Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

ISSN 2544-2147

Publisher:
Fundacja na rzecz promocji nauki i rozwoju TYGIEL
ul. Głowackiego 35/348, 20-060 Lublin
www.fundacja-tygiel.pl

Table of Contents

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

The experience on coronavirus disease 2019 and lung cancer from Regional Center of Pulmonology in Bydgoszcz

Grzegorz Przybylski[1*], Marta Pilaczyńska-Cemel[1], Anna Kozanecka[1]

[1] Department of Respiratory Medicine and Tuberculosis, Nicolaus Copernicus University in Torun, Collegium Medicum in Bydgoszcz

[*]Corresponding author: Dr hab. Grzegorz Przybylski, Prof UMK, Departament of Respiratory Medicine and Tuberculosis, Nicolaus Copernicus University in Torun, Collegium Medicum in Bydgoszcz, Seminaryjna Street 1, 85-326 Bydgoszcz, Phone number: 052-3256781, fax: 052-3256606, Mobile phone: 602-573638

e-mail: gprzybylski@cm.umk.pl

ABSTRACT

In December 2019, an outbreak of 2019 novel coronavirus disease (COVID-19) occurred in Wuhan, Hubei, which has been linked to the severe adult respiratory syndrome coronavirus 2 (SARS-CoV-2). The outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) – related disease (coronavirus disease 2019 [COVID-19]) has spread rapidly to a pandemic proportion. It is characterized by rapid human-to-human transmission from droplet contamination. As of 21May 2020, 20143 COVID-19 cases and 972 deaths have been reported in Poland, representing a global share of 0,41% and 0,29% for incidence and mortality, respectively. We extracted data from the World Health Organization's progress reports. In addition, we retrieved clinical data on patients with cancer and with confirmed COVID-19 in Regional Center of Pulmonology in Bydgoszcz. Here, we report the incidence and outcomes of SARS-CoV-2 infection in cancer patients who were treated at our institution.We reviewed the medical records, including demographic, clinical, and treatment data of 23 patients who were admitted to the Regional Center of Pulmonology, from March 25, 2020, to May 21, 2020 (data cutoff date). COVID-19 pneumonia was diagnosed based on the updated COVID-19 Diagnostic Criteria. Outcomes of COVID-19 among patients with lung cancer were reported and was confirmed in 6 cases (6/23). The median age of infected patients was 69 years (range, 51 to 92 years); 4 of 6 patients (66.7%) were older than 60 years. Cancer has been reported as a major risk factor for adverse outcomes of and death from COVID-19.

Keywords: Cancer, COVID-19, SARS-CoV-2, Coronavirus

INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by the newly identified strain of the coronavirus family severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has rapidly evolved into a worldwide pandemic and caused a public health emergency of major international concern (Shi, Han et al. 2020, Chen, Zhou et al. 2020).

In December 2019, an outbreak of respiratory disease caused by a novel coronavirus was first detected in China and has now spread to more than 150 countries (Woldometer, 2020). The virus was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has a phylogenetic similarity to SARS-CoV-1 that caused the SARS pandemic in 2002 (Guan, Ni, 2020). This new type of respiratory illness is characterized by rapid human-to-human transmission, having achieved pandemic spread (Yu, Ouyang et al. 2020). There are currently no therapeutics or vaccines available and, presumably, no pre-existing immunity in the population.

With the World Health Organization (WHO) declaring the novel coronavirus outbreak a pandemic, focus is needed on the impact of this rapidly spreading viral infection on cancer patients (Anderson, Heesterbeek et al. 2020, WHO Director, 2020, WHO, Coronavirus, 2020). Patients with cancer are more susceptible to infection than individuals without cancer, because malignancy and anticancer therapy result in an immunosuppressive state (Liang, Guan 2020). In a retrospective study during the 2009 influenza A (H1N1) virus pandemic, the cancer patient population was at higher incidence of pneumonia (66%) and 30-day mortality (18.5%) compared with the general population (Dignani, Costantini et al. 2014) 10. A recent small case series study that evaluated SARS-CoV-2 in cancer patients found that patients with cancer had worse outcomes from SARS-CoV-2 than other individuals without cancer (Liang, Guan et al. 2020).

This comprehensive reallocation of health resources is of particular concern in patients such as those with underlying chronic diseases, including cancer. In this context, the threat of COVID-19 infection might also factor into decision making – a role which could possibly be lessened by knowledge of the COVID-19 status of patients suitable for anticancer therapy (Ueda, Martins et al.2020). This already dismal scenario seems to be even more severe for patients with lung cancer because of the high risk of interference of COVID-19 with their effective

diagnostic and therapeutic management by treating physicians. Limited studies and research regarding preparedness plans for the patients with during an infectious pandemic exist (Battershill, 2006, ASCO, 2020). In this review, we aim specifically to address challenges associated patients with COVID-19 and concomitant lung cancer during the COVID-19 pandemic.

MATERIALS AND METHODS

PATIENTS

We reviewed the medical records, including demographic, clinical, and treatment data of 23 patients who were admitted to the Regional Center of Pulmonology, from March 25, 2020, to May 21, 2020 (data cutoff date). COVID-19 pneumonia was diagnosed based on the updated COVID-19 Diagnostic Criteria.

We had patients with pneumonia of unknown cause, which was identified as SARS-CoV-2 soon after.

