methylation data are available for over 10,000 tumours spanning 30 different cancer types. Once developed and established, TBS-seq will be applied to study epigenetic drivers of tumour resistance or recurrence in different cancers as part of the C2c (Cancer to chronic disease) programme.

**Results**: Obtaining ctDNA of sufficient quantity and quality is essential for successful downstream applications. The concentration of ctDNA that can be obtained from plasma/serum samples of mid to late stage cancer patients is usually very low (1–100ng/ml), and is highly fragmented. We have compared several described and novel commercial methods for isolation of ctDNA starting from a standard 10ml draw of peripheral blood, estimating the extraction efficiency,

average DNA yield, DNA integrity, and gDNA contamination to establish optimal standard operating procedure for ctDNA extraction.

Although PCR-based targeted bisulphite sequencing has been successfully used to profile entire human chromosomes, the method had remained labour intensive despite the introduction of next-generation sequencing and inaccessible to the analysis of ctDNA because of the requirement for large amounts of input DNA for target enrichment step. We are evaluating a microfluidics based ThunderStorm platform for target enrichment (RainDance Technologies) coupled with MiSeq Next Generation Sequencing (NGS), and compare its performance to other in-house developed methods for ultra-low input target enrichment and library preparation methods. The ThunderStorm platform allows simultaneous amplification of thousands of target loci in picolitre droplets on fully integrated microfluidic chips, making it the platform of choice for the proposed research. The technology has been extensively tested[8], including RainDrop BS-seq method for targeted bisulphite sequencing. This method is being compared to recently reported Post-BisulfiteAddaptor Tagging (PBAT) method[9], and to an in-house method based on the MMLV-RT enzyme template switching and tailing (SMART)[10].

**Conclusions**: A number of published and commercial ultra-low input whole genome bilulfite sequencing methods are available, however at present there are no established methods for targeted ultra-low input bisulfite sequencing. The method we aim to develop will be of great value to enable cost-effective interrogation of clinical samples for specific epigenetic targets. The opportunity to study DNA methylation variation in these prospective longitudinal efforts will provide new knowledge regarding the dynamics of epigenetic modifications during cancer progression and elucidate new epigenetic mechanisms underlying acquired resistance to cancer therapy, and thus aid the identification of minimally-invasive biomarkers to monitor cancer progression and predict response to chemotherapy.

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**Key words**: Cancer, epigenetics, genomics, biomarkers, DNA methylation

## OP11: Hereditary breast cancer genetics: looking for *BRCA3*?

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Breast cancer remains the second most common cause of cancer mortality in the western world despite ongoing efforts to understand its etiology and improve its outcomes [1]. Analysis of breast cancer genetics gained momentum more than 20 years ago with the identification of the two main breast cancer-associated genes, *BRCA1* and *BRCA2*. 5-10% of all breast cancers have a strong inherited component and 4–5% of these cases arise as a consequence of deleterious mutations in these high penetrance genes [2]–[4]. The risk of breast cancer for *BRCA1* mutation carriers by the age of 70 has been estimated to range from 40 to 87%, and for ovarian cancer from 16 to 68%. The risks for *BRCA2* mutation carriers were estimated to be 40–84% for breast cancer and 11–27% for ovarian cancer [5]. Mutation carriers are candidates for intensive radiological screening or risk reduction measures such as prophylactic surgery or chemoprevention. Hereditary breast cancer is also characterized with a higher frequency of disease among family members, apparent autosomal-dominant transmission, earlier age of onset, multiple primary cancers in individual and bilateral or multifocal cancers. Although important, mutations in *BRCA1/2* genes account for only a small part of inherited susceptibility to breast cancer with more than 60% of breast cancer families not explained by mutations in *BRCA1/2* [6]. In principle, the remaining susceptibility could be due to single genes with rare, strongly predisposing alleles similar to *BRCA1/2*. Another explanation of the hereditary predisposition is that it could be polygenic - the result of the combined effects of moderate or weakly predisposing alleles in a number of genes.

Even though the search for unique *BRCA3* has been going on for more than a decade, thanks to the immense technology development over the last couple of years, we witnessed a big shift towards the idea that breast cancer results from a combined effect of multiple common alleles conferring low risk. Recent development in sequencing technologies and capacities to analyze thousands of single nucleotide polymorphisms (SNPs) and nucleotide sequences through the entire genome has made significant progress in finding new genes that could potentially explain the rest of hereditary predisposition beyond the scope of *BRCA1/2*. However, even a large number of newly discovered allelic variants are still not able to explain complete hereditary predisposition to breast cancer (missing heritability) [7]. So, what is responsible? A high penetrance gene, low penetrance susceptibility genes or alleles? Or perhaps the main cause is not utterly genetic? By 2002, a susceptibility locus in 13q22 gained support for being the hunted *BRCA3*, and consensus was found later by the HUGO Gene Nomenclature Committee (HGNC). Nevertheless, genetic testing and information concerning the putative sought-after gene is still scarce and limited. So, is this the gene we are really looking for? Or perhaps a gene with similar penetrance as *BRCA1* and *BRCA2* does not exist?

Besides *BRCA1*/2, other high penetrance alleles have been identified as part of inherited cancer syndromes (breast cancer lifetime risk 40-85%) [3]. These include germ-line *TP53* mutations found in Li-Fraumeni cancer syndrome. Although *Li-Fraumeni* syndrome, connected to *TP53* gene mutations accounts for only ~0.1% of breast cancers, *TP53* mutation carriers have up to 60-fold increased risk for early onset breast cancer [2]. Germline mutations in tumor suppressor *PTEN* are the cause of *Cowden* syndrome. *PTEN* mutation carriers have lifetime risk for breast cancer of 50%. Although *CDH1* germline mutations predispose for hereditary diffuse gastric cancer, people with mutations in this gene have an increased risk for lobular breast cancer as well (40-54% lifetime risk). Genome-wide linkage studies have failed to map other highly penetrant cancer susceptibility genes, suggesting strongly that no

further high-penetrance genes of comparable importance to *BRCA1* and *BRCA2* exist. In spite of the high risks conferred by high penetrance mutations, these mutations are rare, and are estimated to account for a relatively small percentage (up to 25%) of the familial risk [8].

Another group of genetic variants associated with breast cancer risk are uncommon variants with moderate effects on breast cancer risk. Mutations in moderate penetrance genes have been associated with increased risk for breast cancer (20-40%) but these are rare genes with a population frequency <0,6%. *CHEK2* (increases breast cancer risk 2-fold as well as the risk for bilateral and male breast carcinoma), *ATM* (increases breast cancer risk 2-fold, and in younger than 50 years up to 5-fold), *RAD51C* gene has frequent and highly penetrant mutations especially in families with accumulation of both breast and ovarian cancers. Similar risk pattern for breast cancer has other moderate penetrant genes *BRIP1*, *PALB2*, *XRCC2*, *NBS1*, *RAD50*, *MRE11* and *BARD1* [9]. Because of the modest increases in risk and relatively low frequency of this class of genetic variants, their contribution to familial relative risk is estimated to be less than 3%. Since few genes have been studied in this way, it is likely that additional susceptibility variants of this class exist. However, re-sequencing of large numbers of cases and controls will be required to uncover them.

Most of the unexplained fraction of familial relative risk is likely to be explained by a polygenic model involving a combination of many individual variants with weak associations with risk, the so called low-penetrance variants. Technical advances, coupled with substantial decreases in genotyping cost have enabled investigators to move beyond evaluating a few candidate variants in key genes, to conducting more comprehensive evaluation of common genetic variation in candidate pathways to cancer, and to performing genome-wide association studies (GWAS) in very large study populations. In recent years GWAS studies sucessfuly identified more than 100 low-penetrability loci associated with different diseases, among them at least 17 are associated with breast cancer (*FGFR2*, *TOX3*, *LSP1*, *MAP3K1*, *TGFB1*...). Considerable additional follow-up investigation will be required to establish the relationships between many of the SNPs and the actual causal variant(s) and to further elucidate the role in disease for many of these common genes.

Only a part of hereditary breast cancer predisposition is explained by all genetic variants discovered to date. The remaining heritability, termed as ˝dark matter˝ may be composed of common variants with even smaller effects, or even rare variants that couldn´t be tagged by GWAS. In addition, it is possible that some of the familial risk may be mediated through complex gene-gene or gene-environment interactions. Additional studies require large study populations and, in the case of gene-environment interactions, accurate measurement of lifestyle/environmental factors. Structural variation, such as copy number variants (CNVs), which are not well tagged by SNPs in current arrays, may be a source of missing heritability in breast cancer.

In clinical cancer genetics, variants of unknown significance (VUS) pose significant challenges to genetic counseling even for the *BRCA1*/2 mutation tests. Important clinical question is whether sufficient knowledge will have been gained from all studies on polygenic model to make population-level genetic testing feasible. It is still uncertan if the growing list of genes involved in breast cancer will be integrated into routine clinical testing since prediction risk in the case of common genetic varians is still not clear and no defined clinical decision can be made on the basis of their presence. Given how little has actually been explained of the demonstrable genetic influences on most common diseases, despite identification of hundreds of associated genetic variants, the search for missing heritability provides a potentially valuable path towards further discoveries.

In conclusion, it feels like the two most likely dark matter candidates are common alleles with very small effects and rare alleles with moderate effects (or uncommon variants with effects in-between) [10]. However, when the effect size is as low as it is expected to be (~1.00) it might be impossible to

detect these yet undiscovered alleles no matter how large the sample size. Another limitation is that many rare alleles will be difficult to find especially if many are private or ˝belong˝ to a family. Even though the architecture of inherited susceptibility to breast cancer will be further revealed in the upcoming years, given the mentioned limitations it is quite likely that full understanding of the genetic contribution to cancer will not be achieved in the near future.

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**Key words:** breast cancer, genetic testing, germ-line mutation

## OP12: Cancer epigenetics: from mechanisms to clinical applications

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**Background:** Cancer is associated with a number of genomic aberrations, which can lead to abnormal expression of oncogenes and tumor-suppressor genes. In addition to genomic changes, epigenetic modifications can causestable changes in gene expression that are not coded in the DNA sequence. Major mechanisms of epigenetic control in mammals include DNA methylation, histone modifications and RNA interference (RNA silencing). Once established, epigenetic patterns are stable and mitotically inheritable.

The key epigenetic modification in mammals is the DNA methylation of cytosine in CpG dinucleotides of CpG islands, CG-rich regions in the promoters of tumor suppressor genes, which cause

their transcriptional silencing during tumorigenesis (1). The covalent addition of a methyl group to the cytosine in a CpG dinucleotide is catalyzed by a family of enzymes DNA methyltransferases (DNMTs). WhileDNMT3A andDNMT3B aremostly involved in the de novo methylation, DNMT1 is involved in the maintenance of DNA methylation after replication. Several studies have shown DNMToverexpression (mainly DNMT1and DNMT3B) in cancers(1).

Histones have a dynamic role in the regulation of chromatin structure and gene activity. Histone tails can be modified by acetylation, methylation, phosphorylation, poly-ADP ribosylation, sumoylation, or ubiquitination. A combination of histone modifications and histone variants, referred as a "histone code", determine the interaction of histones with DNA and the interaction of non-histone proteins with chromatin. Histone acetylation by histone acetyltransferases (HATs) neutralizes the positive charge on lysine residues in histone tails, by which the histone tail releases from the negatively charged DNA, chromatin is 'loosened' and accessible to the transcriptional factors. Histone deacetylases (HDAC) deacetylize lysine in histone tails, and nucleosomes are more tightly compacted. Histone methylation, regulated by histone methyltransferases (HMTs), and the histone demethylases (HDMs), does not alter the histone tails charge, but influences the chemical characteristics of histones and their affinity to transcription factors or other regulatory proteins (2).

MicroRNAs have emerged recently as one of the key epigenetic mechanisms underlying malignant transformation. Micro-RNAs (miRNAs) are small (18-22 nucleotides), non-coding RNAs that have a role in posttranscriptional regulation, by binding to the target mRNA RNA. Micro-RNAs function as transcriptional regulators either by complete complimentary base pairing, resulting in the mRNA degradation, or by partial base pairing, which leads to translational inhibition of the targeted mRNA. Micro-RNAs play important roles in cell proliferation, apoptosis, and differentiation.A number of miRNAs are deregulated during tumorigenesis and may act as tumor suppressors or as oncogenes, depending on their targets (3).

Recently, epigenetic changes have been considered one of the most promising tools for the early detection of cancer. In smokers, *p16* and *MGMT* gene hypermethylation was detected in sputum years before clinical manifestation of the lung carcinoma (4). Hypermethylation of APC gene was associated with a high sensitivity and high specificity for cancer on repeat biopsy of prostate carcinoma (5). In addition, DNA methylation of*the GSTP1 gene promoter*, together with the methylation changes in repetitive elements LINE-1 and Sat2 was observed in prostatic preneoplastic lesions (5).

A number of tumor suppressor gene hypermethylation has been associated with worse prognosis in various cancer types. DAPKpromoter hypermethylation has been associated with tumor aggressiveness and poor prognosis in lung cancer (6). p16 methylation in serum samples of colorectal cancer patients correlated with tumor stage, while the presence of methylated DNA in plasma of esophageal patients was associated with decreased survival (5). E-cadherin promoter hypermethylation was the prognostic factor of a worse outcome in a number of carcinomas, including oral cancer (7). In oral carcinomas, the patohistologically negative surgical margins exhibited frequent aberrant DNA methylation changes in a number of cancer-related genes, while DAPKpromoter hypermethylation detected in negative margins was associated the recurrences and poorer overall survival (8).

Hypermethylation of MGMT is the predictor of good response to alkylating therapy in gliomas and in oral carcinoma, and to cyclophosphamide therapy in diffuse B cell lymphoma (9). Acquired hypermethylation of DNA mismatch repair gene hMLH1 (detectable in peripheral blood) during carboplatinum/taxane therapy of ovarian cancer predicts poor outcome (9). Thus, defining the epigenetic profile in predicting the chemosensitivity of individual tumors would contribute to personalized cancer therapy.

Since epigenetic changes are potentially reversible, enzymes involved in the epigenetic regulation are attractive targets for the development of novel epigenetic therapies (10). Epigenetic therapy of

cancer is based mostly on the usage of inhibitors of DNA methyltransferases (DNMTs), histone deacetylase (HDAC) inhibitors and anti-micro-RNA therapy (10).Epigenetic agents might reverse epigenetic changes in tumors and restore normal gene function to a cell. Vidaza (5-azacytidine),a ribonucleoside inhibitor of DNMT1that has already been used in a number of clinical trials, isapproved by the Food and Drug Administration (FDA) for myelodysplastic syndrome and acute myeloid leukemia (10). Accumulating evidence are suggesting that epigenetic agents might serve as a basis for a new therapeutic approach in reactivating tumor suppressors, silencing oncogenes, or acting as chemo- or radiosensitizers, followed by treatment with standard therapy. However, so far this therapy lacks specificity of targeting particular genes.

**Conclusions:** Various studies showed that the number of cancer-related genes that are inactivated by epigenetic modifications equals or even exceeds the number of genes inactivated by genetic changes. Epigenetic markers are emerging as useful markers for early detection, prognosis, and therapy of cancer. Due to the potential reversibility of epigenetic changes, enzymes involved in the epigenetic regulation are attractive targets for the development of novel therapies. In addition, emerging studies suggest that bioactive food compounds can affect gene expression through epigenetic mechanisms, supporting the developing field of Nutritional Epigenetics. A growing number of studies focused on clinical applicability of epigenetic markers and epigenetic agents as anticancer therapy or as chemo and radio sensitizers, withpromisingresults.Further studies are needed tofully elucidateEven though underlying mechanisms of epigeneticchanges and their clinical application.

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**Key words:** epigenetics; DNA methylation; cancer; prognosis

## OP13: Identification and molecular characterization of prostate cancer metastasis founder cells

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**Background:** Metastasis is the cause of more than 90% of cancer-related deaths.[1] In some cases, early detection and treatment of primary tumour can prevent or delay death due to metastasis. However, in most cases, once the metastatic outgrowth is detected, the clinicians can use existing treatments only for palliative purposes. Still, the period between surgical removal of primary tumour and the appearance of lethal metastasis can extend to years and decades. This period, considered by some authors as period of cancer dormancy, offers a time window for adjuvant therapeutic intervention, aiming to prevent or delay outgrowth of metastasis.[2] Disseminated cancer cells (DCC), that remain in the body of patients after surgical removal of primary tumour, are considered to be founder cells of later arising lethal metastasis.[3] Adjuvant cytotoxic therapies in many cases fail to prevent death due to metastasis.[4] On the other hand, current targeted therapies are prescribed upon molecular analysis of each patient's primary tumour. However, even such sophisticated approach only modestly improved the survival of the patients, both with early- stage and metastatic cancer.[5,6] Clearly, current treatments are not able to target metastasis founder cells. One underlying explanation for this could be the vast genetic and phenotypic heterogeneity of cancer cells within the growing tumour. Therefore, it is possible that the majority of cells in the primary tumour and the cancer cells that have disseminated to distant sites will not have the same molecular make-up.[1] Consequently, it is essential to directly characterize metastasis founder cells at the molecular level in order to identify potential therapy targets.[3] DCC can be detected in the bone marrow (BM) of cancer patients, using staining against epithelial markers (cytokeratins [CK] or epithelial cell adhesion molecule [EpCAM]). It was shown in virtually all types of carcinoma that the presence of DCC in BM is associated with worse prognosis.[7] Importantly, bone is often the place where metastatic lesions can be detected in many types of cancer (e.g. breast and prostate), further strengthening the notion that metastasis founder cells are among DCC. However, DCC are rare (1 DCC per $10^6$ bone marrow cells) and often exist as solitary cells. This and the fact that only few DCC can be detected per patient present a challenge to molecular characterization of these cells. In this work, we aimed to analyse the genome and transcriptome of individual EpCAM$^+$ DCC isolated from non-metastatic (M0) prostate cancer (PC) patients.[8] However, it was previously shown that EpCAM is expressed on hematopoietic cells of bone marrow of healthy individuals, thereby confounding the reliable identification of DCC in cancer patients.[9]

**Patients and Methods.** Aiming to identify molecular differences between EpCAM$^+$ DCC and EpCAM$^+$ hematopoietic cells, we obtained BM samples of 105 M0 PC patients and 18 cancer-free males, and searched for EpCAM$^+$ cells. These individual cells were isolated and subjected to transcriptome and genome analysis. We have examined the expression of epithelia-specific (*EPCAM*, *KRT8*, *KRT18*, *KRT19*, *KRT14*, *KRT6a*, *KRT5*), prostate-specific (*KLK3* [PSA]), hematopoietic (*PTPRC* [CD45], *CD33*, *CD34*, *CD19*), and erythroid (*GYPC*, *SCL4A1* [band 3], and haemoglobin [*HBA2*]) transcripts using PCR. Analysis of genomic aberrations was done using comparative genomic hybridization (CGH), which detects unbalanced chromosomal aberrations affecting regions of 10 million base pairs or more.

**Results:** Surprisingly, we detected on average 4 EpCAM$^+$ single cells in 62% of patients and 56% of controls (Figure 1).

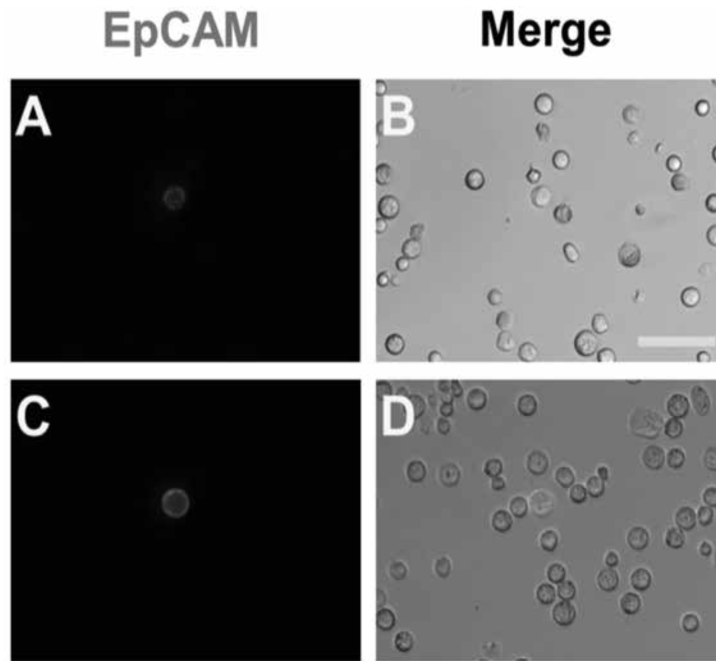


**Figure 1.** Photographs of the EpCAM+ cells isolated from the BM of control (A-B) and M0-stage (C-D) PC patients. Scale bar – 50 µm (modified from [8]).

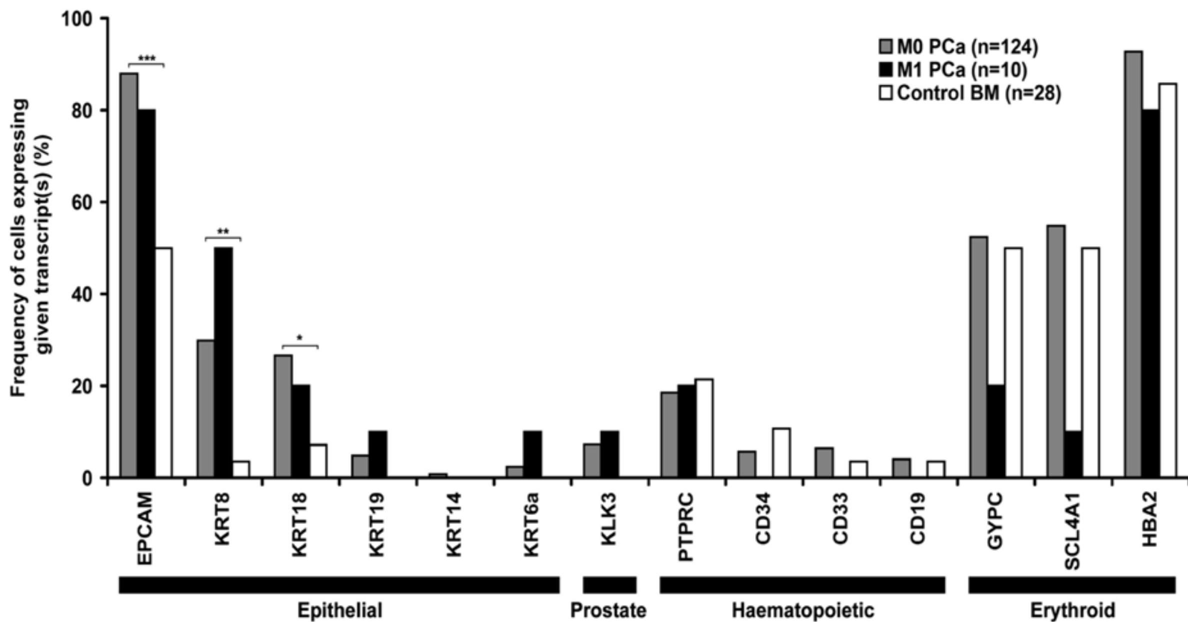


**Figure 2.** Gene expression of EpCAM+ cells isolated from PC patients and controls. The identities of transcripts are on the X-axis. Grey bars - M0-stage PC patients, black bars – M1-stage PC patients, white bars - controls. Asterisks indicate the level of significance: * – $p<0.05$, ** – $p<0.01$, *** – $p<0.001$; n indicates the number of analysed single cells (modified from [8]).

Unexpectedly, EpCAM+ cells from cancer patients were expressing hematopoietic and erythroid transcripts, while EpCAM+ cells from controls were expressing epithelial markers (Figure 2). In order to exclude the possibility that the EpCAM+ cells isolated from PC patients are hematopoietic cells, we have performed genome analyses. The presence of genomic aberrations ensures that the analysed cell is a cancer cell.

