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RESEARCH REPORT 2024

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DEPARTMENT OF EXPERIMENTAL ONCOLOGY

Laboratory of Experimental Anticancer Therapy
Head: Professor Joanna Wietrzyk, Ph.D., D.Sc.

Research on the application of novel biologically active compounds in cancer therapy

A new series of compounds was obtained, including heterocyclic thiosemicarbazones. Their antiproliferative activity was assessed against MCF-7 (human breast adenocarcinoma), SK-N-MC (human neuroepithelioma), MCF10A (normal human breast epithelial), and BALB/3T3 (mouse fibroblast) cell lines. These compounds exhibited high antiproliferative activity. In the case of one compound, the activity was several times higher than that of reference compounds currently in clinical trials. Unfortunately, none of the compounds in these series showed a favourable selectivity ratio between cancer and normal cells. Furthermore, for eight selected N-substituted thiosemicarbazone derivatives, conjugates with metallacarboranes were obtained (in cooperation with the Laboratory of Biomedical Chemistry, IIET). These modifications aimed to improve the pharmacokinetic properties of thiosemicarbazones *in vivo*. However, no improvement in *in vitro* biological activity was observed for the thiosemicarbazone-metallacarborane conjugates compared to the free parent compounds.

The role of regulatory molecules lncRNA and miRNA in the anticancer activity of calcitriol in breast cancer

In breast cancer cell lines MCF-7, T47D, MDA-MB-231, MDA-MB-468, CAL-51, and the normal breast epithelial line MCF-10A treated with calcitriol and tacalcitol for 120 hours, and in the EtOH control (solvent for calcitriol and tacalcitol), the expression levels of full-length lncRNA MALAT1 (using two different primer pairs), two splicing variants (Δ243 and Δ119), and the circular form of MALAT1 (circ-MALAT1) were assessed. Calcitriol and tacalcitol do not affect the levels of full-length MALAT1 or circ-MALAT1. The Δ243 MALAT1 level decreases in MCF-10A and MDA-MB-468 cells after treatment with calcitriol and tacalcitol, while in T47D cells, it increases after calcitriol treatment. No changes are observed in the remaining cell lines. The Δ119 MALAT1 level decreases in MCF-10A cells after treatment with calcitriol and tacalcitol; no changes are observed in the remaining cell lines.

Laboratory of Tumour Molecular Immunobiology
Acting head: Andrzej Rapak, Ph.D., D.Sc.

In 2024, the LIMN laboratory conducted research on the antiproliferative properties of new amine derivatives of betulinic acid.

Betulinic acid and its derivatives have strong antiproliferative effects. Our studies have also shown that betulinic acid in combination with sorafenib induces cell death in lung and pancreatic cancer cells, regardless of the mutations occurring in the cells. However, the use of betulinic acid is limited due to its difficult solubility in water and thus bioavailability in the body. The task aimed to examine the antiproliferative properties of new amine derivatives.

The antiproliferative properties of the obtained conjugates were checked on the human lung cancer A549 and human colon cancer DLD-1 cell lines. Cell viability was examined using the MTS test, and the level of apoptosis was examined by double staining with annexin V and propidium iodide.

Betulinic acid was covalently bound to amine compounds using carbodiimides. The conjugate of betulinic acid with Boc-Ethylenediamine reduced the viability of cancer cells by 15-19%, while after removing the Boc group, the efficiency of the conjugate increased to 75-80%. Betulinic acid alone reduced cell viability by 10-15%. In the apoptosis induction test, the conjugate with the Boc group induced apoptosis by 23-28%, and after removing the Boc group, by 92-97%.

It was shown that the presence of a free amino group in the conjugate significantly increases the antiproliferative potential in cancer cells.

The developed methodology for obtaining soluble derivatives of betulinic acid can lead to obtaining compounds with improved antiproliferative, antibacterial and antiviral properties.

Laboratory of Biomedical Chemistry
Head: Tomasz Goszczyński, Ph.D., D.Sc.

Hydrophobic stabilization of the bacteriophage F8 preparation and its effect on biofilm eradication

Pseudomonas aeruginosa is a multidrug-resistant opportunistic pathogen capable of causing severe infections. Our laboratory investigates the application of bacteriophages as an alternative therapeutic strategy to combat *P. aeruginosa* infections. Phage therapy offers a promising solution for treating antibiotic-resistant bacterial infections. However, the clinical implementation of phage therapy is hindered by the absence of standardized protocols endorsed by health authorities.

A critical requirement in phage therapy is the delivery of an effective concentration of viable phage particles to the infection site. This necessitates the development of formulations capable of maintaining high titers in purified preparations. To address this, we developed a novel phage extraction method employing 1-octanol.

1-Octanol, a naturally occurring fatty alcohol found in citrus oils and widely used in the fragrance industry, demonstrated efficacy in preserving phage infectivity. Its safety for human use is supported by its approval as a food additive by both the U.S. Food and Drug Administration and the Council of Europe.

We confirmed the stabilizing effect of 1-octanol on the storage stability of purified F8 bacteriophage preparations. Compared to crude lysate, both the purified F8 preparation and the formulation stabilized with 1-octanol exhibited enhanced efficacy in eradicating *P. aeruginosa* biofilms. Furthermore, evaluation of phage activity in active human serum revealed no detrimental effect of serum components on bacteriophage antibacterial efficacy.

Although phages represent a powerful tool against antibiotic-resistant bacteria, challenges remain regarding safety and the precise characterization of phage preparations. Our findings provide a foundation for broader investigations into other bacteriophage groups. Future efforts by our team will focus on standardizing phage formulation reproducibility and elucidating the physicochemical properties of phages, which may influence their behavior in diverse environmental conditions.

DEPARTMENT OF CLINICAL IMMUNOLOGY

Laboratory of Clinical Immunogenetics and Pharmacogenetics
Head: Professor Katarzyna Bogunia-Kubik, Ph.D., D.Sc.

NKG2D-MICA/MICB axis in various clinical situations

Proper functioning of NK cells is regulated by a set of activating and inhibitory receptors, which are located on their surface. One of the receptor groups is the C-type lectin-like NKG2 family. Both MICA and MICB are ligands for the activating receptor NKG2D, whose presence is associated with cytolytic and cytotoxic properties of NK cells.

Allogeneic transplantation of hematopoietic stem cells (HSCT)

Studies on serum soluble MICA (sMICA) revealed that its concentration was increased at day +30 after transplantation in recipients who were diagnosed with acute or chronic GvHD (aGvHD/cGvHD). Recipients carrying the *MICA* 1065075 *G* allele, as well as recipients whose donors were carriers of the *MICA* 1065075 *G* allele, were characterized by increased serum sMICA when compared to *AA* homozygous individuals. Among the recipients diagnosed with cGvHD, NKG2D surface expression on NK cells was higher at days +60 and +90 post-transplant, and the percentage of NKG2D+ NK cells differed in recipients based on sMICA concentration. Furthermore, *NKG2D* rs1049174 polymorphism showed potential prognostic properties in the development of aGvHD [Siemaszko et al. *Hum Immunol*.].

Genetic variability studies showed that the *G* allele of two *MICB* polymorphisms, rs1065075 and rs3828903, was less frequently detected among donors for recipients who developed cGvHD. Additionally, the *MICB* rs1065075 *G* allele was less commonly found in donors for recipients with CMV infection. The same relationship was observed in the recipient group. Studies on serum samples collected 30 days after HSCT showed that recipients who developed CMV infection, or those who were diagnosed with cGvHD, were characterized by an increased level of sMICB. Moreover, it was found that sMICB concentration could also be related to *MICB* genetic variants, as the homozygous *AA* genotype of both studied polymorphisms was associated with increased concentration of sMICB [Siemaszko et al. *Arch Immunol Ther Exp*.].

Disease progression and therapy in multiple myeloma

Patients with multiple myeloma carrying the *MICB* rs1065075 *A* allele were less likely to experience disease progression after therapy with the CTD regimen (cyclophosphamide, thalidomide, dexamethasone). Regarding the serum levels of the soluble form of sMICB in patients, however, it was observed that patients in more advanced stages of disease (II-III on the ISS scale) had higher levels of sMICB than patients in the least advanced stage. It was shown that patients with the *MICB* rs3828903 *A* allele had longer disease-free survival than patients without this allele, and this relationship was particularly evident in patients during the first two years after disease remission. On the other hand, these patients were more likely to have a more advanced disease stage (II-III on the ISS scale) at diagnosis.

Axial spondyloarthritis (axSpA) pathogenesis and biological treatment

The genotyping results suggested that *MICA* and *NKG2D* gene polymorphisms may be biomarkers associated with disease susceptibility and clinical outcomes after anti-TNF therapy in axSpA patients and imply a less favourable effect of the *MICA* rs1051792 *A* and *NKG2D* rs1154831 *G* genetic variants [Wielinska et al. *Clin Exp Rheumatol*.].