In this program, all consecutive patients with confirmed COVID-19 admitted to the Regional Center of Pulmonology in Bydgoszcz from March 25, 2020, to May 21, 2020 were enrolled. All patients with COVID-19 enrolled in this study were diagnosed and admitted in accordance with the guideline of the polish recommendations (Flisiak, Horban et al. 2020, Zalecenia, 2020).

The final date of follow-up was May 21 2020.

Of these 23 patients with Covid-19, 6 confirmed lung cancer, which was diagnosed in four patients during current hospitalization, in two with this diagnosis established earlier.

DATA COLLECTION

We reviewed clinical charts, nursing records, laboratory results for all patients.

Epidemiological, clinical, imaging, and serological records and treatment and out-comes data were collected from the electronic medical network of hospital.

REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) TESTS

The confirmation of COVID-19 is achieved by RT-PCR detection of throat swab samples of suspected patients. by the Polish recommendation (Zalecenia, 2020).

STATISTICAL ANALYSIS

Categorical variables were reported as number and percentages, and comparisons between groups were made using the Chi-squared test. Nominal two-tailed statistical significance was set at 0.05. All analyses were performed using programmes Statistica and Excel.

RESULTS

The enrolled 23 patients were all confirmed infected with SARS-CoV-2 with PCR tests of throat swabs. The median age of the patients was 66,6 years (35.0-92.0) (tab. 1). Median age were similiar in both group. The median age of lung cancer group was 64 years, whereas the median age without lung cancer group was 67 years.

9 patients (39%) were men, and 14 patients (61%) were women. In group patients with lung cancer domniated women (83%). Of the 23 patients, almost all had comorbidities (21 [91%]), including malignancy (6 [26%]), hypertension (9 [39%], cardiovascular disease (8 [35%]), and diabetes (7 [30%]), and so forth. Patients of malignancy group showed more underlying comorbidities when compared non malignancy group, such as hypertension (4 [67%] vs 5 [29%], $P < 0,001$). The incidence of other comorbidities was comparable in both groups.

Table 1. Characteristics and Symptoms of Patients Infected With SARS-CoV-2

	All Patients (N = 23)	**With lung cancer (n = 6)**	**Without Lung cancer (n = 17)**	***P* Value**
Age (y)	66,6 (35-92)	64,0 (51- 92)	67 (35 - 90)	NS
Sex				
Male	9 (39%)	1 (17%)	8 (47%)	<.001
Female	14 (61%)	5 (83%)	9 (53%)	
Comorbidity	21 (91%)	6 (100%)	15 (88%)	NS
Malignancy	**6 (26%)**	**6 (100%)**	**0 (0%)**	**<.001**
Hypertension	9 (39%)	4 (67%)	5 (29%)	<.001
Cardiovascular disease	8 (35%)	3 (50%)	9 (53%)	NS
Diabetes	7 (30%)	2 (33%)	6 (35%)	NS
Chronic obstructive Pulmonary disease	4 (17%)	2 (33%)	4 (24%)	NS
hepatopathy	1 (4%)	0 (0%)	1 (6%)	<.001
Fever at onset of illness	17 (74%)	4(67%)	13 (76%)	NS
Symptoms at onset of illness				
Cough	20 (87%)	5 (83%)	15 (88%)	NS
Dyspnea	20 (87%)	5 (83%)	15 (88%)	NS
Fatigue	18 (78%)	3 (50%)	15(88%)	.499
Sputum production	11 (48%)	3 (50%)	8 (47%)	NS
Chest pain	6 (26%)	3 (50%)	3 (18%)	<.001
Oppression in chest	6 (26%)	4 (67%)	2 (12%)	<.001
Anorexia	4 (17%)	3 (50%)	1 (6%)	<.001
Myalgia	4 (17%)	1 (17%)	3 (18%)	NS
Headache	3 (13%)	1(17%)	2 (12%)	NS
Palpitation	3 (13%)	1 (17%)	2 (12%)	NS
Diarrhea	2 (9%)	1(17%)	1 (6%)	NS
Vomiting	2 (9%)	1 (17%)	1 (6%)	NS

The most common clinical feature at the onset of illness was fever (17 [76%]). Other common clinical manifestations included cough (20 [87%]), dyspnea (20 [87%]), and fatigue (18 [78%]). Less common symptoms were sputum production, oppression in chest, dyspnea, diarrhea, headache, and so forth (tab. 1). Compared with the non lung cancer group, patients of the lung cancer group tend to show more frequency of chest pain ,oppresion in chest and anorexia. The frequency of these symptoms among patients with lung cancer can also be explained by the advancement of cancer. The blood counts of patients with lung cancer on admission showed decrease in white blood cells, neutrophils and lymphocytes concurrently to patients of non lung cancer group. The patients with lung cancer showed increase in Lactate dehydrogenase and decrease of aspartate aminotransferase, C-reactive protein compare to patient without lung cancer (tab. 2).