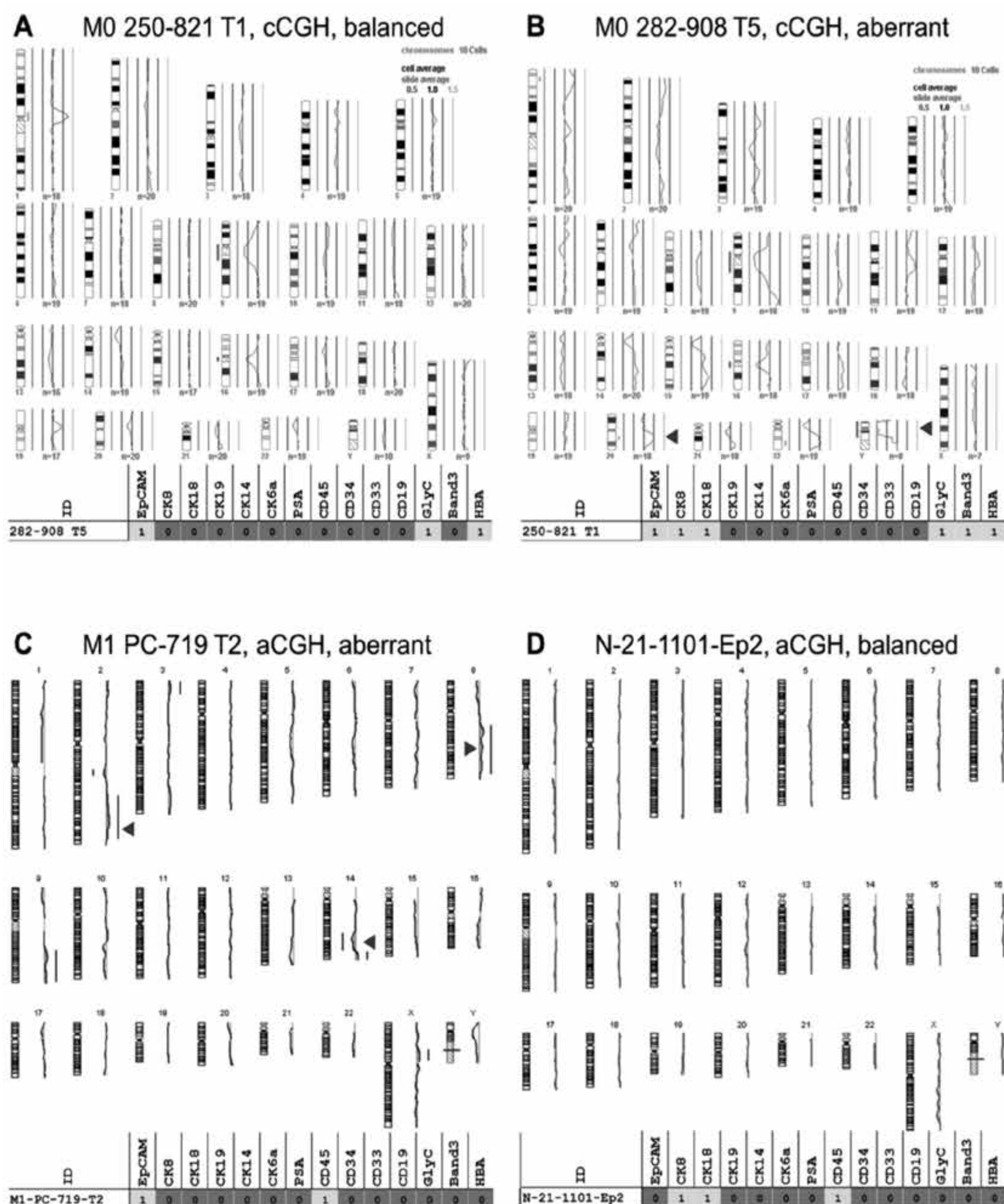


**Figure 3.** Combined genome and transcriptome analysis of single EpCAM+ cells using metaphase- (cCGH) or array- (aCGH) CGH. Profiles of two M0-stage (A,B) and one M1-stage (C), and one control (D) patient are shown. Some of the detected aberrations are indicated by arrowheads. Transcript profiling of each cell is given below the CGH profile (green and 1 indicate that the transcript was detected in the sample, while red and 0 indicate that the transcript was not detected). (modified from [8]).

Using CGH, among EpCAM+ cells from PC patients we have identified 38% of true DCC, of which many expressed transcripts of common hematopoietic marker CD45 or erythroid marker haemoglobin (Figure 3).

**Conclusion.** Our data point to an unexpected transcriptome plasticity of epithelial cancer cells in BM and have important implications for the study of disseminated and circulating tumour cells. First, an assumption that cells expressing one or few epithelial markers are really cancer cells, while the cells that co-express epithelial and hematopoietic markers are not, is most likely incorrect. Second, the observed expression of hematopoietic and erythroid transcripts by BM DCC may reflect adaptation to the specific environment. It is possible that crosstalk between DCC and BM hematopoietic cells, mediated by cell-cell interaction or secreted factors, leads to expression of hematopoietic transcripts

in DCC.[10] Furthermore, the transcriptomes of DCC could be altered by the uptake of mRNA-containing exosomes released from resident BM hematopoietic/erythroid cells or our observations could be the consequence of hypoxic/oxidative stress imposed on DCC within the BM microenvironment. In any case, additional studies are needed to identify the underlying causes, and the observed altered transcriptomes are likely to result in altered phenotypes and cell function, which may be highly relevant for our attempts to tailor cancer- and patient-specific therapies.

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**Key words:** bone marrow, disseminated cancer cells, epithelial cell adhesion molecule, metastasis, prostate cancer, transcriptome analysis

## OP14: Expression of PTCH1b Tumor Suppressor Gene Is Controlled by Different 5' Untranslated Region *Cis*-Regulatory Elements

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**Background:** *PTCH1* tumor suppressor gene encodes for a transmembrane receptor with a negative regulatory role in Hedgehog-Gli signaling pathway, which is a highly conserved cellular mechanism for transducing signals from the cell surface into the nucleus [1]. Because of its important role in many developmental processes, several human syndromes involving congenital malformations are caused by genetic alterations of Hedgehog-Gli pathway genes. For example, *PTCH1* germline mutations cause Gorlin syndrome which is characterized by developmental abnormalities and tumor susceptibility [2]. Most of malformations are caused by *PTCH1* haploinsufficiency, indicative of fine-tuning needed to properly regulate pathway activity. One of the main roles of 5' untranslated region (5'UTR) of mRNA is

a post-transcriptional regulation of gene expression, starting from the initiation of protein translation. The 5'UTRs can differ in length, nucleotide content, secondary structures, and the presence of many different functional elements. So far, many diseases are associated to improper functioning, for example caused by point mutations, of any of those 5'UTR cis-regulatory elements. After identifying 5 to 8 CGG repeats close to translation initiation site, our aim was to examine how 5'UTR regulates the expression of *PTCH1* gene main transcript 1b (*PTCH1b*) [3].

**Material and Methods:** All potential *PTCH1b* 5'UTR cis-regulatory elements were studied by various *in silico* tools and gene reporter assays. We tested the influence of 5'UTR length and CGG-repeat polymorphism by cloning either 188- or 300bp-long *PTCH1b* 5'UTR, each harboring 5 to 8 CGG repeats, in a pGL3-Promoter vector upstream of firefly luciferase (Fluc) reporter gene. Site-directed mutagenesis (SDM) was used to test the significance of predicted upstream open reading frames (uORFs). Bicistronic pRuF vectors were constructed by cloning differently sized *PTCH1b* 5'UTRs between *Renilla* and firefly luciferase genes, since the last 76bp in *PTCH1b* 5'UTR were predicted as an internal ribosome entry site (IRES). Hypoxic cell conditions were used for the activation of putative *PTCH1b* IRES motif, while GLI expression vector pcDNA4nlSMtGLI1 was used for the activation of Hedgehog-Gli signaling pathway. Dual luciferase assays and qPCR quantification of two luciferase genes and different *PTCH1b* 5'UTRs were performed in MCF-7, HCT 116 and HEK 293T cells.

**Results:** Reporter assays showed that shorter 188bp-long 5'UTR significantly increased reporter activity, with a subtle reduction with higher repeat number. Longer 300bp-long 5'UTR led to much reduced reporter activity, without a difference among repeats. At the same time, both 5'UTRs significantly increased Fluc mRNA transcription, which could potentially be explained by the abundance of binding sites for BEN, ZF5 and ETF transcription factors within *PTCH1b* 5'UTR. Two different *in silico* tools predicted two uORFs and one uAUG codon within longer *PTCH1b* 5'UTR only, and those uORFs could interfere with the translation from the main ORF. SDM proved our hypothesis that two out of three potential uORFs might account for severe reduction in Fluc activity which was observed for longer *PTCH1b* 5'UTR transcript. Interestingly, *PTCH1b* 5'UTR quantification showed that longer transcript was virtually exclusive in basal conditions and appeared to be still the most abundant after Hedgehog-Gli pathway activation, although the 188nt-long 5'UTR transcript appeared also to be induced. On the contrary to the results with monocistronic reporter vectors, both 5'UTR sizes significantly increased Fluc activity of bicistronic vectors (proved by PCR and qPCR this was not due to an alternative splicing or presence of a cryptic promoter), while Fluc activity was significantly reduced after removing predicted IRES motif. Firefly/Renilla luciferase mRNA ratios were the same as for empty vector, indicating that observed higher Fluc activity should be due to a post-transcriptional regulation, i.e., cap-independent translation of Fluc mRNA. In addition, we also showed that hypoxia can stimulate *PTCH1b* 5'UTR-mediated cap-independent translation.

**Conclusion:** We showed that the number of CGG repeats could not have a significant impact on either mRNA or protein expression of *PTCH1b* gene, thus supporting the thesis that this *PTCH1* polymorphism could not be a genetic background of a trinucleotide repeat disorder-like disease. The activation of *PTCH1b* IRES motif could allow overcoming the negative effect of uORFs and uAUG within long 5'UTR transcripts. Coupled to our observation that GLI1 overexpression can induce the transcription of *PTCH1b* transcripts containing the short and the longer 5'UTRs, these results could lead to hypothesize that the ensuing negative feedback loop can also be fine-tuned at post-transcriptional level by the competition between uAUG/uORF translation inhibition and IRES-dependent translation initiation [4].

All our results point to the exceptionally complex and so far unexplored levels of *PTCH1b* expression regulation by 5'UTR [5]. So far, we can assert that *PTCH1b* 5'UTR shares its characteristics with mRNAs coding for transcription factors, proto-oncogenes, growth factors and their receptors, or generally for proteins poorly translated under normal condition.

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**Key words:** PTCH1, 5' Untranslated Region, Protein Translational Initiation, Trinucleotide Repeats, Open Reading Frames

## Session: Clinical oncology: Era of molecular markers

### OP15: Lymphonodal metastases in clinically N0 patients with incidental thyroid microcarcinomas

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**Introduction**: Incidental thyroid carcinomasare discovered after thyroid surgery due to benign diseases,such as multinodal goiters, adenomas, Hashimoto's thyroiditis or hyperthyreosis. Commonly, they are referred to as papillary, since serum calcitonin levels and levels of carcinoembryonic antigenare sensitive markers for medullary carcinomas, and these are rarely found incidentally in thyroid specimens.Microcarcinomas are tumors under or 10mm in size. Lymphonodal metastases (LNMs) in papillary thyroid microcarcinomasare common, more likely in tumorsover 5 mm in diameter, tumors with extrathyroid extensionand multifocal carcinomas. Presence of LNMs in central neck compartment, extrathyroid tumor extension and tumors multifocality are distinguished as independent predictive factors for the occurrence of the lateral LNMs. This suggests that lateral neck dissection may be unnecessary in patients without central LNMs, unless lateral LNMs are clinically verified, either by physical examination, or by ultrasound or computerized tomography of the neck. However, "skip metastases" (lateral, without central LNMs) can be found in some patient series in up to 8% of cases.Further, although some authors report high ultrasound specificityin all neck regions, especially in lateral neck compartments, micrometastases can easily be overlookedby imaging methods.

The aim of this researchwas to determinefrequency of LNMs in central neck compartment and jugulo-carotid chain (JCC) in clinically N0 patients with incidental papillary thyroid microcarcinomas (IPTMC), as well as to evaluate accuracy andusefulness of sentinel lymph node (SLN) biopsy in their detection.

**Materials and methods**: Acquisition of data for thyroid cancer database used in this research was performed retrospectively. Out of all patients operated in our Institution from 2004 to 2015 for benign thyroid diseases, 195 were identified to have evident or suspect focus of IPTMC on intraoperative frozen section analysis (later confirmed on standard pathology reports). These patients underwent total thyroidectomy, central neck dissection (CND) and sentinel lymph node (SLN) biopsy of JCC, as

a standard surgical procedure for treatment of malignant thyroid disease. None of the patients had suspect lymph nodes of the central and lateral neck compartments, both on physical and ultrasonical preoperative examination.

SLN mapping was performed injecting 0.2-0.5ml of 1%-methylenebluedye in both lobes prior to their mobilization, directlybeneath thyroid gland capsule, along with coagulation of the capsule to avoid leakage of the vital dye. In cases with clinically evident tumors, methylene blue dye was injected peritumorally.Both JCCs were inspected for blue stained lymph nodes–SLNs, dominantly levels III and IV. In case colored lymph nodes were not identified, those nearest to the colored lymphatic vessel(s) were harvested as SLNs. All SLNs were examined intraoperatively by frozen section analysis. If these findings were positive, immediate modified radical neck dissection (MRND) of the belonging JCC was done. Otherwise, several more lymph nodes, in the proximity of SLNs, were additionally excised, paraffin-embedded and formalin-fixed for standard pathohistological examination as a control group of JCC lymph nodes.

Accuracy of SLN biopsy method was assessed in two levels. Firstly, findings on all SLN specimens obtained by frozen section analysis and standard pathological analysis were compared in order to evaluate quality of rapid, intraoperative pathological analysis of SLNs. Secondly,additionally extirpated lymph nodes from levels III and IV were used as a control group for comparison with frozen section findings on SLNs of the belonging JCC and evaluation of whether excised SLNs were representative for the status of the specific lateral neck region.

Status of central neck lymph nodes was assessed by standard pathohistological examination of paraffin-embedded and formalin-fixed specimens. Frozen section analysis was not performed on these specimenssince central neck dissection is routinely done as a part of extended thyroidectomy for treatment of malignant thyroid disease.

Frequency of LNMs in central, lateral and both neck compartments was assessed.

**Results**: Out of total number of 195 patients, 156 were females and 39 were males, aged from 21 to 73 years, with average of 47.23 years. Mean tumor size was 4.73mm, ranging from 0.5 to 10mm in diameter.IPTMCssmaller than 5mm were more dominant in the series (64.62%, 126/195). Tumors weremulticentric in almost 40% of patients. LNMs were verified in 24% (47/195) of cases. In this sub-group of patients with LNMs, isolated central LNMs were found in 68% (32/47), isolated lateral, JCC, LNMs ("skip metastases")in almost 11% (5/47), while 21% (10/47) of patients had LNMs in both central and lateral neck compartments.

Identification rate of colored SLNs was 70%. Definitive pathohistology on SLNs showed a 97%-match with frozen-section analysis. There were no false positive findings. SLNs were positive on frozen section in 5.64% (11/195) of patients in whom MRND was performed immediately. Results of frozen section on SLNs were inconclusive in 2 patients only, but later on standard pathohistology metastases were found. Sample of surrounding lymph nodes from levels III and IV was metastases-free on definite pathohistology analysis, thus these patients were considered completely surgically cured and no delayed MRND was necessary. In 2 patients, SLNs were defined as benign on frozen section analysis, but on definite pathohistology they were proven to harbor micrometastases. Given that there were no additional LNMs in the sample of excised surrounding lymph nodes from levels III and IV, no further delayed MRND was necessary.

Method's accuracy was 97%.

**Conclusions**: Lymph node metastases in IPTMCs and clinically N0 patients were detected in significant percentage of patients. Frozen section analysis of SLNs was highly precise in detection of LNMs. Micrometastases in SLNs were omitted on intraoperative pathohistological analysis. No patients were falsely selected for immediate MRND. Based on standard pathohistological analysis on control group of lymph nodes from levels III and IV, other than metastases in SLNs (found on frozen section analysis), no

additional metastases were detected in JCCs, thus it can be concluded that SLNs were representative for the status of the specific lateral neck region. SLN biopsy selected precisely 6% (11/195) of clinically N0 patients with JCC metastases for immediate MRND. All these patients were preoperatively staged as clinically N0. This suggests that SLN biopsy is a more precise method for evaluation of disease stage than clinical examination and ultrasonography.Using SLN biopsy, one can avoid unnecessary MRND, as well as prevent under-treatment of patients with good prognosis.

**Key words**: incidental papillary thyroid microcarcinomas, lymphonodal metastases, sentinel lymph node biopsy

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## OP16a: Academic clinical trials in diagnostics and therapy of lung cancer in Serbia - era of advanced technology and molecular biology

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In past few years it was established that non small cell lung cancer (NSCLC) acquires series of molecular, genetic and pathological features with significant influence on carcinogenesis and therapeutic response to targeted therapies. Understanding molecular etiology of NSCLC significantly influences choice of therapy and improves outcome of treatment. Most known driver mutations include mutations in domain of the epidermal growth factor receptor (EGFR), mutations in Kirsten-rous avian sarcoma genes (KRAS), and fusion echinoderm microtubule-associated protein-like4 on anaplastic lymphoma kinase gene (EML4-ALK). Key signaling pathways in carcinogenesis of NSCLC are RAS/RAF/MEK (mitogen-activated protein kinase) pathway which controls cellular proliferation, differentiation and survival, and PI3K/AKT/mTOR (mammalian target of rapamycin) pathway mainly responsible for apoptosis. There are many other important molecular abnormalities which are potential targets for therapy of NSCLC such as system of mesenchimal-epithelial transition (kinases) or MET and Insulin-like growth factor receptor 1. Understanding signaling pathways, driver mutations, target genes and their products enables introduction of personalized therapy in lung cancer treatment. In the near future definitive diagnosis of NSCLC will require genetic profile of the tumor. This will enable application of best therapeutic protocol for each individual patient with advanced NSCLC. One of the most recent concepts in targeted therapy of lung cancer involves use of Heat Shock Protein 90 inhibitors. In a recently published trial "A randomized phase II study of ganetespib, a heat shock protein 90 inhibitor, in combination with docetaxel in second-line therapy of advanced non-small cell lung cancer (GALAXY-1)" we presented the activity and safety of ganetespib in combination with docetaxel in advanced non-small cell lung cancer (NSCLC). The major aim of this lecture is to present the concept of Hsp90 inhibition and discuss the results of Galaxy-1 trial as well as to point the possibilities of investigator initiated trials within the targeted therapy of lung cancer.

**Key words:** clinical trials, Hsp90 inhibitor, lung cancer.

## OP16b: Academic clinical trials

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**Academic clinical trials** are clinical trials that are not funded by the pharmaceutical or biotech companies but by public-good agencies (usually universities or medical trusts) to advance medicine. The trials are a valuable component of the health care system; they benefit patients and help determine the safety and efficacy of drugs and devices, and play an important role in the evidence based medicine.

A typical area of academic clinical trials is the advancement and optimization of already existing therapies. Thus, academic clinical trials may for instance test how a combination of registered drugs may improve treatment outcomes; or they may apply registered treatments in additional, less frequent indications. Such research questions are not a primary focus of for-profit companies, and thus these trials are typically initiated by individual investigators or academic research organizations.

There are many different organizations which have an interest in academic clinical trials and facilitate or take part in their conduct. These organizations include:

- Hospitals, universities, researchers and institutions who view trials as a source of income and prestige, and receive private, charitable and governmental funding.
- Pharmaceutical or biotech companies who view the development and commercialization of treatments as their business.
- Regulators who wish to ensure treatments are safe and work effectively.
- Patients and patients' organizations and associations who want faster access to advanced treatments.

Academic clinical trials are run at academic sites, such as medical schools, academic hospitals, and universities; and non-academic sites which may be managed by so-called site management organizations (SMOs). Site management organizations are for-profit organizations which enlist and manage the physician practice sites that actually recruit and follow patients enrolled in clinical trials.

In spite of the concern raised by academic researchers, there is little hard evidence to support the notion that academic clinical trials are in marked decline. Indeed, in some studies no change has been reported, but this has been attributed to the provision of an adequate infrastructure to support academics through the regulatory process.

And within the investigative centers, there must be awareness and training of personnel at all levels to permit growth and survival of 21st century noncommercial clinical research.

At national levels, positive acknowledgment should be given to recognized centers conducting this type of research through the provision of dedicated funding, with periodic evaluation of the quality of research practice and the quantity of scientific production of such centers.

It has been suggested that investigators organize themselves into networks of centers of research for each pathology. These networks would be investigator-driven and, with adequate funding, free from political and commercial influence.

Ongoing intensive research into the clinical applications of proteomic and genomic advances in medicine will have benefits in the long term for all clinical research. These are still in the early stages of evaluation, but the potential for individualizing patient therapy is enormous. A more targeted selection of participants for clinical trials will mean that in the future there will not be a need for such large numbers, as seen in some of today's mega-sized trials, thus lowering infrastructural and

operational costs. In the meantime, researchers must invest commitment and energy in adapting to their present environment.

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## OP17: The diffuse large B-cell lymphoma – can we at least approach study results in everyday clinical practice


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**Backgrounds:** The diffuse large B-cell lymphoma (DLBCL) is the most common histologic subtype of nonHodgkin's lymphomas (1,2). Still, the disease is quite heterogeneous in terms of morphology, genetics, biologic behaviour, and consequently response to treatment and prognosis (1).The addition of rituximab to standard chemotherapy (CHOP and CHOP-like) in patients with DLBCL has resulted in significant improvements of the disease-free and overall survival rates (3-5). Beside the original international prognostic index (IPI)(6) that has been later on validated also in patients receiving rituximab containing treatment (7) similarly the revised international prognostic index (R-IPI) (8) has been proposed to predict outcome in patients with the DLBCL receiving R-CHOP or R-CHOP-like regimens. Due to superior results with rituximab, the R-CHOP and variants have become the standard initial treatment of patients with the DLBCL (9). However, the conditions of routine treatment are far different from the ones in clinical studies where the study population is highly selected, the histopathology and staging procedures are thoroughly revised and treatment and side effects are strictly controlled. The aim of our retrospective study was therefore to analyse and to compare the treatment results of routinely treated patients with the DLBCL at the Institute of Oncology Ljubljana to results reported by some larger studies.

**Patients and methods:** Two hundred and ninety five patients with the DLBCL were treated between 2004 and 2008 according to the then protocol with R-CHOP or R-CHOP-like regimens at the

Institute of Oncology Ljubljana. The patients' characteristics, patohistological diagnosis, disease stage, response to treatment and survival data were taken from patients' records. Treatment response was evaluated according to Cheson's criteria and the disease-free and overall survival by means of Kaplan Meier survival curves. For the determination of statistical differences the log rank test and Chi-square test were applied.

**Results**

*Patients' characteristics and treatment:* Among 295 patients, there were 44.7% males and 55.3% females. Their median age was 64 years (range 19 to 86 years). Thirty nine point three percent of patients were aged below 60 years and 60.7% were aged 60 or more years. Thirty one point five percent of patients had limited disease and 67.1% had advanced disease at presentation. According to the IPI categories, there were 11.5% patients with IPI 0, 21.4% patients with IPI 1, 22.4% patients with IPI 2, 23.4% patients with IPI 3, 15.6% patients with IPI 4 and 5.7% patients with IPI 5, respectively.All patients were treated with R-CHOP (89.9%) or R-CHOP-like regimens.In just few young poor-prognosis patients (6.4%), the more dose-intensive R-$ACVBP_{21}$ regimen was used instead of the R-$CHOP_{21}$ regimen. In patients with compromised cardiac function, reduced doses of anthracyclines were applied and were sometimes compensated for with the addition of etoposide (reduced intensity R-CHOEP) (3.7%).

*Response to treatment:* The response to treatment for all patients and for distinct IPI categories is presented in Table 1. The difference in response between the low risk group and low intermediate risk group was statistically insignificant as was the difference between the high intermediate and high risk groups. But the significant $Chi^2$ for the entire table (p=0.045) indicates the significant difference between both low risk and both high risk groups.

**Table 1.** Response to treatment according to different IPI categories

| | All patients | | Low risk IPI=0,1 | | Low intermediate risk IPI=2 | | High intermediate risk IPI=3 | | High risk IPI=4-5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | % | N | % | N | % | N | % | N | % |
| CR | 226 | 76,6 | 83 | 85,6 | 54 | 81,8 | 47 | 68,1 | 42 | 66,7 |
| CRu | 4 | 1,4 | 1 | 1,0 | 0 | 0,0 | 0 | 0,0 | 3 | 4,8 |
| PR | 36 | 12,2 | 10 | 10,3 | 4 | 6,1 | 14 | 20,3 | 8 | 12,7 |
| SD | 2 | 0,7 | 0 | 0,0 | 0 | 0,0 | 1 | 1,4 | 1 | 1,6 |
| PD | 13 | 4,4 | 1 | 1,0 | 4 | 6,1 | 4 | 5,8 | 4 | 6,3 |
| Unclassified | 14 | 4,7 | 2 | 2,1 | 4 | 6,1 | 3 | 4,3 | 5 | 7,9 |
| Total | 295 | 100,0 | 97 | 100,0 | 66 | 100,0 | 69 | 100,0 | 63 | 100,0 |

CR – complete response; CRu – complete response unconfirmed; PR- partial response; SD – stable disease; PD – progressive disease; unclassified – unclassified response to treatment; IPI – international prognostic index; N – number of patients

The response to treatment was evaluated also for distinct R-IPI categories. In this case, the difference between the very good and good prognosis groups was statistically insignificant, as well as the entire table $Chi^2$p value (p=0,088).A statistically significant difference in the response was observed between the IPI 2 and IPI 3 categories which was detected by both indexes – namely by the IPI and the R-IPI.