Healthy ageing

Studies on elderly individuals (age 65-99 years) and ethnically matched young people (age 18-64 years) demonstrated significant associations of telomere length with ageing as well as

with the presence of some HLA class I or class II alleles [Marta Dratwa-Kuzmin et al. *Int J Mol Sci*.]. For the *MICA* gene variability, individuals with the rs1051792 *G* allele had shorter telomeres than those with the *AA* genotype. An inverse relationship was observed for the rs1063635 polymorphism. Healthy individuals with the *G* allele of rs1063635 had longer telomeres than *AA* homozygotes.

Laboratory of Immunogenetics and Tissue Immunology
Head: Izabela Nowak, Ph.D., D.Sc.

The role of HLA-E polymorphism and soluble HLA-E isoform in recurrent reproductive failures and male infertility

Human leukocyte antigen (HLA)-E, as a non-classical HLA class I molecule interacting with NK and T cell receptors, may activate or inhibit immune responses. These reactions can impact reproductive success because HLA-E is expressed by trophoblast cells. We investigated the rs1264457 A/G HLA-E polymorphism in couples with reproductive failures such as recurrent implantation failure (RIF) after *in vitro* fertilization (IVF), recurrent spontaneous abortion (RSA), and sporadic spontaneous abortion (SSA) after natural conception. Furthermore, we investigated the role of the soluble HLA-E isoform (sHLA-E) in women's plasma and the seminal plasma of men participating in IVF procedures. We used real-time PCR with a TaqMan probe to study the rs1264457 polymorphism and an ELISA test to measure the soluble HLA-E isoform. Our study indicates that the rs1264457 A/G polymorphism did not influence female infertility or susceptibility to RIF and RSA. However, we noticed that HLA-E 0101 homozygotic men were more prone to have severe, very severe oligozoospermia or azoospermia ($p = 0.013$, OR = 1.70). Moreover, we found a higher concentration of sHLA-E in IVF patients than in control women ($p < 0.0001$/pcorr. = 0.0024). In turn, a lower level of sHLA-E in semen plasma was associated with fewer sperm cells ($p < 0.0001$). In conclusion, the sHLA-E isoform in men is associated with semen parameters, especially with the number of sperm cells in the ejaculate.

The Antigen-Processing Pathway via Major Histocompatibility Complex I as a New Perspective in the Diagnosis and Treatment of Endometriosis

Endometriosis is a debilitating gynecological disease defined as the presence of endometrium-like epithelium and/or stroma outside the uterine cavity. The most commonly affected sites are the pelvic peritoneum, ovaries, uterosacral ligaments, and the rectovaginal septum. The aberrant tissue responds to hormonal stimulation, undergoing cyclical growth and shedding similar to appropriately located endometrial tissue in the uterus. Common symptoms of endometriosis are painful periods and ovulation, severe pelvic cramping, heavy bleeding, pain during sex, urination and bowel pain, bleeding, and pain between periods. Numerous theories have been proposed to explain the pathogenesis of endometriosis. Sampson's theory of retrograde menstruation is considered to be the most accepted. This theory assumes that endometriosis occurs due to the retrograde flow of endometrial cells through the fallopian tubes during menstruation. However, it has been shown that this process takes place in 90% of women, while endometriosis is diagnosed in only 10% of them. This means that there must be a mechanism that blocks the immune system from removing endometrial cells and interferes with its function, leading to implantation of the ectopic endometrium and the formation of lesions. We consider the contribution of components of the Major Histocompatibility Complex (MHC)-I-mediated antigen-processing pathway, such as the ERAP, TAP, LMP, LNPEP, and tapasin, to the susceptibility, onset, and severity of

endometriosis. These elements can induce significant changes in MHC-I-bound peptidomes that may influence the response of immune cells to ectopic endometrial cells.

We tested 30 single-nucleotide polymorphisms (SNPs) in the *ERAP1, ERAP2, LNPEP, TAP1, TAP2, LMP2, LMP7, LMP10, and TAPBP* genes in 391 samples of women with endometriosis and 393 control women. SNP testing was performed by Real-Time PCR using TaqMan probes. Statistical analysis was performed using GraphPad InStat software. Among the polymorphisms studied, the AA and AT genotypes of rs7063 ERAP1 were associated with endometriosis ($p = 0.018$, OR = 1.42; $p = 0.006$, OR = 0.67, respectively). Moreover, the p-value and OR were stronger in stage IV disease for AA ($p = 0.003$, OR = 1.92) and AT ($p = 0.000$, OR = 0.41) genotypes. Preliminary results also indicate differences in the frequencies of TAP2 rs241447 genotypes between women with stage III and IV endometriosis and healthy women ($p = 0.03$, OR = 2.34 and $p = 0.015$, OR = 2.26, respectively).

Laboratory of Clinical Immunology
Head: Professor Andrzej Lange, M.D., FRCP (London)

The primary topic of interest of our group is the use of merrow stem/progenitor cells in clinical activity. Hematopoietic cell transplantation was initially the primary goal for regenerating the marrow or for immunotherapy in patients with leukemia. However, in 2002, the program of regenerating damaged tissues also became clinically valid. The initial interest was devoted to revascularization of the critical ischemic legs. A 15-year follow-up study demonstrates that this approach was successful in 50% of patients after 4 years of surgery, being associated with the content of CD34+ cells but not that of MSC phenotype in the transplanted cell population. It prompted us to use a similar approach in the treatment of avascular necrosis of the femur, which was also successful. Our 2014 activity involved evaluating the effect of intra-articular injection of marrow-derived cells enriched in mononuclear cells, including those of MSC characteristics (CD45-CDw34-, CD90+, CD105+), using centrifugation of the marrow in autologous plasma. Again, this approach was successful. Six to seven years after the surgery, patients who had cells implanted were invited for a control; 30 responded to the invitation. They were physically examined, and the routine blood work was performed. To find out whether the immune system over reactivity may impact the effect of cellular therapy, the levels of soluble cytokines in the blood and expression of the genes encoding immune response-associated cytokines, including IL-6 and KL (Klotho), were measured, A majority of patients (21 out of 30) enjoy long-term benefits for 6 to 7 years. Seven patients still suffered from pain and joint dysfunction, and in two others, the suffering ended with arthroplasty. In response to the cellular treatment, two phases can be distinguished: a short-term response, characterized by pain relief 6 months after cell implantation, and long-lasting effects observed 6 or 7 years after the therapy, with a significant improvement in joint function. The improvement observed tended to be associated with higher expression of the IL-6 and KL genes. Both these gene products are essential for regeneration processes. A positive association was also observed between IL-1β plasma levels and a favorable outcome. In conclusion, cellular therapy with marrow cells enriched in mononuclear cells is effective, providing autologous support, as evidenced by the expression of certain cytokine genes and ultimately, blood levels.

DEPARTMENT OF PHAGE THERAPY
Head: Professor Andrzej Górski, M.D.

Bacteriophage Laboratory
Head: Professor Andrzej Górski, M.D.

Stability studies on *Klebsiella* bacteriophages specific for the *Klebsiella* strains isolated from patients with urinary tract infection (UTI)

The objective of the study was to examine the stability of 11 *Klebsiella* phages in therapeutic phage preparations under various storage conditions (temperature: 4°C, -80°C, -80°C in phage host strain) and different microbiological media (peptone water, LB, BHI, and TSB broth). The study utilized 0.8M trehalose and 15% glycerol as stabilizers over periods of 2, 4, 6, and 12 months.

Stability studies of 11 *Klebsiella* bacteriophages conducted at specific storage periods at a temperature of -80°C and in phage host cells using selected media with the addition of 0.8 M trehalose and 15% glycerol as stabilizers showed significant variation in the degree of lytic activity. The study demonstrated limited stability of preparations with trehalose, while also showing that glycerol cannot be used as a stabilizer for most tested phages stored in all media at -80°C.

Phage lytic activity remained stable for all 11 tested phage preparations in BHI broth after 12 months of storage. These results will help develop optimal microbiological media and storage conditions for therapeutic phage preparations for UTI treatment.

Retrospective analysis of the outcomes of rectal bacteriophage administration in patients with genitourinary infections (continuation of the task from 2021)

This retrospective study evaluated the long-term outcomes of rectal administration of bacteriophage preparations in 34 men with chronic bacterial genitourinary infections, particularly chronic bacterial prostatitis, treated at the Phage Therapy Unit of the Hirszfeld Institute. Follow-up interviews were conducted 1-9 years after phage therapy completion. The most common pathogens were *E. faecalis* and *E. coli* (56%), with others including *K. pneumoniae*, *P. aeruginosa*, and *P. mirabilis*. All patients received phage preparations rectally; some also received them via oral, intravesical, or topical routes.