Table 2. Laboratory Records of Patients Infected With SARS-CoV-2 on Admission

	All Patients (N = 23)	**With lung cancer (n = 6)**	**Without Lung cancer (n = 17)**	***P* Value**
White blood cell count, $\times 10^9$/L	3.82(2.98-3.57)	3.57(2.96-4.93)	6.52 (4.30-7.73)	.006
Neutrophil count, $\times 10^9$/L	2.35(1.62-367)	2.16(1.60-2.70)	5.24 (2.90-6.44)	<.001
Lymphocyte count, $\times 10^9$/L	1.15 (0.82-1.46)	0.61 (0.37-1.00)	1.19 (0.95-1.46)	.002
Monocyte count, $\times 10^9$/L	0.31 (0.23-0.44)	0.31 (0.24-0.46)	0.27 (0.14-0.41)	NS
Eosinophil count, $\times 10^9$/L	0.01 (0.00-0.02)	0.01 (0.00-0.02)	0.00 (0.00-0.01)	NS
Hemoglobin, g/L	130.00 (118.00-140.00)	131.00 (121.00-141.00)	128.00 (117.00-136.00)	NS
Platelet count, $\times 10^9$/L	171.00 (142.00-211.00)	172.00 (138.00-206.00)	167.00 (144.00-215.00)	NS
Alanine aminotransferase, U/L	25.00 (17.00-40.00)	24.00 (16.00-40.00)	31.50 (23.00-52.00)	NS
Aspartate aminotransferase, U/L	28.00 (22.00-42.00)	26.00 (21.00-39.00)	40.50 (24.00-62.00)	.03
Lactate dehydrogenase, U/L	224.00 (183.00-291.00)	517.50 (267.00-549.00)	207.00 (181.00-274.00)	.001
C-reactive protein, mg/L	13.20 (6.78-49.00)	11.30 (6.53-26.30)	81.55 (48.85-105.90)	<.001
Procalcitonin, µg/L	0.13 (0.13-0.15)	0.13 (0.13-0.15)	0.13 (0.13-0.15)	NS

So they were not enrolled in the analysis of treatment and prognosis in table 3. The median time from onset of symptoms to admission was 6.0 days. The all of patients needed oxygen suport. All patients received in accordance with Polish recom-mendations. Death occurred signi-ficantly more frequently in the group of patients with lung cancer.

Table 3. Treatments and Outcomes of Patients Infected With SARS-CoV-2

	All Patients (N = 23)	**With lung cancer (n = 6)**	**Without Lung cancer (n = 17)**	***P* Value**
Onset of symptom to admission	6.0 (4.0-9.0)	6.0 (4.0-9.0)	7.0 (4.0-9.0)	NS
Oxygen support	23 (100%)	6 (100%)	17 (100.0%)	NS
Death	7 (30%)	3 (50%)	4 (24%)	<.001

DISCUSSION

Our experience with these 23 patients confirms that COVID-19 is a kind of epidemic pneumonia with fever, dry cough, and fatigue as the most common onset symptoms. Most patients have mild manifestations and excellent prognosis. However, in our group of patients with lung cancer and COVID, the prognosis was significantly worse.

Cancer patients with SARS-CoV-2 infection may have increased morbidity and mortality from COVID-19 than noncancer patients with SARS-CoV-2 infection. Accumulating evidence suggests that cancer patients are at higher risk of COVID-19 infection and more likely to have higher morbidity and mortality than the general population. In a study with a total of 1,524 patients with cancer, cancer patients had a twofold increased risk of COVID-19 infection when compared with the general population (Yu, Ouyang et al. 2020). In another series from a single institution in the Wuhan region, the infection rate of SARS-CoV-2 in patients with cancer was 0.79% (95% CI = 0.3-1.2), which was higher than the cumulative incidence of all diagnosed COVID-19 cases that was reported over the same time period (0.37%, 41,152/ 11,081,000 cases, data cutoff on February 17, 2020) (Yu,Ouyang et al. 2020). The Chinese

Center for Disease Control and Prevention described the epidemiological characteristics of 72,314 COVID-19 cases in mainland China as of February 11, 2020. They reported that 107 patients (0.5%) had cancer, and 6 of them died. The case fatality was 5.6%, which is higher than the overall reported case fatality (2.3%) from COVID-19 (Novel, 2020). Similarly, the WHO-China Joint Mission on COVID-19 identified significantly higher case fatality amongst patients with pre-existing malignancy (7.6%) compared with patients without comorbid conditions (1.4%) (Report,2020). In the series by Liang et al., cancer was associated with higher risk of severe events (i.e., admission to the intensive care unit, invasive ventilation, or death seen in 7 of 18 patients [39%] with cancer vs. 124 of 1,572 patients [8%] without cancer; p = .0003) (Liang, Guan et al. 2020). These findings have been corroborated internationally, as an Italian study assessing the case fatality of COVID-19 found that amongst 355 patients who died and underwent detailed chart review, 72 (20.3%) had active cancer (Onder, Rezza et al. 2020). While these analyses are preliminary and require validation from larger international cohorts, several factors could account for an elevated risk for acquiring COVID-19 and consequent complications amongst cancer patients, including frequent hospital visits and admissions, immunocom-promised state, advanced age, and poor functional status (Yu, Ouyang et al. 2020). In our study the small study sample size, patients in cancer group characterized similiar results a higher death rate and more severe course.

While having cancer and receiving certain cancer therapies remain plausible risk factors for both contracting SARS-CoV-2 infections and having more severe COVID-19 outcomes, existing data do not yet answer these questions. Notably, the early publi-cations in this area include data from a very small number of patients, but have nonetheless had substantial effects. The cited study by Liang et al. is often used as justification for ‘a possible increased risk’ associated with chemotherapy, despite only two patients in this analysis receiving systemic chemotherapy within the month before COVID-19 diagnosis. Data from Yu et al. have been used to support universal screening of patients with lung cancer for SARS-CoV-2 infections, even though this cohort contained seven patients with lung cancer, six of whom did not have laboratory-confirmed COVID-19 (Yu, Ouyang, 2020). It is difficult to imagine any other context in which data from such small, highly selected, and often flawed case series would be published in major journals and have such a substantial influence on clinical practice and policy.

