Folia Biologica et Oecologica 18: 3–4 (2024)

Acta Universitatis Lodziensis

Folia Biologica et Oecologica 18: 5–6 (2024)

Acta Universitatis Lodziensis

Editorial
9th Molecular Biology Conference

MARCIN RYDZEWSKI[1,2], ŁUKASZ PUŁASKI[3]

[1]University of Lodz, Faculty of Biology and Environmental Protection, Students' Scientific Circle of Young Biophysicists, Pomorska 141/143, 90–236 Lodz, Poland;
[2]University of Lodz, Faculty of Biology and Environmental Protection, Department of Ecology and Vertebrate Zoology, Banacha 12/16, 90–237 Lodz, Poland;
[3]University of Lodz, Faculty of Biology and Environmental Protection, Department of Oncobiology and Epigenetics, Pomorska 141/143, 90–236 Lodz, Poland
E-mail: marcin.rydzewski@biol.uni.lodz.pl, lukasz.pulaski@biol.uni.lodz.pl

The Students' Scientific Circle of Young Biophysicists (Studenckie Koło Naukowe Młodych Biofizyków, SKNMB) is one of the oldest student organisations promoting scientific activity at the Faculty of Biology and Environmental Protection, University of Lodz. Its membership, composed of undergraduate students from various degree courses at the Faculty (but also from other faculties and even universities), dedicates itself to contributing to progress in life sciences by three main avenues of activity: laboratory research by individual members under the supervision of university scientists, coordinated by SKNMB; public outreach and scientific education activities, with experimental demonstrations and workshops at numerous events and science fairs; and finally, organising scientific conferences where undergraduate and graduate students from all over Poland present and discuss their research and findings in an ambitious and nurturing atmosphere. These student conferences have been organised by SKNMB at different scales, locations and scopes throughout the years, but the flagship series of events are indubitably the Molecular Biology Conferences, yearly meetings initiated in 2012 and dedicated to scientific presentations by students of all levels in basic and applied aspects of biology at the molecular (biochemical and biophysical) level. These conferences take place on the premises of the Faculty of Biology and Environmental Protection of UL and involve oral and poster presentations from between 80 and 150 participants affiliated with various universities (including medical and technical universities) in Poland. They also always include plenary invited lectures given by eminent scientists from the best Polish research centres. Its 2024 edition, the 9th Molecular Biology Conference, took place on March 14th–15th and gathered 106 participants.

Organising a nationwide scientific conference after a long pandemic-linked break was an extraordinarily difficult task that involved the time and efforts of all SKNMB members for many months. This corresponds to the core of our organisation's mission, which is to aid the personal and scientific development of student members by doing ambitious things of which we can later be proud. The conference was remarkably successful, with presentations of dozens of speakers and a rich poster session testifying to its high scientific level. In addition, the event attracted the attention of industry

DOI: https://doi.org/10.18778/1730-2366.18.01

companies whose representatives were present in large numbers, enabling both conference participants and University staff to learn about latest technological developments.

As the Chairman of the Organising and Scientific Committee of the conference (M.R.) and scientific supervisor of SKNMB (L.P.), we have the pleasure, together with the editors of Folia Biologica et Oecologica, to present the readers with this special issue of the journal, devoted entirely to student research that had been presented at the 9th Molecular Biology Conference. The articles in this issue are not identical in content to conference presentations, but have been prepared as full manuscripts and have undergone independent peer review separate from acceptance to the conference. With a feeling of pride and encouragement at the quality of research performed by the upcoming generation of Polish life scientists, we hope for the readers appreciation and acknowledgement. We sincerely thank the editors of Folia Biologica et Oecologica for their patronage.

Folia Biologica et Oecologica 18: 7–8 (2024)

Acta Universitatis Lodziensis

Evaluation of the role of oxidative stress in the anticancer effects of CHK1 and PARP-1 inhibitors in HepG2 cells

POST-CONFERENCE REPORT

WIKTORIA BĘBENEK, AGNIESZKA MARCZAK, ANETA ROGALSKA

University of Lodz, Faculty of Biology and Environmental Protection, Pomorska 141/143, 90–236 Lodz, Poland
E-mail: wiktoria.bebenek@edu.uni.lodz.pl

KEYWORDS: CHK1i; olaparib; HepG2; reactive oxygen species, synergism

Olaparib (PARP-1i) is a poly(ADP-ribose) polymerase inhibitor used in the treatment of advanced ovarian cancer, particularly in patients with mutations in the *BRCA1* and *BRCA2* genes, which acts by blocking the repair of single-stranded DNA breaks. CHK1, on the other hand, is a kinase that controls cell cycle and DNA repair. The effect of olaparib in combination with CHK1i on liver cancer cells is unknown. The combined effect of the drugs may prevent metastasis of ovarian cancer to the liver, but also to the bones, lungs, and brain. Indeed, the liver is crucial for detoxification and is therefore more susceptible to damage caused by replication stress, especially because of suppression of intracellular antioxidant defence systems.

The aim of this study was therefore to assess the potential role of oxidative stress in the cytotoxic effects of CHK1 and PARP-1 inhibitors in monotherapy as well as in combination therapy against HepG2 liver cancer cells.

The MTT metabolic activity assay was used to estimate cytotoxicity in the HepG2 line, and the concentrations of the compounds tested (CHK1i and PARPi) used in combinations were determined. Oxidative stress levels in liver cancer cells were also determined using MitoSox Red and H2DCF-DA fluorescent probes. Experiments were performed in variants of pre-incubation with an antioxidant, N-acetylcysteine.

Concomitant application of olaparib with CHK1i induced a cytotoxic effect in liver cancer cells. The greatest synergistic effect was observed with the combination of olaparib 2.5 μM and CHK1i 5 μM, which, after five days of incubation, resulted in a decrease in survival of the HepG2 line to approximately 30% compared with the respective mono-therapies (PARPi approximately 90%; CHK1i approximately 40%). These results clearly indicate a significant increase in the antitumor activity of olaparib in the presence of a CHK1 inhibitor. The experiments performed showed no significant changes in the levels of reactive oxygen species up to 48 hours of incubation with the tested inhibitors.

The combination of olaparib with a CHK1 inhibitor appears to be a promising strategy for the treatment of liver cancer, which, if introduced into clinical practice, would represent a potential benefit for a

DOI: https://doi.org/10.18778/1730-2366.18.02

broad group of ovarian cancer patients previously treated with olaparib, where resistance to olaparib resulted in liver metastasis.

Folia Biologica et Oecologica 18: 9–10 (2024)

Acta Universitatis Lodziensis

MMP-9 as a potential diagnostic marker for urolithiasis

POST-CONFERENCE REPORT

KINGA GRZYBOWSKA[1], PAULINA WIGNER-JEZIORSKA[2]

[1]University of Lodz, Faculty of Biology and Environmental Protection, Biochemical Section of the Student Scientific Circle of Biologists, Pomorska 141/143, 90–236 Lodz, Poland
[2]University of Lodz, Faculty of Biology and Environmental Protection, Department of General Biochemistry, Pomorska 141/143, 90–236 Lodz, Poland
E-mail: kinga.grzybowska@edu.uni.lodz.pl

KEYWORDS: Urolithiasis, single nucleotide polymorphism, mRNA expression, urology, metalloproteinase 9

Urolithiasis is one of the most common disorders of the urinary system classified as civilization diseases. This condition is caused by the presence of insoluble deposits in the urine, which hinder its proper flow in the urinary tract. Nevertheless, its complete etiology still remains unclear. Previous studies suggest that the adhesion of insoluble crystal substances is one of the key stages in the formation of urinary stones. The extracellular matrix, located in close proximity to the forming stone, interacts with various signalling molecules, including matrix metalloproteinases (MMPs). MMP-9 appears to be particularly involved in the processes of adhesion and aggregation of insoluble deposits to urothelial cells.

Therefore, the aim of the presented study was to assess the association between the frequency of occurrence of the single nucleotide polymorphism (SNP) 836 A > G (rs17576) located in the MMP-9 gene and the development of urolithiasis, as well as its impact on the mRNA expression level of this gene.

The material for the research consisted of RNA and DNA isolated using a commercially available kit from whole blood collected from 50 patients diagnosed with urolithiasis, who were hospitalized in the Urology Department of M. Kacprzak Regional Hospital in Płock, as well as from 53 healthy volunteers constituting the control group. Subsequently, profiling of the investigated SNP and determination of MMP-9 mRNA expression were performed using real-time polymerase chain reaction (real-time PCR).

The selected polymorphism for the study did not significantly affect the frequency of urolithiasis occurrence in the examined population. However, the results suggest that a potential increase in the sample size may confirm that heterozygosity of the investigated SNP could be associated with reduced risk of urolithiasis in the female subgroup. On the other hand, a potential increase in risk was observed for G/G homozygotes in the non-smoking subgroup and for A/A homozygotes in the subgroup with normal body mass index. Furthermore, it was

DOI: https://doi.org/10.18778/1730-2366.18.03

confirmed that the development of urolithiasis is associated with decreased expression of the MMP-9 gene ($p < 0.05$), highlighting the significance of this MMP in the molecular pathomechanism of urolithiasis.

Folia Biologica et Oecologica 18: 11–12 (2024)

Acta Universitatis Lodziensis

Dielectric Spectroscopy of Polymers and Colloidal Systems

POST-CONFERENCE REPORT

KAMILA SAMOLEJ, MAKSYMILIAN BARTOSIK, MACIEJ ŚLOT

University of Lodz, Faculty of Physics and Applied Informatics, Department of Solid State Physics, Pomorska 149/153, 90–236, Lodz, Poland
E-mail: maksymilian.bartosik@edu.uni.lodz.pl

KEYWORDS: 3D printing, bioprinting, microwave spectroscopy, quality control

Spectroscopy represents an invaluable tool that facilitates the study of matter through its interaction with electromagnetic radiation. Within the rapidly evolving domain of 3D printing technologies, especially in bioprinting, the application of dielectric spectroscopy becomes particularly compelling. This technique is based on assessing the ability of materials to store and disperse electrical energy. Besides providing crucial information about the dielectric properties of materials, microwave spectroscopy also enables effective detection of material defects in the additive printing process, as evidenced by our use of a custom-built sensor operating within the microwave range (Fig. 1), which is currently under patent application [P.445179].

The research detailed in this abstract was conducted by our group at the department of Solid State Physics at University of Lodz (Ślot *et al*., 2023) and focuses on the dielectric characterization of polymers and colloidal systems using the reflection mode spectroscopy method. Polymers, owing to their low dielectric losses, find application in various fields – from complex electronic components to biomedical uses in 3D printing. Meanwhile, colloidal systems can mimic biofilaments used in laboratory tests of printing with biological substances, such as tissues or entire organs. The results demonstrate the feasibility of employing

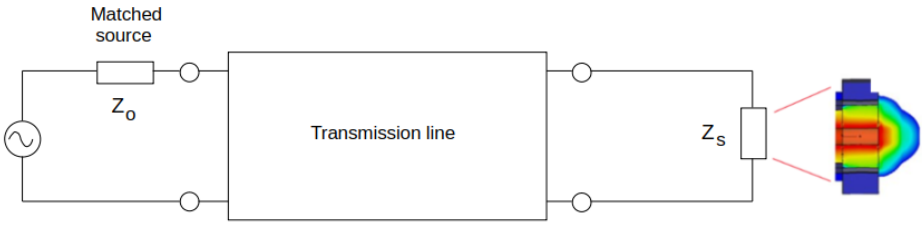

Figure 1. Representation of the sensing system as a circuit based on transmission line theory, along with the electric field distribution of the probe simulated using CST Studio. The sensor operates on the open-ended coaxial probe method, offering non-invasive yet direct contact with the sample under examination. Z_0 is an impedance of Vector Network Analyzer and Z_s is an impedance of the probe.

DOI: https://doi.org/10.18778/1730-2366.18.04

the described microwave sensor to measure low-loss substances (with a dielectric constant <15) using the same measurement parameters applicable to standard 3D printing polymers. Additionally, variations in the noise level of the measured signal amplitude were observed in materials with a high dielectric constant.

The conducted studies confirm that the presented sensor for dielectric spectroscopy can be effectively used not only for traditional measurements of polymer materials but also for biological substances, gels, and food products. As anticipated, it was also noted that colloidal systems exhibit higher dielectric losses, thereby increasing their interactions with microwave radiation. This property broadens the scope of applications for our microwave sensor in studying these materials, potentially enhancing their suitability for quality control in bioprinting.

Reference

Ślot, M., Drabik, P., Bartosik, M., Samolej, K., Zasada, I. 2023. Non-Contact Microwave Sensor for 3d Printing Quality Control. Available at SSRN: https://ssrn.com/abstract=4655382 or http://dx.doi.org/10.2139/ssrn.4655382

Folia Biologica et Oecologica 18: 13 (2024)

Acta Universitatis Lodziensis

Immune checkpoint inhibitors in the treatment of glioblastoma multiforme

POST-CONFERENCE REPORT

ADRIANNA WINNICKA

University of Lodz, Faculty of Biology and Environmental Protection, Pomorska 141/143, 90–236 Lodz, Poland
E-mail: adrianna.winnicka@edu.uni.lodz.pl

KEYWORDS: glioblastoma multiforme, immunotherapy, immune checkpoint inhibitors, monoclonal antibodies, neurooncology

Glioblastoma multiforme (GBM) is among the most common primary tumours of the brain and central nervous system, accounting for about 57% of all diagnosed forms of gliomas. An estimated 5–6 per 100,000 people are diagnosed with GBM each year. Despite extensive research, the median survival of patients diagnosed with GBM is only 12–15 months after diagnosis.

Standard treatment of GBM is based on a multidisciplinary approach – maximal surgical resection, radio-chemotherapy with temozolomide (TMZ) and oral complementary chemotherapy with TMZ. However, due to frequent chemo-resistance and the inability to completely resect the tumour to the exclusion of compromised normal tissue, patients often experience recurrence or progression of the disease. Nevertheless, innovative therapies for GBM are constantly being researched. One of them is immunotherapy, the main goal of which is to mobilize the patient's immune system to recognize and eliminate tumour-transformed cells.

Currently, immunotherapy for GBM includes the use of immune checkpoint inhibitors (ICIs), including mAb anti-PD-1 IgG4, (Niwolumab, Pembrolizumab, Cemiplimab), mAb anti-PD-L1 IgG1 (Atezolizumab, Durvalumab, Avelumab) and mAb anti-CTLA-4 IgG1 (Ipilimumab). ICIs are monoclonal antibodies that, by blocking PD-1 (programmed death receptor 1) or CTLA-4 (cytotoxic T cell antigen 4) proteins, lead to the activation of T cells that fight cancer cells. PD-1 is a protein receptor that inhibits immune activation by binding to PD-L1 and PD-L2 ligands. In contrast, the mechanism by which CTLA-4 inhibits T-cell activation involves competition for ligands with the CD28 molecule.

References

Yasinjan, F., Xing, Y., Geng, H., Guo, R., Yang, L., Liu, Z., Wang, H. 2023. Immunotherapy: a promising approach for glioma treatment. Frontiers in Immunology, 14, 1255611.

Linares, C.A., Varghese, A., Ghose, A., Shide, S.D., Adeleke, S., Sanchez, E., Sheriff, M., Chargari, C., Rassy, E., Boussios, S. 2023. Hallmarks of the Tumour Microenvironment of Gliomas and Its Interaction with Emerging Immunotherapy Modalities. International Journal of Molecular Sciences, 24(17), 13215.

DOI: https://doi.org/10.18778/1730-2366.18.05

Folia Biologica et Oecologica 18: 14–22 (2024)

Acta Universitatis Lodziensis

Assessment of the Impact of Pretreatment of Spent Coffee Grounds with Diluted Sulfuric Acid on the Efficiency of Methane and Lactic Acid Fermentation

SEBASTIAN GOSŁAWSKI, KARINA SKURA

Lodz University of Technology, Department of Environmental Biotechnology, Faculty of Biotechnology and Food Sciences, Wolczanska 171/173, 90–924 Lodz, Poland
E-mail: sebastiangoslawski@interia.pl

ABSTRACT

Lignocellulosic materials are composed of three major biocomponents such as cellulose, hemicellulose and lignin, which form a compact lignocellulosic complex. Characterized by high caloric content, lignocellulosic biomass, including coffee grounds, is a valuable energy source that can be efficiently used in various bioconversion and biotransformation processes. Due to the high consumption of coffee in the world, there is an increasing amount of coffee grounds, which is a rich waste and at the same time a valuable secondary raw material, and its use fits perfectly into a closed-loop economy. Coffee grounds biomass contains polysaccharides, mainly mannans, proteins, lipids, polyphenols, which will allow the development of different biorefinery strategies, the creation of new value-added products with reduced waste generation. The research describes the pre-treatment of coffee grounds with dilute sulfuric acid to evaluate the effect of acid concentration, hydrolysis time on biogas yield, including methane and lactic acid biosynthesis during anaerobic fermentations. A yield of 381.12 mL of CH_4/g-VS methane was obtained, accounting for 72.48% of the total biogas composition. It was found that the most efficient sample in terms of substrate pre-treatment for lactic acid biosynthesis was coffee grounds after 90 min hydrolysis with 1.5% H_2SO_4 at 121 °C.

KEYWORDS: spent coffee grounds, methane fermentation, lactic acid, Fourier transform infrared spectroscopy

Introduction

The world's second most traded commodity after oil is coffee, which is an agro-food commodity. The coffee processing process generates numerous wastes that have significant potential for use as valuable secondary raw materials. By-products include husks, peel, pulp, which together account for about 45% of the coffee fruit. Other by-products, including mucilage and parchment, are

DOI: https://doi.org/10.18778/1730-2366.18.06

also generated during the industrial processing of coffee. As a result, the total amount of waste generated during coffee fruit processing exceeds 50% of its weight (Tsai *et al*., 2012). During the coffee brewing process, additional waste is generated, namely spent coffee grounds (SCG). According to the literature, one ton of green coffee beans produces 650 kg of spent coffee grounds (Murthy *et al*., 2012). Production waste and spent coffee grounds have significant potential in the context of the bioeconomy and sustainable development, including the pursuit of a closed-loop, cradle-to-cradle economy. The bioeconomy is a progressive economic model that deals with the production (using biochemical and biophysical processes) of environmentally friendly renewable biological resources based on the animate world (animals, plants, fungi, *Protista*, *Monera*), biomass and the transformation of these bioresources, as well as the value-added waste generated as a result. Closed-loop economy model on maximizing the use of raw materials and products so as to keep them in circulation for as long as possible. This is in contrast to the linear economy model. In terms of the bioeconomy, which is an integral part of the CE, the use of organic matter like spent coffee grounds (especially lignocellulosic biomass) for the production of biofuels, biopolymers, biosorbents, antioxidants, biochar is not only a way to obtain energy and value-added products, but also a strategy for sustainable development. These processes will make it possible to reduce the carbon footprint (Bevilacqua *et al*., 2023).

Spent coffee grounds are a lignocellulosic material that contains cellulose and hemicellulose in its structure. Spent coffee grounds (SCG) are rich in various polysaccharides, with mannans dominating the composition. Specifically, SCGs consist of 46.8% mannan, 30.4% galactose, 19.0% glucose and 3.8% arabinose, highlighting their complex polysaccharide structure (Mussatto *et al*., 2011). Agro-food waste such as spent coffee grounds contain 13.6% (w/w) of proteins. The protein content of spent coffee grounds exceeds that of coffee beans and fruit due to the accumulation of components not extracted during the manufacture of instant coffee. This can lead to an overestimation of protein content in SCG due to the presence of other nitrogenous compounds such as 1,3,7-trimethyl-xanthine, trigonelline, as well as free amines and, after protein degradation, amino acids (Delgado *et al*., 2008).

In the context of a circular economy, coffee grounds are becoming an important subject of scientific research and practical efforts to find effective solutions for their management. Accordingly, this article aims to increase knowledge about the benefits of using spent coffee grounds and other forms of managing these waste products.

Materials and Methods

Substrate and Inoculum

The biological material used in the study was coffee grounds from *Coffea arabica* L. The coffee grounds were dried at 55 °C for 24 hours, and the ground test material was stored at room temperature with silica gel (25 °C) for further analysis. The chemical composition of the ground coffee was as follows: (TS) dry matter 919.60 ± 4.91 g/kg and (VS) organic dry matter 886.65 ± 3.11 g/kg. The water-soaked liquid containing coffee grounds contained COD 1510 ± 15 mg/dm^3 O_2 and (RS) reducing sugars 10.95 ± 0.08 g/l $C_6H_{12}O_6$ and pH 5.90 ± 0.05.

The inoculum used was digested excess sludge with chemical composition of (TS) 20.02 ± 0.03 g/kg and (VS) 3.42 ± 0.19 g/kg.

Chemical pretreatment of biological material

Two different concentrations of H_2SO_4 were used: a 1.0% and a 1.5% solution in 250 ml flasks with an S:L (solid:liquid) ratio of 1:10. Each of the chemical treatments was carried out both at room temperature and at 121 °C (autoclave). Hydrolysis was performed for 60 and 90 minutes. After autoclaving, the mixture was cooled to room temperature and the solid fraction was separated from the filtrate. It was stored at –7 ± 2 °C until later analysis.

HPLC compound quantification

For carbohydrate analysis, determinations were performed at a mobile phase flow rate of 0.6 ml/min, temperature of 60–70 °C and eluent – dilute acid. Samples were separated on a Hi-Plex H column (7.7 × 300 mm, 8 µm, Agilent Technologies).

Fourier Transform Infrared Spectroscopy Analysis

A Nicolet 6700 FT-IR instrument and the OMNIC analysis program were used for the infrared spectrometric studies. For each measurement, spectra were obtained in the range of 4000 to 400 cm^{-1}. The instrument was cleaned with isopropanol between analyses.

For Methane Production

After careful analysis of various pre-treatment methods, optimal conditions were identified for use in the inoculum-based methane fermentation process. The process was carried out under mesophilic conditions, maintaining a constant temperature of 35 ± 1 °C. The inoculum and the analysed sample were combined in a 2:1 ratio, taking into account the dry organic matter. The weight of the inoculum was 500 g (Figure 1). Mixing of the media contents in the reactors was done manually. Methane was determined with a gas chromatograph.

Culture conditions for Lactobacillus plantarum and Lactobacillus brevis

They were cultured in liquid MRS medium and incubated at 37 °C for 24 hours. Initial cell concentrations for *Lactobacillus plantarum* and *Lactobacillus brevis* were 2×10^9 CFU/ml.

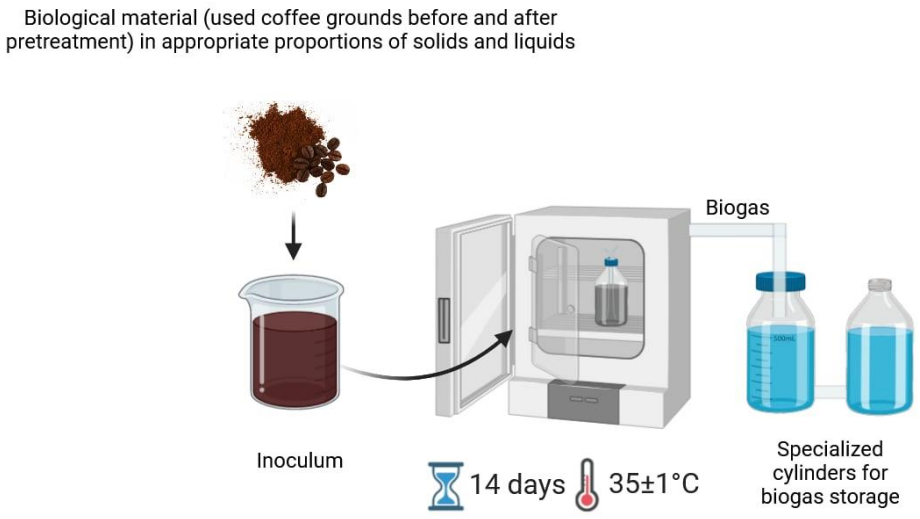

Figure 1. Laboratory set-up.

Lactic fermentation

The experiment was performed in 3 variants: (A) inoculated with *Lactobacillus plantarum*, (B) inoculated with *Lactobacillus brevis*, (C) a consortium of *L. plantarum* and *L. brevis*. The fermentation process was carried out in the SHF system, 5% (v/v) inoculum for each strain was added simultaneously to the suspension of hydrolysed biomass in a ratio of 1:1. For variants (A) and (B), 5% (v/v) inoculum of *L. plantarum* or *L. brevis* was added to the fermentation medium. Samples were incubated at 37 °C for 72 hours. Batch fermentation was performed on a laboratory scale in a 2-liter bioreactor. The anaerobic fermentation process was maintained at a pH of 6.0 ± 0.4 with an optimal process temperature of 37 °C.

Results and Discussion

Products for the pretreatment of spent coffee grounds from Coffea arabica L.

After chemical pretreatment at different parameters of hydrolysis time and temperature, the wet biomass was separated from the liquid. It was shown that the content of dry organic matter decreases with increasing concentration of sulfuric acid (VI) and hydrolysis time. From the results of the chemical oxygen demand by the dichromate method, it can be concluded that the existence of a negative correlation between the analysed variables is indicated by correlation coefficients approaching –1. This confirms that there is a strong negative relationship between the variables. This means that an increase in the concentration of COD in the filtrate is associated with a decrease in the concentration of COD in the biomass. Such a relationship indicates a process of translocation of organic compounds from the biomass to the liquid phase. More favourable results were obtained for hydrolysis at 121 °C. There were more organic compounds in the filtrate that were oxidized. In contrast, there were fewer organic compounds in the biomass after both chemical pretreatment and biological treatment. It is concluded that sulfuric acid (VI) partially hydrolyses hemicellulose to simple sugars and leads to improved cellulose availability by degrading cell structures. The hemicellulose fraction, due to its structure, is a heterobiopolymer that is extremely susceptible to the action of acids, leading to its effective degradation to monosaccharides (Dziekońska-Kubczak *et al.*, 2016). The highest COD value was obtained for the filtrate, where 33.412 mg/dm^3 was obtained for coffee grounds after 90 min hydrolysis with 1.5% H_2SO_4 at 121 °C. This is an increase of 104.21% with respect to the control sample (90 min, 121 °C). When the acid concentration is increased from 1.0% to 1.5%, there is an increase in reaction efficiency of 16.93% after 90 minutes of hydrolysis at 121 °C.

Chemical pretreatment studies have shown that high temperature is not a major factor in the degradation of the lignocellulosic complex, whereas the use of dilute sulfuric acid (VI), according to the analysis, leads to the depolymerisation of hemicellulose (xylose, arabinose and galacturonic acid formed by the oxidation of galactose) and the decrystallisation and depolymerisation of cellulose, leading first to cellobiose and then to glucose. In addition, with increasing temperature, acid concentration and hydrolysis time, there are inhibitors derived from the degradation of pentoses (furfural to formic acid) and hexoses (HMF to formic acid) and from the hydrolysis of acetyl groups of hemicellulose acetic acid (Domański *et al.*, 2016; Janković *et al.*, 2023).

The filtrate was analysed for reducing sugars. The highest value was obtained for coffee grounds after hydrolysis with 1.5% H_2SO_4 at 121 °C for 90 min. 34.02 ± 0.11 g/l $C_6H_{12}O_6$ was obtained, which is an increase of 1128.16% with respect to the control sample, while increasing the acid concentration from 1.0% to 1.5%, there is an increase of 11.55% in the amount of reducing sugars after 90 minutes of hydrolysis at 121 °C. High temperature plays a key role in increasing the yield of reducing sugars, especially when dilute sulfuric acid (VI) is used. This process contributes to the precipitation of some lignin and the efficient hydrolysis of hemicellulose to simple sugars (Galbe and Zacchi, 2012).

Because the results obtained showed a significant amount of organic compounds in the filtrate, which correlated with the results of organic dry matter, which decreased with increasing temperature, hydrolysis time and sulfuric acid (VI) concentration. The filtrate was subjected to HPLC analysis, which showed that the glucose content obtained after chemical hydrolysis of coffee grounds was 7.29 to 25.72 times higher in comparison with samples of grounds treated with water for 60 minutes (control sample) (Table 1). It was shown that the maximum concentration of cellobiose reaches 0.54 g/L when treated with 1.5% H_2SO_4 solution for 60 minutes at 121 °C, while the lowest reaches 0.02 g/L for coffee grounds after 90 minutes of hydrolysis with 1.0% H_2SO_4 at room temperature. This shows that the amount of cellobiose decreases and the amount of glucose increases with hydrolysis time, sulfuric acid concentration, and process temperature (Saini *et al*., 2015). This indicates that H_2SO_4 contributed to the partial depolymerisation of cellulose homopolymer into glucose monomers.

Table 1. Effect of coffee grounds pretreatment with different concentrations of sulfuric acid

Sample Type: H_2SO_4 Concentration, Hydrolysis Time, Hydrolysis Temperature	Cellobiose [g/L]	Glucose [g/L]	Xylose [g/L]	Arabinose [g/L]	Glicerol [g/L]	Acetic acid [g/L]	Formic acid [g/L]	Furfural [g/L]
1.5% H_2SO_4 for 90 minutes at 121 °C	0.50	2.9	9.94	1.22	0.09	1.21	0.13	0.12
1.5% H_2SO_4 for 60 minutes at 121 °C	0.54	2.5	9.61	1.24	0.08	0.58	0.08	0.05
1.0% H_2SO_4 for 90 minutes at 121 °C	0.52	2.6	9.88	0.7	0.06	0.64	0.10	0.05
1.0% H_2SO_4 for 60 minutes at 121 °C	0.53	2.6	8.74	0.8	0.06	0.57	0.06	0.04
1.5% H_2SO_4 for 90 minutes at room temperature	0.11	0.39	0.15	0.17	0.04	0.03	0.01	–
1.5% H_2SO_4 for 60 minutes at room temperature	0.07	0.13	0.11	0.12	0.08	0.01	0.01	–

Table 1(continued). Effect of coffee grounds pretreatment with different concentrations of sulfuric acid

Sample Type: H_2SO_4 Concentration, Hydrolysis Time, Hydrolysis Temperature	Cellobiose [g/L]	Glucose [g/L]	Xylose [g/L]	Arabinose [g/L]	Glicerol [g/L]	Acetic acid [g/L]	Formic acid [g/L]	Furfural [g/L]
1.0% H_2SO_4 for 90 minutes at room temperature	0.03	0.05	0.16	0.01	0.02	0.02	0.01	–
1.0% H_2SO_4 for 60 minutes at room temperature	0.05	0.08	0.24	0.01	0.06	0.02	0.01	–
Control sample for 90 minutes at 121 °C	0.04	0.82	0.46	0.11	0.09	0.19	0.08	0.01
Control sample for 60 minutes at 121 °C	0.01	0.63	0.33	0.04	0.06	0.08	0.04	0.01

The highest amount of glucose was for a 1.5% solution of H_2SO_4 for 90 min at 121 °C and was 2.9 g/L, while 0.4 g/L less glucose was obtained for the sample after 60 min of hydrolysis at 121 °C (Table 1). The highest xylose concentration (9.94 g/L) was observed in samples hydrolysed with 1.5% sulfuric acid for 1.5 h at 121 °C. The highest value for lactic acid was obtained for coffee grounds after 90 min hydrolysis with 1.5% H_2SO_4 at 121 °C (3.07 g/L). This is 168.31% higher than the sample at room temperature (90 minutes, 1.5% H_2SO_4) and 194.05% higher than the control sample (90 minutes, 121 °C, no sulfuric acid).

Samples treated with 1.5% sulfuric acid after 90 min hydrolysis at 121 °C yielded the highest inhibitor concentrations of acetic acid (1.21 g/L), formic acid (0.13 g/L), HMF (0.09 g/L), and furfural (0.12 g/L) of all samples tested. In contrast, for samples hydrolysed for 60 min with the same parameters, fewer inhibitors were obtained with 52.11% acetic acid, 41.01% formic acid, 87.05% HMF and 60.41% furfural (Table 1). The obtained inhibitors inhibit the growth of microorganisms responsible for anaerobic fermentation, including methane and lactic fermentation. The resulting increase in acetic acid was due to the hydrolysis of acetyl groups, which are part of the hemicellulose fraction. The concentration of furfural was shown to increase with the concentration of H_2SO_4, which correlates with the content of xylose and arabinose (simple sugars after hemicellulose depolymerisation). The resulting reaction inhibitors and value added products such as HMF and furfural after liquid phase separation indicate that there has been a loss of sugars (pentoses and hexoses). This can affect the yield and quality of anaerobic fermentation (Zabed *et al*., 2016; Janković *et al*., 2023).

Spent coffee grounds before and after chemical treatment were subjected to FT-IR analysis to confirm efficacy. Figure 2 shows the spectra of the different test samples. To further analyse the raw material of coffee grounds, the coffee beans were analysed after grinding. The physical treatment of coffee beans by grinding increases the availability of the substrate surface, which is critical for

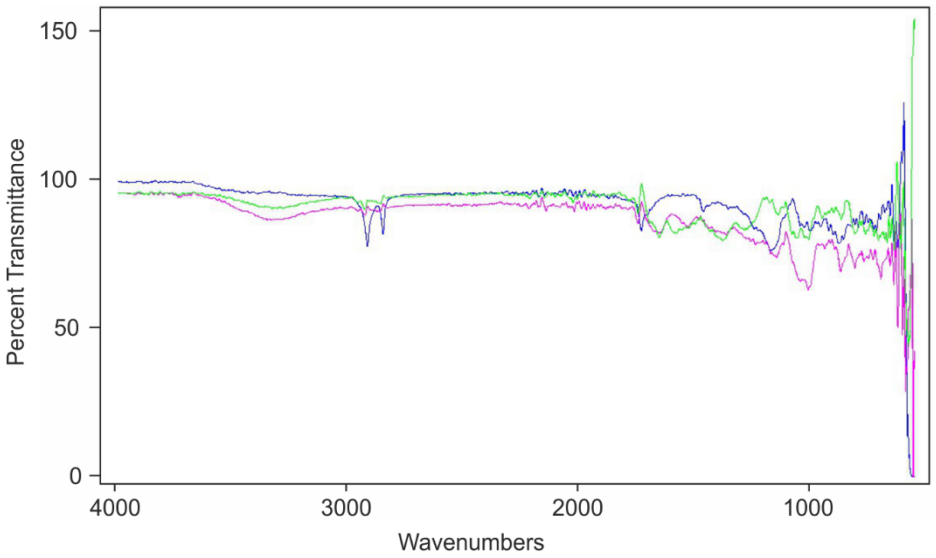

Figure 2. FT-IR spectrum of selected samples after chemical pretreatment. (—) control sample *Coffea arabica*, ground beans, 100% purity.SPA; (—) *Coffee arabica* ground 100% pure, pretreated with 1.5% sulphuric acid (VI), at room temp.SPA; (—) *Coffee arabica* ground 100% pure, pretreated with 1.5% sulphuric acid (VI), at 121 °C.SPA.

further extraction processes. As a result of grinding, the degree of polymerization and crystallization of lignocellulosic materials is reduced, which facilitates access to the structural components of the bean during brewing. When ground coffee is brewed, the extraction process uses conditions analogous to those used in steam explosion and steam pretreatment methods. Both processes are characterized by an intensive thermomechanical treatment, during which hemicellulose and lignin are degraded without the formation of reaction inhibitors, as confirmed by the results of control tests. A significant advantage of the "steam explosion" method is its minimal environmental impact and efficiency in degrading structural polymers, making it an attractive alternative in biomass processing. However, this method requires specialized equipment and strict technical requirements. In the context of home coffee brewing, the process is carried out on a much smaller scale, resulting in coffee grounds as a by-product. These coffee grounds represent a partially processed phytobiomass. In the spectra, characteristic bands for cellulose were observed in the region of 3220–3400 cm^{-1}, corresponding to the bending vibrations of OH groups (Ibrahim *et al*., 2015; Gieparda, 2019). The stretching vibration bands of CH groups were shown at 2916 cm^{-1}. Most of the bands in the range of 1720–890 cm^{-1} were shown at lower intensity, which may indicate that the acid pretreatment process of cellulose leads to a decrease in the degree of crystallinity, an increase in the volume of microfibers, and at the same time intensifies the transformation of the cellulose structure towards an amorphous form (Ibrahim *et al*., 2015). This change may be due to the breaking of hydrogen bonds in the crystalline regions of cellulose, leading to an increase in the mobility of the polymer chains and an increase in their amorphousness. The FTIR absorption spectrum of the

pretreated biomass showed the presence of characteristic stretching bands at 1731 cm^{-1}, typical of carbonyl groups in hemicellulose. This shows a clear correlation with the results obtained from the analysis of the chemical composition of the pretreated biomass.

Conclusions

Biogas, methane yields of selected samples tested

After analysing the biological material after chemical pretreatment with sulfuric acid (VI), the most efficient sample was analysed. The highest yield of biogas and methane after inoculum methane fermentation was obtained for coffee grounds after 90 min hydrolysis with 1.5% H_2SO_4 at 121 °C, amounting to 525.83 mL/g-VS biogas, including 381.12 mL CH_4/g-VS methane, which accounted for 72.48% of the total biogas composition. This is 130.50% higher biogas yield than in the control experiment (90 min, 121 °C) and 116.91% higher biomethane yield than in the control experiment where 175.70 mL CH_4/g-VS was obtained. For the digestion, a pH of 7.60 ± 0.14 was obtained for all test samples. Girotto and co-authors obtained 392 mL of CH_4/g-VS after pretreatment of coffee grounds with NaOH (8% w/w) (Girotto *et al*., 2018). Luz and co-authors co-digested coffee grounds with cow manure without pretreatment, and used cow manure with water as a reference sample. Luz and co-authors showed that for co-digestion of substrate (SCG) with cow manure, it showed a higher biogas yield of 48% and 50% more biomethane compared to the experiment with manure input with water. A yield of 305 ml CH4/g-VS was obtained for the co-digestion (Luz *et al*., 2017; Girotto *et al*., 2018).

The most effective yield of lactic acid during anaerobic lactic fermentation

The study showed that the most efficient sample in terms of substrate pretreatment for lactic acid biosynthesis was coffee grounds after 90 min hydrolysis with 1.5% H_2SO_4 at 121 °C. The highest lactic acid yield was shown for the consortium of *Lactobacillus plantarum* and *Lactobacillus brevis* and is 0.51 ± 0.09 g lactic acid per gram biomass. The consortium data yielded 20 g of lactic acid produced per litter per hour. Lactic fermentation with coffee grounds inoculated only with *Lactobacillus plantarum* showed the most 0.47 ± 0.04 g of lactic acid per gram of coffee grounds for the best pretreatment. This is a 7.84% lower lactic acid gain per gram of substrate.

References

Bevilacqua, E., Cruzat, V., Singh, I., Rose'Meyer, R. B., Panchal, S.K., Brown, L. 2023. The Potential of Spent Coffee Grounds in Functional Food Development. Nutrients, 15(4), 994.

Delgado, P.A., Vignoli, J.A., Siika-aho, M., Franco, T.T. 2008. Sediments in coffee extracts: composition and control by enzymatic hydrolysis. Food Chemistry, 110(1), 168–176.

Domański, J., Marchut-Mikolajczyk, O., Cieciura-Włoch, W., Patelski, P., Dziekońska-Kubczak, U., Januszewicz, B., Bolin, Z., Dziugan, P. 2020. Production of Methane, Hydrogen and Ethanol from *Secale cereale* L. Straw Pretreated with Sulfuric Acid. Molecules, 25, 1013.

Dziekońska-Kubczak, U., Balcerek, M., Patelski, P., Pielech-Przybylska, K. 2016. Ocena wpływu sposobu neutralizacji hydrolizatów lignocelulozowych na wydajność hydrolizy oraz fermentacji etanolowej. Innowacyjne rozwiązania w technologii żywności i żywieniu człowieka.

Galbe, M., Zacchi, G. 2012. Pretreatment: the key to efficient utilization of lignocellulosic materials. Biomass and Bioenergy, 46, 70–78

Gieparda, W. 2019. Obróbka wstępna biomasy konopi w procesie otrzymywania bioetanolu. Przemysł Chemiczny, 1, 124–127. https://doi.org/10.15199/62.2019.12.16

Girotto, F., Lavagnolo, M.C., Pivato, A. 2018. Spent coffee grounds alkaline pre-treatment as biorefinery option to enhance their anaerobic

digestion yield. Waste Biomass Valorization, 9, 2565–2570. https://doi.org/10.1007/s12649-017-0033-8

Ibrahim, M., Maha, F., Fahmy, T., Salaheldin, E., Mobarak, F., Youssef, M., Mabrook, M. 2015. Role of Tosyl Cellulose Acetate as Potential Carrier for Controlled Drug Release. Life Science Journal, 12, 127-133. https://doi.org/10.7537/marslsj121015.16

Janković, T., Straathof, A.J.J., Kiss, A.A. 2023. Enhanced hot-liquid water pretreatment of biomass with recovery and valorization of side products. In A. C. Kokossis, M. C. Georgiadis, & E. N. Pistikopoulos (Eds.), Computer Aided Chemical Engineering (Vol. 52, pp. 2113 –2118). Elsevier. https://doi.org/10.1016/B978-0-443-15274-0.50336-X

Luz, F.C., Cordiner, S., Manni, A., Mulone, V., Rocco, V. 2017. Anaerobic digestion of liquid fraction coffee grounds at laboratory scale: Evaluation of the biogas yield. Energy Procedia, 105, 1096–1101.

Murthy, P.S., Naidu, M.M. 2012. Sustainable management of coffee industry by-products and value addition—A review. Resources, Conservation and Recycling, 66, 45–58. https://doi.org/10.1016/j.resconrec.2012.06.005

Mussatto, S.I., Ballesteros, L.F., Martins, S., Teixeira, J.A. 2011. Extraction of antioxidant phenolic compounds from spent coffee grounds. Separation and Purification Technology, 83, 173–179.

Saini, J.K., Saini, R., Tewari, L. 2015. Lignocellulosic agriculture wastes as biomass feedstocks for second-generation bioethanol production: Concepts and recent developments. 3 Biotech, 5, 337–353.

Tsai, W.-T., Liu, S.-C., Hsieh, C.-H. 2012. Preparation and fuel properties of biochars from the pyrolysis of exhausted coffee residue. Journal of Analytical and Applied Pyrolysis, 93, 63–67. https://doi.org/10.1016/j.jaap.2011.09.010

Zabed, H., Sahu, J.N., Boyce, A.N., Faruq, G. 2016. Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches. Renewable and Sustainable Energy Reviews, 66, 751–774.

Folia Biologica et Oecologica 18: 23–38 (2024)

Acta Universitatis Lodziensis

The posterior hypothalamic area as an independent generator of rhythmic theta oscillatory activity

KLAUDIA PSZCZÓŁKOWSKA*, TOMASZ KOWALCZYK

University of Lodz, Faculty of Biology and Environmental Protection, Department of Neurobiology, Pomorska 141/143, 90–236 Lodz, Poland
E-mail: klaudia.pszczolkowska@edu.uni.lodz.pl

ABSTRACT

Theta rhythm is one of the most prominent examples of rhythmic oscillatory activity in mammalian brain and it is generated mainly in structures of the limbic cortex, including the hippocampal formation. In the 1970s it was shown that theta rhythm may be also recorded in diencephalic region including the posterior hypothalamic nuclei and supramammillary nucleus, together considered as the posterior hypothalamic area (PHa). For decades it was stated that local posterior hypothalamic oscillatory activity is controlled by the descending inputs going to the PHa from the septohippocampal system. However, the latest studies indicated that theta rhythm can be recorded in deafferented PHa in vitro preparations which indicates that the posterior hypothalamic area should be considered as an independent of the other brain structures theta generator. In subsequent research the neurochemical and cellular basis of PHa theta were examined in both *in vivo* and *in vitro* conditions. In the light of multiple evidence obtained in these studies, it is the author's intent to summarize the data concerning the role of the posterior hypothalamic area in hippocampal theta rhythm generation as well as the ability of that brain structure to independently generate theta rhythmicity.

KEYWORDS: theta rhythm, hippocampal formation, hypothalamus, posterior hypothalamic nuclei, supramammillary nucleus

Introduction

The theta rhythm, being one of the model examples of oscillation and synchronization of neuronal activity occurring in the central nervous system (CNS), has been the subject of research since the early 1950s (Buzsáki, 2002). Today, this rhythm is one of the most studied patterns of rhythmic electroencephalographic (EEG) activity recorded in mammalian brain. This type of EEG activity is generated in many regions of the central nervous system, but the main sites of its generation are the structures of the limbic system, including

DOI: https://doi.org/10.18778/1730-2366.18.07

the hippocampal formation (HPC; Bland, 1986). Theta rhythmic activity in humans observed during extracranial EEG examination is recorded from medial regions of the frontal lobe and temporal regions of the cortex. This EEG phenomenon is characterized by a frequency in the range from 4 to 7 Hz and amplitude reaching 80 μV (Mitchell *et al*., 2008). Human theta oscillatory activity is observed during many physiological processes, such as spatial navigation (Caplan *et al*., 2003; Chrastil *et al*., 2022), paradoxical sleep (Cantero *et al*., 2003; Girardeau and Lopes-Dos-Santos, 2021), language processes (Cross *et al*., 2022; Mitchell *et al*., 2008), or performing arithmetic operations and tasks based on working memory (Raghavachari *et al*., 2001).

Interestingly, rhythmic oscillatory activity in the theta band is also observed in human EEG recordings in pathological conditions, thus it may be considered a non-specific indicator of CNS disorders (Kowalczyk *et al*., 2013). One of the most prominent examples of such theta co-occurrence is the inhibition of theta rhythm in patients with temporal lobe epilepsy (TLE). Between seizures, significant additional neuronal firings, called interictal spikes, are observed in EEG recordings taken from patients with the TLE. Interestingly, immediately after those spikes, the power of theta rhythm recorded from anterior hippocampus and the entorhinal cortex (EC) is reduced, and during the prolonged between-spikes periods the inhibitory effect on theta rhythm might sustain (Clemens *et al*., 2021; Fu *et al*., 2018). These observations suggest that the theta inhibitory effect of interictal spikes may be a significant biomarker of the TLE. Another interesting example of changes in theta band oscillations can be observed in EEG recordings taken from patients with Alzheimer's disease (AD). It was shown that theta rhythm increases in its power in AD patients and seems to be more regular compared to the EEG recording of healthy subjects. Furthermore, AD patients show theta frequency-dependent abnormalities in EEG synchrony (Baik *et al*., 2022; Gallego-Jutglà *et al*., 2015). Interestingly, abnormalities in theta rhythm also occur in EEG recordings of patients with posttraumatic stress disorder (PTSD). During the performance of more attentionally-demanding tasks, patients with the PTSD exhibited 'late-stage' theta hyperconnectivity observed in right parietal cortex which contributed to their diminished mental flexibility (Dunkley *et al*., 2015; Toll *et al*., 2020).

Theta rhythm in rodents

The results of human studies presented above, highlight the importance of the theta rhythm in both physiological and pathological states of the CNS, and justify the ongoing research on that oscillatory activity (Kowalczyk *et al*., 2013). Most of those theta rhythm investigations, carried out since the early 1950s, were conducted with the use of rodent models, specifically rats. Rhythmic oscillations in the theta band generated in the HPC of these mammals exhibit a larger frequency band than in humans, ranging from 3 to 12 Hz. Moreover, theta rhythm in rodents is characterized by an almost sinusoidal pattern of regular waves, with a high amplitude of 1 to 2 mV in deep brain recordings (Bland, 1986). In this group of animals, theta activity is associated with physiological processes such as spatial navigation (Hasselmo *et al*., 2002), performance of voluntary movements (Li *et al*., 2021), long-term synaptic potentiation (LTP; Huerta and Lisman, 1995), and sensorimotor integration (Bland and Oddie, 2001). Similar to humans, the hippocampal formation is the main limbic structure in which this EEG

activity is recorded and studied in rodents (Kowalczyk *et al*., 2013).

Considering the different neurochemical basis and the correlation with the animal's behavior, the hippocampal theta rhythm in rodents was divided by Vanderwolf into two types: type I and type II (Vanderwolf, 1969). Type I theta rhythm is generated when an animal is performing voluntary movements, such as walking, running, standing on hind legs, swimming or adjusting posture (Bland, 1986; Vanderwolf, 1969). During the above-mentioned types of behavior, the local hippocampal field potentials occur in the form of waves with a frequency from 6 to 12 Hz. The described type of theta rhythm is resistant to the application of even high doses of atropine sulfate (a cholinergic, muscarinic receptor antagonist). Therefore, that movement-dependent type of theta rhythm has a noncholinergic nature. There are studies suggesting that the generation of type I theta rhythm is related to serotonergic transmission (Bland and Colom 1993).

In contrast, type II theta oscillatory activity, is observed during the absence of the animal's voluntary movements or occasionally, while performing automatic behaviors, such as licking, chewing or cleaning the fur. Rhythmic theta oscillations recorded during an animal's immobility occur in the frequency range from 4 to 9 Hz. It was documented that injection of atropine sulfate results in the abolition of type II theta rhythm, indicating its cholinergic basis (Bland, 1986). This type of theta rhythm can also be recorded in animals under urethane anesthesia, as it is not suppressed by the administration of most anesthetics. In addition, it was also shown that deafferented hippocampal formation preparations maintained *in vitro* are capable of generating type II theta activity after administration of cholinergic receptor agonists such as carbachol or acetylcholine (Kowalczyk *et al*., 2013).

Both types of theta rhythm are recorded from hippocampal formation as a result of characteristic features of its neurons. Those neurons are called theta-related, because they discharge in a specific relationship with the theta rhythm observed extracellularly in the HPC (Fig.1). Widely accepted theta-cell classification was introduced by Colom and Bland in the 1980s. These authors found that the neurons associated with the theta rhythm in the hippocampal formation could be divided into two distinct populations, referred to as theta-on and theta-off cells. Theta-on cells increase their activity when HPC theta rhythm is present, whereas theta-off cells decrease their activity during ongoing hippocampal theta synchronization. Each cell within these two subtypes, can discharge in one of two characteristic patterns. The first pattern, referred to as phasic, is characterized by spike discharges that occur in constant phase correlation to each theta wave cycle. The second pattern, referred to as tonic, is characterized by irregular or regular cell discharges with no consistent relationship to each theta cycle. Both theta-on and theta-off cells include phasic and tonic subtypes (Fig.1; Colom and Bland, 1987; Bland and Colom, 1993). Furthermore, it was shown that theta-on phasic neurons are characterized by the ability to generate rhythmic membrane potential oscillations (MPOs; Artemenko, 1972). The extracellularly recorded theta rhythm is closely correlated with MPOs, which is why they are also referred to as the intracellular theta rhythm (Bland *et al*., 2002). It was accepted, that the spatial summation of fluctuations in membrane potentials of hippocampal theta-on phasic cells is observed as a local field theta oscillation (Bland and Colom, 1993).

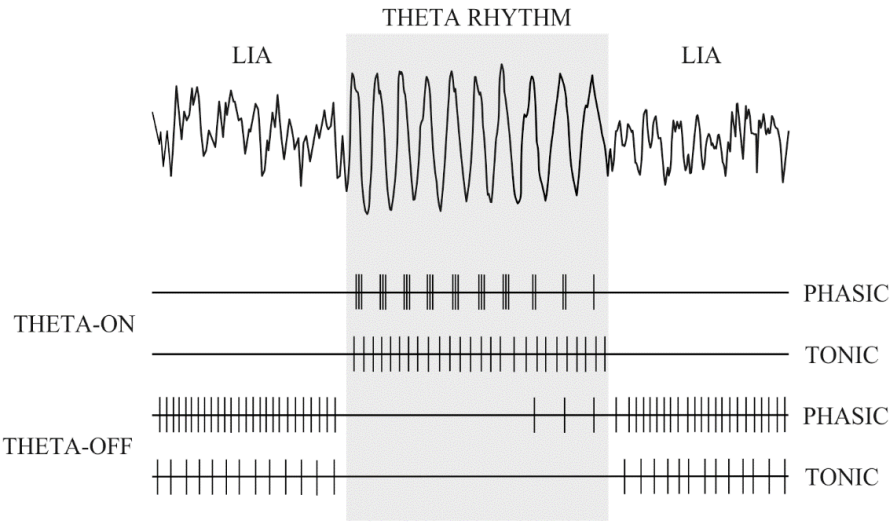

Figure 1. A diagrammatic representation theta-related cells classification. The upper row represents an example of theta rhythm (marked with grey rectangle) and large irregular activity (LIA) recording. The next four rows represent firing patterns of different subtypes of theta cells. Details in the text (Colom and Bland, 1987; Bland and Colom, 1993; modified).

Ascending brainstem-hippocampal synchronizing pathway

The theta field potentials described in the previous section are generated in the hippocampal formation and other structures of the limbic system as a result of extrinsic inputs received from the so-called the ascending brainstem-hippocampal synchronizing pathway or ascending synchronizing system (Fig.2). This pathway comprises numerous structures, located within the pontine region, diencephalon, and basal parts of the forebrain. The process of ascending pathway activation begins with the arousal of specific brainstem nuclei, which send continuous excitatory information through the posterior hypothalamic area to the medial septal area, from where the impulsation is transmitted to the limbic system structures, including the HPC (Kowalczyk *et al*., 2013).

Pontine nuclei

As it was stated previously, the process of activating the ascending brainstem-hippocampal system begins within the specific brainstem nuclei (Fig. 2): the nucleus reticularis pontis oralis (RPO) and the pedunculopontine tegmental nucleus (PPT; Woodnorth *et al*., 2003). Early studies investigated afferents of the PPT by using the examination of retrograde transport combined with the immunohistochemical examinations. These investigations revealed that the PPT provides a widespread innervation to the caudal diencephalon and basal parts of the forebrain (Hallanger *et al*., 1987). Subsequent mapping studies showed that the PPT sends prominent projections to the RPO (Semba *et al*., 1990) which, in turn sends its afferents to the caudal diencephalic region, mainly the supramammillary nucleus (SuM; Vertes *et al*., 1986). In addition to extensive connections with other structures of the

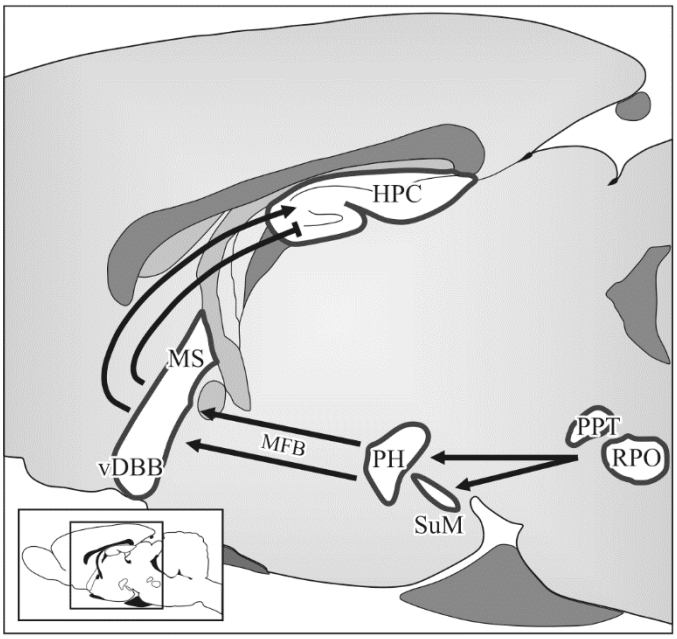

Figure 2. Diagram showing connections between the structures forming the ascending brainstem-hippocampal synchronizing system. RPO – nucleus reticularis pontis oralis, PPT – pedunculopontine tegmental nucleus, SuM – supramammillary nucleus, PH – posterior hypothalamic nuclei, MFB - medial forebrain bundle, MS – medial septum, vDBB – vertical limb of the diagonal band of Broca, HPC – hippocampal formation (Bland and Oddie, 1998; Kowalczyk *et al*., 2021; modified).

ascending system, the significant role of the RPO and PPT in theta rhythm generation in the HPC was indicated by the fact that a prominent population of theta-related cells is present in both of these nuclei. The presence of theta-neurons was confirmed by Kirk *et al*. (1996), who investigated cellular activity recorded from caudal areas of the midbrain including the SuM, posterior hypothalamic nuclei (PH) and medial mammillary nucleus (MM). Recordings were made during a continuous hippocampal theta rhythm induced by an electrical RPO stimulation. The results of this study showed that theta-related neurons can be found in multiple caudal diencephalic sites, including the RPO. Interestingly, all cells recorded from SuM and MM were classified as phasic theta-on, while the theta-on PH and RPO neurons discharged in a tonic manner (Bland *et al*., 1995). Moreover, studies with inhibition of brainstem nuclei confirmed their important contribution to the generation of hippocampal theta rhythm. It was shown that the injection of procaine (local anesthetic) into caudal part of the RPO resulted in inhibition of sensory-elicited theta rhythm in the HPC (Kroplewski *et al*., 2010). Similar results were obtained in the studies on the role of the PPT in hippocampal rhythm generation. The unilateral microinjections of procaine into the PPT successfully blocked tail pinch-elicited hippocampal theta rhythm, thus emphasizing the significant role of the PPT in ascending brainstem-hippocampal synchronizing pathway (Nowacka *et al*., 2002).

Posterior hypothalamic area

The posterior hypothalamic area (PHa) is another important part of the ascending synchronizing pathway (Fig. 2), which is constituted of the posterior hypothalamic nuclei and the supramammillary nucleus. Both these nuclei receive projections from the RPO and PPT (Kirk, 1998) and transmit excitatory impulses to the medial septum (MS; Bland *et al*., 2007) region.

There are multiple experimental evidences indicating the crucial role of the PHa in the generation of hippocampal theta oscillations. Electrolytic lesions of the posterior hypothalamus (including the SuM) in curarized cats resulted in inhibition of HPC theta rhythm even after the application of intense RPO electrical stimulation. Under such conditions only desynchronization and very slow oscillations were present in the hippocampal EEG recordings (Kawamura *et al*., 1961). Another study showing the significant effect of PHa inactivation on rhythmic activity of the hippocampal formation was performed with the use of chemical lesions (procaine administration). When procaine was injected directly into the SuM or to its afferents, a significant reduction in the amplitude and frequency of the theta rhythm in the HPC was observed despite high-frequency electrical stimulation of the RPO (Kirk and McNaughton, 1993).

What is more interesting, the theta-dependent neurons are also present at the level of the posterior hypothalamic area. Kirk and McNaughton were the first to describe these cells in the PHa. In that study, simultaneous EEG recordings were performed in the SuM and hippocampal formation, in urethane-anesthetized rats. It was revealed, that SuM cells discharged rhythmically when the hippocampal theta rhythm occurred spontaneously or when it was evoked by a tail pinch. The firing pattern of SuM neurons was strongly correlated with the frequency and phase of ongoing HPC theta oscillations. Moreover, during periods of EEG desynchronization observed in the HPC the discharge patterns of SuM neurons were irregular (Kirk and McNaughton, 1991; Kowalczyk *et al*., 2021). Interestingly, in subsequent cellular investigations it was documented that neurons recorded from the SuM may be classified as phasic theta-on, whereas PH neurons represent tonic subtype of theta-on neurons (Bland *et al*., 1995; Kirk *et al*., 1996). The results of these studies confirmed the presence of different populations of hippocampal theta-related cells at caudal diencephalic level of the ascending synchronizing system (Kowalczyk *et al*., 2013, 2021).