According to medical assessment, 47% of patients responded well to treatment, with 24% achieving complete eradication of the pathogen. Self-reported outcomes indicated bacterial eradication in 19% and symptom improvement in 42% shortly after therapy. Between 1 and 12 months post-treatment, eradication and symptom relief were observed in 48% and 47% of patients, respectively. At the time of follow-up, 87% reported health improvement, with 42% achieving full clinical recovery. In terms of satisfaction, 48% of patients were very satisfied, and 44% attributed their health improvement directly to phage therapy.

These findings suggest that rectal phage therapy may be effective in managing chronic genitourinary infections and that its therapeutic benefits can persist or emerge long after treatment. This study provides unique and valuable clinical data in a field where such evidence is still limited.

Project founded by the National Science Centre: Studies on the immunomodulatory effects of bacteriophages on functions of immune cells

The conducted studies demonstrated that bacteriophages stimulate the secretion of both pro-inflammatory and anti-inflammatory cytokines in immune cells isolated from peripheral blood. This may be of significant importance in the mechanism underlying their potential immunomodulatory effects. However, this stimulation was markedly weaker than that induced by a pathogenic virus (adenovirus type 5).

For the first time, the impact of bacteriophages/phage therapy on the synthesis of pro- and anti-inflammatory cytokines and on the expression of selected genes regulating the immune response was investigated in peripheral blood mononuclear cells and granulocytes in humans. It was shown that chronically administered bacteriophages/phage preparations did not significantly stimulate such synthesis. This is highly relevant not only for assessing the biological activity of bacteriophages but also for evaluating the safety of phage therapy.

Project funded by the Medical Research Agency: Non-commercial clinical trial to confirm the safety and efficacy of phage therapy in the treatment of chronic rhinosinusitis – RHINOPHAGE

In preparation for the RHINOPHAGE clinical trial, continued collaboration with the CRO focused on clinical documentation development, including analysis of patient data from the Phage Therapy Unit and relevant literature for the Investigator's Brochure and IMPD. A detailed procurement specification was prepared for the manufacturing of the investigational phage cocktail and placebo, followed by a legally binding agreement with the selected manufacturer.

Bacteriophages, bacterial production strains, and manufacturing documentation were developed at HIIET PAS and transferred to the manufacturer, who began production activities. Due to technical challenges in identifying all intended phages, the cocktail composition was modified to include two instead of three phages. Work continued on preparing the clinical trial application for submission in the CTIS system, with the new deadline set for December 31, 2024.

***In vitro* study of the effect of *Enterococcus* phages on the destruction of biofilm in urinary tract infection**

The aim of the study was to extend *in vitro* studies on the effect of selected *Enterococcus* phages on biofilm produced by *Enterococcus* bacteria isolated from patients with urinary tract infection (UTI).

To analyze the effect of phages on bacterial biofilm in UTI, 9 phages from the *Enterococcus* EF group grown on the *Enterococcus* 1679Ł strain devoid of prophages and 7 *Enterococcus* strains from UTI were selected. The conducted studies showed significant destruction of biofilm by 9 *Enterococcus* phages: EF1, EF12, EF15, EF49, EF57, EF62, EF7, EF13 and EF56. The 24-hour biofilm was destroyed most strongly by phages with a titer of 10^7 PFU/ml and 10^9 PFU/ml within 24 hours. Biofilm degradation was observed by phages with a titer of 10^7 PFU/ml in a mean of 51.3% and phages with a titer of 10^9 PFU/ml in a mean of 49.5%. Phages with a titer of 10^6 PFU/ml were less effective in destroying bacterial biofilm, with a mean of 25.1%. The results of the study of the effect of phages on biofilm in UTI will be used to select phages for phage therapy in patients with UTI in the Phage Therapy Unit of Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences.

The optimization of the isolation and amplification of bacteriophages specific to *Acinetobacter baumannii*

We continued our work on the optimization of the isolation and amplification of bacteriophages specific to *Acinetobacter baumannii*, with a particular focus on improving their long-term stability. A total of 189 multidrug-resistant *A. baumannii* strains and 23 environmental samples were screened, leading to the isolation of three new phages. Notably,

two phages were obtained using a newly developed Bait-Phage-Method (BPM), which enables more efficient screening of environmental material, and one was isolated via enriched culture. One phage genome was sequenced using nanopore technology and is undergoing bioinformatic analysis to confirm its therapeutic potential. Importantly, BPM proved versatile, also allowing for the isolation of phages specific to other bacterial species (e.g., *E. coli*).

In parallel, the stability of four *Acinetobacter* phages (Acba_1, Acba_6, Acba_19, Acjo_20) was evaluated under various conditions, including different temperatures (-70°C, 4°C, 24°C, 37°C), light exposure, and storage media (Pluronic, glycerol, trehalose, glass). Long-term storage studies showed that light protection and certain stabilizers significantly improve phage viability, both immediately and after 6 months. These findings support ongoing efforts to establish a 2-year storage protocol. Collectively, our results contribute to expanding the repertoire of therapeutic *A. baumannii* phages and improving their formulation for future clinical use.

Laboratory of Phage Molecular Biology
Head: Professor Krystyna Dąbrowska, Ph.D., D.Sc.

Phageome transfer from gut to circulation and its regulation by human immunity

Bacteriophages are a critical part of the human microbiome, especially in the gastrointestinal tract. While phage translocation beyond the gut is known, its regulation within phageomes and interaction with immune responses remain unclear. This study explores the taxonomic structure of the phageome, lymphatic filtration, and phage-specific IgG induction as factors regulating phage translocation from the gut to the bloodstream. Mucosal intestinal biopsies and serum samples from 38 patients were analyzed to determine gut and blood phageome compositions. Phage epitopes targeted by IgGs were identified via immunoprecipitation of a phageome-wide epitope library and high-throughput sequencing. *In vivo*, phage arrest in lymph nodes was studied using a mouse model.

We identified major phage antigens recognized by specific IgGs in these patients. We sought to explore the phenomenon of translocated phages, like the age-old question of which came first, the chicken or the egg: does the presence of phage induce antibodies that effectively neutralize the phage, thereby preventing the detection of targeted phages in blood samples containing specific IgGs? Or conversely, must phages be present in the blood to sustain detectable levels of phage-specific IgGs?

Conclusions:

Tequatroviruses and Kayviruses have been found among the most represented phage groups in gut mucosa of patients investigated herein, and at the same time they have been found most frequently translocated to the blood circulation.

Overall correlation between phageome composition in gut and translocated phage groups is moderate, thus other factors (than phage concentration in gut) may affect efficacy of phage translocation.

The gut phageome contributes to the composition of the phageome circulating in the blood, but other sources of phages also appear to contribute, including environmental ones.

We propose to consider epithelial transcytosis of phage as the first step of a multi-step phenomenon of phage translocation from gut to blood circulation; since transcytosis is followed by filtration in lymphatic tissue, resulting phage translocation *in vivo* is limited.

Significant levels of phage-specific IgGs were negatively correlated to the presence of targeted phage antigens in blood circulation and in the gut mucus layer, thus phagespecific antibodies are the key limiting factor for translocating phages.

LABORATORY OF BIOLOGY OF STEM AND NEOPLASTIC CELLS
Head: Professor Aleksandra Klimczak, Ph.D., D.Sc.

Characterization of primary and immortalized mesenchymal stem cells (MSCs) from peripheral nerves, creation of organoids with MSCs isolated from skeletal muscles (continuation of the task)

The aim of the research was to optimize the creation of 2D and 3D co-cultures (in the form of organoids) of MSC derived from fragments of the human peripheral nerve: primary PN-MSCs and the immortalized PN-MSC SVT cell line with primary and immortalized MSC cells isolated from skeletal muscles (SM-MSC and SM-MSC SVT, respectively). Functional assessment was performed based on the ability of PN-MSCs growing in co-culture with SM-MSCs to secrete acetylcholine as a mediator of signal transduction to muscle cells.

Both primary and immortalized PN-MSCs have been shown to be able to grow in co-culture with primary and immortalized SM-MSCs under 2D conditions. Immortalized PN-MSC SVT cells in 2D culture are able to secrete acetylcholine only after the process of their differentiation towards nerve cells. However, PN-MSC SVT cells (without prior differentiation) growing in the form of spheroids secrete acetylcholine both in monoculture and in co-culture with SM-MSC SVT cells.

The conducted research allowed the creation of a 2D and 3D cellular model consisting of PN-MSC SVT and SM-MSC SVT as a model for research on the pathogenesis and treatment of neuromuscular diseases.