*The disease-free survival according to IPI and R-IPI categories*

With the median observation period of 22 months, the estimated 3 year disease-free survival rates were 75.3% for low risk, 75.6% for low intermediate risk, 57.2% for high intermediate risk, and 53.1% for high risk group, respectively. The difference between the groups was statistically significant (log rank, p = 0.001).

The progression-free survival was plotted also according to the R-IPI categories - the estimated 4 year disease-free survival rates were 59.4% for very good prognosis, 71.6% for good prognosis, and 51.1% for bad prognosis group, respectively. Again, the difference between the groups was statistically significant (log rank, p= 0.000).

*The overall survival according to IPI and R-IPI categories*

With the median observation period of 31 months, the estimated 3 year overall survival rates were 86.9% for low risk, 81.6% for low intermediate risk, 60.9% for high intermediate risk, and 50.9% for high risk group, respectively. The difference between the groups was statistically significant (log rank, p = 0.000).

The overall survival was plotted also according to the R-IPI categories - the estimated 4 year overall survival rates were 93.7% for very good prognosis, 79.5% for good prognosis, and 55.9% for bad prognosis group, respectively. Again, the difference between the groups was statistically significant (log rank, p= 0.000).

*Treatment outcomes according to clinical categories of patients*

Treatment outcomes were evaluated also according to clinical categories – namely, response to treatment, disease-free survival and overall survival were followed separately for young good prognosis patients (younger than 60 years with IPI 0 or 1), young poor prognosis patients (younger than 60 years with IPI of 2 or more), and older patients (aged over 60 years regardless of IPI), respectively. The difference in the disease-free survival between all three groups was statistically significant (log rank, p = 0.005), but it was insignificant when only young good prognosis and young poor prognosis groups were compared (p = 0.365). Also the difference in the overall survival between all there groups was significant (p = 0.000) as was the difference between young good prognosis and young poor prognosis group (p = 0.005).

**Conclusion:** Response to treatment in our evaluation diverged from the reported one predominately in the low risk group (IPI categorisation) and in the very good prognosis group (R-IPI categorisation). The determined complete response rates in other IPI and R-IPI groups were generally within expectations. Also in the disease-free survival, the largest discrepancy occurred in the low-risk patient group (3 year disease-free survival rate of 75%) and in the very good prognosis group (4 year disease-free survival rate of 59%). In all other IPI risk groups, the disease-free survival at 3 years (low intermediate risk 76%, high intermediate risk group 57%, high risk group 53%) agreed very well with the quoted ones. Slightly worse was the compliance of the 4 year disease-free survival rates (72% in the good prognosis and 51% in the poor prognosis group) with the results from the literature. The 3 year overall survival rates (low risk patients 87%, high intermediate risk 61% and high risk patients 51%) were somewhat worse than the reported ones in all IPI subgroups except in the low intermediate risk group (82%). On the other hand, the 4 year overall survival rates of the R-IPI categories (94% in the very good prognosis group, 80% in the good prognosis group, 56% in the poor prognosis group) were much better correlated with the data from the literature.

In total, the treatment outcomes of routinely treated patient with the DLBCL at our institute are quite encouraging when compared to results of some larger studies. There are probably no dilemmas about how to treat young good prognosis patients and patients aged over 60 years at present. However, the 5 year overall survival rate of 76% for the young poor prognosis group is unsatisfying and needs to be improved. At present, quite a few studies are underway to clarify which of the regimens will perform best in this population.

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**Key words:** diffuse large B-cell lymphoma, R-CHOP, routine treatments,treatment results

## OP18: Breast cancer and brain metastases: clinical, pathological and molecular characteristics

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Brain metastases represent one of the most devastating events in patients with breast cancer predicting short survival after diagnosis [1].

While data from epidemiologic studies estimate that the incidence of brain metastases in women with metastatic breast cancer (MBC) is 10–16 % old but more confident autopsy reports suggest rates as high as 30% [2, 3]. Therefore, in many patients brain metastases remains undiagnosed because there is no routine screening for brain metastases, and diagnosis is made only after symptoms development. Symptomatic brain metastases develop in up to 46% of patients with metastatic breast carcinoma eventually, dependant on a molecular subtype, and in up to 6% of patients who were previously treated for early breast carcinoma, as a first metastatic site, independently of molecular subtype.

Although actually unpredictable, risk for brain metastases varies according to breast carcinoma molecular subtype as it was already mentioned, and is highest in triple negative and Her2 positive subtypes [4].

During the last decade, it has been well established that HER2 overexpression is a risk factor for central nervous system (CNS) metastases, and because there is a specific, targeted therapy for HER2 positive breast cancer with trastuzumab, this became a problem of a special interest. Because trastuzumab is specific targeted therapy in HER2 positive breast carcinoma, brain metastases in that subgroup will be focused in this paper.

Since trastuzumab became available in the treatment of HER2 positive breast cancer prognosis for those patients has been significantly improved. However, it seems that trastuzumab does not cross the intact blood–brain barrier (BBB), and therefore it has been supposed that the CNS serves as a sanctuary

site for metastatic disease, usually while other metastatic sites are well controlled during trastuzumab treatment. [5].

Despite that common belief that trastuzumab does not cross the BBB, overall prognosis for patients with brain metastases as also been improved with prolonged survival, if other metastatic sites are well controlled, trastuzumab continued, and whole brain radiotherapy (WBRT) conducted . Data are obtained retrospectively only, because there are no clinical studies directly examining the impact of trastuzumab on brain metastases.

Although this benefit is most likely due to better systemic control, one study reported a trend toward longer time to CNS progression, suggesting a possible direct CNS effect, although this issue still remains controversial [6]. However, BBB after brain metastases development might not be intact any more, especially after WBRT and trastuzumab still might penetrate to brain at least at some amount, and therefore might have a direct therapeutic effect.

If risk for brain metastases development could be better defined, it could be possible to develop strategies for early diagnosis and prevention eventually. However, there is no routine screening for brain metastases in asymptomatic patients, and there is no proof that early diagnosis of brain metastases can influence definitive prognosis. In metastatic setting, approximately 15% of patients have asymptomatic brain metastases .

In the next section, results from Institute for Oncology and Radiology of Serbia will be briefly described and commented.

**Materials and methods**

Because of centralized oncology organization in Serbia, majority of patients with HER2 3+breast cancer are treated at our Institute.

Also, for the same reason, patients with brain metastases almost exclusively undergo WBRT at our Institute and all patients are recorded in a data base. Therefore, data that we presented are obtained on a large patient population, with confirmed brain metastases developed during various points of BC treatment.

**Results**

We conducted a prospective study on 258 patients with early HER2 positive breast cancer treated with adjuvant trastuzumab and 33% consented to brain CT in the absence of CNS symptoms [7]. Although no brain metastases were diagnosed before symptoms development, during median follow up of 18 months 5/258 patients (1.93%) developed brain metastases, but only 2 (0.77%) while still receiving adjuvant trastuzumab. The median time from breast cancer diagnosis to BM was 24 months (range 14-43).Therefore; we concluded that as brain metastases are a rare event during adjuvant trastuzumab treatment, brain CT screening is not justified in asymptomatic patients with early HER2 3+ breast carcinoma.

In another study, we explored incidence of brain metastases as a first metastatic site in patients with Her2 positive breast cancer [8]. Results were obtained on 230 patients with breast cancer brain metastases, with 73 (31.2%) patients with HER2 positive breast cancer. Amongst that patient subgroup, 34% (25/73) developed brain metastases as a first relapse site. Majority of those patients, (68%;17/25)) have been treated for locally advanced HER2 positive breast cancer, while 32% (8/25) have been treated for early breast cancer. Median time to brain metastases was 18 vs. 41,5 months respectively. Results might imply that patients with locally advanced HER2 breast cancer might be a better selected population for brain screening.

Long term prognosis of patients with brain metastases can seldom be explored because of generally poor prognosis. However, it seems that some patients still might have a prolonged survival even upon brain metastases.

We have recently reported a case of a young patient initially treated for locally advanced HER2 positive breast cancer, who survived more that 10 years, after cerebellar brain metastasectomy, without further metastatic dissemination [9].

In another analysis, we examined the incidence of brain metastases as a first late relapse defined as a relapse after at least 60 months after breast cancer diagnosis. This study shows that the risk for brain metastases in HER2 3+ breast cancer patients is very low or might be even absent as a first late relapse. Absence of late brain metastases in HER2 3+ breast cancer might be attributed to specific biological characteristics of HER2 3+ carcinomas to develop brain metastases mostly in the early course of metastatic disease [10].

**Conclusion:** Research of HER2 positive breast cancer is currently among the most active areas of breast cancer research. Numerous novel HER2-targeted agents are in various phases of clinical development, and many have already shown promising clinical activity. Thus, because of prolonged survival, brain metastases will likely be an increasingly common in patients with HER2-positive MBC. Finding new effective strategies for the treatment, management, and prevention of CNS disease is one of the important priorities.

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**Key words:** brain metastases; cerebellar metastases; HER2 overexpression

## OP19: Metastatic Lung and Colorectal Cancer: Impact of Molecular Markers on Systemic Treatment - Serbian Experience

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**Background:** Biology of malignant tumors represents today an area of the greatest research interest in clinical oncology – a better understanding of tumor molecular biology facilites our understanding of behavior and metastatic propensity of cancer, but also helps us to formulate a more efficient treatment approach.

Advance has been achieved in the last decade in many fields of oncology, and molecular markers and their everyday use has changed practice in the treatment of many solid tumors, but also in the field of haematological oncology.

In lung and colorectal cancer research, some of the most important receptors and signaling pathways involved in these processes have been identified: EGFR - the receptor of epidermal growth factor for lung cancer and KRAS mutational status in colorectal cancer are among the most significant predictive molecular markers.

It has been recognized since 2004 that the mutations of the receptor of epidermal growth factor are those molecular characteristics that allow tyrosine kinase inhibitors (TKI) to achieve clinically significant response in patients who harbor those mutations. In Caucasian population, the frequency of those mutations is 10-15% (85% of them belong to mutations on exons 19 and 21), and in the Asian population 30-40%. These mutations are associated with the histology of adenocarcinoma, female gender, non-smokers and significant response to TKI. In the last decade, several drugs have become the therapeutic standard in patients with advanced non-small cell lung cancer harboring EGFR mutations. In the past few years a lot of effort has been put into the research of resistance to these drugs, which occurs in almost all patients, after 8-12 months of treatment.

In KRAS mutated colon and rectal cancer it has been revealed that the presence of KRAS mutated gene is associated with a lack of response to EGFR inhibition, and even detrimental effect of those drugs on treatment outcome. In contrast, in patients with wild-type KRAS status, there is a good response to EGFR inhibition and treatment outcome is better if EGFR inhibitor (monoclonal antibody) is added to cytotoxic chemotherapy. The activity of these antibodies is neither exclusively associated with particular treatment line, nor expressed only when given with chemotherapy: they can be given in any treatment line, with or without cytotoxic drugs. At the Institute for Oncology and Radiology of Serbia the frequency of wild-type patients and treatment response as well as safety were assessed, in patients who received EGFR inhibition in third line of systemic treatment. Also, in past few years it was demonstrated that among 55-60% of patients with wild-type KRAS status there are 15-20% of patients never responsive to the treatment. Thus, today standard analysis of sensitivity on EGFR inhibitory antibodies comprises extended KRAS and NRAS testing (RAS testing).

**Patients and methods:** EGFR mutation testing of advanced non-small cell lung cancer patients for targeted therapy with tyrosine kinase inhibitors became obligatory in Serbia for adenocarcinoma patients in clinical stage IIIB and IV (TNM 6) and good performance stratus (ECOG 0 and 1) in 2011. EGFR mutations were analyzed by real-time PCR and patients harboring mutations were treated with gefitinib in the first-line of systemic treatment, until progression. A few studies were performed on Serbian patients with advanced lung adenocarcinoma in past four years analyzing EGFR mutation status and molecular targeted therapy against these mutations.

A total of 1874 consecutive chemonaive lung adenocarcinoma patients (stage IIIB and IV, performance status 0 or 1) were included in the analysis from June 2011 until December 2014. The aim of this

study was to determine the frequency and types of EGFR mutations in Serbian lung cancer patients uo to that point and to explore the influence of patients and tumor characteristics on the outcome of gefitinib therapy. The group comprised of 1146 males (61.2%) and 728 females (38.8%) of Caucasian descent, patient age range 20-88 years (median 62 years). Statistical analysis was performed using Chi-square, Fisher's exact, Wilcoxon's and Log-Rank tests; $p < 0.05$ was considered significant. [1]

Overall, 1,148 consecutive patients were included in the analysis of rare EGFR mutations, from June 2011 till April 2014. The group comprised of 697 males (60.7%) and 451 females (39.3%) of Caucasian descent. Patient age range was 20-85 years (median 61 years). Statistical analyses included testing of sample distribution for normality, parameters description and testing the differences between the parameters, with statistical significance set as $P<0.05$. [1]

Regarding metastatic colorectal cancer, K-RAS testing for mutations in codons 12 and 13 has been done at the Institute for Oncology and Radiology of Serbia since 2009. EGFR inhibiting antibody cetuximab has been reimbursed by the Serbian Health Insurance Fund since april 2009. for the use in third line of treatment of mCRC in patients with wild type K-ras gene. We prospectively followed patients treated with cetuximab in third line therapy in a 5-year period. Data concerning demographic characteristics, previous treatment, number of cycles of cetuximab, toxicity and treatment outcome were analysed. [4]

**Results:** In the analysis of advanced adenocarcinoma of the lung, EGFR mutations were detected in 190 out of 1874 patients (10.1%), of which 127 were female (67%) and 63 male (33%) ($p < 0.001$). Apart from del19 (55%) and L858R (32%), 2 patients had L861Q, 8 had G719X, 5 had an insertion in exon 20 and we found 5 double mutants (1 L861Q/ G719X and 4 G719X/S768I). From this group, 46 patients (30 female and 16 male) were treated with gefitinib at the Institute of Oncology and Radiology of Serbia. The follow up period was 2-27 months (median 8 months). Overall survival (OS) median was not reached and progression free survival (PFS) rate was 37.5% after 1 year and 20% after 2 years, with a median of 8 months (95% CI, 6-16 months). Female patients had significantly longer PFS (median 15 vs. 4 months, $p = 0.01$) and OS (median not reached vs. 19 months, $p = 0.04$) than males. Other patient and tumor characteristics did not show statistically significant influence on gefitinib therapy outcome [1,3]

In another study, analyzing the frequency of rare EGFR mutations and outcome of these patients treated with EGFR tyrosine kinase inhibitors, a total of 121 (10.5%) mutated samples for were found of which 69.4% in females ($P=0.00018$), giving the typical EGFR positivity picture of NSCLC patients in Caucasian population: 10-15% of aimed population. Apart from del19 (68 samples) and L858R (39 samples), 14 samples (1.2% of total) with rare mutations were found: L861Q (two samples), G719X (six samples), Ins (four samples) and two double mutants L861Q/G719X and G719X/S768I. Of these 14 patients, eight received gefitinib in first-line treatment. Response to treatment was as followed: two patients with SD and six with PD. [2]

Regarding molecular targeting therapy in metastatic colorectal cancer, between April 2009 and December of 2014. 162 patients with metastatic CRC were treated with cetuximab in the third line setting. Cetuximab was used in combination with irinotecan or as monotherapy, in 14-day cycles, the dose given was 500mg/m$^2$. Of these patients, 95 were male (58,6%) and 67 female (41,2%), with median age at diagnosis 60 years, range 24 to 80. At the beginning of cetuximab treatment, the most common metastatic sites were liver in 132 patients (81%), lungs in 55 (34%), lymph nodes in 36 ( 22%), peritoneum in 31(19%) while 94 patients had more than one metastatic site. [4]

Median number of cycles of cetuximab was 8, range 1 to 33. 44 patients received between 8 and 12 cycles, 53 patients more than 12 cycles, and 44 patients received up to and including 4 cycles. Best response to therapy was partial response in 25 patients (15%), stable disease in 86 patients (53%), and progression in 39 patients (26%).[4]

Median progression-free survival (PFS) was 6.01 months (4.88-7.12, CI 95%). There was no statistically significant difference in PFS between men and women, patients with primary colon or rectal tumors, nor depending on localization of metastatic site(s). Of these patients, 22 are still on cetuximab. [4]

The toxicity was well managed, 120 patients experienced skin rash, gr.1 in 93 patients (57%), gr.2 in 22 (14%), gr.3 in 5 (3%), 42 (26%) patients were without rash. Rash developed after median of 2 cycles of cetuximab. *Paronychia was present in 36 patients, grade 1 in 23 (14%), to grade 3 in 6 (4%). Eye disorders appeared in 12 patients (7%). Cetuximab had to be stopped due to toxicity before progression of disease in 20 patients.*

In this group of patients there was an association between grade of skin toxicity and response to treatment (Spearman's correlation coefficient 0.186, p= 0.023).[4]

**Conclusion:** Advances in knowledge in the field of molecular biology of some of most frequent cancer types today (lung and colorectal cancer) are part of diagnostic and therapeutic routine in clinical practice in Serbia also. By testing advanced adenocarcinoma of the lung in terms of EGFR mutations and metastatic colorectal cancer in terms of K-RAS mutations we facilitate the usage of molecular targeting drugs, such as gefitinib in lung cancer and cetuximab in colorectal cancer. Our analyses revealed that the frequencies of EGFR and K-RAS mutations in Serbia are in the range recorded in Caucasian population, especially in Europe [5]. Female gender was proved to be the determinant of favorable prognosis in advanced lung cancer treated with gefitinib[1,3]. Patients with rare mutations have worse outcome, when treated with standard first-line treatment, gefitinib [2]. Cetuximab in third-line treatment is a good options for patients with metastatic colorectal cancer and wild-type KRAS status, delaying disease progression with good toxicity profile [4].

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**Key words**: Carcinoma, Non-Small Cell Lung; cetuximab; Colorectal Neoplasms; gefitinib; genes, ras; Receptor, Epidermal Growth Factor

## OP20: Palladium (II) based-anticancer compounds: A new hope or an illusion?

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Cancer patients still need more effective therapies, especially chemotherapy-based treatments. According to the recently-published *in vitro* and *in vivo* studies, the combination of a general cytotoxic and a targeted therapy seems to yield much more successful outcomes. Therefore, a need for new general cytotoxics is still desirable. For this aim, the synthesis of new palladium (II) complexes provides a new arsenal. Therefore, the designation and creation of new palladium (II)-based compounds are in increase.

The bioinorganic and medicinal chemistry of 2,2′:6′,2″-terpyridine (terpy) complexes of Pd(II) (1) and Pt(II) (1,2) is an active and growing area of interest. To date, many different compounds including palladium (II) as a metal have been synthesized and tested in terms of their cytotoxic activities against various tumor types. It seems that the ligands attached to the palladium (II) results in big differences in cytotoxic potential of the complexes. In the literature, varying ligands have already been used, such as pyrazoles, oxamate, triphenylphosphine, 2, 6-diacetylpyridine bis (p-chlorophenylthiosemicarbazone, thioures, hydrazine, tetraaza [N 4], sulfur, thiosemicarbazones, hydrazine, oxalato, proflavine, triazole, organoarsenic, curcumin. All these new Pd(II)-based compounds yield different cytotoxic potentials depending on the tumor types.In addition to the ligands above, the bioorganic and medicinal chemistry of 2,29:69,20-terpyridine (terpy) complexes of Pd(II) and Pt(II) is also an active and growing area of interest (3,4).We have investigated the anti-cancer activity of two different Pd (II) complexes of terpy with saccharinate (sac), one being formulated as [Pd(sac)(terpy)](sac)•4H2O and the other being as [Pd(Cl)(terpy)](sac)•2H2O.

These complexes were synthesized by our research group (3) and some publications were already made (5-7). However, the little of them has been tested against the cancer stem cells, which are thought to be responsible drug resistance thereby causes recurrencies in the patients. It is needed to do extensive research on their effects on cancer stem cells either on their own or in combination with the other anticancer drugs/compounds.

Taken together, in this talk, novel Pd(II)-based compounds synthesized by the other groups will be discussed in comparison with our Pd(II)-based compounds *in vitro* and *in vivo*.

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# OP21: Sigma receptors as targets for the development of novel targeted anticancer therapies

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**Background:** Sigma receptors are present in the central nervous system as well as in various peripheral tissues. Their high density in various tumor cell types, and particularly in proliferating cells, makes sigma receptors potential target for diagnostic imaging as well as therapeutic agents Recent data suggest that sigma ligands may induce cell death in various tumor cell lines and xenografts including prostate and breast carcinoma with features consistent with apoptosis. Novel synthetic compounds with sigma binding activities have been tested by our group for antitumor activity *in vitro* and in preclinical cancer models,

**Materials and methods:** The SRB assay was used for the *in vitro* and *ex vivo* cytotoxicity studies. For in *vivo* experiments human tumor xenografts into NOD/SCID mice were used. The effect of selected compounds on the cell cycle was analyzed using flow cytometry. Finally, western blot analysis was used to study the cell death and the expression of the two receptors in patients' tumors and cancer cell lines.

**Results:** Sigma ligands have shown significant *in vitro* cytotoxic activities against human cancer cell lines. Some of them were able to arrest the cell cycle and induce apoptosis to treated cells. Expression studies for the two receptors in tumors from cancer patients showed expression differences between normal and cancer tissues. Finally in efficacy studies in preclinical models (human tumor xenografts) the tested agents found to efficiently delay tumors' growth (colorectal, pancreatic, melanoma).

**Conclusion:** Results suggest that sigma receptors may represent an intriguing target for novel targeted cancer therapeutic approaches.

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**Key words**: cancer, targeted therapies, sigma ligands, xenografts.

## Session: Anticancer drugs investigation I

### OP22: New approaches in overcoming multidrug resistance in cancer

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**Background:** Resistance to chemotherapy is the main obstacle to efficient cancer treatment. The problem of multidrug resistance (MDR) has been intensively studied for the last three decades. Classically defined as resistance to structurally and/or functionally unrelated drugs, MDR is connected to aggressive, untreatable cancers. Selection pressures within the tumor microenvironment favor the development of intrinsic MDR, while the application of chemotherapy induces acquired MDR. In recent years, patient-to-patient variability within each type of cancer has arisen as an unsolved problem. Even more, heterogeneous populations of cancer cells within one patient must be considered as a cause of chemotherapy impediment. Several genes and pathways have been found to contribute to the MDR. MDR phenotype could develop from point mutations, gene amplification or other genetic or epigenetic changes that affect biological functions. Therefore, MDR is driven by similar mechanisms as cancerogenesis. This is also supported by the fact that important characteristics of MDR include abnormal tumor vasculature, regions of hypoxia, aerobic glycolysis, and an elevated apoptotic threshold. Understanding these mechanisms and developing agents to target them are important steps in the design of new therapies. Penetration of drugs into the cancer cell is necessary for their lethal pharmacological effect due to the interaction with intracellular target molecules. Increased activity of membrane efflux pumps reduces the intracellular drug accumulation, thereby preventing drug-target interactions. The discovery of the efflux transporter P-glycoprotein (P-gp) in MDR cancer cells prompted the efforts in overcoming drug resistance by inhibition of P-gp.

**Material and Methods:** Several human cancer cell lines of different origin (non-small cell carcinoma NCI-H460, colorectal carcinoma DLD1, glioblastoma U87 and anaplastic thyroid carcinoma 8505C) as well as rat glioma cell line C6 were employed in order to develop MDR resistant variants (NCI-H460/R, DLD1-TxR, U87-TxR, Rho- and RC6). Different MDR phenotypes were obtained after continuous exposure to DNA damaging drugs such as doxorubicin (DOX) and carmustine (BCNU) as well as mitotic spindle inhibitor – paclitaxel (PTX). Sorting of a small side-population of resistant cells by flow-cytometry was also applied to enrich the heterogenic cancer cells with those that possess MDR characteristics. Newly established MDR cancer cells lines were used as *in vitro* and *in vivo* models for the investigation of different approaches for MDR overcoming. Methodology included cell viability tests MTT and Sulforhodamine B, examination of MDR, stemness, apoptosis, autophagy and antioxidative markers by RT-PCR, western blot, flow-cytometry and confocal microscopy as well as invasion assays such as wound healing, gel degradation and matrigel migration. *In vivo* models included syngenic orthotopic glioma in Wistar rats and subcutaneous xenografts in SCID mice.