Medial septum

Another component of the ascending brainstem-hippocampal synchronizing pathway is the medial septum and the vertical limb of the diagonal band of Broca (vDBB). The MSvDBB plays a significant role in generating hippocampal theta rhythm by combining multiple inputs going from different brainstem and caudal diencephalic sites and distributing rhythmic impulsation into the HPC and other limbic structures (Fig. 2; Bland and Oddie, 1998). It was experimentally proven, that deafferentation of the MSvDBB inhibits the ability of the HPC to theta rhythm generation (Bland, 1986). Moreover, the electrical stimulation of the MSvDBB leads to the production of hippocampal theta and resulting HPC synchronous oscillations follows the frequency of septal stimulation (Kramis and Vanderwolf, 1980). It was also indicated, that MSvDBB neurons discharge pattern is clearly related to the occurrence and time course of HPC theta rhythmic field potentials recorded in urethane-anesthetized rats. The majority of septal theta-related cells were classified

as theta-on phasic neurons (Ford *et al*., 1989).

Multiple studies emphasized the role of the MSvDBB in modulation and peacemaking of hippocampal theta rhythm based on its cholinergic, GABAergic, and glutaminergic inputs to the HPC (Dannenberg *et al*., 2015; Vandecasteele *et al*., 2014). The significance of cholinergic projections was shown in the study, in which the injection of 192 IgG-saporin (the selective eliminator of cholinergic neurons) into the MSvDBB resulted in the absence of hippocampal theta oscillations (Yoder and Pang, 2005). Other studies have shown that MSvDBB GABAergic neurons discharge rhythmically to pace the hippocampal theta oscillations (Ford *et al*., 1989). Based on the firing correlation of these neurons to either the trough (178 degrees) or the peak (330 degrees) of simultaneously recorded hippocampal theta waves, GABAergic medial septal cells were classified into two different populations (Borhegyi *et al*., 2004). It was concluded that the firing pattern of those neurons constitute the basis of the MSvDBB as the main external pacemaker of hippocampal theta oscillations (Hangya *et al*., 2009). Additionally, the results of the latest studies revealed, that the glutamatergic MSvDBB neurons showed a significant increase in firing rate during hippocampal theta oscillations, but without clear relationship to HPC theta phase. This may suggest that these glutamatergic cells contribute to MSvDBB theta pacemaker properties by providing additional tonic excitation necessary to induce HPC theta synchronization (Kocsis *et al*., 2022).

Local theta field activity in the posterior hypothalamic area

The previously described studies emphasized the relevance of the PHa as a part of ascending brainstem-hippocampal synchronizing pathway. Taking into consideration the presence of theta-related cells in both the SuM and PH, especially the subpopulation of theta-on phasic neurons which are highly involved in theta rhythm appearance in a given brain structure, the question arises: is the PHa capable of generating the local theta rhythm by itself? Interestingly, the results obtained from studies conducted since the 1970s suggested that PH and SuM are not just simple modulators of the hippocampal theta rhythm but may also be capable of producing rhythmic oscillatory activity locally.

The first studies addressing the ability of caudal diencephalic region to generate local theta field potentials were performed on freely moving rats by Komisaruk (1970) and concerned the theta oscillatory activity recorded in the mammillary bodies (MB) and hippocampal formation. In both of these structures, the movement-related subtype of theta rhythm was recorded. Theta oscillations in the MB were appearing with constant lag (about 20ms) compared to simultaneously recorded HPC rhythmic theta activity. The same time delay in the appearance of rhythmic oscillations between EEG recordings from the HPC and MB was observed in rats immobilized by a muscle relaxant, gallamine (Komisaruk, 1970). Another interesting results were obtained during simultaneous EEG recordings taken from the dorsomedial-posterior hypothalamus (DMPH) and the hippocampal formation during the animal's spontaneous behavior (Bland and Vanderwolf, 1972). Data obtained in these studies revealed a clear correlation between the animal behavior and synchronous rhythmic EEG activity recorded from the DMPH, and this correlation appeared to be very similar as in the case of HPC theta field potentials. In the same studies, electrical stimulation of hippocampal dentate area led to the

suppression of regular rhythmic activity in both the HPC and DMPH which was associated with the arrest of animal behavior. Only after retrieval of rhythmic oscillatory activity in both structures to its normal amplitude, spontaneous locomotor activity recommenced which indicated the potential involvement of DMPH theta activity in the control of behavior. However, the described results were obtained only from two rats and the detailed characteristic of DMPH rhythmic oscillatory activity was not performed (Bland and Vanderwolf, 1972).

A more detailed analysis of posterior hypothalamic theta activity was compiled during the research conducted by Sławińska and Kasicki (1995). The results were obtained from nine rats, while simultaneous depth EEG signals were recorded from the posterior hypothalamic nuclei and the CA1 pyramidal layer of the HPC in various experimental situations (spontaneous motor activity in a home cage and spontaneous locomotion along a runway). Additionally to EEG recordings, the electrical stimulation of subthalamic and hypothalamic areas was performed in these studies. The authors found that, EEG activity obtained from both structures (i.e. the posterior hypothalamus and hippocampal formation) was related to the animals' spontaneous behavior. What is more interesting, the EEG signals recorded from the posterior hypothalamic nuclei during spontaneous locomotor activity or that evoked by electrical stimulation were characterized by pronounced synchronization and it never occurred when the animal was motionless (Sławińska and Kasicki, 1995).

In subsequent studies, performed on urethane-anesthetized rats, the similarities between hippocampal and posterior hypothalamic theta rhythmic activity were analyzed (Kocis and Vertes, 1997). The authors conducted EEG examination taken from structures in the caudal diencephalon including the posterior hypothalamic nuclei, the supra-mammillary nucleus and the mammillary bodies, along with recordings in the hippocampal formation (specifically the CA1 and dentate gyrus). The obtained results indicated different populations of SuM and MB neurons, which rhythmical firing pattern was strongly correlated with hippocampal theta oscillations at different preferred phases. Furthermore, the posterior hypothalamic area theta field oscillations exhibited a consistent time delay of about one quarter of theta cycle in comparison with simultaneous HPC theta. The authors developed the hypothesis that the theta rhythm observed in the PHa could be controlled by the descending inputs going to the posterior hypothalamic area from the septohippocampal system (Kocis and Vertes, 1997). It was assumed, that these descending inputs, eliciting locally generated PHa theta could be subicular to MB connections (Allen and Hopkins, 1989), or medial septal to posterior hypothalamus projections (Gonzalo-Ruiz *et al*., 1999; Kirk and McNaughton, 1991; Kirk *et al*. 1996). Interestingly, the results of further studies also emphasized an important role of the HPC in generating theta rhythm locally at the level of the posterior hypothalamus. Specifically, during water maze tests, the similarities between theta rhythm recorded from the HPC and SuM were studied along with assessing the level of coherence of theta oscillations recorded simultaneously in both structures. The main finding of that study was that the coherence of HPC and SuM theta rhythm is s not particularly high. Furthermore, it was shown that the SuM was driven by HPC descending activity rather than the opposite (Ruan *et al*., 2011). Subsequent studies by these authors addressed the characteristics of local theta field potentials recorded in the supramammillary/mammillary areas as a

result of bi-directional connections to the HPC through the septal complex (Ruan *et al*., 2017). It was documented that, the inactivation of the MS in freely moving rats did not attenuate MS theta field potentials, but it increased posterior hypothalamic theta frequency simultaneously diminishing its power. The authors stated that the SuM is preferentially involved in coding HPC theta at higher frequencies whereas the MS-HPC neuronal circuit modulates the frequency over the SuM/MM area by its limitation. Furthermore, all of the circuits connecting the SuM through the MS with the HPC, and the HPC descending inputs which innervates the MM, exhibit the bi-directional control of their activity (Ruan *et al*., 2017).

The data presented above strongly suggested that the posterior hypothalamic area is capable of generating theta rhythm locally. However, no systematic analysis of theta rhythmic oscillatory activity observed locally in the PHa was conducted until the 2010s (Bocian *et al*., 2016a, 2016b; Caban *et al*., 2018; Kowalczyk *et al*., 2014). The first studies aimed at providing a more detailed insight into PHa theta rhythmicity, focused on the pharmacological and cellular basis of posterior hypothalamic theta, was conducted by Kowalczyk *et al*. in 2014 (Fig. 3A). The authors have used in their studies both *in vivo* and *in vitro* experimental models. In the urethane-anesthetized rats the simultaneous EEG recordings were performed in both the HPC and the PHa. It was shown that the posterior hypothalamic theta oscillations were 7-8 times lower in amplitude compared to hippocampal theta i.e. spontaneous PHa theta rhythm was characterized by mean amplitude values of approximately 150 μV. The authors concluded, that it was due to the different cellular organization of nuclei forming the posterior hypothalamic area in comparison to the HPC. Specifically, the hippocampal formation is characterized by strict laminar organization which promotes the emergence of synchronous oscillatory phenomena. It is consistent with a statement that a given brain structure, capable of generating local field theta oscillations, should possess a laminar cytoarchitectonic organization of neurons necessary to create electrical dipoles (O'Keefe, 2007). Spontaneous PHa theta activity recorded in anesthetized animals was also examined according to its neurochemical basis. The authors documented cholinergic nature of this EEG phenomenon observed in the PHa since it was blocked after the administration of atropine sulphate (Fig. 3B; Kowalczyk *et al*., 2014). Another important observation was that the time duration of both theta and irregular activity episodes in the posterior hypothalamic area, was different compared to EEG recording taken from the HPC. Interestingly, four different variants of coappearance were observed in rats under urethane anesthesia: the presence of PHa theta oscillations characterized by the same frequency and time course as the hippocampal theta oscillations, the presence of PHa theta with the simultaneous absence of hippocampal theta, the presence of HPC theta with the simultaneous absence of PHa theta, and the simultaneous appearance of theta rhythm in both the HPC and PHa but characterized by different frequencies and time course. That finding was in contrast with previous studies indicating that the theta rhythm generated locally in the PHa is the result of descending information going from the septohippocampal system (Kocis and Vertes, 1997; Ruan *et al*., 2011; 2017). Moreover, in the same studies the ability of posterior hypothalamic in vitro preparations to generate theta oscillations was examined. It was shown that the bath

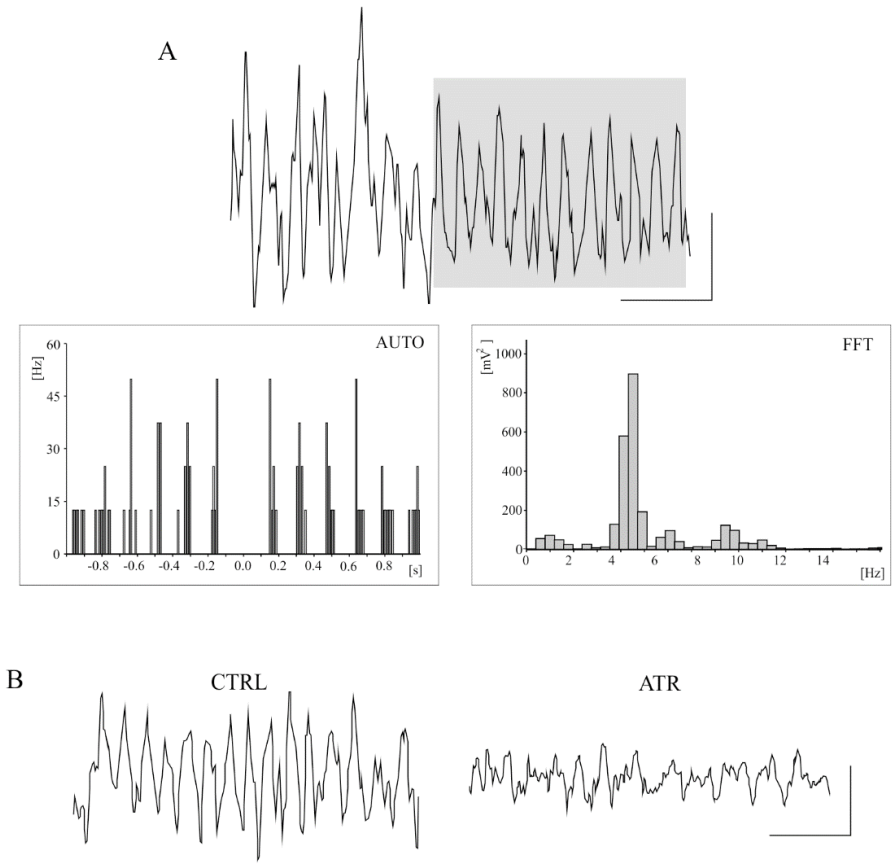

Figure 3. Theta rhythm recorded in the posterior hypothalamic area of a urethane-anesthetized rat. (A) An example of theta field potential recorded in the PHa (upper row). The bottom left row shows an auto-correlation histogram of theta sample, indicated in the upper row with grey rectangle; regularly repeating peaks in the histogram indicate the pronounced rhythmicity of the analyzed signal. The bottom right row shows the power-frequency spectrograph of the theta sample, indicated in the upper row with grey rectangle. (B) An example of PHa field potential recorded before (CTRL; left) and after (ATR; right) the administration of atropine sulphate (10 mg/kg i.v.). Calibration: 1 s, 100 μV (Kowalczyk *et al*., 2014; modified).

perfusion of completely deafferented PHa slices with cholinergic agonist carbachol (CCH) led to the generation of well-synchronized episodes of theta field activity, which again indicated that afferent inputs to the PHa are not essential for theta production in this diencephalic area (Kowalczyk *et al*., 2014).

In the subsequent studies PHa local theta field potentials were analyzed with regard to ongoing local neuronal firing repertoire (Bocian *et al*., 2016b). The PHa theta field potentials were evoked in urethane anesthetized animals by a local injection of carbachol in these studies. According to previously stated criteria of theta-related cells classification (Colom and Bland, 1987), neurons localized in the posterior hypothalamic nuclei and supramammillary nucleus were characterized on the basis of their discharge pattern examined during simultaneously recorded theta rhythm. More than half (specifically 57.7%) of the

total number of theta-related PHa neurons identified in that study were classified as theta-on tonic cells. Smaller number (specifically 33.1%) of theta-related PHa neurons were classified as theta-off tonic cells. Both types of theta-related neurons were present in the SuM as well as in the PH. Interestingly, a new type of firing pattern of posterior hypothalamic neurons was distinguished. Since this discharge pattern was not correlated with ongoing local theta activity, thus according to the authors, these neurons could not be considered as theta-related. It was suggested that these neurons, named "timing cells", and characterized by a constant, long-lasting pattern of discharges, are part of the ascending synchronizing system and provide a regular rhythmic signal that facilitates the transduction of tonic discharges of brainstem cells into theta-frequency rhythmic firing pattern (Bocian *et al*, 2016b). In the same study, additionally to phasic subtype of PHa theta-cells the presence of posterior hypothalamic theta-on phasic cells was also revealed. These neurons constituted most (specifically 81.1%) of theta-related neurons isolated and recorded in PHa in vitro preparations perfused with carbachol (Bocian *et al*., 2016b). The results of the described studies, indicating the presence of local theta-related cells in both the SuM and PH endorsed the results of previous investigations (Kowalczyk *et al*., 2014) showing the ability of PHa to generate theta oscillations independently of other brain structures and thus ruled out the previous hypothesis considering local PHa theta activity as an effect of descending hippocampal projections.

Further studies aimed at estimating the ability of the PHa to the production of oscillatory theta field potentials at different stages of postnatal development (Caban *et al*., 2018). In these studies, the CCH-induced theta rhythm was examined in PHa slices taken from 8 to 24 days old rat pups. The recorded PHa theta rhythm was increasing its probability of occurrence, amplitude values and synchrony in accordance with the preparations taken from pups at higher age. The plateau phase of PHa theta rhythm was reached at age of 22-24 days. Furthermore, the number of theta-related neurons was also increasing in PHa slices correspondingly to the increasing pups' age thus providing evidence for gradually developing the capability of generating theta rhythm (Caban *et al*., 2018).

In subsequent research, conducted in both *in vivo* and *in vitro* conditions it was shown that theta rhythm observed locally in the PHa is not dependent on gap junction communication between neurons as it was previously demonstrated for hippocampal theta (Bocian *et al*., 2016a). Specifically, the effect of broad-spectrum gap junctions (GJs) blocker – carben-oxolone (CBX) was analyzed, and unlike in the HPC, blockage of GJs did not suppress the theta rhythm recorded from the PHa in urethane anesthetized rats indicating that the posterior hypothalamic area theta rhythm's generation does not involve electrical coupling via GJs. Moreover, the administration of trimethylamine (GJs opener), which enhanced hippocampal theta, did not affect posterior hypothalamic oscillations. What is more interesting, the application of CBX resulted in an increase in PHa theta rhythm amplitude, and the observed effect was attenuated by the application of spironolactone (mineralocorticoid receptors antagonist) suggesting that enhancement of posterior hypothalamic theta activity by CBX was mediated by mineralocorticoid receptors (Bocian *et al*., 2016a).

The studies described above concerned the theta rhythm generated in the PHa as a result of cholinergic activation. The next research aimed at examining the

electrophysiological basis and potential involvement of glutamatergic receptors in the production of posterior hypothalamic oscillatory activity. It was shown that glutamatergic stimulation of the PHa with kainic acid induces well-synchronized local theta field potentials in both the supramammillary nucleus and posterior hypothalamic nuclei. The administration of non-NMDA ionotropic glutamate receptor antagonist (DNQX) successfully blocked the ability of the PHa to generate local theta oscillations in response to kainic acid in both in vivo and in vitro conditions (Kowalczyk *et al*., 2023). The results obtained in these studies were consistent with previous experiments indicating the involvement of glutamatergic receptors in the generation of theta oscillations in the limbic cortex studies (Bonansco and Buño, 2003). In the same studies, the firing pattern of glutamatergically-activated PHa theta-related neurons was examined. In *in vivo* conditions, the discharge pattern of PHa neurons activated by kainic acid administration allowed to classify them as theta-on phasic, theta-on tonic, and theta-off subclasses of theta-related neurons. In the same studies the presence of KA-activated theta-on phasic neurons was also revealed in *in vitro* maintained PHa slices. Additionally, as it was shown that as it was in the case of cholinergically-induced theta rhythm (Bocian *et al*., 2016b), also glutamatergic stimulation leads to the emergence of timing cells activity in both *in vivo* and *in vitro* conditions. What is particularly interesting, the results obtained in this study indicated, for the first time in the posterior hypothalamic area, that a subpopulation of theta-related neurons which activity was glutamatergically elicited exhibit clear subthreshold membrane potential oscillations in the theta frequency range (Kowalczyk *et al*., 2023).

Summary

The experimental evidences presented in this review clearly show, that the posterior hypothalamic area including the supramammillary nucleus and posterior hypothalamic nuclei plays significant functions in the ascending synchronizing pathway. The role of the PHa as modulator and transducer of excitatory impulsation running from the brainstem regions to the limbic structures was widely discussed and demonstrated (Bland and Vanderwolf, 1972; Kowalczyk *et al*., 2021). However, the results obtained over the last decade have shown that the impact of the posterior hypothalamic area on theta rhythm production appeared to be much more complex than it was previously thought.

Multiple studies have shown the ability of the PHa to the generation of theta oscillations and the association between these local theta oscillations with animals' movement and behavior was indicated (Bland and Vanderwolf, 1972; Sławińska and Kasicki 1995). While the presence of local posterior hypothalamic theta activity was recorded in numerous studies, the basis of this EEG phenomenon was suggested to be an effect of descending inputs from the HPC and MSvDBB (Kocis and Vertes, 1997; Ruan *et al*., 2011; Ruan *et al*., 2017). However, the latest studies have clearly shown, that the local theta field potentials recorded in the PHa could be generated independently of the HPC in both anesthetized animals and the deafferented PHa slice preparations. What is more interesting, the analysis of both SuM and PH neuronal firing pattern revealed the presence of theta neurons, related to local, posterior hypothalamic oscillatory activity (Bocian *et al*., 2016a; 2016b; Kowalczyk *et al*., 2014), which exhibit clear membrane potential oscillations (Kowalczyk *et al*., 2023). This finding is specifically important since MPOs,

referred to an intracellular theta rhythm, seem to be a crucial electrophysiological phenomenon for theta rhythm to appear in a given brain structure (Bland *et al*., 2002). In subsequent studies it was also revealed that unlike in the hippocampal formation, PHa local theta oscillations are not dependent on electrical coupling via GJs, this activity may be induced by glutamatergic stimulation, and it is modulated by corticosteroids (Bocian *et al*., 2016a; Kowalczyk *et al*., 2023).

In the light of ability of the posterior hypothalamic area to generate local theta field oscillations, it seems to be particularly important to consider this EEG pattern not only in the context of animal's locomotor activity (Bland and Vanderwolf, 1972; Sławińska and Kasicki 1995), but in the wider context of CNS functions. It was shown that, supramammillary involvement in modulation of HPC excitability indirectly influences the place-learning ability and associative memory processing (Pan and McNaughton, 1997). In addition, according to its projection to the association cortex, the PHa significantly contributes to motivated behaviors, emotion, and arousal (Pan and McNaughton, 2004). Furthermore, presented above the possibility of modulating posterior hypothalamic theta rhythm by activation of mineralocorticoid receptors may indicate the importance of this EEG pattern in the organism's response to stress and adaptation to stressful stimuli, both at the behavioral and metabolic levels (Hunter *et al*., 2009). However, the possible contribution of the PHa to the animal's stress response along with others mentioned in this review OUN functions has not been yet sufficiently examined and thus it should state a goal of future posterior hypothalamic research.

References

Allen, G.V., Hopkins, D.A. 1989. Mamillary body in the rat: topography and synaptology of projections from the subicular complex, prefrontal cortex, and midbrain tegmentum. The Journal of Comparative Neurology, 286(3), 311 –336.

Artemenko, D.P. 1972. Uchastie neĭronov gippokampa v generatsii teta-voln [Participation of hippocampal neurons in the generation of theta waves]. Neirofiziologiia = Neurophysiology, 4(5), 531–539.

Baik, K., Jung, J.H., Jeong, S.H., Chung, S.J., Yoo, H.S., Lee, P.H., Sohn, Y.H., Kang, S.W., Ye, B.S. 2022. Implication of EEG theta/alpha and theta/beta ratio in Alzheimer's and Lewy body disease. Scientific Reports, 12(1), 18706.

Bland B.H. 1986. The physiology and pharmacology of hippocampal formation theta rhythms. Progress in Neurobiology, 26(1), 1–54.

Bland, B.H., Colom, L.V. 1993. Extrinsic and intrinsic properties underlying oscillation and synchrony in limbic cortex. Progress in Neurobiology, 41(2), 157–208.

Bland, B.H., Declerck, S., Jackson, J., Glasgow, S., Oddie, S. 2007. Septohippocampal properties of N-methyl-D-aspartate-induced theta-band oscillation and synchrony. Synapse (New York, N.Y.), 61(3), 185–197.

Bland, B.H., Konopacki, J., Dyck, R.H. 2002. Relationship between membrane potential oscillations and rhythmic discharges in identified hippocampal theta-related cells. Journal of Neurophysiology, 88(6), 3046–3066.

Bland, B.H., Konopacki, J., Kirk, I.J., Oddie, S.D., Dickson, C.T. 1995. Discharge patterns of hippocampal theta-related cells in the caudal diencephalon of the urethan-anesthetized rat. Journal of Neurophysiology, 74(1), 322–333.

Bland, B.H., Oddie, S.D. 1998. Anatomical, electrophysiological and pharmacological studies of ascending brainstem hippocampal synchronizing pathways. Neuroscience and Biobehavioral Reviews, 22(2), 259–273.

Bland, B.H., Oddie, S.D. 2001. Theta band oscillation and synchrony in the hippocampal formation and associated structures: the case for its role in sensorimotor integration. Behavioural Brain Research, 127(1–2), 119–136.

Bland, B.H., Vanderwolf, C.H. 1972. Electrical stimulation of the hippocampal formation: behavioral and bioelectrical effects. Brain Research, 43(1), 89–106.

Bocian, R., Caban, B., Kłos-Wojtczak, P., Konopacki, J., Kowalczyk, T. 2016a. Is electrical coupling involved in the generation of posterior hypothalamic theta rhythm? The

European Journal of Neuroscience, 44(6), 2324 –2333.

Bocian, R., Kłos-Wojtczak, P., Caban, B., Kowalczyk, T., Kaźmierska, P., Konopacki, J. 2016b. Cell discharge correlates of posterior hypothalamic theta rhythm recorded in anesthetized rats and brain slices. Hippocampus, 26(10), 1354–1369.

Bonansco, C., Buño, W. 2003. Cellular mechanisms underlying the rhythmic bursts induced by NMDA microiontophoresis at the apical dendrites of CA1 pyramidal neurons. Hippocampus, 13(1), 150–163.

Borhegyi, Z., Varga, V., Szilágyi, N., Fabo, D., Freund, T.F. 2004. Phase segregation of medial septal GABAergic neurons during hippocampal theta activity. The Journal of Neuroscience: the official journal of the Society for Neuroscience, 24(39), 8470–8479.

Buzsáki G. 2002. Theta oscillations in the hippocampus. Neuron, 33(3), 325–340.

Caban, B., Staszelis, A., Kazmierska, P., Kowalczyk, T., Konopacki, J. 2018. Postnatal Development of the Posterior Hypothalamic Theta Rhythm and Local Cell Discharges in Rat Brain Slices. Developmental Neurobiology, 78(11), 1049–1063.

Caplan, J.B., Madsen, J.R., Schulze-Bonhage, A., Aschenbrenner-Scheibe, R., Newman, E.L., Kahana, M.J. 2003. Human theta oscillations related to sensorimotor integration and spatial learning. The Journal of Neuroscience: the official journal of the Society for Neuroscience, 23(11), 4726–4736.

Cantero, J.L., Atienza, M., Stickgold, R., Kahana, M.J., Madsen, J.R., Kocsis, B. 2003. Sleep-dependent theta oscillations in the human hippocampus and neocortex. The Journal of neuroscience: the official journal of the Society for Neuroscience, 23(34), 10897–10903.

Chrastil, E.R., Rice, C., Goncalves, M., Moore, K. N., Wynn, S.C., Stern, C.E., Nyhus, E. 2022. Theta oscillations support active exploration in human spatial navigation. NeuroImage, 262, 119581.

Clemens, B., Emri, M., Csaba Aranyi, S., Fekete, I., Fekete, K. 2021. Resting-state EEG theta activity reflects degree of genetic determination of the major epilepsy syndromes. Clinical Neurophysiology: official journal of the International Federation of Clinical Neurophysiology, 132(9), 2232–2239.

Colom, L.V., Bland, B.H. 1987. State-dependent spike train dynamics of hippocampal formation neurons: evidence for theta-on and theta-off cells. Brain Research, 422(2), 277–286.

Cross, Z.R., Corcoran, A.W., Schlesewsky, M., Kohler, M.J., Bornkessel-Schlesewsky, I. 2022. Oscillatory and Aperiodic Neural Activity Jointly Predict Language Learning. Journal of Cognitive Neuroscience, 34(9), 1630–1649.

Dannenberg, H., Pabst, M., Braganza, O., Schoch, S., Niediek, J., Bayraktar, M., Mormann, F., Beck, H. 2015. Synergy of direct and indirect cholinergic septo-hippocampal pathways coordinates firing in hippocampal networks. The Journal of Neuroscience: the official journal of the Society for Neuroscience, 35(22), 8394 –8410.

Ford, R.D., Colom, L.V., Bland, B.H. 1989. The classification of medial septum-diagonal band cells as theta-on or theta-off in relation to hippocampal EEG states. Brain Research, 493(2), 269–282.

Fu, X., Wang, Y., Ge, M., Wang, D., Gao, R., Wang, L., Guo, J., Liu, H. 2018. Negative effects of interictal spikes on theta rhythm in human temporal lobe epilepsy. Epilepsy and Behavior: EandB, 87, 207–212.

Gallego-Jutglà, E., Solé-Casals, J., Vialatte, F.B., Dauwels, J., Cichocki, A. 2015. A theta-band EEG based index for early diagnosis of Alzheimer's disease. Journal of Alzheimer's Disease: JAD, 43(4), 1175–1184.

Girardeau, G., Lopes-Dos-Santos, V. 2021. Brain neural patterns and the memory function of sleep. Science (New York, N.Y.), 374(6567), 560–564.

Gonzalo-Ruiz, A., Morte, L., Flecha, J.M., Sanz, J.M. 1999. Neurotransmitter characteristics of neurons projecting to the supramammillary nucleus of the rat. Anatomy and Embryology, 200(4), 377–392.

Hallanger, A.E., Levey, A.I., Lee, H.J., Rye, D.B., Wainer, B.H. 1987. The origins of cholinergic and other subcortical afferents to the thalamus in the rat. The Journal of Comparative Neurology, 262(1), 105–124.

Hangya, B., Borhegyi, Z., Szilágyi, N., Freund, T.F., Varga, V. 2009. GABAergic neurons of the medial septum lead the hippocampal network during theta activity. The Journal of Neuroscience: the official journal of the Society for Neuroscience, 29(25), 8094–8102.

Huerta, P.T., Lisman, J.E. 1995. Bidirectional synaptic plasticity induced by a single burst during cholinergic theta oscillation in CA1 in vitro. Neuron, 15(5), 1053–1063.

Hunter, R.G., Bellani, R., Bloss, E., Costa, A., McCarthy, K., McEwen, B.S. 2009. Regulation of kainate receptor subunit mRNA by stress and corticosteroids in the rat hippocampus. PloS one, 4(1), e4328.

Karakaş, S. 2020. A review of theta oscillation and its functional correlates. International Journal of Psychophysiology: official journal of the

International Organization of Psychophysiology, 157, 82–99.

Kawamura, H., Nakamura, Y., Tokizane, T. 1961. Effect of acute brain stem lesions on the electrical activities of the limbic system and neocortex. The Japanese Journal of Physiology, 11, 564–575.

Kirk, I.J., McNaughton, N. 1991. Supramammillary cell firing and hippocampal rhythmical slow activity. Neuroreport, 2(11), 723–725.

Kirk, I.J., McNaughton, N. 1993. Mapping the differential effects of procaine on frequency and amplitude of reticularly elicited hippocampal rhythmical slow activity. Hippocampus, 3(4), 517–525.

Kirk, I.J., Oddie, S.D., Konopacki, J., Bland, B.H. 1996. Evidence for differential control of posterior hypothalamic, supramammillary, and medial mammillary theta-related cellular discharge by ascending and descending pathways. The Journal of Neuroscience: the official journal of the Society for Neuroscience, 16(17), 5547–5554.

Kocsis, B., Martínez-Bellver, S., Fiáth, R., Domonkos, A., Sviatkó, K., Schlingloff, D., Barthó, P., Freund, T.F., Ulbert, I., Káli, S., Varga, V., Hangya, B. 2022. Huygens synchronization of medial septal pacemaker neurons generates hippocampal theta oscillation. Cell Reports, 40(5), 111149.

Kocsis, B., Vertes, R.P. 1997. Phase relations of rhythmic neuronal firing in the supramammillary nucleus and mammillary body to the hippocampal theta activity in urethane anesthetized rats. Hippocampus, 7(2), 204–214.

Kowalczyk, T., Bocian, R., Caban, B., Konopacki, J. 2014. Atropine-sensitive theta rhythm in the posterior hypothalamic area: in vivo and in vitro studies. Hippocampus, 24(1), 7–20.

Kowalczyk, T., Bocian, R., Konopacki, J. 2013. The generation of theta rhythm in hippocampal formation maintained in vitro. The European journal of neuroscience, 37(5), 679–699.

Kowalczyk, T., Staszelis, A., Bocian, R., Siwiec, M., Sowa, J.E., Tokarski, K., Kaźmierska-Grębowska, P., Caban, B. 2023. Posterior hypothalamic theta rhythm: Electrophysiological basis and involvement of glutamatergic receptors. Hippocampus, 33(7), 844–861.

Kowalczyk, T., Staszelis, A., Kaźmierska-Grębowska, P., Tokarski, K., Caban, B. 2021. The Role of the Posterior Hypothalamus in the Modulation and Production of Rhythmic Theta Oscillations. Neuroscience, 470, 100–115.

Kramis, R., Vanderwolf, C.H. 1980. Frequency-specific RSA-like hippocampal patterns elicited by septal, hypothalamic, and brain stem electrical stimulation. Brain Research, 192(2), 383–398.

Kroplewski, M., Orzel-Gryglewska, J., Nowacka, A., Trojniar, W., Jurkowlaniec, E. 2010. Differential effect of procaine injection into the rostral and caudal part of the nucleus pontis oralis on hippocampal theta rhythm in urethane-anesthetized rats. Acta Neurobiologiae Experimentalis, 70(3), 261–270.

Li, J.Y., Kuo, T.B.J., Hung, C.T., Yang, C.C.H. 2021. Voluntary exercise enhances hippocampal theta rhythm and cognition in the rat. Behavioural Brain Research, 399, 112916.

Mitchell, D.J., McNaughton, N., Flanagan, D., Kirk, I.J. 2008. Frontal-midline theta from the perspective of hippocampal "theta". Progress in Neurobiology, 86(3), 156–185.

Nowacka, A., Jurkowlaniec, E., Trojniar, W. 2002. Microinjection of procaine into the pedunculopontine tegmental nucleus suppresses hippocampal theta rhythm in urethane-anesthetized rats. Brain Research Bulletin, 58(4), 377–384.

O'Keefe, J. 2007. Hippocampal neurophysiology in the behaving animal, in: The Hippocampus Book. Oxford University Press, New York, NY, US, pp. 475–548.

Pan, W.X., McNaughton, N. 1997. The medial supramammillary nucleus, spatial learning and the frequency of hippocampal theta activity. Brain Research, 764(1–2), 101–108.

Pan, W.X., McNaughton, N. 2004. The supramammillary area: its organization, functions and relationship to the hippocampus. Progress in Neurobiology, 74(3), 127–166.

Raghavachari, S., Kahana, M.J., Rizzuto, D.S., Caplan, J.B., Kirschen, M.P., Bourgeois, B., Madsen, J.R., Lisman, J.E. 2001. Gating of human theta oscillations by a working memory task. The Journal of Neuroscience: the official journal of the Society for Neuroscience, 21(9), 3175–3183.

Ruan, M., Young, C.K., McNaughton, N. 2011. Minimal driving of hippocampal theta by the supramammillary nucleus during water maze learning. Hippocampus, 21(10), 1074–1081.

Ruan, M., Young, C.K., McNaughton, N. 2017. Bi-Directional Theta Modulation between the Septo-Hippocampal System and the Mammillary Area in Free-Moving Rats. Frontiers in Neural Circuits, 11, 62.

Semba, K., Reiner, P.B., Fibiger, H.C. 1990. Single cholinergic mesopontine tegmental neurons project to both the pontine reticular formation and the thalamus in the rat. Neuroscience, 38(3), 643–654.

Sławińska, U., Kasicki, S. 1995. Theta-like rhythm in depth EEG activity of hypothalamic areas during spontaneous or electrically induced locomotion in the rat. Brain Research, 678(1–2), 117–126.

Toll, R.T., Wu, W., Naparstek, S., Zhang, Y., Narayan, M., Patenaude, B., De Los Angeles, C., Sarhadi, K., Anicetti, N., Longwell, P., Shpigel, E., Wright, R., Newman, J., Gonzalez, B., Hart, R., Mann, S., Abu-Amara, D., Sarhadi, K., Cornelssen, C., Marmar, C., Etkin, A. 2020. An Electroencephalography Connectomic Profile of Posttraumatic Stress Disorder. The American Journal of Psychiatry, 177(3), 233 –243.

Vandecasteele, M., Varga, V., Berényi, A., Papp, E., Barthó, P., Venance, L., Freund, T.F., Buzsáki, G. 2014. Optogenetic activation of septal cholinergic neurons suppresses sharp wave ripples and enhances theta oscillations in the hippocampus. Proceedings of the National Academy of Sciences of the United States of America, 111(37), 13535–13540.

Vanderwolf, C.H. 1969. Hippocampal electrical activity and voluntary movement in the rat. Electroencephalography and Clinical Neurophysiology, 26(4), 407–418.

Vertes, R.P., Martin, G.F., Waltzer, R. 1986. An autoradiographic analysis of ascending projections from the medullary reticular formation in the rat. Neuroscience, 19(3), 873 –898.

Woodnorth, M.A., Kyd, R.J., Logan, B.J., Long, M.A., McNaughton, N. 2003. Multiple hypothalamic sites control the frequency of hippocampal theta rhythm. Hippocampus, 13(3), 361–374.

Folia Biologica et Oecologica 18: 39–47 (2024)

Acta Universitatis Lodziensis

The role of the cytokinin biosynthesis pathways in the rate of tobacco leaf senescence

PAULINA WĘZIGOWSKA[1], MAGDALENA TROJAK[2], ERNEST SKOWRON[2]

[1]Jan Kochanowski University of Kielce, Biotechnology, Uniwersytecka 7, 25-406 Kielce, Poland;
[2]Jan Kochanowski University of Kielce, Department of Environmental Biology, Uniwersytecka 7, 25-406 Kielce, Poland
E-mail: ernest.skowron@ujk.edu.pl

ABSTRACT

The regulation of leaf senescence depends on endogenous and exogenous factors, among them phytohormones like cytokinins (CKs). CKs are key players regulating the senescing process, as their endogenous concentration is linked to the onset and rate of senescence progression. Thus, this study aimed to identify the relationship between the activity of endogenous CKs biosynthesis pathways - the cytosolic mevalonate (MVA) and the plastid methyl-erythritol phosphate (MEP) and the rate of leaf senescence. To this end, three distinct tobacco (*Nicotiana tabacum* L.) cultivars – Xanthi, Golden Virginia and Monte Calme Yellow were analysed. The study involved treatment with exogenous CK – benzyladenine – and two different CK synthesis inhibitors: lovastatin and clomazone. The progression of senescence was induced by light deprivation and monitored with chlorophyll level (SPAD), photosynthetic activity (PAM) and changes in the Rubisco protein profile (SDS-PAGE). Analyses showed that the Xanthi cultivar was characterized by delayed onset of senescence and *stay-green* phenotype, while Golden Virginia, and particularly Monte Calme Yellow showed rapid leaf senescence. The studies provided valuable information regarding the role of MEP and MVA pathway of CK synthesis in the regulation of tobacco leaf senescence.

KEYWORDS: dark-induced leaf senescence, cytokinin biosynthesis, stay-green phenotype

Introduction

The final stage of a plant's life is senescence, which results in the ultimate death of and organ or whole organism. At the same time, however, during the ontogenesis, we observe that the rate of metabolic processes systematically decreases because of gradual aging. Environmental factors such as temperature, humidity and photoperiod influence the rate of senescence (Thomas,

DOI: https://doi.org/10.18778/1730-2366.18.08

2013). Also, light, which is essential for the growth and development of plants, plays a crucial role. Prolonged reduction of light intensity or its complete deprivation are very efficient activators of leaf senescence. Therefore, dark-induced leaf senescence (DILS) assay is widely accepted as a rapid and effective method for synchronously inducing the onset of senescence (Guo *et al*., 2021).

Leaves are the most important assimilatory organs (donor-type organs) (Woo *et al*., 2013), thus their senescence is orchestrated and involves changes in the expression patterns of numerous genes as well as physiological responses. Consequently, the leaf's cellular structures and biological molecules are broken down in and ordered manner and remobilised to other, acceptor-type organs (Mayta *et al*., 2019). Moreover, the leaf senescence is related to the decrease in cytokinin (CKs) concentration and activity. Their application is effective in delaying chlorophyll breakdown and maintaining the integrity of chloroplasts (Hönig *et al*., 2018). Higher plants possess two pathways of CK biosynthesis, including the cytosolic mevalonate pathway (MVA) and the plastid-localized 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway. Clomazone and lovastatin are inhibitors of the MEP and MVA pathway, respectively.

Clomazone specifically inhibits 1-deoxy-D-xylulose-5-phosphate synthase (DXS), the enzyme catalysing the first step of the MEP pathway, while lovastatin is a specific inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), blocking the rate-limiting step of the MVA pathway (Kobayashi *et al*., 2007) (Fig. 1).

Thus, the aim of the study is to identify the relationship between the activity of endogenous CKs biosynthesis pathway of

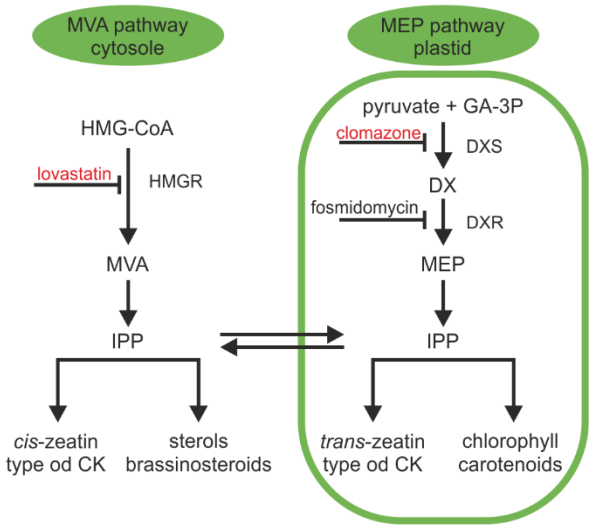

Figure 1. The alternative pathways of isoprenoid compound synthesis in plant cells are the cytosolic mevalonate pathway (MVA) and the plastidial methylerythritol phosphate pathway (MEP). The action of the applied inhibitors, lovastatin and clomazone, are indicated in red. MVA – mevalonic acid; MEP – 4-phosphate 2-methyl-D-erythritol; HMG-CoA reductase – 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMGR –3-hydroxy-3-methylglutaryl-CoA reductase; IPP – isopentenyl diphosphate; DXS – 1-deoxy-D-xylulose-5-phosphate synthase; DX – 1-deoxy-D-xylulose-5-phosphate; DXR – 1-deoxy-D-xylulose-5-phosphatereductoisomerase (according to Kobayashi *et al*., 2007; Añorga *et al*., 2020, modified).

MVA and MEP, and the rate of leaf senescence, to provide solutions to delay the onset of senescence. To this end, three distinct tobacco (*Nicotiana tabacum* L.) cultivars – Xanthi, Golden Virginia and Monte Calme Yellow were analysed. Tobacco is a plant belonging to the *Solanaceae* family, including such economically important species as tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.). In addition to tobacco industry application, tobacco is used as an important plant species in metabolic engineering and genetic manipulation research. Studies are being conducted on tobacco chloroplasts for their use in producing human proteins, while leaves are successfully utilized for the synthesis of recombinant proteins with significance in the pharmaceutical, medical, and cosmetic industries (Tsaballa *et al*., 2020). The study involved treatment with exogenous CK – benzyl-adenine – and two different CK synthesis inhibitors: lovastatin and clomazone. The progression of senescence was induced by light deprivation and monitored with chlorophyll level (SPAD), photosynthetic activity (PAM) and changes in the Rubisco protein profile (SDS-PAGE). Analyses showed that the Xanthi cultivar was characterized by delayed onset of senescence and *stay-green* phenotype, while Golden Virginia, and particularly Monte Calme Yellow showed rapid leaf senescence.

Methods

Plant material and growth conditions

The seeds of tobacco (*N. tabacum* L.) cultivars Xanthi (X), Golden Virginia (V) and Monte Calme Yellow (M) presenting different leaf pigmentation – dark (X) and pale (V and M), were sown into seedling trays with single-cell dimensions of 65 × 55 × 60 mm. Seedlings at the age of 20 DAS (*days after sowing*) were placed individually in P9 (9 × 9 × 10 cm) containers, filled with the substrate (white and black peat, perlite, and N:P:K = 9:5:10; pH 6.0–6.5), divided into groups, and transferred to environmentally controlled chambers and grown under LED RhenacM12 lamps (PXM, Podleze, Poland) delivering 200 µmol m^{-2} s^{-1} of the RGB spectrum (R:G:B; 661:633:520:434 nm) for next 25 days (Fig. 2).

Leaf senescence induction and chemical treatment

Leaf discs (10 mm in diameter) of each tobacco cultivar were cut off and analysed before treatment (day 0, "0") or incubated in Petri dishes over 72 h under the previous light regime, floating on water (negative control of senescence, "–") or without light (light deprivation) for dark-induced senescence (DILS) according to Sobieszczuk-Nowicka *et al*. (2018),

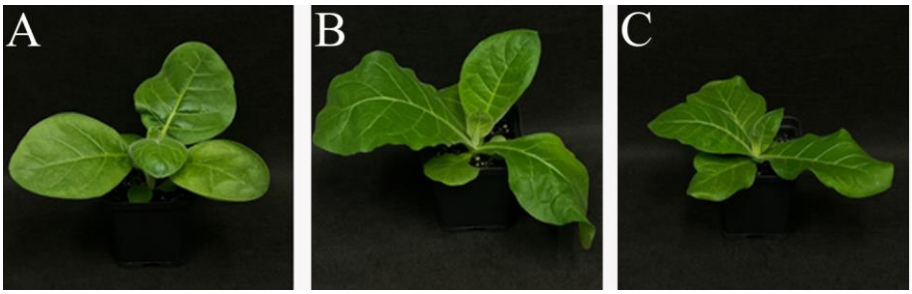

Figure 2. Morphology of 45-DAS plants of tobacco (*Nicotiana tabacum* L.) cultivars – Xanthi (A), Golden Virginia (B) and Monte Calme Yellow (C).

floating on water (positive control of senescence, "+") or with cytokinin 50 µM (benzyladenine, "B"), 50 µM lovastatin ("L") or 50 µM clomazone solution ("C").

Chlorophyll and photosynthetic activity analyses

Chlorophyll level was monitored with a non-invasive chlorophyll meter (SPAD-502, Konica Minolta, Tokyo, Japan). Photosynthetic activity was analysed with the chlorophyll a fluorescence induction curve analysis (IMAGING-PAM M-Series, Walz, Germany) and changes in the Rubisco protein profile with electrophoretic separation of proteins on a polyacrylamide gel (SDS-PAGE) stained with Coomassie Brilliant Blue R-250 Staining Solution according to Skowron and Trojak (2021) and quantified within densitometric analysis by ImageJ software (ImageJ v.1.53t., National Institutes of Health, Bethesda, USA).

Statistical analysis

Statistical analyses were performed using Statistica 13.3 software (StatSoft Inc., Oklahoma, OK, USA). The normal distribution of variables was verified using the Shapiro-Wilk test, and the equality of variances was evaluated using Levene's test. One-way ANOVA and post hoc Tukey's HSD tests were employed to analyse the differences between the investigated groups. The data are presented as mean with standard deviation (± SD). Statistical significance was determined at the 0.05 level ($p = 0.05$).

Results

Senescing leaf phenotype. Chlorophyll SPAD

It was demonstrated that the tested tobacco varieties exhibit different sensitivity to the applied compounds and rates of leaf senescence (Fig. 3).

The analysis of relative changes in chlorophyll content in leaves using the *leaf greenness index* (SPAD) for the Xanthi cultivar (X) shows SPAD value decreases after DILS compared to the control (day 0). It was noted that the

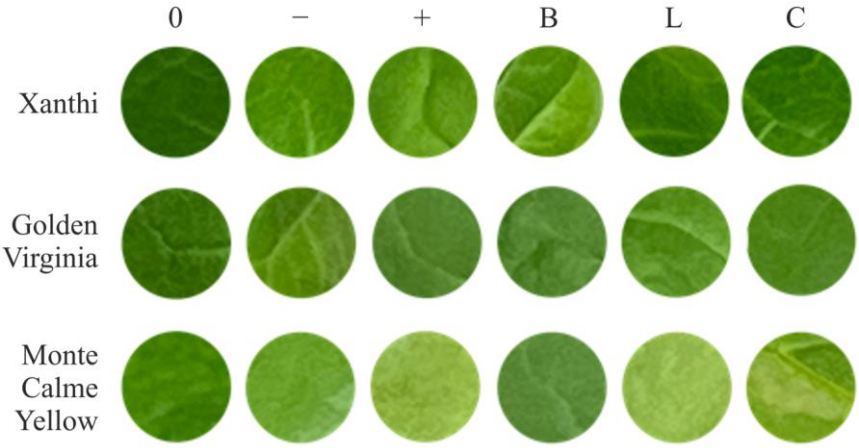

Figure 3. Phenotype observations of leaf discs cut out from 4th leaf of 45 DAS tobacco cultivars (*Nicotiana tabacum* L.): Xanthi, Golden Virginia and Monte Calme Yellow, analysed before treatment (day 0, "0") or incubated in Petri dishes over 72 h under the previous light regime (yellow area), floating on water (negative control of senescence, "−") or without light (light deprivation, grey area) for dark-induced senescence (DILS), floating on water (positive control of senescence, "+") or with cytokinin 50 µM (benzyladenine, "B"), 50 µM lovastatin ("L") or 50 µM clomazone solution ("C").

decrease in SPAD values for groups X–, X+, XB, XL, and XC amounted to 13%, 13%, 24%, 18%, and 8% respectively, relative to X0 (Fig. 4A). The SPAD analysis for the Golden Virginia cultivar (V) (Fig. 4B), like the Xanthi cultivar, indicated that chlorophyll content decreases in each treatment over the course of leaf senescence relative to day zero. However, this decrease was more pronounced, with respective reductions for V–, V+, VB, VL, and VC treatments of 35%, 27%, 22%, 31%, and 28%. The average SPAD values for groups V+, VB, VL, and VC did not exhibit statistically significant differences among them (Fig. 4B). In the case of the Monte Calme Yellow cultivar (M), the SPAD analysis (Fig. 4C) revealed that this cultivar is characterized by the lowest values at M0 compared to X0 and V0. Similar to the Xanthi and Golden Virginia cultivars, values decrease with leaf senescence in each case relative to plants at day zero. The most significant decrease (61%) was recorded for leaf disc incubation with lovastatin (Fig. 4C).

Photosynthetic activity

The effective quantum yield of photosystems II – Y(II), was measured with pulse amplitude modulation (PAM) chlorophyll a fluorometer. The analysis of the Y(II) confirmed that the most significant decrease in photosynthetic activity for Xanthi was recorded in the case of discs incubated with BA under light deprivation conditions (Fig. 5A). Statistically significant reduction in the Y(II) parameter values was noted in all groups compared to X0. For X–, X+, XL, and XC, this decrease was 13%, 25%, 39%, and 30%, respectively (Fig. 5A). In the case of Golden Virginia showed that Y(II) values of the parameter obtained in the case of the negative control V– showed 15% decrease compared to V0 (Fig. 5B). The greatest decrease in Y(II) was observed for treatment with clomazone - a decrease of 42%, and VL – a decrease of 38% compared to V0 (Fig. 5B). In the case of Y(II) in M+ plants, a decrease of 61% compared to M0 was noted. A significant decrease in Monte Calme Yellow photosynthetic activity

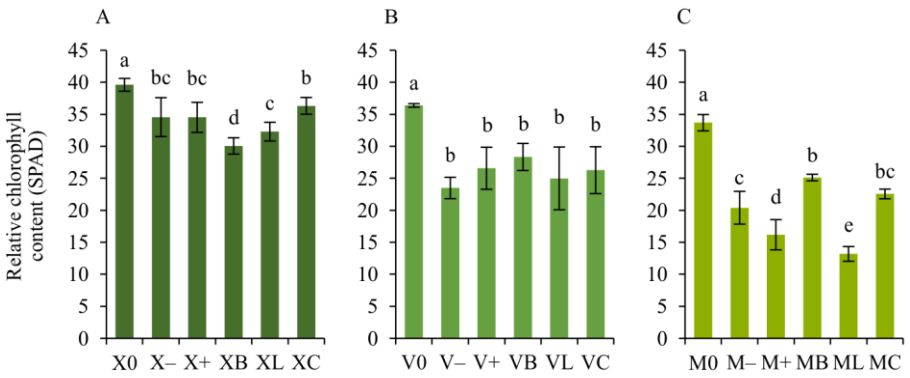

Figure 4. Analysis of relative chlorophyll content (SPAD) in leaves of tobacco (*N. tabacum* L.) cultivars – Xanthi (X) (A), Golden Virginia (V) (B) and Monte Calme Yellow (M) (C) at 45 days after sowing (DAS). 1/ "0" – material from day zero; 2/ "–" – negative senescing control, leaves kept under light in distilled water; 3/ "+" – positive senescing control, distilled water and 72 h dark incubation (DILS); 4/ "B" – 50 µM of benzyladenine (CKs) and DILS for 72 h; 5/ "L" – 50 µM lovastatin and DILS for 72 h; 6/ "C" – 50 µM clomazone and DILS for 72 h. Each bar represents the average ± SD of six independent measurements (n = 6). Different letters (a–e) indicate significant differences between treatments at p = 0.05 with a Tukey's HSD test.

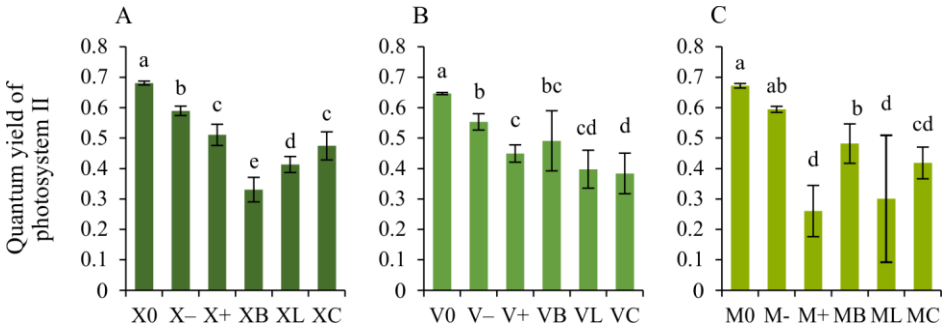

Figure 5. Analysis of photosynthetic activity with effective quantum yield of photosystem II – Y(II) in leaves of tobacco (*N. tabacum* L.) cultivars– Xanthi (X) (A), Golden Virginia (V) (B) and Monte Calme Yellow (M) (C) at 45 days after sowing (DAS). 1/ "0" – material from day zero; 2/ "–" – negative senescing control, leaves kept under light in distilled water; 3/ "+" – positive senescing control, distilled water and 72h dark incubation (DILS); 4/ "B" – 50 µM of benzyladenine (CKs) and DILS for 72h; 5/ "L" – 50 µM lovastatin and DILS for 72h; 6/ "C" – 50 µM clomazone and DILS for 72h. Each bar represents the average ± SD of six independent measurements (n = 6). Different letters (a–e) indicate significant differences between treatments at p = 0.05 with a Tukey's HSD test.

was also noted after incubation with lovastatin and clomazone. Conversely, incubation with BA exerted a protective effect on the activity of PSII despite DILS (Fig. 5C).

RuBisCO LSU and SSU

Densitometric analysis of the gel-separated protein bands (Fig. 6) revealed that the highest content of RuBisCO large (LSU) and small (SSU) subunit was noted in the control samples "0" of all cultivars tested. At the same time, leaf detachment and incubation under the previous light regime were very efficient factors in inducing senescence-related protein degradation (negative senescing control). Surprisingly, DILS incubation slowed down this process in Xanthi and Golden Virginia samples. The most effective for delaying leaf senescence was incubation with BA, which for LSU showed only a 5% lower level when compared to X0 and XB. In the case of the Golden Virginia cultivar, combined effects of light deprivation and treatment with clomazone, resulted in LSU and SSU decreases by 53% and 15%, respectively, when compared to V0 (Fig. 6). We also showed that in Monte Calme Yellow cultivar both DILS as well as clomazone treatment decrease LSU by 66%, whereas for SSU by 47% and 41%, respectively.

Discussion

The study presents preliminary results of investigation of the impact of exogenous cytokinin – benzyladenine, as well as inhibitors of CKs' alternative synthesis pathways – lovastatin and clomazone, on the leaf senescing process of *N. tabacum* L. cultivars: Xanthi, Golden Virginia, and Monte Calme Yellow induced by light deprivation (DILS). Surprisingly, the study revealed that leaf detachment and subsequent incubation under light was an even more effective factor to induce the onset of senescence than DILS itself. Similarly, Zhao *et al*. (2012) showed that mechanical leaf detachment from the plant was a sufficient factor to induce the senescing rate of wheat leaves (*Triticum aestivum* L. var. Shiluan 02-1). This phenomenon can be explained by the decrease in endogenous CK levels, which are no longer exported from the parent plant to

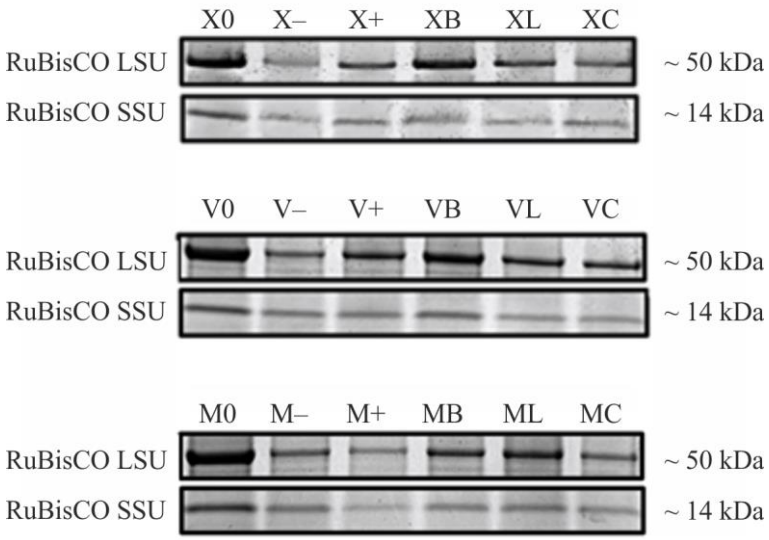

Figure 6. Analysis of total soluble proteins resolved in polyacrylamide 4–20% TGX gel stained with Coomassie Brilliant Blue reagent. The large (LSU) and small (SSU) subunits of RuBisCO isolated from tobacco (*N. tabacum* L.) leaves of Xanthi (X), Golden Virginia (V) and Monte Calme Yellow (M) at 45 days after sowing (DAS). 1/ "0" – material from day zero; 2/ "–" – negative senescing control, leaves kept under light in distilled water; 3/ "+" – positive senescing control, distilled water and 72h dark incubation (DILS); 4/ "B" – 50 μM of benzyladenine (CKs) and DILS for 72h; 5/ "L" – 50 μM lovastatin and DILS for 72h; 6/ "C" – 50 μM clomazone and DILS for 72h.

leaf tissues due to mechanical separation (Janečková *et al*., 2018).