Assessment of the impact of microvesicles derived from mesenchymal stem cells in inhibiting the proliferative activity of ovarian cancer cell lines (continuation of the task)

The influence of the mesenchymal stem cell (MSC) secretome on the biological activity of ovarian cancer cell lines has been examined. For this purpose, tumor cell proliferation and apoptosis tests were performed in the presence of conditioned medium (CM) and microvesicles (MVs), obtained from immortalized human MSCs originating from adipose tissue (HATMSC2).

In the first stage of work, conditioned medium (CM) was collected from the HATMSC2 cell culture and microvesicles (MVs) were isolated. Experiments were prepared using the following test groups: culture medium with serum, culture medium without serum, CM with microvesicles, and microvesicles alone. DLS and protein electrophoresis confirmed serum-free samples of the prepared CM and MVs. The biological activity of CM and MVs was examined on selected reference ovarian cancer lines: OAW-42 (cystadenocarcinoma) and ES-2 (clear cell carcinoma).

The performed analyzes show that treatment of ES-2 cells with MVs obtained from HATMSC2 cells does not affect the proliferation rate of the tested cell line, while the proliferation rate of OAW-42 cells decreases after their treatment with MVs after the 3rd day of culture. In both cell lines treated with HATMSC2-MVs, a decrease in the percentage of live cells and an increase in the proportion of apoptotic and necrotic cells was observed.

These results indicate the anti-proliferative and pro-apoptotic nature of MVs isolated from HATMSC2 cells in the reference ovarian cancer cell lines.

DEPARTMENT OF ANTHROPOLOGY
Head: Professor Sławomir Kozieł, Ph.D., D.Sc.

The association between maternal stress and human milk concentrations of cortisol and prolactin

Psychosocial stress affects the relationship between prolactin (PRL) and cortisol (CORT). The dynamics of PRL and CORT changes under stress in human milk (HM) are largely unknown. We investigated how maternal stress related to recent life changes affects milk CORT and PRL concentrations. The study involved 116 mothers exclusively breastfeeding 5-month-old infants. Maternal psychological stress was evaluated using the Recent Life Changes Questionnaire (RLCQ). Stress response was determined by administering the cold pressor test and measuring CORT in saliva taken during and in milk collected after the test. Hormone concentrations were assayed using the ELISA method. The hierarchical regression models were run to test the association between maternal RLCQ, salivary CORT, and PRL, and CORT in milk. Maternal RLCQ correlated positively with the CORT in saliva; however, no direct association was found between RLCQ and PRL. After controlling for covariates, a positive association was found between salivary and milk CORT. A negative relationship was observed between salivary CORT and milk PRL. The results of the present study indicate that maternal psychological stress may affect the relationship between CORT and PRL in HM. In response to psychological stress, both hormones transported via milk can program infant development in the early postnatal period.

Prosocial reputation and stress among contemporary hunter-gatherers: the Hadza case

It has been suggested that having a reputation for being prosocial is a critical part of social status across all human societies. It has also been argued that prosocial behaviour confers benefits, whether physiological, such as stress reduction, or social, such as building allies or becoming more popular. Here, we investigate the relationship between helping reputation (being named as someone others would go to for help) and hair-derived chronic stress (hair cortisol concentration). In a sample of 77 women and 62 men, we found that perceived helping reputation was not related to chronic stress. Overall, the results of our study suggest that, in an egalitarian society with fluid camp membership and widely practised generosity, such as the Hadza, helping reputation does not necessarily boost stress-related health benefits through prestige-signalling mechanisms observed in hierarchical, large-scale societies.

DEPARTMENT OF MICROBIOLOGY

Laboratory of Molecular Biology of Microorganism (LMBM)
Head: Anna Pawlik, Ph.D., D.Sc.

Secondary metabolism in *Streptomyces*
Antimicrobial therapy
Bacterial response to stress

The research activity of LMBM is dedicated to addressing three crucial scientific issues: i/ secondary metabolism in *Streptomyces*, a key area in understanding microbial behaviour; ii/ the development of new compounds for antimicrobial therapy, a pressing need in the face of

increasing antibiotic resistance, and iii/bacterial response to stress, a fundamental aspect of bacterial survival and adaptation.

i. Our laboratory is at the forefront of studying the regulation of coelimycin synthesis in *Streptomyces coelicolor* A3(2). We are particularly intrigued by the autoregulation of the coelimycin biosynthesis gene cluster (CPK BGC) and the roles of two SARP activators, CpkO and CpkN, an orphan kinase CpkM, gamma-butyrolactone receptors, and the transporter CpkF in coelimycin production. We aim to unravel the regulatory connection between coelimycin synthesis, which occurs at the switch from primary to secondary metabolism, and the control of other secondary metabolite biosynthetic gene clusters. In particular, we have shown that intracellular CPK precursor(s) (preCPK) is/are involved in a negative feedback loop repressing the CPK BGC. We characterized the cluster-encoded efflux pump CpkF, showing that CpkF is essential for the extracellular CPK production. These findings could potentially lead to the development of improved industrial *Streptomyces* strains.
ii. Bacterial resistance to antibiotics is becoming increasingly problematic. As a result, new antimicrobial compounds and therapies are needed to eradicate pathogenic bacteria effectively. Our primary focus is on using flexible organic light-emitting diodes (OLED) and new photosensitizing chemicals with potential bactericidal activity to eradicate bacteria in skin infections photochemically. We are investigating new photosensitizing compounds using *Staphylococcus aureus* as a model species and clinical strains of bacteria isolated from diabetic foot infections. A biomimetic model of cell membranes has been developed to study the molecular phenomena occurring during photodynamic therapy within bacterial biological structures. We have also identified species infecting wounds of diabetic patients and characterised their antimicrobial resistance. Based on the data obtained, we are developing concepts for the effect of APDT on bacteria.
iii. We study bacterial factors that regulate stress response in *Campylobacteria: Helicobacter pylori, Campylobacter jejuni,* and *Arcobacter butzleri*. Specifically, we focus on CemR atypical response regulators, which control gene transcription and chromosome replication initiation. We have shown that the CemR proteins are pleiotropic regulators, controlling the expression of many, often species-specific genes (about 30-35%) of multiple categories. However, central carbon metabolism, particularly glycolysis/gluconeogenesis, the tricarboxylic acid cycle, and oxidative phosphorylation, is controlled by CemRs in response to oxygen availability in all species analysed. We have therefore named these regulators *Campylobacteria* energy and metabolism regulators, CemR. Such a regulator, controlling the oxygen-related metabolism of Campylobacteria species, was unknown, and our results will enable further progress on the physiology and pathogenicity of these bacteria.

Laboratory of Microbiome Immunobiology
Head: Sabina Górska, Ph.D., D.Sc.

The ability of extracellular vesicles (OMVs) produced by E. coli strain 083 to regulate innate immune mechanisms

The research aimed to investigate whether *E. coli* 083 extracellular vesicles (EcO83-EV) could induce the proliferation of mouse bone marrow-derived macrophages and to examine their impact on nitric oxide (NO) production and secretion, as well as activation of the inducible NO synthase (iNOS), under physiological conditions.

The study was performed on a mouse bone marrow-derived macrophage (BMDM) model. Firstly, we assessed the impact of EcO83-EVs and EcO83 bacteria on BMDM viability via

the MTT assay. The results showed that neither EcO83-EVs nor EcO83 bacteria were toxic to macrophages, even at high concentrations. We demonstrated that both EcO83-EVs and EcO83 stimulate nitric oxide (NO) production in BMDMs in a dose-dependent manner. Specifically, 1×10^{10} vesicles and 1×10^{7} CFU/ml bacteria induced NO levels comparable to those induced by the administration of 1 μg LPS. To characterize the role of specific TLRs in EcO83-EV-induced NO production, NO levels produced by BMDMs lacking TLR2, TLR4, TLR7, and TLR9 expression were quantified. Our results indicate a significant reduction in NO production in BMDMs lacking TLR4 expression only.

To determine whether the induction of NO by EcO83-EV in BMDMs depends on iNOS, we used the specific iNOS inhibitor, i.e. S-MIU. Administering S-MIU before exposure to EcO83-EV or EcO83 was found to completely inhibit NO production, indicating that NO induction by the tested EcO83-EV or EcO83 depends on iNOS. We also investigated the effect of EcO83-EV on cytokine production. BMDMs incubated with EcO83-EV or EcO83 for 24 hours secreted IL-6, TNF-α and IL-10 in a dose-dependent manner.

We demonstrated the effect of EcO83-EV on the expression of iNOS, the production and secretion of NO by BMDM macrophages, as well as its impact on the regulation of pro- and anti-inflammatory cytokine production by these cells.

DEPARTMENT OF TUMOR IMMUNOLOGY

Laboratory of Molecular and Cellular Immunology
Head: Małgorzata Cebrat, Ph.D., D.Sc.