Patients with lung cancer usually have compromised lung function with associated dyspnea, cough, and polypnea. They might be at higher risk of severe forms of COVID-19 infection due to decreased pulmonary function. In the aforementioned studies that evaluated COVID-19 in a small cohort of patients with cancer, the investigators found that lung cancer was the most frequent type of malignancy in this cohort of COVID-19 – infected patients (five [28%] of 18 patients). Whether this reflects a true increase in the susceptibly of the lung cancer population to SARS-CoV-2 infection or is simply due to the fact that lung cancer is the most cancer in China is yet to be determined (Feng, Zong, 2019). Lastly, other potential causes for the respiratory deterioration of NSCLC patients, which could mimic COVID-19 symptoms, must be considered; these may include obstructive pneumonia, pleural or pericardial effusion, pulmonary embolism, and heart failure; therefore, rapid access to SARS-CoV-2 assays is of utmost importance. Postponing anticancer treatment should be considered according to the patient's risk of infection, performance, and clinical status. Currently, there are no clinical trials to examine the safety and efficacy of antiviral prophylaxis for SARS-CoV-2 in cancer patients.

COVID-19 and the cancer patient is based on the latest information and knowledge available to the medical community at this time. As the COVID-19 pandemic continues to evolve and unfold, it is likely that the health care community will be faced with additional, yet unknown challenges. It is imperative that we stay abreast of all deve-lopments with COVID-19 to provide our most vulnerable COVID-19 and the cancer patient with the care needed for their best chance of to optimal health care.

As the size of this cohort is limited, the statistical analysis results should be interpreted with caution, and the P value without statistical significance does not necessarily reflect the exact situation of the whole population. Larger sample size of clinical studies is needed to

elucidate the epidemiology, clinical characteristics and prognostic factors of COVID-19. Moreover, due to the outcomes data of patients have shown that, the prognosis comparation between the lung cancer group and the nonlung cancer group is much worse.

CONCLUSIONS

The study groups infected with SARS-CoV-2 (with and without lung cancer) differed significantly in several aspects. In group of patients with lung cancer dominated women. Patients of malignancy group showed more underlying comorbidities, such as hypertension and hepatopathy. Patients of the lung cancer group tend to show more frequency of chest pain, oppression in chest and anorexia. Another difference was blood laboratory analysis. The lung cancer group showed decrease in white blood cells, neutrophils, lymphocytes, aspartate aminotransferase and C-reactive protein compared to the non-lung cancer group. The most important difference is the mortality rate in the studied groups. Death occurred significantly more frequently in the group of malig-nancy patients.

Considering the above aspects, it is safe to say that the coexistence of COVID-19 and lung cancer may change the diagnosis, treatment and prognosis of both diseases.

SUMMARY

Our review on COVID-19 and the cancer patient is based on the latest information and knowledge available to the medical community at this time. As the COVID-19 pandemic continues to evolve and unfold, it is likely that the health care community will be faced with additional, yet unknown challenges. Lung cancer associated with COVID-19 is a disease that significantly worsens prognosis. Therefore, patients should be isolated and protected against potentially possible coronavirus disease infection (COVID-19). Early diagnosis, timely isolation, and appropriate treatment are the keys in fighting this infection.

References

Anderson R.M., Heesterbeek H., Don Klinkenberg, Déirdre Hollingsworth T., **How will country-based mitigation measures influence the course of the COVID-19 epidemic?,** Lancet, 2020; 395: 931-934.

ASCO COVID-19 Clinical Oncology Frequently Asked Questions (FAQs), Last updated – March 12, 2020. Available at https://www.asco.org/sites/new-www.asco.org/files/content-files/blog-release/pdf/COVID-19-Clinical%20Oncology-FAQs-3-12-2020.pdf. Accessed March 14, 2020.

Battershill P.M.. **Influenza pandemic planning for cancer patients**, Curr Oncol, 2006; 13: 119-120.

Chen N., Zhou M., Dong X., **Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study**, Lancet, 2020;395:507-513.

Dignani M.C., Costantini P., Salgueira C., Jordán R., Guerrini G., Valledor A., et al. **Pandemic 2009 influenza A (H1N1) virus infection in cancer and hematopoietic stem cell transplant recipients; a multicenter observational study,** F1000Res, 2014; 3: 221.

Feng R.M., Zong Y.N., Cao S.M., Xu R.H., **Current cancer situation in china: Good or bad news from the 2018 global cancer statistics?,** Cancer Commun (Lond), 2019; 39: 22.

Flisiak R., Horban A., Jaroszewicz J., Kozielewicz D., Pawłowska M., Parczewski M., et al. **Management of SARS-CoV-2 infection: recommendations of the Polish Association of Epidemiologists and Infectiologists as of March 31, 2020**, Pol Arch Intern Med., 2020; 130: 352-357. doi:10.20452/pamw.15270.

Guan W.J., Ni Z.Y., Hu Y., Liang W.H., Ou Ch., He J., et al. **Clinical characteristics of coronavirus disease 2019 in China,** N Engl J Med., 2020 Feb 28 : NEJMoa2002032. Published online 2020 Feb 28. doi: 10.1056/NEJMoa2002032.