The actual effect of clomazone on leaf senescence induced by light deprivation and/or mechanical damage, and the identification of cultivar-specific sensitivity, was realized by XC, VC, and MC comparison within corresponding X+/X–, V+/V–, and M+/M– treatments. These additional controls indicate that among the analysed cultivars Xanthi shows low sensitivity to clomazone treatment, while more clomazone sensitive is Golden Virginia and Monte Calme Yellow. Results are consistent with previous reports. Darwish *et al*. (2015) documented that the Xanthi cultivar exhibits greater tolerance to clomazone compared to the Golden Virginia type variety (Virginia vk51). In the case of the Monte Calme Yellow, although there are no direct literature analyses specific to this cultivar, studies have been conducted on the same tobacco group – Burley (Yellow Burley). Li *et al*. (2017) and Li *et al*. (2021) demonstrated that Burley group varieties, such as Monte Calme Yellow, are characterized by a light green leaf colour, resulting from a chloroplastic mutation leading to a reduction in photosynthetic pigment content – both carotenoids and chlorophyll *a* and *b*. This formed the basis for selecting this variety for analysis due to the application of selective inhibitors of MEP and MVA pathways, which, besides CK synthesis, are responsible for the synthesis of photosynthetic pigment precursors. However, results show that Monte Calme Yellow at day zero control showed a similar pigments concentration to Golden Virginia, due to enhanced nitrogen fertilization. Yet cultivar is still more

sensitive to pigment loss under the senescing progression.

Conclusions

Preliminary results of our study shows that the photosynthetic activity of the studied tobacco cultivars is not directly correlated with the leaf senescence pattern. The Xanthi exhibits the highest resistance, while Monte Calme Yellow shows the lowest resistance to factors inducing leaf senescence – mechanical detachment and light deprivation. Secondly, tobacco cultivars classified as bright types (Golden Virginia and Monte Calme Yellow) present faster progression of leaf senescence compared to cultivars with darker leaf pigmentation (Xanthi), as indicated by the SPAD analysis. All analysed tobacco cultivars exhibit different sensitivity to clomazone, lovastatin, and benzyladenine, which is significant in the context of further research. For the Xanthi, which displayed *stay-green* phenotype trait, leaf incubation with exogenous cytokinin resulted in decreased photosynthetic activity, yet this variety was insensitive to MVA and MEP pathway inhibitors. Sensitivity to clomazone was confirmed for the Golden Virginia, while a delay in leaf senescence was observed for the Monte Calme Yellow following lovastatin application.

Acknowledgements

This research was funded by the Polish Ministry of Science and Higher Education (Grant No. SUPB.RN.24.211, E.S, M.T.) and the Polish Agency for Restructuring and Modernisation of Agriculture (Grant No. DDD.6509.00044.2022. 13, M.T., E.S.).

References

Añorga, M., Pintado, A., Ramos, C., De Diego, N., Ugena, L., Novák, O., Murillo, J. 2020. Genes ptz and idi, coding for cytokinin biosynthesis enzymes, are essential for tumorigenesis and in planta growth by P. syringae pv. savastanoi NCPPB 3335. Frontiers in Plant Science, 11: 1294.

Darwish, M., Vidal, V., Lopez-Lauri, F., Alnaser, O., Junglee, S., El Maataoui, M., Sallanon, H. 2015. Tolerance to clomazone herbicide is linked to the state of LHC, PQ-pool and ROS detoxification in tobacco (Nicotiana tabacum L.). Journal of Plant Physiology, 175: 122–130.

Guo, Y., Ren, G., Zhang, K., Li, Z., Miao, Y., Guo, H. 2021. Leaf senescence: Progression, regulation, and application. Molecular Horticulture, 1(1): 1–25.

Hönig, M., Plíhalová, L., Husičková, A., Nisler, J., Doležal, K. 2018. Role of cytokinins in senescence, antioxidant defence and photosynthesis. International Journal of Molecular Sciences, 19(12): 4045.

Janečková, H., Husičková, A., Ferretti, U., Prčina, M., Pilařová, E., Plačková, L., Pospíšil, P., Dolezal, K., Špundová, M. 2018. The interplay between cytokinins and light during senescence in detached Arabidopsis leaves. Plant, Cell & Environment, 41(8): 1870–1885.

Kobayashi, K., Suzuki, M., Tang, J., Nagata, N., Ohyama, K., Seki, H., Kiuchi, R., Kaneko, Y., Nakazawa, M., Matsui, M., Matsumoto, S., Yoshida, S., Muranaka, T. 2007. Lovastatin insensitive 1, a novel pentatricopeptide repeat protein, is a potential regulatory factor of isoprenoid biosynthesis in Arabidopsis. Plant and Cell Physiology, 48(2): 322–331.

Li, Y., Chang, D., Zhang, X., Shi, H., Yang, H. 2021. RNA-Seq, physiological, and biochemical analysis of burley tobacco response to nitrogen deficiency. Scientific Reports, 11(1): 16802.

Li, Y., Yang, H., Chang, D., Lin, S., Feng, Y., Li, J., Shi, H. 2017. Biochemical, physiological and transcriptomic comparison between burley and flue-cured tobacco seedlings in relation to carbohydrates and nitrate content. Molecules, 22(12): 2126.

Mayta, M.L., Hajirezaei, M.R., Carrillo, N., Lodeyro, A.F. 2019. Leaf senescence: The chloroplast connection comes of age. Plants, 8(11): 495.

Skowron, E., Trojak, M. 2021. Effect of exogenously-applied abscisic acid, putrescine and hydrogen peroxide on drought tolerance of barley. Biologia, 76(2): 453–468.

Sobieszczuk-Nowicka, E., Wrzesiński, T., Bagniewska-Zadworna, A., Kubala, S., Rucińska-Sobkowiak, R., Polcyn, W., Misztal, L., Mattoo, A. K. 2018. Physio-genetic dissection of dark-induced leaf senescence and timing its reversal in barley. Plant Physiology, 178(2): 654–671.

Thomas, H. 2013. Senescence, ageing and death of the whole plant. New Phytologist, 197(3): 696 –711.

Tsaballa, A., Sarrou, E., Xanthopoulou, A., Tsaliki, E., Kissoudis, C., Karagiannis, E., Michailidis, M., Martens, S., Sperdouli E., Hilioti, Z., Fotopoulos, V., Nianiou-Obeidat, I., Tsaftaris, A., Madesis, P., Kalivas, A., Ganopoulos, I. 2020. Comprehensive approaches reveal key transcripts and metabolites highlighting metabolic diversity among three oriental tobacco varieties. Industrial Crops and Products, 143: 111933.

Woo, H.R., Kim, H.J., Nam, H.G., Lim, P.O. 2013. Plant leaf senescence and death–regulation by multiple layers of control and implications for aging in general. Journal of Cell Science, 126(21): 4823–4833.

Zhao, L., Zhang, H., Zhang, B., Bai, X., Zhou, C. 2012. Physiological and Molecular Changes of Detached Wheat Leaves in Responding to Various Treatments. Journal of Integrative Plant Biology, 54(8): 567–576.

Folia Biologica et Oecologica 18: 48–65 (2024)

Acta Universitatis Lodziensis

Selected biological properties of quercetin, curcumin, and kaempferol

MAŁGORZATA WIELGUS, NIKOLA ZANIEWICZ

University of Lodz, Faculty of Biology and Environmental Protection, Biochemical Section of the Student Biologists' Scientific Club, Pomorska 141/143, 90–236 Lodz
E-mail: malgorzata.wielgus@edu.uni.lodz.pl

ABSTRACT

Polyphenols are a large group of organic compounds present in plants, where they play various roles pivotal to their proper physiological functioning. Polyphenols are ubiquitous in many dietary sources such as fruits, vegetables, beverages, seeds, and honeys. Diet plays a crucial role in sustaining overall well-being of the organism and preventing diseases, including cancer. Despite broad spectrum of health promoting activity of polyphenols, such as antioxidant, anti-inflammatory and antimicrobial, many of them are also potent anti-cancer compounds. In this review we focused on presentation of three polyphenols such as quercetin, curcumin, and kaempferol. We discussed recent studies concerning their beneficial impact on human health and potential as anticancer agents.

KEYWORDS: polyphenols, anticancer, anti-inflammatory, neuroprotection

Introduction

Polyphenols are a large group of organic compounds which chemical structure is characterized by presence of at least one hydroxyl group affixed to an aromatic ring. They are found in the tissues of many plants, where they play crucial role in defending from environmental stress, such as unfavourable temperature and light (Lattanzio, 2013). Numerous studies investigated various health benefits of dietary intake of polyphenols. They are known to have anti-inflammatory, antioxidant, and antimicrobial properties. Moreover, polyphenols have an immense potential for development of anticancer drugs (Zhou *et al*., 2016). Quercetin, curcumin and kaempferol are well-known phenolic compounds ubiquitous in human diet, especially sources such as fruits and vegetables (Fig. 1).

Bioavailability

Even though polyphenols are immensely common in human diet, their impact on health is intricate due to their relatively low bioavailability, with estimated rate of absorption from 0.3 to 43%. Moreover, human body reacts to phenolic compounds such as polyphenols in the same way it reacts to xenobiotics, thus they are being rapidly excreted (Albuquerque *et al*., 2021). The term

DOI: https://doi.org/10.18778/1730-2366.18.09

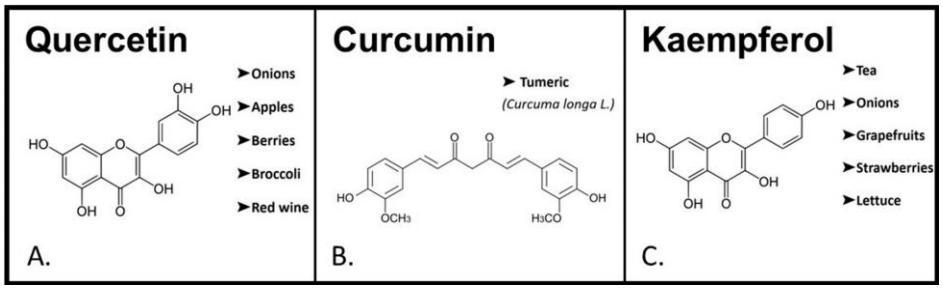

Figure 1. Chemical structures of quercetin (A), curcumin (B), and kaempferol (C) and their main dietary sources.

"bioavailability" derives from pharmacology and refers to the time and amount to which a drug reaches its target of action. Currently the most accurate definition of this term is probably "that fraction of an ingested nutrient or compound that reaches the systemic circulation and the specific sites where it can exert its biological action" meaning simply how much of ingested polyphenol will reach target tissue and perform its beneficial action (D'Archivio *et al*., 2010).

Bioavailability of polyphenols depends on their concentration in food, kind of food matrix, its preparation and interactivity with other compounds such as protein bonding (Visioli *et al*., 2014). Moreover, despite their large bioactivity, they might perform poor effectivity in the human body due to their lower intrinsic activity or poor absorption from the intestine or expeditious elimination. Interestingly, although detailed mechanisms of intestinal absorption and metabolism of are not investigated, it is assumed that most of the polyphenols might be too hydrophilic to infiltrate the gut wall by passive diffusion (Manah *et al*., 2004). Furthermore, absorption of phenolic compounds depends on the release of microbial metabolism and activity of digestive enzymes localized in epithelial cells of small intensine, such as lactase phloridzin hydrolase (LPH) and cytosolic b-glucosidase (CBG), which facilitates absorption of polyphenols through hydrolysation of their glycosides. However, it is assumed that compounds with high level of polymerization are not able to be absorbed properly the in small intensine, as a result of which only small part of them undergo further metabolism and reaches circulatory system (Hui Teng and Lei Chen, 2018).

Quercetin

Quercetin (3,3',4',5,7-pentahydroxy-flavone) (Fig. 1A) draws its name from the Latin word *Quercetum*, which means "Oak Forest" and belong to one of the six subclasses of flavonoids called flavonols. It is a plant pigment present in high concentrations in onions, grapes, berries, and broccoli (Anand David *et al*., 2016). In plants, quercetin plays various tasks in facilitating their proper physiological functioning, through regulating ROS (reactive oxygen species) level. Quercetin also takes part in modulating auxin signalling and, therefore has an impact on the growth of the plant (Singh *et al*., 2021). Selected biological properties of quercetin are presented in Figure 2.

Occurrence

Quercetin is present in many dietary and medical plants. It is abundant in onions, apples, tea, red wine, and *Ginkgo biloba* (Williamson *et al*., 2005). It might

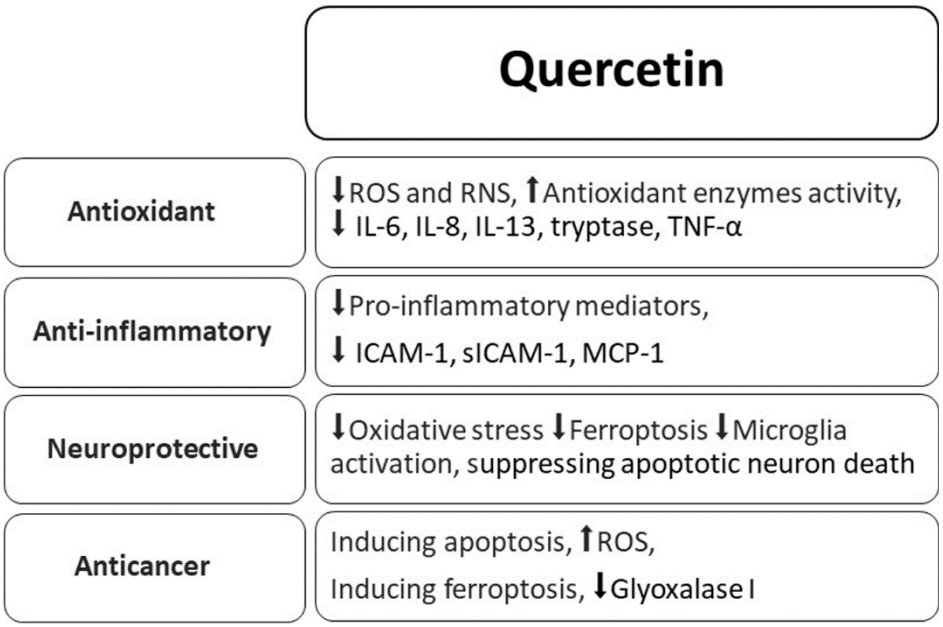

Figure 2. Summary of the most important biological properties of quercetin discussed in the review.

also be found in berries. Häkkinen *et al*. (1999) analysed 16 species of cultivated berries and 9 species of wild berries and found quercetin in all of them, unlike other flavonols examined in the study. The highest concentration of quercetin was found in the wild bog whortleberry *Vaccinium uliginosum* (158 mg/kg, fresh weight). A high concentration of quercetin was also noted in cranberries (83 and 121 mg/kg). Numerous glucoside forms and quercetin aglycone were found in onion bulbs (*Allium cepa* L.), which belong to the richest sources of flavonoids in human diet (Slimestad *et al*., 2007). Interestingly, the kind of food matrix is important, when it comes to concentration and bioavailability of polyphenols, including quercetin. Wiczkowski *et al*. (2008) examined, that in dry shallot skin total content of quercetin was more than 20 times higher than in the flesh. Moreover, authors suggest that quercetin aglycone which is abundant in dry shallot skin is more bioavailable due to its low hydrophilicity than quercetin glucosides present in the flesh. Furthermore, quercetin was also detected in 19 honeys of different floral and non-floral sources (Petrus *et al*., 2011).

Anti-inflammatory Activity

Quercetin is known for its anti-inflammatory properties (Oršolić *et al*., 2004). Inflammation is a complex immune response of the organism triggered by the harmful biological, chemical of physical stimuli. Immune mechanisms include the involvement of immune cells, for example, basophils, mast cells, macrophages, monocytes, and inflammatory mediators. Examples of these mediators are inflammatory cytokines, especially interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), and ROS. Excess of ROS might offset and exacerbate inflammation via degradation of the IκB – inhibitor associated with transcription factor NF-

κB. As a result of that, active NF-κB upregulates expression of genes associated with inflammatory response, increasing production of proinflammatory cytokines (Morgan *et al*., 2011). With that being said, the antioxidative properties of flavonoids such as quercetin are important factors in reducing inflammation. Carullo *et al*. (2017) compared the effects of quercetin and its derivatives derived from different plant extracts. These compounds performed anti-inflammatory actions via e.g., ROS reduction, inhibition of NF-κB and decreasing levels of proinflammatory mediators. Extracts containing quercetin appeased inflammation in gastrointestinal disorders, obesity, gout, and atherosclerosis.

Multiple in vitro and in vivo studies examined mechanism of inflammation reduction as a result of quercetin intake. Quercetin was confirmed to suppress release of pro-inflammatory mediators such as IL-6, IL-8, IL-13, tryptase, and TNF-α in human mast cells. Moreover, quercetin inhibited activation of the calcium-insensitive protein kinase C theta (PKC θ), suggesting its potential in the treatment of allergies (Kempuraj *et al*., 2005). Mast cells products such as tryptase might also be associated with the process of neurodegenerative disease, which indicates quercetin's potential in their treatment since it suppresses mast cells exocytosis (Chirumbolo *et al*., 2010). Additionally, Bureauet *et al*. (2008) investigated that treatment with quercetin suppressed the apoptotic death of PC12 neurons mediated by microglial inflammatory activation. It also inhibited IL-1a and TNF-α gene expression in N9 microglia cells treated with lipopolysaccharide (LPS). Furthermore, Cheng *et al*. (2019) examined that quercetin suppressed protein and gene expression of intercellular adhesion molecule-1 (ICAM-1), soluble ICAM-1 (sICAM-1), monocyte chemoattractant protein-1(MCP-1), IL-6 and IL-8, which level increased as a result of stimulation of ARPE-19 cells with proinflammatory cytokine IL-1β. Several *in vivo* studies showed various health effects of quercetin, such as regulating immune response in obese rats and improving retrieval of motor functions in rats with severe spinal cord injury (Di Petrullo *et al*., 2022; Li *et al*., 2016)

Anti-cancer Activity

'Cancer' is a generic term that embraces a wide spectrum of diseases affecting different parts of the body. It is an effect of a multi-step and multi mechanism process called oncogenesis (Nakamura *et al*., 2005). Heavy issue of this disease is the rapid formation of abnormal cells which grow beyond their usual size and invade vicinal areas, eventually causing the process called metastasis (invasion of other organs and generating secondary tumours), which is a final stage of the cancer leading to death. As already mentioned above, multiple studies demonstrated potential of quercetin in regulating inflammation. Prolonged or chronic inflammation might lead to carcinogenesis.

First to make connection between inflammation and cancer after noting presence of lymphocytes in neoplastic tissue was Rudolf Virchow in 1863 (Balkwill and Mantovani, 2001). Certain chronic infections, such as autoimmune disease and microbial infections are associated

with certain types of cancer (for instance, inflammatory bowel disease with colon cancer and infection with *Helicobacter pylori* with gastric cancer) (Mantovani *et al*., 2008). Moreover, infection with *Schistosomiasis* is associated with bladder malignancy and infection with *Papillomavirus* is linked to cervical cancer (Balkwill and Mantovani, 2001).

Chronic inflammation results in excessive generation of ROS. Macrophages produce reactive oxygen and nitrogen species in order to eradicate harmful stimuli such as pathogens. This immoderate production of inflammatory mediators taking place during chronic infection might lead to mutations and DNA damage. Tumour and tumour-associated cells might produce major pro-inflammatory cytokines and chemokines which might lead to malignant progression. Many of them are induced by hypoxia, which significantly differentiates tumours from normal tissue (Singh *et al*., 2019).

Diet plays pivotal role in prevention and treatment of cancer (Narimatsu and Yaguchi, 2022). Quercetin is the most common flavonoid in the human diet and one of the most sufficient antioxidants (Formica and Regelson, 1995; Prior, 2003). Thus, it is a potent compound in anti-cancer therapy due to its health beneficial properties. Quercetin inhibits glyoxalase I which plays crucial role in production and regeneration of key factors of tumour growth, such as D-lactase and glutathione (Formica and Regelson, 1995). Quercetin also inhibits ferroptosis which is a special kind of programmed cell death, connected with excessive production of ROS and accumulation of iron, characteristic for many diseases including diabetes, renal and liver injuries, and neurodegenerative diseases such as Parkinson's disease and epilepsy. In all of these injuries, quercetin alleviated ferroptosis by preventing ROS production and other mechanisms like preventing iron accumulation, increasing level of glutathione (GSH), and glutathione peroxidase 4 (GPX4) (Cruz-Gregorio and Aranda-Rivera, 2023). GSH is a key molecule used by many enzymes to neutralize ROS (Ferreira *et al*., 2023). GPX4 is an important antioxidant enzyme which plays crucial role in alleviating ferroptosis via reduction of phospholipid hydroperoxides (Xue *et al*., 2023).

Quercetin also inhibits ferroptosis present during lung inflammation and asthma through decreasing levels of pro-inflammatory mediators. On the other hand, quercetin promotes ferroptosis in cancer cells by increasing the iron level leading to excessive production of ROS. Moreover, quercetin decreases level of GPX4 and induces ferritinophagy, which facilitates iron 'recycling' and induces apoptosis (Cruz-Gregorio and Aranda-Rivera 2023). Interestingly, in comparison with normal cells, cancer cells are more iron-dependent and vulnerable to ferroptosis. Wang and colleagues examined that quercetin induces cancer cell death via ferroptosis induced by ROS and lysosome activation mediated by transcription factor EB (TFEB) (Wang *et al*., 2021). Among cancers, lung cancer is the main cause cancer-related deaths worldwide (Ferlay *et al*., 2015). Non-small cell lung cancer (NSCLC) is a histological subtype of lung cancer involving adenocarcinoma, squamous cell carcinoma and large-cell carcinoma histosubtypes, referring to about 85% of new cases of lung cancer (Gridelli *et al*., 2015). Recent studies reveal that quercetin and its derivatives perform therapeutic effects on NSCLC (Alsharairi, 2023). In the research conducted by Zhou *et al*. (2023) quercetin reduced proliferation in A549 and H1299 NSCLC cells while not having such effect on normal lung epithelial BEAS-2B cells. Moreover, authors concluded that quercetin via SIRT5/PI3K/AKT pathway induces apoptosis and DNA damage in NSCLC cells but not in normal cells. In another recent study, quercetin inhibited glucose-6-phosphate dehydrogenase (G6PD) which level is increased in many cancers and is associated with drug resistance. Via inhibiting G9PD quercetin had an impact on degradation of

EGFRT790M, a common mutation in NSCLC (Ge *et al*., 2023).

Curcumin

Curcumin (1,7-Bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) (Fig. 1B) also known as diferuloyl methane, is a compound extracted from the rhizomes of turmeric (*Curcuma longa* L.). Curcumin is one of the curcuminoids, which are a subclass of non-flavonoid polyphenols. Curcumin has multiple applications worldwide and is being used in food, beverages, cosmetics, as a colorant, and as antiseptic (Hewlings and Kalman, 2017). It has been used in medicine for centuries due to its various health promoting properties (Fig. 3) (Priyadarsini, 2014). For example, in traditional Indian medicine turmeric is being used for healing diabetic wounds, rheumatism, and hepatic disorders (Eigner and Scholz, 1999). In recent years curcumin has been the subject of many studies and it has been examined to have antioxidative, anti-inflammatory, anti-cancer, anti-aging, antimutagenic, antimicrobial, cardioprotective, hepato-protective, anti-diabetic, and anti-aging properties (Hewlings and Kalman, 2017; Kotha and Luthria, 2019; Monroy *et al*., 2013).

Antioxidant Activity

Curcumin is a strong, lipid soluble antioxidant that, contrary to most natural antioxidants, has both phenolic and a b-diketone group on the same molecule (Priyadarsini, 1997). Important property of curcumin is the ability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS), thus preventing lipid membrane peroxidation which has deleterious results for the whole organism (Visioli *et al*., 2014; Wright, 2002). Curcumin also increases the activity of catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD) and heme oxygenase-1 (OH-1), important antioxidant enzymes (Pulido-Moran *et al*., 2016). Moreover, curcumin can inhibit the of expression ROS-generating enzymes such as cyclooxygenase-2 (COX-2) and 5-lipooxygenase (5-LOX), as well as vascular endothelial growth factor (VEGF), phosphorylated signal transducers and activators of transcription 3 (STAT3) and matrix metalloproteinase-9 (MMP-9), factors directly associated with tumorigenesis (Lin *et al*., 2007). Due to its lipophilic character, curcumin is often compared to vitamin E and considered as a chain-breaking antioxidant (Hewlings and Kalman, 2017; Priyadarsini *et al*., 2003).

Anti-Inflammatory Activity

As already mentioned in this review, oxidative stress is associated with exacerbation of inflammation, which leads to development of numerous chronic illnesses. Curcumin performed therapeutic effect on many of them, including such as neurodegenerative disease, allergy, metabolic syndrome, cancer, asthma, diabetes, obesity, depression, epilepsy, cerebral injury arthritis, and acquired immune deficiency syndrome (AIDS). Supplementation of curcumin was proved to significantly decrease serum levels of TNF-a, IL-6, MCP-1 and transforming growth factor beta (TGF-β) in subjects with metabolic syndrome (Panahi *et al*., 2016). Moreover, curcumin inhibited NF-κB-related upregulation of cardiac pro-inflammatory genes, which is involved in inflammation causing cardiomyocytic injury in cardiopulmonary bypass (CPB) and cardiac and global ischemia and reperfusion (I/R). Furthermore, curcumin is able to regulate glucose levels in blood and increase plasma insulin levels in diabetes via decreasing oxidative stress and lipid peroxidation (Aggarwal and

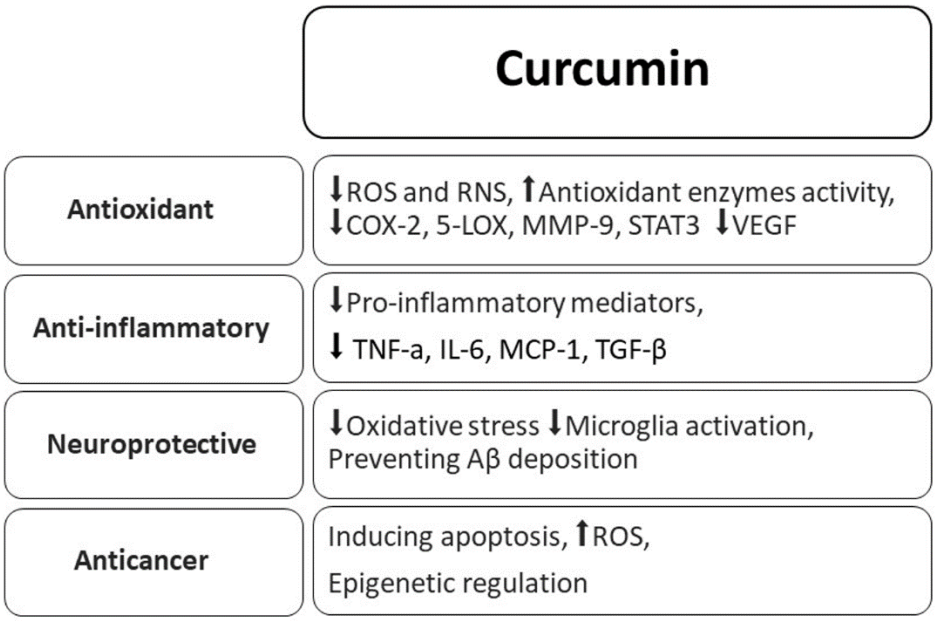

Figure 3. Summary of the most important biological properties of curcumin discussed in the review.

Harikumar, 2009). Additionally, curcumin alleviates skin disorders via moderating inflammation. For example, in psoriasis curcumin decreased levels of TNF-α, IL-2, IL-12, IL-22, IL-23, and IFN-gamma. Due to its antimicrobial properties, curcumin also performed therapeutic effects in bacterial skin infections by inhibiting growth and disruption of bacterial cell membrane and was efficient even against multi drug resistant bacteria (Vollono *et al*., 2019).

Curcumin also has potential as a drug in neurodegenerative disease treatment and neuroprotection. It is able to reduce inflammation in central nervous system (CNS) by inhibiting expression of pro-inflammatory cytokines (IL-1α, IL-6 and TNF-α) by microglial cells, innate macrophages of CNS, which activation plays crucial role in pathogenesis of neurodegenerative disorders such as Alzheimer's disease (AD) (Hansen *et al*., 2018; Monroy *et al*., 2013). Moreover, curcumin may stimulate microglia to phagocytize β-amyloid (Aβ) aggregates, which formation is involved in the pathogenesis of Alzheimer's disease and Huntington's disease. Interestingly, curcumin itself is able to inhibit formation of β-amyloid fibrils due to its high affinity to this protein (Monroy *et al*., 2013). Furthermore, curcumin inhibits neuronal death and mitochondrial dysfunctions induced by various factors such as stimulating and neurotoxic compounds, lifestyle- and excitotoxicity- induced neurodegeneration, and pathologies associated with protein aggregation, thus it has an excellent potential to protects CNS against neurodegenerative disease (Bagher *et al*., 2020).

Anticancer Activity

On the other hand, depending on the concentration and presence of metal ions, curcumin might selectively perform pro-oxidative activities in malignant cells, which may indicate her potential as an

anti-cancer drug (Pulido-Moran *et al*., 2016). In recent years, numerous studies investigated anti-cancer properties of curcumin in many cancers such as breast and lung cancer, haematological cancers, cancers of digestive system and in other kinds of cancer, such as prostate cancer and head and neck cancers (Giordano and Tommonaro, 2019). In the study conducted by Kim *et al*. (2016), curcumin reduced proliferation and induced apoptosis in human cervical cancer cells via generating endoplasmic reticulum (ER) stress resulting in unfolded protein response (UPR), which increased level is a marker of cell death. Interestingly, curcumin did not show any of these actions on normal cells. Moreover, curcumin induced apoptosis small cell lung cancer (SCLC) cells. In comparison with NSCLC already mentioned in this review, SCLC occurs less frequently but is more aggressive and has much higher fatal rate. Yang and colleagues examined that curcumin caused ROS overproduction, decrease of mitochondrial membrane potential, and activation of apoptosome, thus led to apoptosis of NCI-H446 cancer cells (Yang *et al*., 2012). Curcumin is a potential therapeutic agent in colorectal cancer (CRC), which is second the most fatal cancer worldwide (Ionescu *et al*., 2023). Studies suggest the role of curcumin in epigenetic changes in cancer cells. Epigenetic changes, such as DNA methylation and histone modifications which cause remodelling of the chromatin and result in phenotype changes, are involved in the pathogenesis of many diseases, including cancer (Rajendran *et al*., 2022). Treatment with curcumin caused alterations in gene expression and DNA methylation in HCT116, RKO and HT29 colorectal cancer cells (Link *et al*., 2013). In recent study it was examined that curcumin increased ROS levels in HCT116 cancer cells and therefore activated KEAP1/NRF2/miR-34a/b/c pathway leading to suppression of the tumour (Liu *et al*., 2023).

Kaempferol

Kaempferol, (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) (Fig. 1C), owning its name after Engelbert Kaempfer who was a German doctor, naturalist, and historian thanks to which Europe could acquaint with traditional methods of Asian medicine (Periferakis *et al*., 2020), is an organic chemical compound belonging to the group of flavonoids which are a class of polyphenols (Chen *et al*., 2022). It is a tetrahydroxyflavone in which the four hydroxy groups are located at positions 3, 5, 7, and 4' (Imran *et al*., 2019). Kaempferol is immensely substantial in most edible plants such as tea, fruits and vegetables including species: onion (*Allium cepa*) (Rodríguez Galdón *et al*., 2008), tea (*Camellia sinensis*) (Lee *et al*., 2008), grapefruit (*Citrus paradisi*) (Gupta *et al*., 2018), strawberry (*Fragaria vesca*) (Sun *et al*. 2014), and lettuce (*Lactuca* sativa) (Złotek *et al*., 2014) as well as in medicinal plants for instance *Kaempferia galanga* (L.) (Huang *et al*., 2008), *Acacia nilotica* (L.) (Al-Nour *et al*., 2019), *Aloe vera* (L.) (Keyhanian *et al*., 2007), *Crocus* sativus (L.) (Mokhtari-Zaer *et al*., 2015), *Ginkgo biloba* (L.) (Zhang *et al*., 2008), *Hypericum perforatum* (L.) (Silva *et al*., 2008), and *Rosmarinus officinalis* (L.) (Bai *et al*., 2010).

Traditionally, plants rich in kaempferol were used to treat the symptoms of hypertension, abdominal pains, headache, rheumatism, toothache, dyspepsia, coughs, and inflammatory tumour. Currently, many studies have shown kaempferol's much broader spectrum of health-promoting effects (Fig. 4). In particular, antioxidant, anti-inflammatory, antimicrobial, anticancer,

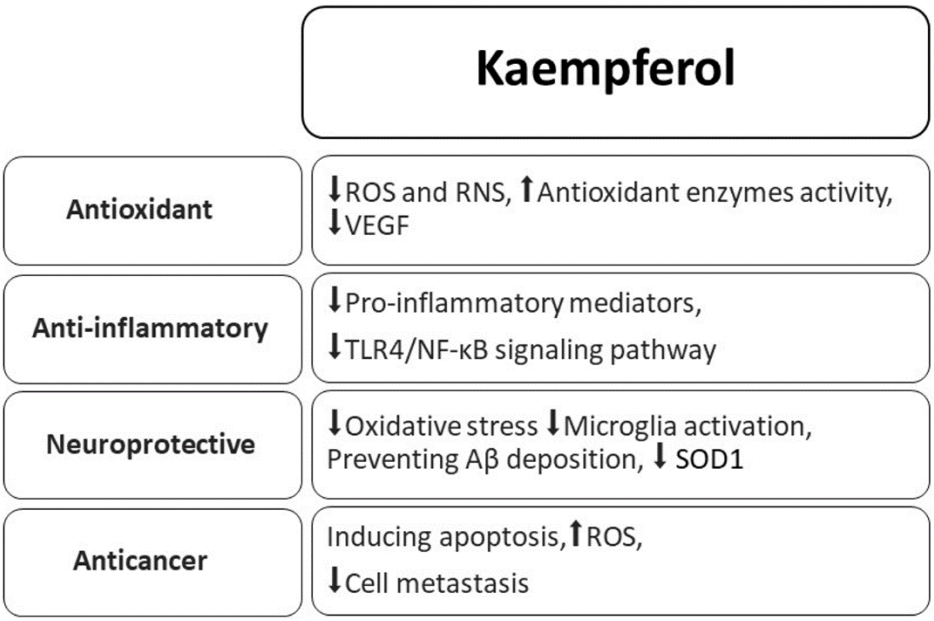

Figure 4. Summary of the most important biological properties of kaempferol discussed in the review.

cardioprotective, neuroprotective, anti-diabetic, anti-osteoporotic, estrogenic/ antiestrogenic, antiviral, anxiolytic, analgesic, and antiallergic activities (Calderón-Montaño *et al*., 2011).

As a result, because of its pervasiveness and comprehensiveness in pharmacological properties this compound is once again gaining importance in modern medicine. Furthermore, nowadays there is essential hope in kaempferol's cytotoxic activity against multiple types of human cancer cells while showing high selectivity for tumour cells and little or non-effect on normal cells (Matsuda *et al*., 2002; Zhang *et al*., 2008). The abovementioned qualities make kaempferol an ideal nominee for an agent associated with cancer prevention or cancer co-therapy as chemotherapeutic (Lim *et al*., 2007; Ninomiya *et al*., 2013; Sak, 2014; Szliszka *et al*., 2011).

Antiviral Activity

Kaempferol has shown antiviral action against both DNA and RNA viruses. Moreover, it is also confirmed that compound has effects on enveloped viruses, such as hepatitis B (Yang, 2014). In an *in vitro* study carried out by Parvez *et al*. this compound exhibited its anti-hepatitis B activity by inhibiting HBsAg and HBeAg (HBe which is a marker for cccDNA replication) synthesis (Parvez *et al*., 2022). Furthermore, the research shows kaempferol's ability to form stable complexes with HBV- polymerase binding-pocket amino acids, therefore it could be potentially used as a therapeutic agent against HBV virus (Parvez *et al*., 2022).

Another virus in which kaempferol may be used in the treatment is African Swine Fever Virus. African Swine Fever (ASF) caused by mentioned virus in a repeatedly fatal disease which targets monocytes and macrophages (Njau *et al*.,

2021). It is entering the cells principally through endocytosis, mediated by receptors, and via micropinocytosis. Kaempferol is said to inhibit the endocytosis, by such means the virions are prevented from releasing to the cell. It is resulting in the suppression of viral infection even over 90% (Arabyan *et al*., 2021).

A fortiori, studies have evaluated the efficacy of kaempferol in action against SARS-CoV-2 (Anand *et al*., 2021; Khazdair *et al*., 2019). It exhibited promising molecular docking parameters on the N3 side in the Covid-19 main protease, which shows its promising use in the treatment (Owis *et al*., 2020).

Interestingly, in many cases kaempferol has a treatment effectiveness comparable to the specific drugs, which appears to be important considering the side effects of a drug therapy. Thus, supplanting or supplementation a drug with kaempferol, seems to be a good alternative (Periferakis *et al*., 2023).

Inhibition of low-density lipoprotein (LDL) oxidation

Flavonoids in general are considered as potent inhibitors od LDL oxidation, which is a complex process during which both the protein and the lipids undergo oxidative alterations causing in formation of complex products. Inhibitory effect is shown by cell protection against damage induced by reactive oxygen species (ROS) and copper ion-induced oxidation, exhibition of radical-scavenging activity and scavenging free radicals, exhibition of affinity to ATP-binding proteins (associated with their structural analogy with ATP) (Fuhrman *et al*., 2002; Tomás-Barberán *et al*., 2012). This activity is prominently important taking into consideration how many diseases are connected with oxidative stress and the oxidation of low-density lipoprotein, e.g. atherosclerosis linked by various studies to those processes since last century (Quinn *et al*., 1987; Steinberg *et al*., 1989; Steinbrecher *et al*., 1984)

Neuroprotective activity

Kaempferol has presented a neuroprotective action via the modulation of some proinflammatory signalling pathways including the nuclear factor kappa B (NF-kB), p38 mitogen-activated protein kinases (p38MAPK), serine/ threonine kinase (AKT), and β-catenin cascade (Silva dos Santos *et al*. 2021)., This compound has shown its value in potential neuropathic pain (NP) treatment via regulating the activation of TLR4/NF-κB signalling pathway, which hyperactivity has been proven to cause chronic inflammation (Chang *et al*., 2022). Moreover, research to date have suggested that kaempferol and its derivatives possess neuroprotective properties and may have potential therapeutic benefits in neurodegenerative diseases (NDDs), such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Kaempferol has a positive effect on the nervous system and structures directly related to it. Specifically, said compound influence prevention of the deposition of amyloid fibrils (e.g. amyloid β-protein (Aβ), tau, α-synuclein),inhibition of microglia activation, reduction of the release of inflammatory factors, scavenging free radicals, restoration of the mitochondrial membrane (which prevent oxidative stress), protection of the blood-brain barrier, and inhibition of specific enzyme activities (e.g. cholinesterase which is an enzyme responsible for catalysation of the hydrolysis of the neurotransmitter acetylcholine (ACh) into choline and acetic acid (Colović *et al*., 2013).

ALS is a fatal, progressive NDD that selectively affects motor neurons (Yang *et*

al., 2021). Characteristic changes occur in these neurons under the influence of the disease, i.e., aggregation and accumulation of ubiquitinated protein inclusions (Bendotti *et al*., 2012). Studies have proven that there is a correlation between the activity of the SOD1 enzyme and the pathogenesis of ALS and the progression of the disease. For example, transgenic mice expressing human ALS-related variants SOD1G93A, SOD1G37R, and SOD1G85R exhibited a distinct ALS-like phenotype, such as SOD1 aggregation, mitochondrial dysfunction, death of motor neurons, and overall motor disability (Chen *et al*., 2021; Bk *et al*., 2019; Pambo-Pambo *et al*., 2009). In contrast, loss of SOD1 function significantly improved motor system dysfunction. Kaempferol protects against neurotoxicity caused by mutant SOD1 in an ALS model, furthermore, inhibits mutant SOD1-induced cell death, and also reduces intracellular aggregation of SOD1 mutants and significantly inhibits SOD1-induced mitochondrial superoxide mutants, suggesting that kaempferol is a candidate for naturopathic treatment of ALS (Jin *et al*., 2023).

Anticancer activity

It is said that diet rich in vegetables and fruits (particularly those rich in flavonoids such as kaempferol) significantly reduces the risk of multiple diseases, such as cancer. Interestingly, there is a monitored low cases of cancer disease in the population of vegetarians (Petrick *et al*., 2015; WHO, 2014). Deducing, that diet is closely related to the incidence and prevention of different cancer types. Moreover, convincing epidemiological evidence suggests that ingestion of foods saturated in the kaempferol could lead to reduction of development of certain cancers, which has been proven by numerous in vitro studies. Besides this aspect, there are much research showing the inhibitory ability of this flavonoid on the growth of different cancers, among others glioma/glioblastoma (Sharma *et al*., 2007), breast adenocarcinoma (Diantini *et al*., 2012), leukaemia (Ren *et al*., 2010), lung cancer (Leung *et al*., 2007), colorectal carcinoma (Li *et al*., 2009).

Furthermore, kaempferol display a direct effect on the apoptosis extrinsic pathway, due to the presence of death receptors on the cell surface able to recognize substances responsible for death induction. Mentioned death receptors include tumour necrosis factor alpha (TNF-α), FAS and TRAIL (Thorburn, 2004). The TRAIL receptor is particularly taken into account because of its specification, such as induction of apoptosis in human colon cancer cells and a deficiency in the expression on cell surface which explains resistance of cancer cells to apoptosis (Jin *et al*., 2004). Kaempferol is likely to up-regulate said TRAIL receptors by reducing cancer cells' resistance to apoptosis and sensitization of those cells onto TRAIL-dependent apoptosis (Yoshida *et al*., 2008). Several in vitro and in vivo research showcase kaempferol's impact in induction of apoptosis in cancer cell in various tissues, for instance lung (Conforti *et al*., 2009; Leung *et al*., 2007), breast (Kang *et al*., 2009; Kim *et al*., 2008), colon (Li *et al*., 2009), prostate (Brusselmans *et al*., 2005), liver (Mylonis *et al*., 2010), pancreas (Zhang *et al*., 2008), blood/lymph (Benyahia *et al*., 2004), skin (Li *et al*. 2007), brain (Jeong *et al*., 2009b), uterus (Li *et al*., 2007), and ovary (Luo *et al*., 2010).

Research shows that kaempferol is demonstrating antiangiogenic activities (generation of new blood vessels) (Kim and Choi, 2013). The main mediator of this process is vascular endothelial growth factor (VEGF) (Ferrara, 2004). Kaempferol can significantly reduce

VEGF expression in ovarian cancer cells, which leads to reduction of tumour proliferation. Moreover, kaempferol is able to inhibit HIF-1 transcription factor at a low micromolar range (Luo *et al*., 2009; Mylonis *et al*., 2010), which could be a potential therapeutic target, as the overexpression of HIF-1 causes the induction of tumour aggressiveness.

Metastasis is a process during which cancer cells spread from their original site to other areas of the body. For this process to occur, cancer cells must degrade extracellular matrix (ECM), which allows them to reach the blood vessels and then proliferate throughout the organism. For this purpose, cancer cells use various enzymes, such as the matrix metalloproteinases (MMPs), which give rather poor clinical prognoses in cancer patients (Guan, 2015). For that reason, there are studies testing the usage of various substances in therapy against this process. Kaempferol has exhibited inhibitory effect on cell metastasis through ERK-p38, JNK, and AP-1 signalling pathways in human osteosarcoma cells (Chen *et al*., 2013). This compound has the ability to reduce protein phosphorylations at ERK, p38, and JNK, thus decreasing the DNA binding activity of AP-1, and causing reduction in expression of MMP-2, MMP-9, and uPA (urokinase-type plasminogen activator), therefore overall reduction of metastatic potential (Li *et al*., 2014; Lin *et al*., 2013).

Conclusions

Polyphenols are important compounds in the human diet characterized by their various properties beneficial for health (Fig. 5). Kaempferol, quercetin, and curcumin are strong antioxidants performing health-promoting activities, including anti-oxidative, anti-inflammatory, antimicrobial, antiviral, and neuroprotective properties. These compounds have recently been intensively examined in terms of their anti-cancer activity. Numerous in vivo and in vitro studies investigated their ability to inhibit proliferation and promote cell death via inducing apoptosis in cancer

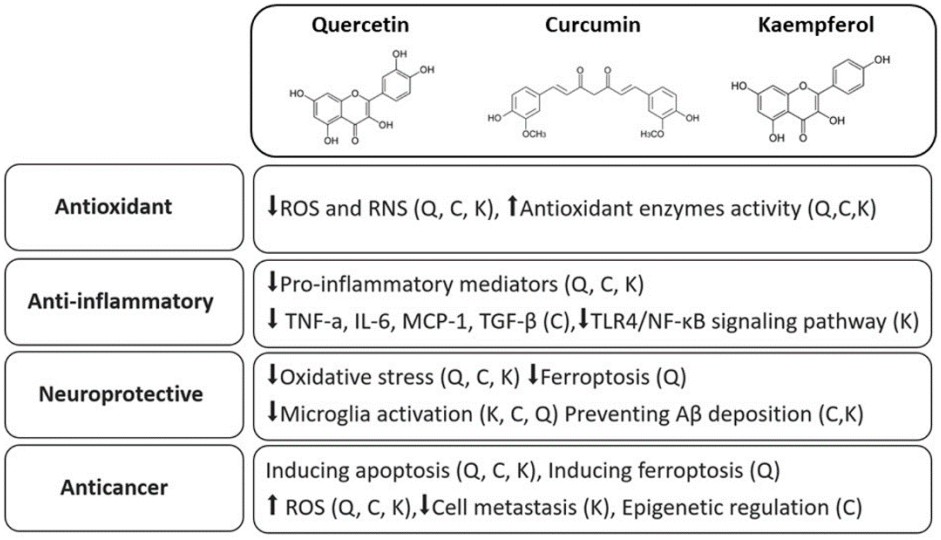

Figure 5. Summary of the most important biological properties of quercetin (Q), curcumin (C), and kaempfrrol (K) discussed in the review.

cells. However, due to the low bioavailability of polyphenols, their health beneficial effect as dietary compounds remains indeterminate. Thus, absorption and interactions with other compounds potentially increasing bioavailability of polyphenols need to be further studied to thoroughly utilize their therapeutic potential. Moreover, anticancer potential of quercetin, curcumin, and kaempferol and their ability to abolish resistance in cancer cells appear to be important areas for further research.

References

Aggarwal, B. B., Harikumar, K. B. 2009. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune, and neoplastic diseases. The International Journal of Biochemistry Cell Biology, 41(1): 40–59.

Al-Nour, M.Y., Ibrahim, M.M., Elsaman, T. 2019. Ellagic Acid, Kaempferol, and Quercetin from Acacia nilotica: Promising Combined Drug With Multiple Mechanisms of Action. Current Pharmacology Reports, 5(4): 255–280.

Alsharairi, N.A. 2023. Quercetin Derivatives as Potential Therapeutic Agents: An Updated Perspective on the Treatment of Nicotine-Induced Non-Small Cell Lung Cancer. International Journal of Molecular Sciences, 24(20): 15208.

Anand David, A.V., Arulmoli, R., Parasuraman, S. 2016. Overviews of Biological Importance of Quercetin: A Bioactive Flavonoid. Pharmacognosy Reviews, 10(20): 84–89.

Anand, A.V., Balamuralikrishnan, B., Kaviya, M., Bharathi, K., Parithathvi, A., Arun, M., Senthilkumar, N., Velayuthaprabhu, S., Saradhadevi, M., Al-Dhabi, NA., Arasu, M.V., Yatoo, M.I., Tiwari, R., Dhama, K. 2021. Medicinal Plants, Phytochemicals, and Herbs to Combat Viral Pathogens Including SARS-CoV-2. Molecules (Basel, Switzerland), 26(6): 1775.

Arabyan, E., Hakobyan, A., Hakobyan, T., Grigoryan, R., Izmailyan, R., Avetisyan, A., Karalyan, Z., Jackman, J.A., Ferreira, F., Elrod, C.C., Zakaryan, H. 2021. Flavonoid Library Screening Reveals Kaempferol as a Potential Antiviral Agent Against African Swine Fever Virus. Frontiers in Microbiology, 12: 736780.

Bagheri, H., Ghasemi, F., Barreto, G.E., Rafiee, R., Sathyapalan, T., Sahebkar, A. 2020. Effects of curcumin on mitochondria in neurodegenerative diseases. BioFactors (Oxford, England), 46(1): 5–20.

Bai N., He K., Roller M., Lai C.S., Shao X., Pan M.H., Ho C.T. 2010. Flavonoids and phenolic compounds from Rosmarinus officinalis. Journal of Agricultural and Food Chemistry, 58(9): 5363–5367.

Balkwill, F., Mantovani, A. 2001. Inflammation and cancer: back to Virchow?. Lancet (London, England), 357(9255): 539–545.

Bendotti C., Marino M., Cheroni C., Fontana E., Crippa V. , Poletti A., De Biasi S. 2012. Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: implication for protein aggregation and immune response. Progress in Neurobiology, 97: 101–126.

Benyahia, S., Benayache, S., Benayache, F., Quintana, J., López, M., León, F., Hernández, J. C., Estévez, F., Bermejo, J. 2004. Isolation from Eucalyptus occidentalis and identification of a new kaempferol derivative that induces apoptosis in human myeloid leukemia cells. Journal of Natural Products, 67(4): 527–531.

Bk, B., Skuntz, S., Prochazkova, M., Kesavapany, S., Amin, N.D., Shukla, V., Grant P., Kulkarni A.B., Pant H.C. 2019. Overexpression of the Cdk5 inhibitory peptide in motor neurons rescue of amyotrophic lateral sclerosis phenotype in a mouse model. Human Molecular Genetics, 28(19): 3175–3187.

Brusselmans, K., Vrolix, R., Verhoeven, G., Swinnen, J.V, 2005. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. The Journal of Biological Chemistry, 280(7): 5636–5645.

Bureau, G., Longpré, F., Martinoli, M.G. 2008. Resveratrol and quercetin, two natural polyphenols, reduce apoptotic neuronal cell death induced by neuroinflammation. Journal of Neuroscience Research, 86(2): 403–410.

Calderón-Montaño, J.M., Burgos-Morón, E., Pérez-Guerrero, C., López-Lázaro, M. 2011. A review on the dietary flavonoid kaempferol. Mini Reviews in Medicinal Chemistry, 11(4): 298–344.

Carullo, G., Cappello, A. R., Frattaruolo, L., Badolato, M., Armentano, B., Aiello, F. 2017. Quercetin and derivatives: useful tools in inflammation and pain management. Future Medicinal Chemistry, 9(1): 79–93.

Chang, S., Li, X., Zheng, Y., Shi, H., Zhang, D., Jing, B., Chen, Z., Qian, G., Zhao, G. 2022. Kaempferol exerts a neuroprotective effect to reduce neuropathic pain through TLR4/NF-κB

signaling pathway. Phytotherapy Research: PTR, 36(4): 1678–1691.

Chen H.J., Lin C.M., Lee C.Y., Shih N.C., Peng S.F., Tsuzuki M., Amagaya S., Huang W.W., Yang J.S. 2013. Kaempferol suppresses cell metastasis via inhibition of the ERK-p38-JNK and AP-1 signaling pathways in U-2 OS human osteosarcoma cells. Oncology Reports, 30:925–932.

Chen J, Huang Z, Cao X, Zou T, You J, Guan W. 2022. Plant-derived polyphenols in sow nutrition: An update. Animal Nutrition, 12: 96–107.

Chen, H.J., Lin, C.M., Lee, C.Y., Shih, N.C., Peng, S.F., Tsuzuki, M., Amagaya, S., Huang, W.W., Yang, J.S. 2013. Kaempferol suppresses cell metastasis via inhibition of the ERK-p38-JNK and AP-1 signaling pathways in U-2 OS human osteosarcoma cells. Oncology Reports, 30(2): 925–932.

Cheng, S.C., Huang, W.C., Pang, J.H., Wu, Y.H., Cheng, C.Y.2019. Quercetin Inhibits the Production of IL-1β-Induced Inflammatory Cytokines and Chemokines in ARPE-19 Cells via the MAPK and NF-κB Signaling Pathways. International Journal of Molecular Sciences, 20(12): 2957.

Chirumbolo S. 2010. The role of quercetin, flavonols and flavones in modulating inflammatory cell function. Inflammation Allergy Drug Targets, 9(4): 263–285.

Colović M.B., Krstić D.Z., Lazarević-Pašti T.D., Bondžić A.M., Vasić V.M. 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. Current Neuropharmacology, 11(3): 315–335.

Conforti, F., Menichini, F., Rigano, D., Senatore, F. 2009. Antiproliferative activity on human cancer cell lines after treatment with polyphenolic compounds isolated from Iris pseudopumila flowers and rhizomes. Zeitschrift für Naturforschung C, 64: 490–494.

Cruz-Gregorio, A., Aranda-Rivera, A.K. 2023. Quercetin and Ferroptosis. Life (Basel, Switzerland), 13(8): 1730.

D'Archivio, M., Filesi, C., Varì, R., Scazzocchio, B., Masella, R. 2010. Bioavailability of the polyphenols: status and controversies. International Journal of Molecular Sciences, 11(4): 1321–1342.

Di Lorenzo, C., Colombo, F., Biella, S., Stockley, C., Restani, P. 2021. Polyphenols and Human Health: The Role of Bioavailability. Nutrients, 13(1): 273.

Di Petrillo, A., Orrù, G., Fais, A., Fantini, M.C. 2022. Quercetin and its derivates as antiviral potentials: A comprehensive review. Phytotherapy Research: PTR, 36(1): 266–278.

Diantini, A., Subarnas, A., Lestari, K., Halimah, E., Susilawati, Y., Supriyatna, Julaeha, E., Achmad, T. H., Suradji, E.W., Yamazaki, C., Kobayashi, K., Koyama, H., Abdulah, R. 2012. Kaempferol-3-O-rhamnoside isolated from the leaves of Schima wallichii Korth. inhibits MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway. Oncology Letters, 3(5): 1069–1072.

Eigner, D., Scholz, D. 1999. Ferula asa-foetida and Curcuma longa in traditional medical treatment and diet in Nepal. Journal of Ethnopharmacology, 67(1): 1–6.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., Bray, F. 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International Journal of Cancer, 136(5): E359–E386.

Ferrara N., 2004. Vascular endothelial growth factor as a target for anticancer therapy. Oncologist, 9, Supplement, 1: 2–10.

Ferreira, M.J., Rodrigues, T.A., Pedrosa, A.G., Silva, A.R., Vilarinho, B.G., Francisco, T., Azevedo, J.E. 2023. Glutathione and peroxisome redox homeostasis. Redox Biology, 67: 102917.

Formica, J.V., Regelson, W. 1995. Review of the biology of Quercetin and related bioflavonoids. Food and Chemical Toxicology: An International Journal published for the British Industrial Biological Research Association, 33(12): 1061–1080.

Fuhrman, B., Aviram, M. 2002. Polyphenols and flavonoids protect LDL against atherogenic modifications. In: Handbook of Antioxidants, Marcel Dekker, Inc., New York, USA.

Ge, Z., Xu, M., Ge, Y., Huang, G., Chen, D., Ye, X., Xiao, Y., Zhu, H., Yin, R., Shen, H., Ma, G., Qi, L., Wei, G., Li, D., Wei, S., Zhu, M., Ma, H., Shi, Z., Wang, X., Ge, X., Qian, X. 2023. Inhibiting G6PD by quercetin promotes degradation of EGFR T790M mutation. Cell Reports, 42(11): 113417.

Giordano, A., Tommonaro, G. 2019. Curcumin and Cancer. Nutrients, 11(10): 2376.

Gridelli, C., Rossi, A., Carbone, D.P., Guarize, J., Karachaliou, N., Mok, T., Petrella, F., Spaggiari, L., Rosell, R. 2015. Non-small-cell lung cancer. Nature Reviews. Disease Primers, 1: 15009.

Guan X. 2015. Cancer metastases: challenges and opportunities. Acta Pharmaceutica Sinica B, 5: 402–418.

Gupta V, Sharma R, Bansal P, Kaur G. 2018. Bioactivity-guided isolation of potent anxiolytic compounds from leaves of Citrus paradisi. An

International Quarterly Journal of Research in Ayurveda, 39(1): 21–28.

Häkkinen, S.H., Kärenlampi, S.O., Heinonen, I.M., Mykkänen, H.M., Törrönen, A.R. 1999. Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. Journal of Agricultural and Food Chemistry, 47(6): 2274–2279.

Hansen, D.V., Hanson, J.E., Sheng, M. 2018. Microglia in Alzheimer's disease. The Journal of Cell Biology, 217(2): 459–472.

Hewlings, S.J., Kalman, D.S. 2017. Curcumin: A Review of Its Effects on Human Health. Foods (Basel, Switzerland), 6(10): 92.

Huang, L., Yagura, T., Chen, S. 2008. Sedative activity of hexane extract of Keampferia galanga L. and its active compounds. Journal of Ethnopharmacology, 120(1): 123–125.

Imran M., Salehi B., Sharifi-Rad J., Aslam Gondal T., Saeed F., Imran A., Shahbaz M., Tsouh Fokou P.V., Umair Arshad M., Khan H., Guerreiro S.G., Martins N., Estevinho L.M. 2019. Kaempferol: A Key Emphasis to Its Anticancer Potential. Molecules, 24(12): 2277.

Ionescu, V.A., Gheorghe, G., Bacalbasa, N., Chiotoroiu, A.L., Diaconu, C. 2023. Colorectal Cancer: From Risk Factors to Oncogenesis. Medicina (Kaunas, Lithuania), 59(9): 1646.

Jeong, J.C., Kim, M.S., Kim, T.H., Kim, Y.K. 2009. Kaempferol induces cell death through ERK and Akt-dependent down-regulation of XIAP and survivin in human glioma cells. Neurochemical Research, 34: 991–1001.

Jin, Z., McDonald, E.R., Dicker, D.T., El-Deiry, W.S. 2004. Deficient tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor transport to the cell surface in human colon cancer cells selected for resistance to TRAIL-induced apoptosis. The Journal of Biological Chemistry, 279: 35829–35839.

Jin, S., Zhang, L., Wang, L. 2023. Kaempferol, a potential neuroprotective agent in neurodegenerative diseases: From chemistry to medicine. Biomedicine Pharmacotherapy, 165: 115215.