The Impact of IFTAP Protein on the Course of MHV1 Virus Infection in Mice – A Pilot Study

The hIFTAP protein (Intraflagellar Transport Associated Protein, also known as Hepis or NWC) has been molecularly identified as a binding partner of the non-structural protein 10 (nsp10) of the SARS-CoV virus. To date, no studies have confirmed the actual involvement of IFTAP in the course of infection by this pathogen. Using a unique mouse model lacking IFTAP expression (NWC), we undertook a preliminary analysis of the impact of IFTAP deficiency on the course of coronavirus infection in mice.

As a model for SARS-CoV, we used the murine coronavirus MHV1, for which the nsp10 protein has been shown to play a similarly essential role in viral replication. A pilot experiment was conducted using sibling pairs of mice: WT (wild-type C57BL/6) and IFTAP-KO (IFTAP knockout). The mice were intranasally infected with a standardized suspension of the MHV-1 murine virus. Five days post-infection, the animals were euthanized, and viral titers were measured in various tissues.

Preliminary data from infections in three pairs of WT and IFTAP-KO mice revealed consistent differences in viral loads in the liver for each pair. This outcome supports further continuation of the project. However, we also observed significant intra-group variability in viral loads, which prompted us to modify the experimental protocol. Continuing the study under the original design could lead to a situation where—despite detecting differences in infection progression between WT and IFTAP-KO mice—demonstrating statistical significance when formally testing the hypothesis would require an extremely large sample size (estimated at 260 mice, based on statistical analysis).

DEPARTMENT OF EXPERIMENTAL THERAPY
Head: Professor Michał Zimecki, Ph.D., D.Sc.

Laboratory of Immunobiology
Head: Professor Michał Zimecki, Ph.D., D.Sc.

Effect of lactoferrin on alpha-1 antitrypsin production

Alpha-1 antitrypsin (AAT), an acute-phase protein, is the main inhibitor of serine proteases. The protein is produced by hepatocytes, neutrophils, macrophages and lung epithelium. The aim of the investigation is to establish whether some immunomodulatory proteins, such as Lactoferrin (LF), yolkin and glycomacropeptide (GMP), may affect AAT levels in hepatocyte and leukocyte cultures. In the cultures of the hepatocyte HepG2 line, no effects of the proteins on AAT production were found. In addition, inclusion to our studies of interleukin 6 (IL-6), known as a regulator of acute phase protein levels or a combination of IL-6 and dexamethasone, was also without effect on the studied proteins on AAT level. However, in the case of human peripheral blood mononuclear cells (PBMC), preincubation of cells with yolkin led to a higher AAT level. On the other hand, preincubation of PBMC with yolkin caused a decrease in AAT production in cultures treated with LPS. The investigations are continued and confirm the immunoregulatory action of yolkin.

Virucidal and antiviral properties of yolkin in a homologous model

Yolkin was tested for its potential virucidal and antiviral properties against RAV-2 (VR-1828^{TM}; ATCC CRL-12203) virus using DF1 (ATCC CRL-3586^{TM}) hen fibroblasts, defined as reduction of virus titer and the cytopathic effect, following 96h culture on DF-1 infected cells. The co-culture of yolkin with the virus led to a concentration-dependent virus inactivation by 4 log at 50 μg/ml of yolkin and by 2 log at 10 μg/ml. On the other hand, the treatment of infected DF-1 cells with yolkin caused a diminution of the virus titer by 3 log for a concentration of 50 μg/ml and by 2 log at 10 μg/ml of yolkin. The results indicate that yolkin exhibits both direct virucidal properties and the capability to interfere with virus replication in the homologous model. Thus, yolkin may play an antiviral role in a developing bird embryo.

Effects of yolkin on restoration of the immune system function in cyclophosphamide (CP) treated mice

The aim of this project was to evaluate the potential capability of yolkin to restore immune function in CP-immunocompromised mice in the models of oxazolone-induced contact sensitivity (CS) and the humoral immune response to ovalbumin (OVA). In parallel, the changes in the content of immune cells bearing respective phenotypes in the lymphoid organs were analyzed. The mice were administered a sublethal dose of CP and given yolkin in drinking water for 15 days before sensitization with oxazolone or for 26 days before immunization with OVA. The lymphatic organs were isolated for the determination of cell number and phenotype one day before each immunization. The results showed a significant suppression of CS following CP treatment, in the measurement of antygen-specific auricle thickness, and restoration of this parameter in yolkin-treated mice. In turn, both suppression of the antibody response to OVA and the yolkin effect were not significant. In mice treated with CP, a 49% loss in splenic T cell content was found in those bearing the CD4+ phenotype. The renewal of CS was correlated with an increase of CD3+, CD4+ and CD8+

lymphocytes to 88%, 89% and 76%, respectively, of the values registered in control mice. A significant depletion of B cells and total cell number in the bone marrow following CP treatment was completely restored after 26-day but not after 15-day administration of yolkin. We conclude that the immunorestoring property of yolkin may have potential application in the renewal of the immune function of immunocompromised patients undergoing chemotherapy.

Laboratory of Immunopathology
Head: Edyta Pawlak, M.D., Ph.D., D.Sc.

I. Beyond the essential role of p27Kip1 and cyclin D2 in cell cycle progression, they are also shown to confer an anti-apoptotic function in peripheral blood (PB) lymphocytes. Although the aberrant longevity and expression of p27Kip1 and cyclin D2 in leukemic cells are well documented, the exact mechanisms responsible for this phenomenon have yet to be elucidated. This study was undertaken to determine the associations between polymorphisms in the *CDKN1B* and *CCND2* genes (encoding p27Kip1 and cyclin D2, respectively) and susceptibility to chronic lymphocytic leukemia (CLL), as well as their influence on the expression of both cell cycle regulators in PB leukemic B cells and non-malignant T cells from untreated CLL patients divided according to the genetic determinants studied. Three *CDKN1B* single-nucleotide polymorphisms (SNPs), rs36228499, rs34330, and rs2066827, and three *CCND2* SNPs, rs3217933, rs3217901, and rs3217810, were genotyped using a real-time PCR system. The expression of p27Kip1 and cyclin D2 proteins in both leukemic B cells and non-malignant T cells was determined using flow cytometry. We found that the rs36228499A and rs34330T alleles in *CDKN1B* and the rs3217810T allele in the *CCND2* gene were more frequent in patients and were associated with increased CLL risk. Moreover, we observed that patients possessing the CCND2rs3217901G allele had lower susceptibility to CLL (most pronounced in the AG genotype). We also noticed that the presence of the *CDKN1B*rs36228499CC, *CDKN1B*rs34330CC, *CDKN1B*rs2066827TT, and *CCND2*rs3217901 AG genotypes shortened the time to CLL progression. Statistically significant functional relationships were limited to T cells and assigned to *CDKN1B* polymorphic variants; carriers of the polymorphisms rs34330CC and rs36228499CC (determining the aggressive course of CLL) expressed a decrease in p27Kip1 and cyclin D2 levels, respectively. We indicate for the first time that genetic variants at the *CDKN1B* and *CCND2* loci may be considered as a potentially low-penetrating risk factor for CLL, and determine the clinical outcome.

II. Adverse childhood experiences (ACEs) are a well-known risk factor of schizophrenia. Moreover, individuals with schizophrenia are likely to use maladaptive stress coping strategies. Although it has been reported that a history of ACEs might be associated with a pro-inflammatory phenotype in patients with schizophrenia, the interacting effect of coping styles on this association has not been tested so far. In the present study, we aimed to investigate the levels of immune-inflammatory markers in patients with schizophrenia and healthy controls (HCs), taking into consideration a history of ACEs and coping strategies. Participants included 119 patients with schizophrenia and 120 HCs. Serum levels of 26 immune-inflammatory markers were determined. A history of any category of ACEs was significantly more frequent in patients with schizophrenia. Moreover, patients with schizophrenia were significantly more likely to use emotion-focused coping and less likely to use active coping strategies compared to HCs. The levels of interleukin(IL)-6, RANTES, and tumor necrosis factor-α (TNF-α) appeared to be elevated in patients with schizophrenia after adjustment for potential confounding factors in all tested models. Participants reporting

a history of any ACEs had significantly higher levels of TNF-α and IL-6. No significant main and interactive effects of active strategies as the predominant coping on immune-inflammatory markers with altered levels in patients with schizophrenia were found. Findings from the present study indicate that ACEs are associated with elevated TNF-α and IL-6 levels regardless of schizophrenia diagnosis and predominant coping styles.

Laboratory of Reproductive Immunology
Head: Professor Anna Chełmońska-Soyta, Ph.D, D.V.M.

Immunological mechanisms associated with reproductive processes in health and disease

The effect of IL-33 on the expression of transcription factors in B lymphocytes

The planned study aimed to determine the role of regulatory B lymphocytes (Bregs) in normal pregnancies and after miscarriage, and analyse ST2 receptor and nIL-33 expression in these cells.