**Results:** In the search for new modulators of MDR in cancer, different approaches were examined: (i) natural products; (ii) modified natural products i.e. semi-synthetic derivatives; (iii) compounds inspired and synthetized according to structures found in nature; (iv) antimetabolites i.e. purine nucleoside and nucleotide analogs and (v) compounds synthetized by molecular modeling i.e. signal transduction inhibitors. Natural products that first drown the attention for anti-MDR research were ***jatrophanes***. They are major constituents of the diterpenoid fraction isolated from the whole plant and the latex of widely spread spurge - *Euphorbia dendroides*. Jatrophane compounds, named euphodendroides were non-toxic to

normal cells and potent MDR modulators with new anticancer features that included disturbance of cell cycle kinetics and inhibition of vascular endothelial growth factor (VEGF) secretion [1]. Moreover, these compounds enhanced the effects of PTX by reversing the existing resistance in NCI-H460/R, DLD1-TxR and U87-TxR cells [2]. The most promising euphodendrophanes isolated from the latex completely blocked the P-gp transporter demonstrating higher activity than well-known P-gp inhibitors Dex-verapamil and tariquidar [3]. Oxidated flavonoid derivatives - ***protoflavones*** were found to act selectively towards P-gp overexpressing human cancer cells (NCI-H460/R and U87-TxR) but they were less active against the rat MDR cancer cells (RC6) [4]. ROS level in human P-gp overexpressing cells was lower in comparison with parallel sensitive cells while rat MDR cells were adapted to higher ROS level. The increase of ROS induces a compensatory upregulation of antioxidant systems as an adaptive response especially in cancer cells. This mechanism can be applied to rat MDR cells that we have examined [5]. In case of tested human P-gp overexpressing cells, lower level of ROS indicate inactivity of antioxidant systems. To prove this concept, we assessed the level of glutathione (GSH) as the main intracellular antioxidant, the expression of superoxide dismutase (SOD) involved in quenching of active oxygens, glutathione-S-transferase π (GST π) responsible for the decomposition of lipid hydroperoxides and hypoxia inducible factor-1α (HIF-1α) regulated by NO concentration in the cell. Obtained results suggested that collateral sensitivity (CS) of human MDR cancer cells to protoflavone derivatives is mediated by hypersensitivity to ROS, which is not directly caused by P-gp overexpression, but other adaptive changes during the evolution of P-gp-mediated resistance [4]. The synergy between these isomers and PTX was also observed. The influence of lipophilic ***antioxidant coenzyme-Q 10 (CoQ)*** on temozolomide (TMZ) efficacy was assessed in resistant rat glioma cells (RC6) *in vitro* and *in vivo*. CoQ enhanced the sensitivity of RC6 cells and synergistically interacted with TMZ. The development of drug resistance in RC6 was followed by significant increase in ROS production [5], while CoQ was able to decrease it. Addition of CoQ resulted in a substantial decrease in RC6 migration, evaluated by quantification of lamellipodia positive (migrating) cells. Moreover, CoQ decreased invasiveness according to gelatin degradation assay and syngenic orthotopic model in Wistar rats. The effects of ***NK-2 cationic peptide*** were also evaluated in human MDR cancer cells [6]. NK-2 was synthesized according to NK-lysin structure found in porcine natural killer (NK) cells. It is a membrane-active agent, attracted by negatively charged cancerous plasma membrane. Considering that P-gp contributes to cell membrane composition and increases the net negative charge on cancer cell surfaces, the interaction between a cationic and amphipathic NK-2 and P-gp was studied. The results indicated that NK-2 preferentially bound to P-gp high-expressing cancer cells since it was able to distinguish them from cancer cells expressing low levels of P-gp. Importantly, P-gp expression was decreased in MDR cancer cells recovered after NK-2 treatment. Therefore, NK-2 was able to exploit the presence of P-gp and consequently sensitize MDR cancer cells by selective killing of the P-gp high-expressing cell population [6]. Recently, two purine analogs ***sulfinosine (SF)*** as nucleoside and ***8-Cl-cAMP*** as cyclic nucleotide showed promising activity in human MDR cancer cell lines. SF anticancer effects in resistant NCI-H460/R and U87-TxR cell lines included depletion of GSH, reversion of MDR through inhibition of P-gp expression and activity, and modulation of VEGF intracellular pool [7, 8]. The results implicated that SF action was mediated through modification of redox status and HIF-1α regulation. Pretreatment with 8-Cl-cAMP improved the sensitivity to DOX more than verapamil [9]. The increased accumulation of DOX observed after 8-Cl-cAMP treatment resembled the results obtained with verapamil. Even more, 8-Cl-cAMP considerably decreased the P-gp coding mRNA expression. The effects of ***dual mTOR inhibitor (AZD2014)*** were examined in the model of resistant anaplastic thyroid carcinoma Rho- *in vitro* and *in vivo*. Recent data showed that AZD2014 synergized with both DOX and PTX in anaplastic thyroid carcinoma cells [10]. Rho- cells were significantly resistant to PTX. However, the combination of PTX and AZD2014 was synergistic and equally effective in both MDR Rho- and non-MDR 8505C cells. This was shown by cytotoxicity and invasion assays and confirmed *in vivo* using preclinical

model - xenografts in SCID mice. Novel ***c-Src inhibitor LDS10*** showed 2 fold higher inhibitory potential in human MDR cancer cell lines than in their sensitive counterparts suggesting the presence of CS. In addition, LDS10 enormously decreased P-gp activity. After prolonged treatment LDS10 was even able to decrease the P-gp expression and activity. Further studies confirmed MDR sensitizing potential of LDS10 in combination with DOX and PTX. **Conclusion.** The search for nontoxic anticancer agents with the potential to overcome MDR has been an imperative in the field of drug design and discovery for many years. This study contributed to the aforementioned efforts by identifying new compounds able to: (i) preserve cytotoxic activity towards MDR cancer cells; (ii) restore the cytotoxicity of classic anticancer drugs and (iii) exploit the presence of MDR.

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**Key words:** multidrug resistance; anticancer drugs; drug development; collateral sensitivity; preclinical study

## OP23: Fullerenes: biological activity and potential biomedical application

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Until discovery of fullerenes in 1985, two forms of carbon, graphite and diamond, were known. Some years later, in 1991, carbon nanotubes were characterized, and in 2004, the third form of " new carbon"- graphene, one-atom-thick flat sheet of carbon, was isolated from graphite. Since their discovery, carbon nano-alltrops attracted significant attention of researches in various scientific fields including biomedicine.

Fullerenes are defined as polyhedral closed cages made up entirely of carbon atoms. They contain 12 pentagons and different number of hexagons. The most known fullerene is C60, which contains 12 pentagons and 20 hexagons and has geometry of truncated icosahedrons. Fullerenes are entirely insoluble in water and polar solvents. Several fundamental properties of fullerenes (extremely high hydrophobicity, high cohesive force between fullerene molecules, photoactivity, ability to accept and release electrons, and relatively high reactivity that allows structural modifications) allowed functionalization of fullerenes that made them interesting for biological research. Surface chemical modifications of fullerenes resulted in a wide variety of biologically active water-soluble fullerene derivatives. Water-soluble fullerene derivatives may effectively interact with DNA, proteins and living cells.

Thus far, both non-derivatized and derivatized fullerenes showed various biological activities. They induce oxidative, genotoxic and cytotoxic response while their antioxidative/cytoprotective properties has been considered for utilization in treatment of oxidant-mediated disease. Fullerenes have shown potential application in photodynamic therapy, phototheramal treatment, radiotherapy and chemotherapy. They are also used as a novel contrast agent in MR (magnetic resonance) imaging. Besides fullerenes, graphene and their derivatives have also raised interest for biomedical application.

Here, we briefly report on basic physical and chemical properties of carbon nano-clusters: fullerenes, nanotubes and graphene, their specificities, activities, and potential application in biological systems. Special emphasis will be given to our the most important results obtained in *in vitro* and *in vivo* using polyhydroxylated fullerene derivative $C_{60}(OH)_{24}$.

**Key words**: biological activity, biomedicine, fullerene, fullerene derivative

## OP24: Platinum complexes as anticancer drugs: properties and perspectives

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Since the discovery of cisplatin, cis-diamminedichloroplatinum(II), CDDP, as anticancer agent, metal-containing coordination compounds such as cisplatin, carboplatin and oxaliplatin became the basis of combination chemotherapy regimens for solid tumors. However, main drawbacks to their successful application, such as development of resistance and severe side toxic effects, prompted extensive research in the field of medicinal chemistry toward the effective alternative to cisplatin, with improved pharmacological properties. In the last 40 years, thousands of platinum analogues have been synthesized by varying structural parameters such as: carrier and/ or living ligands structure, *cis-/trans-* geometrical isomerism, coordination sphere around platinum and its oxidation state, resulting in complexes with cytotoxicity equal or better to that of cisplatin. It is known that the mechanism of cytotoxic action

of cisplatin and analogues rely on formation of stabile DNA adducts, though yet exact mechanism of DNA-lesions processing, as well as the mechanism that governs platinum agents accumulation in the cell, are not completely elucidated. Numerous investigations pointed toward interaction of platinum complexes with cellular proteins or biomolecules containing S- or N- donor groups as a step that sometimes competes and sometimes integrates DNA-targeted mechanism. Class of platinum complexes with planar amine ligands (*trans-* platinum aromatic heterocycles-TPA), initially developed by Farrell et al., were reported to exhibit unique mechanism of action, and circumvent cisplatin resistance in preclinical or even clinical models (1).

In our previous studies, we have investigated *cis-/trans-* platinum(II) or platinum (IV) complexes, with carrier ligands such as aromatic planar amines: pyridine, acetylpyridine, and leaving ligands such as: chloride, iodide. Platinum(II) complexes with the carrier ligands such as: ethylenediamine-N,N-diacetate (edda)-type of esters, were also investigated. Analysis of the mechanism of action *in vitro*, on panel of tumor cell lines, revealed significant cytotoxicity, comparable to that of cisplatin and better affinity for intracellular accumulation or DNA binding. In addition some complexes exhibited advantageous *in vitro* antitumor properties such as: antiangiogenic effect at sub toxic doses; citoselective action toward tumor cells and small toxicity in MRC-5 (human fetal lung fibroblast cells); synergistic action in combination with PARP (Poly-ADP ribose polymerase) inhibitors. Improvement of pharmacological effect of cisplatin and analogues can be addressed partially by alterations of the signal transduction pathways, and by alterations of mechanism that protect against genomic instability such as DNA repair. New targets such as the kinases in the pathways of DNA repair or signaling transduction pathway and compounds that inhibit them will expand the capabilities in confronting cancer. The molecularly targeted agents generally benefit from co-administration with cytotoxic agents. Therefore, for the foreseeable future, there will be a need for both existing and new cytotoxic platinum-based agents with novel carrier groups which would provide more stability, novel modalities of action and better selectivity toward tumor cells.

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**Key words:** DNA platination, intracellular accumulation, *in vitro* antiangiogenic effect, *trans*-platinum complexes

## Session: Anticancer drugs investigation II

### OP25: Development of Ru(II)-arene complexes with pyridine derivatives as anticancer agents

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**Background:** Clinical success of cisplatin and its derivatives directed extensive investigations to develop novel platinum- and non-platinum metal–based antitumor drugs in order to improve clinical effectiveness, to reduce general toxicity and to broaden the spectrum of activity (1). Among non-platinum compounds, ruthenium complexes have become particularly interesting because of their variety of modes of action involving both extra- and intracellular processes (2). Interestingly, some ruthenium complexes demonstrate greater efficacy against cancer metastasis than against primary tumors. Exam-

ple of such complexes is NAMI-A, the first ruthenium complex that underwent clinical trials (3). The most numerous group of investigated ruthenium complexes is Ru(II)–arene complexes, developed primarily by Dyson and co-workers and Sadler and co-workers, although none of these compounds has yet entered clinical trials (2). All these complexes have typical "piano-stool" geometry, where the arene ligand forms the seat and the chelating ligand along with chlorido ligand are the legs of the piano stool. Variations in the three building blocks, arene ligand identity, as well as the nature of monodentate or bidentate ligand influence the pharmacological properties of these complexes (4).

**Materials and Methods:** Our studies of ruthenium(II) complexes started with series of complexes containing the p-cymene ligand (as arene) and a pyridine derivatives (dicarboxylic, acetyl or amino pyridine derivatives) coordinated in a monodentate or bidentate manner (4,5). Analysis of cell growth inhibition caused by Ru(II)-arene complexes was determined on: six tumor cell lines (HeLa, MDA-MB-361, MDA-MB-453, FemX, B16, LS-174), two transformed endothelial lines (EA.hy 926, MS1) and one normal human cell line (MRC-5) using MTT assay (4-6). For further examination and comparison of structure and activity we selected four complexes, from which two were with monodentate bonded pyridine ligand and two with bidentate bonded pyridine ligand, which didn´t have any important cytotoxic activity and picolinate ruthenium(II)-p-cymene complex, as complex with important activity. The effect of the selected ruthenium(II)-p-cymene complexes on cell cycle progression of HeLa and EA.hy 926 cells was examined by flow cytometry using staining with PI. Potential of most active picolinate ruthenium(II)-p-cymene complex to induce apoptosis was determined after two colors staining with Annexin V-FITC and PI and analysis on flow cytometer. Ru(II) distribution among the DNA and protein fractions in HeLa cells treated with investigated complexes was determined using inductive coupled plasma with optical emission spectrometry (ICP-OES). In this study, we also evaluated whether DNA-repair-dependent signaling, as a result of interaction with DNA, which includes components of NER or MMR is utilized in cell response to ruthenium(II)-p-cymene complexes, by following expression of ERCC1 (mRNA and protein level) and MSH2 (protein level) using Quantitative Real-Time PCR (RQ-PCR) and Western blot. Potential of our Ru(II)-p-cymene complexes to modulate several steps of metastatic progression like adhesion, migration, invasion through matrigel, proteolitic degradation of extracellular matrix (through influence on matrix metalloproteinase activity) and formation of new blood vessels has been studied *in vitro*. We also performed combined treatment of HeLa cells with most active picolinate ruthenium(II)-p-cymene complex and PARP inhibitor (3-aminobenzamide) and this complex with clinically relevant anticancer agents, cisplatin and paclitaxel.

**Results:** Investigated series of complexes revealed low antiproliferative activity (IC50 >200 μM), except for compound with picolinic acid as ligand with IC50 being 81.97 μM on HeLa cells. Knowing that few Ru complexes which failed traditional anticancer drug screening programs based on their level of genotoxicity and cytotoxicity *in vitro*, have demonstrated antimetastatic activity, we further investigated mechanism of action of four selected Ru(II)-p-cymene complexes. Analysis of ruthenium(II) accumulation in DNA and protein fractions of HeLa cells, using ICP-OES revealed significantly higher content of complex with picolinic acid in DNA fraction in comparison to the other tested compounds. Examination of the cell cycle progression of HeLa and EAhy.926 cells after 24 h treatment with selected complexes indicated that only picolinate ruthenium(II)-p-cymene complex induced perturbations of cell cycle of HeLa cells, other complexes had no effect on cell cycle phase progression in both HeLa and EA.hy926 cells. Picolinate ruthenium(II)-p-cymene complex induced decrease of percent of cells in G1 and slight arrest in the S phase of cell cycle. Slower progression through replication phase indicates its preferential binding to DNA. Complex with picolinic acid affected expression of DNA repair enzymes ERCC1 and MSH2. Data obtained on HeLa cells after 24 h continual treatment showed that this complex significantly increased expression of ERCC1 on mRNA level. Other ruthenium compounds showed no effect or negatively modulated relative expression levels of ERCC1 mRNA, compared to the non treated control. Picolinate ruthenium(II)-p-cymene complex induced apoptosis as mode of cell death

and showed enhanced activity in combination with 3-aminobenzamide (PARP inhibitor). Investigated Ru(II)-p-cymene complexes, additionally exerted antimetastatic effect on several tumor cell lines *in vitro*, achieved mostly by the effect on cell adhesion, migration and angiogenesis, while picolinate ruthenium(II)–arene additionally exerted inhibitory effect on extracellular matrix degradation.

**Conclusion:** Based on their *in vitro* anticancer properties, investigated complexes have one complicated mechanism of action, involving both extra- and intra-cellular processes. Variations of the nature of monodentate or bidentate ligand significantly influenced pharmacological properties of the complexes. Picolinate ruthenium(II)–p-cymene complex distinguished among the tested ruthenium complexes, according to its highest antiproliferative and antimetastaic potential *in vitro* and become promising candidate for further structural modifications. Based on the results of the antiproliferative activity of the first series of complexes, where additional ionizing groups within ligands had negative impact on the activity of complexes, further investigation ongoing in our laboratory, include second series of ruthenium(II)-p-cymene complexes with picolinic acid derivatives with additional non ionisable functional groups.

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**Key words:** antimetastatic, antiproliferative activity, ruthenium(II)–arene

## OP26: *In vitro* antitumor cytotoxicity of some macrofungi extracts

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**Background:** The medicinal use of fungi has been documented in China, Russia, Japan and Korea, as well as in the United States and Canada (1). For example entire fungi and fungi extracts are used in traditional Chinese medicine for the treatment of cancer (2-4). Lentinan, derived from *Lentinula edodes*, schizophyllan from *Schizophyllum commune*, the Maitake D-fraction (β glucan) from *Grifola frondosa* and krestin from *Trametes versicolor*, are in clinical use for this purpose.

Nowadays, constituent molecules of macrofungi organelles and secondary metabolites are known as bioactive compounds that belong to polysaccharides, glycoproteins, proteoglycans, terpenoids, fatty acids, proteins, lectins that possess certain medicinal properties.

Immunomodulating activity of compounds isolated from medicinal macrofungi, related their effects to act on immune effecter cells such as hematopoietic stem cells, lymphocytes, macrophages, T cells, DCs, and NK cells involved in the innate and adaptive immunity, resulting in the production of cytokines. The therapeutic effects of these compounds such as antitumor and antiinfective activity and

suppression of autoimmune diseases have been associated in many cases with their immunomodulating effects.

Epidemiological and pharmacological studies have shown that the intake of fungi is associated with many beneficial effects, such as antioxidative, antiviral, antitumor, anti-inflammatory, and hepatoprotective activities. In particular, the antioxidative activity has gained the most interest, and the biological effects of edible fungi are believed to come, in large part, from their antioxidative properties. In contrast to beneficial effects, many compounds from fungi also have been found to be pro-oxidant or mutagenic and to produce toxicity.

Access to a limited number of cancer chemotherapies, their deleterious side effects and high cost of most of these drugs, make disease treatment especially difficult. Furthermore, many existing therapies do not effectively treat certain cancers, and multi-drug-resistant tumors exacerbate treatment challenges. The discovery of new chemotherapeutic agents is a key goal for natural product and medicinal chemists.

In this study water extracts, hot alkali extracted polysaccharides as well as purified and enzyme modified extracts of the fruiting bodies of *Auricularia auricula-judae, Fomes fomentarius and Sparassis crispa* collected at various localities in Serbia were screened for cytotoxic activity against three cancer cell lines and normal control lung fibroblasts.

**Material and methods:** Fungi were identified by authors from an examination of macro- and micromorphology in comparison to standard descriptions in the monographs and taxonomic treatments. The aerial parts of the fungi were extracted with hot water or hot alkaline water solution. Several extracts were modified with enzymes, in order to reduce molecular mass of polysaccharides, and eventually cut off side chains and purification of extracts was done with ethanol. Extracts were dissolved directly in nutrient medium at concentrations in the range from 0.125 mg/mL to 2 mg/mL. Target cells were malignant human breast cancer MDA-MB-453, cervical adenocarcinoma HeLa, myelogenous leukemia K562 and normal lung fibroblast MRC-5 cells. Antiproliferative activity of investigated compounds was assessed, measuring cell survival in standard, 72 h MTT test. The $IC_{50}$ reflected the concentration of an extract that inhibited 50% of cell proliferation and was used as a measure of cytotoxicity of the extracts.

**Results:** Modified hot alkaline extracts derived from *Fomes fomentarius* and *Sparassis crispa* and purified enzyme modified water extract from *Fomes fomentarius* displayed significant cytotoxic activity and a dose dependent antiproliferative action towards all investigated tumor cell lines with very good selectivity in activity toward tumor cells in comparison to normal human fibroblast cells, with approximately two times higher $IC_{50}$ values for normal cells. Moderate cytotoxic effects showed hot water and purified water extract from *Auricularia auricula-judae* and hot water and alkaline extracts from *Fomes fomentarius*. The most cytotoxic effect showed modified alkaline extract of the *Fomes fomentarius* ($IC_{50}$=0.328 mg/mL for K562 cells, and 0.391 mg/mL for HeLa cells) and modified purified water extract of *Fomes fomentarius* ($IC_{50}$=0.364 mg/mL for K562 cells). No cytotoxic activity shoved alkaline and modified alkaline extracts from *Auricularia auricula-judae*, water and modified water extracts from *Fomes fomentarius* and water and alkaline extracts from *Sparassis crispa*.

**Conclusion:** Results obtained showed that some compounds of extracts from *Fomes fomentarius* and *Sparassis crispa*, could be promising agents for the treatment of human tumors and are candidates for further analyses on experimental animals, *in vivo*. Our study provides a second evaluation of the cytotoxicity of various Serbian fungi species and forms an important basis for the isolation and structural elucidation of cytotoxic compounds from these species in the future.

Concentrations of the extracts which induced 50% decrease ($IC_{50}$) in malignant and normal cells survival.

| | extract | $IC_{50}$ mg/mL HeLa | K562 | MDA-MB-453 | MRC-5 |
|---|---|---|---|---|---|
| *Auricularia auricula-judae* | hot alkali extracted polysaccharides | 1.567 | 0.879 | 1.621 | >2 |
| | enzyme modified hot alkali extracted polysaccharides | >2 | >2 | >2 | >2 |
| | purified enzyme modified extract | >2 | >2 | >2 | >2 |
| | enzyme modified water extract | 1.414 | >2 | >2 | >2 |
| | purified | 0.978 | 0.746 | 1.529 | 1.924 |
| | water extract | 1.2 | 1.001 | 1.564 | >2 |
| *Fomes fomentarius* | hot alkali extracted polysaccharides | 1.2 | 0.838 | 1.983 | >2 |
| | enzyme modified hot alkali extracted polysaccharides | 0.391 | 0.328 | 0.414 | 0.723 |
| | purified enzyme modified extract | 0.475 | 0.364 | 0.455 | 0.783 |
| | enzyme modified water extract | >2 | >2 | >2 | >2 |
| | purified | >2 | >2 | >2 | >2 |
| | water extract | 0.914 | 0.619 | 1.862 | >2 |
| *Sparassis crispa* | hot alkali extracted polysaccharides | >2 | >2 | >2 | >2 |
| | enzyme modified hot alkali extracted polysaccharides | 0.71 | 0.455 | 0.784 | 1.462 |
| | enzyme modified water extract | >2 | 1.895 | >2 | >2 |
| | water extract | >2 | >2 | >2 | >2 |

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**Key words**: fungi, extracts, cytotoxicity, tumor

## OP27: G protein-coupled receptor heteromers as new pharmacological targets in lung cancer

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**Background:** Neuroendocrine tumors (NETs) of the lung represent a heterogeneous group of tumors ranging from typical (TC) and atypical (AC) carcinoids to high grade small cell lung cancer and large cell neuroendocrine carcinoma, all of which have different prognosis and treatment options[1]. They are characterized by neuroendocrine morphology and expression of neuroendocrine markers. Small cell lung cancer and large cell carcinomas grow very rapidly, usually in a peripheral localization, and occur almost exclusively in patients with a history of smoking. Carcinoids however usually occur in never-smokers and are mostly centrally located in the lungs[2]. The incidence of pulmonary neuroendo-

crine carcinoids is relatively low, with TCs accounting for approximately 1–2% and ACs only 0.1–0.2% of all pulmonary neoplasms[1]. However, a dramatic increase of their incidence has been reported in the last 30 years, which is probably mainly due to improved awareness, detection methods and diagnostic protocols[3]. Compared to high grade lung NETs, carcinoids have a relatively favorable prognosis with a 5-year overall survival of over 90% for TCs and around 60–90% for ACs[4]. Carcinoids occur slightly more often in women than in men, in Caucasians than in African Americans or other ethnicities including Hispanic and Asian people, and they usually occur in the fourth to sixth decades of life (mean age at diagnosis 45 years for TC, 55 years for AC)[4]. They do not have a high number of genetic alterations, but are therapeutically challenging because of frequent reoccurrence of systemic metastases even decades after diagnosis[5]. Surgical resection is often followed by chemo-, radio- or biological therapy, but specific guidelines are lacking. Also, inoperable tumors are difficult to treat because of their insensitivity to both radiation and chemotherapy. In comparison with non-small cell lung cancer, little is known about the potential prognostic and/or predictive biomarkers of lung NETs, although there is a constant progress in the field[6]. G-protein-coupled receptors (GPCRs) comprise a diverse protein family of receptors that transduce signals from the extracellular milieu to many intracellular signaling molecules evoking cellular responses. The chemical diversity of ligands that bind and activate GPCRs is exceptional and they use GPCRs to stimulate targets through heterotrimeric G-protein-dependent and -independent pathways. GPCRs are no more considered as single functional units, but as forming part of multimolecular aggregates localized in the plasma membrane which contains other interacting proteins[7]. Heteromerization has proven to be essential for the function of many receptors. Moreover, heteromers have different biochemical and pharmacological characteristics from the monomers, thus increasing the number of functional entities in the cell. Targeting the purinergic signaling pathways, where adenosine 5′-triphosphate (ATP) and adenosine act as extracellular signaling molecules through GPCRs, is becoming very popular in molecular oncology, as activation or inhibition of various purinergic GPCRs can induce cancer cell death or growth inhibition[8]. Numerous studies have shown the beneficial action of extracellular nucleotides in colorectal cancer, leukemia, oesophageal cancer, lung cancer, cervical cancer etc. As tumor cells have very high ATP content compared to most healthy cells, ATP-depleting strategies enhance anticancer agent activity. Dopamine GPCRs mediate the effects of dopamine and dopaminergic drugs via a number of different signal transduction pathways. The most important of these is the modulation of adenylyl cyclase activity resulting in either stimulation or inhibition of cyclic AMP accumulation. In recent years there has been considerable interest in novel effects of dopamine receptor activation and, in particular, in the control of cell growth and differentiation. Recently, adenosine and dopamine receptors have been recognized as targets for the treatment of NETs [9,10]. It has also previously been shown that adenosine $A_{2A}$ and dopamine $D_2$ G-protein coupled receptors form functional heteromers with different biochemical and pharmacological characteristics from the monomers, depending on the cell type[7]. In order to fully recognize the potential of $A_{2A}R$-$D_2R$ heteromer signaling in neuroendocrine lung cancer therapy, a more detailed characterization of the presence and heteromerization of these receptors in neuroendocrine tumor cells is needed. The aim of this project was to evaluate the differences in genetic and protein expression and potential heteromerization of $A_{2A}R$ and $D_2R$ receptors in typical and atypical neuroendocrine carcinoids of the lung.