Liang W., Guan W., Chen R., **Cancer patients in SARS-CoV-2 infection: A nationwide analysis in China,** Lancet Oncol, 2020; 21: 335-337.

Novel Coronavirus Pneumonia Emergency Response Epidemiology Team. The epidemiological characteristics of an outbreak of 2019 novel coronavirus diseases (COVID-19) in China [in Chinese], Zhonghua Liu Xing Bing Xue Za Zhi 2020;41:145-151.

Onder G, Rezza G, Brusaferro S., **Case-fatality rate and characteristics of patients dying in relation to COVID-19 in Italy**, JAMA, 2020 [Epub ahead of print].

Report of the WHO–China Joint Mission on Coronavirus Disease 2019 (COVID-19), Available at https://www.Who.Int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf. Accessed March 12, 2020.

Shi H., Han X., Jiang N., **Radiological findings from 81 patients with COVID-19 pneumonia in Wuhan, China: a descriptive study,** Lancet Infect Dis, 2020; 20:425-434.

Ueda M., Martins R., Hendrie P.C., **Managing cancer care during the COVID-19 pandemic: agility and collaboration toward a common goal,** J Natl Compr Canc Netw. 2020 doi: 10.6004/jnccn.2020.7560. published online March 20.

World Health Organization. WHO Director-General's opening remarks at the media briefing on COVID-19, March 11, 2020. Available at https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—11-march-2020. Accessed March 11, 2020.

World Health Organization. Coronavirus disease (COVID-19) Pandemic, Available at https://www.who.int/emergencies/diseases/novel-coronavirus-2019, Accessed March 11, 2020.

Worldometer. COVID-19 Coronovirus Pandemic, Available at https://www.worldometers.info/coronavirus/#countries. Accessed March 16, 2020.

Yu J., Ouyang W., Chua M.L.K., **SARS-CoV-2 transmission in cancer patients of a tertiary hospital in Wuhan,** JAMA Oncol, 2020 [Epub ahead of print].

Zalecenia w COVID-19 Polskie zalecenia diagnostyczno-terapeutyczne oraz organizacyjne w zakresie opieki nad osobami zakażonymi lub narażonymi na zakażenie SARS-CoV-2, Agencja Oceny Technologii Medycznych i Taryfikacji z 25 kwietnia 2020 r.

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

Photodynamic therapy as alternative therapy for prostate cancer and colorectal carcinoma as well as an antimicrobial treatment – a systematic review

Hanna Doleżych-Teister[1], Katarzyna Komoniewska[1], Iwona Wilk[1], Gayane Martirosian[1]

Katedra i Zakład Mikrobiologii lekarskiej, Wydział Lekarski w Katowicach Śląskiego Uniwersytetu Medycznego

ABSTRACT

INTRODUCTION: Photodynamic therapy (PDT) is used in many different oncologic fields. This study includes PDT in prostate cancer (the second most common neoplasm after lung cancer in men in the European Union), colorectal cancer (third in men, second in women after breast cancer,) as well as a therapy for infectious problems accompanying these oncologic diseases. This review aims to give a general overview of the PDT application to those diseases in the field of clinical trials to emphasize its curative, and insufficiently exploited potential.

MATERIAL AND METHODS: Literature on PDT for cancer treatment with the following medical subject headings search terms: colorectal cancer, prostate cancer , photodynamic therapy, clinical trials, antimicrobial photodynamic therapy and bacterial infection was reviewed. The articles were selected by their relevance to the topic.

RESULTS: There are many positive and promising trial results from I to II/III phase for the use of PDT in colorectal cancer both in less advanced tumors as well as in the palliative therapy of advanced lesions. As well in prostatic cancer some studies had evaluated a negative biopsy rate after PDT. The most common adverse events were haematuria, erectile dysfunction, and dysuria. It also has been proven that PDT can be used as an adjuvant for the treatment of infectious diseases. The use of photosensitizer methylene blue, toluidine blue O (TBO), indocyanine green with light diode laser centered at (630±10 nm) and (650±10 nm) wavelengths have been shown to have significant results for the treatment of infectious diseases because of bactericidal properties. In the skin diseases, a PDT has been tested with promising results in different infections. Therefore, it is presented as a possible treatment option against antibiotic resistant microbes.

CONCLUDING REMARKS: PDT seems to be a safe and a feasible treatment option for colorectal cancer. Theoretical assumptions confirmed by many results of preclinical studies taking into consideration an increasing number of analyzed clinical trials, should lead to the development of optimized standards by using PDT in colorectal cancer treatment.

Review results show that PDT for patients with prostate cancer can be considered as effective based on single-arm clinical trials. Meanwhile, this study reveals that there are not only low levels of side effect rates but also insignificant effect on both urinary and erectile function.

These findings also suggest that a PDT can be an efficient method in the treatment of localized and superficial infections.