Description of the completed work: A series of multicolour cytometric stainings was carried out to determine the frequency of the Breg subpopulation: B1a, B10, immature B lymphocytes, plasma blasts and memory B cells. The expression of the ST2 receptor on these cells was analysed in the blood of pregnant women and of women after miscarriage. Serum levels of IL-33 and its receptor sST2 were also analysed in these patients.

Description of major achievements: Compared to controls, women after miscarriage had an increased percentage of B cells (CD19+), immature B cells, plasmablasts and memory B cells expressing the ST2 receptor within total lymphocytes, but a reduced percentage of B10 ST2+ cells within CD19+ cells. The median number of B10 ST2+ and B1a ST2+ cells was significantly higher in patients who had experienced a miscarriage. No significant differences were observed in serum IL-33 and sST2 levels.

Advanced analytical tools such as t-SNE and FlowSOM were used to perform detailed comparative analyses of samples using two-dimensional gating methods. These analyses revealed no differences in B-cell cluster complexity between pregnant and post-pregnancy women.

Advanced analysis using the FlowSOM tool enabled visualisation of B cell populations characterised by the highest ST2 receptor expression (CD19+ CD27+ IgM+/-; memory B cells). Analysis of the frequency of these cells within the CD19+ population revealed a significant decrease in the CD19+ CD27+ IgM+ subset and an increase in the CD19+ CD27+ IgM- subset in patients after miscarriage, compared to the control group.

The results indicate that the ST2 receptor is present on regulatory B cells in pregnant and postpartum women, but in different proportions depending on the subpopulation of these cells. The B1a, B10 and memory B cell subpopulations appear to be involved in IL-33/ST2-mediated immune regulation. Manuscript in preparation.

Laboratory of Genetics and Epigenetics of Human Diseases
Head: Professor Lidia Karabon, Ph.D., D.Sc.

Challenge in Optimization of Lymphocyte Transduction and Culture – Laboratory Experience with CAR-T Programming and Manufacturing

Multiple myeloma(MM) is a heterogeneous disease characterized by the progressive proliferation of malignant altered plasmocytes in the bone marrow. Despite a number of

treatment options with the latest generation of drugs, it remains an incurable disease. CART anti-BCMA therapy has been proven to be effective in MM, but it is not available to patients in Poland. In 2024, a consortium consisting of the Technology Reassurance Company, our Institute, and the Lower Silesian Oncology, Hematology and Pulmonology Center obtained funding from the Medical Research Agency for the project ”Development of a therapeutic product based on genetically modified T cells for the treatment of relapsed and refractory forms of plasmocytic myeloma: from CAR receptor DNA vector production to phase I/II studies, BECAME - B(e)CMA CAR-T in MM THERAPY”.

The task of our laboratory is to determine the optimal culture and expansion conditions of CAR-T cells. The main goal was to achieve a high level of transduction and differentiation of cells toward the most desirable cellular subpopulations, i.e. naive-like and central memory T-cells, while minimizing effector memory and terminal effector T-cells, as well as to obtain the least exhausted cells possible.

We analyzed various T-cell activation and expansion strategies, comparing different activation systems (e.g., CD3/CD28 or OK-T-3 activation) and cytokine cocktails (IL-2, IL-7, IL-15, IL-21 alone or in combination) to achieve optimal cell proliferation, differentiation, and persistence. Results of our study allowed us to find the best combination of cytokines that enhances T-cell expansion while maintaining a favorable T-cells phenotype. Our results underscore the need for precise control over *ex vivo* culture parameters to optimize the functional fitness of CAR-T cells before clinical application.

Despite significant progress in optimizing transduction and culture conditions, manufacturing a clinically effective CAR-T product remains a challenge due to variability in T-cell quality, vector production, and *ex vivo* expansion protocols. Further refinement of GMP-compliant manufacturing protocols and preclinical validation are essential to enhance CAR-T therapy's therapeutic potential. This work is supported by the Medical Research Agency.

TIGIT receptor in non-small cell lung cancer (NSCLC)

The introduction of immune checkpoint blockade as a treatment option for solid cancers has revolutionized anti-cancer treatment. NSCLC patients benefit from immune checkpoint inhibitors targeting the PD-1/PD-L1, as this treatment extends survival time and improves quality of life. Unfortunately, not all patients respond to immunotherapy. Therefore, research into new potential targets for immunotherapy, such as TIGIT, is essential.
TIGIT (T cell immunoglobulin and ITIM domain) is an inhibitory receptor expressed on T lymphocytes and natural killer cells (NK cells), which interacts with its ligand CD155 present on antigen-presenting cells (APCs) or tumor cells and suppresses anti-tumor response.

In this study, we investigated the expression of TIGIT and CD155: i) at the mRNA levels in tumor tissues (droplet digital PCR); ii) at the protein levels in tumor and non-tumour tissue (immunohistochemistry, IHC); iii) on different subpopulations of immune cells in blood and tumor tissues (multiparametric flow cytometry).

We analysed 61 resected NSCLC specimens for TIGIT and 68 specimens for CD155 by IHC and found that 54 tumour tissues were positive for TIGIT (more than 5% of TILs positive for TIGIT) and 63 tumour specimens were positive for CD155 (H score above 10). We observed a significantly higher percentage of $CD4^+$, $CD8^+$, T_{regs} and NK cells positive for TIGIT in tumour tissues of NSCLC in comparison to corresponding blood samples. Stage III of NSCLC was associated with the higher expression of TIGIT on NK cells, both in blood and tumour tissues of NSCLC, as well as increased expression of CD155 on tumour cells in relation to stages I and II.

Our study confirmed that TIGIT and CD155 are overexpressed in NSCLC and may be considered as targets for immunotherapy. (This work was supported by the National Science Centre, Poland, Grant number 2019/33/B/NZ5/03029).

DEPARTMENT OF IMMUNOLOGY OF INFECTIOUS DISEASES
Head: Professor Andrzej Gamian, Ph.D., D.Sc.

Laboratory of Medical Microbiology
Head: Professor Andrzej Gamian, Ph.D., D.Sc.

Lipidomic analysis of propionic bacteria, characterization of extracellular vesicles secreted by *Cutibacterium* and studies on peptide vaccine against bacterial dysentery, as well as thermal stability of proteins

The aim of the study was lipidomic analysis of representatives in the genus *Cutibacterium*. Results revealed the presence of lipids in the genus *Cutibacterium*, which belong to amides of fatty acids and cardiolipins. The chemical composition of cellular glycolipids has been determined in *Cutibacterium* spp. Such glycolipids were also identified in extracellular vehicles produced by these bacteria. The analysis has been performed for 8 strains of *Cutibacterium:* four filotypes *C. acnes*: IA1, IB, II and III, two strains *C. granulosum*, as well as *C. avidum* and *C. namnetense*. Results are the basis for further investigation of antigenic properties for vaccine and diagnostic construction. In the frame of the second task, the method has been optimized for the construction of the conjugate of the epitope peptide RYDERY with glycine linker and human serum albumin as carrier protein. The peptide was found to be responsible for inducing the protective response of the immune system for infections caused by enterobacterial strains. Results of *in vitro* experiments indicate that the received conjugate as an anti-diarrhea vaccine prototype is not cytotoxic towards cell lines. Antigenic properties of the obtained conjugate have also been specified. The third task concerned the determination of the heat tolerance of different normal and cancer cells, treated with heat pulses and cytokine profiles for their immune status. Research indicates that cancer cells are susceptible to temperatures higher than 37-42°C but are resistant to 37-42°C. Nevertheless, the experiments will be continued for practical purposes with the determination of other parameters of the effect of temperature on normal and cancer cells.

Laboratory of Virology
Acting head: Beata Orzechowska, Ph.D., D.Sc.