Kang, G.Y., Lee, E.R., Kim, J.H., Jung, J.W., Lim, J., Kim, S.K., Cho, S.G., Kim, K.P. 2009. Downregulation of PLK-1 expression in kaempferol-induced apoptosis of MCF-7 cells. European Journal of Pharmacology, 611(1–3): 17–21.

Kempuraj, D., Madhappan, B., Christodoulou, S., Boucher, W., Cao, J., Papadopoulou, N., Cetrulo, C.L., Theoharides, T.C. 2005. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. British Journal of Pharmacology, 145(7): 934–944.

Keyhanian, S., Stahl-Biskup, E. 2007. Phenolic constituents in dried flowers of aloe vera (Aloe barbadensis) and their in vitro antioxidative capacity. Planta Medica, 73: 599–602.

Khazdair, M., Anaeigoudari, A., Agbor, G. 2021. Anti-viral and anti-inflammatory effects of kaempferol and quercetin and COVID-2019: A scoping review. Asian Pacific Journal of Tropical Biomedicine, 11: 327–334.

Kim, B.W., Lee, E.R., Min, H.M., Jeong, H.S., Ahn, J.Y., Kim, J.H., Choi, H.Y., Choi, H., Kim, E.Y., Park S.P. 2008. Sustained ERK activation is involved in the kaempferol-induced apoptosis of breast cancer cells and is more evident under 3-D culture condition. Cancer Biology Therapy, 7: 1080–1089.

Kim, S.H., Choi, K.C. 2013. Anti-cancer Effect and Underlying Mechanism(s) of Kaempferol, a Phytoestrogen, on the Regulation of Apoptosis in Diverse Cancer Cell Models. Toxicological Research, 29: 229–234.

Kim, B., Kim, H.S., Jung, E.J., Lee, J.Y., Tsang, B.K., Lim, J.M., Song, Y.S. 2016. Curcumin induces ER stress-mediated apoptosis through selective generation of reactive oxygen species in cervical cancer cells. Molecular Carcinogenesis, 55(5): 918–928.

Kotha, R.R., Luthria, D.L. 2019. Curcumin: Biological, Pharmaceutical, Nutraceutical, and Analytical Aspects. Molecules (Basel, Switzerland), 24(16): 2930.

Chen, L., Na, R., McLane K.D., Thompson, C.S., Gao, J., Wang, X., Ran, Q. 2021. Overexpression of ferroptosis defense enzyme Gpx4 retards motor neuron disease of SOD1G93A mice. Scientific Report, 11(1): 12890

Lattanzio, V. 2013. Phenolic Compounds: Introduction. In Ramawat, K.G., Mérillon J.-M. (Eds.), Natural Products: 1543–1580.

Lee, V.S., Chen, C.R., Liao, Y.W., Tzen, J.T., Chang, C.I. 2008. Structural determination and DPPH radical-scavenging activity of two acylated flavonoid tetraglycosides in oolong tea (Camellia sinensis). Chemical Pharmaceutical Bulletin, 56(6): 851–853.

Leung, H.W., Lin, C.J., Hour, M.J., Yang, W.H., Wang, M.Y., Lee, H.Z. 2007. Kaempferol induces apoptosis in human lung non-small carcinoma cells accompanied by an induction of antioxidant enzymes. Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 45(10): 2005–2013.

Li, C., Zhao, Y., Yang, D., Yu, Y., Guo, H., Zhao, Z., Zhang, B., Yin, X. 2015. Inhibitory effects

of kaempferol on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-9. Biochemistry and Cell Biology, 93(1): 16–27.

Li, W., Du, B., Wang, T., Wang, S., Zhang, J. 2009. Kaempferol induces apoptosis in human HCT116 colon cancer cells via the Ataxia-Telangiectasia Mutated-p53 pathway with the involvement of p53 Upregulated Modulator of Apoptosis. Chemico-Biological Interactions, 177(2): 121–127.

Li, Y., Fang, H., Xu, W. 2007. Recent advance in the research of flavonoids as anticancer agents. Mini Reviews in Medicinal Chemistry, 7(7): 663–678.

Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M.T., Wang, S., Liu, H., Yin, Y. 2016. Quercetin, Inflammation and Immunity. Nutrients, 8(3): 167.

Lim, D.Y., Jeong, Y., Tyner, A.L., Park, J.H. 2007. Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound luteolin. American Journal of Physiology, Gastrointestinal and Liver Physiology, 292(1): G66–G75.

Lin, C.W., Chen, P.N., Chen, M.K., Yang, W.E., Tang, C.H., Yang, S.F., Hsieh, Y.S. 2013. Kaempferol reduces matrix metalloproteinase-2 expression by down-regulating ERK1/2 and the activator protein-1 signaling pathways in oral cancer cells. PloS One, 8(11): e80883.

Lin, Y.G., Kunnumakkara, A.B., Nair, A., Merritt, W.M., Han, L.Y., Armaiz-Pena, G.N., Kamat, A.A., Spannuth, W.A., Gershenson, D.M., Lutgendorf, S.K., Aggarwal, B.B., Sood, A.K. 2007. Curcumin inhibits tumor growth and angiogenesis in ovarian carcinoma by targeting the nuclear factor-kappaB pathway. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research, 13(11): 3423–3430.

Link, A., Balaguer, F., Shen, Y., Lozano, J.J., Leung, H.C., Boland, C.R., Goel, A. 2013. Curcumin modulates DNA methylation in colorectal cancer cells. PloS One, 8(2): e57709.

Liu, C., Rokavec, M., Huang, Z., Hermeking, H. 2023. Curcumin activates a ROS/KEAP1/NRF2/miR-34a/b/c cascade to suppress colorectal cancer metastasis. Cell Death and Differentiation, 30(7): 1771–1785.

Luo, H., Daddysman, M.K., Rankin, G.O., Jiang, B.H., Chen, Y.C. 2010. Kaempferol enhances cisplatin's effect on ovarian cancer cells through promoting apoptosis caused by down regulation of cMyc. Cancer Cell International, 10: 1.

Mantovani, A., Allavena, P., Sica, A., Balkwill, F. 2008. Cancer-related inflammation. Nature, 454(7203): 436–444.

Matsuda, H., Ninomiya, K., Shimoda, H., Yoshikawa, M. 2002. Hepatoprotective principles from the flowers of Tilia argentea (linden): structure requirements of tiliroside and mechanisms of action. Bioorganic Medicinal Chemistry, 10: 707–712.

Mokhtari-Zaer, A., Khazdair, M.R., Boskabady, M.H. 2015. Smooth muscle relaxant activity of Crocus sativus (saffron) and its constituents: possible mechanisms. Avicenna Journal of Phytomedicine, 5: 365.

Monroy, A., Lithgow, G. J., Alavez, S. 2013. Curcumin and neurodegenerative diseases. BioFactors (Oxford, England), 39(1): 122–132.

Morgan, M.J., Liu, Z.G., 2011. Crosstalk of reactive oxygen species and NF-κB signaling. Cell Research, 21(1): 103–115.

Mylonis, I., Lakka, A., Tsakalof, A., Simos, G. 2010. The dietary flavonoid kaempferol effectively inhibits HIF-1 activity and hepatoma cancer cell viability under hypoxic conditions. Biochemical and Biophysical Research Communications, 398: 74–78.

Nakamura, Y., Chang, C.C., Mori, T., Sato, K., Ohtsuki, K., Upham, B.L., Trosko, J.E. 2005. Augmentation of differentiation and gap junction function by kaempferol in partially differentiated colon cancer cells. Carcinogenesis, 26: 665–671.

Narimatsu, H., Yaguchi, Y.T. 2022. The Role of Diet and Nutrition in Cancer: Prevention, Treatment, and Survival. Nutrients, 14(16): 3329.

Ninomiya, M., Nishida, K., Tanaka, K., Watanabe, K., Koketsu, M. 2013. Structure-activity relationship studies of 5,7-dihydroxyflavones as naturally occurring inhibitors of cell proliferation in human leukemia HL 60 cells. Journal of Natural Medicines, 67: 460–467.

Njau, E.P., Machuka, E.M., Cleaveland, S., Shirima, G.M., Kusiluka, L.J., Okoth, E.A. 2021. Pelle R. African Swine Fever Virus (ASFV): Biology, Genomics and Genotypes Circulating in Sub-Saharan Africa. Viruses, 13: 2285.

Owis, A.I., El-Hawary, M.S., El Amir, D., Aly, O.M., Abdelmohsen, U.R., Kamel, M.S. 2020. Molecular docking reveals the potential of Salvadora persica flavonoids to inhibit COVID-19 virus main protease. RSC Advances, 10(33): 19570–19575.

Pambo-Pambo, A., Durand, J., Gueritaud, J.P. 2009. Early excitability changes in lumbar motoneurons of transgenic SOD1G85R and SOD1G(93A-Low) mice. Journal of Neurophysiology, 102(6): 3627–3642.

Panahi, Y., Hosseini, M.S., Khalili, N., Naimi, E., Simental-Mendía, L.E., Majeed, M., Sahebkar, A. 2016. Effects of curcumin on serum cytokine concentrations in subjects with metabolic syndrome: A post-hoc analysis of a randomized controlled trial. Biomedicine Pharmacotherapy, 82: 578–582.

Parvez, M.K., Al-Dosari, M.S., Basudan, O.A., Herqash, R.N. 2022. The anti-hepatitis B virus activity of sea buckthorn is attributed to quercetin, kaempferol and isorhamnetin. Biomedical Reports, 17(5): 89.

Periferakis A., Periferakis K. 2020. On the Dissemination of Acupuncture to Europe. JournalNX, 6: 201–209.

Periferakis, A., Periferakis, A.T., Troumpata, L., Periferakis, K., Scheau, A.E., Savulescu-Fiedler, I., Caruntu, A., Badarau, I.A., Caruntu, C., Scheau, C. 2023. Kaempferol: A Review of Current Evidence of Its Antiviral Potential. International Journal of Molecular Sciences, 24(22): 16299.

Petrick, J.L., Steck, S.E., Bradshaw, P.T., Trivers, K.F., Abrahamson, P.E., Engel, L.S., He, K., Chow, W. H., Mayne, S.T., Risch, H.A., Vaughan, T.L., Gammon, M.D. 2015. Dietary intake of flavonoids and oesophageal and gastric cancer: incidence and survival in the United States of America (USA). British Journal of Cancer, 112(7): 1291–1300.

Petrus, K., Schwartz, H., Sontag, G. 2011. Analysis of flavonoids in honey by HPLC coupled with coulometric electrode array detection and electrospray ionization mass spectrometry. Analytical and Bioanalytical Chemistry, 400(8): 2555–2563.

Prior, R.L. 2003. Fruits and vegetables in the prevention of cellular oxidative damage. The American Journal of Clinical Nutrition, 78(3 Suppl.): 570S–578S.

Priyadarsini, K.I. 1997. Free radical reactions of curcumin in membrane models. Free Radical Biology Medicine, 23(6): 838–843.

Priyadarsini, K.I. 2014. The chemistry of curcumin: from extraction to therapeutic agent. Molecules (Basel, Switzerland), 19(12): 20091–20112.

Priyadarsini, K.I., Maity, D.K., Naik, G.H., Kumar, M.S., Unnikrishnan, M.K., Satav, J.G., Mohan, H. 2003. Role of phenolic O-H and methylene hydrogen on the free radical reactions and antioxidant activity of curcumin. Free Radical Biology Medicine, 35(5): 475–484.

Pulido-Moran, M., Moreno-Fernandez, J., Ramirez-Tortosa, C., Ramirez-Tortosa, M. 2016. Curcumin and Health. Molecules (Basel, Switzerland), 21(3): 264.

Quinn, M.T., Parthasarathy, S., Fong, L.G., Steinberg, D. 1987. Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis. Proceedings of the National Academy of Sciences of USA, 84: 2995–2998.

Rajendran, P., Abdelsalam, S.A., Renu, K., Veeraraghavan, V., Ben Ammar, R., Ahmed, E.A. 2022. Polyphenols as Potent Epigenetics Agents for Cancer. International Journal of Molecular Sciences, 23: 11712.

Ren, H.J., Hao, H.J., Shi, Y.J., Meng, X.M., Han, Y.Q. 2010. Apoptosis-inducing effect of quercetin and kaempferol on human HL-60 cells and its mechanism. Zhongguo Shi Yan Xue Ye Xue Za Zhi, 18(3): 629–633.

Rodríguez Galdón, B., Rodríguez Rodríguez, E., Díaz Romero, C. 2008. Flavonoids in onion cultivars (Allium cepa L.). Journal of Food Science, 73: C599–C605.

Roszkowski, S. 2023. Application of Polyphenols and Flavonoids in Oncological Therapy. Molecules (Basel, Switzerland), 28(10): 4080.

Sak, K. 2014. Cytotoxicity of dietary flavonoids on different human cancer types. Pharmacognosy Reviews, 8: 122–146.

Sharma, V., Joseph, C., Ghosh, S., Agarwal, A., Mishra, M.K., Sen, E. 2007. Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. Molecular Cancer Therapeutics, 6: 2544–2553.

Silva Dos Santos, J., Gonçalves Cirino, J.P., de Oliveira Carvalho, P., Ortega, M.M. 2021. The Pharmacological Action of Kaempferol in Central Nervous System Diseases: A Review. Frontiers in Pharmacology, 11: 565700.

Silva, B., Oliveira, P.J., Dias, A., Malva, J.O. 2008. Quercetin, kaempferol and biapigenin from Hypericum perforatum are neuroprotective against excitotoxic insults. Neurotoxicity Research, 13(3–4): 265–279.

Singh, N., Baby, D., Rajguru, J.P., Patil, P.B., Thakkannavar, S.S., Pujari, V.B. 2019. Inflammation and cancer. Annals of African Medicine, 18(3): 121–126.

Singh, P., Arif, Y., Bajguz, A., Hayat, S. 2021. The role of quercetin in plants. Plant Physiology and Biochemistry: PPB, 166: 10–19.

Slimestad, R., Fossen, T., Vågen, I.M. 2007. Onions: a source of unique dietary flavonoids. Journal of Agricultural and Food Chemistry, 55(25): 10067–10080.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., Witztum, J.L. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. The New England Journal of Medicine, 320(14): 915–924.

Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L., Steinberg, D. 1984. Modification

of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proceedings of the National Academy of Sciences of USA, 81(12): 3883–3887.

Sun, J., Liu, X., Yang, T., Slovin, J., Chen, P. 2014. Profiling polyphenols of two diploid strawberry (Fragaria vesca) inbred lines using UHPLC-HRMS(n.). Food Chemistry, 146: 289–298.

Szliszka, E., Helewski, K.J., Mizgala, E., Krol, W. 2011. The dietary flavonol fisetin enhances the apoptosis-inducing potential of TRAIL in prostate cancer cells. International Journal of Oncology, 39(4): 771–779.

Teng, H., Chen, L. 2019. Polyphenols and bioavailability: an update. Critical Reviews in Food Science and Nutrition, 59(13): 2040–2051.

Thorburn, A. 2004. Death receptor-induced cell killing. Cellular Signalling, 16:139–144.

Tomás-Barberán, F.A., Ferreres, F. 2012. Analytical methods of flavonols and flavones. In: Analysis of Antioxidant-Rich Phytochemicals, Xu Z., Howard L.R., Eds.; John Wiley Sons Ltd, Hoboken, NJ, USA.

Visioli, F., De La Lastra, C.A., Andres-Lacueva, C., Aviram, M., Calhau, C., Cassano, A., D'Archivio, M., Faria, A., Favé, G., Fogliano, V., Llorach, R., Vitaglione, P., Zoratti, M., Edeas, M. 2011. Polyphenols and human health: a prospectus. Critical Reviews in Food Science and Nutrition, 51(6): 524–546.

Vollono, L., Falconi, M., Gaziano, R., Iacovelli, F., Dika, E., Terracciano, C., Bianchi, L., Campione, E. 2019. Potential of Curcumin in Skin Disorders. Nutrients, 11(9): 2169.

Wang, Z.X., Ma, J., Li, X.Y., Wu, Y., Shi, H., Chen, Y., Lu, G., Shen, H.M., Lu, G.D., Zhou, J. 2021. Quercetin induces p53-independent cancer cell death through lysosome activation by the transcription factor EB and Reactive Oxygen Species-dependent ferroptosis. British Journal of Pharmacology, 178(5): 1133–1148.

WHO, 2014. World Cancer Report 2014. (Stewart, B.W. and Wild, C.P., Eds.). IARC.

Wiczkowski, W., Romaszko, J., Bucinski, A., Szawara-Nowak, D., Honke, J., Zielinski, H., Piskula, M.K. 2008. Quercetin from shallots (Allium cepa L. var. aggregatum) is more bioavailable than its glucosides. The Journal of Nutrition, 138(5): 885–888.

Williamson, G., Manach, C. 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. The American Journal of Clinical Nutrition, 81(1 Suppl): 243S–255S.

Wright, J.S. 2002. Predicting the Antioxidant Activity of Curcumin and Curcuminoids. Journal of Molecular Structure: THEOCHEM, 591: 207×217.

Yang, X., Ji, Y., Wang, W., Zhang, L., Chen, Z., Yu, M., Shen, Y., Ding, F., Gu, X., Sun, H. 2021. Amyotrophic lateral sclerosis: molecular mechanisms, biomarkers, and therapeutic strategies. Antioxidants, (Basel), 10 (7): 1012.

Xue, Q., Yan, D., Chen, X., Li, X., Kang, R., Klionsky, D.J., Kroemer, G., Chen, X., Tang, D., Liu, J. 2023. Copper-dependent autophagic degradation of GPX4 drives ferroptosis. Autophagy, 19(7): 1982–1996.

Yang, C.L., Ma, Y.G., Xue, Y.X., Liu, Y.Y., Xie, H., Qiu, G.R. 2012. Curcumin induces small cell lung cancer NCI-H446 cell apoptosis via the reactive oxygen species-mediated mitochondrial pathway and not the cell death receptor pathway. DNA and Cell Biology, 31(2): 139–150.

Yang, Z.F., Bai, L.P., Huang, W.B., Li, X.Z., Zhao, S.S., Zhong, N.S., Jiang, Z.H. 2014. Comparison of in vitro antiviral activity of tea polyphenols against influenza A and B viruses and structure-activity relationship analysis. Fitoterapia, 93, 47–53.

Yoshida, T., Konishi, M., Horinaka, M., Yasuda, T., Goda, A.E., Taniguchi, H., Yano, K., Wakada, M., Sakai, T. 2008. Kaempferol sensitizes colon cancer cells to TRAIL-induced apoptosis. Biochemical and Biophysical Research Communications, 375(1): 129–133.

Zhang, Y., Chen, A.Y., Li, M., Chen, C., Yao, Q. 2008. Ginkgo biloba extract kaempferol inhibits cell proliferation and induces apoptosis in pancreatic cancer cells. The Journal of Surgical Research, 148(1): 17–23.

Zhou, B., Yang, Y., Pang, X., Shi, J., Jiang, T., Zheng, X. 2023. Quercetin inhibits DNA damage responses to induce apoptosis via SIRT5/PI3K/AKT pathway in non-small cell lung cancer. Biomedicine pharmacotherapy, 165: 115071.

Złotek, U., Świeca, M., Jakubczyk, A. 2014. Effect of abiotic elicitation on main health-promoting compounds, antioxidant activity and commercial quality of butter lettuce (Lactuca sativa L.). Food Chemistry, 148:253 260.

Folia Biologica et Oecologica 18: 66–71 (2024)

Acta Universitatis Lodziensis

Simple approach to bacterial genomes comparison based on Average Nucleotide Identity (ANI) using fastANI and ANIclustermap

KLAUDIA MUSIAŁ *, LEON PETRUŃKO, DAWID GMITER

Jan Kochanowski University of Kielce, Faculty of Natural Sciences, Institute of Biology, Department of Microbiology, Uniwersytecka 7, 25–406 Kielce, Poland
E-mail: musial.klaudia0805@gmail.com

ABSTRACT

The Average Nucleotide Identity (ANI) was proposed as a standard for taxonomic affiliation of newly sequenced bacterial genomes. However, usage of ANI value as a means of strains phenotypic diversity offers a relatively easy way for studding bacterial phylogeny. Here we present a simple approach to bacterial genomes comparison based on ANI using fastANI and ANIclustermap. Both programs are available as an open-source tools and can be run using simple command lines. We present protocol for programs installation as a conda packages, that facilitate it utilization. Further, we explain how to prepare commands to perform the analysis. We believed our work could be useful for young scientist that begin their experience with bioinformatics.

KEYWORDS: bacterial genomes comparison, bacterial phylogeny, Average Nucleotide Identity (ANI), fastANI, ANIclustermap

Introduction

Growing accessibility of bacterial genomes sequences through recent development on next generation sequencing (NGS) technology result in higher number of studies exploited this data (Buermans and den Dunnen, 2014; Edwards and Holt, 2013; Gmiter *et al*., 2021; Hodkinson and Grice, 2015). Use of bacterial whole genome sequences shed a new light on our understanding of bacterial diversity, evolution and mechanisms of virulence and environmental adaptation (Deurenberg *et al*., 2017; Gmiter *et al*., 2021; Kobras *et al*., 2021). However, use of NGS data might be challenging, as it requires not only knowledge about microbial genetics, but also appropriate hardware and, more importantly, at least basic computational skills (Edwards and Holt, 2013; Gmiter *et al*., 2021). Much software is shared as an open-source tools, that based on usage of relatively simple, but not always intuitive, commands (Edwards and Holt, 2013; Gmiter *et al*., 2021). The programs usage might be problematic, especially from the point of view of young scientist who have

DOI: https://doi.org/10.18778/1730-2366.18.10

just started they journey with bioinformatics.

Previously, we presented a short review of programs used for pan-genome analysis with a beginner's guide of how to work with them. We believe that it might be good introduction into the studies focused on bacterial pan-genomes (Gmiter *et al*., 2021).

Within this paper we would like to present a simple approach to bacterial genomes comparison based on Average Nucleotide Identity (ANI) using fastANI (Jain *et al*., 2018) and ANIclustermap (https://github.com/moshi4/ANIclustermap). The ANI was proposed as a standard for taxonomic affiliation of newly sequenced genomes. It is a similarity index between a given pair of genomes that can be applicable to prokaryotic organisms independently of their G+C content, and a cut-off score of > 95% indicates that they belong to the same species (Figueras *et al*., 2014) Nevertheless, the usage of ANI value as a mean of strains phenotypic diversity offers a relatively easy way for studding bacterial phylogeny. The proposed programs can be used for study the phylogeny of complete as well as DRAFT bacterial genomes. The biggest advantage of the programs is their relative simplicity in use. However, programs allow for basic phylogenetic analysis, and do not consider the differences between coding and non-coding regions or recombination regions. More detailed analysis will require another approach.

Average Nucleotide Identity (ANI)

As mentioned, ANI is generally used to confirm the affiliation of new genome to proposed genus. A general rule of 95% cut-off of ANI similarity closely reflects the traditional microbiological concept of DNA–DNA hybridization relatedness for defining species, where recommended cut-off point is 70% (Goris *et al*., 2007; Jain *et al*., 2018). As it is based on comparison of multiple genes it provides higher resolution comparing to other standard methods, such as 16S rRNA sequence comparison (Arahal, 2014). Online tools for calculation ANI value might be used, however, they not always offer flexibility in terms of data curation. Therefore, we propose the usage of fastANI and ANIclustermap, depending on a need of the researcher.

Installation as a conda packages

The presented programs are open-source tools, which means they are available for download free of charge. Their use by the PC (Windows) users requires the installation of the latest version of Ubuntu (Linux program based on Debian), which we strongly recommend. It can be installed as a dual boot with Windows (Gmiter *et al*., 2021). On the others hand, both programs can be used also on the computers with the macOS.

The easiest way for installing the programs is through their installation as a conda packages. Conda is an open-source package and environment management system that works on Windows, Linux and macOS. For more details about conda installation please see (Gmiter *et al*., 2021). After conda is properly installed, you can simply download and install fastANI and ANIclustermaps by typing following commands typed into the Terminal (Fig. 1):

```
conda install bioconda::fastani
```

or

```
conda install bioconda/label/cf201901::fastani
```

and

```
conda install -c conda-forge -c bioconda
  aniclustermap
```

Usage protocol

For presented work, we use 10 complete genome sequences of

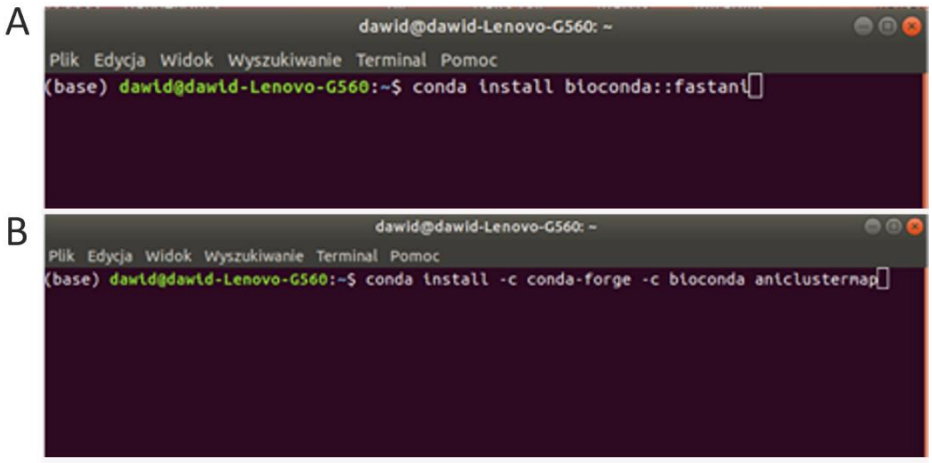

Figure 1. The command line required for installation of (A) fastANI and (B) ANIclustermap as conda packages on PC with Linux system.

Escherichia coli strains obtained from National Centre of Biotechnological Information (NCBI) (Availability: 04.10.2024) – see Table 1.

Table 1. *Escherichia coli* genome sequences used in this work.

Strain	ID
91H1	NZ_CP149810.1
2023CK-01687	NZ_CP149850.1
11128	AP010960.1
EH53	NZ_CP146512.1
JS01	NZ_CP148986.1
LF82	CU651637.1
MDP9-27	NZ_CP146515.1
SLDY13	NZ_CP149967.1
Z1322HEC0001	NZ_CP148583.1
Z1323CEC0007	NZ_CP148463.1

fastANI

The fastANI allows for the one-to-one, one-to-many and many-to-many genomes comparison. For one-to-one analysis simply type following command:

```
fastANI -q [QUERY_GENOME] -r
    [REFERENCE_GENOME] -o
    [OUTPUT_FILE]
```

The **-q** flag (flags provides the software the options to be used) stands for the query genome, a newly sequenced genome. Meanwhile, **-r** and **-o** flags provide the reference genome and localization of output files (results of analysis), respectively. It is expected that the input files will be genome assemblies in FASTA or multiFASTA format. To inform the program about localization of files, one must simply type the pathway to the file, for example:

```
-q /user/Desktop/genomes/new_genome.fasta
```

For one-to-many and many-to-many genomes comparison users must provide a pathway to text file containing directory paths to reference and/or query genomes, one per line:

```
/user/Desktop/genomes/genome1.fasta
/user/Desktop/genomes/genome2.fasta
/user/Desktop/genomes/genome3.fasta
```

In this situation, the flags **--ql** and **--rl** are required. It means that users must provide a path to the localization of genome list:

```
--ql or--rl /user/Desktop/geneme_list.txt
```

Users might use different or the same genome list as query and reference. To run the analysis the following commands should be used:

```
fastANI -q [QUERY_GENOME] --rl
  [REFERENCE_LIST] -o [OUTPUT_FILE]
fastANI --ql [QUERY_LIST] --rl
  [REFERENCE_LIST] -o [OUTPUT_FILE]
```

In all above cases, OUTPUT_FILE will contain tab delimited row(s) with query genome, reference genome, ANI value, count of bidirectional fragment mappings, and total query fragments. Alignment fraction (wrt. the query genome) is simply the ratio of mappings and total fragments.

Optional, to use the fastANI as a tool of genomes similarity investigation, users might supply **–matrix** parameter, which generate identity values arranged in a PHYLIP-formatted lower triangular matrix. The result of analysis of used *E. coli* genome sequences is presented as a similarity matrix in Table 2. The fastANI generates matrix in .txt format, which might be used in publication after manual correction (e.g. deletion of paths to genome sequences is required). The fastANI offers some option to be modified and the possible parameters are available after usage of **-h** flag. For more details, please see https://github.com/ParBLiSS/FastANI (Availability: 04.10.2024).

ANIclustermap

The fastANI provides only a numerical data, which are useful in many cases. However, ANI values might be visualized as a heat map of all-vs-all microbial genomes to better insight using ANIclustermap (https://github.com/moshi4/ANIclustermap). When ANIclustermap is used, ANI values are calculated by fastANI and clustermap is drawn using Seaborn. Additionally, ANIclustermap generate Newick format clustering dendrogram.

The usage of ANIclustermap requires following basic command:

```
ANIclustermap -i [Genome fasta directory] -o
  [output directory]
```

However, in contrary to fastANI, where input data is provided as a direct path to genome file or genomes list, here the path to folder containing all studied genome sequences is expected. ANIclustermap outputs 3 types of files:

- **ANIclustermap.[png|svg]** – ANI clustermap result figure,
- **ANIclustermap_matrix.tsv** – Clustered all-vs-all ANI matrix,
- **ANIclustermap_dendrogram.nwk** – Newick format clustering dendrogram.

Example of basic command required to run ANIclustermap is presented below:

```
ANIclustermap -i /user/Desktop/genomes -o
  /user/Desktop/genomes_results
```

Table 2. Matrix presenting ANI similarity between 10 used *E. coli* genome sequences obtained with fastANI.

Strain	ANI Values								
91H1									
2023CK-01687	98.88								
11128	98.20	98.14							
EH53	98.35	98.27	98.75						
JS01	98.34	98.33	98.83	99.37					
LF82	96.80	96.65	96.45	96.72	96.71				
MDP9-27	98.33	98.30	98.79	99.72	99.45	96.70			
SLDY13	99.04	98.85	98.22	98.23	98.27	96.85	98.28		
Z1322HEC0001	98.36	98.26	98.87	99.06	99.05	96.67	99.05	98.27	
Z1323CEC0007	99.04	98.77	98.00	98.22	98.30	96.69	98.16	99.34	98.20

The resulted ANI clustermap of the analysis of 10 *E. coli* sequences is presented on Figure 3. Obtained heat map can be easily modified. Program offers possibility to alter map colours, its size, addition of annotation (drawing ANI values) by simple modification of command line. All parameters are available with **-h** flag. For more details, please see https://github.com/moshi4/ANIclustermap (Availability: 04.10.2024).

Conclusions

In this work we presented a simple protocol for utilization of fastANI and ANIclustermap as tools allowing direct approach to study biodiversity of bacterial genome sequences. We understand that even though both programs are relatively easy in performance, their usage might be problematic for people without previous bioinformatics experience. We believe that this review and protocol will be a solid introduction in the issue.

Acknowledgements

The realization of this work was supported by the Polish National Science Centre Grant 2019/32/T/NZ1/00515 (D.G.).

Reference

Arahal, D.R. 2014. Whole-genome analyses: Average nucleotide identity. [In:] Methods in Microbiology, Vol. 41, pp. 103–122.

Buermans, H.P.J., den Dunnen, J.T. 2014. Next generation sequencing technology: Advances and applications. Biochimica et Biophysica Acta – Molecular Basis of Disease, 1842(10), 1932–1941.

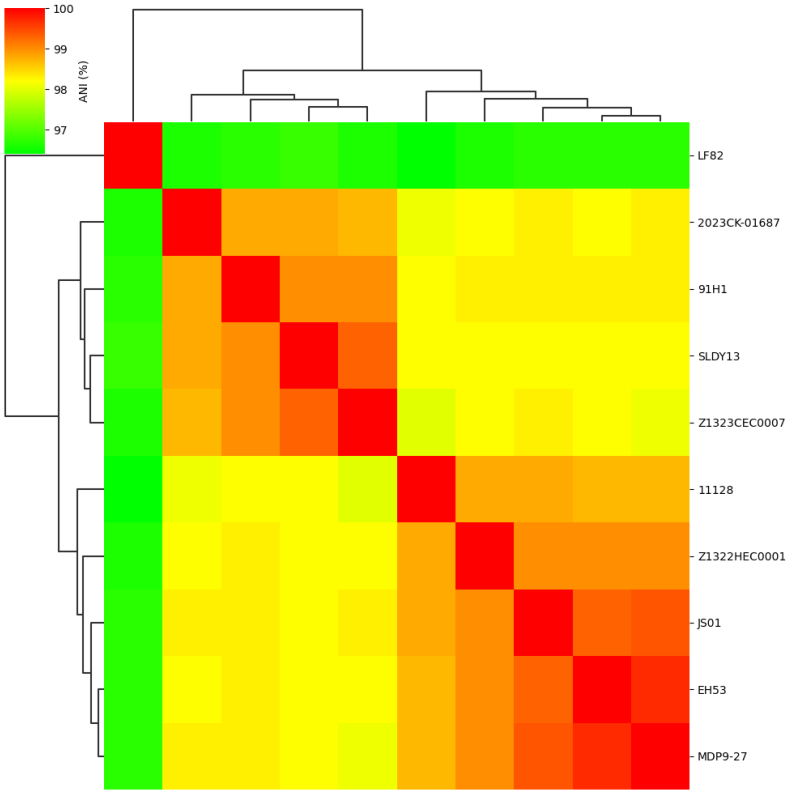

Figure 3. Results of ANIclustermap analysis of 10 *E. coli* genome sequences diversity.

Deurenberg, R H., Bathoorn, E., Chlebowicz, M.A., Couto, N., Ferdous, M., García-Cobos, S., Kooistra-Smid, A.M. D., Raangs, E.C., Rosema, S., Veloo, A.C. M., Zhou, K., Friedrich, A.W., Rossen, J.W.A. 2017. Application of next generation sequencing in clinical microbiology and infection prevention. Journal of Biotechnology, 243, 16–24.

Edwards, D.J., Holt, K.E. 2013. Beginner's guide to comparative bacterial genome analysis using next-generation sequence data. Microbial Informatics and Experimentation, 3(1), 2.

Figueras, M.J., Beaz-Hidalgo, R., Hossain, M.J., Liles, M.R. 2014. Taxonomic affiliation of new genomes should be verified using Average Nucleotide Identity and Multilocus Phylogenetic Analysis. Genome Announcements, 2(6), e00927-14-e00927-14.

Gmiter, D., Nawrot, S., Pacak, I., Zegadło, K., Kaca, W. 2021. Towards a better understanding of the bacterial pan-genome. Acta Universitatis Lodziensis. Folia Biologica et Oecologica, 17, 84–96.

Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P., Tiedje, J.M. 2007. DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. International Journal of Systematic and Evolutionary Microbiology, 57(1), 81–91.

Hodkinson, B.P., Grice, E.A. 2015. Next-Generation Sequencing: A Review of technologies and tools for wound microbiome research. Advances in Wound Care, 4(1), 50–58.

Jain, C., Rodriguez-R, L.M., Phillippy, A.M., Konstantinidis, K.T., Aluru, S. 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nature Communications, 9(1), 1–8.

Kobras, C.M., Fenton, A.K., Sheppard, S.K. 2021. Next-generation microbiology: from comparative genomics to gene function. In Genome Biology (Vol. 22, Issue 1). BioMed Central Ltd.

Folia Biologica et Oecologica 18: 72–84 (2024)

Acta Universitatis Lodziensis

The characterization of genome sequences diversity of *Pseudomonas aeruginosa* strains from international reference panel using wide range of *in silico* techniques

LEON PETRUŃKO*, KLAUDIA MUSIAŁ, DAWID GMITER

Jan Kochanowski University of Kielce, Faculty of Natural Sciences, Institute of Biology, Department of Microbiology, Uniwersytecka 7, 25-406 Kielce, Poland
E-mail: leonpetrunko@gmail.com

ABSTRACT

Pseudomonas aeruginosa is an important pathogen in patients suffering from Cystic Fibrosis as well as acute opportunistic infections in people without it. For the reason of *P. aeruginosa* having a broad range of habitats it's diversity and adaptability lead it to be a very diverse species. Previous attempts of classifying *P. aeruginosa* strains based on their biochemical and genetic characteristics were made. In presented studied we performed additional characteristic of P. aeruginosa panel strains genomes using wide range of in silico approaches, including Single Nucleotide Polymorphisms (SNPs) - based phylogeny, as well as pan-genome and Intergenic Regions (IGRs) investigation. We shed light on strains diversity, expanding our knowledge about the strains assembled in this international panel. The results of our study may become the basis for further research aimed at fully understanding the pathogenesis of *P. aeruginosa*.

KEYWORDS: *Pseudomonas aeruginosa*, reference panel, pan-genome, intergenic regions (IGRs)

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium which causes many opportunistic infections, including wound, urinary tract, and respiratory tract. This pathogen is especially important in patients with Cystic Fibrosis (CF) with over half of patients suffering from CF chronically infected by adulthood. Given the incredible diversity of its habitats (soil, still water, plants), which leads to huge biochemical and genetical diversity, it is to no surprise that classifying *P. aeruginosa* strains had been difficult. A large part of the research of *P. aeruginosa* was based on PAO1 strain which is considered a laboratory strain and may have diversified greatly throughout its existence. Considering the diversity of bacterial strains within the species, this may have led to conclusions not relevant to clinical scenario of *P. aeruginosa* infections. To felicitate the research, the

DOI: https://doi.org/10.18778/1730-2366.18.11

international reference panel of *P. aeruginosa* strains was developed. The strains were previously well characterized based on their biochemical, phenotypic, and genotypic characteristics (Cullen *et al*., 2015; De Soyza *et al*., 2013; Freschi *et al*., 2018).

However, additional genomic studies will allow better understanding of the diversity of mentioned strains. Therefore, the purpose on the presented work was the phylogenetic analysis of panel *P. aeruginosa* strains using wide range of *in silico* methods, including SNPs and pan-genome based phylogeny. For wider insight, our study focused as well on diversity of non-coding regions present in strains genomes.

Materials and methods

Genome sequences

All 40 *P. aeruginosa* raw sequences were obtained from the National Center for Biotechnology Information in a FASTA format. Detailed information about the sequences could be found in the work by (Freschi *et al*., 2018).

SNPs-based phylogeny

The REALPHY webserver (Bertels *et al*., 2014) was used to perform SNPs-based phylogenetic analysis. The default options were used and the genome of *P. aeruginosa* strain PAO1 was used as a reference sequence. The loci containing elevated densities of base substitutions, which are marked as recombinations, were identified from REALPHY generated alignment file using Gubbins v3.3.0 (Croucher *et al*., 2015) and visualized using Phandango v1.3.1 (Hadfield *et al*., 2018). The obtained maximum likelihood (ML) phylogenetic tree was midpoint-rooted and visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Functional annotation with Prokka

The Prokka, (Galaxy Version 1.14.6+galaxy1), (Seemann, 2014) available online on the Galaxy web Server (https://usegalaxy.org/) was used with the default options to annotate the sequences, resulting in a GFF3-formated files.

Pan-genome and IGRs investigation

Initially *P. aeruginosa* pan-genome was analysed using Roary with the following setups: -e -n (to perform alignments using MAFFT), -i 90 (90% sequence identity cut-off) (Page *et al*., 2015). Next the -s option (to not split paralogs into separate clusters) was used in second analysis. The output files from the second analysis were subjected for the IGRs analysis using Piggy with the default parameters (Thorpe *et al*., 2018). Data visualization (genes and IGR presence/absence) was performed using Phandango. The phylogenetic trees based on core genes and core IGRs alignment were created using SeaView (Gouy *et al*., 2010) using ML method (substitution model GTR, branch support: aLTR – SH-like)). To reduce the memory and run time, the alignment files were pre filter using snp_sites v2.5.1 (Page *et al*., 2016). The visualization of trees was done using FigTree. The R package phytools was used for tree comparison (Revell, 2012).

Results and Discussion

P. aeruginosa is a pathogenic bacteria know from its phenotypic and genotypic diversity. To felicitate studies, the panel of reference strains was developed – collected strains represent isolates obtained from different sources, mostly from cystic fibrosis patients, from all over the word (De Soyza *et al*., 2013). So far, the detailed phenotypic characteristic of strains was performed (Cullen *et al*., 2015), together with genomic studies (Freschi *et al*., 2018). However, many aspects of the genomics of panel strains

are yet to be studied. Therefore, within this work, we performed phylogenetic and pan-genome characteristic of mentioned strains. Our work also focused on evaluation of the diversity of non-coding regions within the genome, known as intergenic regions (IGRs).

In first step SNPs-based phylogenetic analysis was performed using online tool REALPHY based on core genomic content. The obtained ML and minpoint-rooted tree are presented on Figure 1. Results revealed clear separation of strains into two major phylogenetic groups: first (green) includes eight strains, meanwhile remaining (n = 32) form wider group (yellow). Within the groups, we observed the presence of closely related strains. These results correspond to the previous one (Freschi *et al*., 2018), were panel strains also grouped in similar way based on core genome single nucleotide variant (SNV), expect for the strain Mi162, that in our analysis fall into wider group.

Next, the Gubbins software was used to investigate putative events of recombination based on alignment file provided by REALPHY. The Gubbins uses spatial scanning statistics to identify loci containing elevated densities of base substitutions suggestive of horizontal sequence transfer while concurrently constructing a maximum likelihood

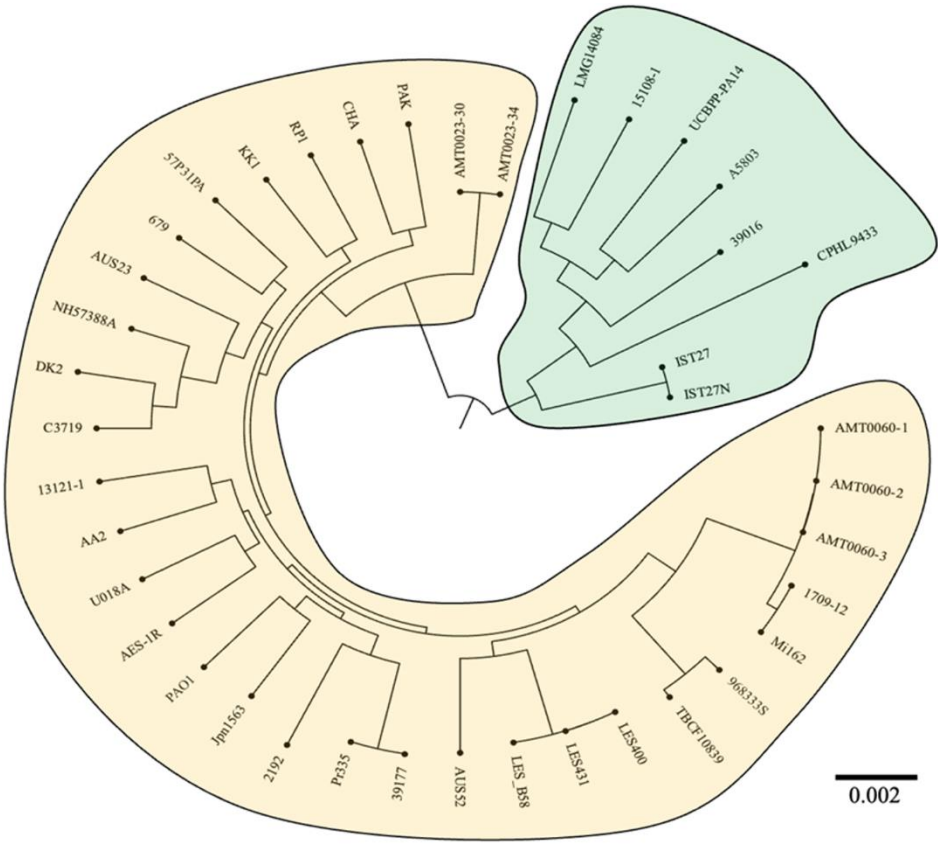

Figure 1. SNPs-based phylogenetic tree of panel *P. aeruginosa* strains genome sequences obtained using REALPHY webserver. Tree was visualized and midpoint-rooted using FigTree.

phylogeny based on the putative point mutations outside these regions of high sequence diversity (Croucher *et al*., 2015) (Fig. 2.).

To better explore the impact of putative recombination events on *P. aeruginosa* panel strains phylogeny, the trees obtained using REALPHY and Gubbins were compared using R package

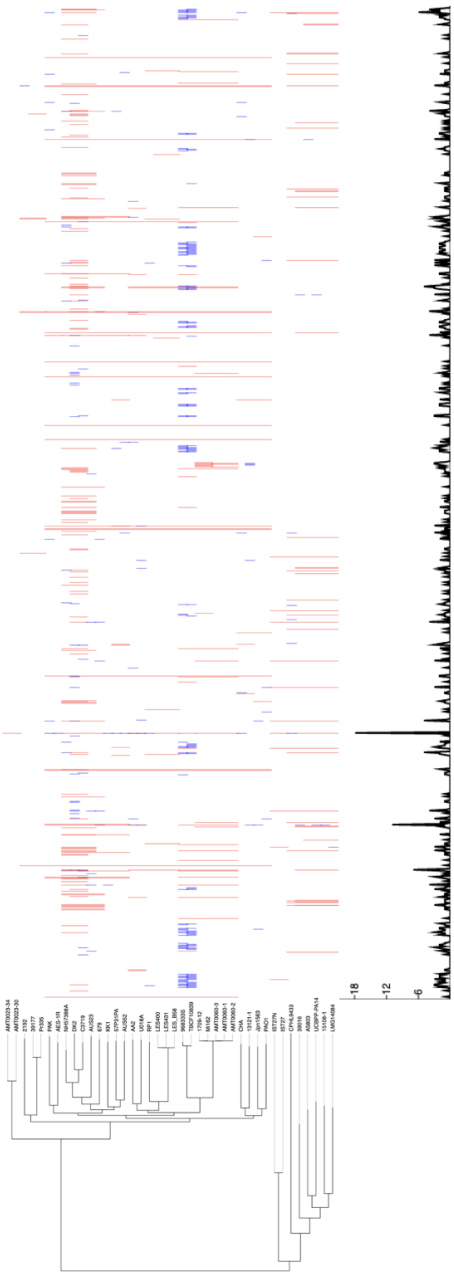

Figure 2. Phylogenetic tree after exclusion of putative recombination regions and matrix showing the recombination pattern within the *P. aeruginosa* genomes. Each column of the matrix refers to a base in the analysed sequence; each row represents an isolate in the phylogeny. Red blocks indicate predicted recombination regions occurring in the internal branch, therefore common to many isolates – common origin. Blue blocks represent recombinations occurring at terminal branches that are unique to individual isolates.

phytools (Fig. 3). Results shown similar clustering of strains into two major groups, consistent in the same strains. Strains from green group cluster identically, however exclusion of regions identified by Gubbins impacted the pattern within orange group. This suggests that horizontal gene transfer occurs at higher level among these strains.

Further, we focused on panel strains pan-genome investigation. It is well recognized that genes within bacterial pan-genomes are categorized as core, additional and unique genes when present in all, most of or only one of the studied genomes, respectively (Gmiter *et al*., 2021). For pan-genome investigation we used Roary, a frequently used software, that allows downstream analysis based on the generated files (Gmiter *et al*., 2021). The Roary software performs pan-genome analysis based on GFF3-formatted input files, containing the annotated genomes sequences (Page *et al*., 2015; Sitto and Battistuzzi, 2020). In step one, software counts identified genes and categorizes them, based on their frequency of occurrence between studied

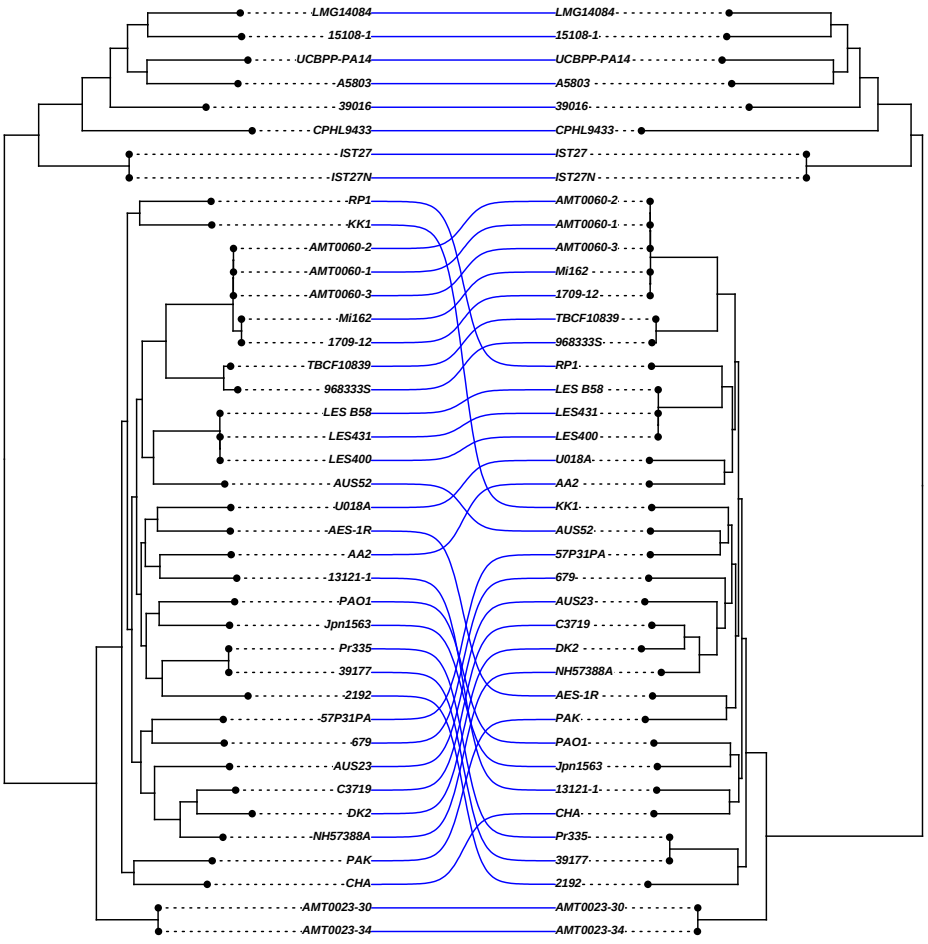

Figure 3. Comparison of phylogenetic trees of *P. aeruginosa* panel strains based on SNPs obtained using REALPHY (left) and Gubbins (right).

strains, into four groups, defined by the software designers; for example, if a gene could be found in 99 to 100% of genomes it is called the core gene (Page *et al*., 2015; Sitto and Battistuzzi, 2020). The Table 1 present the count of genes within categories identified by the Roary, used with the default options and after including the -s parameter (option needed for further analysis). Generally, approximately 5,000 genes can be found in core and soft-core gene categories. Meanwhile, Shell and cloud gene categories, which correspond to the additional and unique genes, accounts greater number of genes. Despite the used parameters of the analysis, the resulted reretraction curves (Fig. 4) indicates that the pan-genome of studied set of *P. aeruginosa* strain is open. It is indicated by the fact that Total genes curve on Fig 4. does not reach plateau (Gmiter *et al*., 2021). Corresponding results were obtained previously (Freschi *et al*., 2019; Mosquera-Rendón *et al*., 2016), indicating similar level of panel strains diversity in comparison to the other isolates.

Roary also creates a gene presence/absence table and then provides phylogenetic tree based on it (so-called pan-genome based phylogeny). The tree together with the matrix is presented on Figure 5. Sequences clustered into two major pan-genome groups, indicated with red and purple.

Table. *Pseudomonas aeruginosa* pan-genome count.

Gene type	Frequency between strains	Default	With -s parameter
Core genes	(99% <= strains <= 100%)	4313	4502
Soft core genes	(95% <= strains < 99%)	884	764
Shell genes	(15% <= strains < 95%)	1680	1382
Cloud genes	(0% <= strains < 15%)	6469	5098
Total genes	(0% <= strains <= 100%)	13346	11746

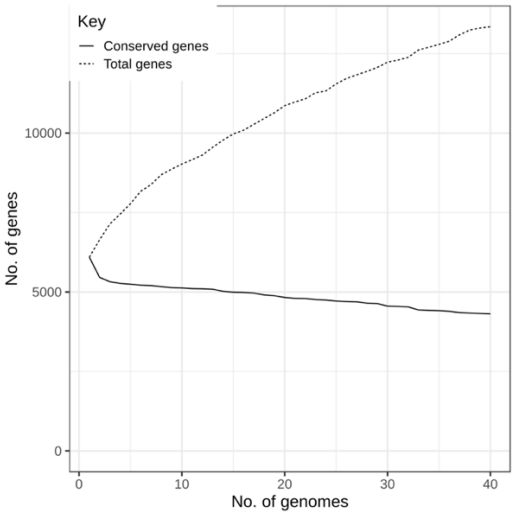

Figure 4. Gene accumulation curves of the *P. aeruginosa* panel strains pan-genome (dashed) and core genes (solid).

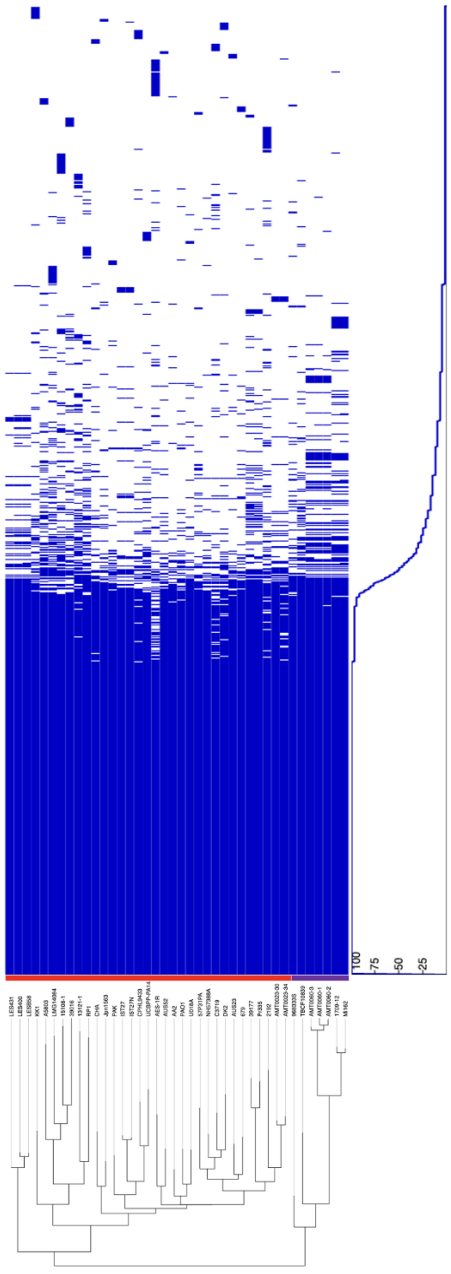

Figure 5. Pan-genome based phylogenetic tree of 40 genomes from *P. aeruginosa* reference panel together with the gene presence/absence matrix. Visualized using Phandango.

To better explore patterns of strains clustering based on SNPs and genomic content, trees from Fig. 1 and Fig. 5 were compared using R package phytools. The results are presented on Figure 6. It could be seen that both phylogenetic approaches provide different pattern of strains clustering. That indicates different evolution of strains genomes content in comparison to the genome sequences. A

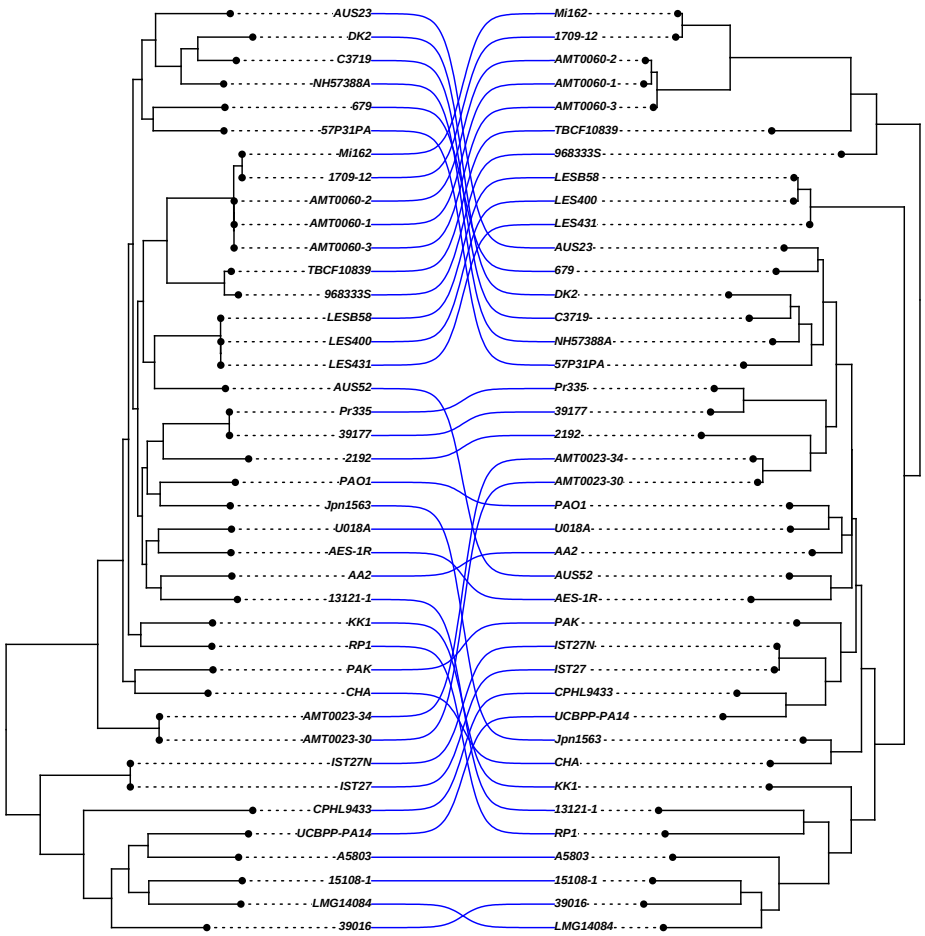

Figure 6. Phylogenetic trees of panel *P. aeruginosa* strains based on SNPs obtained using REALPHY (left) and pan-genome content (right).

similar variability in the way strains were grouped, depending on which one of the above phylogenetic methods was used, was observed in the case of strains belonging to the genus *Aeromonas* (Science *et al*., 2019).

Much of the research analyzing *P. aeruginosa* pan-genome focused on genes often overlooked non-coding intergenic regions (IGRs). IGRs sequence contains a significant amount of crucial genomic information, being biologically relevant elements such as promoter, terminators, and regulatory binding sites (Nielsen *et al*., 2023). For the linkage between genotypes and phenotypes of microorganisms to be effective, IGRs must be taken into consideration. IGRs may also undergo Horizontal Regulatory Transfer (HRT) (Matus-Garcia *et al*., 2012; Ragan and Beiko, 2009). It is thought that as much as 32% of core regulatory regions in *Escherichia coli* and 51% of overall core IGRs were acquired via HRT (Oren *et al*., 2014). Thus, in some cases IGRs may lead to more genetical diversity than genes themselves.