**Material (Patients) and Methods**: The patient groups consisted of 26 TC and 26 AC samples, each group consisting of both good and poor responders to standard chemotherapy. TC patient group comprised 15 females and 11 males of Caucasian descent, age range 17-75 (median 50). AC patient group consisted of 12 females and 14 males of Caucasian descent, age range 29-72 (median 53). Tumor localizations ranged from lower left lobe, upper left lobe, left principal bronchus, upper right lobe, lower right lobe, middle lobe to the lingual trahea. RNA and proteins were isolated from FFPE tumors samples using RNeasy® FFPE kit (Qiagen) and Qproteome® FFPE Tissue kit (Qiagen), and cDNA synthesized from RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™). Evaluation of

genetic and protein expression in TC and AC samples was performed by RT-PCR on and Western blot, using GAPDH as a reference. Detection of $A_{2A}$R-$D_2$R heteromers in tumor samples was performed by Proximity ligation assay (PLA), using the Duolink II *in situ* PLA detection Kit (OLink, Bioscience). Microscopic observations of heteromerization were made using Olympus FV 300 confocal scanning laser microscope (Leica Lasertechnik, Leica Microsystems). Red fluorescent images were processed with Image J software, and statistical analysis performed using GraphPad Prism V.6, with significance set at $p<0.05$.

**Results:** We did not confirm a statistically significant difference in the expression of $A_{2A}$R and $D_2$R between TC and AC at the genetic level (p=0.6164 and p=0.6914). At the protein level, we did not confirm a statistically significant difference in the expression of $D_2$R between TC and AC (p=0.5464). However, AC had statistically significant higher expression of $A_{2A}$R compared to TC (p= 0.0458). PLA experiments showed that no $A_{2A}$R-$D_2$R heteromers were present in TC samples, while 80% of the analyzed AC samples showed $A_{2A}$R-$D_2$R heteromerization. **Conclusions:** Significant differences in the expression and heteromerization of $A_{2A}$R and $D_2$R were detected between typical and atypical neuroendocrine carcinoids. Ongoing pharmacological characterization of these tumor-specific heteromers and correlation with patient and tumor characteristics and therapy response is expected to discover new targetable biomarkers for neuroendocrine lung carcionoids. This might ultimately lead to the development of heteromer-receptor-selective drugs or dual drugs able to interact simultaneously with both units of a tumor-specific receptor heteromer, thus enabling an effective targeted therapy.

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**Key words:** adenosine, dopamine, GPCR, lung cancer

## Session: Molecular changes in cancer development and progression II

### OP28: p53/p63/p73 protein network

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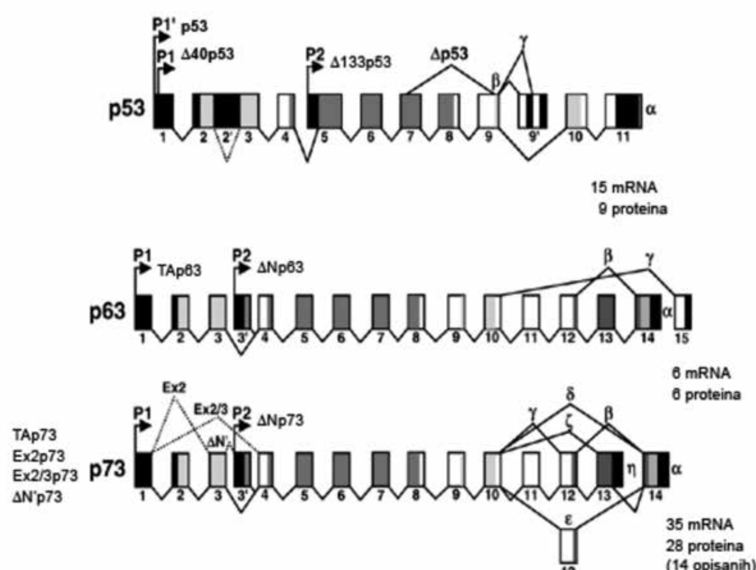
**Background:** The tumor suppressor protein p53 regulates cellular response to stress signals, by inducing DNA repair and, if DNA is not repairable, transient or permanent cell cycle arrest, apoptosis or senescence. p53 function is frequently compromised in cancer cells, as over 50% of the human cancers contain mutations and other alterations of the gene. Two sibling genes- p63 and p73 have been found to encode several proteins whose structures and functions are similar to those of p53 but not identical. p53 is important in the prevention of cancer, while p63 and p73 are in addition crucial for normal development. p53 family members share significant similarity at gene levels as well at protein level within the functional domains. The complexity of the family has been enriched by the use of alternative promoters, splicing and translational sites. Consequently, several protein isoforms with distinct N- and C- termini are encoded. Transcribing from P1 promoter principally gives rise to full length isoforms with transactivation domain (p53, TAp63 and TAp73), while using the alternative P2 promoter produces amino-terminal truncated isoforms without TAD (ΔNp53 (Δ133/160p53), ΔNp63, ΔNp73). Combining the alternative splicing on both N and C end with different promoter usage, additional protein isoforms of p53, p63 and p73 arise.

There is an important cross-talk between family members in tumors.Certain isoforms of p53/p63/p73 proteins can form heterotetramers. The formation of such mixed heterocomplexes correlates with functional transdominance – loss of transactivation of target genes and proapoptotic abilities.

A subset of p53 mutant proteins,as well as ΔNp73, negatively regulates wild type p53/TAp73/TAp63 through direct interaction. Functionally, such stable heterocomplexes cause loss of wild type p53/TAp73/TAp63 tumor suppressor functions. However, there are still unexplained relationships between some mutant p53 and TAp73/TAp63 proteins and particularly it is still unknown whether p53 isoforms interfere with TAp73 activity.

***Human p53, p63 and p73 gene structure.Alternative splicing (α, β, γ) and alternative promoters (P1 and P2) are indicated.***



**Materials and methods:** All experiments were performed using p53 null human lung cancer cell line H1299, transiently transfected with Lipofectamine 2000. Immunoblot Analyses was performed according to standard procedure. The antibodies used are as follows: ER15 IMG-246 for p73α, GC15 for p73β, DO-1 for p53, p53β and p53γ, PAb421 for Δ133p53α and Δ40p53α, KJC8 (J.C. Bourdon) for Δ133p53β, DO-12 for Δ133p53γ, 4A4 and H129 for TAp63α, anti-EGFP and anti-α vimentin. For co-immunoprecipitation assay we used Dynabeads® Protein G (Life Technologies). Luciferase assay was performedusing Dual-Glo Luciferase Assay System (Promega Corporation) as described by the supplier. Apoptotic cells were determined using Annexin-V-FLUOS Staining Kit (Roche Diagnostics) and evaluated by flow cytometer. Half life of p53 isoforms was determined upon treating H1299 cells with 40 μg/ml of cycloheximide for 24 hours post transfection, and followed by standard western blot analysis and quantification by ImageMaster VDS Software (Pharmacia Biotech).

**Results**

*1. Heterotetramer analysis between TAp73α or TAp73β and p53 isoforms*

To determine whether p53 isoforms form complexes with TAp73α or TAp73β, we performed the co-immunoprecipitation assay with ectopic proteins. All examined p53 isoforms (p53β, p53γ, Δ133p53α, Δ133p53β, Δ133p53γ, Δ40p53α) form mixed complex with TAp73β. In contrast, TAp73α tetramerizes only with Δ133 isoforms.

*2. Effects of p53 isoforms on TAp73 transcriptional activity*

To test the hypothesis that some p53 isoforms could be dominant-negative inhibitors of human TAp73 we performed reporter assays. First we determined the transcriptional activity of p53 isoforms and have found that p53β by itself showed the highest transcriptional activity comparing to all other isoforms examined. When transfected together with TAp73β, all p53 isoforms suppress the transcriptional activity of TAp73β but with different effect - Δ133p53α and Δ133p53γ are the strongest inhibitors of TAp73β transcriptional activity (41% and 50%, respectively), followed by Δ133p53β and Δ40p53α (35% and 38%, respectively), while p53β and p53γ inhibit only 28% and 21%, respectively of the transcriptional activity of TAp73β. Furthermore, we determined the specific effect of p53 isoforms on the transcription of specific target genes, *bax* and *p21*, and their effect on TAp73β transcriptional activity. We have shown that all p53 isoforms counteract TAp73β transactivation function in a promoter-dependent manner.

*3. Effect of p53 isoforms on apoptosis induced by TAp73β*

To assess proapoptotic activity of p53 isoforms comparing to TAp73β, H1299 cells were transfected with either pcDNA3 vector or plasmids expressing TAp73β or each of p53 isoforms, and percentage of apoptotic cells was determined after Annexin V and propidium iodide staining by FACS analysis. All isoforms, except p53β, have strongly reduced proapototic activity upon DNA damage comparing to TAp73β. When cotransfected together with TAp73β, apoptotic activity of TAp73β was augmented by coexpression of p53β, while Δ133p53α and β inhibit its apoptotic activity most efficiently.

*4. Stabilization of TAp73β by p53 isoforms*

We have also determined the half-life of different p53 isoforms: p53γ isoform has the shortest while Δ133p53γ has the longest half-life. Furthermore, inhibitory interactions of two proteins in complex often lead to their stabilization. However, only three isoforms (Δ133p53α, Δ133p53β and Δ40p53) stabilize the expression of TAp73β.

*5. Effects of p53 mutants on TAp73 and TAp63 transcriptional activity*

We have confirmed the heterocomplex formation between mutant p53 (R175H, L194F, R280K, R282W) and TAp73β or TAp63α by co-immunoprecipitation assay. Additionally, we have shown that common po-

lymorphism in mutant p53 R72P, 72Arg binds more efficiently to TAp73β than the equivalent 72Pro, but there is no difference in binding to TAp63α. We also tested mutant p53 to stabilize TAp73β and the results have shown that all tested mutant p53 can stabilize TAp73β but with different effect.

In order to analyze the effect of p53 mutants on TAp73β and TAp63α transcriptional activity, we performed luciferase reporter assay and the results have shown that all mutant p53, except R280K inhibit both TAp73β and TAp63α transcriptional activity but with different efficiency and in a promoter-dependent manner.

**Conclusions:** Here we report the inhibition of TAp73β-mediated transcriptional activity and apoptosis through the formation of heterocomplexes between p53 isoforms (p53β, p53γ, Δ133p53α, Δ133p53β, Δ133p53γ, Δ40p53α) and TAp73β. Furthermore, we analyzed the inhibitory effect of p53 mutants on TAp73β and TAp63α. We have found that not all p53 mutants have inhibitory effect on p73/p63 tumor suppressor activity.

Our results suggest (summarized in Table below) that the biochemical and biological functions of p53 family member, p73, can be mediated by the interplay between p53 isoforms and TAp73 protein. Thus, regulation of p73 function in normal and tumor human tissues is likely to be more complex than has been so far considered. It is obvious that p53 isoforms can modulate p53 family tumor suppressor activities, and the expression ratio between members of the family is probably the most important factor in determining the cell destiny.

*Summary table of the effect of various p53 isoforms on TAp73β activities.*

| | Complex formation (Co-IP) | Inhibition of TAp73β transcriptional activity | | | TAp73β-mediated apoptosis | Stabilization of TAp73β | **Half-life** |
|---|---|---|---|---|---|---|---|
| | | **PG13** | **Bax No camp/ camp** | **p21 No camp/ camp** | | | |
| **p53β** | +TAp73β | + | ++/- | ++/- | enhance | - | ++++ |
| **p53γ** | +TAp73β | + | ++/- | ++/+ | reduce | - | + |
| **Δ133p53α** | +TAp73α<br>+TAp73β | +++ | ++/- | ++/+ | reduce | + | ++++ |
| **Δ133p53β** | +TAp73α<br>+TAp73β | ++ | +++/++ | +++/- | reduce | + | ++++ |
| **Δ133p53γ** | +TAp73α<br>+TAp73β | +++ | +++/- | +/++ | modest | - | +++ |
| **Δ40p53** | + TAp73β | ++ | +++/- | ++/++ | modest | + | + |

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**Key words:** apoptosis, p53 protein, p63 protein, p73 protein, protein-protein interaction, transactivation

## OP29: Image Analysis of Breast Tumour Histology in Prognosis of Distant Metastasis Risk

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**Introduction:** The importance of breast cancer metastasis risk prognostication derives from the fact that metastasis occurrence is exceptionally variable and that death by breast cancer is mainly caused by metastatic relapse at distant sites. Such importance of metastasis for the disease outcome means that the prognostication of metastasis risk is the central factor for decisions on the individual therapeutic regime.

Breast cancer metastasis risk biomarkers have been a field of intensive research in the past decade with a focus on molecular prognosticators including transcriptional profiling [1], microRNA analysis [2], detection of circulating tumour cells in blood [3], proliferation [4], and stem cell markers [5]. The need for new prognostic approaches derives from the fact that molecular risk biomarkers often outperform the established clinicopathological prognosticators, but regrettably still exhibit insufficient prognostic accuracy, with the remaining unreliable therapeutic guidance.

The accurate prognosis of breast cancer outcome is exceptionally challenging based on the fact that malignant transformation is a random process rendering every tumour unique. A demand for improvement of breast cancer risk prognosis has triggered the idea to implement alternatives to molecular approaches, with a resulting expansion of the digital pathology as a structure examination tool for medical images.

Diagnostic and prognostic value of texture analysis has been experimentally established for mammographs [6], breast ultrasound [7], and breast magnetic resonance images [8]. Surprisingly, image analysis has rarely been implemented for characterization of tumours at the microscopic histology level as the primary tumour of patients with early breast cancer is generally used as the main source of information for assessment of distant metastasis risk. Image texture reflects spatial distribution of

pixel grey levels, very much as elevation of highs and lows define rough and smooth physical touch texture. Gray level co-occurrence matrix (GLCM) evaluates visual texture of the image based on pixel intensity spatial arrangement statistics [9] and is reportedly the most common and sensitive texture descriptor [10].

Taken together, based on the pressing need to improve breast cancer prognosis this study was undertaken to explore the metastasis risk prognostic value of GLCM tumour histology image analysis.

**Patients and methods:** Selection of invasive breast tumour histology samples was retrospective, based on the absence of hormonal, chemotherapeutic or any other systemic treatment (natural course of disease). The prospective power calculation, a pilot experiment on 35 samples, avoidance of missing data and the rarity of systemically untreated patients resulted in the final sample size of 78. The metastasis event rate was 27%, median follow up time was 147 months, ranging between 77 - 165 months.

**Hematoxylin&Eosin (H&E) and Immunostaining:** Samples of invasive primary breast tumours were obtained during surgery. Tissue was formalin-fixed, paraffin-embedded and immediately cut to produce 4 μm whole sections which were mounted on glass slides. Immunostaining was performed without Haematoxylin counterstain in order to highlight only epithelial cells. The employed mAE1/AE3 antibody cocktail (Dako, Glostrup, Denmark, #M3515) identifies two epitopes present on a majority of epithelial cytokeratins.

**Image acquisition:** Representative histological sections for each tumor were chosen by a pathologist. Digital microscopic images were acquired at ×400 magnification using Olympus BX-51 light microscope and a mounted Olympus digital camera at 3638 × 2736 pixel resolution.

**Texture Analysis:** Image texture features were extracted by use of the GLCM algorithm and freely available ImageJ software (http://imagej.nih.gov/ij/). Five GLCM features were calculated: entropy and contrast as measures of tissue heterogeneity, while angular second moment (ASM) and inverse difference moment (IDM) measure homogeneity. Correlation as a fifth feature is not directly associated with homogeneity or heterogeneity, but measures correlation of pixels to their neighbours over the whole image.

**Statistical Analyses:** Cox proportional hazards regression or log-rank Mantel-Cox test were used to relate predicted and actual metastasis outcome. Analyses were performed by SPSS version 22 (Chicago, IL) and Statistica 8 (Statsoft, Tulsa, OK). Adjuvant! Online score was calculated as a 10 year risk of relapse based on age, tumour grade, ER status and tumour size at https://www.adjuvantonline.com/breastnew.jsp

A bootstrap with 1000 data resamples was performed to quantify the over optimism by correcting the confidence intervals of hazard risks (95% CI) and *P*-values. Split-sample cross-validation estimated the generalizeability of the prognostic model by randomly splitting the original sample into a training set of 44 patients and a validation set of 34 patients in five validation cycles.

**Results:** Of the standard clinicopathological parameters, pathological tumour size (pT), age (60 years cutpoint), ER status (51 fmol/mg cutpoint) and Adjuvant! Online score significantly associated with metastasis risk.

Texture was analysed with an emphasis on either non-specific or specific tumour histology features, depending on the type of staining. H&E non-specifically stains nuclei and cytoplasm of all cells, while pan-CK epithelial staining specifically demarcates malignant cells.

Several texture variables associated with metastasis risk already by raw continuous data, while subsequent value categorization (0 – below; 1 – above the optimal cutpoint with minimal *P*-value) mainly improved the prognostic performance. Data categorization was necessary for multiparametric Cox regression and Kaplan Meier analyses.

Figure 1 clearly illustrates the excellent discrimination efficiency of the Contrast texture variable obtained on tumour sections stained for malignant cells pan-cytokeratin in comparison to the Adjuvant! metastasis risk score as the major clinicopathological risk prognosticator.

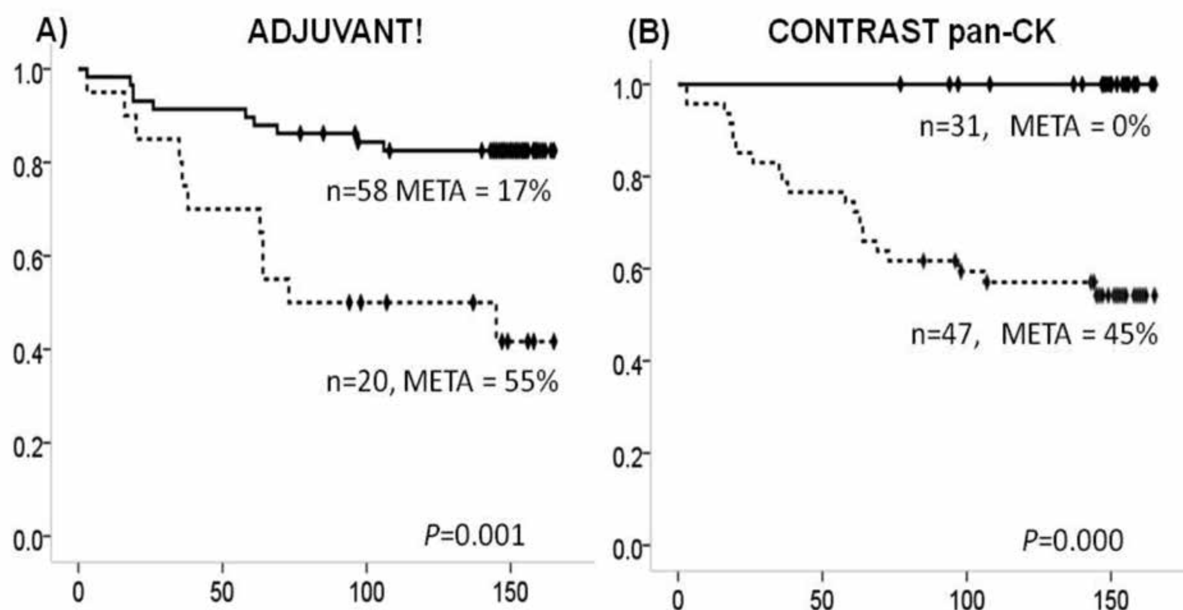


**Figure 3. Kaplan–Meier analysis of distant metastasis occurrence in relation to prognostic variables.** Patients were divided into poor and good prognostic subgroups according to optimal cutpoints. (**A**) Adjuvant! Online is the standard clinicopathological composite score. (**B**) Contrast as the parameter of GLCM texture analysis obtained on pan-cytokeratin tumour-tissue sections. The time from primary breast tumour surgery until occurrence of the first distant metastasis is noted in months.

**Conclusion:** On the basis of obtained results it is concluded that parameters of tumour microscopic histology GLCM texture strongly and independently correlate with biological properties of a tumour and present a promising strategy to assess the risk of distant metastasis. The original contribution of this study to the field of prognosis of breast cancer outcome is based on the first evidence that texture analysis of tumour microscopic histology produces superior performance in prognostication of distant metastasis in comparison to standard clinicopathological prognosticators. Furthermore, the texture prognostic biomarkers are simple and inexpensive to measure from routine archived H&E-stained histology, thus permitting both prospective and retrospective validation studies as well as possible clinical application without additional costs.

Usefulness and reliability of GLCM texture parameters is based on their high prognostic effect size, accuracy, and cost-effectiveness. Internal validation results were exceptionally good and while this work provides analytical validity, future larger studies with validation in external cohorts will be required to prove clinical utility as a guide to adjuvant therapy decisions in breast cancer.

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**Key words:** breast neoplasms; image processing, computer-assisted; histology; neoplasm metastasis; prognosis

## OP30: C-KIT signaling in cancer

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Cancer development and progression are strongly associated with the activity of receptor tyrosine kinases (RTKs) and their intracellular signal transduction pathways, which regulate several cell functions including proliferation, apoptosis, motility, adhesion and angiogenesis. Detailed structural and functional studies of RTKs, including the stem cell factor (SCF) receptor c-KIT, revealed the complexity of these receptor systems and contributed to development of targeted clinical approaches with relevance in both prognosis and therapy. C-KIT mutations and aberrant pathway activation are found in numerous solid and hematological malignancies, with gain-of-function mutations being the most common abnormality (1-2). C-KIT signaling network has been the subject of intense research and pharmaceutical strategies to identify novel target-based approaches for cancer treatment. Evidence that c-KIT signaling promotes cell proliferation and survival, along with the frequency in which this pathway is aberrantly activated in cancer, support the current efforts to identify approaches for its efficient inhibition. C-KIT mutations are associated with several human malignancies, such as gastrointestinal stromal tumors (GIST), acute myeloid leukemia (AML), mast cell leukemia (MCL), and melanoma. Through a complex series of interactions, activation of the c-KIT receptor tyrosine kinase by its ligand SCF, leads to cell survival, evasion of apoptosis, angiogenesis, dysregulated cell cycle, adhesion and chemotaxis control and promotion of tumorigenesis (1-3).

Gain-of-function mutations in c-KIT gene affect the function of c-KIT/SCF signaling pathway due to altered hyperactivated RTK function, causing pleiotropic phenotypes, including association with several human malignancies. Activating mutations in C-KIT protein confer the ligand-independent activa-

tion and signaling, thus positively regulating the growth and tumorigenicity, cluster in key locations within the c-KIT protein. A variety of oncogenic mutations were found in c-KIT ectodomain including inframe deletions, point mutations, in-frame duplications, and insertions that collectively lead to formation of activated forms of c-KIT (1-4).

Bivalent binding of SCF to c-KIT leads to receptor dimerization, autophosphorylation, creates binding sites for SH2-containing phosphotyrosine-binding proteins, and include kinase activation. Several pathways are involved in cell proliferation and inhibition of apoptosis, including the PI3K/Akt, PLC-γ, Ras/MAPK, Janus kinase (JAK)/signal transducer and activator (STAT), and the Src signaling cascades. A large number of signaling molecules have been identified as either interacting or as substrate proteins for c-KIT, including the p85 subunit of phosphatidylinositol 3′ kinase (PI3K), phospholipase Cγ (PLCγ), Src family tyrosine kinases, and the adaptor proteins Grb2, Grb7, and APS. PI3K-mediated activation of protein kinase B (PKB, Akt), the Ras-mitogen-activated protein kinase (MAPK) cascade, the Jak-Stat pathway, and $Ca^{2+}$ signaling cascades (5).