1. Previous studies have highlighted the beneficial properties of plants rich in polyphenols, such as *Lonicera caerulea* var. *Kamtschatica* Sevast. (LCK), *Aronia melanocarpa* (AM) and *Echinacea purpurea* (EP). These plants have demonstrated antioxidant, immunomodulatory, and potential antiviral effects. Thus, the objective of this study was to investigate the impact of the ELA blend, a polyphenol-rich blend containing EP, LCK, and AM, on the cellular mechanisms involved in viral infection. To assess the effects of the ELA blend, various experiments were conducted using A549 cells and a mucociliary tissue 3D model called EpiAirway™. Inflammation and oxidative stress induced by LPS were evaluated through measurements of SOD activity, ELISA, and qPCR analysis. Additionally, antiviral assays were performed in a cell-present environment to examine the blend's effectiveness against HCoV-OC43. The results showed that the ELA blend-treated

group exhibited reduced expression of *IL1B*, *CXCL8*, *ICAM1*, *MCP1*, and *RELA* in both A549 cells and EpiAirway™. Moreover, the blend enhanced the expression of *CAT*, *HMOX1*, *SOD1*, and *SOD2* in A549 cells. The antiviral activity of the ELA blend was also investigated, i.e. its influence on the viral replication cycle, to determine the potential as an antiviral preparation. At the highest non-cytotoxic concentration, the ELA blend demonstrated an 87.5% reduction in viral titer when administered simultaneously with HCoV-OC43. It emphasize the potential ability of the preparation to block viral entry to the host cells. At the same time, the ELA blend did not express virucidal activity, i.e. inactivation of free viral particles, against HCoV-OC43. In conclusion, ELA blend displayed antiviral activity and exhibited immunomodulatory and antioxidant effects. Based on these findings, it can be concluded that the ELA blend has potential for the prevention and treatment of viral infections. Results were published in: Zima K, Khaidakov B, **Sochocka M, Ochnik M**, Lemke K, Kowalczyk P. Exploring the potency of polyphenol-rich blend from *Lonicera caerulea* var. *Kamtschatica* sevast., *Aronia melanocarpa*, and *Echinacea purpurea*: Promising anti-inflammatory, antioxidant, and antiviral properties. The results have been published in Heliyon. 2024; 10(15):e35630.

2. Upper respiratory tract infections (URTIs) are a prevalent health issue, causing considerable morbidity. Despite the availability of conventional treatments, there is an increasing interest in natural products due to their potential antiviral and immunomodulatory benefits. This study aims to evaluate the efficacy of an ELA blend (E-*Echinacea purpurea*, L-*Lonicera cerulea*, A-*Aronia melanocarpa*) in preventing and alleviating the symptoms of URTIs. Additionally, the study examines the blend's antiviral and immunomodulatory effects both in vitro and through a clinical trial. A randomized, double-blind, placebo-controlled trial involved 61 participants prone to URTIs, with a 60-day treatment and follow-up period. A placebo group later received the ELA blend for 60 days. The ELA blend significantly reduced the incidence of URTIs during the observation period (2 vs. 8; $p = 0.044$) and, in particular, throat-related symptoms (8 vs. 16; $p = 0.038$). Analyses of PBMCs showed that baseline production of the cytokines IFN-γ ($p = 0.020$), IL-1β ($p = 0.004$), IL-2($p < 0.001$), IL-6 ($p < 0.001$), and TNF-α ($p < 0.001$) increased after ELA blend treatment. Moreover, the ELA blend modulated cytokine production in response to PHA-L stimulation, decreasing IFN-γ ($p = 0.008$) and IL-2 ($p = 0.012$) while increasing IL-1β ($p = 0.005$). Following R848 stimulation, the ELA blend enhanced the production of INF-α ($p = 0.012$) and IL-2 ($p = 0.025$), and decreased IL-1β ($p < 0.001$), IL-6 ($p < 0.001$), and TNF-α ($p = 0.049$). The blend suppressed VSV replication and significantly increased cytokine levels, with IFN-γ increasing by 98 pg/mL ($p = 0.002$), IL-1β rising by 233.0 pg/mL ($p = 0.004$), and TNF-α showing an increase of 2905 pg/mL ($p = 0.002$). These findings highlight the ELA blend's potential to alleviate URTI symptoms, modulate inflammatory and antiviral immune responses, and inhibit viral replication. Further investigations should aim to validate these findings through large-scale studies and explore the ELA blend's long-term safety and efficacy in diverse populations. Additionally, research should investigate optimal dosing strategies and explore potential synergistic effects with conventional treatments to maximize clinical outcomes. Trial registration: retrospectively registered under NCT06020001. Results were published in: Zima K, **Sochocka M, Ochnik M**, Khaidakov B, Lemke K, Kowalczyk P. Therapeutic Potential of a Natural Blend of *Aronia melanocarpa*, *Lonicera caerulea*, and *Echinacea purpurea* Extracts in Treating Upper Respiratory Tract Infections: Preliminary Clinical and *In Vitro* Immunomodulatory Insights. The results have been published *in Int J Mol Sci*. 2024;25(24):13436.

Laboratory of Genomics & Bioinformatics
Head: Łukasz Łaczmański, Ph.D., D.Sc.

Molecular characteristics of cancer cells - analysis of transcriptomes and epigenetic data

Three key groups of genes: proto-oncogenes, suppressor genes, and mutator genes- play a critical role in tumour transformation, the process by which a normal cell becomes cancerous. To enhance treatment effectiveness, identifying genetic alterations that influence individual drug responses and potential prognostic and predictive markers is crucial. This enables the tailoring of therapies, such as adjusting treatment duration and doses, which can improve prognosis and quality of life. Recent advances include technologies such as whole-genome, exome, and transcriptome sequencing, alongside studies of protein-DNA/RNA interactions, miRNA profiles, and methylation patterns, leading to more precise molecular diagnoses and better-informed treatment plans. The molecular biology technique NGS (Next-Generation Sequencing) is a practical tool that enables comprehensive analysis, including sequencing specific gene groups and entire genomes or transcriptomes, thereby revealing genetic relationships. Additionally, methods to assess genome stability, such as TMB, are being developed to understand tumourigenesis and treatment responses.

Project objectives include identifying transcriptomic changes as predictive or prognostic markers, integrating in silico transcriptomic and epigenetic data to develop a tumour cell expression model, exploring correlations between genome-wide mutational stability and tumour grade or treatment response, and analysing genome stability as a mechanism of tumour development.

During the reporting period, efforts focused on creating an *in silico* model of breast cancer tumour cells. Data from patients with both transcriptomic and mutational profiles- collected from LGiB and the Lower Silesian Centre for Oncology, Pulmonology, and Haematology- were used. Additional data on treatment response from the Cancer Genome Atlas database supplemented this.

Collaboration with the Institute of Computer Science at Wrocław University of Technology (Dr Adrianna Kozierkiewicz, Prof. PWr) continues, employing deep learning algorithms to classify the data and develop the *in silico* model.

Efficacy and safety profile of base editors delivered as mRNA and protein used to create an in vitro model of cutaneous squamous cell carcinoma associated with a dystrophic recessive form of bullous epidermal detachment (RDEB-SCC)

Bullous epidermal detachment (RDEB) is an inherited skin disease caused by mutations in the COL7A1 gene that encodes type VII collagen. This type of disease is dystrophic and recessive, and it is rare. Patients affected by RDEB experience extensive blistering and non-healing skin wounds that cause them pain and intractable itching. These symptoms significantly reduce their quality of life. Also, excessive scarring and recurrent infections lead to reduced mobility and limb deformity. Patients with RDEB have an increased risk of developing aggressive and metastatic squamous cell carcinoma (SCC) of the skin. According to the US National EB Registry, the risk of SCC in this group increases with age, reaching more than 90% after 55 years of age.

The study of rare diseases like RDEB is challenging because of the limited availability of patient samples. There is a lack of universal cellular and animal models that can accurately reflect the phenotype caused by specific mutations in a patient. This makes it difficult to search for potential targeted therapies for RDEB-associated cancer (RDEB-SCC). To address this issue, an easily obtainable and patient-adaptable model of RDEB-SCC needs to be

developed. Such a model could be useful for screening anti-cancer drugs and pre-testing gene therapies.

CRISPR/Cas9 is a highly accurate gene editing tool that uses a Cas9 nuclease to cut double-stranded DNA. It also includes a 20-nucleotide guide RNA (gRNA) molecule that directs the Cas9 protein to the complementary DNA sequence in the genome. The cytosine base editor (BE3) is a modified version of CRISPR/Cas9 that has an attached cytosine deaminase subunit. This subunit catalyses the conversion of cytosine to uracil, which the cell reads as thymine during replication. According to data from the ClinVar database, there are 77 recorded pathogenic point non-sense mutations in the COL7A1 gene, of which 57 are C>T or G>A mutations that can be inserted using a cytosine base editor. Therefore, BE3 was selected as the precise tool to insert mutations causing the most severe, generalized form of RDEB into cells from a commercially available SCC.

Cas9 (or BE3) can be introduced into the cell in the form of: a plasmid (fully expressed in the cell), an mRNA (subject to translation in the cytoplasm) or a protein-RNA complex. It has been observed that the mode of Cas9 delivery affects the efficiency of editing, as well as its specificity - delivery in plasmid form results in the most unscheduled editing.

Main hypothesis:

By using a cytosine editor (BE3) to introduce RDEB-causing mutations into a commercially available SCC cell line (MET1), we can establish a clinically relevant model of RDEB-SCC.

Supporting hypothesis:

The delivery of BE3 in the form of mRNA or protein may affect the efficiency and safety of editing at the genome, epigenome and/or transcriptome level.