The analysis of IGRs in *P. aeruginosa* panel strains genomes was performed using Piggy, based on Roary files generated with -s option, to not split paralogs into separate clusters (Thorpe *et al.*, 2018). IGRs can be denoted as core and additional, depending on frequency. Figure 7 presents the matrix of core and additional IGRs among strains.

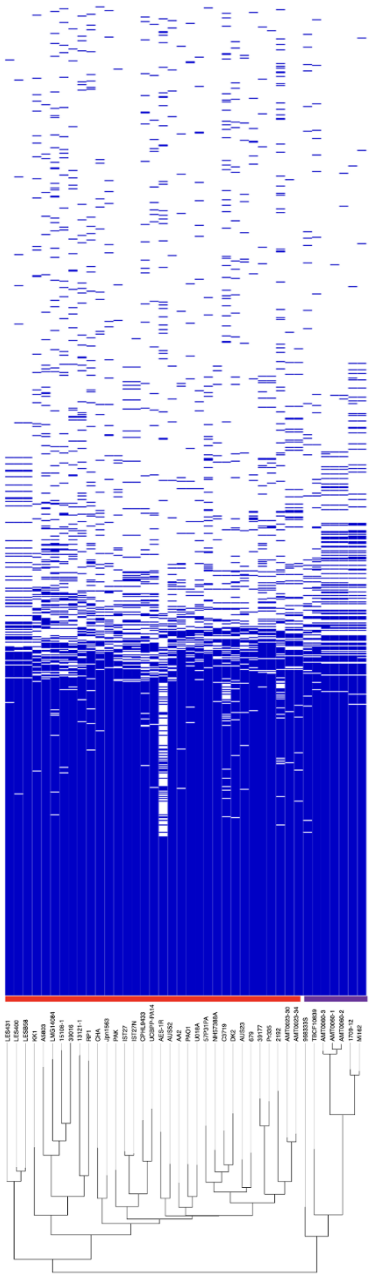

Figure 7. Distribution of IGRs among studied 40 genome sequences of panel *P. aeruginosa* strains. The pan-genome based phylogenetic tree was used for visualization using Phandango.

Piggy provides a core IGRs alignment file – similar to the way Roary provides the core genes alignment file. Both files can be used to perform phylogenetic analysis. Here we used SeaView to obtain ML phylogenetic trees. Figure 8 presents comparison of the mentioned trees based on core genes and core IGRs. Both approaches generated similar patterns of strains clustering into two major groups. However, the groups differ in terms of strains diversity within. Smaller group of eight strains (green group, Fig 1.) cluster almost identical, except of strain 15108-1, which shown more similarities to LMG14084 in IGRs based phylogeny, and strain 39016, for which phylogenetic distance to LMG14084 is higher in IGRs based phylogeny. Meanwhile, strains from second group shown higher level of core genes and core IGRs dissimilarities. These results suggest different evolutionary pressure on genomic regions in a particular panel strain.

Finally, Figure 9 presents the characteristic of genes and IGRs frequency and distribution in *P. aeruginosa* panel strains pan-genome. We

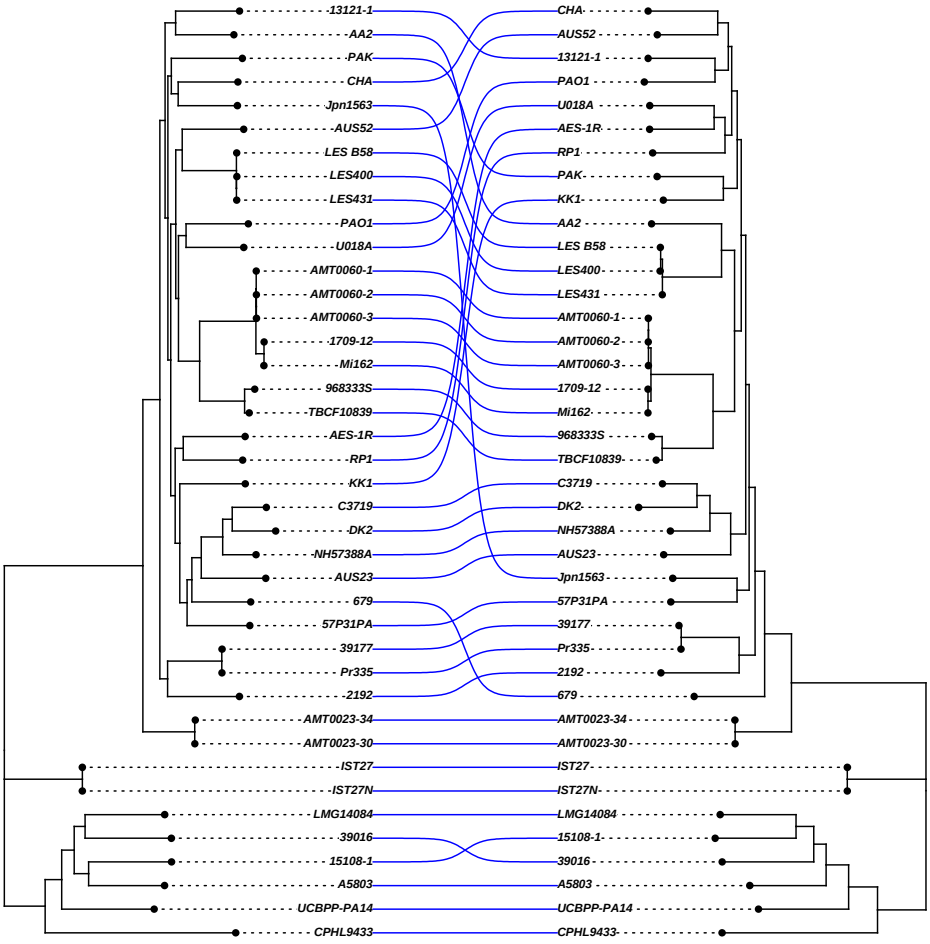

Figure 8. The phylogenetic trees of *P. aeruginosa* panel strains based on core gene sequences (left) and core IGRs sequences (right).

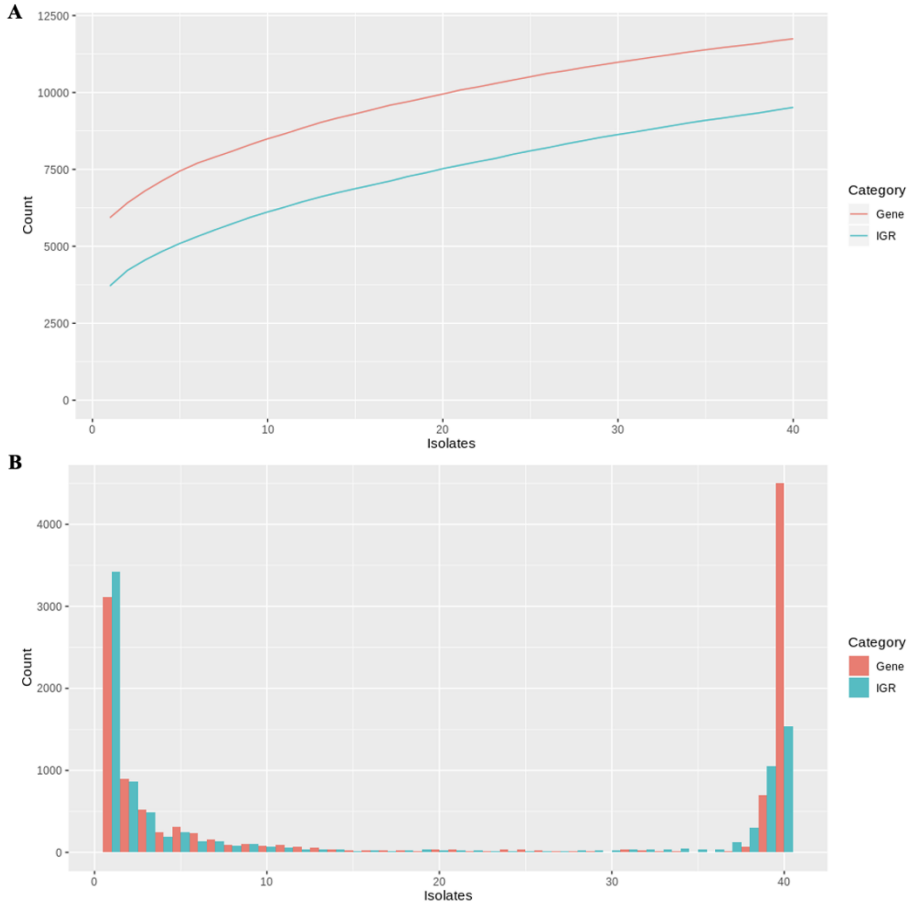

Figure 9. Properties of the panel *P. aeruginosa* strains pan-genome and its IGRs. (A) Number of unique IGRs (green) and genes (orange) as a function of the number of isolates included in the pangenome. (B) Distribution of unique IGRs (green) and genes (orange) across the pangenome, illustrated with a frequency histogram (number of IGRs/genes present in the given number of genomes).

observed that the number of unique IGRs increases with the number of analysed genomes, with a similar pattern in case of the number of unique genes (Fig. 9A). Moreover, the majority of IGRs was present in either most of the strains or only a few of them, which means that they were either very common or very rare, with only few exceptions (Fig. 9B). The obtained results remain in agreement with previous observations for other bacterial species (Nielsen *et al*., 2023; Thorpe *et al*., 2018).

Conclusions

P. aeruginosa represents species that is known for its genetic and phenotypic diversity. To better explore mechanisms of its pathogenicity, the reference panel of word-wide isolates was developed and primarily characterized. Within presented study we performed additional characteristic of panel strains genomes using wide range of *in silico* approaches, including SNPs- based phylogeny, as well as pan-genome and IGRs investigation. We shed light on strains diversity,

concluding that strains form two major phylogenetic groups, when used methods based on comparison of DNA sequences. Further, these groups are consistent with the same strains. However, strains from these groups might be characterized by different level of diversity. We observed different pattern of potential recombination events, as well as dynamics of coding and non-coding regions evolution. On the other hand, studied strains shown distinct patter of clustering in pan-genome based phylogeny comparing to previous. Obtained results suggest evolutionary pressure from the environment of their isolation source. This diversity, in turn, most likely affect strains pathogenicity.

Acknowledgements

Study was supported by the Polish National Science Centre Grant 2019/32/T/NZ1/00515 (D.G.).

Reference

Bertels, F., Silander, O.K., Pachkov, M., Rainey, P. B., Van Nimwegen, E. 2014. Automated reconstruction of whole-genome phylogenies from short-sequence reads. Molecular Biology and Evolution, 31(5).

Croucher, N.J., Page, A.J., Connor, T.R., Delaney, A.J., Keane, J.A., Bentley, S.D., Parkhill, J., Harris, S.R. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Research, 43(3), e15.

Cullen, L., Weiser, R., Olszak, T., Maldonado, R.F., Slachmuylders, L., Brackman, G., Paunova-Krasteva, T.S., Zarnowiec, P., Czerwonka, G., Reilly, J., Drevinek, P., Kaca, W., Melter, O., De Soyza, A., Perry, A., Winstanley, C., Stoitsova, S.R., Lavigne, R., Mahenthiralingam, E., Sá-Correia, I., Coenye, T., Drulis-Kawa, Z., Augustyniak, D., Valvano, M.A., McClean, S. 2015. Phenotypic characterization of an international Pseudomonas aeruginosa reference panel: strains of cystic fibrosis (CF) origin show less in vivo virulence than non-CF strains. Microbiology, 161(10), 1961–1977.

De Soyza, A., Hall, A.J., Mahenthiralingam, E., Drevinek, P., Kaca, W., Drulis-Kawa, Z., Stoitsova, S.R., Toth, V., Coenye, T., Zlosnik, J.E.A., Burns, J. L., Sá-Correia, I., De Vos, D., Pirnay, J.P., Kidd, T.J., Reid, D., Manos, J., Klockgether, J., Wiehlmann, L., Tümmler, B., McClean, S., Winstanley, C. 2013. Developing an international Pseudomonas aeruginosa reference panel. MicrobiologyOpen, 2(6).

Freschi, L., Bertelli, C., Jeukens, J., Moore, M.P., Kukavica-Ibrulj, I., Emond-Rheault, J.G., Hamel, J., Fothergill, J.L., Tucker, N.P., McClean, S., Klockgether, J., De Soyza, A., Brinkman, F.S.L., Levesque, R.C., Winstanley, C. 2018. Genomic characterisation of an international Pseudomonas aeruginosa reference panel indicates that the two major groups draw upon distinct mobile gene pools. FEMS Microbiology Letters, 365(14).

Freschi, L., Vincent, A.T., Jeukens, J., Emond-Rheault, J.G., Kukavica-Ibrulj, I., Dupont, M.J., Charette, S.J., Boyle, B., Levesque, R.C. 2019. The *Pseudomonas aeruginosa* pan-genome provides new insights on its population structure, horizontal gene transfer, and pathogenicity. Genome Biology and Evolution, 11(1), 109–120.

Gmiter, D., Nawrot, S., Pacak, I., Zegadło, K., Kaca, W. 2021. Towards a better understanding of the bacterial pan-genome. Acta Universitatis Lodziensis. Folia Biologica et Oecologica, 17, 84–96.

Gouy, M., Guindon, S., Gascuel, O. 2010. Sea view version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution, 27(2), 221–224.

Hadfield, J., Croucher, N.J., Goater, R.J., Abudahab, K., Aanensen, D.M., Harris, S.R. 2018. Phandango: An interactive viewer for bacterial population genomics. Bioinformatics, 34(2), 292–293.

Matus-Garcia, M., Nijveen, H., Van Passel, M.W.J. 2012. Promoter propagation in prokaryotes. Nucleic Acids Res. 40, 10032–10040.

Mosquera-Rendón, J., Rada-Bravo, A.M., Cárdenas-Brito, S., Corredor, M., Restrepo-Pineda, E., Benítez-Páez, A. 2016. Pangenome-wide and molecular evolution analyses of the Pseudomonas aeruginosa species. BMC Genomics, 17(1), 1–15.

Nielsen, F.D., Møller-Jensen, J., Jørgensen, M.G. 2023. Adding context to the pneumococcal core genes using bioinformatic analysis of the intergenic pangenome of Streptococcus pneumoniae. Frontiers in Bioinformatics, 3.

Oren, Y., Smith, M.B., Johns, N.I., Kaplan Zeevi, M., Biran, D., Ron, E.Z., Corander, J., Wang, H.W., Alm, E.J., Pupko, T. 2014. Transfer of noncoding DNA drives regulatory rewiring in Bacteria. Proc. Natl. Acad. Sci. U. S. A. 111, 16112–16117.

Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T.G., Fookes, M., Falush, D., Keane, J.A., Parkhill, J. 2015. Roary: Rapid large-scale prokaryote pan-genome analysis. Bioinformatics, 31(22), 3691–3693.

Page, A.J., Taylor, B., Delaney, A.J., Soares, J., Seemann, T., Keane, J.A., Harris, S.R. 2016. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. Microbial Genomics, 2(4), e000056.

Ragan, M.A., Beiko, R.G. 2009. Lateral genetic transfer: Open issues. Philos. Trans. R. Soc. B Biol. Sci. 364, 2241–2251.

Revell, L.J. 2012. Phytools: An R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution, 3(2), 217–223.

Science, E., Zhong, C., Han, M., Yang, P., Chen, C., Yu, H., Wang, L., Ning, K. 2019. Comprehensive analysis reveals the evolution and pathogenicity of *Aeromonas*, Viewed from Both Single Isolated Species and Microbial Communities. mSystems, 4(5), 1–19.

Seemann, T. 2014. Prokka: Rapid prokaryotic genome annotation. Bioinformatics, 30(14), 2068–2069.

Sitto, F., Battistuzzi, F. U. 2020. Estimating Pangenomes with Roary. Molecular Biology and Evolution, 37(3), 933–939.

Thorpe, H.A., Bayliss, S.C., Sheppard, S.K., Feil, E.J. 2018. Piggy: A rapid, large-scale pan-genome analysis tool for intergenic regions in bacteria. GigaScience, 7(4), 1–11.

Folia Biologica et Oecologica 18: 85–90 (2024)

Acta Universitatis Lodziensis

Probiotics as a potential tool for gynecological cancers prevention and therapy support

OLGA KUŹMYCZ[1], WIKTORIA WIERZBIŃSKA[1,2]

[1]University of Lodz, Faculty of Biology, Department of Molecular Microbiology, Banacha 12/16, 90–237 Lodz, Poland;
[2]BioMedChem Doctoral School of the University of Lodz and Lodz Institutes of the Polish Academy of Sciences, Matejki 21/23, 90–237 Lodz, Poland
E-mail: olga.kuzmycz@biol.uni.lodz.pl, wiktoria.wierzbinska@edu.uni.lodz.pl

ABSTRACT

The human body is host to a variety of microbial species that are essential for maintaining general health. About nine percent of these microbial species inhabit the urogenital tract. It is therefore important to establish a symbiotic relationship between the host's immune system and the microbiota in this region. Disruptions of this symbiosis can contribute to the development of pelvic inflammatory disease (PID), bacterial vaginosis (BV), vaginal candidiasis and/or even gynaecological cancers by promoting the proliferation of infectious agents. The chemotherapeutic agents currently used in cancer treatment are associated with undesirable side effects. Therefore, there is an urgent need for antitumor agents that have minimal toxicity compared to conventional treatments. Furthermore, the use of drugs to prevent or alleviate the side effects of cervical cancer treatment could significantly improve the quality of life of patients undergoing such therapy. In this review, we focus our attention on the potential efficacy of probiotics in both the prevention and therapeutic intervention of neoplasms affecting the genital tract.

KEYWORDS: *Lactobacillus*, *Saccharomyces cerevisiae* var. *boulardii*, cervical cancer, microbiome

Introduction

Probiotics are living microorganisms, usually bacteria, which provide health benefits when consumed in sufficient quantities. Similar beneficial microorganisms occur naturally in certain foods such as yogurt, kefir, sauerkraut, kimchi and kombucha, as well as in dietary supplements. Probiotic microorganisms commonly used in the human diet mainly belong to the genera: *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus* and *Enterococcus*. Some strains of *Bacillus* and *Saccharomyces* are also used (Markowiak and Śliżewska, 2017). These microorganisms are able to produce anticarcinogenic, antioxidant and

DOI: https://doi.org/10.18778/1730-2366.18.12

antimutagenic agents and provide protection against various bacterial diseases, which makes them an interesting tool in cancer research.

In the uterine tract of healthy women, *L. crispatus*, *L. gasseri*, *L. iners* and/or *L. jensenii* have been found to dominate the microbiome and maintain a physiological pH range (3.8–4.5) through the production of lactic acid, which inhibits the proliferation of pathogenic species (Garcia-Grau *et al*., 2018). In addition, lactobacilli are involved in the production of hydrogen peroxide (H_2O_2), which has been shown to have a protective role against bacterial vaginosis (BV) associated bacteria (e.g. *Gardnerella vaginalis*, *Prevotella bivia*, *Atopobium vaginae*) and sexually transmitted infections (Yarbrough *et al*., 2015). In studies by Ravel and colleagues (2011), the 16S rRNA marker gene was used to categorize the vaginal microbiota into four distinct low-diversity CSTs (community state types) dominated by a single *Lactobacillus* spp.: CST-I, in which *L. crispatus* dominates, CST-II with a predominance of *L. gasseri*, CST-III – *L. iners* and CST-V – *L. jensenii*. About 80% of women of reproductive age have a simple vaginal microbiota dominated by *Lactobacillus*, while the remaining 20% have a more diverse community type (CST-IV) with several subgroups (Ravel *et al*., 2011). Studies have shown that women who are not dominated by lactobacilli have an increased susceptibility to various genital infections, an increased risk of unfavourable reproductive outcomes, a poor pregnancy rate, early spontaneous abortion, late miscarriage and premature birth (Pendharkar *et al*., 2023). Current research indicates that the microbiome is integral to the female reproductive endocrine system, and interacts with estrogen, androgen, insulin, and others (Qi *et al*., 2021). Brooks *et al*. (2017) suggested that the oral hormonal contraceptives can positively impact the reproductive system by increasing the abundance of *Lactobacillus* species and reducing bacterial texa associated with bacterial vaginosis. Furthermore, progesterone may enhance the α-diversity of both vaginal and endometrial microbiomes.

Microbiome of gynecological cancer

The precise involvement of the uterine microbiome in pathophysiological processes remains unclear. Recent advances in metagenomic and sequencing technologies offer promising opportunities to solve this mystery. A cross-sectional study of 31 women revealed a significant correlation between the co-occurrence of *Atopobium* and *Porphyromonas* in the lower and upper genital tract, coupled with abnormal vaginal pH (> 4.5), and the incidence of endometrial cancer (Walther-António *et al*., 2016). Subsequently, a separate study examined the microbiome composition in ovarian tissue and normal tissue samples from 25 patients diagnosed with ovarian cancer. The results showed a significant reduction in the abundance of lactobacilli and an increase in anaerobic bacterial populations in ovarian carcinoma tissue, compared to adjacent normal tissue (Zhou *et al*., 2019). Furthermore, a robust case-control study of 360 women, including 176 individuals with ovarian cancer and 184 controls, demonstrated a reduced presence of lactobacilli (<50%) in the cervicovaginal microbiome of women with ovarian cancer (Nené *et al*., 2019). In cervical cancer, dysbiosis has been shown to influence human papillomavirus (HPV) infection by affecting HPV acquisition, clearance and persistence, as well as the host immune response by affecting levels of immune system proteins such as TGF-β1 (transforming growth factor β 1) (Łaniewski *et al*., 2020). Collectively,

these studies provide compelling evidence linking disruption of the genital microbiome to the pathogenesis of gynaecological malignancies. Therefore, probiotics may serve as adjunctive agents to enhance or modulate other diagnostic and therapeutic approaches.

Kailasapathy *et al*. (2000) have proposed mechanisms by which probiotics may exert their role as antitumour agents. These mechanisms include activation of the host immune system, alteration of colonic transit time and motility, suppression of pro-carcinogens and carcinogens, inhibition of bacteria involved in the conversion of pro-carcinogens to carcinogens, and lowering of intestinal pH (Kailasapathy *et al*., 2000). Research suggests that probiotics have a significant impact on several biological processes involved in cancer, including apoptosis, oxidative stress, proliferation, inflammation and metastasis (Johanshahi *et al*., 2020).

Probiotics and their use in gynecological cancer treatment

Cervical cancer is one of the most common malignancies affecting women's health worldwide, although its incidence is significantly lower in developed countries. Approximately 80% of cases are reported in developing or less developed countries. It is typically asymptomatic in its early stages, but as the disease progresses, symptoms may manifest, such as dyspareunia, pelvic pain, irregular vaginal bleeding, fatigue and leg swelling (Simms *et al*., 2020). Findings have shown that *Lactobacillus plantarum*, sourced from vaginal secretions of young adult and adolescent women, has been demonstrated to possess probiotic attributes and exhibit anticancer properties against the HeLa line of cervical cancer (Nami *et al*., 2014). In another study involving the HeLa cell line, research on human milk-derived strains of *Lactobacillus* (*L. casei* SR1, *L. casei* SR2, and *L. paracasei* SR4) demonstrated notable probiotic effects. Results indicated that supernatants from cell-free cultures exhibited anticancer activities, including the BCL-2 downregulation and upregulation of genes of such proteins as caspase 3, caspase 8, caspase 9, BAX (BCL2-associated X protein), and BAD (BCL2-associated agonist of cell death) (Rajoka *et al*., 2018). Treating HeLa cells with supernatants of *L. rhamnosus* and *L. crispatus* has been observed to decrease the expression of the *CASP3* (caspase-3) gene, as well as *MMP2* (matrix metalloproteinase 2) and *MMP9* (matrix metalloproteinase 9), resulting in an inhibitory effect on metastasis (Nouri *et al*., 2016). Research findings of Cha *et al*. (2012) indicate that *Bifidobacterium adolescentis* SPM1005-A exhibits antiviral activity in the SiHa cervical cell line, which expresses HPV type 16 (oncogenic), potentially contributing to the prevention of cervical cancer. Treatment with this bacterium has been shown to decrease the expression of the *E6* and *E7* oncogenes at both mRNA and protein levels. *L. gasseri* 3396 and *L. crispatus* 2743 had an inhibitory effect on the expression of *E6* and *E7* at the mRNA level (Li *et al*., 2019). The same effect was noticed in the case of cell-free supernatants (CFS) of *L. crispatus*, *L. jensenii* and *L. gasseri* treatment of Ca Ski cells (Wang *et al*., 2018).

It has been observed that cisplatin pro-apoptotic and cytotoxic effects against cervical cancer cells are enhanced by co-treatment with *Lactobacillus* bacteria. In a recent study, Negi *et al*. (2020) proposed a drug delivery strategy involving the application of probiotic strains. They concluded that pessaries containing cisplatin and probiotic biomass could offer an improved therapeutic approach for cervical cancer, as probiotic

strains exhibited favourable effects such as free radicals' elimination. Conversely, Kim *et al*. (2015) reported that the extract of *L*. *casei* did not demonstrate synergistic effects with anticancer drugs in suppressing the growth of HeLa and Ca Ski cancer cells. Despite numerous studies investigating the role of probiotics in cervical cancer, little is understood about their synergistic effects with drugs used to treat this cancer. Nonetheless, probiotics have the potential to enhance the antitumor effects of other drugs (Villeger *et al*., 2019). The knowledge of exact mechanisms associated with antitumour actions of probiotic bacteria is still limited and requires further research. Little is known about the effects of probiotics in other types of gynaecological cancer.

Yeast probiotics and their potential use in cancer therapy support

A wide array of yeasts, such as *Saccharomyces cerevisiae* var. *boulardii*, *Kluyveromyces*, *Debaryomyces*, *Candida*, *Pichia*, *Hanseniaspora*, and *Metschnikowia*, have been demonstrated to exhibit probiotic properties. Similarly, the anticancer properties of yeast probiotics have been extensively examined through various methods in numerous studies, including cell-based investigations, animal models, and clinical trials (Sambrani *et al*., 2021). The promotion of traditional fermented foods enriched with a spectrum of yeast probiotic strains holds promise in mitigating the risk of colorectal cancer (CRC) development significantly. Multiple investigations have underscored the favourable impact of yeast probiotics in cancer therapy support, highlighting their capacity to engage apoptotic pathways and modulate immune responses (Shamekhi *et al*., 2020). *In vivo* studies, coupled with high-throughput metagenomic analysis of 281 stool samples, have substantiated efficacy of *S. cerevisiae* var. *boulardii* use in significantly impeding colorectal cancer metastasis. The inhibition of cancer cells development was achieved through the stimulation of cancer cell apoptosis and the promotion of gastrointestinal health via immunomodulation. Notably, *S. cerevisiae* var. *boulardii* demonstrates a capacity to downregulate the expression of various genes encoding proteins implicated in tumour genesis, including TNF-α (tumour necrosis factor alpha), interleukin-1β, and interleukin-17. *S. cerevisiae* var. *boulardii*-based probiotics has been also used in vaginal candidiasis treatment, which is caused by *Candida albicans*. The yeast-based probiotics and fungus interaction results in prohibiting the cohesion of *Candida albicans* to the vaginal epithelial cells (Pericolini *et al*., 2017). Despite reports suggesting the advantageous effects of yeast probiotics in support of cancer treatment, the range of their application in the treatment and prevention of gynecological malignancies remains incompletely investigated. A summary of the presented probiotics and their effect on cancer is shown in Table 1.

Conclusion

Because of their ability to restore and maintain a healthy microbiome in the gynaecological tract and modulate the immune system, probiotics hold promise for the treatment of several gynaecological conditions, including bacterial vaginosis, vaginal candidiasis, sexually transmitted infections, and human papillomavirus (HPV) infection. While numerous studies have highlighted the potential benefits of probiotics in cancer prevention and treatment support, research into their impact on endometrial and ovarian cancer prevention remains limited. Furthermore, the therapeutic potential of yeast in the treatment of

Table 1. Studies investigating probiotics on chosen cancer types.

Probiotics	Cancer type	Cell line/ Research material	Findings	Publication
Lactobacillus plantarum	Cervical cancer	HeLa	Inhibition of cancer cells growth	Nami *et al*. 2014
Lactobacillus casei SR1, *Lactobacillus casei* SR2, *Lactobacillus paracasei* SR4		HeLa	Apoptosis-related genes upregulation	Rajoka *et al*. 2018
Lactobacillus rhamnosus, *Lactobacillus crispatus*		HeLa	Inhibitory effect on metastasis	Nouri *et al*. 2016
Bifidobacterium adolescentis SPM1005-A		SiHa	Antiviral properties against HPV infection	Cha *et al*. 2012
Lactobacillus gasseri 3396, *Lactobacillus crispatus* 2743		SiHa	HPV oncogene downregulation	Li *et al*. 2019
Lactobacillus crispatus, *Lactobacillus jensenii, Lactobacillus gasseri* CFS		Ca Ski	HPV oncogene downregulation	Wang *et al*. 2018
Saccharomyces cerevisiae var. *boulardii*	Colorectal cancer	Metagenomic analysis of stool samples	Downregulation of expression of the genes implicated in tumor genesis	Shamekhi *et al*. 2020

gynaecological cancers has been largely overlooked.

Further investigation of the anticancer properties of specific probiotic strains and their underlying mechanisms is essential. Randomized, double-blind, placebo-controlled clinical trials are needed to gain approval from the medical community and validate probiotics as a viable assistance in cancer therapy (Śliżewska *et al*., 2020). In conclusion, further research is needed to clarify the precise mechanisms of action of probiotics, although the existing evidence strongly supports their use to improve women's health.

References

Brooks, J.P., Edwards, D.J., Blithe, D.L., Fettweis, J.M., Serrano, M.G., Sheth, N.U. 2017 Effects of combined oral contraceptives, depot medroxyprogesterone acetate and the levonorgestrel-releasing intrauterine system on the vaginal microbiome. Contraception, 95, 405–413.

Cha, M.K., Lee, D.K., An, H.M., Lee, S.W., Shin, S.H., Kwon, J.H. 2012. Antiviral activity of Bifidobacterium adolescentis SPM1005-a on human papillomavirus type 16. BMC Medicine, 10, 72.

Garcia-Grau, I., Simon, C., Moreno, M. 2018. Uterine microbiome low biomass and high expectations. Biology of Reproduction, 101(6), 1102–1114.

Jahanshahi, M., Maleki Dana, P., Badehnoosh, B., Asemi, Z., Hallajzadeh, J., Mansournia, M.A., Yousefi, B., Moazzami, B., Chaichian, S. 2020. Anti-tumor activities of probiotics in cervical cancer. Journal of Ovarian Research, 13(1), 68.

Kailasapathy, K., Chin, J. 2000. Survival and therapeutic potential of probiotic organisms with reference to Lactobacillus acidophilus and Bifidobacterium spp. Immunology & Cell Biology, 78, 80–88.

Kim, S.N., Lee, W.M., Park, K.S., Kim, J.B., Han, D.J., Bae, J. 2015. The effect of *Lactobacillus casei* extract on cervical cancer cell lines. Contemporary Oncology (Poznan), 19, 306 –312.

Laniewski, P., Ilhan, Z.E., Herbst-Kralovetz, M.M. 2020. The microbiome and gynaecological cancer development, prevention and therapy. Nature Reviews Urology, 17, 232–250.

Li, C., Jia, L., Yu, Y., Jin, L. 2019. Lactic acid induced microRNA-744 enhances motility of SiHa cervical cancer cells through targeting ARHGAP5. Chemico-Biological Interactions, 298, 86–95.

Markowiak, P., Śliżewska, K. 2017. Effects of probiotics, prebiotics, and synbiotics on human health. Nutrients, 9, 1021.

Nami, Y., Abdullah, N., Haghshenas, B., Radiah, D., Rosli, R., Khosroushahi A.Y. 2014. Assessment of probiotic potential and anticancer activity of newly isolated vaginal bacterium *Lactobacillus plantarum* 5BL. Microbiology and Immunology, 58(9), 492–502.

Negi, D., Singh, A., Joshi, N., Mishra, N. 2020. Cisplatin and Probiotic Biomass Loaded Pessaries for the Management of Cervical Cancer. Anti-cancer Agents in Medicinal Chemistry, 20(5), 589–98.

Nené, N.R., Reisel, D., Leimbach, A., Franchi, D., Jones, A., Evans, I. 2019. Association between the cervicovaginal microbiome, BRCA1 mutation status, and risk of ovarian cancer: a case-control study. The Lancet Oncology, 20(8), 1171–1182.

Nouri Z., Karami F., Neyazi N., Modarressi M.H., Karimi R., Khorramizadeh M.R. 2016. Dual anti-metastatic and anti-proliferative activity assessment of two probiotics on HeLa and HT 29 cell lines. Cell Journal, 18(2):127.

Pendharkar, S., Skafte-Holm, A., Simsek, G., Haahr, T. 2023. Lactobacilli and Their Probiotic Effects in the Vagina of Reproductive Age Women. Microorganisms, 11(3):636.

Pericolini, E., Gabrielli, E., Ballet, N., Sabbatini, S., Roselletti, E., Cayzeele Decherf, A., Pélerin, F., Luciano, E., Perito, S., Jüsten, P. 2017. Therapeutic activity of a *Saccharomyces cerevisiae*-based probiotic and inactivated whole yeast on vaginal candidiasis. Virulence, 8:74–90.

Qi, X., Yun, C., Pang, Y., Qiao, J. 2021. The impact of the gut microbiota on the reproductive and metabolic endocrine system. Gut Microbes, 13(1):1-21.

Rajoka M.S.R., Zhao H., Lu Y., Lian Z., Li N., Hussain N. 2018. Anticancer potential against cervix cancer (HeLa) cell line of probiotic *Lactobacillus casei* and *Lactobacillus paracasei* strains isolated from human breast milk. Food & Function, 9(5), 2705–2715.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S.K., McCulle, S.L., Karlebach, S., Gorle, R., Russell, J., Tacket, C.O. 2011. Vaginal Microbiome of Reproductive-Age Women. Proceedings of the National Academy of Sciences USA, 108 (Suppl. 1), 4680–4687.

Sambrani, R., Abdolalizadeh, J., Kohan, L., Jafari, B. 2021. Recent advances in the application of probiotic yeasts, particularly Saccharomyces, as an adjuvant therapy in the management of cancer with focus on colorectal cancer. Molecular Biology Reports, 48(1), 951–960.

Shamekhi ,S., Lotfi, H., Abdolalizadeh, J., Bonabi, E., Zarghami, N. 2020. An overview of yeast probiotics as cancer biotherapeutics: possible clinical application in colorectal cancer. Clinical and Translational Oncology, 22(8), 1227–1239.

Simms, K.T., Steinberg, J., Caruana, M., Smith, M.A., Lew, J.B., Soerjomataram, I. 2019. Impact of scaled up human papillomavirus vaccination and cervical screening and the potential for global elimination of cervical cancer in 181 countries. The Lancet Oncology, 20(3), 394–407.

Śliżewska, K., Markowiak-Kopeć, P., Śliżewska, W. 2020. The Role of Probiotics in Cancer Prevention. Cancers (Basel), 23, 13(1):20.

Villeger, R., Lopes, A., Carrier, G., Veziant, J., Billard, E., Barnich, N. 2019. Intestinal microbiota: a novel target to improve anti-tumor treatment? International Journal of Molecular Sciences, 20, 4584.

Walther-António, M.R., Chen, J., Multinu, F., Hokenstad, A., Distad, T.J., Cheek, E.H. 2016. Potential contribution of the uterine microbiome in the development of endometrial cancer. Genome Medicine, 8(1):122.

Wang, K. D., Xu, D. J., Wang, B. Y., Yan, D. H., Lv, Z., Su, J. R. 2018 Inhibitory effect of vaginal Lactobacillus supernatants on cervical Cancer cells. Probiotics and Antimicrobial Proteins, 10, 236–242.

Yarbrough, V.L., Winkle, S., Herbst-Kralovetz, M.M. 2015 Antimicrobial Peptides in the Female Reproductive Tract: A Critical Component of the Mucosal Immune Barrier with Physiological and Clinical Implications. Human Reproduction Update, 21(3):353-77.

Zhou, B., Sun, C., Huang, J., Xia, M., Guo, E., Li, N. 2019 The biodiversity composition of microbiome in ovarian carcinoma patients. Scientific Report, 9(1), 1691.

Folia Biologica et Oecologica 18: 91–109 (2024)

Acta Universitatis Lodziensis

Epigenetic Landscapes of Endometriosis: From Pathogenesis to Precision Medicine

ALICJA WIĘCEK, ALEKSANDRA BEZUBIK, VERANIKA KANANOVICH, KACPER PIETRZYK, TADEUSZ PIETRUCHA

Medical University of Lodz, Department of Medical Biotechnology, Zeligowskiego 7/9, 90–752 Lodz, Poland
E-mail: alicja.wiecek@stud.umed.lodz.pl

ABSTRACT

Endometriosis, a challenging gynecological disorder characterized by the ectopic presence of endometrial-like tissue, presents significant diagnostic and therapeutic hurdles due to its complex etiology and diverse clinical manifestations. Recent advancements in understanding its pathogenesis have underscored the pivotal role of epigenetic alterations, offering new insights into disease mechanisms and therapeutic targets. Epigenetic changes in endometrial cells significantly contribute to endometriosis pathogenesis, disrupting normal physiology and hormone responsiveness, particularly to progesterone. Dysregulation of histone modifications, DNA methylation, and non-coding RNA expression disrupts cellular homeostasis and promotes disease progression. Histone modifications, notably methylation and acetylation, influence chromatin structure and gene expression, affecting progesterone responsiveness and disease progression. Epigenetic regulators such as Cfp1 modulate progesterone receptor expression and downstream signalling pathways, presenting potential therapeutic targets. Non-coding RNAs, including miRNAs and lncRNAs, exert regulatory effects on gene expression and are implicated in endometriosis pathogenesis. Dysregulated expression disrupts cellular homeostasis and promotes disease progression. Biomarker studies have identified specific miRNAs and lncRNAs associated with endometriosis, offering avenues for non-invasive diagnosis and targeted therapies. siRNA-based therapies targeting key genes involved in endometriosis pathogenesis show promise as novel treatment modalities. By modulating gene expression and cellular functions, siRNA-based therapies offer a targeted approach to mitigate pathological processes. In this review, we summarize recent findings in the molecular mechanisms and regulatory pathways of endometriosis, offering valuable insights into pathology and therapeutic interventions. Future research efforts aimed at elucidating the complex interplay between epigenetic regulators and disease pathways hold promise for innovative diagnostic tools and targeted therapies.

DOI: https://doi.org/10.18778/1730-2366.18.13

KEYWORDS: endometriosis, epigenetic, infertility, pathogenesis of endometriosis

Introduction

Endometrium, the lining of the uterine cavity, is, undoubtedly, an extraordinary tissue. Its growth and maturation, which are extremely important for the embryo implantation, depend intimately on estrogen and progesterone concentrations. In case of the absence of conception, hormonal changes lead to the hypoxia and cell death in its functional (outer) layer, resulting in menstrual bleeding. Starting from the very first day of the menstrual cycle, the cells in the basal (inner) layer of endometrium start to mature and proliferate, in order to build a new functional layer in a few days, just in time for ovulation. This makes endometrium one of the fastest growing tissues in the human body (Maenhoudt *et al*., 2022).

Another striking characteristic of endometrium is the fact that in some patients it can be found outside its natural location. This benign pathological condition, called endometriosis, is defined as a presence of functional endometrium outside the uterus (MeSH database). Most often, the endometriotic foci, or ectopic endometrium, are found in the peritoneal cavity (Saunders and Horne, 2021) but can be found in more distant organs as well (Andres *et al*., 2020). Just like the eutopic endometrium, the ectopic one goes through a hormone-dependent cycle of growth and bleeding. Additionally, processes such as inflammation (Machairiotis *et al*., 2021), epithelial -to-mesenchymal transition (EMT) (Proestling *et al*., 2015), angio- (Rocha *et al*., 2013) and neurogenesis (Asante and Taylor, 2011) are observed in endometriosis. Several cancer-driving mutations have been discovered in the ectopic lesions of patients with this condition (Anglesio *et al*., 2017), although it's important to mention, the observed changes might be a result of the endometriosis progression rather than a reason for its development (Guo, 2018).

Endometriosis affects about 10% of women worldwide and is associated with pain (Bellelis *et al*., 2010), infertility (Filip *et al*., 2020) and increased predisposition to cancer (Pearce *et al*. 2012; Kok *et al*., 2015). Despite this disease being a social-economic burden (Missmer *et al*., 2021; Darbà and Marsà, 2022), mechanisms responsible for its pathogenesis are still unknown; this slows down both the process of discovering new, more effective treatments that do not interfere with patients' ability to conceive and the non-invasive yet reliable diagnostic tests.

The theory of implantation through retrograde menstruation proposed almost a century ago till date remains the most well-known and the best-supported in the scientific community (Sampson, 1927). It suggests that during menstruation some amount of endometrium cells can travel with blood through fallopian tubes to the peritoneal cavity, then attach and form the endometriotic lesions. This theory, however, ignores cases of endometriosis in men (Martin and Hauck 1985; Schrodt *et al*., 1980), same as the presence of ectopic endometrium in species that don't menstruate, such as guinea pigs (Baldi *et al*., 2017). It also does not necessarily explain why only a portion of women experiencing retrograde menstruation develops endometriosis (Halme *et al*., 1984).

Epithelial to mesenchymal transition of endometrium cells can be another way to explain the onset of the disease. During this process EMT transcription factors, such as zinc finger E-box-binding proteins (Zeb1/Zeb2), repress the expression of epithelial cell junction elements, for example E-cadherin, encoded by

Cadherin-1 (*CDH1)* gene (Du *et al*., 2019). Furthermore, TET1 activation leads to simultaneous *CDH1* downregulation and enhanced synthesis of N-cadherin. Obtained mesenchymal phenotype of cells allows for migration and invasion of other organs (Wu *et al*., 2020).

For the progression of endometriosis, the endometrial implant must be protected from apoptosis and immune response. Upregulated expression of cyclooxygenase-2 (COX-2) promotes the production of prostaglandins, consequently leading to immune evasion (Wang *et al*., 2012). Increased prostaglandins' levels promote angiogenesis via stimulating vascular endothelial growth factor (VEGF) overexpression, which increases the supplies of nutrients (Tamura *et al*., 2006). That, alongside the increased estrogen concentration, leads to endometrial tissue growth stimulation. The estrogen action on ectopic endometrium is regulated by progesterone, which by binding to its receptor induces downregulation of the estrogen receptors. However, the loss of progesterone receptors allows for endometrial cell proliferation, independent of progesterone (Reis *et al*., 2020). Furthermore, obtained ability to either synthesize estrogen, acquired via steroidogenic factor-1 (SF-1) activation, or convert extracellular testosterone to estrogen, due to aromatase production, accelerates the growth of endometriotic tissue (Xue *et al*., 2007b).

The overexpression of *Insulin-like growth factor 1 receptor (IGF1R)* in ectopic endometrium positively affects both endometrium cells migration and proliferation (Bai *et al*., 2021). This effect is enhanced by decreased levels of *Homeobox A10 (Hoxa10)* expression product, which allows for epithelial- to mesenchymal transition and estrogen synthesis (Elias *et al*., 2023) (Fig. 1).

Endometriosis is considered a complex disease, where there is no specific gene responsible for its development, but a set of genomic and environmental alterations. Given that, data from Genome Wide Association Study (GWAS) can serve as a valuable source of information for possible etiopathology (Cano-Gamez and Trynka, 2022). As some of the single nucleotide polymorphisms identified through GWAS are located in independent non-coding DNA fragments involved in gene expression regulation, it was suggested that epigenetic modifications contribute to endometriosis development (Zondervan *et al*., 2016).

Epigenetics studies gene expression changes without interfering in DNA sequence. Those changes are possible because of the epigenetic modifications. They can activate or inhibit gene transcription by chromatin modifications. Genetic modifications involve several mechanisms. There are DNA methylation, histone modification and non-coding RNA. DNA methylation interferes with gene expressions connected with implantation. Histone modifications are important in pathogenesis in endometriosis. It suggests that inhibitors of histone modifications enzymes may have their role in endometriosis treatment. In endometriosis there are over 50 different expressions of miRNA. MiRNA is type of non-coding RNA (Adamczyk *et al*., 2022). Such changes can be passed on to offspring and might explain a partially hereditary nature of endometriosis (Simpson *et al*., 1980). Role of epigenetics in endometriosis is mainly referring to development of endometriosis (Adamczyk *et al*., 2022).

In recent years, numerous studies on epigenetic regulation of endometriosis have been published, revealing the striking differences in epigenetic

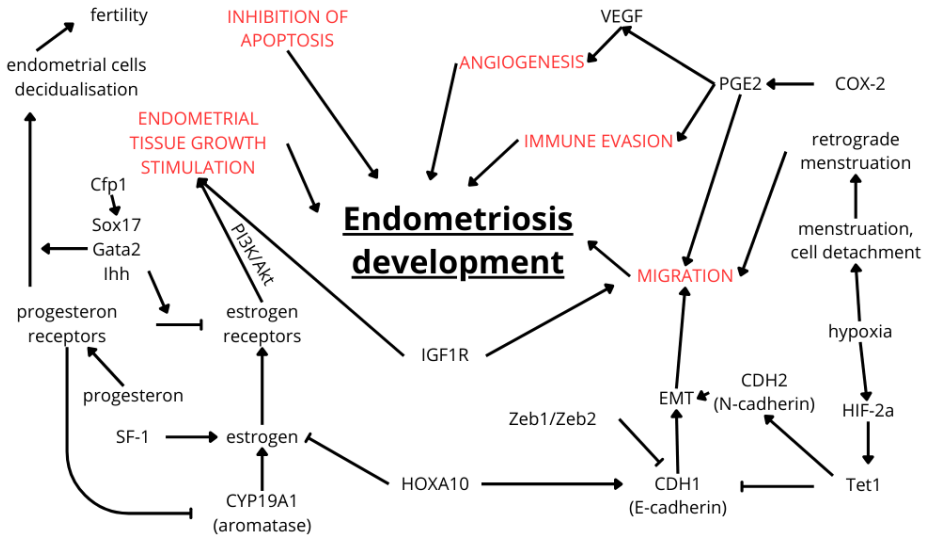

Figure 1. Processes leading to endometriosis development. Endometriosis is characterized by several processes which comprise the pathogenic phenotype. These include migration, endometrial tissue growth stimulation, inhibition of apoptosis, angiogenesis and immune evasion (enumerated and described respectively in the following text). I) Migration is fostered by the following factors. Physiological hypoxia during menstruation, which allows for cell detachment, upregulates *Tet1* through *HIF-2a* transcription activation. This promotes EMT through inhibition of *CDH1* (encoding E-cadherin) and activation of *CDH2* (encoding N-cadherin) expression. HOXA10 promotes *CDH1* expression. Conversely, Zeb1 and Zeb2 downregulate it. Migration can be promoted by IGF1R and also by PGE2 action, the level of which is elevated after COX-2 overexpression. II) Endometrial tissue growth is regulated by estrogen receptor activation (ER) followed by PI3K/Akt activation. ER activation is inhibited by progesterone receptor (PR) action, which is promoted by Cfp1. PR downstream pathway inhibits aromatase function, which is to convert androstendione to estrogen. The latter can be produced intracellularly after SF-1 expression activation in endometrial cells. HOXA10 inhibits the aforementioned process. IGF1R promotes the growth of endometrial tissue. III) Inhibition of apoptosis that can be introduced by downregulation of suppressor genes like *TP53* (not included on the figure). IV) Angiogenesis is induced by VEGF overexpression that can be caused by PGE2 action. V) Immune evasion also is promoted by PGE2. Additionally, Cfp1 provides proper decidualization by acting on PR downstream pathway, what is crucial for maintaining fertility.

regulation of patients with endometriosis and healthy women. In this review, we discuss and streamline some of that work, to illuminate possible molecular mechanisms underlying causes of endometriosis and discuss potential treatment and diagnostic opportunities.

Deacetylation and methylation of histones may have a critical role in endometriosis progression

Epigenetics plays a key role in regulating gene expression without simultaneously affecting the DNA sequence. One of the well-studied mechanisms included in epigenetics is post-translational modifications of histones (Weinhold, 2006). On their N-terminal and C-terminal tails are amino acid residues such as lysine, arginine, serine or threonine (Alaskhar Alhamwe *et al*., 2018). These amino acid residues undergo acetylation, methylation and phosphorylation as modification processes of histones (Zhang *et al*., 2021).

The mechanisms of histone acetylation and deacetylation mainly occur via two enzymes: histone acetyltransferases

(HATs) and histone deacetylases (HDACs). The role of the mechanism of histone acetylation is not known yet. However, the role of the deacetylation mechanism and its occurrence in pathological endometriosis tissue has been demonstrated. Decreased level of histone 3 (H3) acetylation has been found in the endometrial lesions; in particular, the decreases level of acetylation of histone 3 on lysine 9 (H3K9) is mainly indicated here. H3K9 has influence on expression of *p16*, *MLH1* and *HOX* genes. High expression of *HOXA10* has influence of endometriosis progression. Hypoacetylation of H3 causes lower transcription of *HOXA10*. These genes are related to the endometriosis progression (Monteiro *et al*., 2014). However, despite the general lack of differences in histone 4 (H4), it is worth noting that the same study also presented clear evidence of decreased level of acetylation of histone 4 on lysine 16 (H4K16) in patients diagnosed with endometriosis. Deacetylation of the above histone lysine residues highlights the role of HDACs in the development of endometriosis (Monteiro *et al*., 2014) and inhibition of HDACs that causes a reduction of invasion and proliferation on endometrial cells in an animal model (Wu *et al*., 2007).

The study to understand the changes in HDAC expression levels in endometriosis started with histone deacetylase 1 (HDAC1) and histone deacetylase 2 (HDAC2). The HDAC1 expression was higher in endometriosis samples compared to control samples, however, the origin of the tissue showing the changes present in endometriosis was important (Colón-Diaz *et al*., 2012). The HDAC2 has also been identified as a factor that affects the development of endometriosis, as it has been shown to be highly expressed in the endometriosis cells. Furthermore, silencing the gene of HDAC2 induced apoptosis for cells and inhibition of endometriosis progression. Activation of the HNFA4/ARID1A axis has been found in endometriosis progression (Fig. 2). HNF4A is a

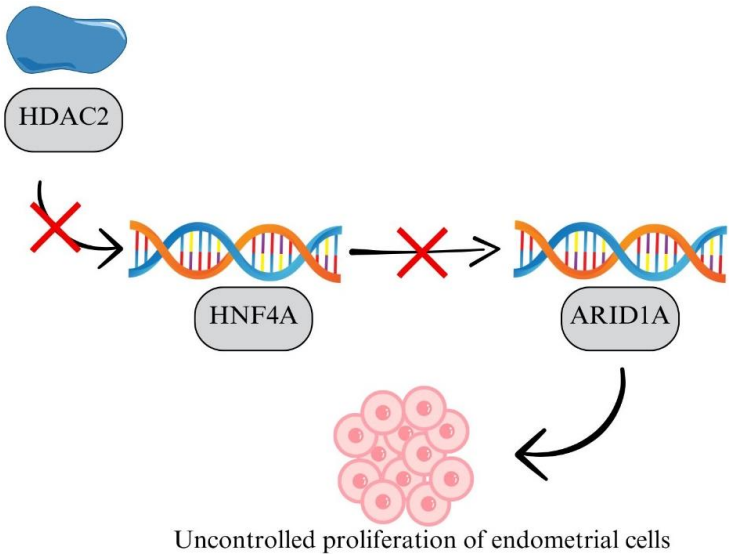

Figure 2. High level of histone deacetylase 2 (HDAC2) may lead to progression of endometriosis by inhibition of HNF4A/ARID1A pathway.

transcriptional factor promoting the expression of other genes such as *ARID1A* and others that affect endometrial cells progression. The study shows that higher level of HDAC2 effects on silencing of *HNFA4*. That means, the lower level of *HNFA4* may silence the expression of *ARID1A*. That means uncontrolled proliferation of endometrial cells and progression of endometriosis (Mai *et al*., 2021).

In the HDAC family there is also a Sirtuin 1, whose elevated expression has been correlated with endometriosis and formation of inflamed tissue, particularly with the ovarian endometriosis type (Taguchi *et al*., 2016; Mvunta *et al*., 2017). On the other hand, abnormal expressions of HDAC1 and histone deacetylase 6 (HDAC6) were clearly noted in deeply infiltrating endometriosis (Zheng *et al*., 2023). At the same time, attention was drawn to histone deacetylase 8 (HDAC8), whose inhibition of expression in deeply infiltrating endometriosis by using an HDACs inhibitor reduced lesion development and endometriosis progression by two-thirds (Zheng *et al*., 2023). HDAC and its role of endometriosis progression is not well studied. They seem to be very successful therapeutic targets. However, their influence on certain genes needs to be found. Still there are not many studies about this topic, which is important for better understanding of endometriosis progression.

Methylation also occurs predominantly on the lysine residues of histones, mainly H3 and H4. It is mainly catalysed by histone methyltransferase (HMT). This leads to chromatin compression and regulation of gene expression (Zhang *et al*., 2021). However, there is a mechanism of histone demethylation carried out by the enzyme histone demethylase 1 (LSD1) (Li *et al*., 2024). H3K9 and H3K4 demethylation are linked with LSD1. LSD1 influences on *CDH1* causing its downregulation of expression. It means that there is a high chance of starting EMT in endometriosis (Ding *et al*., 2014).

Higher levels of histone methylation were observed in endometriosis than in normal tissue (Monteiro *et al*., 2014). Level of methylation of histone 3 on lysine 4 (H3K4) is a much higher in the endometriotic than in the control samples, whereas an increase in methylation of H3K9 was also noticed in the same samples but comparably not as high. H3K9 has influence on *HOXA10* gene expression. Hypermethylated *HOXA10* is involved in endometriosis progression. By far the most interesting result was obtained for the levels of methylation of histone 3 on lysine 27 (H3K27), which was found in both eutopic and ectopic lesions in patients diagnosed with endometriosis (Monteiro *et al*., 2014). Further investigations showed that the highest expression in endometriosis was from 3-methylated lysine 27 of histone 3 (H3K27me3). This methylation model is catalysed by the enhancer homologue of zeste 2 (EZH2), whereas increased expression in endometriosis (Colón-Caraballo *et al*. 2015). Furthermore, many H3K27me3 are in the promoter regions of candidate genes as biomarkers in endometriosis, including genes of estrogen receptor 1 (*ESR1*), cadherine 1 (*CDH1*), and progesterone receptor (*PGR*). Especially there is focus on the *CDH1* and the CDH1 methylation in this case (Colón-Caraballo *et al*., 2018). *CDH1* is hypermethylated in endometriosis lesions, which means this gene's expression is lower. Downregulation of *CDH1* promotes the development of EMT (Monteiro *et al*. 2014). There is still too little information about histone methylation of *ESR1* and *PGR* and its influence on development of endometriosis.

All these facts render the HDAC inhibitor a new therapeutic target, whereas an understanding of the effect of HDACs in the pathogenesis of endometriosis is required. However, it is important to remember that epigenetic aberrations basis of endometriosis is highly dependent on the development of endometriosis (Zheng *et al*. 2023, Colón-Diaz *et al*. 2012). H3K27me3 has also increased in endometriosis. That makes H3K27me3 the major epigenetic marker for endometriosis. Furthermore, inhibition of EZH2 is considered as a target in the treatment of endometriosis (Colón-Caraballo *et al*., 2015).

Women with endometriosis have an altered DNA methylation profile

The most abundant DNA modification is methylation of cytosine to 5C-methylcytosine (5-mC). Other oxidated forms of 5-mC (5-hydroxy-methylcytosine [5-hmC], 5-formyl-cytosine [5-fC], 5-carboxylcytosine [5-caC]) and methylation of adenine to N6-methyladenine (6-mA) are less common and therefore less examined, yet their role in transcription regulation is pivotal (Klungland and Robertson, 2017). Cytosine modification takes place mainly within regions of high cytosine (C) and guanine (G) density sequences named CpG islands.

CpG islands have been found in about 70% of human gene promoters and their role in transcription regulation has been proven robustly (Al Aboud *et al*., 2023). Gene transcription can be both silenced by CpG 5mC methylation within promoters and, less commonly, silenced or enhanced by CpG methylation in other functional parts of genes. These changes can be recovered thanks to DNA active demethylation. The process of methylation is guided by DNA methylotransferases (DNMTs) and the demethylation is conducted by ROS1 and also by Tet and TDG pathways (He *et al*. 2011; Onodera *et al*., 2021). The 5-hmC, 5-fC, and 5-caC serve as intermediate states preceding demethylation but independent biological functions of 5-hmC and 5-fC are also suspected (Kumar *et al*., 2018). While methylation of gene promoters and first exon in gene body is percieved as transcription downregulating factor, methylation within rest of gene body can be actually an enhancer of its transcripton in in dividing cells (Moore *et al*., 2013) Taking into consideration that endometrial and endometriotic cells proliferate in high rate, this dependence may apply to them.

TET methylcytosine dioxygenase genes were claimed to be downregulated in endometrium of women with endometriosis and endometriotic lesions compared to control samples. However, at the same time 5-hmC content in tissue was increased, whereas decreased in blood (Roca *et al*., 2016). This wasn't confirmed in other study (Yotova *et al*., 2017), therefore aforementioned contradictory results could have arisen from some mistake. Another study indicates that in general Tet family expression is dysregulated in endometriotic cells (Wu *et al*., 2020). Representants of TET family are expected to exhibit specificity in their targets of demethylation, therefore it is important to examine the gene expression individually for its members. The study concentrated on methylcytosine dioxygenase TET1, encoded by *TET1*, function and expression in endometriotic tissue. Protein encoded by this gene has been shown to upregulate N-cadherin (mesenchymal adhesive protein) while downregulating E-cadherin (epitelial adhesive protein) in epitelium of eutopic endometriotic cells. This trait points to TET1 protein as a crucial factor of EMT. Authors present results indicating upregulation of *TET1* expression in epitelium of eutopic endometriotic cells.

It is suggested that hypoxia induced *HIF-2α* (Hypoxia induced factor 2α) transcription factor expression upregulates *TET1* gene. Hypoxic conditions arise while endometrial epitelial cells detach during menstruation. Then, during retrograde menstruation, they change adhesion profile from epithelial to mesenchymal, which allows for cell survival while not attached to basal layer. E-cadherin is a part of pathway that leads to anoikis during disconnection from the layer (Kumar *et al*., 2011). Substitution of this adhesive protein with N-cadherin helps to avoid anoikis during cell migration.