INTRODUCTION

Prostate cancer is a major cause of disease and mortality among men, and each year 1.6 million men are diagnosed with and 366,000 men die of prostate cancer (Siegel et al. 2017). Current treatment options for men with localized PC include active surveillance and radical therapy. The optimal treatment should provide cancer control with only few side effects (Kasivisvanathan et al. 2013). Radical prostatectomy is the first-line therapy for patients with prostate cancer. However, considering the morbidity and prognosis, the risks and efficacy of radical therapy were frequently not identified (Kawczyk-Krupka et al. 2015).Photodynamic therapy (PDT) is one of the focal therapies used for prostate cancer. This treatment modality uses laser of a specific wavelength in the presence of oxygen to activate a photosensitizing medication. This process causes localized cell death or tissue necrosis (Zhu et al. 2005). PDT has been used for neoplasms including cancers of lung, head and neck, pancreas, esophagus, and bladder (Gheewala et al. 2017). Since the 1990s, studies of PDT for localized PC have been reported (Windahl et al.1990).

Colorectal cancer is the third most commonly diagnosed cancer and fourth leading cause of cancer-related deaths, it accounts for almost 10% of cancer-related deaths in Western countries. This cancer is associated with a high risk of metastasis and recurrence despite an increased availability of diagnostic and therapeutic strategies. To treat patients with colorectal cancer, an approach that selectively targets cancer cells without damaging normal cells and which minimizes the risk of perforating the intestinal barrier is needed (Kawczyk-Krupka et al. 2015). Early detection of precan-cerous polyps may prevent the onset of colorectal neoplasm or increase the chances of a successful treatment. Currently, several different screening tests are available including endoscopy, stool-based blood tests, and radiology-

based tests. Colorectal cancer is commonly treated by tumor resection, as chemotherapy and radiation have proven to be less effective, especially if the tumor has metastasized. Resistance to therapies occurs in almost all patients with colorectal cancer, especially in those with metastatic tumors. Cancer stem cells have the ability to self-renew, and their slow rate of cycling enhances resistance to treatment and risk of tumor recurrence. Most metastatic tumors are unable to be surgically removed, thus creating a need for treatment moda-lities that target cancers directly and can destroy cancer stem cells. Photodynamic therapy involves a photosensitizer that when exposed to a light source of a particular wavelength becomes excited and produces a form of oxygen that kills cancer cells. Photodynamic therapy is currently being investigated as a treatment modality for colorectal cancer, and new studies are exploring enhancing photodynamic therapy efficacy with the aid of drug carriers and immune conjugates. These modifications could prove effective in targeting cancer stem cells that are thought to be resistant to photodynamic therapy. In order for photodynamic therapy to be an effective treatment in colorectal cancer, it requires treatment of both primary tumors and the metastatic secondary disease that is caused by colon cancer stem cells. This review focuses on current photodynamic therapy treatments available for colorectal cancer and highlights proposed actively targeted photosynthetic drug uptake mechanisms specifically mediated towards colon cancer stem cells, as well as identify the gaps in research which need to be investigated in order to develop a combinative targeted photodynamic therapy regime that can effectively control colorectal cancer primary and metastatic tumor growth by eliminating colon cancer stem cells.

PDT is considered as a complementary therapy aimed at preventing tumor recurrence after surgical resection of colorectal cancer (Shishkova et al. 2013) making a suitable approach for continuous removal of small fractions of tumors (Barr et al.1990). It has also been reported that PDT is an effective alternative treatment for drug-resistant colorectal cancer (Halaburková et al. 2017). PDT uses a modality-based photo-sensitizer, which selectively affects cancer cells, using excitation and light-absorption in the presence of oxygen to produce a high con-centration of reactive oxygen species (ROS), such as singlet oxygen and other free radicals (Kwiatkowski et al. 2018). The resulting damage cannot be overcome by the antioxidant system to protect the cell from oxidative damage, leading to necrosis, apoptosis, or autophagy of the target cell and tissue (Broekgaarden et al. 2015).

The broad antibiotic resistance of hospital pathogens is inducing human morbidity and mortality, as well as hospital costs (Geralde et al. 2017). The antibiotic resistant strains of bacteria imply the demand for alternative treatments for infectious disease. One strategy that may lead to improved antimicrobial treat-ment is the application of anti-microbial photo-dynamic therapy (aPDT) (Nakonechny et al. 2010). a PDT involves the use of a chemical photosensitizer or a nontoxic photoactivatable dye, visible light, and reactive oxygen. The therapy is based on the energy (absorbed as light via the intracellular photosensitizer) trans-ferred to the oxygen molecules producing extremely reactive mediation, such as singlet oxygen and superoxide, that are noxious to the cells(Mahmoudi et al. 2018). The spread of multi-resistant bacterial strains is one of the most worrying threats to public health in recent years and has arisen due to the excessive use of antibiotics (Fotinos et al. 2008). In view of the prediction of the "end of the antibiotic era" (Nordmann et al. 2011), antimicrobial photo-dynamic therapy is starting to be considered as a promising alternative approach to resistant infections and has the further advantage of not leading to the selection of resistant strains (Sperandio et al. 2013).Antimicrobial PDT is particularly good for dental (Sprendido et al. 2013, Dai et al., 2009) and dermatological (Choudhary et al. 2009) applications, involving the light irradiation of a tissue containing microorganisms that were previously exposed to a photosensitizing dye (PS). This PS should be able to generate reactive oxygen species (ROS) in the presence of light and oxygen (Kawczyk-Krupa et al. 2015). In order to be suitable for antimicrobial PDT, the ideal PS should possess low levels of dark toxicity and the presence of absorption bands in the so-called optical window (600-900 nm) for sufficient tissue penetration of light (Sharma et al. 2011). The PS should have a high yield of excited electronic triplet state and of singlet oxygen (Sharma et al. 2011). The PS should be excited by visible light of the correct wavelength