Oncogenic KIT mutations play an important "driver" role in GIST, acute myeloid leukemias, and melanoma, among other cancers. The mutations of the KIT gene have pathogenic role in various neoplasms, with important impacts in therapy and clinical patient outcome (1).

The biology of GIST has been widely investigated since mutations of proto-oncogene *c-KIT* coding for the SCF receptor were demonstrated as a pathogenic mechanism of GIST. C-KIT tyrosine kinase activity is regulated by its juxtamembranous (JM) domain, which inhibits c-KIT kinase activity in the absence of c-KIT ligand SCF. The principal genetic event responsible for the pathogenesis of GIST has been identified to be a gain-of-function mutation in the *c-KIT* proto-oncogene or, occasionally, in the platelet-derived growth factor alpha (*PDGFRA*) gene. Oncogenic *c-KIT* mutations result in ligand-independent kinase activation. The mutations usually affect the hot-spot *c-KIT* region at the 5′ end of exon 11 (65%, involving codons 550-560), that encodes the juxtamembrane domain of the protein (6).

The last decade marked an important era in the history of GIST, culminating from the advancement of diagnosis of GIST and our understanding of its pathogenesis, to the development of prognostic indicators and personalized treatment based on translation of laboratory success into biologically relevant therapeutics, dramatically improving patient outcomes. The inhibition of c-KIT oncoprotein by imatinib is the example of posttranslational inhibition through competitively binding the ATP-binding pocket of c-KIT, affecting c-KIT phosphorylation and subsequently inactivating downstream molecules, such as MAP kinase and PI3 kinase-AKT pathways. At this time, there is a clear benefit to adjuvant imatinib administered for 3 years, and relapse of disease is highly likely upon discontinuation (1,7).

Although many studies have contributed to the analyses of the c-KIT receptor in normal and malignant melanocytes, the precise role of the c-KIT-mediated intracellular signaling in melanocyte migration, survival, proliferation, and differentiation remains incompletely understood. Intracellular receptor tyrosine kinases such as c-KIT are providing the critical transforming signals that drive tumor growth and survival, whereas their inactivation results in selective apoptosis and tumor regression. This dependence induced the postulation of the concept of "oncogene addiction", which argues that cancer cells rely more heavily on hyperactivated intracellular signaling pathways than do normal cells, and therefore rely critically on activated oncogenes that drive those pathways of cellular survival (1,8).

Over 21 different mutations of the *c-KIT* gene have been found in melanoma, mainly in exons 9, 11, 13, 17 and 18. C-KIT activating mutations or amplifications have been reported in ~20–25% of melanomas arising in mucosal, acral or chronically sun-damaged skin. The most common melanoma c-KIT somatic mutation is L576P. The activation of this tyrosine kinase results in the stimulation of the MAPK and PI3K-AKT pathways, producing both proliferation and survival advantages. In the melanoma types

in which c-KIT mutations are found, BRAF mutations are relatively uncommon, and therefore, the two mutation spectra represent somewhat of a mirror image of each other (9).

Receptor tyrosine kinase signaling pathways play a central role in the pathogenesis of acute myeloid leukemia. Thus, various approaches focus on targeting RTKs, e.g., the FMS-like tyrosine kinase (FLT3), c-KIT, and signal transduction via the PI3K and MAPK pathways. Aberrations in genes coding for RTKs represent the most frequent genetic alterations in hematopoietic malignancies. More than 50 different RTKs have been identified, classified into 20 subfamilies based on their structural and functional characteristics (10).

Murine models of AML and next-generation sequencing of patient's leukemic cells genomes suggested that cooperating mutations in genes involved in signaling pathways that enhance the cellular proliferation, are necessary for leukemogenesis. These findings, and other lines of evidence, led to the proposal of a "two-hit" model of leukemogenesis, involving the cooperation between mutations that fall into two broad complementation groups. Class I mutations, are represented by activating mutations, such as those of *C-KIT*, *FLT3* and *N-RAS*, associated with activated signal transduction providing a proliferative and survival advantage to the hematopoietic progenitors. The class II gene mutations interfere with transcription and lead to impaired differentiation – either by direct alteration of transcription factors due to gene fusions, such as in the CBF leukemias or in PML-RARA-positive AML, or by indirect interference with transcription processes, e.g. MLL rearrangements (1). The correlation of these molecular changes further strengthens the importance of epigenetics as a key molecular pathway for leukemic transformation. Possible use of targeted treatment options (eg, demethylating agents such as 5-azacytidine or decitabine) specifically within this group of patients will be an important question for future studies. Thus, modifications of the current scheme of "two-hit" model, indicate that at least 3 major pathways are affected in adult AML, that is, proliferation, differentiation and epigenetic modification, with the possibility of further extension of mutational classes, to mutations in adhesion/cell-cell interaction or DNA repair/RNA splicing pathways (10).

The coexistence of t(8;21) and c-KIT mutations may accelerate the AML progression, with c-KIT as a secondary oncogenic event that confers proliferative/survival advantages to leukemic cells. These results support the concept of two-hit leukemogenesis, with requirement of aberrations affecting both transcription factors and RTKs for leukemic phenotype expression. This observation provides an important insight in pathogenesis of leukemia and suggests therapeutic approaches, such as combination of cytotoxic and TKI therapy.

The inhibitors of c-KIT tyrosine kinase receptor have the potential to slow the progression of c-KIT-positive tumors and may contribute to design of novel and alternative targets for drug development. These encouraging results warrant further preclinical investigations and clinical trials on the use of the c-KIT inhibitors, aiming to identify novel anticancer drugs protocols targeting signaling molecules implicated in critical oncogenic pathways, alone or combined with conventional chemotherapy. It is expected from further laboratory and clinical research to assess the efficacy of other molecular approaches including anti-sense, receptor antagonist and/or antibody to inhibit activated c-KIT mutants and/or SCF in resistant malignant cells. Such novel molecular-targeted therapies could provide promising solutions for therapy in patients with c-KIT-positive resistant malignancies (1).

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**Key words**: c-KIT, cancer, signaling, GIST, melanoma, leukemia, therapy

## PP01: Inflammatory cytokines-primed adipose tissue mesenchymal stem cells increase malignancy of breast cancer cells via transforming growth factor β

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**Background:** Adipose tissue mesenchymal stem cells (ASCs) and inflammatory conditions are important factors in breast tumor microenvironment, although crosstalk of ASCs and tumor cells is still not well understood. We investigated whether inflammatory cytokines interferon-γ (IFN-γ) and/or tumor necrosis factor-α (TNF-α) prime human ASCs (hASCs) to enhance MCF-7 breast cancer cell line malignancy. **Material and Methods:** Conditioned media (CM) of hASCs isolated from breast adipose tissues pre-exposed to IFN-γ and/or TNF-α were used for MCF-7 cells treatment. Epithelial to mesenchymal transition (EMT) of MCF-7 cells was followed by immunofluorescence labeling of E-cadherin and vimentin. Migratory capability of MCF-7 cells was assessed by wound healing assay. Enzyme activity of cell-associated urokinase type-plasminogen activator (uPA) was determined by zymography. Protein expression was analyzed by western blot analysis, while gene expression levels were determined by RT-PCR. **Results:** Our results showed that inflammatory cytokines increase TGF-β expression in hASCs. CM of inflammatory cytokines primed hASCs induced EMT of MCF-7 cells by reducing E-cadherin and inducing vimentin expression. Induced EMT was accompanied by increased MCF-7 cell migration and uPA expression. Chemical inhibitor of type I transforming growth factor-β (TGF-β) receptor (SB505124), was used to elucidate whether TGF-β mediates the effects of CM on MCF-7 cells EMT and migration. SB505124 inhibitor disabled the CM derived by hASCs to increase MCF-7 EMT, cell migration and uPA expression. **Conclusions:** Results suggested that the role of TGF-β expression induced by inflammatory cytokines in hASCs was essential for the enhancement of malignancy in MCF-7 cells, with potentially important implications in breast cancer progression.

**Key words:** adipose tissue, breast cancer, epithelial to mesenchymal transition, inflammation, migration, stem cells

## PP02: Regulation of mitotic factors by HN1

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**Background:** Ubiquitously expressed hematological and neurological expressed 1 (HN1) level was reported to be high in various carcinomas, while low in benign tumors of yolk sac, gliomas, and teratomas. Moreover, depletion of HN1 in both melanoma and prostate cells results in delayed G1/S-phase

transition and increases the p21, cyclin D1, and cyclin B1 levels, suggesting that HN1 significantly contributes to the cell cycle regulation. In our studies with PC-3 tumor cell line, we aimed to investigate how HN1 influences the APC/C mitotic cyclins. **Results:** We demonstrated that the effects of HN1 on APC/C-mediated cyclin B1 degradation, in addition to cell cycle progression. We also found that the HN1 acts as a Cdh1-interacting partner and stabilizes the level of Cdh1 regardless of its phosphorylation state, consistent with the higher levels of HN1 expression, which was observed in prostate cancer samples. Also, overexpression of HN1 to levels similar or higher of those observed in human carcinomas decreased Cdk1(T14/Y15) phosphorylation as well as Cyclin B1 and Aurora B expression levels. **Conclusion:** Thus, increased HN1 level promotes an early exit from mitosis by facilitating the proteosomal degradation of the cyclin B1-Cdk1 complex, also leads to S phase arrest, presumably by extending the duration of Cdh1-mediated APC/C activation. Hence, our results reveal the role of HN1 in mitosis regulation in human cancer cell lines.

**Key words:** cancer, cell cycle, HN1

## PP03:Targeting PI3K-mTOR pathway to modulate autophagy - a comparative analysis in cancer, normal and developing cells

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**Background:** Autophagy is connected with multiple cancer-related pathways including PI3K-mTOR, and represents a defense mechanism for cancer cells under therapeutic stress. Apoptosis and autophagy share multiple molecular components and crosstalk between these processes is essential for embryonic development. The autophagic and apoptotic mechanisms in zebrafish are analogous to those in mammals, with significant gene homology. **Material and Methods:** Autophagy and cell survival under stress were studied in human *non-small cell lung carcinoma* (NCI-H460) and normal keratinocytes (HaCaT). Influence of autophagy on stress-induced cell death during development was studied in zebrafish (Danio rerio). Apoptosis and autophagy were detected by caspase-3 and LC3 immunohistochemical staining in cells and western blot analysis in zebrafish. Apoptosis in live embryos was evaluated by acridine orange assay. **Results:** We investigated cell survival in *pro-apoptotic conditions while* modulating autophagy. mTOR inhibition by AZD2014 induced autophagy and antagonized the pro-apoptotic effect of doxorubicin (DOX) in cancer and normal cells. PI3K inhibition via wortmannin (WORT) blocked autophagosome formation and synergized with pro-apoptotic agents. WORT/DOX combination increased cleaved caspase 3 levels compared to DOX treatment, while AZD2014/DOX co-treatment had the opposite effect. Autophagy induction in zebrafish embryos prevented Cpt-induced apoptosis. Moreover, autophagic flux inhibition increased cell death during embryogenesis in Cpt-treated animals. **Conclusion**: When autophagy is manipulated for therapeutic purposes, it is crucial to recognize its function in both cytoprotection and cytotoxicity. Considering the evolutionary conservation of autophagic and apoptotic mechanisms, zebrafish serves as a valuable model for gaining insights on their important role in human disease.

**Key words:** autophagy, cancer, PI3K-mTOR pathway, zebrafish

## PP04:The overexpression of *SOX3* gene: effects on proliferation, viability and migration of U87 and U251 glioblastoma multiforme cell lines

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**Background:** Gliomas are the most common primary brain tumors in humans. Grade IV of glioma tumors, glioblastoma multiforme (GBM), is one of the most aggressive and deadly forms of cancer with the median survival of 15 months despite intensive therapeutic strategies. *SOX3* together with *SOX1* and *SOX2* genes *belong to a SOXB1* subgroup of a *SOX* family of genes which encode a transcription factors with important roles in embryonic development and carcinogenesis. Co-expression of *SOXB1* genes was revealed in GBM. While function of *SOX2* was well studied in gliomas, the role of *SOX1* and *SOX3* in GBM has not yet been determined. **Material and Methods:** We overexpressed *SOX3* gene in human glioblastoma multiforme cell lines U87 and U251 which are widely used as *in vitro* model system for studying glioblastoma pathobiology. Afterward, we investigated how elevated *SOX3* expression affects certain characteristics of tumor cells, such as proliferation, viability and migration. Proliferation and viability of transfected cells were studied by MTT and *crystal violet staining* assays. The migratory capability was tested by the scratch wound healing assay. **Results:** We have detected SOX3 expression in U87 and U251 cell lines where it acts as a transcription activator. Overexpression of *SOX3* gene increased proliferation and viability of these cell lines and altered migration. **Conclusion:** The obtained results contribute to understanding of the role of *SOX3* gene in GBM which potentially could form the basis for the developing new molecular approaches to targeting glioblastoma cells.

**Key words:** brain neoplasms, glioblastoma, SOX3 transcription factor

## PP05: Translational study: Overcoming anaplastic thyroid carcinoma resistance by mTOR inhibition

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**Background:** Anaplastic thyroid carcinoma (ATC) is an aggressive and chemoresistant tumor with poor prognosis. There are two possible causes for ATC chemoresistance: activation of the PI3K/AKT/mTOR and RAS/MAPK/ERK pathways and multidrug resistance (MDR). The major mechanism of MDR includes overexpression of ATP Binding Cassette (ABC) transporters. We found that inhibition of mTOR is the most effective way for the reinstatement of ATC cells' sensitivity towards classic chemotherapeutics such as paclitaxel (PTX). **Material and Methods:** We used 12 ATC samples obtained from patients and human ATC cell line 8505C. Patients' samples were analyzed using immunohistochemistry. All *in vitro* results were obtained by: MTT assay, flow-cytometry, western blot, invasion and gelatine degradation assay. *In vivo* studies in human-to-mouse xenografts (SCID mice) were performed to confirm the *in*

*vitro* results. **Results:** ABC transporters were detected in 66.7 % of ATC patients' samples. We employed 8505C cell line to establish a resistant ATC model system similar to patients' phenotype. Rho- subline was generated by sorting the small portion of 8505C cells with low rhodamine123 accumulation. Rho- cells had higher ABC transporters and lower pAKT expression compared to 8505C cells and they were resistant to PTX. Combination of PTX and dual mTOR inhibitor AZD2014 was synergistic and equally effective on both cell lines. Using preclinical models of ATC (i.e. xenografts in SCID mice) we confirmed the *in vitro* results. **Conclusion**: According to the obtained results, we propose the use of combination of PTX and dual mTOR inhibitor as a novel therapeutic strategy for ATC treatment.

**Key words**: anaplastic thyroid carcinoma (ATC), chemoresistance, mTOR inhibitor, xenografts

## PP06: Expression and heteromerization of adenosine $A_{2A}$ and dopamine $D_2$ G protein-coupled receptors in neuroendocrine tumors of the lung

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**Background:** Neuroendocrine tumors (NETs) of the lung are relatively rare, contributing with 1-5% to all lung tumors. Recently, dopamine and adenosine receptors have been recognized as new pharmacological targets for lung NETs, and their expression linked with NET aggressiveness. The aim of this project was to evaluate the expression and heteromerization of $A_{2A}R$ and $D_2R$ receptors in Serbian patients with typical (TC) and atypical (AC) carcinoids of the lung. **Patients and Methods:** The patient groups consisted of 26 TC and 26 AC samples. TC group comprised 15 females and 11 males, age range 17-75 (median 50). AC group comprised 12 females and 14 males, age range 29-72 (median 53). Evaluation of genetic and protein expression was performed by RT-PCR and Western blot. $A_{2A}R$-$D_2R$ heteromerization was evaluated by Proximity ligation assay. Statistical analysis was performed to determine differences in expression between TC and AC samples, with significance set at $p<0.05$. **Results:** No significant differences in expression of $A_{2A}R$ and $D_2R$ were detected between TC and AC at the genetic level, but at the protein level, AC had statistically significant higher expression of $A_{2A}R$ than TC ($p=0.0458$). No $A_{2A}R$-$D_2R$ heteromers were detected in TC samples, while 80% of AC samples showed heteromerization. **Conclusions:** Marked differences were observed in the expression and heteromerization of $A_{2A}R$ and $D_2R$ between typical and atypical neuroendocrine lung tumors. Ongoing correlation of this data with patient and tumor characteristics, therapy response and recurrence might soon discover new targetable biomarkers for neuroendocrine carcinoids of the lung.

**Key words:** adenosine, dopamine, GPCR, lung cancer

## PP07: Histone deacetylase (HDAC) 1 and 2 integrate the expression of p53 mutants in pancreatic cancer

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**Background**: Pancreatic cancer (PaC) is so far unmet clinical challenge. Mutation of the tumor-suppressor gene *TP53* is one of the most frequent genetic lesions in PaC. Mutated p53 proteins lose their tumor-suppressive functions. In addition, p53 mutants often gain tumor-promoting functions, qualifying them as important therapeutic targets. Here, we show that the class I histone deacetylases HDAC1 and HDAC2 contribute to maintain the expression of p53 mutants in human and murine PaC cells. **Materials and Methods:** We used human pancreatic ductal adenocarcinoma (PDAC) cell lines. To reduce the HDAC1/2/3 expression we used siRNA-mediated knock-down and HDAC inhibitors (MS275, SAHA and Marbostat) as well as murine genetically engineered HDAC1/2-deficient PDAC cell lines. Protein detection was performed by western-blot and immunocytochemistry. Gene expression was analyzed using qPCR and microarray analysis, while promoter occupancy was analyzed by chromatin-immunoprecipitation (ChIP). **Results:** Our data reveal that the inhibition of HDACs and the genetic elimination of HDAC1/2 leads to reduction of p53 mRNA and protein levels. We further show that HDAC1, HDAC2, and MYC directly bind to the *TP53* gene *in vivo*. We observed that MYC binding to the gene was regulated by HDACs. Moreover, our data demonstrate that HDACs regulate the expression of MYC at the post-transcriptional level. **Conclusion:** Our results illustrate a previously unrecognized class I HDAC-dependent control of the *TP53* gene and provide evidence for a contribution of MYC. A combined approach targeting HDAC1/HDAC2 and MYC may present a novel and molecularly defined strategy to target expression of mutant p53 in pancreatic cancer.

**Key words:** HDAC1, HDAC2, MYC, pancreatic cancer, p53

## PP08: A novel factor HN1 contributes to centrosome assembly and control of mitotic progression

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**Background:** Hematological and Neurological expressed 1 (*HN1*) is an evolutionary conserved gene in vertebrates with its ubiquitous expression in various cells. In our studies, we aimed to investigate the effect of HN1 on cell cycle regulation, microtubule formation and cytokinesis failure in tumor cell lines. **Results:** Here, we show that HN1 localizes to centrosomes to suppress aberrant spindle formation to ensure the fidelity of cytokinesis. In PC-3 prostate and MDA-MB-231 mammary carcinoma cells, high HN1 levels influenced the cell cycle, cyclin and cyclin dependent kinase associations and microtubule formation. Knockdown of HN1 using specific siRNA is accompanied by disruption of centrosome formation and PLK1 localization involving the spontaneous emergence of short monopolar spindles leading to extensive cytokinesis failure. In addition, micro-nucleated cells, chromosome number alteration, early exit from mitosis and nuclear anomalies with polyploidy was observed in both cell lines studied. **Conclusion:** Hence, our results reveal the role of HN1 in centrosome nucleation and spindle formation in human cells that is deregulated in cancer. We further conclude that biphasic HN1 expression augments both the timely completion of mitosis and centrosome maturation at S phase, and contributes to mitotic fidelity.

**Key words:** cancer, cell cycle, centrosome, HN1, microtubules, ploidy

## PP09: Association between genomic instability and pathological/clinical parameters in invasive breast cancer

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**Background:** Breast cancer is the most commonly diagnosed cancer in women worldwide (1). The major pathological types of breast cancer are invasive lobular breast cancer (ILC) and invasive ductal breast cancer (IDC) (2). Common characteristic of these tumor types is an increased frequency of mutations within the genome of tumor cells, referred to as genomic instability (GI) (3). Genomic instability is believed to be the driving force of tumorigenesis (4, 5). Based on the nature of genetic alterations, genomic instability can be classified as chromosomal instability (CIN) and microsatellite instability (MIN). MIN is, generally, less frequent in breast tumors than CIN (3). Another characteristic of breast cancers is age-dependent incidence rate: it is well known that breast cancer incidence rises with advancing age. However, breast cancers arising in older patients are, generally, less aggressive, with slower growth rates. The impact of age, as an independent factor, on the biology of cancer cells has been speculated for a long time. Mechanisms by which aging could affect the biology of cancer cells remain poorly

understood (6). The aim of this study was to investigate the impact of GI on pathogenesis of invasive breast cancer and association of GI and standard clinical parameters.

**Patients and methods:** To investigate the presence and nature of GI in invasive breast cancer, 26 samples of IDC and 21 sample of ILC were analyzed. The level of GI was assessed by comparing the paired profiles of tumor and normal DNA, generated by AP-PCR using three different primers. AP-PCR method allows analysis of anonymous genomic regions and simultaneous detection of CIN and MIN differences between paired DNA profiles (7).

**Results:** Genomic instability was detected in all of the tested samples and it ranged from 0.237 to 0.573. While CIN was found in all tumor samples, the presence of MIN was detected in 92.3% of IDC and 85.7% of ILC. The contribution of CIN in total level of genomic instability ranged from 50 to 100%. Hence, the contribution of MIN ranged from 0 to 50%. Based on the calculated levels of GI, tumor samples were divided into three groups: (**i**) 14 tumor samples with high levels of GI (>0,45), (**ii**) 23 tumor samples with medium levels of GI (0,35-0,45) and (**iii**) 10 tumor samples with low levels of GI (<0,35). Using $\chi^2$ test, we showed that ILC are associated with high and IDC with medium level of GI ($p$=0.042). Analysis of Kaplan-Meier survival curves associated high and low levels of GI with longer patient survival, while medium level was associated with poor prognosis ($p$=0.04). Statistically significant negative correlation between the level of GI and patient age was found by Spearman test (r=-0.366, $p$=0.0161). In addition, t-test showed that statistically significant difference in the level of GI exist between tumors of premenopausal and postmenopausal women ($p$=0.013).

**Conclusion:** This study confirms that CIN is predominant form of genomic instability in breast cancer, but opposes the opinion that MIN is uncommon, given that the presence of this form of instability was detected in most of the analyzed tumor samples. Association of medium level of GI with reduced patient survival, via Kaplan-Meier survival curves, may be reflecting the role of GI in breast cancer progression: certain level of GI generates a number of different genotypes/phenotypes large enough to enable the population of tumor cells to overcome selective barriers. Association of high level of GI with longer patient survival indicates the existence of threshold above which further alterations in genome become lethal. Our study showed that the number of changes in the primary structure of DNA is negatively correlated with patient age.

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**Key words:** age, breast cancer, chromosomal instability, genomic instability, microsatellite instability

## PP10: Quantitating 8-OHdG damage in prostate cancer as a marker of genomic heterogeneity

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**Background:** Oxidative stress plays a significant role in all major steps of carcinogenesis such as promotion, initiation, and progression. During promotion, reactive oxygen species (ROS) induce DNA mutations and structural modifications in a great variety of molecules leading to genomic instability. One of the most prevalent damage types is the generation of 8-hydroxydeoxyguanosine (8-OHdG), which leads to G:T transversion mutations and is used as a novel oxidative DNA damage marker. Since ROS formation is a secondary effect of chronic inflammation, we aimed to investigate the effect of inflammation-induced proteasomal degradation of androgen receptor (AR) and tumor suppressor NKX3.1 on 8-OHdG levels in prostate cancer cells. **Material and Methods:** Total 8-OHdG levels were detected by LC/MS/MS. In addition, immunofluorescence staining of 8-OHdG was performed and foci were counted by imageJ analysis and/or Aklides. NUK system on single cell level. Western blot was performed to show overexpression or depletion of interested proteins. **Results and Conclusion:** LC/MS/MS was used as the reference method for comparing 8-OHdG concentrations to total 8-OHdG foci numbers/100 cells obtained by Aklides.NUK counting. Consistently, the data showed similar alterations in 8-OHdG levels induced by AR and NKX3.1 silencing as well as NKX3.1 overexpression on both settings. Since the Aklides.NUK system is capable to detect the 8-OHdG levels in every single cell as "average foci number/cell" and "% of cells with more than 3 foci", we suggest that the Aklides.Nuk can quantitate 8-OHdG levels as a measure of genomic instability in prostate cancer.