Numerous studies present genes that show different methylation patterns between control and endometriosis samples. These include: hypomethylation of coding region in gene coding the aromatase (*CYP19A1*) what leads to upregulation of gene expression (Izawa *et al*., 2011); homeobox A10 (*HOXA10*) promoter hypermethylation (Elias *et al*. 2023); estrogen receptor 2 (*ESR2*) (Xue *et al*. 2007a), steroidogenic factor-1 (*SF-1*) (Xue *et al*., 2007a) and cyclooxygenase-2 (*COX-2*) (Wang *et al*., 2012) promoter hypomethylation.

Aromatase is an androstendione to estradiol converting enzyme. As the most potent from all estrogens, estradiol promotes proliferation of cells expressing estrogen receptor, including endometriotic ones. Overexpression of this enzyme's gene in ectopic stromal cells allows for more aromatase production and higher levels of estradiol inside the cells, leading to increased cell division and implant growth.

Homeobox protein Hox-A10 (homeobox A10) encoded by *HOXA10* is a DNA-binding transcription factor which is responsible for controlling the expression of genes in stromal cells of endometrial tissue during secretory phase, when differentiation from fibroblast-like to decidual cells takes place. The hypermethylation of *HOXA10* promoter has been discovered in eutopic but not ectopic endometriotic cells. This may indicate that silencing *HOXA10*, which results in undifferentiated cellular development state, is essential for cell detachment and migration, though hypermethylation has to be retreated to let for ectopic implantation. It is consistent with homeobox A10's function as a repressor of Snail gene (*SNAI1*), product of which is a repressor of E-cadherin (Yoshida *et al*., 2006). *HOXA10* has been also connected with downregulation of enzymes for cholesterol synthesis, which is a substrate in estrogen production (Yu *et al*., 2022). Therefore, *HOXA10* silencing may increase estrogen production in endometriotic cells.

Estrogen receptors are required for both endometrial and endometriotic cells to proliferate. Functional particles are dimers formed by subunits α and β as homo or heterodimers. ERβ, encoded by ESR2 gene, expression is upregulated in ectopic tissue cells compared to eutopic ones. This is not the case with ERα encoded by ESR1 gene on the other hand. Methylation of CpG islands within promoters of these genes reflect the mRNA expression level. This result is intriguing, as ERβ homodimers show anti-proliferating activity after binding the estrogen in a murine cellular model (Song *et al*., 2022). ERα is the estrogen receptor subunit homologue, which is overexpressed in cancers like breast cancers, and it exhibits a proliferation stimulating trait. There is a possibility of the model not being translative to human cells *in vivo*, but currently the hypomethylation of ERβ does not seem to be beneficial to endometriotic cells but the real gain may yet to be discovered.

Steroidogenic factor-1 is a transcription factor responsible for expression of machinery for estrogen

biosynthesis. Even though the process is one of physiological sources of estrogen, it does not happen in endometrial stromal cells due to SF-1 promoter hypermethylation. Hypomethylation of CpG island within this locus allows for endometriotic cells for self-synthesis of estrogen, which through estrogen receptors promote the proliferation of the cell.

Cyclooxygenase-2 takes part in enzymatic conversion of arachidonic acid to prostaglandins. CpG island within promoter of the gene it is encoded by in normal endometrium is hypermethylated. Expression of *COX-2* leads to inflammation through prostaglandins production. Further activation of molecular pathways promotes cell survival, invasion, angiogenesis and immune evasion. These traits allow endometriotic cells to migrate beyond uterus, when *COX-2* expression is elevated in case of their promoter hypomethylation. PGE2 also mediate the angiogenesis through *VEGF* overexpression, leading to enhanced nutrition of intensively proliferating tissue of endometrial implants (Tamura *et al*., 2006).

As shown, many genes, particularly those connected with proliferation, cell signalling, migration, immunity, angiogenesis, undergo changed methylation content in endometriosis compared to control (Mortlock *et al*., 2023). This stresses the importance of methylation level in endometriosis pathomechanism. In the epithelial cells of endometrium of women with endometriosis, 5-hmC contents are lower than in control, although parallel to 5-mC, indicating general trend of demethylation (Yotova *et al*., 2017).

Although not commonly seen in DNA, N6-methyladenine (6-mA) has been classified as an RNA modification. Shen *et al*. proved also the influence that 6-mA can have on endometriosis development. Functionality of methyltransferases conducting methylation of adenosine is crucial for post transcriptional use of mRNA. It has been proven that 6-mA content in mRNAs coding proteins from PI3K/Akt pathway affects translation performance. The pathway is responsible for cell survival, proliferation, estrogen signalling. Therefore, enhanced translation caused by hypermethylation of adenosine in these transcripts can promote endometriotic lesion growth (Shen *et al*., 2023).

DNA methylation is a double-edged sword when it comes to functional traits – it can silence transcription of genes both beneficial and unfavorable for disease development. Therefore, to utterly understand the molecular basis behind probable impacts of DNA modifications on the mechanisms of selectivity of this phenomenon need to be uncovered.

Enhancer methylation has been proven more correlated with gene expression than promoters' methylation in breast cancer (Aran and Hellman, 2013). This study stresses the importance of enhancer sequence methylation. It is therefore crucial not to confine research to mere promoters, but to also study the role of enhancers and the coding regions also in endometriosis pathology.

Some studies focus on discovering the epigenetic differences between samples of eutopic and ectopic foci (Mortlock *et al*., 2023), which at first glance is the proper way to evaluate the shift which the endometrial cell must make to migrate to ectopic loci. Unfortunately, the rate of specific cell types differs in constitution of eutopic endometrium compared to ectopic lesion. Therefore, while analysing any markers with purpose of comparison between these two sources, this inequality has to be taken into consideration (Barjaste *et al*., 2019).

Etiopathological changes may well be present already in the cells of endometrium (Li *et al*., 2019). Changes in expression of genes from pathways which play a key role in endometriosis can be found throughout the surface of patients' uteri (Brosens *et al*., 2012). This phenomenon has a twofold consequence. Firstly, it allows us to diagnose the disease early and in a non-invasive way, thanks to biomarkers detected in aspiration biopsy material from eutopic endometrial tissue (Żeberkiewicz *et al*., 2022). Secondly, it sheds more light on the character of endometriosis: when pathogenic changes are present in the progenitor cells throughout uterus, a foundation of the disease has been inherited or developed up to the moment of organogenesis. Then the pathogenic traits get activated at the onset of puberty with the change in sex hormones release. Alternatively, the pathogenic traits were gained due to environmental factors which have driven epigenetic change in most endometrial cells or cells that control their functioning. Even taking into consideration a delayed diagnosis (Nnoaham *et al*., 2011), substantial part of cases onset after the age of 30 (Gunawardena *et al*., 2020). Therefore, it is possible that the inherited mutations but also the modifications gained through individual's life can modulate the pathogenic phenotype (Afshar *et al*., 2013). This points to epigenetics as the probable driver of these changes (Martin and Fry, 2018).

Epigenetic changes are responsible for progesterone resistance in endometriosis pathogenesis

Understanding of the epigenetic changes such as methylation or acetylation of DNA that underlie endometriosis requires comprehension of the dynamic physiology of endometrium, which is widely regulated by female sex hormones such as progesterone and estrogen. The first one – progesterone (P4) is very important both for monthly development of endometrium and during pregnancy, and especially for the embryo implementation into the uterine wall. The molecular mechanisms of P4-driven regulations depend on activation of its receptors (progesterone receptors; [PGRs]). The PGRs control expression of local factors such as those responsible for epithelium-stroma interactions which play a clue role in endometriosis pathogenesis. The well-known methylation and acetylation patterns of H3/H4 in women with endometriotic lesions are highly distinct in endometrium of the healthy patients. These changes can interrupt the physiological response to progesterone and estrogen (Yang *et al*., 2023).

One of the genes indirectly affecting the uterus response to progesterone is *Cfp1*, which encodes the zinc finger protein 1. Due to its numerous binding domains, *Cfp1* induces epigenetic changes in two ways: At the DNA level by interfering with DNA methyl transferase 1 (DNMT1) activity, and at the histone level by attaching the SET1 subunit to the H3K4 methyltransferases, which enables its trimethylation. The CXCC domain binds DNA at the unmethylated CpG islands; this prevents the formation of inappropriate H3K4 methylation patterns outside these regions and thus maintains the appropriate epigenetic structure of chromatin (Fan *et al*., 2023). *Cfp1* is an evolutionarily conserved gene and its silencing in the embryonic cells is closely related to a decrease in cytosine methylation, by decreasing the translation efficiency and the methyltransferase stability. In addition, *Cfp1* affects the levels of expression of *Gata2*, *Sox17* or *Ihh* genes involved in the signalling pathways of cellular response to progesterone (Yang *et al*., 2023).

The loss of the *Cfp1* gene and ergo lack of that gene's translated protein was

correlated with mouse infertility, which was caused by several disorders from embryo transport to subsequent implantation, including disorders of endometrial cell proliferation (Yang *et al*., 2023). Additionally, abnormalities in the inhibition of ectopic endometrial growth by P4 were observed in a mouse model of endometriosis with *Cfp1* deletion. The deletion is associated with a significant decrease in expression of *Gata2, Sox17* and *Ihh* genes (Yang *et al*., 2023). These observations became the basis for the study of Cfp1-dependent progesterone resistance in human endometrial foci cells. For this purpose, transcripts of healthy patients and patients with endometriosis were compared (Yang *et al*., 2023). Despite the lack of association between the level of *Cfp1* expression and the levels of expression of the progesterone receptor gene, a similar decline in expression of the *Gata2, Sox17* and *Ihh* genes was observed in the cells of women with endometriosis, similarly to the mouse model (Yang *et al*., 2023). However, noticeable differences in the expression of these genes were found only in women with severe disease (Yang *et al*., 2023). These findings indicated the role of *Cfp1* in the pathogenesis of endometriosis and helped formulate the hypothesis of disruption of signalling pathways in the cellular responses to progesterone in cells following the epigenetic changes (Yang *et al*., 2023).

Altered non-coding RNA expression profile can lead to endometriotic lesions

The term 'non-coding RNA' (ncRNA) refers to the RNA fragments that do not encode for a protein, but are involved in other important processes, to which the post-transcriptional gene expression regulation belongs (Hombach and Kretz, 2016) Some ncRNA, such as microRNA (miRNA), small interfering RNA (siRNA), and long non-coding RNA (lncRNA or lnRNA) have been identified as potential key players in endometriosis development (Abbaszadeh *et al*., 2023).

MicroRNA are short RNA sequences, predominantly 22 nucleotides in length; when bound to mRNA, microRNAs serve as a signal for its degradation by ribonucleases. In that way miRNA is able to influence the expression level of targeted genes (Hombach and Kretz, 2016).

The 2020 case-control study showed the difference in the miR-125b levels between endometrium (eutopic or ectopic) in patients with endometriosis and the healthy controls. It was proposed that the increased activity of miR-125b interferes with TP53 expression, resulting in inhibition of apoptosis (Hajimaqsoudi *et al*., 2020).

A microRNA profile analysis of patients' ectopic endometrial tissue using new generation sequencing (NGS) (Hawkins *et al*., 2011) resulted in 10 upregulated (miR-202, 193a-3p, 29c, 708, 509-3-5p, 574-3p, 193a-5p, 485-3p, 100, 720) and 12 downregulated (miR-504, 141, 429, 203, 10a, 200b, 873, 200c, 200a, 449b, 375, 34c-5p) miRNAs when compared to healthy endometrium of the controls.

Interestingly, a different study confirmed an increase in expression of miR-29c, this time in eutopic endometrium of endometriosis patients; miR-29c involvement in endometrial tissue progesterone resistance through downregulation of its targeted gene's level (FK506-binding protein 4) was shown as well (Joshi *et al*., 2017). Additionally, both the miR-200 family and the miR-34c-5p have been associated with epithelial-to-mesenchymal transition. miR-200s inhibit Zeb1 and Zeb2 transcription factors, as well as MALAT lncRNA (Du *et al*., 2019), while miR-34c-5p inhibits the Notch1 signaling. Downregulation of those microRNAs

results in the loss of epithelial phenotype and increases cells' migration and invasion abilities in the in vitro models of endometriosis (Luo *et al*., 2020).

Several studies have focused on serum miRNA profiling with the aim of creating a new non-invasive biomarker for endometriosis (Cosar *et al*., 2016; Chico-Sordo *et al*., 2024). For example, serum levels of circulating miR-31 and miR-145 turned out to be significantly different in women with and without endometriosis (Bashti *et al*., 2018).

Combining microRNA research and artificial intelligence allowed to differentiate between patients with endometriosis and controls with exceptional accuracy and test's AUC (area under the curve) value reaching up to 98.4% (Bendifallah *et al*., 2022).

Long non-coding RNAs define the RNA sequences exceeding 200 nt in length which do not undergo translation. But, lncRNAs undergo splicing, polyadenylation, and capping, similar to mRNA (Guttman *et al*., 2009). The lncRNA are able to act on gene expression by employing four main molecular mechanisms, including of modifications protein: protein interactions, small RNA binding, miRNA inhibition through competition, and guiding RNAs to RNA-binding proteins (Statello *et al*., 2021). The way lnRNAs can serve as an epigenetic agent is therefore through interference with functioning of small RNAs and proteins that control gene expression. Recently, lnRNA was proven to regulate chromatin structure, thus influencing transcription process (Yan and Bu, 2021).

Based on bioinformatics analysis, 3 lnRNAs were found as strongly associated with endometriosis development, allowing for functional explanations of pathological traits they supply (Bai *et al*., 2021). One of them, H19, inhibits miRNA let-7 leading to IGF1R upregulation and, in consequence, to enhanced proliferation and migration of endometrial stromal cells (Bai *et al*., 2021). Another one, GS1-358P8.4, interrupts the Rap1 signalling pathway, thus providing cancerous traits. The last one – RP11-96D1.10, comparably the least studied, seems to supply a trait common with H19.

In another study, SNHG4 lnRNA that promotes proliferation, migration, epithelial – mesenchymal transition, and suppresses apoptosis, has been proven to be overexpressed in uteri of women with endometriosis, suggesting possibility of implementing this lnRNA in diagnostic assays (Szaflik *et al*., 2023).

Small interfering RNA (siRNA) plays a pivotal role in defence against RNA viruses. Its ability to complementarily guide the RNA degradation by utilizing RISC allows it to overpower the invader but also control the copy number of transcripts (mRNA) (Zhang, 2023). This feature can be applied to treat diseases that result from overexpression of specific genes. Therefore, such treatments can find a potential use in endometriosis therapy. For example, vascular endothelial growth factor (VEGF) and ribonucleotide reductase subunit M2 (RRM2) have been proposed as therapeutic targets as proof-of-concept for novel treatments for endometriosis (Kiisholts *et al*., 2021). The VEGF provides the cell with the ability to induce angiogenesis in its environment, whereas the RRM2 promotes proliferation and epithelial-mesenchymal transition, among other processes (Kiisholts *et al*., 2021). Constraining the concentration of their mRNA in endometriotic cells cytoplasm restrains overproduction of proteins; this in turn endows the pathogenic cells with traits useful for migration to and survival in ectopic locus (Kiisholts *et al*., 2021).

Therapeutic and diagnostic perspectives for targetting the epigenetic changes in endometriosis

Discovering the epigenetic mechanisms underlying endometriosis provides us not only with the knowledge itself. Awareness of specific molecules and pathways involved in pathogenesis can be utilized as treatment targets and diagnostic markers. Among the former there are HDAC family and EZH2. The silencing of these genes and inhibition of proteins they encode have shown a potentially therapeutic effect, also on animal models. Another possibility of treatment had an advent along with exploration of siRNA translation downregulating effect on mRNA coding proteins that promote endometriosis development. Implementation of siRNA which silent expression of genes, for instance VEGF and RRM2, can revolutionize the state of the art in endometriosis treatment.

Lack of effective treatment of endometriosis is not the only problem that remains yet unsolved. Non-invasive tests for endometriosis diagnosis are still not implemented as standard procedure on behalf of invasive laparoscopy and USG which lacks sensitivity (Allaire *et al*., 2023). Minimally invasive tests employing biomarkers acquire increasingly relevant performance. DNA modification is another epigenetic mechanism which impacts gene transcription efficiency. Basing on differences in specific mRNA levels in tissue samples of endometrium, a diagnostic test has already been developed (Żeberkiewicz *et al*., 2022). There are promising results of studies which put histone methylation under examination. `3-methylated lysine 27 of histone 3 is significantly more abundant in patients compared to control samples. This fact places it among potential endometriosis markers.

Non-coding RNAs, particularly microRNAs and long non-coding RNAs, have emerged as key regulators of gene expression and potential non-invasive biomarkers for disease diagnosis and prognostication, and novel therapeutic targets for intervention. Tests that measure the concentration of lnRNAs and miRNA prove their utility while showing significant performance.

Conclusions

For over a century endometriosis's etiopathology remains an unsolved mystery. However, recent advancement in molecular biology techniques provided researchers with tools to investigate the disease's underlying causes. There is a growing body of evidence that epigenetic modifications might play a crucial role in endometriosis development. Studies have shown that patients with endometriosis resemble significantly different patterns in histone modifications, DNA methylation and ncRNA expression. Epigenetic alterations emerge as critical determinants in endometriosis, influencing hormone responsiveness, gene expression profiles, and disease progression.

Histone acetylation patterns, mainly of H3K9, introduced by HATs and rescued by HDACs differentiate normal from endometriosis's epigenetic landscape. HDAC members level in endometriosis are elevated leading to survival (HDAC2), inflammation (Sirtuin 1) and infiltration of peritoneal layer (HDAC1, HDAC6 and HDAC8).

Methylation level of H3K27me3 in histones associated with promoters of genes important in endometriosis development was elevated in ectopic endometrium in patients with endometriosis and control endometrium in secretory phase compared to control in proliferative phase. The same dependency was claimed in the case of EZH2 protein

level, which catalyses this type of methylation (Colón-Caraballo *et al*., 2015). It is claimed that H3K27me3 repressing mark was put in regions of promoters of *PGR* and *ESR1* (Colón-Caraballo *et al*., 2018), *HOX* and *COX-2* (Colón-Caraballo *et al*., 2015) genes, which seems to be the contradictory results considering endometriosis promoting roles of *ESR1* and *COX-2* and repressing roles of *PGR* and *HOX* (precisely *HOXA10*) genes. Nevertheless, reduced migration and proliferation followed by EZH2 pharmacological inhibition stresses its importance in endometriosis development and substantiates the need for further research.

CFP1 maintains the proper methylation, thus expression of downstream elements in PGR signalling. Its loss or downregulation can abolish the progesterone induced decidualization, causing embryo implantation disorders. This mechanism may contribute to infertility and endometriosis coincidence.

DNA modifications, predominantly cytosine methylation, introduced by enzymes, including DNMT and TET1, regulate expression of genes involved in pathological processes. Among discovered potential drivers of endometriosis development are: hypomethylation of coding region in CYP19A1 gene encoding aromatase (Izawa *et al*., 2011); homeobox A10 (HOXA10) promoter hypermethylation (Elias *et al*., 2023); estrogen receptor 2 (ESR2) (Xue *et al*. 2007a), steroidogenic factor-1 (SF-1) (Xue *et al*., 2007b) and cyclooxygenase-2 (COX-2) (Wang *et al*., 2012) promoter hypomethylation.

Non-coding RNAs, act on multiple stages including mRNA (microRNA and siRNA), protein-protein interactions (lnRNA) and RNA-protein interaction (lnRNA). Their influence on endometriosis development has been studied intensively in recent years. The levels of specific non-coding RNAs' sets in peripheral blood disclose a promising role in non-invasive endometriosis diagnosis. Moreover, the ability of these molecules to control specific genes expression and protein-protein interactions, makes them proper candidates for endometriosis therapies. The landscape of epigenetic changes responsible for endometriosis development presented in this paper can be targeted in such treatments. Combined with not invasive, therefore earlier, diagnosis non-coding RNAs can make a breakthrough in the field of endometriosis healthcare.

Whereas advancements have been made towards identifying epigenetic signatures and potential therapeutic targets, further investigations are warranted to elucidate the mechanistic underpinnings of epigenetic dysregulation in endometriosis. In the future larger cohort studies, interdisciplinary approaches, integrated multi-omics analyses and translational research efforts will be essential for improving outcomes for patients affected by endometriosis.

Acknowledgements

We would like to thank Dr Marcin Nowicki at the University of Tennessee for helpful discussion and his support in our work.

List of abbreviations

5-caC – 5-carboxylcytosine
5-fC – 5-formylcytosine
5-hmC – 5-hydroxymethylcytosine
5-mC – 5C-methylocytosine
6-mA – N6-methyladenine
C – cytosine
CDHI – cadherine 1
COX-2 – cyclooxygenase-2
DNMT – DNA methylotransferase
DNMT1 – DNA methyl transferase 1
EMT – epithelial-to-mesenchymal transition
ERβ – estrogen receptor 2
ESR1 – estrogen receptor 1
EZH2 – homologue of zeste 2
G – guanine
GWAS – Genome Wide Association Study

H3 – histone 3
H3K27 – histone 3 on lysine 27
H3K27me3 – `3-methylated lysine 27 of histone 3
H3K4 – histone 3 on lysine 4
H3K9 – acetylation of histone 3 on lysine 9
H4 – histone 4
H4K16 – acetylation of histone 4 on lysine 16
HAT – histone acetylotransferase
HDAC – histone deacetylases
HDAC1 – histone deacetylase 1
HDAC2 – histone deacetylase 2
HMT – histone methyltransferase
HOXA10 – homeobox 10A
lncRNA – long non-coding RNA
LSD1 – histone demethylase 1
miRNA – microRNA
ncRNA – non-coding RNA
NGS – new generation sequencing
P4 – progesterone
PGR – progesterone receptor
RRM2–ribonucletide reductase subunit M2
SF-1 – steroidogenic factor-1
siRNA – small interfering RNA
VEGF – endothelial growth factor

References

Abbaszadeh, M., Karimi, M., Rajaei, S. 2023. The landscape of non-coding RNAs in the immunopathogenesis of Endometriosis. Frontiers in Immunology, 14, 1223828.

Adamczyk, M., Wender-Ozegowska, E., Kedzia, M. 2022. Epigenetic Factors in Eutopic Endometrium in Women with Endometriosis and Infertility. International Journal of Molecular Sciences, 23(7), 3804.

Afshar, Y., Hastings, J., Roqueiro, D., Jeong, J.W., Giudice, L.C., Fazleabas, A.T. 2013. Changes in eutopic endometrial gene expression during the progression of experimental endometriosis in the baboon, Papio anubis. Biology of Reproduction, 88(2), :44.

Al Aboud, N.M, Tupper, C., Jialal, I. 2023. Genetics, Epigenetic Mechanism. [In:] StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available from: https://www.ncbi.nlm.nih.gov/books/NBK532999.

Alaskhar Alhamwe, B., Khalaila, R., Wolf, J., von Bülow, V., Harb, H., Alhamdan, F., Hii, C.S., Prescott, S.L., Ferrante, A., Renz, H., Garn, H., Potaczek D.P. 2018 Histone modifications and their role in epigenetics of atopy and allergic diseases. Allergy, Asthma & Clinical Immunology, 14, 39.

Allaire, C., Bedaiwy, M.A., Yong, P.J. 2023 Diagnosis and management of endometriosis. Canadian Medical Association Journal, 195(10), E363–E371.

Andres, M.P., Arcoverde, F.V.L., Souza, C.C.C., Fernandes, L.F.C., Abrão, M.S., Kho, R.M. 2020. Extrapelvic Endometriosis: A Systematic Review. Journal of Minimally Invasive Gynecology, 27(2), 373–389.

Anglesio, M.S., Papadopoulos, N., Ayhan, A., Nazeran, T.M., Noë, M., Horlings, H.M., Lum, A., Jones, S., Senz, J., Seckin, T., Ho, J., Wu, R.C., Lac V., Ogawa, H., Tessier-Cloutier, B., Alhassan, R., Wang, A., Wang, Y., Cohen, J.D., Wong, F., Hasanovic, A., Orr, N., Zhang, M., Popoli, M., McMahon, W., Wood, L.D., Mattox, A., Allaire, C., Segars, J., Williams, C., Tomasetti, C., Boyd, N., Kinzler, K.W., Gilks, C.B., Diaz, L., Wang, T.L., Vogelstein, B., Yong, P.J., Huntsman, D.G., Shih, I.M. 2017. Cancer-Associated Mutations in Endometriosis without Cancer. The New England Journal of Medicine, 376(19), 1835–1848.

Aran, D., Hellman, A. 2013, DNA methylation of transcriptional enhancers and cancer predisposition. Cell, 154(1), 11–13.

Asante, A., Taylor, R.N. 2011. Endometriosis: The role of neuroangiogenesis. Annual Review of Physiology, 73, 163–182.

Bai, J., Wang, B., Wang, T., Ren, W. 2021. Identification of Functional lncRNAs Associated With Ovarian Endometriosis Based on a ceRNA Network. Frontiers of Genetics, 12, 534054.

Baldi, A., Lanza, A., Menicagli, F., Signorile, P.G., Spugnini, E,P. 2017. Histological and Immunohistochemical Characterization of a Case of Endometriosis in a Guinea Pig (Cavia tschudii). Case Reports in Veterinary Medicine, 4594510.

Barjaste, N., Shahhoseini, M., Afsharian, P., Sharifi-Zarchi, A., Masoudi-Nejad, A. 2019. Genome-wide DNA methylation profiling in ectopic and eutopic of endometrial tissues. Journal of Assisted Reproduction and Genetics, 36(8), 1743–1752.

Bashti, O., Noruzinia, M., Garshasbi, M., Abtahi, M. 2018. miR-31 and miR-145 as Potential Non-Invasive Regulatory Biomarkers in Patients with Endometriosis. Cell Journal, 20(1), 84–89.

Bellelis, P., Dias, J.A.Jr, Podgaec, S., Gonzales, M., Baracat, E.C., Abrão, M.S. 2010. Epidemiological and clinical aspects of pelvic endometriosis – a case series. Revista da Associação Médica Brasileira, 56(4), 467–471.

Bendifallah, S., Dabi, Y., Suisse, S., Jornea, L., Bouteiller, D., Touboul, C., Puchar, A., Daraï, E. 2022. MicroRNome analysis generates a blood-based signature for endometriosis. Scientific Reports, 12(1), 4051.

Brosens, I., Brosens, J.J., Benagiano, G. 2012. The eutopic endometrium in endometriosis: are the changes of clinical significance? Reproductive BioMedicine Online, 24(5), 496–502.

Cano-Gamez, E., Trynka, G. 2020. From GWAS to Function: Using Functional Genomics to Identify the Mechanisms Underlying Complex Diseases. Frontiers in Genetics, 11, 424.

Chico-Sordo, L., Ruiz-Martínez, T., Toribio M, González-Martín, R., Spagnolo E, Domínguez F., Hernández, A., García-Velasco JA. 2024 Identification of miR-30c-5p microRNA in Serum as a Candidate Biomarker to Diagnose Endometriosis. International Journal of Molecular Sciences, 25(3), 1853.

Colón-Caraballo M, Torres-Reverón A, Soto-Vargas JL, Young SL, Lessey B, Mendoza A, Urrutia R, Flores I. 2018 Effects of histone methyltransferase inhibition in endometriosis. 1;99(2):293-307.

Colón-Caraballo, M., Monteiro, J.B., Flores, I. 2015. H3K27me3 is an Epigenetic Mark of Relevance in Endometriosis. Reproductive Sciences, 22(9), 1134–1142.

Colón-Díaz, M., Báez-Vega, P., García, M., Ruiz, A., Monteiro, J.B., Fourquet, J., Bayona, M., Alvarez-Garriga, C., Achille, A., Seto, E., Flores, I. 2012. HDAC1 and HDAC2 are differentially expressed in endometriosis. Biology of Reproduction, 19(5), 483–492.

Cosar, E., Mamillapalli, R., Ersoy, G.S., Cho, S., Seifer, B., Taylor, H.S. 2016. Serum microRNAs as diagnostic markers of endometriosis: a comprehensive array-based analysis. Fertility and Sterility, 106(2), 402 –409.

Darbà, J., Marsà, A. 2022. Economic Implications of Endometriosis: A Review. Pharmacoeconomics, 40(12), 1143–1158.

Ding, D., Liu, X., Guo, S.W. 2014. Overexpression of lysine-specific demethylase 1 in ovarian endometriomas and its inhibition reduces cellular prolifeartion, cell cycle progression and invasivness. Fertility and Sterility, 101(3), 740 –749.

Du, Y., Zhang, Z., Xiong, W., Li, N., Liu, H., He, H., Li, Q., Liu, Y., Zhang, L. 2019. Estradiol promotes EMT in endometriosis via MALAT1/miR200s sponge function. Reproduction, 157(2), 179–188.

Elias, M.H., Lazim, N., Sutaji, Z., Abu, M.A., Abdul Karim, A.K., Ugusman, A., Syafruddin, S.E., Mokhtar, M.H., Ahmad, M.F. 2023. HOXA10 DNA Methylation Level in the Endometrium Women with Endometriosis: A Systematic Review. Biology (Basel), 12(3), 474.

Fan, T., Xiao, C., Liu, H., Liu, Y., Wang, L., Tian, H., Li, C., He, J. 2023. CXXC finger protein 1 (CFP1) bridges the reshaping of genomic H3K4me3 signature to the advancement of lung adenocarcinoma. Signal Transduction and Target Therapy, 8(1), 369.

Filip, L., Duică, F., Prădatu, A., Crețoiu, D., Suciu, N., Crețoiu, S.M., Predescu, D.V., Varlas, V.N., Voinea, S.C. 2020. Endometriosis Associated Infertility: A Critical Review and Analysis on Etiopathogenesis and Therapeutic Approaches. Medicina (Kaunas), 56(9), 460.

Gunawardena, S., Dior, U.P., Cheng, C., Healey, M. 2021. New Diagnosis of Endometriosis is Less Common in Women over Age Forty Presenting with Pelvic Pain. Journal of Minimally Invasive Gynecology, 28(4), 891–898.e1.

Guo, S.W. 2018. Cancer driver mutations in endometriosis: Variations on the major theme of fibrogenesis. Reproductive Medicine and Biology, 17(4), 369–397.

Guttman, M., Amit, I., Garber, M., French, C., Lin, M.F., Feldser, D., Huarte, M., Zuk, O., Carey, B.W., Cassady, J.P., Cabili, M.N., Jaenisch, R., Mikkelsen, T.S., Jacks, T., Hacohen, N., Bernstein, B.E., Kellis, M., Regev, A., Rinn, J.L., Lander, E.S. 2009. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature, 458, 223–227.

Hajimaqsoudi, E., Darbeheshti, F., Kalantar, S.M., Javaheri, A., Mirabutalebi, S.H., Sheikhha, M.H. 2020, Investigating the expressions of miRNA-125b and *TP53* in endometriosis. Does it underlie cancer-like features of endometriosis? A case-control study. International Journal of Reproductive BioMedecine, 18(10), 825–836.

Halme, J., Hammond, M.G., Hulka, J.F., Raj, S.G., Talbert, L.M. 1984. Retrograde menstruation in healthy women and in patients with endometriosis. Obstetrics & Gynecology, 64(2), 151–154.

Hawkins, S.M., Creighton, C.J., Han, D.Y., Zariff, A., Anderson, M.L., Gunaratne, P.H., Matzuk, M.M. 2011. Functional microRNA involved in endometriosis. Molecular Endocrinology, 25(5), 821–832.

He, Y.F., Li, B.Z., Li, Z., Liu, P., Wang, Y., Tang, Q., Ding, J., Jia, Y., Chen, Z., Li, L., Sun, Y., Li, X., Dai, Q., Song, C.X., Zhang, K., He, C., Xu, G.L. 2011. Tet-mediated formation of 5-carboxylcytosine and its excision by TDG in mammalian DNA. Science, 33(6047), 1303 –1307.

Hombach, S., Kretz, M. 2016. Non-coding RNAs: Classification, Biology and Functioning. Non-Coding RNAs in Colorectal Cancer. Advances in Experimental Medicine and Biology, 937, 3 –17.

Izawa, M., Taniguchi, F., Uegaki, T., Takai, E., Iwabe, T., Terakawa, N., Harada, T. 2011. Demethylation of a nonpromoter cytosine-phosphate-guanine island in the aromatase gene may cause the aberrant up-regulation in endometriotic tissues. Fertility and Sterility, 95(1), 33–39.

Joshi, N.R., Miyadahira, E.H., Afshar, Y., Jeong, J.W., Young, S.L., Lessey, B.A., Serafini, P.C., Fazleabas, A.T.2017. Progesterone Resistance in Endometriosis Is Modulated by the Altered Expression of MicroRNA-29c and FKBP4. Journal of Clinical Endocrinology and Metabolism, 102(1), 141–149.

Kiisholts, K., Kurrikoff, K., Arukuusk, P., Porosk, L., Peters, M., Salumets, A., Langel, Ü. 2021. Cell-Penetrating Peptide and siRNA-Mediated Therapeutic Effects on Endometriosis and Cancer In Vitro Models. Pharmaceutics, 13(10), 1618.

Klungland, A., Robertson, A.B. 2017, Oxidized C5-methyl cytosine bases in DNA: 5-Hydroxymethylcytosine; 5-formylcytosine; and 5-carboxycytosine. Free Radical Biology and Medicine, 107, 62–68.

Kok, V.C., Tsai, H.J., Su, C.F., Lee, C.K. 2015. The Risks for Ovarian, Endometrial, Breast, Colorectal, and Other Cancers in Women With Newly Diagnosed Endometriosis or Adenomyosis: A Population-Based Study. International Journal of Gynecological Cancer, 25(6), 968–976.

Kumar, S., Chinnusamy, V., Mohapatra, T. 2018. Epigenetics of Modified DNA Bases: 5-Methylcytosine and Beyond. Frontiers in Genetics, 9, 640.

Kumar, S., Park, S.H., Cieply, B., Schupp, J., Killiam, E., Zhang, F., Rimm, D.L., Frisch, S.M. 2011. A pathway for the control of anoikis sensitivity by E-cadherin and epithelial-to-mesenchymal transition. Molecular and Cellular Biology, 31, 4036–4051.

Li, L., Chen, Q., Fan, Q.B., Wang, S., Shi, H.H., Zhu, L., Sun, D.W., Leng, J.H., Lang, J.H. 2019. Pathogenetic gene changes of eutopic endometrium in patients with ovarian endometriosis. Chinese Medical Journal (Engl), 132(9), 1107–1109.

Li, M., Dai, M., Cheng, B., Li, S., Guo, E., Fu, J., Ma, T., Yu, B. 2024. Strategies that regulate LSD1 for novel therapeutics. Acta Pharmaceutica Sinica B, 14(4), 1494–1507.

Luo, Y., Wang, D., Chen, S., Yang, Q. 2020. The role of miR-34c-5p/Notch in epithelial-mesenchymal transition (EMT) in endometriosis. Cellular Signalling, 72, 109666.

Machairiotis, N., Vasilakaki, S., Thomakos, N. 2021. Inflammatory Mediators and Pain in Endometriosis: A Systematic Review. Biomedicines, 9(1), 54.

Maenhoudt, N., De Moor, A., Vankelecom, H. 2022. Modeling Endometrium Biology and Disease. Journal of Personalized Medicine, 12(7), 1048.

Mai, H., Liao, Y., Luo, S., Wei, K., Yang, F., Shi, H. 2021. Histone deacetylase HDAC2 silencing prevents endometriosis by activating the HNF4A/ARID1A axis. Journal of Cellular and Molecular Medicine, 25(21), 9972–9982.

Martin, E.M., Fry, R.C. 2018. Environmental Influences on the Epigenome: Exposure-Associated DNA Methylation in Human Populations. Annual Review of Public Health, 39, 309–333.

Martin, J.D. Jr., Hauck, A.E.1985. Endometriosis in the male. American Surgeon, 51(7), 426–430.

Missmer, S.A., Tu, F.F., Agarwal, S.K., Chapron, C., Soliman, A.M., Chiuve, S., Eichner, S., Flores-Caldera, I., Horne, A.W., Kimball, A.B., Laufer, M.R., Leyland, N., Singh, S.S., Taylor, H.S., As-Sanie, S. 2021. Impact of Endometriosis on Life-Course Potential: A Narrative Review. International Journal of General Medicine, 14, 9–25.

Monteiro, J.B., Colón-Díaz, M., García, M., Gutierrez, S., Colón, M., Seto, E., Laboy, J., Flores, I. 2014. Endometriosis is characterized by a distinct pattern of histone 3 and histone 4 lysine modifications. Reproductive Sciences, 21(3), 305–318.

Moore, L., Le, T., Fan, G. 2013. DNA Methylation and Its Basic Function. Neuropsychopharmacology, 38, 23–38.

Mortlock, S., Houshdaran, S., Kosti, I., Rahmioglu, N., Nezhat, C., Vitonis, A.F., Andrews, S.V., Grosjean, P., Paranjpe, M., Horne, A.W., Jacoby, A., Lager, J., Opoku-Anane, J., Vo, K.C., Manvelyan, E., Sen, S., Ghukasyan, Z., Collins, F., Santamaria, X., Saunders, P., Kober, K., McRae, A.F., Terry, K.L., Vallvé-Juanico, J., Becker, C., Rogers, P.A.W., Irwin, J.C., Zondervan, K., Montgomery, G.W., Missmer, S., Sirota, M., Giudice, L. 2023. Global endometrial DNA methylation analysis reveals insights into mQTL regulation and associated endometriosis disease risk and endometrial function. Communication Biology, 6, 780.

Mvunta, D.H., Miyamoto, T., Asaka, R., Yamada, Y., Ando, H., Higuchi, S., Ida, K., Kashima, H., Shiozawa, T. 2017. Overexpression of SIRT1 is Associated With Poor Outcomes in Patients With Ovarian Carcinoma. Applied Immunohistochemistry & Molecular Morphology, 25(6), 415–421.

Nnoaham, K.E., Hummelshoj, L., Webster, P., d'Hooghe, T., de Cicco Nardone, F., de Cicco Nardone, C., Jenkinson, C., Kennedy, S.H.,

Zondervan, K.T. 2011. World Endometriosis Research Foundation Global Study of Women's Health consortium. Impact of endometriosis on quality of life and work productivity: a multicenter study across ten countries. Fertility and Sterility, 96(2), 366–373.e8.

Onodera, A., González-Avalos, E., Lio, C.J., Georges, R.O., Bellacosa, A., Nakayama, T., Rao, A. 2021. Roles of TET and TDG in DNA demethylation in proliferating and non-proliferating immune cells. Genome Biology, 22(1), 186.

Pearce, C.L., Templeman, C., Rossing, M.A., Lee, A., Near, A.M., Webb, P.M., Nagle, C.M., Doherty, J.A., Cushing-Haugen, K.L., Wicklund, K.G., Chang-Claude, J., Hein, R., Lurie, G., Wilkens, L.R., Carney, M.E., Goodman, M.T., Moysich, K., Kjaer, S.K., Hogdall, E., Jensen, A., Goode, E.L., Fridley, B.L., Larson, M.C., Schildkraut, J.M., Palmieri, R.T., Cramer, D.W., Terry, K.L., Vitonis, A.F., Titus, L.J., Ziogas, A., Brewster, W., Anton-Culver, H., Gentry-Maharaj, A., Ramus, S.J., Anderson, A.R., Brueggmann, D., Fasching, P.A., Gayther, S.A., Huntsman, D.G., Menon, U., Ness, R.B., Pike, M.C., Risch, H., Wu, A.H., Berchuck, A. 2012. Ovarian Cancer Association Consortium. Association between endometriosis and risk of histological subtypes of ovarian cancer: a pooled analysis of case-control studies. Lancet Oncology, 13(4), 385–394.

Proestling, K., Birner, P., Gamperl, S., Nirtl, N., Marton, E., Yerlikaya, G., Wenzl, R., Streubel, B., Husslein, H. 2015. Enhanced epithelial to mesenchymal transition (EMT) and upregulated MYC in ectopic lesions contribute independently to endometriosis. Reproductive Biology and Endocrinology, 13, 75.

Reis, F.M., Coutinho, L.M., Vannuccini, S., Batteux, F., Chapron, C., Petraglia, F. 2020. Progesterone receptor ligands for the treatment of endometriosis: the mechanisms behind therapeutic success and failure. Human Reproduction Update, 26(4), 565–585.

Roca, F.J., Loomans, H.A., Wittman, A.T., Creighton, C.J., Hawkins, S.M. 2016. Ten-Eleven Translocation Genes are Downregulated in Endometriosis. Current Molecular Medicine, 16(3), 288–298.

Rocha, A.L., Reis, F.M., Taylor, R.N. 2013. Angiogenesis and endometriosis. Obstetrics and Gynecology International, 859619.

Sampson, J.A. 1927. Metastatic or Embolic Endometriosis, due to the Menstrual Dissemination of Endometrial Tissue into the Venous Circulation. American Journal of Pathology, 3(2), 93–110.

Saunders, P.T.K., Horne, A.W. 2021. Endometriosis: Etiology, pathobiology, and therapeutic prospects. Cell, 184(11), 2807–2824.

Schrodt, G.R., Alcorn, M.O., Ibanez, J. 1980. Endometriosis of the male urinary system: a case report. Journal of Urology, 124(5), 722–723.

Shen, L., Zhang, C., Zhang, Y., Yang, Y. 2023. METTL3 and METTL14-mediated N^6-methyladenosine modification promotes cell proliferation and invasion in a model of endometriosis. Reproductive BioMedicine Online, 46(2), 255–265.

Simpson, J.L., Elias, S., Malinak, L.R., Buttram, V.C. Jr. 1980. Heritable aspects of endometriosis. I. Genetic studies. American Journal of Obstetrics and Gynecology, 137(3), 327–331.

Song, S.Y., Jung, Y.W., Shin, W., Park, M., Lee, G.W., Jeong. S., An, S., Kim, K., Ko, Y.B., Lee KH, Kang, B.H., Lee, M., Yoo, H.J. 2023. Endometriosis-Related Chronic Pelvic Pain. Biomedicines, 11(10), 2868.

Statello, L., Guo, C.J., Chen, L.L., Huarte, M. 2021. Gene regulation by long non-coding RNAs and its biological functions. Nature Reviews Molecular Cell Biology, 22, 96–118

Szaflik, T., Romanowicz, H., Szyłło, K., Smolarz, B. 2023. Long Non-Coding RNA *SNHG4* Expression in Women with Endometriosis: A Pilot Study. Genes (Basel), 14(1), 152.

Taguchi, A., Wada-Hiraike, O., Kawana, K., Koga, K., Yamashita, A., Shirane, A., Urata, Y., Kozuma, S., Osuga, Y., Fujii, T. 2014. Resveratrol suppresses inflammatory responses in endometrial stromal cells derived from endometriosis: a possible role of the sirtuin 1 pathway. Journal of Obstetrics and Gynaecology Research, 40(3), 770–778.

Tamura, K., Sakurai, T., Kogo, H. 2006. Relationship between prostaglandin E2 and vascular endothelial growth factor (VEGF) in angiogenesis in human vascular endothelial cells. Vascular Pharmacology, 44(6), 411–416.

Wang, D., Chen, Q., Zhang, C., Ren, F., Li, T. 2012. DNA hypomethylation of the COX-2 gene promoter is associated with up-regulation of its mRNA expression in eutopic endometrium of endometriosis. European Journal of Medical Research, 17(1), 12.

Weinhold, B. 2006. Epigenetics: the science of change. Environmental Health Perspectives, 114(3), A160–A167.

Wu, J., Li, X., Huang, H., Xia, X., Zhang, M., Fang, X. 2020. TET1 may contribute to hypoxia-induced epithelial to mesenchymal transition of

endometrial epithelial cells in endometriosis. PeerJ, 15, 8.

Wu, Y., Starzinski-Powitz, A., Guo, S.W. 2007. Trichostatin A, a histone deacetylase inhibitor, attenuates invasiveness and reactivates E-cadherin expression in immortalized endometriotic cells. Reproductive Sciences, 14(4), 374–382.

Xue, Q., Lin, Z., Cheng, Y.H., Huang, C.C., Marsh, E., Yin, P., Milad, M.P., Confino, E., Reierstad, S., Innes, J., Bulun, S.E. 2007a. Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis. Biology of Reproduction, 77(4), 681–687.

Xue, Q., Lin, Z., Yin, P., Milad, M.P., Cheng, Y.H., Confino, E., Reierstad, S., Bulun, S.E. 2007b. Transcriptional activation of steroidogenic factor-1 by hypomethylation of the 5' CpG island in endometriosis. Journal of Clinical Endocrinology & Metabolism, 92(8), 3261 –3267.

Yan, H., Bu, P. 2021. Non-coding RNA in cancer. Essays in Biochemistry, 65, 625–639.

Yang, S.C, Park, M., Hong, K.H., La, H., Park, C., Wang, P., Li, G., Chen, Q., Choi, Y., DeMayo, F.J., Lydon, J.P., Skalnik, D.G., Lim, H.J., Hong, S.H., Park, S.H., Kim, Y.S., Kim, H.R., Song, H. 2023. CFP1 governs uterine epigenetic landscapes to intervene in progesterone responses for uterine physiology and suppression of endometriosis. Nature Communications, 14(1), 3220.

Yoshida, H., Broaddus, R., Cheng, W., Xie, S., Naora, H. 2006. Deregulation of the HOXA10 homeobox gene in endometrial carcinoma: role in epithelial-mesenchymal transition. Cancer Research, 66(2), 889–897.

Yotova, I., Hsu, E., Do, C., Gaba, A., Sczabolcs, M., Dekan, S., Kenner, L., Wenzl, R., Tycko, B. 2017. Epigenetic Alterations Affecting Transcription Factors and Signaling Pathways in Stromal Cells of Endometriosis. PLoS One. 12(1), 0170859.

Yu, M., Tang, J., Huang, Y., Guo, C., Du, P., Li, N., Quan, Q. 2022. HOXA10 Regulates the Synthesis of Cholesterol in Endometrial Stromal Cells. Frontiers in Endocrinology (Lausanne), 13, 852671.

Zhang, J., Chen, B., Gan, C., Sun, H., Zhang, J., Feng, L. 2023. A Comprehensive Review of Small Interfering RNAs (siRNAs): Mechanism, Therapeutic Targets, and Delivery Strategies for Cancer Therapy. International Journal of Nanomedicine, 18, 7605–7635.

Zhang, Y., Sun, Z., Jia, J., Du, T., Zhang, N., Tang, Y., Fang, Y., Fang, D. 2021.. Overview of Histone Modification. [In:] Fang, D., Han, J. (eds.) Histone Mutations and Cancer. Advances in Experimental Medicine and Biology, 1283, pp. 1–16. Springer, Singapore

Zheng, H., Liu, X., Guo, S.W. 2023. Aberrant expression of histone deacetylase 8 in endometriosis and its potential as a therapeutic target. Reproductive Medicine and Biology, 22(1), e12531.

Zondervan, K.T., Rahmioglu, N., Morris, A.P., Nyholt, D.R., Montgomery, G.W., Becker, C.M., Missmer, S.A. 2016. Beyond Endometriosis Genome-Wide Association Study: From Genomics to Phenomics to the Patient. Seminars in Reproductive Medicine, 34(4), 242–254.

Żeberkiewicz, M., Hyc, A., Iwan, A., Zwierzchowska, A., Ścieżyńska, A., Kalaszczyńska, I., Barcz, E., Malejczyk, J. 2022. Expression of Fucosyltransferase 4 (*FUT4*) mRNA Is Increased in Endometrium from Women with Endometriosis. Journal of Clinical Medicine, 11(19), 5606.

Folia Biologica et Oecologica 18: 110–121 (2024)

Acta Universitatis Lodziensis

The use of UV-A radiation for biofortification of lettuce and basil plants with antioxidant phenolic and flavonoid compounds

ILONA PACAK[1], MAGDALENA TROJAK[2], ERNEST SKOWRON[2]

[1]Jan Kochanowski University of Kielce, Biotechnology, Uniwersytecka 7, 25–406 Kielce, Poland;
[2]Jan Kochanowski University of Kielce, Department of Environmental Biology, Uniwersytecka 7, 25–406 Kielce, Poland.
E-mail: ernest.skowron@ujk.edu.pl

ABSTRACT

Stable plant production is a crucial concern of modern agriculture facing increasing food demands and the risk of less predictable weather conditions in the open field. Alternative approaches for plant production are greenhouses and indoor farming. Modern LED-based artificial lighting indoor facilities allow not only to fill the gap in the supply chain of food production, but to produce plants characterized with higher concentration of essential phytochemicals. Thus, in this study, we attempt to analyse the efficiency of short-term supplementation of spectrum within ultraviolet A light (UV-A, 365 nm) to increase the antioxidant potential of leafy plants, assessed by total phenolic (TPC) and flavonoid (TFC) content. To this end, two distinct cultivars of *baby leaf* lettuce (*Lactuca sativa* var. *crispa* L.) and *microgreens* basil (*Ocimum basilicum* L.) plants were grown under red-green-blue spectrum supplemented prior to harvest with low doses of UV-A radiation. Analyses showed that UV-A exposition enhanced TPC and TFC with green leaf cultivars, compared to dark-pigmented ones. The analysis also proved that plants exposed to UV-A presented higher overall antioxidant potential measured with 1,1-diphenyl-2-picrylhydrazil (DPPH). Results are crucial for better understanding the potential of UV-A supplementation to produce functional plants which are natural sources of dietary antioxidants.

KEYWORDS: indoor farming, spectrum optimization, baby leaf, microgreens, antioxidant potential

Introduction

The sustainability of resources and safety in the plant food production is a major issue globally. It is expected that by 2050, the global population will reach 9.8 billion people. Thus, it will force the necessity to enhance the total area of crop production (Avgoustaki and Xydis, 2020). At the same time, however, the

DOI: https://doi.org/10.18778/1730-2366.18.14

availability of land area for agriculture is projected to be restricted due to purposes such as urbanization, energy production, infrastructure, and predicted effects of global temperature rise, causing sea level increase (Despommier, 2011). Fortunately, indoor farming (IF) approaches have recently been identified as a potential alternative to contribute to sustainable plant food production (Specht *et al*., 2014). IF also provides the opportunity to create desired conditions for crop growth such as temperature, relative humidity, carbon dioxide level, air circulation, and artificial lighting quantity and quality, and at the same time mitigates open field disadvantages such as weather extremes or pathogens and pests (Ampim *et al*., 2022). Moreover, for most plant production lighting systems with non-saturating light intensity are sufficient (Trojak and Skowron, 2021), thus the light quality rather than quantity is more crucial during plant production.

Different light wavelengths have a significant impact on the nutritional content of food crops. Both visible and ultraviolet (UV) radiation can notably affect the secondary metabolism leading to the accumulation of health-promoting phytochemicals vital for human health (Lee *et al*., 2022). UV region (100–400 nm) is divided into three sub-regions, UV-A (315–400 nm), UV-B (280–315 nm) and UV-C (100–280 nm) (Darré *et al*., 2022). Plants cultivated in open-field conditions are naturally exposed to sunlight, which contains UV radiation (Surjadinata *et al*., 2017). As a result, plants evolved various metabolic and biochemical responses to UV exposition, among them increased antioxidant secondary metabolites synthesis (Brazaityte *et al*., 2015).

The underlying mechanism of plant UV responses is related to different photoreceptors – blue light/UV-A ones such as cryptochromes (crys) or UV Resistance Locus 8 (UVR8) photoreceptors, which operates through UV-B light (D'Amico-Damião and Carvalho 2018). Perception and response to UV-C light are associated with the redox state of cells and reactive oxygen species (ROS) generation (Artés-Hernández *et al*., 2022). The master regulator of UV responses is the elongated hypocotyl 5 (HY5) transcription factor. Its UV-depended accumulation induces phenolic compounds biosynthesis, with largest class of flavonoids (Vanhaelewyn *et al*., 2020; Xiao *et al*., 2022). Consequently, UV radiation is considered as a tool to biofortify IF-grown crops with nutraceuticals (Jacobo-Velázquez *et al*., 2022).

Up to date, however, data on the UV-A effect on the accumulation of phenolic compounds is scarce and incomplete (Verdaguer *et al*., 2017). Thus, the aim of the study was to examine the efficiency of short-term supplementation of spectrum within UV-A (365 nm) in increasing the antioxidant potential of leafy plants, assessed by total phenolics (TPC) and flavonoids (TFC) content. To this end, *baby leaf* lettuce (*Lactuca sativa* var. *crispa* L.) cultivars with green (cv. Lollo Bionda) and red leaf (cv. Lollo Rossa), as well as *microgreens* basil (*Ocimum basilicum* L.) cultivars with green (cv. Sweet Large) and purple leaf (cv. Dark Opal), were grown in a growth chamber under red-green-blue (RGB) spectrum, supplemented prior to harvest with and increasing doses of UV-A. Spectrophotometric analyses clearly showed that UV-A exposition enhanced TPC and TFC as well as total antioxidant capacity (TAC) in both green-leaf cultivars, while both dark-pigmented cultivars presented slightly lower TPC and TFC content, compared to control plants. The study provides valuable insight into the role of UV-A supplementation in standard RGB lighting

systems, mostly devoid of UV components, to improve the quality of plant leafy products.

Methods

Plant material and growth conditions

Baby leaf lettuce (*L. sativa* var. *crispa* L.) cultivars with green (cv. Lollo Bionda, LB) and red leaf (cv. Lollo Rossa, LR), as well as *microgreens* basil (*O. basilicum* L.) cultivars with green (cv. Sweet Large, SL) and purple leaf (cv. Dark Opal, DO), seeds were sown in P9 containers (9 × 9 × 10 cm) and filled with the substrate (white and black peat, perlite, and N:P:K = 9:5:10; pH 6.0–6.5), divided into groups, and transferred to environmentally controlled growth chambers, with non-reflective black separators to eliminate light contamination. The plants were grown for the next 20 consecutive days (20 DAS, days after sowing) (Fig. 1) under LED RhenacM12 lamps (PXM,

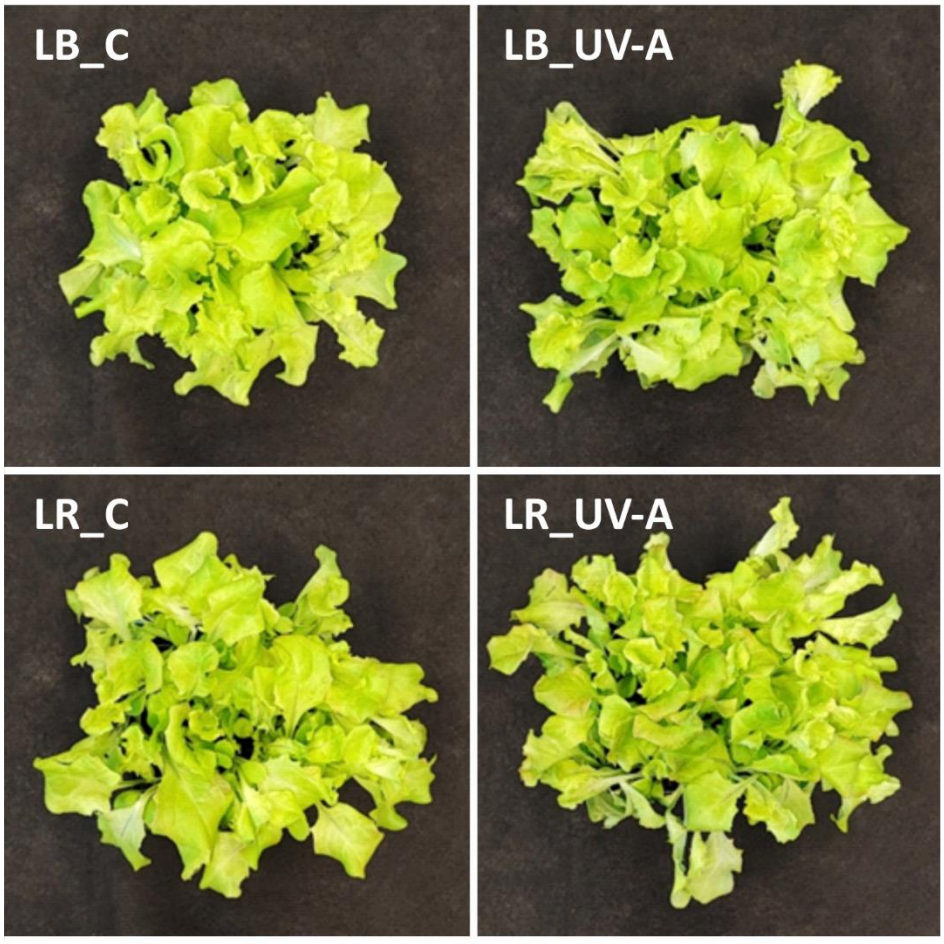

Figure 1. Morphology of 20-DAS plants of baby leaf lettuce (*Lactuca sativa* var. *crispa* L.) cultivars with green (cv. Lollo Bionda, LB) and red leaf (cv. Lollo Rossa, LR) as well as *microgreens* basil (*Ocimum basilicum* L.) cultivars with green (cv. Sweet Large, SL) and purple leaf (cv. Dark Opal, DO) grown under RGB (C, control) or RGB+UV-A (UV-A supplemented) spectrum.