(wavelength absorbed by the PS) to enter a long-lived triplet state. This particular state of the PS can then interact with molecular oxygen by energy transfer or by electron transfer processes. (Castano et al. 2004). After being excited to the short-lived singlet state the PS can lose energy by fluorescence, heat conversion or can undergo intersystem crossing to the long-lived triplet state. In case the PS is a fullerene, energy loss by fluorescence is negligible, and in the absence of oxygen fullerene triplet states lose energy by phosphorescence (Sharma et al. 2011). mAntimicrobial PS should be able to kill multiple classes of microbial cells at relatively low concentrations and low fluences of light. The PS should also be reasonably nontoxic in the dark and should show selectivity for microbial cells over host mammalian cells. In fact, the microbial uptake process of PS with cationic substituents such as quaternary ammonium groups is rapid when compared to the uptake of these PS by host mammalian cells, which slowly occurs over time (Soncin et al. 2002). Therefore, if light is delivered soon after applying the PS to the infected area, microbial cells can be killed without causing harm to the host tissue (Sperandio et al. 2013).

BACTERIA AND COLORECTAL CANCER

The growing evidence suggests that bacteria can play an important role in the initiation and progression of colorectal neoplasm by inducing chronic inflammation and by the release of carcinogenic metabolites (Marchesi et al. 2011). Some of the bacteria most commonly associated with colon cancer include: *Fuso-bacteium nucleatum* (Shang et al. 2018), *Bacteroides fragilis,* and *Escherichia coli* (Dejea et al. 2018). Inflammatory bowel disease (IBD)-associated colorectal cancer is a classic example of an inflam-mation-induced cancer (Choi et al,2017).

The intestinal tract acts as a reservoir for various microbial species, together known as the intestinal microbiota (Thursby et al. 2017). In the last two decades, strong evidence has indicated that the gut microbiota plays a critical role in providing nutrients to the gut mucosa, in the development of the mucosal immune system, and in preventing pathogen colonization (Kang et al. 2017). The mucosal immune system's main tasks are to mount an immune response against pathogenic microbes and to maintain tolerance against food and commensal microbial antigens. Loss of tolerance to commensal enteric microorganisms finally leads to uncontrolled chronic inflammation like that seen in patients with IBD. In addition, since the colon carries 10^{12} bacteria/mL, compared to 10^{2} bacteria/mL in the small intestine, the colon presents a 12-fold higher risk of developing tumors (Tjalsma et al. 2012). Recent findings suggest that microbes such as *F. nucleatum,* (Kostic et al. 2013) enterotoxigenic *B. fragilis*, *Streptococcus bovis*, *E. coli*, and *Klebsiella pneumoniae* can play an important role in colon cancer development (Antonic et al. 2013). These gut-associated bacteria can increase the risk of tumor malignancy by several mechanisms including secretion of mutagenetic metabolites and promoting inflammation. Lately, a link between gut bacteria and the efficacy of anti-PD-1 immunotherapy has also been uncovered (Routy et al. 2018). Collectively, these studies show important link between bacteria and colorectal cancer pathogenesis. Although several studies have demonstrated the involvement of micro-bes in IBD and cancer progression, the mechanistic insights into how these bacteria actually lead to these conditions or their potential role in relapse of disease are yet to be discovered.

URINARY MICROBIOME IN MEN WITH PROSTATE CANCER

Studies profiling the urinary microbiome in men with and without a biopsy proven diagnosis of prostate cancer revealed that the urinary microbiota of most men is predo-minated by a single genus and notably by species of *Coryne-bacterium*, *Staphylococcus* and S*treptococcus*. While similar trends have been reported in urine samples from women, female urine samples differ in that the predominant microorganisms are *Lactobacillus* and *Gardnerella* (Pearce et al. 2014, Shrestha et al. 2018). Interestingly we identified a subset of men with predominant urine representation from *Lacto-bacillus* or *Gardnerella* species. The presence *G. vaginalis* was associated with chronic inflammation in corresponding prostate biopsies. This raises the intriguing possibility that some men may harbor urinary microbiota associated with inflammatory conditions in women (eg bacterial vaginosis – BV).

Several additional species of pro-inflammatory bacteria and/or known uropathogens were differentially represented in men with prostate cancer. Notable examples included *A. schaalii*, an emerging uropathogen of potentially under-estimated clinical significance due to difficulty with phenotypic identification (Chu et al. 2009). *A. schaalii* was found in men with and without prostate cancer but this species was included in the cluster of pro-inflammatory bacteria more prevalent in men with cancer (Shrestha et al. 2018). As mentioned, species of Ureaplasma were also differentially abundant in the urinary microbiota of men with prostate cancer. In Shresta's study, the cancer on SB samples had the highest average number of OTUs and none showed a predominance of *Corynebacterium*, *Staphylococcus* or *Streptococcus* (Shrestha et al. 2018). There were several limitations to the current studies, including the fact that all men were being seen for some indication for prostate biopsy. Although men in the benign group were biopsy negative for prostate cancer, they nevertheless represented a population with elevated PSA and were likely to have BPH and/or prostatic inflammation. Indeed, the men in the benign group had a larger average prostate TRUS volume than the men with cancer or cancer on SB, indicating prostate enlargement in this group. As BPH is also associated with chronic inflammation, (Gandaglia et al. 2013, Shrestha et al. 2018) future studies warrant an association of urinary microbiota with the presence of BPH. In addition, it is likely that a fraction of the men with a negative biopsy actually had prostate cancer because the false-negative rate of TRUS guided prostate biopsy is commonly reported to be up to 30%. Future followup studies will necessitate a true control population of men without an indication for prostate cancer to determine whether the urinary microbiome profile is unique in those without prostate disease or rather consistent with the control group in the current study (Shrestha et al. 2018). Several studies have shown that there is an increased risk of prostate cancer in men with a history of prostatitis (Shrestha et al. 2018, Cheng et al. 2010).The key hypothesis that emerged from the current study is that pro-inflammatory species that reside in the urinary tract may serve as a potential source of inciting chronic inflammation in the prostate. Ultimately establishing the link between the urinary microbiome and chronic inflammation in the prostate may be keenly important in terms of developing strategies for prostate cancer prevention.