**Key words:** 8-OHdG, Aklides NUK, genomic heterogeneity oxidative DNA damage

## PP11:Association between miR-21 and TIMP-3 mRNA expression levels in breast carcinomas

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**Background:** Initial step for the metastasis of breast carcinomas is the invasion of cancerous cells into the surrounding tissue. Decrease of TIMP-3 mRNA and increase of miR-21 levels might be important event that enables migration of malignant cells. Protein TIMP-3 may function as the regulator of homeostasis of the extracellular matrix. Association between miR-21 and TIMP-3 mRNA expression levels is poorly investigated. Our goal was to examine association between TIMP-3 mRNA and miR-21 expression levels, grouped according to standard diagnostic and prognostic tumor parameters, which might be useful factor of breast carcinoma invasiveness. **Patients and Methods:** The study analyzed

miR-21 and TIMP-3 expression levels in 47 breast cancer samples using TaqMan RT-qPCR technology. **Results:** Statistically significant negative correlation (Spearman's test) between TIMP-3 mRNA and miR-21 expression levels was detected among following breast cancer groups: ER positive (n=38, ρ=-0,398, *p*=0,0133), PR positive (n=30, ρ=-0.5533, *p*=0.0015), Ki-67≤20% (n=19, ρ=-0.4667, *p*=0.0440), histological grade II (n=26, *p*=-0.05145, *p*=0.0072) and diameter ≤ 20 mm (n=18, *p* =-0.6305, *p*=0.0050). **Conclusion:** We found significant negative correlation between TIMP-3 mRNA and miR-21 in only ER positive, PR positive, Ki-67 ≤ 20%, histological grade II and diameter ≤ 20 mm tumor groups, which are the groups with relatively increased miR-21 expression levels. In those groups, TIMP-3 mRNA expression might be regulated due to miR-21 interference, rather than other mechanisms. Listed tumor groups could be potential targets for early screening of invasive phenotype based on those two markers, and for antagomiR gene therapy.

**Key words:** breast cancer invasion, gene expression, miR-21, TIMP-3

## PP12: Methylation status *of TIMP3* gene in breast cancer

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**Background:** Breast cancer is a leading cancer in women worldwide. The incidence of breast cancer is increasing in the developing world due to the increase of life expectancy, the increase of urbanization and the adoption of western lifestyles. Tissue inhibitor of metalloproteinase 3 (TIMP3) is an extracellular matrix-bound protein which regulates matrix composition and affects tumor growth, invasion, angiogenesis and metastasis. Inactivation of *TIMP3* gene has been related to the appearance of tumorigenesis. Hypermethylation of the promoter region is one of the most common mechanisms of *TIMP3* tumor suppressor gene inactivation. The aim of this study was to determine the aberrant hypermethylation of the promoter CpG islands of *TIMP3* gene in breast cancer patients. **Patients and Methods:** This study included 38 patients with lobular and ductal breast carcinomas. Methylation status of the promoter region of *TIMP3* gene was analyzed. The analyses included DNA bisulfite modification followed by methylation specific PCR (MS PCR). In MS PCR, both specific methylated and unmethylated primer forms were used. The methylation status of *TIMP3* was then compared with corresponding patient's clinicopathologic characteristics. **Results and Conclusion:** Hypermethylation was detected in 50% (19/38) of samples. Previous investigations have shown a low level of iRNA expression in the same samples, therefore, we may conclude that there is a strong correlation between the methylation status and the level of *i*RNA expression. Also, the results indicate that hypermethylation of the promoter region has important role in TIMP3 gene expression and may provide evidence for the molecular diagnosis of breast cancer.

**Key words:** breast cancer, inactivation, methylation status, *TIMP3* gene

## PP13: *XRCC1* Arg399Gln genotyping in platinum-treated Serbian lung adenocarcinoma patients

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**Background:** XRCC1 protein is involved in DNA base excision repair. In non-small cell lung cancer, *XRCC1* Arg399Gln polymorphism can affect the survival of platinum-treated patients. This study aimed to evaluate the influence of *XRCC1* Arg399Gln polymorphism on the toxicity and progression-free survival (PFS) of advanced lung adenocarcinoma patients in Serbia. **Patients and Methods:** The study included 165 patients, stage IIIB or IV, performance status 0, 1 or 2, of Caucasian descent. The group consisted of 106 males (64%) and 59 females (36%), median age at diagnosis 61 years (range 37-84). DNA was extracted from FFPE tissue samples. Patients without EGFR mutations were treated with a standard platinum-based doublet. *XRCC1* genotyping was done by PCR-RFLP. Statistical analysis was performed using Chi-square, Fisher's exact, Wilcoxon rank sum test, with $p<0.05$. Survival methodology was used for PFS. **Results:** The follow up period was 1-50 months (median 6 months), during which 56 patients (34%) progressed, and 37 (22%) experienced chemotherapy-related toxicity of grade 3 and 4. Median (95%CI) PFS was 5 (3-6) months. The genotype frequencies for *XRCC1* Arg399Gln were 56% for Arg/Arg, 41% for Arg/Gln and 3% for Gln/Gln genotype. Statistically significant difference in PFS according to the patients' *XRCC1* genotypes was not confirmed, but *XRCC1* homozygotes Arg/Arg were statistically more susceptible to chemotherapy-induced high-grade toxicity ($p=0.027$). Also, carriers of the *XRCC1* Gln allele were more susceptible to progression during chemotherapy ($p=0.025$). **Conclusion:** In the Serbian population, *XRCC1* Arg399Gln genotyping might predict the clinical outcome and toxicity of platinum-treated advanced lung adenocarcinoma patients.

**Key words:** drug-related toxicity, lung adenocarcinoma, polymorphism, XRCC1

## PP14: Importance of copy number alterations of *EGFR* and *c-MYC* genes in triple-negative breast cancer

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**Background:** Triple negative breast cancer (TNBC) is defined by the lack of expression of the three most commonly targeted receptors in human breast cancer: estrogen, progesterone and human epidermal growth factor receptor 2 (HER2). There is a lack of specific therapeutic options for this type of breast tumor. Identifying molecular targets and devising new therapeutics for these targets is an ongoing effort and imperative for the development of successful therapy for TNBC. Epidermal growth factor receptor (EGFR) and c-MYC are frequently overexpressed in TNBC and are emerging as potential

therapeutic targets. **Patients and Methods:** We examined *EGFR* and *c-MYC* gene copy number alterations on archive TNBC samples using Real Time PCR. Our aim was to determine the impact of *EGFR* and *c-MYC* copy number gain on clinicopathologic parameters and clinical outcome in TNBC. **Results:** Our results showed that 93% (73 out of 78) of samples had increased EGFR copy number and 77% (60 out of 78) had increased *c-MYC* copy number. These findings, although without any correlation with clinicopathological variables, indicate that *EGFR* copy number status may be used as a marker for triple negative tumor phenotype. In addition, our results showed significant correlation between *c-MYC* copy number gain and grade of TNBCs. However, we did not observe significant association between *c-MYC and/*or *EGFR* copy number status and patient survival. **Conclusion:** In conclusion, *c-MYC* copy number gain may be associated with risk of high-grade disease in TNBC patients, however, *EGFR* and *c-MYC* increased copy number status may not be informative prognostic factors for TNBCs.

**Key words**: c-MYC, EGFR, gene copy number alterations, survival, TNBC

## PP15: *SMAD4* gene promoter analysis in patients with solid tumors

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**Background:** The SMAD family member 4 (SMAD4) is an intracellular mediator of the transforming growth factor beta (TGFB) signal transduction pathway, which regulates numerous cellular processes, such as cell proliferation, cell differentiation, apoptosis, cell fate and migration (1). The loss of SMAD4 gene heterozigosity combined with inactivating mutations is known to be involved in tumorigenesis (2). Mutations in the SMAD4 coding region were found with high frequency in pancreatic adenocarcinoma and to a lesser extent in colorectal cancer, while complex pattern of SMAD4 mutations was found in thyroid tumors (2,3). However, SMAD4 is currently not considered to be a classic tumor suppressor gene and decrease in SMAD4 gene expression levels was found to have serious consequences for the cell (4). Therefore, the phenomenon of haploinsufficiency, or dosage dependence, should be taken into consideration when evaluating functional relevance of the SMAD4 genotype in cancer cells. Structure and function of the SMAD4 gene promoter and its role in health and disease are severely under investigated. Previous studies indicated that some variants in the SMAD4 gene promoter, region encompassing 500bp upstream from the transcription start site, may have functional relevance and potentially contribute to SMAD4 haploinsufficiency (5-7). Mutations located in the transcription factors' binding sites and affecting functionality of this DNA segment were found in endometrial carcinoma (8). Decrease in SMAD4 gene promoter activity may contribute to the disease development and malignant potential of the tumor, since the loss of SMAD4 expression is known to be involved in late carcinogenesis and metastases (9). Also, there is evidence that loss of TGFB signaling due to loss of SMAD4 expression may confer resistance to some types of antitumor agents (10). Therefore, SMAD4 gene promoter mutations may be of potential relevance for clinical management of solid tumors as biomarkers for disease diagnosis, prognosis and choice of therapy.

This study was aimed to investigate the presence of genetic variants in SMAD4 gene promoter in malignant tissue of four types of solid tumors: pancreatic, colorectal, endometrial and thyroid carcinoma.

**Patients and Methods:** The study was performed on genomic DNA isolated from malignant tissue samples obtained on surgery from patients with solid tumors. The study has included 50 patients with pancreatic adenocarcinoma, 110 patients with colorectal cancer, 36 patients with endometrial carcinoma and 42 thyroid papillary carcinomas. Periprheral blood samples from the same patients were also analyzed. Screening for mutations was performed within an 800bp-long fragment of the SMAD4 gene promoter, encompassing 668 bp to +131 bp from the transcription start site. The analysis was performed by PCR and DNA sequencing using the following oligonucleotides: GCGCGCTATACTCGAGATTTCTCGGCTTCTTCAATA and CCGCCGAATTAAGCTTTGTTCAAGTTTTTCCTTTTA. The obtained PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and sequenced using the ABI Prism BigDye Terminator Kit (Applied Biosystems). The presence of mutations in the SMAD4 gene promoter was analyzed using Sequencing Analysis Software v5.2 Patch 2 (Applied Biosystems).

**Results:** Analysis of the SMAD4 gene promoter in malignant tumor tissue revealed the presence of two mutations. Mutation -10delTT in 88% of pancreatic cancer cases, 3% of endometrial carcinoma cases and 2% of colorectal cancer cases. The somatic origin of this mutation was confirmed by analysis of DNA isolated from peripheral blood of same patients. This mutation affects the region in the close proximity of the transcription start site in the basal promoter and most likely not recognized by transcription factors. However, it was shown to decrease activity of promoter *in vitro* by 40% in comparison to the wild type, indicating that it may be relevant for transcriptional deregulation. Possible mechanism by which this variant disrupts transcription process may be by affecting interactions between transcription factors that bind upstream and downstream from this element. Mutation -180delA was detected in 5% of patients with thyroid tumors and it was confirmed to be of germline origin, since it was detected in DNA isolated from both tumor tissue and peripheral blood. This variant affects predicted binding site for homeobox protein HoxA5, involved in cell cycle regulation in development and cancer. The functional effect of the mutation -180delA was not shown by SMAD4 immunostaining of the thyroid tissue sections from patients with thyroid tumors who carried this mutation compared to the normal thyroid tissue. A possibility remains that this mutation alters SMAD4 expression to a lesser extent, which should be investigated by more sensitive methods. Since this variant affects predicted binding site of transcription factor involved in cell cycle regulation, it should be further characterized functionally.

**Conclusion:** The SMAD4 gene promoter is polymorphic in the population and can be affected by somatic mutations in malignant tissue of different solid tumors. Variants -4delTT and -180delA appear not to be directly involved in tumorigenesis, but since they may alter SMAD4 transcriptional regulation to some extent, they should be further functionally characterized in order to elucidate the role of SMAD4 gene promoter in health and disease. Considering that dosage dependence is of great importance for the role of SMAD4 protein in tumorigenesis, potential clinical significance of SMAD4 gene promoter mutations is also worth further investigation.

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**Key words**: gene promoter, mutation, SMAD4, solid tumor

## PP16: First large deletion in *BRCA1* in Croatian breast cancer patient

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Breast cancer is the most common cancer in women after non-melanoma skin cancer, and it is the leading cause of cancer related deaths in Croatia. Ovarian cancer is in the fifth place, both in incidence and mortality. About 5-10% of all breast and/or ovarian cancer cases are hereditary, and heterozygous germline *BRCA1* and *2* mutations are responsible for the majority of hereditary breast and/or ovarian cancers. In the most cases, the mutations are small nucleotide alterations leading to premature stop of translation. Large rearrangements of *BRCA1* gene and less often, *BRCA2* gene have been described in recent years, but haven't been found in Croatia so far.

Here we describe a case of a Croatian breast cancer patient with no apparent family history of cancer, who developed a triple negative breast cancer at the age of 29, with return of the disease at the age of 33. No mutation was found by HRM or sequencing, but the 5-7 exon deletion of the *BRCA1* gene was determined with a Quantitative Multiplex PCR method and confirmed with MLPA analysis.

## PP17: Semi-synthetic *Astragalus* saponins inhibit inflammation-induced carcinogenic mechanisms in LNCaP prostate cancer cells

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**Background:** Chronic inflammation leads carcinogenesis by abrogation of the cellular mechanisms such as cell proliferation, apoptosis and angiogenesis. Since it has been determined that use of anti-inflammatory drugs reduces the cancer incidence and risk of having prostate cancer decreases 15-20% by regular usage of aspirin and non-steroidal anti-inflammatory drugs, inhibition of inflammation-induced carcinogenic mechanisms is an efficient therapeutic strategy in anticancer drug development studies. Therefore, we aimed to investigate the inhibition effects of semi-synthetic derivatives of cycloastragenol and astragenol molecules isolated from *Astragalus* species on inflammation-related tumorigenic mechanisms in prostate cancer cells. **Material and Methods:** Inflammatory microenvironment was stimulated by either inflammatory conditioned media (CM) or LPS. Griess assay was used to measure NO release. Intracellular reactive oxygen species (ROS) were measured by DCFH method. Expression levels of proteins were determined by western blot and flow cytometer analysis of M30 staining was used to measure apoptosis. **Results and Conclusion:** LPS-induced NO release was determined to be inhibited by *Astragalus* saponins in RAW264.7 macrophages. Level of intracellular ROS induced by $H_2O_2$ treatment was suppressed to nearly basal levels in LNCaP prostate cancer cells. Proteasomal degradations of androgen receptor (AR) and tumor suppressor NKX3.1 due to inflammatory microenvironment were partially suppressed. In addition, enhanced vascular endothelial growth factor (VEGF) expression by CM treatment was totally inhibited. However, apoptosis was not induced at NF-kB inhibitory concentrations. Therefore, we suggested that semi-synthetic derivatives of cycloastragenol and astragenol molecules have a high potential for use of anti-inflammatory drugs in the inhibition of inflammation-induced tumorigenic mechanisms.

**Key words:** androgen receptor, inflammation, NKX3.1, prostate cancer, saponin

## PP18: Addition of curcumin to a palladium (II)-barbiturate complex enhances the cytotoxic/apoptotic activity on non-small cell lung carcinoma cells

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**Background:** Palladium(II) (Pd) complexes have been reported to possess antitumor activity at least comparable to cisplatin [1]. Our group already published some studies regarding the Pd(II) complex [2-5], but this is the first time to explore the activity of Pd(II) a complex and curcumin treatment. However, enhanced cytotoxic effect is still desirable due to unsatisfactory treatment outcomes of lung

cancer patients. Curcumin (*Curcuma longa*) is therefore explored here for potentiation of the activity of Pd(II) complex. **Material and Methods:** The human non small cell lung cancer cell line H1299 was treated with different concentrations of Pd(II) complex (0,39-50μM), curcumin (0,78-100μM), and their combination. Cytotoxicity was analyzed by SRB (sulforhodamine B) assay which is recommended by NCI. Nuclear morphology and plasma membrane integrity were visualized respectively by Hoechst 33342 (blue) and propidium iodide (red) staining. Apoptosis (Caspase 3/7 activity, Annexin-V assay, qPCR) and DNA Damage (γH2AX assay) were determined. **Results:** Following 48 h of treatment, the combination of Pd(II) with curcumin exhibited increased apoptosis compared to Pd(II) or curcumin alone. The well-known apoptosis markers (pyknosis and nuclear fragmentation) were evident. Annexin-V staining, activity of caspase 3/7 and DNA damage were found to have further increased in a dose dependent manner by the combinatorial effect. The genes related to apoptosis were up-regulated while those related to autophagy were surprisingly downregulated compared with control. **Conclusion:** The present study has demonstrated that the combination of curcumin with Pd (II) complex effectively suppressed lung cancer cells' viability *in vitro* through inducing apoptosis (+ supressing autophagy). Thereby, this combination may provide an effective treatment for lung cancer, warranting *in vivo* experiments for proof of concept.

**Key words:** apoptosis; combination drug therapy; curcumin; lung cancer

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## PP19: Blocking autophagy enhanced cytotoxicity induced by doxorubicin in the metastatic breast cancer cell line

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**Background**: 3-methyladenine (3-MA) inhibits autophagy through suppression of class III PI3K activity. Here we evaluate the effects of 3- MA to enhance the cytotoxic activity of the anticancer drug doxorubicin (DXR). **Material and Methods**: MDA-MB-231 cells were treated with DXR (0,92 μM) alone or in combination with 3-MA (5μM). MTT and ATP assay were perfomed to determine cytotoxicity after 48h of treatment. To determine the cell death mechanism, M65 and M30 ELISA was performed at 48h. AnnexinV-FITC was performed to confirm apoptosis. Western blot was employed to detect the protein expression of LC3, Pro-caspase 8, JNK, Beclin 1, Atg5, PARP, p62/SQSTM1,RIP1 at 48h. The expression

levels of apoptosis, necroptosis and autophagy-related genes were analyzed by Real-time quantitative PCR at 24h. **Results and Conclusion:** 3-MA enhanced the cytotoxicity of DXR causing necroptosis. Combination treatment also showed decreased LC3-II protein levels compared to DXR-treated cells, indicating that the inhibition of autophagy enhances cytotoxicity. Genes related to necroptosis were up-regulated under combinatorial treatment in comparison to control. Autophagy is a mechanism of resistance to doxorubicin and inhibiting autophagy might be a promising therapeutic strategy in metastatic breast cancer.

**Key words:** autophagy, breast cancer, 3- methyladenine

## PP20: *In vitro* evaluation of the cytotoxic potential of *Mahonia aquifolium* extracts and their influence on sensitization of MDA-MB-231 breast cancer cells to doxorubicin

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**Background:** *Mahonia aquifolium* is a typical species of *Mahonia* genus. We investigated a potential of *M. aquifolium* on inhibition of cancer cell growth, their synergistic effect and sensitization of cells to doxorubicin. **Material and Methods:** The cytotoxicity *M.aquifolium* ethanol extract (MAE) and water extract (MAW) was tested *in vitro* against: colon (LS174), cervix (HeLa), lung (A549) and breast (MDA-MB-231) adenocarcinoma cells using the MTT test. The cytotoxicity of doxorubicin and sensitizing cells to doxorubicin with extracts was tested against MDA-MB-231. Cell cycle was assessed by cytofluorimetric analysis. Investigated cell lines were incubated with IC50 or 2xIC50 value of extracts and analyzed after 24 h and 48 h of treatment. **Results:** The IC50 values in the MTT assay ranged from 38.96 to >200 µg/ml, for MAE, and range from 39.45 to 191.98 µg/ml, for MAW against all cell lines tested. Also, it is shown that sub-toxic concentrations (50 µg/ml) of extracts in combination with doxorubicin demonstrated possibility of sensitizing MDA-MB-231 cells and decreased the IC50 of doxorubicin from 2.84 µg/ml to 0.45 µg/ml and 0.30 µg/ml for MAE and MAW respectively. We observed that treatment of LS174 cells with MAE and MAW for 24 hours leads to an increase in sub-G1 and accumulation in the G1 phase of the cell cycle. After 48 h, treatment of both extracts induced significant accumulation of cells in sub-G1 peak. **Conclusion:** Extracts of *M. aquifolium* showed inhibition of cell growth, induced apoptosis, and manifested possibility to save/increase effect of lower doxorubicin concentrations on cells.

**Key words:** cytotoxicity, doxorubicin, *Mahonia*

## PP21: Cytotoxic activity of alcoholic extract of *Berberis vulgaris*

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**Background:** *Berberis vulgaris* is traditionally considered as an anticancer drug. In this study we investigated the biological activity and anticancer properties of alcoholic extract of *Berberis vulgaris*.

The highest biological activity of the *Berberis vulgaris* comes from its isoquinoline alkaloid, berberine. **Material and Methods:** The cytotoxic activity of alcoholic extract of *Berberis vulgaris* was tested on cell lines: human cervix adenocarcinoma HeLa, human colonic cancer LS174, human lung adenocarcinoma A549, and normal human fetal lung fibroblasts MRC-5, using the MTT test. Changes in the cell cycle of LS174 cells treated with the extract were analyzed by flow cytometry. **Results:** The investigated *Berberis vulgaris* alcoholic extract expressed cytotoxic activity on used cancer cell lines. The best activity of the extract is shown toward LS174 cell line and the $IC_{50}$ values in the MTT test in HeLa, LS174 and A549 cells were 55.32 ± 0.66 µg/ml, 44.67 ± 6.61 µg/ml, 124 ± 3.9 µg/ml, respectively. To the contrary, investigated extract showed a weak to moderate activity against MRC-5 cell line. For analysis of distribution of the cell cycle, sensitive LS174 cells were treated for 24 and 48 hours with an extract at $IC_{50}$ or $2IC_{50}$ value established by MTT. Treatment of LS174 with $IC_{50}$ or $2IC_{50}$ of the investigated extract, induced accumulation of cells in the sub-G1 peak and slight decrease of cells in G2/M phase of cell cycle upon 48 hours of treatment. **Conclusion:** *Berberis vulgaris* may represent an important source of novel potential antitumor agents. These results indicate the need for further *in vitro* and *in vivo* investigations.

**Key words:** *Berberis vulgaris,* berberine, cytotoxicity, cancer cells

## PP22: *In vitro* investigation of anticancer activity and mechanism of action of novel 1,2,4-triazoles

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**Background**: Triazoles have a five-membered ring of two carbon and three nitrogen atoms, and represent attractive pharmaceutical molecules. 1,2,4-triazoles are found in a wide variety of novel drug candidates, including sedatives, antianxiety, anti-inflammatory, antimicrobial and antimycotic agents. Two novel 1,2,4-triazoles have been synthesized with the aim of investigating their biological activity and selectivity against malignant cell lines, and elucidating these compounds' mechanism of action. **Material and Methods**: The compounds were evaluated for cytotoxicity against the target cell lines: human cervix carcinoma (HeLa), human lung adenocarcinoma cells (A549) and normal epithelial lung cells (MRC5). MTT assay was used to measure the cell survival rate after applying dilution of the compounds that varied from 12.5 µg/ml to 200 µg/ml. The mechanism of action was examined by flow cytometry using propidium iodide to label DNA. After treatment of A549 cells with IC50 of the compounds, cells were harvested and analyzed. **Results**: Compound 1, containing one phenyl ring, showed weak cytotoxic effects, with IC50 around 200 µg/ml on all tested cell lines, while Compound 2 showed a significantly higher activity toward malignant cell lines, especially toward HeLa cells (21,52 µg/ml). Also, this compound showed significant selectivity towards the normal transformed cell line. Cell cycle analysis of A549 cells treated with Compound 2 indicated a decrease in G1 phase and a moderate increase in G2/M phase, suggesting that this compound may affect mitotic division entry. **Conclusion**: Results obtained in this study indicate that Compound 2 may be considered for potential further development and investigations.

**Key words:** anticancer, cytotoxicity, triazoles

## PP23: *In vitro* validations of the anticancer potential of the brown alga extract *Bifurcaria bifurcata*

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**Background:** Seaweeds are important part of the human diet. Species that produce antifouling substances like *Bifurcaria bifurcata* present excellent source of compounds with potential anticancer activity and high phenolic content might be responsible for the antioxidant and antidiabetic action. **Material and Methods:** The cytotoxicity of the dichloromethane-methanol (50:50) extract of the alga was evaluated by the MTT test. Cell cycle distribution of the HeLa cells was evaluated using flow cytometry. Acridine orange/ethidium bromide was used to observe cell death modes. Total phenolic content was assessed using modified Folin-Ciocalteu method. Antioxidant activity was analyzed based on the free radical scavenging activity on the stable DPPH radical. Antidiabetic properties were assessed using α-glucosidase inhibitory enzyme assay. Antibacterial and antifungal activities were assessed by determining MIC. **Results:** Extract exerted cytotoxic activity toward malignant HeLa and K562 cell lines with moderate to high selectivity comparing to normal MRC5 cells. The $IC_{50}$ values of the extract from the MTT assay ranged from 31.45±3.75(μg/ml) for K562 to 55.57±1.66(μg/ml) for HeLa cells. Accumulation of the HeLa cells in sub G1 phase was dose- and time-dependent. Fluorescence microscopy showed dose dependent early apoptotic signs for $IC_{50}$, late apoptotic marks for $2xIC_{50}$ concentrations of the extract in treated HeLa cells. Total phenolic content was 12.10±0.60(mg GAE/g) and $IC_{50}$ value for DPPH scavenger activity was 27.51±0.72(mg/ml). Extract exerted moderate antibacterial, antifungal activity and strong antidiabetic (16.58±0.44μg/ml) potential compared to acarbose (59.8±12.3μg/ml). **Conclusions:** *B. bifurcata* showed promising *in vitro* anticancer action, with high potential benefits for overall health of the patients.