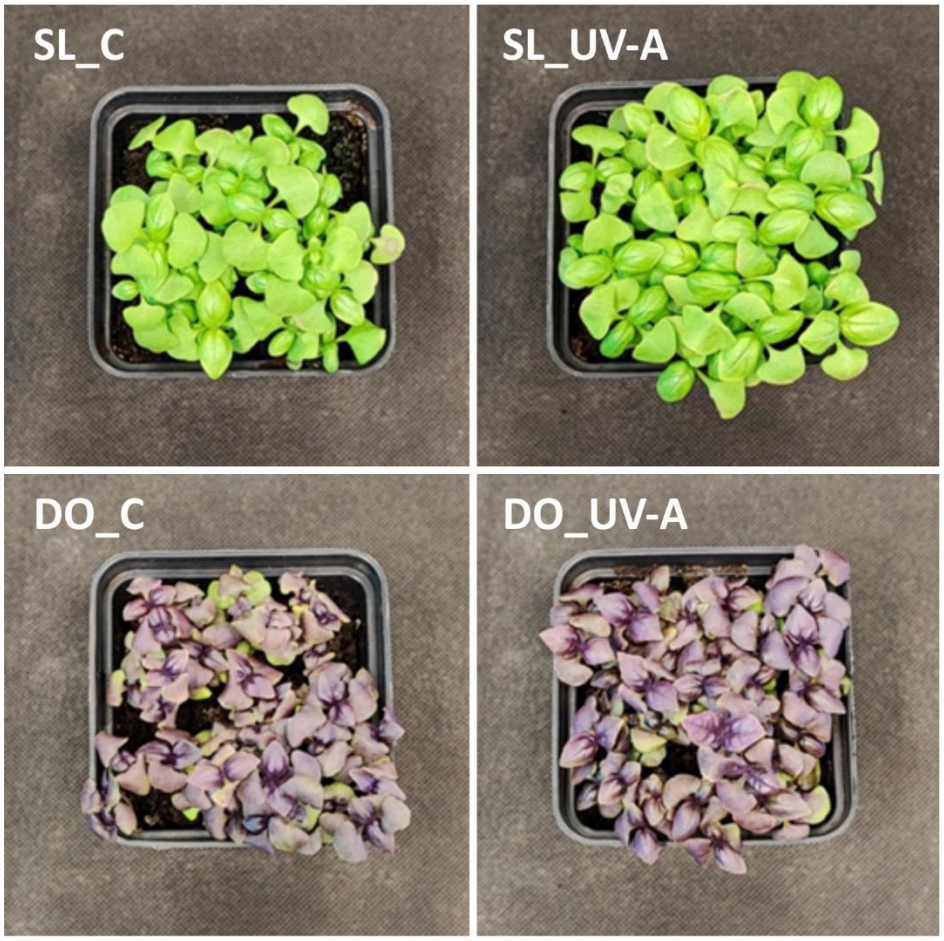

Figure 1 (continued). Morphology of 20-DAS plants of baby leaf lettuce (*Lactuca sativa* var. *crispa* L.) cultivars with green (cv. Lollo Bionda, LB) and red leaf (cv. Lollo Rossa, LR) as well as *microgreens* basil (*Ocimum basilicum* L.) cultivars with green (cv. Sweet Large, SL) and purple leaf (cv. Dark Opal, DO) grown under RGB (C, control) or RGB+UV-A (UV-A supplemented) spectrum

Podleze, Poland) delivering 200 µmol m^{-2} s^{-1} of the RGB spectrum (R:G:B; 661:633:520:434 nm) (Fig. 2A) or RGB+UV-A supplemented for 4 days prior to harvest with and gradually increasing daily doses – 60, 120, 240 and 480 min, respectively of UV-A lamp with peak wavelength 365 nm (9 W m^{-2}) (Figs. 2B,C) (Actinic BL PL-S 9W/10/2P, Philips lighting, Eindhoven, The Netherlands). RGB treatment was used as the control group. Light composition and photosynthetic photon flux density (PPFD) were monitored by a calibrated spectroradiometer GL SPECTIS 5.0 Touch (GL Optic Lichtmesstechnik GmbH, Weilheim/Teck, Germany).

The containers with plants were turned in twice a day. The photoperiod was 16/8 h (day/night; day 6:00 a.m.–10:00 p.m.), the average air temperature was maintained at 23/20 °C (day/night), relative air humidity was kept at 50–55% and 420 ± 10 µmol mol^{-1} of CO_2. The

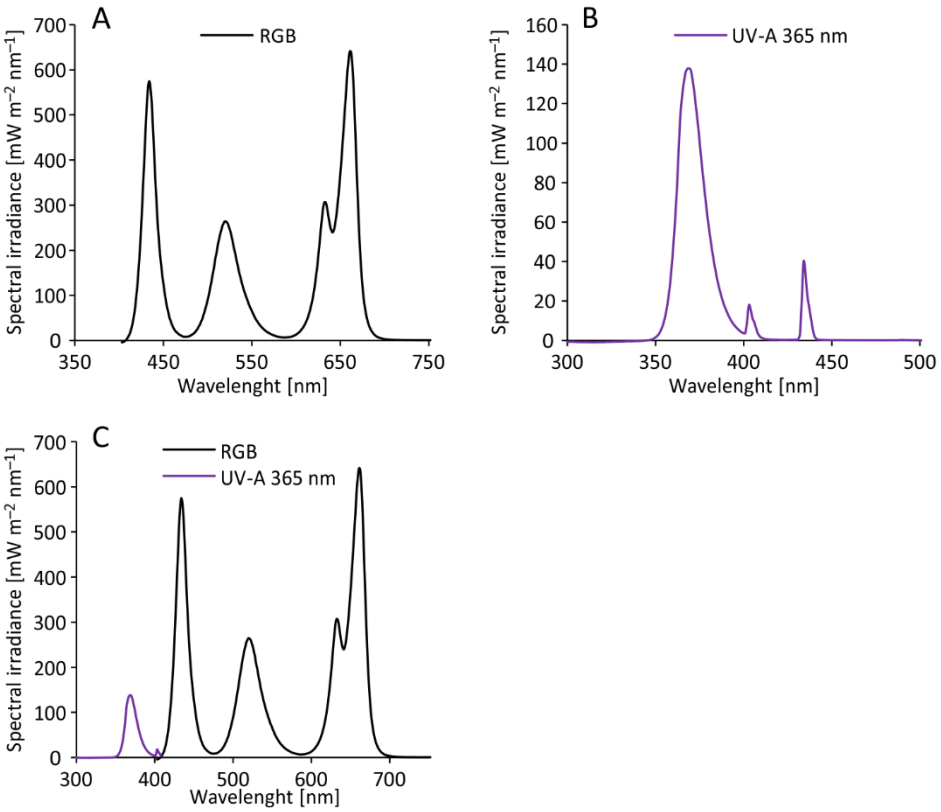

Figure 2. The light spectra of growth chambers were recorded with a spectroradiometer at six locations and then averaged. Plants in the RGB (red–green–blue) chamber were grown under 200 µmol m^{-2} s^{-1} of the RGB spectrum (R:G:B; 661:633:520:434 nm) (A) or RGB supplemented with UV-A (B, C) (365 nm, 9 W m^{-2}) for 4 days prior to harvest with increasing doses. RGB states for the control plants (R:G:B = 1:1:1).

plants were watered with tap water when necessary and fertilized once a week with 1% (w/v) fertilizer (N:P:K = 9:9:27; Substral Scotts, Warszawa, Poland). The top leaves from the plants 20 DAT were used for subsequent analyses. All analyses were conducted between 8:00 a.m. and 12:00 p.m. Ten plants (two repetitions with five plants per light condition) were grown with each kind of light treatment.

Estimation of total phenolic content with Folin-Ciocalteu assay

Estimation of total phenolic content (TPC) was conducted, as described by Ainsworth and Gillespie (2007). In brief, 100 mg of fresh weight (FW) leaf tissue (four replicates per light treatment) was placed in tubes with 1.0 ml of methanol. Samples, kept in dim light, were vortexed for 20 s and incubated for 30 min at 60 °C with inversion every 10 min to improve extraction. Then, the sample mixture was centrifuged at 10,000 × *g* for 2 min, and then the supernatant was carefully collected without disturbing the plant tissue, transferred to a new tube, and mixed once again for 15 s. Then 100 µl of each extract, cooled down to room temperature (RT), was mixed with 200 µl 10% (v/v) Folin-Ciocalteu reagent (F-C) and vortexed twice for 10 s. Then 800 µl of 700 mM Na_2CO_3 was added, vortexed

twice for 10 s, and incubated for 30 min at 40 °C, protected from light. After incubation mixture was centrifuged at 10,000 × *g* for 1 min and transferred to a clear 96-well microplate with 200 µl per well. For TPC determination the absorbance at 765 nm was estimated with a microplate spectrophotometer (Mobi, MicroDigital Co., Ltd., Republic of Korea) with four replicates. The standard curve with gallic acid (0–200 nmol) was used to estimate nanomoles of phenolic compounds (gallic acid equivalents) in a sample.

Estimation of total flavonoid content

For the measurement of total flavonoid (TFC) assay proposed by Shraim *et al*. (2021) with modification was applied. The 60 µl of methanol extract obtained previously for TPC assay was mixed with 680 µl of 30% (v/v) methanol: water and 30 µl of 0.5M $NaNO_2$, vortexed for 20 s and incubated at RT for 3 min without light. Then 30 µl of 0.3M $AlCl_3$ x $6H_2O$ was added to each sample, vortexed for 20 s and incubated at RT for 3 min, and then mixed with 200 µl of 1M NaOH, vortexed and left for the next 40 min at RT without light. After incubation, samples were mixed, shortly centrifugated (5,000 × *g* for 1 min) and 200 µl aliquot of each sample were transferred to 96-well microplate. For TFC determination the absorbance at 506 nm was estimated with a microplate spectrophotometer with four replicates. The flavonoid content in the sample extracts was quantified using calibration curves of flavonoid standards of rutin.

Antioxidant activity by DPPH assay

The antioxidant activity of each extract of tested plants was measured by the 1,1-diphenyl-2-picrylhydrazil (DPPH) scavenging assay according to the method proposed by Mehmood *et al*. (2022). For DPPH assay the 60 µl of plant methanol extract obtained previously for TPC assay was mixed with 904 µl of methanol and 576 µl of 0.125mM DPPH in methanol, vortexed for 20 s and incubated for 30 min at 37 °C. Using a microplate spectrophotometer, the absorbance of each sample was measured at 517 nm with four replicates. To determine sample radical scavenging activity, the calibration curve with a synthetic antioxidant – butylated hydroxytoluene (BHT) (0–400 µg per ml) and 0.125mM DPPH was plotted.

The following formula was used to calculate the percentage of DPPH scavenging activity:

$$\text{DPPH inhibition } \% = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100\%$$

– control states for DPPH mixture incubate with 0 µg BHT solution.

Models for fitting and statistical analysis

The fitting of experimental data of DPPH inhibition by BHT used for DPPH radical scavenging activity rate was performed using OriginPro version 2024 (OriginLab Corporation, Northampton, MA, USA).

Statistical analyses were performed using Statistica 13.3 software (StatSoft Inc., Oklahoma, OK, USA). The normal distribution of variables was verified using the Shapiro–Wilk test, and the equality of variances was evaluated using Levene's test. One-way ANOVA and post hoc Tukey's HSD tests were employed to analyse the differences between the investigated groups. The data are presented as mean with standard deviation (±SD). Statistical significance was determined at the 0.05 level ($p = 0.05$).

Results

Total phenolic content

Estimated total phenolic content (TPC) is expressed as nmol gallic acid equivalents per mg of fresh weight (FW) (Fig. 3). Analysis showed that dark-

pigmented cultivars of both species presented higher phenolics content under the control RGB spectrum. In lettuce leaves TPC is almost 3 times higher for LR than LB cultivar (Fig. 3A), while for basil the difference is about 17% higher for DO than SL (Fig. 3B). Yet, basil leaves have significantly higher TPC than lettuce ones. The short-term exposition of leaves to additional UV-A light increased TPC significantly in green-leaf cultivars, as we observed 170% and 16% higher TPC for LB and SL, respectively. In contrast, UV-A decreased TPC by 27% and 24% in LR and DO, respectively.

Total flavonoid content

Total flavonoid content (TFC) is expressed as µg rutin equivalents per g of fresh weight (FW) (Fig. 4).

Similarly to TPC, also flavonoids content was significantly higher in dark-pigmented cultivars. Under RGB spectrum, we documented 2.7 times higher TFC level in the LR compared to

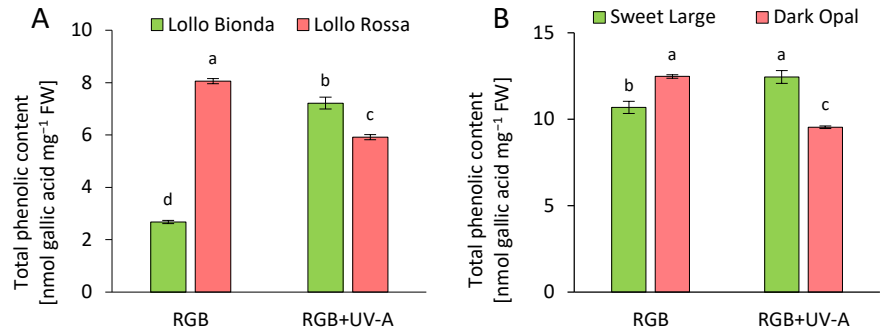

Figure 3. Total phenolic content (TPC) of control (RGB) or UV-A treated (RGB+UV-A) plants of *baby leaf* lettuce (*Lactuca sativa* var. *crispa* L.) cultivars with green (cv. Lollo Bionda) and red leaf (cv. Lollo Rossa) (A) or *microgreens* basil (*Ocimum basilicum* L.) cultivars with green (cv. Sweet Large) and purple leaf (cv. Dark Opal) (B) 20 DAS, estimated as nmol gallic acid equivalents per mg of fresh weight (FW). Each bar represents the average ± SD of four independent measurements (n = 4). Different letters (a–d) indicate significant differences between treatments at p = 0.05 with a Tukey's HSD test.

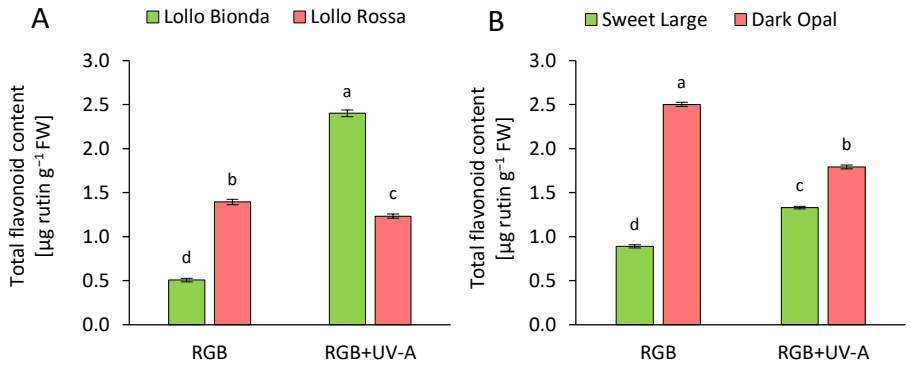

Figure 4. Total flavonoid content (TFC) of control (RGB) or UV-A treated (RGB+UV-A) plants of *baby leaf* lettuce (*Lactuca sativa* var. *crispa* L.) cultivars with green (cv. Lollo Bionda) and red leaf (cv. Lollo Rossa) (A) or *microgreens* basil (*Ocimum basilicum* L.) cultivars with green (cv. Sweet Large) and purple leaf (cv. Dark Opal) (B) 20 DAS, estimated as µg rutin equivalents per g of fresh weight (FW). Each bar represents the average ± SD of four independent measurements (n = 4). Different letters (a–d) indicate significant differences between treatments at p = 0.05 with a Tukey's HSD test.

the LB cultivar, and approximately 2.8 times higher in DO than in the SL basil cultivar. Supplementation of RGB spectrum within UV-A light increased flavonoids content by almost 4.7- and 1.5-fold in green-leaf cultivars of lettuce (Fig. 4A) and basil (Fig. 4B), respectively. UV-A also showed negative impact noticed with 12% and 28% TFC decrease in LR and DO, respectively.

Overall antioxidant capacity

The total antioxidant capacity (TAC) was expressed as µg equivalents of butylated hydroxytoluene (BHT) per mg of FW (Figs. 5A,B) and DPPH radical scavenging activity rate (Figs. 5C,D) was assessed based on fitted experimental data of BHT calibration curve, as described in Methods. As expected, analysis proved that overall antioxidant capacity complies with TPC and TFC. Under spectrum depleted in UV-A radiation green leaf cultivars showed 1.5- and 6.5-times lower antioxidant capacity compared to dark-pigmented ones in lettuce and basil plants, respectively. Despite short-term exposition of plants within UV-A light, it was efficient in antioxidant capacity increase. In the LB lettuce TAC was increased by 162% (Fig. 5A), while in the SL basil by 460% compared to RGB-grown plants (Fig. 5B). In the case of LR cultivar presenting reddish leaf phenotype UV-A light treatment increased TAC by 12%. On the other hand, UV-A lowered TAC by 7% in DO basil. Estimated DPPH radical scavenging activity rate shows that UV-A exposition increased its value by approximately 14% and 45% in LB and

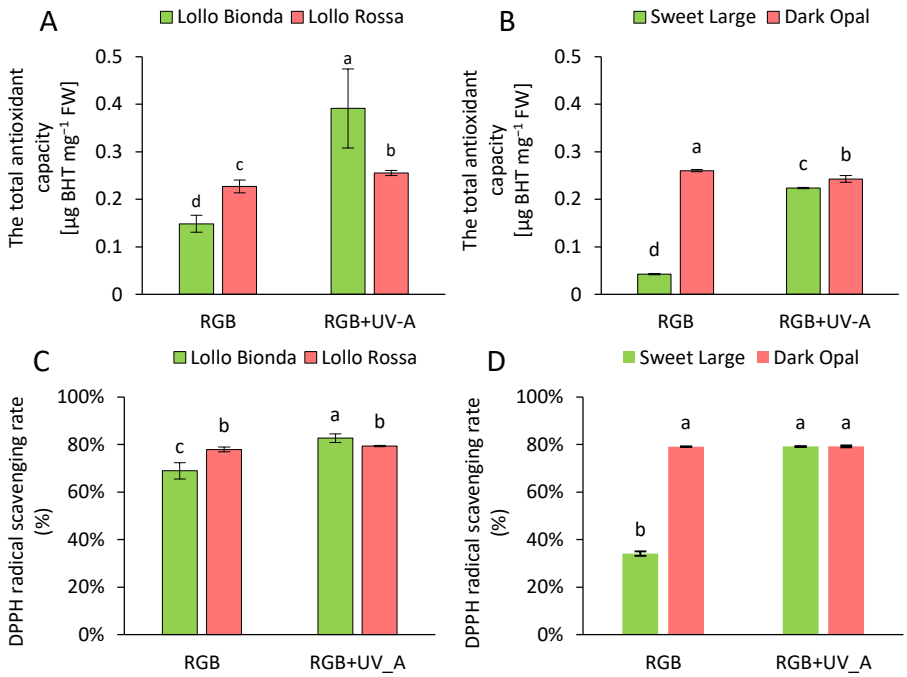

Figure 5. The total antioxidant capacity (A, B) and DPPH radical scavenging activity rate (C, D) of control (RGB) or UV-A treated (RGB+UV-A) plants of *baby leaf* lettuce (*Lactuca sativa* var. *crispa* L.) cultivars with green (cv. Lollo Bionda) and red leaf (cv. Lollo Rossa) or *microgreens* basil (*Ocimum basilicum* L.) cultivars with green (cv. Sweet Large) and purple leaf (cv. Dark Opal) 20 DAS, estimated as µg BHT equivalents per mg of fresh weight (FW). Each bar represents the average ± SD of four independent measurements (n = 4). Different letters (a–d) indicate significant differences between treatments at p = 0.05 with a Tukey's HSD test.

SL cultivars (Figs. 5C,D), respectively. At the same time, dark-pigmented cultivars show no effect on scavenging rate after exposition (Figs. 5C,D).

Discussion

The consumption of fruits and vegetables is related to the prevention of many diseases due to the antioxidant activity of plants' secondary metabolites (Jacobo-Velázquez *et al.*, 2022). However, the postulated expansion of indoor farming agriculture, employing strictly controlled, stable grown conditions, results in the restriction of health-promoting compound levels, as they accumulate mostly in response to abiotic stresses. Thus, the aim of the study is to elucidate an efficient, easy-to-operate and non-invasive method to biofortify plant tissue with secondary metabolites intended to be applied within indoor farming. The proposed application is based on the short-term exposition of plants to low doses of UV-A radiation directly prior to harvest. We analysed the influence of UV-A treatment on two popular leafy species of lettuce and basil in the form of *baby leaf* (leafy greens) and *microgreens*, respectively. Lettuce and basil plants were grown in green- and red-leaf cultivars.

Similar approaches have been previously analysed in paper of Ordidge *et al*. (2010), which evaluated the effect of plant cultivation in open-sided tunnels under a plastic film of three different UV light transparency. The mentioned authors, documented that the application of films with higher UV transparency induced accumulation of total phenolics and flavonoids (quercetin) in red lettuce, but was ineffective in the case of green lettuce cultivar. In the case of our study, we documented opposite results, as the green leaf cultivars of both lettuce (cv. Lollo Bionda) as well as basil (cv. Sweet Large) present significantly higher responsiveness to UV-A-depended phenolic compounds synthesis than dark-pigmented cultivars (Lollo Rossa and Dark Opal). In leaves, phenolics accumulation protects photosynthetic apparatus against UV damage, thus the green cultivars presented significantly lower TPC when grown without stressors such as UV, while it made them more vulnerable to UV-A exposition that activates phenolics synthesis and deposition. In the case of dark-pigmented cultivars UV-A is ineffective to stimulate further accumulation of TPC above the high, initial level. Moreover, the UV-A exposition exerted a negative influence on TPC, as a result of partial degradation of phenolics absorbing UV-A or restricted penetration of UV-A due to low doses applied. The latter explanation agrees with analyses of Qin *et al*. (2023), who documented that UV-A induced enhanced TPC accumulation in purple lettuce cultivars when applied at higher intensity, while plants grown under low-doses of UV-A showed control-like TPC. Also, Kang *et al*. (2022) documented that higher doses of UV-A light can improve TPC accumulation in basil.

In addition to TPC, we also analysed flavonoids concentration (TFC) as TPC and TFC synthesis share a mutual biochemical pathway. It has been documented that UV-mediated changes in phenolics and flavonoids levels may be attributed to its ability to induce the gene expression of phenylalanine ammonia lyase (PAL), a key enzyme involved in the first step of the phenylpropanoid pathway (Wong *et al*., 2020). Like phenolics, also flavonoids may benefit consumer health due to their anti-oxidative and presumed anti-carcinogenic effect (Rodriguez *et al*., 2014). Consequently, we also analysed the total antioxidant capacity of plants extracts with and without UV-A exposition. In a previous study (He *et al*., 2021) it has been reported that UV-A

exposition of lettuce increased the radical-scavenging rate measured with DPPH, due to enhanced accumulation of TFC and TPC. Also, Lee *et al*. (2013) documented that plants of sowthistle (*Ixeris dentata* Nakai) exposed to UV-A for 5 days presented 50% higher flavonoid content than the control. In this study, we documented that the accumulation pattern of flavonoids follows that of phenolics, namely UV-A exposition exerts a stimulating effect in green leaf cultivars of lettuce and basil, while in dark-pigmented cultivars we observed slightly decreased the TFC. Moreover, we noted that the TAC of plant extract is strictly related to actual TFC and TPC, and consequently was significantly enhanced in green cultivars: Lollo Bionda and Sweet Large. Basil cv. Dark Opal presents a consequent decrease of TAC. However, an exception to this was red leaf lettuce – Lollo Rossa, which shows a little TAC increase after UV-A treatment. As we documented that the Lollo Rossa cultivar showed decreased levels of both TPC and TFC it might be other antioxidant compounds such as anthocyanins, that accumulated after UV-A treatment that enhanced TAC.

Taken together, our results are in accordance with Lee *et al*. (2014) documented that low-dose UV-A treatment enhanced phenolic compound and antioxidant properties in lettuce particularly between days 1 and 4 after exposure. However, prolonged UV-A exposition attenuated this effect and concentration of antioxidative compounds decreased to the control level. The authors explain that such an effect may be a consequence of continuous quenching of ROS with antioxidant phytochemicals. Such a reaction may be expected as increased accumulation of UV-A highly absorbing compounds such as phenolics and flavonoids make more the UV radiation to be absorbed and generates ROS. Presumably, such a response was also a consequence of the unresponsiveness of darked-pigmented cultivars of lettuce and basil to induce further antioxidant accumulation, as they already contain high TPC and TFC.

Conclusions

The results demonstrate that low-dose (9 W m^{-2}) UV-A short-term (4 days, total 15h) exposure applied within a red-green-blue light spectrum background allowed to induce accumulation of health-promoting phytochemicals such as phenolics and flavonoids and overall antioxidants capacity measured as ability to scavenging the DPPH radical. Analyses were conducted on lettuce and basil plants, grown in short crop time - *baby leaf* and *microgreens*, respectively. However, the positive effect was observed only for green leaf cultivars – Lollo Bionda of lettuce and Sweet Large of basil. Dark-pigmented cultivars presented significantly higher antioxidant compounds under the control spectrum (RGB), whereas after UV-A treatment both cultivars presented slightly decreased of phenolic and flavonoids. The study provides an approach for indoor farming spectrum optimization, mostly devoid of UV components, to improve the quality of plant leafy products.

Acknowledgements

This research was funded by the Polish Ministry of Science and Higher Education (Grant No. SUPB.RN.24.211, E.S, M.T.) and the Polish Agency for Restructuring and Modernisation of Agriculture (Grant No. DDD.6509.00044.2022.13, M.T., E.S.).

References

Ainsworth, E.A., Gillespie, K.M. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nature protocols, 2(4), 875–877.

Ampim, P.A., Obeng, E., Olvera-Gonzalez, E. 2022. Indoor vegetable production: An alternative approach to increasing cultivation. Plants, 11(21), 2843.

Artés-Hernández, F., Castillejo, N., Martínez-Zamora, L. 2022. UV and visible spectrum led lighting as abiotic elicitors of bioactive compounds in sprouts, microgreens, and baby leaves – A comprehensive review including their mode of action. Foods, 11(3), 265.

Avgoustaki, D.D., Xydis, G. 2020. How energy innovation in indoor vertical farming can improve food security, sustainability, and food safety? Advances in Food Security and Sustainability, 5, 1–51.

Brazaityte, A., Virsile, A., Jankauskiene, J., Sakalauskiene, S., Samuoliene, G., Sirtautas, R., Novičkovas, A., Dabašinskas, L., Miliauskienė, J., Vaštakaitė, V., Bagdonavičienė, A., Duchovskis, P. 2015. Effect of supplemental UV-A irradiation in solid-state lighting on the growth and phytochemical content of microgreens. International Agrophysics, 29(1), 13–22.

D'Amico-Damião, V., Carvalho, R.F. 2018. Cryptochrome-related abiotic stress responses in plants. Frontiers in plant science, 9, 431291.

Darré, M., Vicente, A.R., Cisneros-Zevallos, L., Artés-Hernández, F. 2022. Postharvest ultraviolet radiation in fruit and vegetables: Applications and factors modulating its efficacy on bioactive compounds and microbial growth. Foods, 11(5): 653.

Despommier, D. 2011. The vertical farm: controlled environment agriculture carried out in tall buildings would create greater food safety and security for large urban populations. Journal für Verbraucherschutz und Lebensmittelsicherheit, 6, 233–236.

He, R., Zhang, Y., Song, S., Su, W., Hao, Y., Liu, H. 2021. UV-A and FR irradiation improves growth and nutritional properties of lettuce grown in an artificial light plant factory. Food Chemistry, 345, 128727.

Jacobo-Velázquez, D.A., Moreira-Rodríguez, M., Benavides, J. 2022. UVA and UVB radiation as innovative tools to biofortify horticultural crops with nutraceuticals. Horticulturae, 8(5), 387.

Kang, S., Kim, J.E., Zhen, S., Kim, J. 2022. Mild-intensity UV-A radiation applied over a long duration can improve the growth and phenolic contents of sweet basil. Frontiers in Plant Science, 13, 858433.

Lee, M.J., Son, J.E., Oh, M.M. 2013. Growth and phenolic content of sowthistle grown in a closed-type plant production system with a UV-A or UV-B lamp. Horticulture, Environment, and Biotechnology, 54, 492–500.

Lee, M.J., Son, J.E., Oh, M.M. 2014. Growth and phenolic compounds of *Lactuca sativa* L. grown in a closed-type plant production system with UV-A, -B, or-C lamp. Journal of the Science of Food and Agriculture, 94(2), 197–204.

Lee, M., Kim, J., Oh, M.M., Lee, J.H., Rajashekar, C.B. 2022. Effects of supplemental UV-A LEDs on the nutritional quality of lettuce: accumulation of protein and other essential nutrients. Horticulturae, 8(8), 680.

Mehmood, A., Javid, S., Khan, M.F., Ahmad, K.S., Mustafa, A. 2022. In vitro total phenolics, total flavonoids, antioxidant and antibacterial activities of selected medicinal plants using different solvent systems. BMC chemistry, 16(1), 1–10.

Ordidge, M., García-Macías, P., Battey, N.H., Gordon, M.H., Hadley, P., John, P., Lovegrove, J.A., Vysini, E., Wagstaffe, A. 2010. Phenolic contents of lettuce, strawberry, raspberry, and blueberry crops cultivated under plastic films varying in ultraviolet transparency. Food Chemistry, 119(3), 1224–1227.

Qin, H., Xu, Y., Liu, B., Gao, Y., Zheng, Y., Li, Q. 2023. UV-A supplement improved growth, antioxidant capacity, and anthocyanin accumulation in purple lettuce (*Lactuca sativa* L.). Horticulturae, 9(6): 634.

Rodriguez, C., Torre, S., Solhaug, K.A. 2014. Low levels of ultraviolet-B radiation from fluorescent tubes induce an efficient flavonoid synthesis in Lollo Rosso lettuce without negative impact on growth. Acta Agriculturae Scandinavica, Section B–Soil & Plant Science, 64(2), 178–184.

Shraim, A.M., Ahmed, T.A., Rahman, M.M., Hijji, Y.M. 2021. Determination of total flavonoid content by aluminum chloride assay: A critical evaluation. LWT, 150, 111932.

Specht, K., Siebert, R., Hartmann, I., Freisinger, U.B., Sawicka, M., Werner, A., Thomaier, S., Henckel, D., Walk, H., Dierich, A. 2014. Urban agriculture of the future: an overview of sustainability aspects of food production in and on buildings. Agriculture and Human Values, 31, 33–51.

Surjadinata, B.B., Jacobo-Velázquez, D.A., Cisneros-Zevallos, L. 2017. UVA, UVB and UVC light enhances the biosynthesis of phenolic antioxidants in fresh-cut carrot through a synergistic effect with wounding. Molecules, 22(4), 668.

Trojak, M., Skowron, E. 2021. Light quality-dependent regulation of non-photochemical quenching in tomato plants. Biology, 10(8), 721.

Vanhaelewyn, L., Van Der Straeten, D., De Coninck, B., Vandenbussche, F. 2020. Ultraviolet radiation from a plant perspective: The plant-microorganism context. Frontiers in Plant Science, 11, 597642.

Verdaguer, D., Jansen, M.A., Llorens, L., Morales, L.O., Neugart, S. 2017. UV-A radiation effects on higher plants: Exploring the known unknown. Plant Science, 255, 72–81.

Wong, C.E., Teo, Z.W.N., Shen, L., Yu, H. 2020. Seeing the lights for leafy greens in indoor vertical farming. Trends in Food Science & Technology, 106, 48–63.

Xiao, Y., Chu, L., Zhang, Y., Bian, Y., Xiao, J., Xu, D. 2022. HY5: A pivotal regulator of light-dependent development in higher plants. Frontiers in Plant Science, 12, 800989.

Folia Biologica et Oecologica 18: 122–132 (2024)

Acta Universitatis Lodziensis

Potential role of bacterial pathogens in the immunopathogenesis of ovarian cancer

WIKTORIA WIERZBIŃSKA [1,2], OLGA KUŹMYCZ [2]

[1]BioMedChem Doctoral School of the University of Lodz and Lodz Institutes of the Polish Academy of Sciences, Matejki 21/23, 90–237 Lodz, Poland,
[2]University of Lodz, Faculty of Biology, Department of Molecular Microbiology, Banacha 12/16, 90–237 Lodz, Poland
E-mail: wiktoria.wierzbinska@edu.uni.lodz.pl, olga.kuzmycz@biol.uni.lodz.pl

ABSTRACT

The development of next-generation sequencing (NGS) techniques allowed conducting research with greater efficiency and determining the microbial pattern of niches in the human body that were previously considered sterile. Observed changes in the microbiome composition of patients with cancer lesions increasingly indicate the role of microorganisms in the tumour induction and progression. Overgrowth of certain pathogenic strains within the tissue may cause inflammation, which in its chronic form may lead to destabilization of host genome. Such changes may result in altering the expression of genes encoding proteins involved in significant metabolic pathways and promote pathogenic cell functions such as proliferation stimulation, apoptosis inhibition and modulation of inflammatory response. Consequently, these events may lead to tissue destruction, disruption of physiological processes and development of disease states including cancer. In light of emerging reports on the role of changes in the composition of the microbiota in tumorigenesis induction and the presence of pathogenic strains in the ovarian cancer (OC) tumour microenvironment (TME), the hypothesis of a potential role for bacteria in the pathogenesis of this cancer is also gaining interest. The following review presents a summary of scientific research indicating potential role of TME bacteria in the immunopathogenesis of OC.

KEYWORDS: microbiota, TME, OC microbiota, ovarian microbiota

Introduction

Ovarian cancer is the deadliest disease of the female upper reproductive tract (URT) with a cancerous origin, with more than 300 000 new cases and more than 150 000 deaths reported worldwide each year. Due to the lack of specific symptoms, most patients are diagnosed at an advanced stage of the disease, which, combined with a poor prognosis and frequent drug resistance, translates into a

DOI: https://doi.org/10.18778/1730-2366.18.15

high mortality rate. Therefore, it is extremely important to identify potential indicators that would allow early detection and diagnosis of these cancers, which would imply an improved prognosis for patients (Pathak *et al*., 2020). Particular attention of the scientific community has been drawn in recent years to the relationship between the presence of microorganisms in specific locations of the human body and the genesis of neoplastic processes (Łaniewski *et al*., 2020). The presence of certain bacterial species can contribute to altering the expression of genes encoding proteins involved in the inflammatory response, proliferation, apoptosis, cytotoxicity, as well as modifying the secretion of inflammatory factors. Consequently, these events can disrupt physiological processes and promote tumorigenesis (Francescone *et al*., 2014; Di Tucci *et al*., 2023). Relationships between the presence of specific strains and carcinogenesis have previously been demonstrated for several cancers (including gastric, colorectal cancers), and there are also reports of the possible involvement of a dysbiotic microbiota in some female URT cancers, including endometrial cancer (Walther-António *et al*., 2016; Walsh *et al*., 2019; Li *et al*., 2021; Lu *et al*., 2021; Chen *et al*., 2021). In recent years, the role of changes in the composition of patients' microbiota has gained increasing support from the scientific world in terms of potentially promoting carcinogenesis. Also, in the case of ovarian cancer (OC), the first reports of the presence of pathogenic strains in the TME are emerging (Zhou *et al*., 2019; Banerjee *et al*., 2017; Wang *et al*., 2020; Asangba *et al*., 2023). However, determining their potential role in the induction or progression of carcinogenic processes requires further research.

The female URT microbiota

For almost a century female URT was divided into three areas; the non-sterile vagina, the cervical mucus plug, which was believed to form a barrier to microorganisms, and the sterile endometrium, fallopian tubes, and ovaries (Baker *et al*., 2018). That is until the second part of the 20-th century when the first reports of microorganisms isolated from other than the vagina parts of the female reproductive tract (FRT) appeared. However, since these studies were conducted using culture methods, they did not reflect the biodiversity or abundance of microorganisms harbouring these niches, as only 1% of microorganisms residing in the human body are capable of growing on synthetic media. Moreover, the material used in these studies was mostly obtained from swabs, resulting in a high risk of contamination and thus inaccurate results (Tao *et al*., 2017). A breakthrough in case of understanding microbial composition within previously inaccessible areas of the human body was the development of NGS, which not only allowed more precise identification of the material, but also increased the efficiency of the process (Human Microbiome Project Consortium, 2012). Thanks to NGS, today without a doubt, we can say that not only vagina, but also other parts of FRT harbour their own microbiota (Pelzer *et al*., 2011).

Years of research into the origins of microorganisms within the URT indicate that they are most likely bacteria ascending from the vagina (Swidsinski *et al*., 2013). The argument supporting this thesis is the fact that the cervical mucus plug, wrongly considered an impermeable barrier for microorganisms, is not effective against all bacteria (Hansen *et al*., 2014). Transport of microorganisms down the FRT can be supported by the physiological uterine contractions, as well as the peristaltic pump which promotes

the sperm transport. This mechanism can also promote the transport of other associated macromolecules including bacteria, especially during the follicular and luteal phases of the menstrual cycle, which are characterized by increased frequency of contractions (Kunz and Leyendecker 2002; Zeryomanolakis *et al*., 2007).

Regardless numerous research results, we are still unable to conclusively determine the species composition of microorganisms residing in particular parts of the female URT. There are several limitations that we must consider in the context of interpreting the results of studies on the healthy microbial pattern of female URT. First of all, in majority of studies, the analysed material was obtained transcervically which is associated with a high risk of contamination. A potential solution to this problem could be to take material directly from the tissues of patients undergoing hysterectomy or oophorectomy, however, neither of these procedures is performed on healthy patients, so the results obtained could also be inaccurate. Moreover, most studies were performed on relatively small groups of patients, which can lead to false conclusions (Franasiak *et al*., 2020).

Despite the variations in the results obtained and multiple factors that must be considered when interpreting them, we can make some general conclusions based on the known data. While vagina is considered one of the most microbial-rich niches in female body, the available data indicate that the URT differs from it fundamentally in terms of both quantity and biodiversity. Comparing to vagina URT is considered as an environment with a lower bacterial abundance as it harbours up to 10 000 times less microorganisms, yet according to available data it exhibits a higher bacterial biodiversity. The study by Chen *et al*., showed that in the deeper parts of the reproductive tract, the number of *Lactobacillus* bacteria decreases sharply accounting for 97% of the microbiota at the cervical level, 30% at the uterine level and only 1.7% at the ovarian level, while the number of species with more diverse populations increases (Chen *et al*., 2017). However, it is worth noting that the study was conducted on material collected from patients with gynecologic conditions such as: hysteromyoma, adenomyosis, endometriosis and salpingemphraxis, whose microbiota composition may differ from healthy patients.

Ovarian microbiota

While the composition of the vaginal microbiota is considered relatively well understood, and studies of the endometrial microbial pattern provide new reports that bring us closer to determining its composition, we still do not know relatively much about the microorganisms residing in the ovary. Pelzer *et al*., screened follicular fluid collected from 262 *in vitro* fertilized (IVF) women for the presence of microorganisms. Study confirmed that the human follicular fluid contains bacteria, which allows us to hypothesize that the bacteria are also present in the ovary (Pelzer *et al*., 2013). Confirmation of this thesis may be a study conducted by Brewster *et al*. (2022) on a group of 10 postmenopausal women scheduled for salpingo-oophorectomy. Sequencing results confirmed the bacterial presence within proximal fallopian tube, fimbriae, and ovary of examined patients. Moreover, a significant difference between the microbiota of different parts of URT were observed. Microbial composition between the fallopian tube and the ovary differed, which confirmed the thesis that each part of the female URT harbours its own, unique microbiological pattern. Similar observations were made after comparing

the microbiota of the fallopian tube and the fimbriated end. The most abundant species detected in the ovaries were *Proteobacteria* (69%), *Bacteroides* (19%), *Actinobacteria* (10%) and *Firmicutes* (2%) (Brewster *et al*., 2022). In another study Miles *et al*. (2017) collected swabs and tissue samples from 10 patients undergoing total hysterectomy and bilateral salpingo-oopherectomy. The sequencing results obtained indicated greater biodiversity within the URT compared to the vaginal environment which is consistent with the observations of Chen *et al* (Miles *et al*., 2017; Chen *et al*., 2017). In another study Wang *et al*.(2020) compared the microbiome of 6 patients diagnosed with OC and 10 patients diagnosed with noncancerous ovarian conditions, which formed the control group in this study. Results revealed the presence of bacterial LPS both in oncologic and non-cancerous group, which once again confirmed the bacterial presence within the ovaries. Similarly to previous reports *Proteobacteria* was the most abundant phylium both in cancer (67.2%) and the control group (67.1%). Unlikely previous reports the second most abundant phylium in this study was *Firmicutes* (23.77% in the control group and 23.82% in the cancer group) and the third most abundant phylium was *Bacteroidetes* (3.26% in the control group and 3.41% in the cancer group) (Wang *et al*., 2020). The above studies undoubtedly provide us with valuable scientific evidence of microbial presence in the ovaries, but they cannot establish a scientific basis for determining the composition of a healthy ovarian microbiota. The available data comes from studies conducted on relatively small groups of patients who have been diagnosed with gynecological abnormalities such as ovarian cancer or fertility disorders, which can lead to false conclusions.

Role of TME in the immunepathogenesis of cancer

Tumour microenvironment, which is a complex, dynamic, and continuously evolving entity, consists of, among other factors; immune cells, extracellular matrix elements, stroma, and endothelial cells (Anderson and Simon, 2020). Considering recent reports indicating the presence of microorganisms in niches previously considered sterile and their potential role in carcinogenesis, microbes residing in the TME are increasingly being included among other elements forming this specific environment (Niño *et al*., 2022). The presence of microorganisms within a certain niche, can be safe and beneficial for the host, as long as a certain limit of bacterial invasion is maintained. When the microbial load is too high, or enriched in representatives of certain taxa, induction of abnormal pathological processes within the tissue may occur. Disruption of microbal homeostasis may contribute to excessive tissue destruction, immune stimulation, and disruption of key metabolic pathways (Punzón-Jiménez and Labarta, 2021).

Most cases of pathogen-induced inflammation, results in intruders' complete elimination by the immune system and restoration of homeostasis. Unfortunately, in case of some infections, complete elimination of pathogens does not occur, and their presence within the tissue results in the excessive secretion of inflammatory mediators, which over time results in chronic inflammation (Łaniewski *et al*., 2020). Toll-like receptors (TLRs) play a key role in detecting the presence of pathogens within the tissue, through both receptor and activation functions (Janeway and Medzhitov, 2002). Once bacterial molecular patterns are recognized by TLR receptors, the inflammatory cascade within the infected tissue is activated (Fig. 1). The released cytokines stimulate the

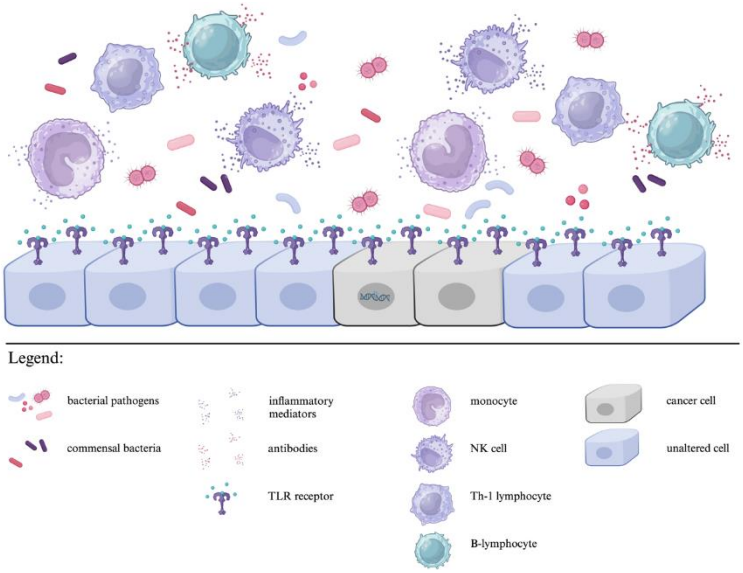

Figure 1. Progression of the inflammatory response in ovarian tissue.

differentiation of type 1 T helper (Th-1) lymphocytes, as well as the activation of monocytes and natural-killer (NK) cells, which secrete further inflammatory mediators including interleukin-2 (IL-2), IL-12, IL-15, IL-18, tumour necrosis factor α (TNF-α), interferons α (IFN-1) and β (IFN-2). Another line of defence activated by TLRs are B lymphocytes, which under activation transform into plasmatic cells secreting antibodies, which are a precise tool to fight foreign microorganisms. TLR receptors thus represent an activating factors for both specific and non-specific immunity (Majewska and Szczepanik, 2006; Bossowska-Nowicka *et al*., 2015).

Under physiological conditions, the inflammatory response persists until the intruder is eliminated, and then undergoes self-silencing. Unfortunately, in the case of a chronic reaction, which may be driven by a persistent bacterial infection, constant activation of TLR receptors, may result in their excessive mobilization, and thus further stimulation of the inflammatory response, or promotion of cellular angiogenesis (Basith *et al*., 2012). TLR receptors may express their role in promoting angiogenesis by stimulating the secretion of vascular endothelial growth factor (VEGF), which is responsible for the vascularization of tumour cells, allowing for significant tumour growth due to its supply in oxygen and nutrients (Carmeliet, 2005). Moreover, TLR activation may result in the activation of protein complex nuclear factor kappa B (NF-κB). NF-κB expresses its role in the inflammatory response by stimulating the production of pro-inflammatory cytokines, which, unfortunately, can also result in inhibition of apoptosis, stimulation of proliferation or enhancement of angiogenesis – processes which are the hallmarks of tumorigenesis (Liu *et al*., 2017). Moreover, TLR activation results in production of oxygen free radicals (ROS), which in excessive amount contributes to

lipids, proteins and nucleic-acid damage. Such changes within cell metabolism may contribute to tumour initiation, development and growth as well as therapy-resistance development (Ding *et al*., 2021).

Presence of certain bacterial taxa within the ovaries and persistent chronic inflammation can also lead to destabilization of host genome. Chronic inflammation, as well as bacterial second metabolites, provide an environment in which damage, resulting in changes to the host genome is more likely to occur. Among other things, the changes can be associated with overexpression of cyclooxygenase 1 (COX1) and 2 (COX2), nitric oxide, and ROS, which in high concentration within the TME may promote damage to the genetic material of host cells, potentially resulting in mutations (Vakkila and Lotze, 2004). Carcinogenesis requires at least two allelic mutations in a single gene, according to model proposed by Knudson. Such single mutations could lead to the development of cancer, but usually more mutations are required. However, chronic inflammation provides a highly mutagenic environment in which DNA damage is more likely to happen. The resulting mutations can lead to abnormalities in cellular metabolism such as proliferation or apoptosis – key processes in the pathogenesis of cancer (Knudson, 1971)

Even up to 13–20% of cancers diagnosed each year may have an inflammatory basis (Shahanavaj *et al*., 2015). However, identification of specific species and the mechanisms responsible for processes underlying carcinogenesis has been a challenge. To date, International Agency for Research on Cancer has classified only one bacterial species as a human carcinogen. Infection with bacteria *Helicobacter pylori*, referred to results in an inflammation of gastric mucosa (Rudnicka *et al*., 2019). The chronic inflammation can lead to peptic ulcer disease which if sustained for a long time, may also lead to stomach cancer (Nomura *et al*., 1991). Similar relationship has been demonstrated in case of colon cancer and the *Fusobacterium sp*. infection, in particular *F. mortiferum*, *F. nucleatum* and *F. necrophorum* species. Activated by *F. nucleatum* NF-κB factor, may result in development of chronic inflammation (Kostic *et al*., 2012).

Similar relationships were observed in case of URT cancers such as endometrial cancer (EC). In experiment conducted by Walther-António *et al*. (2016), presence of *Atopobium vaginae* and *Porphyromonas sp*. bacteria in combination with high vaginal pH was linked with EC. Walsh *et al*. (2019), also identified *Porphyromonas somerae* as the most predative marker for uterine cancer. In another study, Li *et al*. (2021), confirmed that increasing *Prevotella* abundance in endometrial tissue, especially when correlated with elevated serum levels of D-dimers (DD) and fibrin degradation products (FDP), may be associated with carcinogenesis. In study conducted by Lu *et al*. (2021), correlation between increased *Micrococcus sp*. abundance and IL-6 and IL-17 micro-RNA levels in EC patients was observed. In another study enrichment of *Firmicutes*, *Proteobacteria*, *Tenericutes*, *Actinobacteria* and *Bacteroidete* was observed in group of EC patients (Chen *et al*., 2021). The results presented, despite the variation, indicate that the role of changes in the composition of the microbiota not only of the stomach or colon, but also of the female URT should not be underestimated. In addition, some results point to a potentially inflammatory role of pathogens present in the tumour environment. Unfortunately, there is still a lack of evidence indicating whether the presence of microorganisms

in altered tumour tissue is an effect or a cause of neoplastic changes.

Pathogens associated with ovarian cancer

Ovarian cancer is the third most common female cancer with the highest mortality rate worldwide. Every year 300 000 women receive a diagnosis of ovarian cancer, which causes up to 152 000 deaths worldwide each year. The high mortality is caused mostly by the asymptomatic nature of OC, which results in late diagnosis, usually at an advanced stage of cancer (Sung *et al*., 2021). The high mortality rate of this cancer is also influenced by frequent resistance to chemotherapy. OC therapy most often includes optimal surgical reduction of tumour-altered tissue in combination with chemotherapy, as well as neoadjuvant chemotherapy, radiotherapy, and immunotherapy. Unfortunately, for more than 50% of patients with OC, the cancer recurs, usually in a form that is refractory to the chemotherapy used (Ding *et al*., 2021).

Autosomally inherited mutations in the BRCA breast cancer susceptibility (BRCA) genes, also known as hereditary breast and ovarian cancer (HBOC) syndrome, and in the DNA mismatch repair (MMR) genes, also known as hereditary nonpolyposis colorectal cancer (HNPCC) syndrome or Lynch II syndrome, are assumed to be responsible for about 15% of OC cases. However, mutations in the BRCA 1/2 genes, which account for up to 90% of hereditary OC cases, are most often responsible for HBOC syndrome. BRCA genes are a group of cancer suppressor genes, and mutations that are associated with them are predisposed to various cancers, including OC (Pan and Xie, 2017). Among other risk factors the most commonly discussed are high body mass index (BMI), smoking cigarettes, nulliparity and the family history. It is also supposed that a specific factor may be linked to the particular type of ovarian cancer (Fortner *et al*., 2019). Based on the genetic, clinical, and histopathological factors, OC is divided into type I and type II. Type I tumours are characterized by a good prognosis accounting for only 10% OC-related deaths. In this type of tumour cancer cells develop from benign extraovarian precursor lesions which are present in the ovary (Ducie *et al*., 2017). Type II cancers represent the majority of OC cases, characterized as highly aggressive neoplasms. The mortality of type II OC is reaching up to 90% of OC-related deaths, of which high-grade serous carcinoma (HGSC) is considered the most common form of diagnosis, resulting in 70–80% of deaths from OC (Bowtell *et al*., 2017). Scientific evidence over the past decade has challenged the theory that serous ovarian cancer originates in the surface epithelium of the ovary and has identified the fallopian tube as the source of HGSC. The distal region of the fallopian tube, also known as the fimbria, is exposed to the pelvic cavity, which is the most common location of serous carcinoma in BRCA-positive women with p53 mutations (Crum *et al*., 2007). Accordingly, Zhou *et al*. (2019) hypothesized that OC carcinogenesis may be promoted or driven by several factors, including but not limited to pelvic inflammatory disease (PID), local tumour immune microenvironment, hormonal fluctuations, and spontaneous mutations. The research team also points to disruptions in microbial composition as a potential driver of chronic inflammation, and thus perhaps carcinogenesis. In a conducted study, they compared the microbiota composition of tissue samples collected from 25 patients diagnosed with ovarian cancer and 25 normal distal fallopian tube tissue samples. High-throughput sequencing results revealed

that both diversity and richness indexes were significantly reduced in ovarian cancer tissues compared to tissues from normal distal fallopian tubes, which represented a control group in this study. At the phylum level, *Proteobacteria* and *Firmicutes* were the most dominant taxa in both groups. Whereas at the genus level, *Acinetobacter*, *Sphingomonas* and *Methylobacterium* were significantly enriched in the group of cancer patients, while *Lactobacillus* was the outstandingly dominant genus in the control group. Moreover, study revealed that transcriptional profiles of ovarian cancer tissues differed from the normal fallopian tube tissues. Inflammation-associated signalling pathways including NF-κB signalling pathway, cytokine-cytokine receptor interaction and chemokine signalling pathway were dramatically activated in OC tissues. Overall, presented results suggest role of changes within the ovarian microbiome in the stimulation of inflammatory processes, which may have a key role in the induction of carcinogenic processes (Zhou *et al*., 2019). Banerjee *et al*. (2017) screened 99 ovarian cancer samples using pan-pathogen array (PatoChip), combined with capture-next generation sequencing. In contrast to the results of Zhou *et al*., the above study showed greater biodiversity in the material collected from cancer patients, compared to the non-ovarian control group. The predominant bacterial phyla in ovarian cancer samples were *Proteobacteria* (52%), followed by *Firmicutes* (22%). Other phylla including *Bacteroidetes, Actinobacteria, Chlamydiae, Fusobacteria, Spirochaetes* and *Tenericutes* were also present in OC samples (Banerjee *et al*., 2017). Another study conducted by Wang *et al*. (2020) revealed that ovarian bacterial communities in ovarian cancer group were dominated by *Gemmata obscureglobus* (13.89%), followed by *Halobacteroides halobius* (11.99%) and *Methyloprofundus sedimenti* (11.12%). An interesting conclusion was reached by the research team of Asangba and colleagues, they analysed the microbiome of all body sites, with the exception of the stool and peritoneal network, which were not collected from the control group of patients with benign non-cancerous lesions in this study and compared them with the microbiome of patients diagnosed with cancer ovary (Asangba *et al*., 2023). The study showed significant differences between the microbiome of patients with OC, compared to a group of patients with benign gynecological conditions. In addition, the team also observed an overall enrichment of several microbial taxa, including *Dialister*, *Corynebacterium*, *Prevotella* and *Peptoniphilus* in all body sites of patients with unfavourable ovarian cancer outcomes, which may suggest a role for these microbes in modulating the body's response to therapy and provide an indicator in predicting the efficacy of treatment (Asangba *et al*., 2023). This result is also consistent with the thesis that microbial-induced carcinogenesis is often associated with global changes in the microbiome, rather than attributed to individual pathogens (Schwabe and Jobin, 2013).

Conclusions

With the development of research on the human microbiota, more and more evidence point to the undeniable role of microorganisms in the induction and progression of diseases that have posed a challenge to modern medicine for years. Among them, the role of disorders within the microbial composition in the context of cancer is increasingly being discussed. In recent years, numerous studies have shown that female URT, for years wrongly considered sterile, also has its own unique microbiota. Moreover, with

more reports of the presence of pathogenic microorganisms, among patients with established cancerous lesions, there is growing support for the thesis according to which bacteria may play an important role in the induction, and perhaps progression of cancer. Such relationships have previously been well established for gastric cancer, as well as for colorectal cancer. There is also growing evidence supporting the involvement of microorganisms in the induction of neoplastic lesions in the female URT, including the EC. Emerging evidence of the presence of pathogenic strains in the tissues of patients with OC, indicates that a similar relationship may also exist for this cancer. The presence of certain bacterial strains within the tissue can induce inflammation, which, persisting for a long time, develops into a chronic form. Chronic inflammation, in turn, can provoke can provoke changes within the host's genetic material that result in disruption of key metabolic pathways, posing a risk of cancer development.

Ovarian cancer is now one of the most frequently diagnosed and deadliest female cancers in the world. Due to its late diagnosis and lack of specific symptoms, it is most often detected at an advanced stage, when treatment options are severely limited. Determining the microbiological pattern of the ovary, as well as identifying strains with a potentially carcinogenic role, gives hope for the development of specific biomarkers that would allow an early detection of pathological changes in the tissue. However, further research is needed, not only to indicate the composition of the ovarian microbiome in a state of eubiosis and potentially pathogenic strains, but also to detect mechanisms underlying bacterial-induced carcinogenesis.

Literature

Anderson, N.M., Simon, M.C. 2020. The tumor microenvironment. Current biology: CB, 30(16), R921–R925.

Asangba, A.E., Chen, J., Goergen, K.M., Larson, M.C., Oberg, A.L., Casarin, J., Multinu, F., Kaufmann, S.H., Mariani, A., Chia, N., Walther-Antonio, M.R.S. 2023. Diagnostic and prognostic potential of the microbiome in ovarian cancer treatment response. Scientific Reports, 13(1), 730.

Baker, J.M., Chase, D.M., Herbst-Kralovetz, M.M. 2018. Uterine Microbiota: Residents, Tourists, or Invaders? Frontiers in immunology, 9, 208.

Banerjee, S., Tian, T., Wei, Z., Shih, N., Feldman, M.D., Alwine, J.C., Coukos, G., Robertson, E.S. 2017. The ovarian cancer oncobiome. Oncotarget, 8(22), 36225–36245.

Basith, S., Manavalan, B., Yoo, T.H., Kim, S.G., Choi, S. 2012. Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. Archives of Pharmacal Research, 35(8), 1297–1316.

Bossowska-Nowicka M., Dembele K., Toka F. 2015. Udział receptorów Toll-podobnych w patogenezie atopowego zapalenia skóry u ludzi i zwierząt. Cz. 1 Rola receptorów Toll-podobnych w odporności. Życie Weterynaryjne. 2015:789–792.

Bowtell, D.D., Böhm, S., Ahmed, A.A., Aspuria, P.J., Bast, R.C., Jr, Beral, V., Berek, J.S., Birrer, M.J., Blagden, S., Bookman, M.A., Brenton, J.D., Chiappinelli, K.B., Martins, F.C., Coukos, G., Drapkin, R., Edmondson, R., Fotopoulou, C., Gabra, H., Galon, J., Gourley, C., Heong V, Huntsman, D.G., Iwanicki, M., Karlan, B.Y., Kaye, A., Lengyel, E., Levine, D.A., Lu, K.H., McNeish, I.A., Menon, U., Narod, S.A., Nelson, B.H., Nephew, K.P., Pharoah, P., Powell, D.J Jr., Ramos, P, Romero, I.L., Scott C.L., Sood, A.K., Stronach, E.A., Balkwill, F.R. 2015. Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer. Nature Reviews. Cancer, 15(11), 668–679.

Brewster, W.R., Burkett, W.C., Ko, E.M., Bae-Jump, V., Nicole McCoy, A., Keku, T.O. 2022. An evaluation of the microbiota of the upper reproductive tract of women with and without epithelial ovarian cancer. Gynecologic Oncology Reports, 42, 101017.

Carmeliet P. 2005. VEGF as a key mediator of angiogenesis in cancer. Oncology, 69 Suppl 3, 4–10.

Chen, C., Song, X., Wei, W., Zhong, H., Dai, J., Lan, Z., Li, F., Yu, X., Feng, Q., Wang, Z., Xie, H., Chen, X., Zeng, C., Wen, B., Zeng, L., Du, H., Tang, H., Xu, C., Xia, Y., Xia, H., Yang H, Wang J, Wang J, Madsen L, Brix S, Kristiansen

K, Xu X, Li J, Wu R, Jia, H. 2017. The microbiota continuum along the female reproductive tract and its relation to uterine-related diseases. Nature Communications, 8(1), 875.

Chen, P., Guo, Y., Jia, L., Wan, J., He, T., Fang, C., Li, T. 2021. Interaction Between Functionally Activate Endometrial Microbiota and Host Gene Regulation in Endometrial Cancer. Frontiers in Cell and Developmental Biology, 9, 727286.

Crum, C.P., Drapkin, R., Miron, A., Ince, T.A., Muto, M., Kindelberger, D.W., Lee, Y. 2007. The distal fallopian tube: a new model for pelvic serous carcinogenesis. Current Opinion in Obstetrics & Gynecology, 19(1), 3–9.

Di Tucci C, De Vito I, Muzii L. 2023. Immune-Onco-Microbiome: A New Revolution for Gynecological Cancers. Biomedicines. 2023;11(3):782.