Results:

1) The mRNA production process has been optimised to enable consistent synthesis of 50-100 µg of purified mRNA per batch. The quality of each batch is verified regularly using capillary electrophoresis.
2) New mRNA constructs have been developed that include a codon-optimised sequence for the BE3 protein, along with a gene for green fluorescent protein (EGFP) linked via a self-cleaving P2A peptide. The EGFP marker enables monitoring of transfection efficiency and facilitates sorting the transfected cells' fluorescent population through FACS. Using the optimised BE3 protein sequence markedly improved transfection efficiency, resulting in 60-80% editing in HEK293T cells and 10-20% in SCC A431 cells.
3) A431 cells transfected with optimised mRNA and gRNA to introduce the COL7A1 c.1732C>T mutation were isolated into single cells through serial dilutions, creating a fully modified monoclonal line. Twenty-one lines were established, with an overall editing efficiency of 11%. Further genotyping of individual lines using Sanger sequencing or ARMS PCR will confirm if the desired modification was successful, indicating whether an in vitro model of squamous cell carcinoma associated with dystrophic, recessive epidermolysis bullosa has been achieved.
4) Additionally, the new mRNA constructs obtained have interchangeable untranslated UTR regions that can regulate the translation rate and influence the stability of the mRNA molecule within the cell. To date, three sets of flanking UTRs, selected based on literature data, have been tested:

a. 5' and 3' UTRs of Xenopus globin (Xen);
b. 5'UTR from the Moderna mRNA1273 vaccine and 3' UTR from human alpha-globin (Mod);
c. Aptamer binds translation initiation factors eIF4G as 5'UTR and the mutated PRE of the hepatitis B virus as 3'UTR (Apt).

5) A fourth construct containing the new AES-mtRNR1 3' UTR to increase mRNA stability was produced, but no mRNA has yet been synthesised for it.
6) Constructs differing only in their UTR sequences were found to result in reproducible differences in transfection efficiency (assessed as the number and intensity of fluorescent cells under a fluorescence microscope and by flow cytometry) and in editing efficiency (estimated by Sanger sequencing and confirmed by NGS amplicon sequencing). The Mod construct proved to be the least effective, while the Xen construct was the most effective.
7) RNA collected on an ongoing basis from transfected cells will be used to evaluate the impact of the UTRs used on the efficacy and safety profile of base editors delivered in the form of mRNA.

DEPARTMENT OF IMMUNOCHEMISTRY

Laboratory of Glicobiology
Head: Professor Marcin Czerwiński, Ph.D., D.Sc.

General project: Blood group antigens: molecular biology, interactions with pathogens.
Title of 2025 project: Structures of glycans in patients with congenital glycosylation disorders

Congenital disorders of glycosylation (CDG) are genetically determined diseases caused by mutations in genes responsible for glycosylation of proteins and lipids. Since glycosylation may play an important role in protein folding, even one missing glycan may cause a decrease in protein activity, leading to clinical effects. The clinical presentation of CDG is usually multi-organ, although the nervous system is often the most affected. Over 150 different variants of CDG have been described so far.

Since the clinical effects of CDG are often diverse, the diagnosis of a particular CDG may be quite challenging. The most widely used test (isoelectric focusing of transferrin) usually does not give a clear answer about the variant of CDG. Thus, the diagnosis of CDG by analysis of N-glycan structures is getting increasingly popular. To this end, in cooperation with Dr Patryk Lipiński (Warsaw Medical University), we analyzed structures of N-glycans and glycosphingolipids from the blood plasma of patients with three variants of CDG. These diseases are rare, and the cooperation is ongoing as we acquire more patient samples. The results so far are in the table below.

CDG	Patient(s)	Mutation in gene/ Encoded protein	N-glycans–results	Glycosphingolipids–results
ALG13-CDG	KS	*ALG13* (X) UDP-GlcNAc-transferase subunit ALG13	Increase of short, fucosylated complex structures (Fuc_1Gn_2, $Fuc_1Hex_1Gn_2$)	No changes
ATP6AP1-CDG	MSz	*ATP6AP1* (X) Subunit Ac45 of the V ATPase complex	Increase of monosialylated structure $Hex_1Gn_2NeuAc_1$	No changes
SSR4-CDG	AS, TI	*SSR4* (X) Translocon-associated protein subunit delta	Decrease of monosialylated structures $Fuc_{(0-1)}Hex_2Gn_3NeuAc_1$.	Increased LacCer, possibly increased globotriaosylceramid(Gb3)

Laboratory of Microbial Immunochemistry and Vaccines
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Biochemical characteristics of macromolecules involved in immunological processes. Immunochemical studies of bacterial endotoxins

The expertise of the Laboratory of Microbial Immunochemistry and Vaccines covers a variety of preparative, chromatographic, chemical, and instrumental analytical techniques (mass spectrometry, nuclear magnetic resonance spectroscopy, surface plasmon resonance) to elucidate structures of biologically relevant carbohydrates, especially lipopolysaccharides (LPS, endotoxins) of Gram-negative bacteria. The expertise has been extended by the analysis of genes involved in LPS biosynthesis recently. Our research concerns Gram-negative species, such as *Klebsiella pneumoniae*, *Escherichia coli*, *Shigella sonnei*, *Bordetella* spp., *Plesiomonas shigelloides*, and *Edvarsiella* spp. that represent opportunistic human pathogens associated with extraintestinal and nosocomial infections in humans and animals. Moreover, *K. pneumoniae*, particularly ESBL- and KPC-strains, has been singled out in 2017 as *"priority 1. critical pathogen"* for health care by the WHO, CDC, and the UK Department of Health. *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, and *B. holmesii* are mammalian respiratory pathogens, having substantial economic impact on human health and agriculture. *B. pertussis* is responsible for whooping cough (pertussis), and *B. holm*esii is the second pertussis etiological factor; however, the current anti-pertussis vaccines do not provide cross-protection. Major virulence factors and surface antigens of the studied species are: lipopolysaccharide (LPS, endotoxin, O antigen), capsules (antigen K, e.i. capsular polysaccharide - CPS and exopolysaccharide (EPS), and fimbriae. LPS is built up of lipid A, core oligosaccharide (OS), and O-specific polysaccharide (O-PS). LPS and CPS determine the O serotype or the K serotype, respectively. Especially, O serotype is defined by the O-PS region, which is built up of carbohydrate repeating units. O-antigens and other conserved surface antigens of bacteria are of interest as targets for antibodies protective against infections.

In 2024, we advanced the knowledge regarding the structure of O antigens of *K. pneumoniae*. A new O13 serotype structure was associated with the OL101 locus responsible for O antigen biosynthesis. Four clinical isolates predicted by the Kaptive tool as OL101 were characterized and found to have the O antigen structure composed of β-Kdo*p*-[→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*-(1→]n, representing a novel serotype O13. Identification of the β-Kdo*p* terminus was based on the analysis of the complete LPS molecule by HR-MAS NMR spectroscopy. The bioinformatic analysis of 71,377 *K. pneumoniae* genomes from public databases (July 2023) revealed a notable OL101 prevalence of 6.55 %. Importantly, inventors of the Kaptive bioinformatic tool used our results to improve algorithms predicting the *K. pneumoniae* O serotype (*Artyszuk D. et al., Carbohydrate Polymers, 2024. 326: 121581*).

In collaboration with the Faculty of Natural Sciences, Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice (Poland), we have demonstrated for the first time the O-antigen structure of *A. bogorensis* ATCC BAA-21 LPS. It was concluded that its repeating unit is a branched trisaccharide with the following structure: →6)-α-D-Glc*p*-(1→2)-[β-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→. *Asaia bogorensis* is a Gram-negative bacterium isolated from flowers and fruits growing in tropical climate, the reproductive system of mosquitoes, and rarely isolated from immunocompromised patients. In Europe, *A. bogorensis* is responsible for the contamination of flavoured mineral waters (*Kaczmarek A, Carbohydrate Research, 2024. 5:545:109266*).

Furthermore, we have elucidated O-antigen structures from 28 *Aeromonas salmonicida* strains isolated from farmed fish. The new O-polysaccharide has been identified in two isolates: →3)-α-L-Rha*p*-(1→3)-α-D-Man*p*NAc-(1→2)-β-D-Glc*p*-(1→3)-α-L-Rha*p*2OAc4OAc-(1→3)-β-D-Man*p*NAc-(1→3)-α-D-Glc*p*-(1→ (Ucieklak K. *et al.* Microorganisms, 2024).

Additionally, N-glycosylation of yolkin was determined, combining with characteristics of its storage conditions, thermal stability, aggregation ability, and antioxidant, antihypertensive, and antidiabetic potential. As a result, yolkin can be considered to have significant therapeutic potential and represents a valuable tool for the development of novel nutraceuticals (collaboration with the Laboratory of Microbiome Immunobiology of the IIET PAS; *Zambrowicz A. et al. Food Funct. 2024, 15(21):10746-10760*).