**Key words**: alga, antibacterial, anticancer, antidiabetic, antifungal, antioxidant, drug

## PP24: Real-time monitoring of cytotoxic effects of electroporation on breast and colon cancer cell lines

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**Background:** Despite progress in treatment, breast and colon cancers are still the leading causes of death worldwide. Electroporation is promising method used in cancer therapy. **Material and**

**Methods:** The effects of electroporation on human breast cancer (MDA-MB-231), human colon cancer (SW-480 and HCT-116), and human healthy fibroblast cell line (MRC-5) have been studied. Real-time technology for cell viability monitoring has been used. Acridine orange/ethidium bromide (AO/EB) double staining assay was used for cell death type determination. A numerical model has been used to describe the behavior of cancer cells after electroporation treatment. **Results:** Electroporation treatment mainly induced inhibition of cell viability in a dose (voltage) dependent way. Application of low voltages, up to 100 V increased cell viability, while voltages over 200 V had significant cytotoxic effect on all tested cell lines. Voltage of 150-175 V caused irreversible electroporation of cancer cells and reversible electroporation of healthy cells. AO/EB method suggested that the use of lower voltage rating (150 V) lead to apoptosis as the predominant type of cell death, while the use of higher voltage (270 V) mainly caused necrosis. **Conclusion:** We have found a "therapeutic window" for the application of electroporation of 150-175 V, because in this range electroporation caused permanent damage to cancer cells, while healthy cells completely recovered after 72 h, achieving the highly desired selectivity, which is the ideal treatment outcome pursued by modern oncology. A good agreement of numerical and experimental results shows that this approach can be successfully used for the next analysis of phenomena related to electroporation treatment.

**Key words:** breast cancer, cell viability, colon cancer, electroporation, numerical model

## PP25: Antiproliferative activity of heteropentanuclear oxalato-bridged nd-4f (n = 4, 5) ruthenium complexes with NO ligand

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**Background**: Following our interest in metal nitrosyl compounds with promising biological properties, the series of lanthanide-labelled ruthenium-nitrosyl complexes, as potential anticancer agents, were synthesized and characterized by elemental analysis, IR spectroscopy, ESI mass spectrometry and X-ray crystallography. Heteropentanuclear oxalate-bridged Ru(NO)–Ln (4d-4f) metal complexes with the general formula $(n\mathrm{Bu_4N})_5[\mathrm{Ln\{RuCl_3(\mu\text{-}ox)(NO)\}_4}]$, where Ln = Y (**2**), Gd (**3**), Tb (**4**), Dy (**5**) and ox=oxalate anion, were obtained by reaction of $(n\mathrm{Bu_4N})_2[\mathrm{RuCl_3(ox)(NO)}]$ (**1**) with the respective lanthanide salt. **Material and Methods**: The antiproliferative activity of the ruthenium lanthanide complexes **1**–**5** was evaluated using colorimetric MTT assay for 48 h of continuous drug action in human neoplastic cell lines (HeLa, A549), and human foetal lung fibroblast cell line (MRC-5). Based on obtained $IC_{50}$ values, complex **4** was chosen for ICP-MS analysis in order to investigate intracellular distribution and accumulation of Ru in A549 cells. **Results**: The results showed that all tested compounds exhibited dose-dependent cytotoxicity in the range of concentrations up to 100 μM. Comparison of antitumor activity of **2**–**5** with that of **1** indicates slightly higher activity of the former species.The highest antiproliferative activity with $IC_{50}$ values of 20.0 and 22.4 μM was found for **4** in HeLa and A549 cell line, respectively. ICP-MS results showed that ruthenium exhibited significant intracellular accumulation/ distribution in A549, which increased in a time-dependent manner. **Conclusion**: These compounds showed promising anticancer properties. Further studies on elucidating mechanisms underlying diffe-

rent anticancer activity and cellular accumulation are required in order to ascertain their potential for the development as anticancer agents.

**Key words:** cytotoxicity, intracellular distribution, lanthanide, nitrosyl, ruthenium

## PP26: Semi-synthetic *Astragalus* saponins inhibit inflammation-induced Β-catenin pathway activation and migration in LNCaP prostate cancer cells

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**Background:** Since deregulation of inflammatory response leads to tumorigenic alterations in cellular mechanisms, inhibition of inflammation-induced carcinogenic mechanisms is an efficient therapeutic strategy in anticancer drug development studies. It has been determined that regular usage of anti-inflammatory drugs reduces the cancer incidence as in the case that use of aspirin and non-steroidal anti-inflammatory drugs decreases prostate cancer risk 15-20%. Since saponin molecules exhibit potential anti-inflammatory properties, it is aimed to investigate the effects of semi-synthetic derivatives of cycloastragenol and astragenol molecules isolated from *Astragalus* species on tumorigenic Akt/PKB and Β-catenin mechanisms in prostate cancer cells. **Material and Methods:** Expression levels of proteins were determined by western blot. Xcelligence real-time cell proliferation system was used to measure the alterations in cell proliferation and scratch assay was performed to determine cell migration. Inflammatory microenvironment was stimulated by either inflammatory conditioned media (CM) or LPS. **Results and Conclusion:** Inhibitory effects of *Astragalus* saponins on Β-catenin pathway was determined through decreased levels of $pAkt^{(S473)}$ and Β-catenin in inflammatory microenvironment induced by both LPS and CM treatments. Akt-induced phosphorylation of Β-catenin at S552, which enhances nuclear translocation of Β-catenin and further transcriptional activation of proliferative and migrative target genes was also found to be decreased by the molecules. Further, saponin molecules suppressed proliferation and migration of prostate cancer cells. Therefore, it is suggested that semi-synthetic derivatives of cycloastragenol and astragenol molecules have a high potential for inhibition of tumorigenic Β-catenin activation.

**Key words:** Β-catenin, inflammation, prostate cancer, saponin, xcelligence

## PP27: The effects of CXCR4 and FAK inhibition on resistance reversal and suppression of invasion in non-small cell lung carcinoma cell lines

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**Background:** Non-small cell lung carcinoma (NSCLC) is the most common type of lung cancer. Its very high death rate is related to development of metastasis and therapeutic resistance. Promising approach in the treatment of lung cancer, that targets both invasion and therapy resistance, is inhibition of CXC chemokine receptor type 4 (CXCR4) and its downstream elements, such as focal adhesion kinase (FAK). Therefore, we examined the impact of CXCR4 and FAK inhibitors on reversal of doxorubicin (DOX) resistance, both intrinsic and acquired, and their effects in combined treatment with DOX on the inhibition of metastatic processes. **Material and Methods:** We used human NSCLC cell lines: COR-L23, NCI-H460 and NCI-H460/R. MTT assay, DOX efflux assay, quantitative real-time PCR, western blot and cell cycle analysis to determine the level and type of resistance to DOX and the effect of inhibitors on the reversion of resistance. Additionally, wound healing, invasion assay and gelatine degradation analysis were employed to evaluate the effects of inhibitors of DOX on cell migration and invasion. **Results**: COR-L23 cells showed intrinsic resistance to DOX, while NCI-H460/R cells exhibited multidrug resistant phenotype. CXCR4 and FAK inhibitors strongly synergized with DOX in reducing viability of both resistant cell lines with cell cycle arrest in S or G2/M phase. Additionally, DOX enhanced anti-metastatic characteristics of CXCR4 and FAK inhibitors, by reducing the cell invasiveness. **Conclusion**: Targeting CXCR4 and FAK in NSCLC could help to overcome both intrinsic and acquired resistance, while simultaneously DOX could potentiate their anti-invasive capacity.

**Key words**: CXCR4, FAK, invasion, non-small cell lung carcinoma, resistance

## PP28: Autophagy inhibition with chloroquine enhances cytotoxicity of palladıum (II)-barbiturate bomplex in prostate cancer

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**Background:** Palladium (Pd) (II) complexes were shown to have significant anti-tumor activities against different types of cancer cells. Chloroquine (CQ) is an inhibitor of autophagy that is a prosurvival and resistance mechanism against chemotherapeutics. Therefore, the combination of these two may provide a new treatment modality. **Material and Methods:** The human metastatic prostate cancer cell line PC-3 and the human normal prostate cell line PNT1A were treated with different concentration of Pd (II) complex (1,56-100μM), CQ (0,6-40μM) alone or in combination with CQ. Viability was detected by MTT and ATP viability assays at 24 h and 48 h. Nuclear morphology and plasma membrane integrity were visualized by Hoechst 33342 (blue) and Propidium Iodide (red) staining for 12 and 24h. Apoptosis

(Caspase 3/7 activity, Annexin-V assay, mitochondrial membrane potential) and DNA Damage (γH2AX assay) were determined. **Results:** The combination of Pd (II) complex with CQ dramatically decreased the viability, relative to either compound alone in PC-3 cell line. Moreover, it exhibited higher cytotoxic activity against normal prostate cell line, PNT1A. Also, the combination further increased the level of caspase 3/7 (+) cells, Annexin-V (+) cells, the number of pyknotic nuclei, and depolarization of mitochondria membrane in PC-3 cell line. Surprisingly, higher levels of DNA damage were observed after Pd (II)-treatment compared to the combinatorial treatment, suggesting the DNA-damage activating role of autophagy. **Conclusion:** The combination of Pd (II) complex and CQ enhances apoptotic cell death, possibly via the inhibition of autophagy. Therefore, this novel combination deserves further attention for proof of concept in the treatment of metastatic prostate cancer.

**Key words:** apoptosis, autophagy, chloroquine, prostate cancer

## PP29: *In vitro* evaluation of intravesical gel formulations of gemcitabine HCl-loaded nanoparticles on bladder cancer cell lines

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**Background:** The aim of this study was to evaluate the *in vitro* cytotoxic effects of intravesical gel formulations [mucoadhesive chitosan gel / *in-situ* Poloxamer (Plx) gel] containing Gemcitabine hydrochloride (GemHCl) loaded nanoparticles (NPs) on T24 (human bladder carcinoma) and RT4 (human bladder papilloma) bladder cancer cells. **Material and Methods:** The NPs were prepared by ionotropic gelation of Protosan with sodium tripolyphosphate (TPP). For the preparation of chitosan gel, chitosan was dissolved in diluted lactic acid solution and then NPs were dispersed in chitosan gel. *In situ* Plx gel was prepared according to cold method. Plx was added to distilled water at 4°C and NPs were suspended. RT4 and T24 cells were cultured and maintained according to ATCC's instructions. The cytotoxic effects of formulations containing NPs were evaluated by live cell counting. Cells were treated at different concentrations (0.1-100 μM) of aqueous GemHCl solution, equivalent amount of GemHCl NP loaded gels and empty NP gels. After 48 hours treatment, cells were harvested and then counted by using trypan blue exclusion staining. For each sample, cell viability was calculated. **Results and Conclusion:** The cell-growth inhibition potency of formulations were expressed as IC50 values and % inhibition. GemHCl was more potent on RT4 cells than T24 cells in terms of cytotoxicity. Both chitosan and Plx gel formulations containing GemHCl loaded NPs showed potent cytotoxicity and chitosan gel formulation was found to be more potent than the Plx gel on RT4 cells. However, only Plx gel formulation exhibited an effective cell death on T24 cells. Empty nanoparticles and gels had no effect on cells.

**Key words:** bladder cancer, cytotoxicity, gemcitabine HCl, intravesical administration, RT4 cells, T24 cells

# PP30: Synthetic cyclic KTS peptides, vimocin and vidapin as dual antagonists of α1β1/α2β1 integrins with antiangiogenic activity

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**Background:** Angiogenesis, the generation of new capillaries, is a complex, yet tightly regulated process. Integrins play an important physiological role and are also involved in pathological blood vessel formation. Two major collagen binding integrins are α1β1 and α2β1 [1]. Inhibition of the early steps of angiogenesis has been identified as the attractive approach for the treatment of human cancers. Peptides have been proposed as important therapeutics for cancer and angiogenesis-dependent diseases [2]. Disintegrins, snake venom peptides are useful tools in cancer research because they inhibit integrins with relative selectivity. The short disintegrins Viperistatin and Obtustatin, are the41 amino acids polypeptides, which are cross-linked with four conserved disulfide bonds and having KTS sequence in active site of integrin binding loop [3]. These disintegrins selectively bind α1β1integrin, in contrast to partial selectivity of RGD disintegrins [4]. Viperistatin and Obtustatin were used as a structural template for synthesis of the peptides containing KTS motif and required conformation governed by cyclization via disulfide bridges, in order to develop antiangiogenic inhibitors by blocking α1/α2 integrins.

**Material and Methods:** Anti-adhesive properties of the cyclic pepides were investigated by cell adhesion assay using α1/α2 integrin-overexpressing cells [4]. Molecular dinamic (MD) simulations were performed using the Gromacs Molecular Dynamics package [5] with the AMBER99SB-ILDN force field [6]. The effect of the cyclic peptides on endothelial cells proliferation was assessed using the BrdU assay using human aortic endothelial cells (HAEC). The cyclic peptides effect on human umbilical cord vein endothelial cells (HUVEC) migration were investigated using wound healing assay [7]. The ability of the peptides to inhibit endothelial cells tube formation was investigated in Matrigel angiogenesis assay [8]. The effect of the cyclic peptides on angiogenesis in the quail embryonic chorioallantoic membrane (CAM) assay was tested using fertilized Japanese quail eggs [9]. The antiangiogenic properties of the cyclic peptides were additionally investigated in *in vivo* angiogenesis model, corneal micropocket assay [10]. Stability of the peptides was tested in human serum [11]. Anti metastatic effect of the peptides was meaasured in a mouse model using B16 melanoma cells [12].

**Results:** Linear peptides, analoques of Viperistatin and Obtustatin (Fig. 1,1A,B) containing the KTS motif were synthesized and their *in vitro* anti-adhesive potency was assessed. Linear peptide, Compound 3 ($^{19}$CW**KTS**RTSHYC$^{29}$) was found to be the most active, yet significantly less active than Viperistatin. With the aim to design more potent peptides, cyclization by disulfide and amide bonds was used, while Compound 3 linear sequence served as a basic structure for peptide synthesis [13]. Based on the results obtained with an antiadhesive screening assay, two peptides were selected as potential lead compounds: Vimocin (Fig. 1,1C), with one intra-disulfide bond and Vidapin (Fig. 1,1D) with one additional Cys inserted at position 18 and containing two disulfide bonds. Both cyclic peptides demonstrated the same high potency ($IC_{50}$ = 0.17 nM) and intermediate efficacy (20% and 40%) in inhibition of α1/α2 integrin overexpressing cells adhesion to the respective collagen I and IV ligands [13].

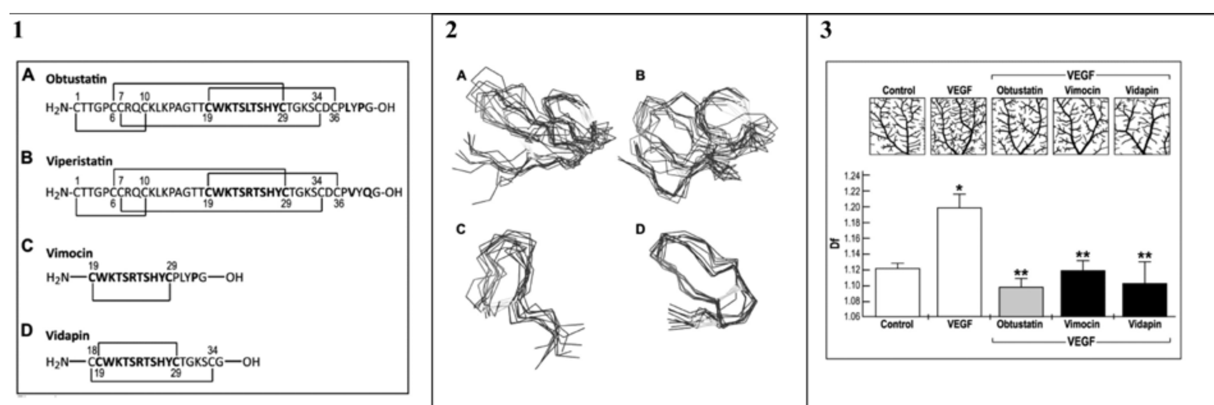


**Figure 1.** Cyclic KTS peptides. 1. Chemical structures of cyclic peptides; 2. Conformational space sampled by molecular dynamics simulations of (A) Obtustatin, (B) Viperistatin, (C) Vimocin, and (D) Vidapin; 3. Inhibitory effect of the peptides on VEGF-induced angiogenesis in the quail CAM model.

To examine the conformational similarity of the designed peptides, Vimocin and Vidapin to the control compounds, Viperistatin and Obtustatin, MD simulations for these compounds were run (Fig. 1,2). Results for Viperistatin (Fig. 1,2A) and Obtustatin (Fig. 1,2B) show that the KTS loop (in red) region is mobile. Vimocin, which has one disulfide bond, is less flexible (Fig. 1,2C), while Vidapin, with 2 disulfide bonds, is quite rigid (Fig. 1,2D). Apparently a larger numbers of disulfide bonds restrict the sampled conformational space of the designed compounds [13].

The effect of Vimocin and Vidapin was investigated in several *in vitro* and *in vivo* angiogenesis assays representing the different phases of the neovascularization process i.e. endothelial cells proliferation, migration, tube formation and capillary sprouting. Vimocin and Vidapin inhibited proliferation of HAEC by 90%, similarly to Obtustatin [13]. In migration assay Vimocin blocked wound closure by 53%, two fold stronger than Vidapin, which blocked the wound closure by 26% [13]. Thereafter, Vimocin and Vidapin completely inhibited tube formation indicating angiostatic effect of these KTS cyclic peptides. When vascular endothelial growth factor (VEFG)-induced angiogenesis was investigated VEGF-induced capillary formation was strongly inhibited by Vimocin and Vidapin, the same as with Obtustatin (Fig. 1,3) [13]. The results obtained for Vimocin and Vidapin inhibition of bFGF-induced angiogenesis in naturally avascular tissue rodent cornea, by comparison of quantified angiogenesis area, showed that only Vimocin significantly inhibited angiogenesis by 20% [13]. These data indicated that synthesized peptides possess antiangiogenic properties. Stability of the peptide is an important prerequisite for a successful drug. The linear peptide, Compound 3 was completely degraded after only 5 hours with a half-life of 3 hours. The half-life of Vimocin is 10 hours and it was completely degraded after 24 hours. Vidapin is the most stable KTS cyclic peptide with half-life of 30 hours, indicating that cyclization of the peptides prolonged their stability in human serum [13]. The safety of Vimocin and Vidapin was demonstrated in *in vitro* and *in vivo* experiments. The peptides did not showed any toxicity toward either endothelial cells or mice [13]. To investigate the relevance of the antiangiogenic effects of Vidapin and Vimocin for tumor therapy, we measured their anti metastatic effect in a mouse model. Vidapin significantly increased the survival of mice with B16 melanoma up to 73 days, whereas median survival time of animals in this tumor experimental model is 40 days [13].

**Conclusion:** Vimocin and Vidapin are dual antagonists of α1β1/α2β1 integrins with antiangiogenic activity and can be further used as lead compounds for drug development. They may also serve as preclinical tools for investigations into α1β1/α2β1 biological function in cancer and angiogenesis.

**Key words:** antiangiogenesis inhibitor, anticancer agents, cyclic peptides, integrin α1β1, integrin α2β1.

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## PP31: MDR cancer phenotype modulates the efficacy of novel tubulin destabilizing agent DTA0100

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**Background:** Microtubule targeting agents (MTAs) are widely used in cancer chemotherapy. However, development of drug resistance limits their efficacy in cancer treatment. In the present study, we evaluated the effects of propargylic enol ether derivative DTA0100, a microtubule destabilizing agent on multi-drug resistant (MDR) cancer cell lines. **Material and Methods:** The effects of DTA0100 were studied in MDR cancer cell lines and their sensitive counterparts (colorectal carcinoma DLD1-TxR and DLD1, glioblastoma U87-TxR and U87). Cytotoxicity of MTAs was assessed by Sulforhodamine B assay. Fluorescent microscopy was used to determine the effects of DTA0100 on mictrotubule network. Changes in the expression of β tubulin isotypes were analyzed by semi-quantitative RT-PCR. Flow cytometric analysis was used to examine the effect of DTA0100 on cell cycle distribution and cell death.

**Results:** Both MDR cancer cell lines demonstrated resistance to various MTAs. Importantly, DTA0100 was 2-fold more efficient towards MDR colon cancer cells. However, its efficacy against U87-TxR cells was reduced in comparison to their sensitive counterparts. DTA0100 caused microtubule disruption in all cell lines. DTA0100 increased a portion of apoptotic cells in U87 and DLD1-TxR cells. U87-TxR cells underwent G2/M cell cycle block after DTA0100 treatment. The expression of II, III and IVb - β tubulin was increased in U87-TxR cells, while the class III was decreased in DLD-TxR. **Conclusion:** The observed changes in the expression pattern of β tubulin isotypes between MDR glioblastoma and colon cancer cells may confer to their different sensitivity to DTA0100.

**Key words:** DTA0100, microtubule targeting agents, multi-drug resistance, β tubulin

## PP32: Evaluation of carvacrol derivatives on treatment of glioblastoma multiforme

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**Background:** Glioblastoma Multiforme (GBM) is one of the most common aggressive malignant cancer types. Surgery, chemotherapy and radiotherapy approaches are not sufficient to treat the GBM. On the other hand, natural compounds and their derivatives are very crucial research area for the treatment of cancer, nowadays. Taken in this context, in this study, U87-MG cell line was chosen as GBM model and carvacrol has proved to be very effective on cancer treatment. **Material and Methods:** Eight different carvacrol derivatives were synthesized and their anticancer effects were evaluated for the first time. Anticancer effects of these carvacrol derivatives were assessed with 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrasodium bromide (MTT) and lactate dehydrogenase (LDH) assays. **Results and Conclusion:** Our results demonstrated that the carvacrol derivatives exhibited cytotoxicity effects at high concentrations (100 and 50 µg/ml) and there is no toxic effects at low concentrations. In conclusion, our results suggest that 8. carvacrol derivative ($C_{14}SNO_2H_{15}$) is the best compound for GBM as compared to other carvacrol derivatives. Therefore, 8. carvacrol derivative can be a new therapeutic agent for the treatment of GBM.

**Key words:** carvacrol derivatives, cell viability, glioblastoma, LDH, MTT, U87-MG cell line

## PP33: Synthesis, characterization of novel NSAIDs and their cytotoxic effects on human glioblastoma cell line

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**Background:** Glioblastoma Multiforme (GBM) is one of the most malignant brain tumors classified as grade IV by WHO (World Health Organization) accounts for one in four of all primary brain tumors. The treatment options of GBM is so limited and the median survival is still short. Thus, new treatment approaches are necessary to prolong this limited life time. In this study we aimed to synthesize new

NSAID prodrugs (Ibuprofen, Naproxen and Flurbiprofen) and investigate *in vitro* cytotoxic effects of these NSAID prodrugs on U-87 MG human GBM cell line. **Material and Methods:** U-87 MG GBM cell cultures were treated with 9 different NSAIDs (3,125; 6,25; 12,5; 25; 50 and 100 μg/ml) for 48 hours. Cell viability was investigated with MTT assay. Then, membrane integrity was determined with LDH assay. **Results:** According to the our results NSAID prodrugs exhibited dose-dependent cytotoxic effects on U-87 MG GBM cell line when compared with the control group. The cytotoxic and anti-cancer effects of novel NSAID prodrugs on U-87 MG human GBM cell line were investigated for the first time. **Conclusion:** The current study presents an insight into the use of novel NSAIDs for developing treatment strategies for GBM.

**Key words**: cytotoxicity, glioblastoma multiforme, LDH release, MTT, non-steroidal anti-inflammatory drugs, U87-MG cell line

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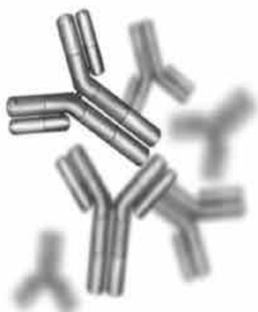
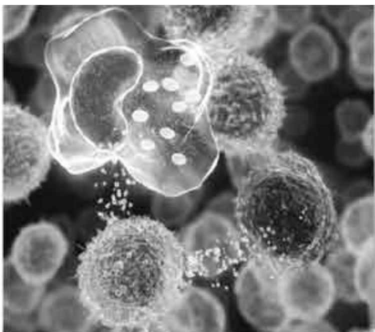

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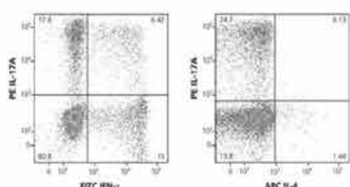


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