Ding, D.N., Xie, L.Z., Shen, Y., Li, J., Guo, Y., Fu, Y., Liu, F. Y., Han, F. J. 2021. Insights into the Role of Oxidative Stress in Ovarian Cancer. Oxidative Medicine and Cellular Longevity, 2021, 8388258.

Ducie, J., Dao, F., Considine, M., Olvera, N., Shaw, P.A., Kurman, R.J., Shih, I.M., Soslow, R.A., Cope, L., Levine, D. A. 2017. Molecular analysis of high-grade serous ovarian carcinoma with and without associated serous tubal intra-epithelial carcinoma. Nature Communications, 8(1), 990.

Fortner, R.T., Poole, E.M., Wentzensen, N.A., Trabert, B., White, E., Arslan, A.A., Patel, A.V., Setiawan, V.W., Visvanathan, K., Weiderpass, E., Adami, H.O., Black, A., Bernstein, L., Brinton, L.A., Buring, J., Clendenen, T.V., Fournier, A., Fraser, G., Gapstur, S.M., Gaudet, M.M., Giles, G.G., Gram, I.T., Hartge, P., Hoffman-Bolton, J., Idahl, A., Kaaks, R., Kirsh, V.A., Knutsen, S., Koh, W.P., Lacey, J.V.Jr., Lee, I.M., Lundin E., Merritt, M.A., Milne, R. L., Onland-Moret, N.C., Peters, U., Poynter, J. N., Rinaldi, S., Robien, K., Rohan, T., Sánchez, M.J., Schairer, C., Schouten, L.J., Tjonneland, A., Townsend, M.K., Travis, R.C., Trichopoulou, A., van den Brandt, P.A., Vineis, P., Wilkens, L., Wolk, A., Yang, H.P., Zeleniuch-Jacquotte, A., Tworoger, S.S. 2019. Ovarian cancer risk factors by tumor aggressiveness: An analysis from the Ovarian Cancer Cohort Consortium. International Journal of Cancer, 145(1), 58–69.

Francescone R, Hou V, Grivennikov S.I. 2014. Microbiome, inflammation, and cancer. Cancer Journal. 2014;20(3):181-189.

Galeano Niño, J.L., Wu, H., LaCourse, K.D., Kempchinsky, A.G., Baryiames, A., Barber, B., Futran, N., Houlton, J., Sather, C., Sicinska, E., Taylor, A., Minot, S.S., Johnston, C.D., Bullman, S. 2022. Effect of the intratumoral microbiota on spatial and cellular heterogeneity in cancer. Nature, 611(7937), 810–817.

Hansen L.K., Becher N., Bastholm S., Glavind J., Ramsing M., Kim C.J., Romero R., Jensen J.S., Uldbjerg N. 2014. The cervical mucus plug inhibits, but does not block, the passage of ascending bacteria from the vagina during pregnancy. Acta Obstetricia et Gynecologica Scandinavica, 93(1):102–118.

Human Microbiome Project Consortium. 2012. Structure, function and diversity of the healthy human microbiome. Nature, 486(7402), 207 –214.

Janeway, C.A. Jr, Medzhitov, R. 2002. Innate immune recognition. Annual Review of Immunology, 20, 197–216.

Knudson, A.G. Jr. 1971. Mutation and cancer: statistical study of retinoblastoma. Proceedings of the National Academy of Sciences of the United States of America, 68, 820–823.

Kostic A.D., Gevers D., Pedamallu C.S., Michaud M., Duke F., Earl A. M., Ojesina A.I., Jung J., Bass A.J., Tabernero J., Baselga J., Liu C., Shivdasani R.A., Ogino S., Birren B.W., Huttenhower C., Garrett W.S., Meyerson M. 2012. Genomic analysis identifies association of Fusobacterium with colorectal carcinoma. Genome Research, 22(2), 292–298.

Kunz G., Leyendecker G. 2002. Uterine peristaltic activity during the menstrual cycle: characterization, regulation, function, and dysfunction. Reproductive BioMedicine Online, 4, 5–9.

Łaniewski, P., Ilhan, Z.E., Herbst-Kralovetz, M.M. 2020. The microbiome and gynaecological cancer development, prevention and therapy. Nature Reviews. Urology, 17(4), 232–250.

Li C., Gu Y., He Q., Huang, J., Song Y., Wan X., Li Y. 2021. Integrated Analysis of Microbiome and Transcriptome Data Reveals the Interplay Between Commensal Bacteria and Fibrin Degradation in Endometrial Cancer. Frontiers in Cellular and Infection Microbiology, 11:748558.

Liu, T., Zhang, L., Joo, D., Sun, S.C. 2017. NF-κB signaling in inflammation. Signal transduction and targeted therapy, 2, 17023.

Lu, W., He, F., Lin, Z., Liu, S., Tang, L., Huang, Y., Hu, Z. 2021. Dysbiosis of the endometrial microbiota and its association with inflammatory cytokines in endometrial cancer. International Journal of Cancer, 148(7), 1708 –1716.

Majewska M, Szczepanik M. 2006. Rola receptorów toll-podobnych (TLR) w odporności wrodzonej

i nabytej oraz ich funkcja w regulacji odpowiedzi immunologicznej. Postępy Higieny i Medycyny Doświadczalnej 60(null):52–63.

Miles, S.M., Hardy, B.L., Merrell, D.S. 2017. Investigation of the microbiota of the reproductive tract in women undergoing a total hysterectomy and bilateral salpingo-oopherectomy. Fertility and Sterility, 107(3), 813–820.e1.

Nomura A., Stemmermann G.N., Chyou P.H., Kato I., Perez-Perez G.I., Blaser M.J. 1991. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. New England Journal of Medicine, 17, 325(16), 1132–1136.

Pan, Z., Xie, X. 2017. BRCA mutations in the manifestation and treatment of ovarian cancer. Oncotarget, 8(57), 97657–97670.

Pelzer, E.S., Allan, J A., Waterhouse, M.A., Ross, T., Beagley, K.W., Knox, C.L. 2013. Microorganisms within human follicular fluid: effects on IVF. PloS one, 8(3), e59062.

Pelzer, E.S., Allan, J.A., Cunningham, K., Mengersen, K., Allan, J.M., Launchbury, T., Beagley, K., Knox, C.L. 2011. Microbial colonization of follicular fluid: alterations in cytokine expression and adverse assisted reproduction technology outcomes. Human Reproduction (Oxford, England), 26(7), 1799–1812.

Punzón-Jiménez P., Labarta E. 2021. The impact of the female genital tract microbiome in women health and reproduction: a review. Journal of Assisted Reproduction and Genetics, 38(10), 2519–2541.

Rudnicka K., Backert S., Chmiela M. 2019. Genetic Polymorphisms in Inflammatory and Other Regulators in Gastric Cancer: Risks and Clinical Consequences. Current topics in microbiology and immunology, 421, 53–76.

Schwabe, R.F., Jobin, C. 2013. The microbiome and cancer. Nature Reviews. Cancer, 13(11), 800 –812.

Shahanavaj K., Gil-Bazo I., Castiglia M., Bronte G., Passiglia F., Carreca A.P., del Pozo J.L., Russo A., Peeters M., Rolfo C. 2015. Cancer and the microbiome: Potential applications as new tumor biomarker. Expert review of anticancer therapy, 2015; 15:317–330.

Sudipta P., Jacek R. Wilczyński J.R., Paradowska E. 2020. Factors in Oncogenesis: Viral Infections in Ovarian Cancer. Cancers, 12(3), 561.

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F. 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA:–A Cancer Journal for Clinicians, 71(3), 209–249.

Swidsinski A., Verstraelen H., Loening-Baucke V., Swidsinski S., Mendling W., Halwani Z. 2013. Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. PLOS One, 8(1): e53997.

Tao X., Franasiak J. M., Zhan Y., Scott R. T., Rajchel J., Bedard J., Newby R. J., Treff N. R., Chu T. 2017. Characterizing the endometrial microbiome by analyzing the ultra-low bacteria from embryo transfer catheter tips in IVF cycles: next generation sequencing (NGS) analysis of the 16S ribosomal gene. Human Microbiome Journal, 3: 15-21.

Vakkila, J., Lotze, M.T. 2004. Inflammation and necrosis promote tumour growth. Nature Reviews. Immunology, 4(8), 641–648.

Walsh, D.M., Hokenstad, A.N., Chen, J., Sung, J., Jenkins, G. D., Chia, N., Nelson, H., Mariani, A., Walther-Antonio, M.R.S. 2019. Postmenopause as a key factor in the composition of the Endometrial Cancer Microbiome (ECbiome). Scientific Reports, 9(1), 19213.

Walther-António, M.R., Chen, J., Multinu, F., Hokenstad, A., Distad, T.J., Cheek, E.H., Keeney, G.L., Creedon, D.J., Nelson, H., Mariani, A., Chia, N. 2016. Potential contribution of the uterine microbiome in the development of endometrial cancer. Genome Medicine, 8(1), 122.

Wang, Q., Zhao, L., Han, L., Fu, G., Tuo, X., Ma, S., Li, Q., Wang, Y., Liang, D., Tang, M., Sun, C., Wang, Q., Song, Q., Li, Q. 2020. The differential distribution of bacteria between cancerous and noncancerous ovarian tissues in situ. Journal of Ovarian Research, 13(1), 8.

Zervomanolakis, I., Ott, H.W., Hadziomerovic, D., Mattle, V., Seeber, B.E., Virgolini, I., Heute, D., Kissler, S., Leyendecker, G., Wildt, L. 2007. Physiology of upward transport in the human female genital tract. Annals of the New York Academy of Sciences, 1101, 1–20.

Zhou, B., Sun, C., Huang, J., Xia, M., Guo, E., Li, N., Lu, H., Shan, W., Wu, Y., Li, Y., Xu, X., Weng, D., Meng, L., Hu, J., Gao, Q., Ma, D., Chen, G. 2019. The biodiversity Composition of Microbiome in Ovarian Carcinoma Patients. Scientific Reports, 9(1), 1691.

Folia Biologica et Oecologica 18: 133–141 (2024)

Acta Universitatis Lodziensis

Analysis of furin (*FURIN*) gene expression in the U-87 MG cell line as a potential target for gene inhibiting therapies in (auto-) immune diseases

KAROLINA ZAPLETAL-PUDEŁKO[1], GRZEGORZ MACHNIK[1], KATARZYNA STEC-GROSMAN[2], ŁUKASZ BUŁDAK[1], BOGUSŁAW OKOPIEŃ[1]

[1]Medical University of Silesia, Faculty of Medical Science in Katowice, Department of Internal Medicine and Clinical Pharmacology, Medykow 18, 40–752 Katowice, Poland;
[2]Medical Laboratory Synevo, Ujastek 3, 31–752 Kraków, Poland
E-mail: karolinazapletal@yahoo.com

ABSTRACT

Purpose: Furin is a proprotein convertase commonly found in the human body. The enzymatic activity of furin is necessary for the activation of numerous substrates including e.g. hormones and growth factors. Nevertheless, furin is involved in various pathological conditions caused by, among others, chronic inflammation. Therefore furin is considered as a potential target in autoimmune diseases therapy.
We performed an experiment in which the expression of *FURIN* gene in U-87 MG astrocytoma cells was investigated. Additionally, this cell line contains some sequences coding human endogenous retroviruses (HERVs), including *ERVW-1* and its receptor- *SLC1A5*. Deregulation of HERV expression has been observed in some neurodegenerative diseases as well as in inflammatory process.
Material and Methods: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot (WB) techniques were utilized for analysis. Phorbol 12-myristate 13-acetate (PMA) were used for cell stimulation. Short interfering RNAs (siRNA) were used for gene expression inhibition in U-87 MG cells *in vitro*.
Results: Cell stimulation by PMA strongly increased *FURIN* expression, simultaneously downregulating *ERVW-1* ($p<0.01$). Moreover addition of PMA significantly stimulates the autocatalytic action of cellular furin itself resulting in the dissociation of its propeptide that was clearly visible in a time-dependent manner. SiRNA-mediated expression inhibition of *ERVW-1* and *FURIN* influenced the mRNA level for *SLC1A5 (ASCT2)* - primary syncytin-1 receptor, that was significantly lower. *FURIN* inhibition by siRNA caused strong upregulation of *ERVW-1* expression ($p<0.01$).
Conclusion: Our results showed that stimulation by PMA and inhibition expression by siRNA affects the expression of *FURIN* in U-87 MG

DOI: https://doi.org/10.18778/1730-2366.18.16

astrocytoma cells. Moreover, furin shows a complex relationship on the expression of *ERVW-1* and *SLC1A5* genes, as well as on the form (precursor or mature) and the amount of the final translation products of the transcripts. The regulation of *FURIN* may pose a potential therapeutic strategy in the treatment of neurodegenerative diseases caused by autoimmunity.

KEYWORDS: neuroinflammation, *FURIN*, HERVs, neurodegenerative diseases, gene therapies

Introduction

Inflammation is a defensive process that is activated by the appearance of a damaging factor(s) in the body. The goal of inflammation is to restore the body's homeostasis. Unfortunately, due to the deregulation of defence mechanisms, an excessive inflammatory reaction is often observed, which causes damage to adjacent tissues. In some cases inflammation process becomes chronic, which is the cause of many life-threatening diseases like atherosclerosis and rheumatoid arthritis (Roe, 2021). In regard to its location, there is a specific type of inflammation namely neuroinflammation. Neuroinflammation involves neuronal tissue and plays a significant role in the development and progression of neurodegenerative diseases such as Alzheimer's disease and multiple sclerosis (MS). Neurodegenerative diseases are a group of diseases in which the degradation and atrophy of cells from the nervous system occur (Oliveira *et al*., 2015). WHO reports from 2007 estimated that approximately 6.8 million people die from neurodegenerative diseases annually. Unfortunately, despite the passage of time, there is still no effective therapy for the treatment of neurodegenerative diseases (Vardi and Merrick, 2007). Hence, there is an urgent need for continuous development in understanding the mechanisms of development of these diseases, which will enable programming of effective treatment. There are numerous indications that two proteins: furin and syncytin-1 play a significant role in the initiation and progression of neurodegenerative diseases mainly due to their pro-inflammatory potential (Dolei, 2018; Valli *et al*., 2019).

Furin is a proprotein convertase that is commonly present within human body. This enzyme is coded by *FURIN* gene that is located on chromosome 15 (15q26.1). The enzymatic activity of furin is necessary for the activation of numerous substrates including e.g. hormones (proinsulin, proparathyroid hormone) and growth factors (transforming growth factor beta 1 precursor, pro-nerve growth factor) (Zhang *et al*., 2022). Despite its crucial role in human physiology furin has been found to be involved in various pathological conditions that are principally caused by chronic inflammatory state. Therefore furin is extensively examined as a potential target in autoimmune diseases therapy both at the level of its enzymatic activity inhibition and, more currently, at the level of gene expression silencing (Valli *et al*., 2019).

The human genome carries approximately 8% of sequences of retroviral origin, that has been incorporated millions of years ago as a result of exogenous retroviral infections. The sequences of human endogenous viruses (HERVs) are mostly unexpressed due to numerous mutations caused them permanently inactive. However, some of HERVs, such as *ERVW-1*, are still transcribed under specific conditions. The

ERVW-1 expression product is a glycoprotein syncytin-1 that plays an important physiological role in the early stages of pregnancy being involved in the formation of the placenta (Noorali *et al*., 2009; Wang *et al*., 2018a). Nevertheless, numerous studies prove that syncytin-1 exhibits some pro-inflammatory properties, mainly observed in brain tissue. The initiation and progression of neuronal inflammation that was related to syncytin-1 activation causes cells damage that has been subsequently demonstrated as a major factor in multiple sclerosis (MS) course (Dolei, 2018). The pathological neuroinflammatory and neurodegenerative potential of syncytin-1 prompt the search for a therapy involving the inhibition of the effective expression of *ERVW-1*. An important fact in the syncytin-1 synthesis is that furin activity is necessary for the transformation of the inactive form of prosyncytin-1 into a functional one. Therefore the regulation of furin gene expression is an important topic to consideration in any pathological conditions that are directly or indirectly responsible for causing autoimmune diseases, including neurodegenerative ones (Dolei *et al*., 2015).

Materials and Methods

In order to investigate the neuronal/glial *FURIN* and *ERVW-1* activity we performed a gene expression analysis using U-87 MG astrocytoma cell line (U-87 MG Cell Line human, No. Cat. 89081402-1VL, Merck, Poznań, Poland). A primary syncytin-1 receptor: the neutral amino acid transporter SLC1A5 coded by *SLC1A5* gene has also been found in this cell line as an expressed mRNA. While *ERVW-1* gene codes for syncytin-1 glycoprotein, furin activity is essential to achieve the syncytin-1 mature form (Machnik *et al*., 2024). Therefore we investigated the *ERVW-1* gene expression behaviour along that for *FURIN* in U-87 MG cells. To date, an altered HERVs expression has been observed in some neurodegenerative diseases as well as in an inflammatory processes (Dolei *et al*., 2019).

RNA extraction

Total RNA was extracted from U-87 MG cells using TRI Reagent (MRC Inc. Cincinnati, OH, USA) according to one-step RNA extraction method described by Chomczynski and Sacchi (1987). Finally RNA was dissolved in nuclease-free water and its concentration was estimated spectrometrically. Next, 500 ng of total RNA was reverse transcribed using GoScript Reverse Transcription System (Promega Corporation, Madison, WI, USA). Reverse transcription (RT) reaction mixture was diluted 1:4 with water before further analysis.

Reverse transcription and real time quantitative polymerase chain reaction

Quantitative analysis of *FURIN*, *ERVW-1* and *SLC1A5* mRNA expression was carried out by real-time quantitative polymerase chain reaction (real time QPCR). An amount of 5 µl of reverse transcription reaction mixture (i.e. an equivalent of 50 ng of total RNA) was used as a template in each reaction. Real-time QPCR was performed using GoTaq qPCR Master Mix (Promega). Reaction mixture (total volume of 25 µl) contained 12.5 µl 2× Master Mix, and 200 nM of each forward and reverse primer. Primer sequences for *ERVW-1 FURIN*, *SLC1A5* as well as for *GAPDH* were listed in our previous paper (Machnik *et al*., 2024). We calculated the relative mRNA quantity of genes of interest based on SYBR-Green I chemistry with a parallel amplification of *GAPDH* mRNA that served as endogenous normalizer. Quantitative PCR was performed on Roche LightCycler 480 Real-Time PCR system (Roche Diagnostics Polska Warsaw,

Poland) as described previously (Schmittgen and Livak, 2008).

Western blotting

After PMA or siRNA treatment, U87-MG cells were lysed directly in wells of 12-well culture plate. To lyse cells, 150 µl of cold RIPA buffer supplemented with 1/100 volume of Halt Protease Inhibitor Cocktail was added (Thermo Fisher Scientific, Warsaw, Poland) and cells were incubated for 10 minutes on ice under shaking. Thereafter protein extracts were centrifuged at 13,000 RPM and total protein concentration was estimated by BCA assay (Thermo Scientific, Warsaw, Poland). Proteins from cell lysates (20 µg) were separated by means of electrophoresis in 10% polyacrylamide gel in the presence of ColorPlusPrestained Protein Marker (New England Biolabs, Lab-Jot, Warsaw, Poland). After separation, proteins were blotted onto PVDF membrane (Merck Millipore, Poznań, Poland).

Immunodetection of specific proteins

Unspecific binding sites were blocked by incubation of membrane in 3% bovine serum albumin (BSA) solution for two hours and then membranes were placed in 3% BSA/1xTTBS containing each of syncytin-1, furin or SLC1A5 -specific antibody at a final dilution of 1:1000. Parallel, an anti-GAPDH antibody (dilution of 1:2000) was used. Incubations were performed for one hour with continuous rocking. Then an Anti-rabbit IgG (whole molecule)-peroxidase antibody (Merck Sigma Aldrich, Poznań, Poland) was added (antibody dilution of 1:10,000) and membranes were incubated for one hour. Finally a specific chemiluminescent signal was developed (Pierce ECL Western Blotting Substrate, Thermo Scientific, Warsaw, Poland) and membranes were scanned using ChemiDoc-It Imaging System (Analytik Jena, Jena, Germany). Measurements of integrated optical density (IOD) representing the amount of the protein of interest in a sample were done using ImageJ software while IOD for GAPDH served as a normalizer (Schneider *et al*., 2012).

Inhibition of gene expression by RNA interference

Small interfering RNA (siRNA) molecules were from Thermo Fisher Scientifics (Warsaw, Poland). For *ERVW-1* and *FURIN* a Silencer Select siRNA was used (siRNA ID: s26921 and s9989, respectively). About 5 nM of each siRNA was delivered to the cells by means of adding of 3 µl of cationic lipid-based cargo (Lipofectamine RNAiMAX Transfection Agent, Thermo Fisher Scientific, Warsaw, Poland) in the 12-well plate wells (Tissue Culture Testplate, SPL Life Sciences, Pocheon-si, Korea). The inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression was performed as a control assay (Ambion Silencer GAPDH Positive control siRNA, Thermo Fisher Scientifics, Warsaw, Poland).

Cells stimulation by phorbol myristate acetate (PMA)

U87-MG astrocytoma cell line was stimulated using 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) which is a specific activator of Protein Kinase C (PKC) and hence of NF-kB. PMA has been previously used for induction of ERVW-1 mRNA in monocytoid (MDM) and astrocytic (U373) cell lines (Amos *et al*., 2005). Cells were treated with PMA by adding of the agent directly into cell culture medium at various time intervals ranging from 15 minutes to 5 hours. Thereafter culture medium was replaced with the fresh one and cells incubation was continued for consecutive 24 hours.

Results

We found that cell stimulation by phorbol 12-myristate 13-acetate (PMA) strongly increased *FURIN* expression measured as a relative mRNA expression level (Fig. 1). The addition of PMA stimulated the expression level of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) ($p < 0.01$) as well as influenced the level of mRNA expression for *SLC1A5 (ASCT2)* – a primary syncytin-1 receptor ($p > 0.5$). Interestingly, we observed that the relative mRNA level for *ERVW-1* was significantly reduced after 30 minutes of PMA treatment or in longer time span ($p < 0.01$).

Treatment the astrocytoma cells with of PMA significantly stimulated the autocatalytic activity of cellular furin resulting in the dissociation of its propeptide that was clearly visible in a time-dependent manner (Fig. 2).

FURIN expression inhibition by Small interfering RNA (siRNA) resulted in strong upregulation of *ERVW-1* expression ($p < 0.01$) (Fig. 3A). SiRNA -mediated inhibition of *ERVW-1*

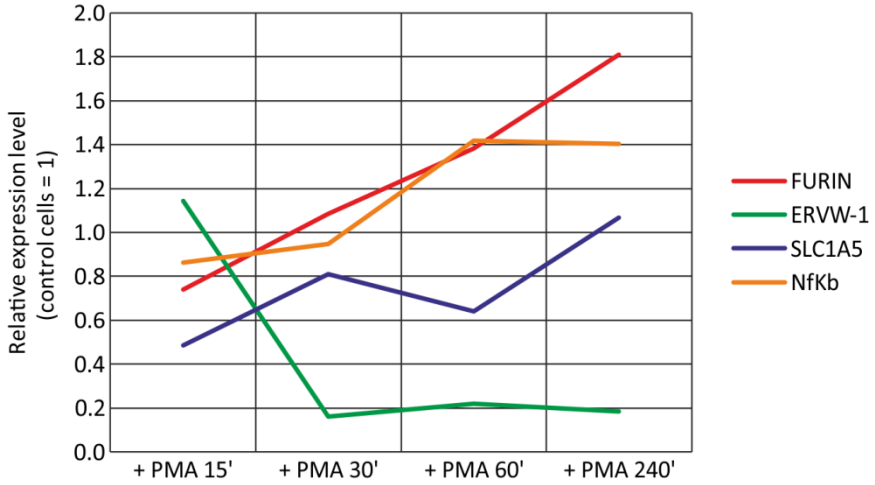

Figure 1. Phorbol 12-myristate 13-acetate (PMA) treatment of U87-MG astrocytoma cells. The relative expression level of *FURIN*, *ERVW-1*, *SLC1A5* as well as *NFKB1* genes is shown at each time point. The expression level that was observed in control (non PMA treated) cells was assumed as a value of 1.

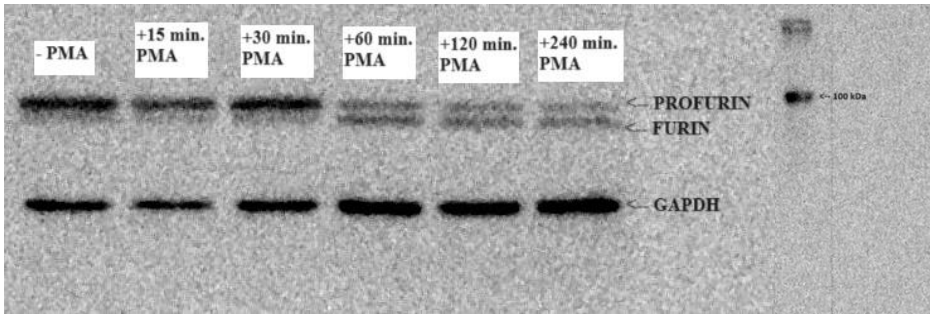

Figure 2. An autocatalytic activity of furin in U87-MG astrocytoma cells after treatment with PMA. The dissociation of profurin to furin was clearly visible in a time-dependent manner. The autocatalytic activity was compared to that observed in control cells (non PMA treated) and normalized with a control protein (GAPDH).

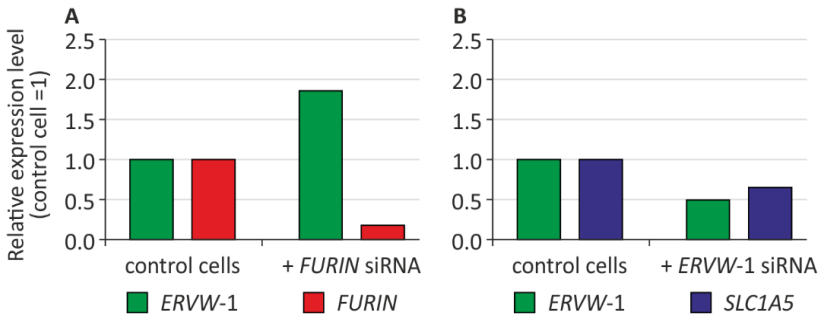

Figure 3. A. Expression level of *ERWV-1* and *FURIN* genes after addition of *FURIN*- specific small interfering RNA (siRNA). B. Expression level of *ERWV-1* and *SLC1A5* genes after addition of *ERVW-1*-specific siRNA. The expression level observed in control, non siRNA-treated cells was assumed as a value of 1.

expression subsequently influenced the mRNA level for *SLC1A5* (*ASCT2*) gene coding for the primary syncytin-1 receptor. It's expression level was lower than that of control cells albeit with no significancy (Fig. 3B).

Discussion

Furin is a proprotein convertase that is responsible for transforming numerous inactive proteins into their active forms thus it is necessary to maintain a proper physiology in vertebrates. Nevertheless in addition to regulating physiological processes, an overexpression of the *FURIN* gene has been observed in many pathological conditions such as atherosclerosis or rheumatoid arthritis (Valli *et al*., 2019).

Among others, furin is responsible for the activation of the protein product of the *ERVW-1* gene coding for syncytin-1 glycoprotein, belonging to the HERV family. HERVs are sequences of human endogenous retroviruses, which constitute approximately 8% of the human genome, but most number of them is transcriptionally inactive. Numerous scientific reports have proven that deregulation of HERV expression occurs in neurodegenerative diseases and also as a result of inflammation (Singh *et al*., 2024).

Antony and colleagues conducted an extensive analysis of the expression of syncytin-1 (*ERVW-1)* gene in multiple sclerosis patients, in which they observed a significant increase in the number of *ERVW-1* mRNA copies in the brain tissue of people with multiple sclerosis compared to healthy controls (Antony *et al*., 2006). In turn, Wang *et al*. observed an excessive expression of the gene encoding syncytin-1 and of CRP (C-reactive protein) in patients suffering from schizophrenia. Scientists confirmed that syncytin-1 activates the CRP protein, which presents a new mechanism for the regulation of inflammation by syncytin-1 in schizophrenia (Wang *et al*., 2018b). In addition to reports indicating the direct involvement of furin- activated syncytin-1 in the course of neurodegenerative diseases associated with inflammation of nervous tissue. There are some indications that furin is involved in the development of these diseases through different mechanisms of action and possibly at another biochemical level. Bennett *et al*. proved that furin activates BACE (beta-site amyloid precursor protein cleaving enzyme) protease, which is a beta-secretase, and that is responsible for the transformation of the precursor form of amyloid into beta-amyloid that forms plaques in Alzheimer's (Bennett *et al*., 2000).

Taking into consideration aspects mentioned above we investigated the connections between the expression of *FURIN*, *ERVW-1*, and *SLC1A5* (syncytin-1 receptor) genes. The understanding of the mechanisms or the relationship between activity of those genes may allow the creation of potential therapy in neurodegenerative diseases that may involves e.g. the regulation of the expression of *FURIN* gene. The use of PMA as a stimulator in cell culture and siRNA as an expression silencer, indicated that *FURIN* gene exhibited an increased or decreased expression level, respectively (Fig. 1, Fig. 3A). That results confirmed the possibility of intentional control of the *FURIN* expression depending on the desirable needs. Moreover, in our study the inhibition of the *ERVW-1* by siRNA resulted in a significant expression reduction of *SLC1A5* gene, which indicates a relationship between mentioned genes (Fig. 3B). In turn, inhibition of the *FURIN* gene by a specific siRNA resulted in strong overexpression of the *ERVW-1* gene, which can be explained as a compensatory mechanism in that a decrease amount of furin protein led to the limited transformation of the precursor form of syncytin-1 protein into its active form, that, in turn initiated cell processes toward the increased transcription of the *ERVW-1* gene that eventually increase the amount of syncytin-1 protein in the cell (Fig. 3A).

After incubating U-87 MG cells with PMA, we also measured the expression levels of *SLC1A5* and *ERVW-1* genes. In the tested time frame, the expression level of the *SLC1A5* gene increased significantly compared to the control sample, but there was a significant decrease in the *ERVW-1* gene expression (Fig. 1). It should be also borne in mind that the protein product of *SLC1A5*, i.e. a neutral amino acid transporter B(0), is not specific only for syncytin-1, but it is the main transporter of amino acids like glutamine, alanine, serine, hence the increase in the expression of *SLC1A5* after using a cellular stimulator should be understood as a result of an activation of cellular metabolism (Ni *et al*., 2023).

To deeper investigate the topic of inflammation we decided to include the NF-κB protein complex in our research. NF-κB is a protein complex that functions as a transcription factor. NF-κB occurs in almost all animal cells and is involved in the cell's response to stimuli such as stress, cytokines, free radicals, or antigens. NF-κB plays a key role in regulating the immune response to infection. Disturbances in the regulation of NF-κB are associated with cancer, inflammation and autoimmune diseases (Poma, 2020). Incubation of U-87 MG cells in culture medium supplemented with PMA resulted in a significant increase in the expression level of the *NFKB1* gene (Fig. 1). An increase in the expression level of *NFKB1* was observed already within the first 15 minutes and was most strongly stimulated for an hour, after which the expression was maintained at the maximum level until the end of the experiment (Fig. 1). This study confirmed the stimulating effect of PMA on the expression of the NF-κB factor gene. It is worth to mention that Abu El-Asrar and colleagues confirmed that furin induces excessive activation of the p65 subunit of NF-κB, which indicates a direct interaction between furin and the NF-κB transcription factor (Abu El-Asrar *et al*., 2022).

In addition we noticed the effect of PMA on the synthesized furin present in the cell. Furin is synthesized in the form of a zymogen, i.e. as profurin which must be activated to fulfill its role in the organism. In the case of profurin, its activation occurs only by means of autocatalysis where the auto-inhibitory

domain is removed (Gawlik *et al*., 2009). It turned out that PMA stimulates the profurin-to-furin autocatalysis and that process was clearly visible in a time-dependent manner (Fig. 2).

Obtained results confirmed findings published previously that syncytin-1 abundance rely on the activity of furin. We showed that either stimulation by PMA or expression inhibition by siRNA enable an intentional regulation of furin in U-87 MG astrocytoma cells. Moreover we noted that furin exhibits a multi-faceted relationship with syncytin-1 (*ERVW-1)* and *SLC1A5* genes, as well as on the form (precursor or mature) and on the amount of the final products.

Due to the fact that there is still ineffective treatment of autoimmune and neurodegenerative diseases, it is important to conduct more investigations in this research topic.

References

Amos, S., Martin, P.M., Polar, G.A., Parsons, S.J., Hussaini, I.M. 2005. Phorbol 12-myristate 13-acetate induces epidermal growth factor receptor transactivation via protein kinase Cdelta/c-Src pathways in glioblastoma cells. The Journal of Biological Chemistry, 280, 7729–7738.

Antony, J.M., Izad, M., Bar-Or, A., Warren, K.G., Vodjgani, M., Mallet, F., Power, C. 2006. Quantitative analysis of human endogenous retrovirus-W env in neuroinflammatory diseases. AIDS Research and Human Retroviruses, 22, 1253–1259.

Bennett, B.D., Denis, P., Haniu, M., Teplow, D.B., Kahn, S., Louis, J.C., Citron, M., Vassar, R. 2000. A furin-like convertase mediates propeptide cleavage of BACE, the Alzheimer's β-secretase. The Journal of Biological Chemistry 275, 37712–37717.

Chomczynski, P., Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochememistry, 162, 156–159.

Dolei, A. 2018. The aliens inside us: HERV-W endogenous retroviruses and multiple sclerosis. Multiple Sclerosis (Houndmills, Basingstoke, England) 24, 42–47.

Dolei, A., Ibba, G., Piu, C., Serra, C. 2019. Expression of HERV Genes as Possible Biomarker and Target in Neurodegenerative Diseases. International Journal of Molecular Sciences, 20.

Dolei, A., Uleri, E., Ibba, G., Caocci, M., Piu, C., Serra, C. 2015. The aliens inside human DNA: HERV-W/MSRV/syncytin-1 endogenous retroviruses and neurodegeneration. Journal of Infection in Developing Countries, 9, 577–587.

Abu El-Asrar, A.M., Nawaz, M.I., Ahmad, A., Siddiquei, M.M., Allegaert, E., Gikandi, P.W., De Hertogh, G., Opdenakker, G. 2022. Proprotein convertase furin is a driver and potential therapeutic target in proliferative diabetic retinopathy. Clinical & Experimental Ophthalmology, 50, 632–652.

Gawlik, K., Shiryaev, S.A., Zhu, W., Motamedchaboki, K., Desjardins, R., Day, R., Remacle, A.G., Stec, B., Strongin, A.Y. 2009. Autocatalytic activation of the furin zymogen requires removal of the emerging enzyme's N-terminus from the active site. PloS One, 4, e5031.

Machnik, G., Bułdak, Ł., Zapletal-Pudełko, K., Grabarek, B.O., Staszkiewicz, R., Sobański, D., Okopień, B. 2024. The impact of wound-healing assay, phorbol myristate acetate (PMA) stimulation and siRNA-mediated FURIN gene silencing on endogenous retroviral ERVW-1 expression level in U87-MG astrocytoma cells. Advances in Medical Sciences, 69, 113–124.

Ni, J., Dai, W., Liu, C., Ling, Y., Mou, H. 2023. A pan-cancer analysis of SLC1A5 in human cancers. Heliyon, 9, e17598.

Noorali, S., Rotar, I.C., Lewis, C., Pestaner, J.P., Pace, D.G., Sison, A., Bagasra, O. 2009. Role of HERV-W syncytin-1 in placentation and maintenance of human pregnancy. Applied Immunohistochemistry & Molecular Morphology, 17, 319–328.

Oliveira, A.C.P. de, Candelario-Jalil, E., Fiebich, B.L., Santos, M. da S., Palotás, A., Reis, H.J. dos 2015. Neuroinflammation and Neurodegeneration: Pinpointing Pathological and Pharmacological Targets. BioMed Research International, 2015, 487241.

Poma, P. 2020. NF-κB and Disease. International Journal of Molecular Sciences, 21.

Roe, K. 2021. An inflammation classification system using cytokine parameters. Scandinavian Journal of Immunology 93, e12970.

Schmittgen, T.D., Livak, K.J. 2008. Analyzing real-time PCR data by the comparative C(T) method. Nature Protocols, 3, 1101–1108.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods, 9, 671–675.

Singh, S., Borkar, M.R., Bhatt, L.K. 2024. Transposable Elements: Emerging Therapeutic

Targets in Neurodegenerative Diseases. Neurotoxicity Research 42, 9.

Valli, A., Ranta, N., Grönholm, A., Silvennoinen, O., Pesu, M., Isomäki, P. 2019. Increased expression of the proprotein convertase enzyme FURIN in rheumatoid arthritis. Scandinavian Journal of Rheumatology 48, 173–177.

Vardi, G., Merrick, J. 2007. Neurological Disorders. Public Health Challenges. Journal of Policy and Practice in Intellectual Disabilities 4.

Wang, X., Huang, J., Zhu, F. 2018a. Human Endogenous Retroviral Envelope Protein Syncytin-1 and Inflammatory Abnormalities in Neuropsychological Diseases. Frontiers in Psychiatry, 9, 422.

Wang, X., Liu, Z., Wang, P., Li, S., Zeng, J., Tu, X., Yan, Q., Xiao, Z., Pan, M., Zhu, F. 2018b. Syncytin-1, an endogenous retroviral protein, triggers the activation of CRP via TLR3 signal cascade in glial cells. Brain, Behavior, and Immunity, 67, 324–334.

Zhang, Y., Gao, X., Bai, X., Yao, S., Chang, Y.-Z., Gao, G. 2022. The emerging role of furin in neurodegenerative and neuropsychiatric diseases. Translational Neurodegeneration, 11, 39.

Folia Biologica et Oecologica 18: 142–147 (2024)

Acta Universitatis Lodziensis

Cytoplasmic male sterility in plants with special emphasis on sugar beet

GABRIELA SADZIK

University of Agriculture in Krakow, Faculty of Biotechnology and Horticulture, Department of Plant Biology and Biotechnology, 29 Listopada 54, 31–425 Kraków, Poland
E-mail: gabriela.sadzik@student.urk.edu.pl

ABSTRACT

The phenomenon of cytoplasmic male sterility (CMS) in plants is characterized by a disturbance in the development of functional pollen. The cause of this disorder is the incompatibility of the mitochondrial and nuclear genome. Determinants of CMS are found in the mitochondrial genome and are inherited maternally. Nuclear *Rf* genes are responsible for restoring male fertility in CMS plants most often by posttranscriptional and/or posttranslational activity on mitochondrial gene expression. Two fertility restoring genes have been identified in sugar beet plants, of which *Rf1* is the best characterized. It is concluded that the translation product of this gene acts as a molecular chaperone leading to inactivation of a specific mitochondrial protein that is a marker for the CMS trait in sugar beet. The CMS phenomenon is applied in the commercial breeding of hybrid varieties of many crop species. Exploiting heterosis is easier with the knowledge of cytoplasmic (mitochondrial) determinants of sterility and corresponding restorer genes.

KEYWORDS: *Beta vulgaris* L., cytoplasmic male sterility, mitochondrial DNA, open reading frame, fertility restorer genes

Introduction

The phenomenon of cytoplasmic male sterility (CMS) has been described in more than 150 species of monocotyledonous and dicotyledonous plants (Sofi *et al*., 2007), including sugar beet (*Beta vulgaris* L.) (Kubo *et al*., 1999).

CMS includes a number of processes leading to disturbances in the microsporogenesis process, resulting in formation of non-functional microspores or pollen grains (Majewska-Sawka and Sadoch, 2003). Disturbances in stamen production have also been observed (Andersen, 1965; Rohrbach, 1965). These disorders result from mitochondrial and nuclear genome incompatibility (Chase, 2007). CMS is a trait inherited in the female line – the determinants of this trait are in the mitochondrial genome, which is

DOI: https://doi.org/10.18778/1730-2366.18.17

transferred to the embryo by the egg cell (Zhiwen *et al*., 2017). For masking of CMS fertility restorer genes are responsible for, which are present in the nuclear genome.

Cytoplasmic male sterility has found wide application in breeding of many crops. CMS forces plants to pollinate with foreign pollen, which promotes the increase in heterozygosity and genetic recombination in the offspring and, as a result, leads to an increase in its vigour (Kaul, 1988; Święcicki *et al*., 2011).

CMS symptoms

Cytoplasmic male sterility manifests itself at the level of flower morphology, flower tissues, the structure of mitochondria and the process of microsporogenesis. Changes may also affect the structure of the flower itself (Ivanov and Dymshits, 2007). The literature describes cases of the transformation of stamens into petals or carpels and stigmas (Laser and Lersten, 1972). Cases of complete absence of inflorescences are also known (Chase, 2007). Structural changes were found in stamen filaments (primarily in the vascular bundle), as well as in anthers, which was associated with abortion of microspores in plants characterized by CMS (Wasiak, 2019). It has been shown that the filaments of normal beet (*Beta vulgaris*) stamens were characterized by elongated cells with a large amount of cytoplasm as opposed to the cells in the filaments of sterile plant stamens (Rohrbach, 1965).

Cytoplasmic male sterility also affects mitochondria. In tapetum and microspores of CMS plants, it was observed that mitochondria are often much smaller and also characterized by a smaller area of the internal membrane forming the crest system (Majewska-Sawka and Sadoch, 2003). Cell division and differentiation are processes that require large amounts of ATP. In the tapetum of CMS plants, a reduced number of mitochondria was observed, and thus also production of ATP at a lower level (Jańska and Wołoszyńska, 1996; Majewska-Sawka and Sadoch, 2003). As a result of recombination in mtDNA, abnormal open reading frames encoding transmembrane proteins may be created that alter the permeability and potential of the internal mitochondrial membrane (Zhiwen *et al*., 2017; Wasiak, 2019). Based on numerous studies of enzyme activity present in mitochondria and structural analyses of mitochondrial DNA, mitochondria were identified as organelles responsible for phenotypic changes observed in CMS plants' pollen (Majewska-Sawka and Sadoch, 2003).

Disorders of microsporogenesis result in the formation of non-functional microspores or pollen grains (Jańska and Wołoszyńska, 1996). Abnormalities may appear at different stages of differentiation – during meiotic division of the pollen stem cell or just after the end of meiosis (Bino, 1985), at the tetrad stage (Horner and Rogers, 1974), as well as during other phases of development of haploid microspores (Majewska-Sawka *et al*., 1993). The most common cause of CMS is cytological disorders in pollen stem cells and tapetum cells (Majewska-Sawka and Sadoch, 2003). Symptoms of abnormal tapetum development include excessive vacuolation, loss of cellular character of tissue and formation of multicellular syncytia, and premature or delayed death (Scoles and Evans, 1979). Electron microscopy techniques have shown structural abnormalities of some cellular organelles in pollen stem cells and tapetum. In the tapetum structure of sterile anthers of sugar beet (*Beta vulgaris* L.) plants, fusion of cells into multi-nucleus syncytia and concentric forms of endoplasmic reticulum was observed (Majewska-Sawka and Sadoch, 2003).

Fertility restorer genes

In many plant species, it is possible to restore male fertility with one or more nuclear *Rf* genes (Tan *et al*., 2015), which are responsible to varying degrees for the variability of the trait (Wang *et al*., 2013). These genes compensate or inactivate the effect of sterilizing mitochondrial factors (Touzet and Meyer, 2014), correcting thus disturbed pollen development (Ivanov and Dymshits, 2007). Many studies show that most fertility restoring genes act posttranscriptionally and/or posttranslationally (Jańska and Wołoszyńska, 1996). As a result of the action of these genes, transcript ends of regions correlated with CMS (Kennell and Pring, 1989) and/or changes occur in quantitative relationships between transcripts (Pruitt and Hanson, 1991). Consequently, a reduction (Nivison and Hanson, 1989) or absence (Song and Hedgcoth, 1994) of proteins characteristic of male sterile cytoplasms is observed.

Most fertility restorer genes belong to the PPR (pentatricopeptide repeat protein) family. Genes of the PPR family are present in the nuclear, mitochondrial and chloroplast genomes (Nakamura *et al*. 2012), and their expression products are responsible for regulating the transcription process (Schmitz-Linnerweber and Small, 2008). PPR-type RF proteins contain 35-amino acid repeating motifs, and by binding specifically to the mitochondrial transcripts that condition the CMS feature, they promote their degradation (Barkan and Small, 2014). This results in a strong reduction in the production of mitochondrial CMS-inducing proteins (Chen and Liu, 2014).

The phenomenon of cytoplasmic male sterility in sugar beet (*Beta vulgaris* L.) was discovered by Owen in 1945 and is now used in breeding (McGrath and Panella, 2018). CMS in sugar beet is linked to a specific hydrophobic mitochondrial protein with a molecular weight of 39 kDa, preSATP6, which forms a 250 kDa complex (Matsuhira *et al*., 2012). Two restorers have been identified in *B. vulgaris* – *X* (*Rf1*) and *Z* (*Rf2*), the latter having little effect on fertility restoration (Honma *et al*., 2014). The use of genetic mapping allowed the localization of these genes – on chromosomes III and IV respectively (Owen, 1945). The *Rf1* gene is best characterized. Analysis of the nucleotide sequence of the *Rf1* gene revealed a cluster consisting of four copies of an open reading frame encoding a yeast metallopeptidase-like protein OMA1, which is involved in quality control of mitochondrial inner membrane proteins (Matsuhira *et al*., 2012; Arakawa, 2020). One of the genes forming the cluster, *bvORF20*, showed partial ability to restore fertility in an experiment using transgenic sugar beet plants (Matsuhira *et al*., 2012). When *bvORF20* was expressed in sugar beet suspension cells characterized by CMS, binding of the expression product of this gene to the preSATP6 protein and formation of a new 200 kDa complex was observed (Arakawa *et al*., 2019). The presence of bvORF20-preSATP6 complex was also detected in the anthers of sugar beet plants with previously restored fertility (Kitazaki *et al*., 2015). Together with the appearance of the bvORF20-preSATP6 complex in the anthers of plants with restored fertility, it was observed that the amount of the preSATP6 complex was significantly reduced, while the total amount of the monomeric protein preSATP6 remained almost unchanged. This phenomenon has been interpreted as a modification of the higher order structure of preSATP6 by the chaperone-like molecular activity exerted by bvORF20 (Matsuhira *et al*., 2012; Arakawa, 2020).

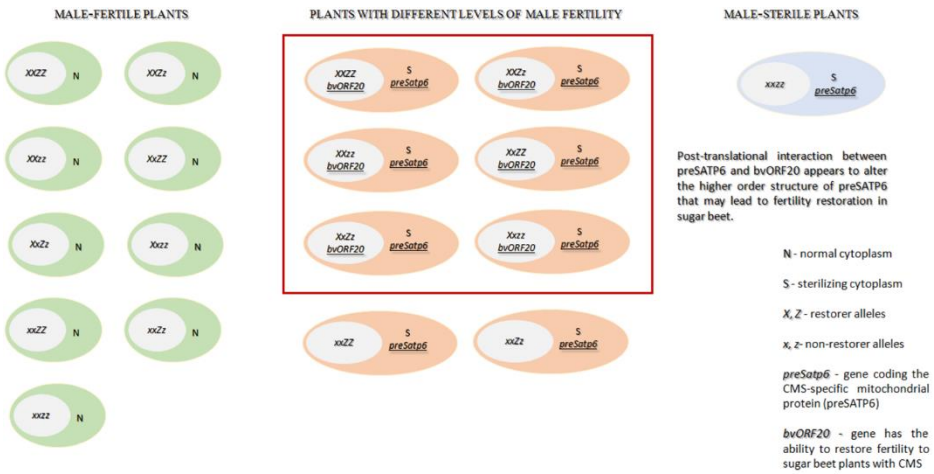

Figure 1. Genetics of cytoplasmic male sterility (CMS) in sugar beet (Owen, 1945; Matsuhira *et al*., 2012; Kitazaki *et al*., 2015)

Practical application of the CMS

The production of hybrid varieties is one of the methods that allows to increase the yield of plants. Such varieties are obtained by pollination of inbred lines with foreign pollen, and the resulting F1 generation shows heterosis (Tsaftaris, 1995). Currently widely used in commercial breeding of hybrid varieties of many crop species is the phenomenon of cytoplasmic male sterility (Stojałowski *et al*., 2019).

The phenomenon of heterosis is not fully understood (Wasiak, 2019). Heterosis is manifested by increased biomass, increased growth rate, increased fertility and better resistance to pests and diseases (Wolko *et al*., 2019), thanks to which hybrid forms give better breeding results than parent forms (Hochholfinger and Hoecker, 2007).

The cultivation of sugar beet owes much to cytoplasmic male sterility, since all current varieties are hybrids produced using CMS (Bosemark, 2006). Production of hybrid seeds using CMS requires three lines – maternal, complementary and paternal (Chen and Liu, 2014). Maternal lines must have a cytoplasm inducing male sterility (S) and so-called complement alleles (*rf*) (Schnable and Wise, 1998). The complement line differs from the CMS line in the presence of normal cytoplasm (N) that determines male fertility. Such cytoplasm should also be present in the paternal line. Restorer alleles (*Rf*) that condition male fertility despite the presence of S cytoplasm and occur in the same locus as the complement alleles (*rf*) make it difficult to obtain CMS and complement lines.

Conclusions

Cytoplasmic male sterility (CMS) favours crosspollinations and is widely used to obtain hybrid seeds. The phenomenon of CMS is masked by fertility restorer genes (Rf) genes located in the nuclear genome, therefore it is important to identify mitochondrial determinants of sterility and responsible Rf genes. The molecular characterization of these sequences also includes studies of their expression at the protein level.

References

Andersen, W.R. 1965. Cytoplasmic male sterility in hybrids of *Lycopersicon esculentum* and

Solanum pennelli. Journal of the Minnesota Academy of Science, 32, 93–94.

Arakawa, T., Ue, S., Sano, C., Matsunaga, M., Kagami, H., Yoshida, Y., Kuroda, Y., Taguchi, K., Kitazaki, Kazuyoshi, K., Kubo, T. 2019. Identification and characterization of a semi-dominant restorer-of-fertility 1 allele in sugar beet (*Beta vulgaris*). Theoretical and Applied Genetics, 132, 227–240.

Arakawa, T., Matsunaga, M., Matsui, K., Itoh, K., Kurodaa, Y., Matsuhira, H., Kitazaki, K., Kubo, T. 2020. The molecular basis for allelic differences suggests *Restorer-of-fertility 1* is a complex locus in sugar beet (*Beta vulgaris* L.). BMC Plant Biology, 20, 503.

Barkan, A., Small, I. 2014. Pentatricopeptide repeat proteins in plants. Annual Review of Plant Biology, 65, 415–442.

Bino, R.J. 1985. Ultrastructural aspects of cytoplasmic male sterility in *Petunia hybrida*. Protoplasma, 127, 230–240.

Bosemark, N.O. 2006. Genetics and breeding. (In:) Draycott, A.P. (ed.), Blackwell, Oxford, pp. 50 –88.

Chen, L., Liu, Y.G. 2014. Male Sterility and Fertility Restoration in Crops. Annual Review of Plant Biology, 65, 579–606.

Hochholdinger, F., Hoecker, N. 2007. Towards the molecular basis of heterosis. Trends in Plant Science, 12, 427–432.

Honma, Y., Taguchi, K., Hiyama, H., Yui-Kurino, R., Mikami, T., Kubo, T. 2014. Molecular mapping of *restorer-of-fertility 2* gene identified from a sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) homozygous for the non-restoring *restorer-of-fertility 1* allele. Theoretical and Applied Genetics, 127, 2567–2574.

Horner, H.T., Rogers, M.A. 1974. A comparative light and electron microscopic study of microsporogenesis in male-fertile and cytoplasmic male-sterile pepper (*Capsicum annuum*). Canadian Journal of Botany, 3, 435 –441.

Ivanow, M.K., Dymshits, G.M. 2007. Cytoplasmic male sterility and restoration of pollen fertility in higher plants. Russian Journal of Genetics, 43, 354–368.

Jańska, H., Wołoszyńska, M. 1996. Molekularne podstawy cytoplazmatycznej męskiej sterylności u roślin wyższych. (In:) Postępy biochemii. Zielińska Z. (ed.), Polskie Towarzystwo Biochemiczne, Warszawa, 253 –259.

Kaul, M.L.H. 1988. Male sterility in higher plants. Monographs on Theoretical and Applied Genetics, vol. 10. Springer-Verlag, Berlin-Heidelberg-New York.

Kennel, J.C., Pring, D.R. 1989. Initiation and processing of *atp6*, *T-urf13* and *ORF221* transcripts from mitochondrial of T cytoplasm maize. Molecular Genetics and Genomics, 216, 16–24.

Kitazaki, K., Arakawa, T., Matsunaga, M., Yui-Kurino, R., Matsuhira, H., Mikami, T., Kubo, T. 2015. Post-translational mechanisms are associated with fertility restoration of cytoplasmic male sterility in sugar beet (*Beta vulgaris*). The Plant Journal, 83, 290–299.

Kubo, T., Nishizawa, S., Mikami, T. 1999. Alterations in organization and transcription of the mitochondrial genome of cytoplasmic male sterile sugar beet (*Beta vulgaris* L.). Molecular and General Genetics, 262, 283–290.

Laser, K.D., Lersten, N.R. 1972. Anatomy and cytology of microsporogenesis in cytoplasmic male sterile angiosperms. The Botanical Review, 38, 425–454.

Majewska-Sawka, A., Rodriguez-Garcia, M.I., Nakashima, H., Jassem, B. 1993. Ultrastructural expression of cytoplasmic male sterilityin sugar beet (*Beta vulgaris* L.). Sexual Plant Reproduction, 6, 22–32.

Majewska-Sawka, A., Sadoch, Z. 2003. Cytoplazmatyczna męska sterylność roślin – mechanizmy biologiczne i molekularne. Kosmos, 52, 413–423.

Matsuhira, H., Kagami, H., Kurata, M., Kitazaki, K., Matsunaga, M., Hamaguchi, Y., Hagihara, E., Ueda, M., Harada, M., Muramatsu, A., Yui-Kurino, R., Taguchi, K., Tamagake, H., Mikami, T., Kubo, T. 2012. Unusual and typical features of a novel restorer-of-fertility gene of sugar beet (*Beta vulgaris* L.). Genetics, 192, 1347–1358.

McGrath, J.M., Panella, L. 2018. Sugar Beet Breeding. (In:) Plant Breeding Reviews. Golgman I. (ed.), Wiley, USA, 167–218.

Nakamura, T., Yagi, Y., Kobayashi, K. 2012. Mechanistic insight into pentatricopeptide repeat proteins as sequence-specific RNA-binding proteins for organellar RNAs in plants. Plant and Cell Physiology, 53, 1171–1179.

Nivison, H.T., Hanson, M.R. 1989. Identification of a mitochondrial protein associated with cytoplasmic male sterility in petunia. The Plant Cell, 1, 1121–1130.

Owen, F.V. 1945. Cytoplasmically inherited male sterility in sugar beet. Journal of Agricultural Research, 71, 423–440.

Pruitt, K.D., Hanson, M.R. 1991. Transcription of the *Petunia* mitochondrial CMS-associated *Pcf* locus in male sterile and fertility-restored lines. Molecular Genetics and Genomics, 227, 348 –355.

Rohrbach, U. 1965. Beitrage zum Problem der Pollensterilitat bei *Beta voulgaris* L. Untersuchungen uber die Ontogenese des Phanotyps. Zeitschrift fur Pflanzeuchtung, 52, 105–104.

Schmitz-Linnerweber, C., Small, I. 2008. Pentatricopeptide repeat proteins, a socket set for organelle gene expression. Trends in Plant Science, 13, 663–670.

Schnable, P.S., Wise, R.P. 1998. The molecular basis of cytoplasmic male sterility and fertility restoration. Trends in Plant Science, 3, 175–180.

Scoles, G.J., Evans, L.E. 1979. Pollen development in male-fertile and cytoplasmic male-sterile rye. Canadian Journal of Botany, 57, 2782–2790.

Sofi, P.A., Rather, A.G., Wani, S.A. 2007. Genetic and molecular basis of cytoplasmic male sterility in maize. Communications in Biometry and Crop Science, 2, 49–60.

Song, J., Hedgcoth, C. 1994. A chimeric gene (orf256) is expressed as protein only in cytoplasmic male-sterile lines of wheat. Plant Molecular Biology, 26, 535–539.

Stojałowski, S., Orłowska, M., Bienias, A., Myśków, B., Tomczak, P., Wesołowski, W., Szklarczyk, M., Brukwiński, W., Banaszak, K., Hanek, M., Krysztrofik, R., Zając, M. 2019. Poszukiwanie wspólnych mechanizmów dziedziczenia płodności roślin z cytoplazmą CMS-C oraz cytoplazmą CMS-Pampa. Biuletyn Instytutu Hodowli i Aklimatyzacji Roślin, 286, 151–154.

Święcicki, W.K., Surma, M., Koziara, W., Skrzypczak, G., Szukała, J., Bartkowiak-Broda, I., Zimny, J., Banaszak, Z., Marciniak, K. 2011. Nowoczesne technologie w produkcji roślinnej – przyjazne dla człowieka i środowiska. Polish Journal of Agronomy, 7, 102–112.

Tan, Y., Xu, X., Wang, C., Cheng, G., Li, S., Liu, X. 2015. Molecular characterization and application of a novel cytoplasmic male sterility-associated mitochondrial sequence in rice. BMC Medical Genetics, 16, 45.

Tsaftaris, A.S. 1995. Molecular aspects of heterosis in plants. Physioligia Plantarum, 94, 362–370.

Wang, Z.W., Wang, C., Gao., L., Mei S.Y., Zhou, Y., Xiang, C.P., Wang, T. 2013. Heterozygous alleles restore male fertility to cytoplasmic male-sterile radish (*Raphanus sativus* L.), a case of overdominance. Journal of Experimental Botany, 64, 2041–2048.

Wasiak, M. 2019. Genetyczne podstawy cytoplazmatyczno-jądrowej męskiej sterylności (CMS) u roślin oraz jej wykorzystanie w hodowli. Agronomy Science, 74, 15–30.

Wolko, J., Dobrzycka, A., Bosianowski, J. 2019. Ocena efektu heterozji cech struktury plonu mieszańców pojedynczych i mieszańców trójliniowych rzepaku (*Brassica napus L.*). Biuletyn Instytutu Hodowli i Aklimatyzacji Roślin, 287, 21–22.

Zhiwen, C., Nan, Z., Shuangshuang, L., Corrinne, E. G., Hushuai, N., Wendel, J.F., Jinping, H. 2017. Plant mitochondrial genome evolution and cytoplasmic male sterility. Critical Reviews in Plant Sciences, 36, 55–69.