INTESTINAL MICROBIOME AND PROSTATE CANCER RELATIONSHIP

Prostate cancer is the second leading cause of death in the United States and accounts for 1 in 5 new diagnoses in the male population (Siegel et al. 2019). The lifetime risk for prostate cancer is about 16%, with 276,000 new cases in 2018 (The Global Cancer Observatory ,2018). In Europe, the statistics are similar (Sha et al. 2020). Standard treatment for prostate cancer include androgen-based therapies; however, this treatment does not change other risk factors for prostate cancer, such as bacterial infections, environmental stimuli, or inflammatory markers. Due to prostate cancer's high prevalence, these alternate risk factors are explored in recent years (Sha et al. 2020). With an increasing under-standing of microbial effects on carcinogenesis, studies have been conducted exploring specific GI microbes and prostate cancer outcomes. The composition of intestinal microbiome may influence the metabolism of certain compounds that may be associated with increased prostate cancer risk (Sha et al. 2020). Intake of calcium in dairy products (Lampe 2011), red meat (Punnen et al. 2011), and fat (Sonn et al. 2005) have been linked to increase prostate cancer risk or progression. This may relate to the micro-biome's role in phytochemical digestion (Musso et al. 2011), dairy product digestion (Masood et al. 2011), and the generation of inflammatory molecules (Sha et al. 2020, Arthur et al. 2012), which can influence neoplastic development, not only locally in gut mucosa but also in distant locations.

Antibiotics commonly used in hospital and outpatients environment lead to microbial selection, in most cases with adverse result. A reduced diversity profile can lead to an overgrowth of bacteria that promote inflam-mation and neoplasia. Studies have shown that antibiotic usage increases likelihood of bacterial infections from *Clostridioides difficile* and methicillin-resistant *Staphylococcus aureus* (Hunter et al. 2010). These bacterial species are typically present in the GI microbiome, but are able to proliferate under conditions of microbial disruption. The association between prostate cancer risk has been investigated in the context of antibiotic exposure. Antibiotic-induced

changes in microbiota form changes in intestinal permeability, introducing risk of neoplastic changes (Tulstrup et al. 2011). Another paper shows that an antibiotic would cause a change in the bacterial diversity of the GI and induce chronic inflammation. The risk of prostate cancer increased moderately with the use of penicillins, quinolones, sulphonamides, and tetracyclines (Boursi et al. 2015). When descry-bing how the micro-biome affects distant carcinogenesis from the GI, as in the case of prostate cancer, a functional estrobiome, or enteric bacterial genes that are able to meta-bolize estrogen were postulated (Plottel et al. 2011). β-Glucuronidases and β-glucuronides are particularly important in the metabolism of estrogen by conjugation and deconjugation. Elevated estrogen levels were reported in patients with prostate cancer compared to healthy con-trols (Althuis et al. 2004). Estrogen promotes carcinogenesis by activating polycyclic aromatic hydrocarbons (PAHs) which involve the forma-tion of carcinogenic metabolites, diol epoxides and radical cations. Diol epoxides and radical cations react with DNA that can lead to cancer-promoting mutations. This estrogen mechanism is linked to Plottel's hypothesis of the estrobiome, or estrogen-metabolizing bacteria, and therefore when disturbed would cause an increase in serum estrogen (Plottel et al. 2011).

In addition to the estrogen-driven carcino-genesis hypothesis, chronic inflammation has been described to create dysbiosis and subse-quently increase cancer risk. *In vivo* studies showed that GI tract bacterial infection is sufficient to enhance prostate intraepithelial neoplasia (PIN) and microinvasive carcinoma (Poutahidis et al. 2013). Induction of neoplasia was started by the prior neutralization of inflam-matory molecules such as tumor necrosis factor α, suggesting that GI microbial-based inflam-mation plays a large role in tumor formation and progression. There are certain microbes that increase the risk of prostate cancer *in vivo*. *Campylobacter jejuni* was found to induce cell cycle arrest, chromatin fragmentation, and cell death from its toxin termed cytolethal distending toxin (Lara-Tejero et al. 2000). Clostridium was found to convert gluco-corticoids in the gut to androgens by side-chain cleavage, which could contribute to prostate cancer development (Ridlon et al. 2013). *Escherichia coli* is common in the human gut and is typically in symbiosis with the host; however, it was (Cuevas-Ramos et al. 2010) noted that *in vivo* infection of *E. coli* induced DNA damage response with signs of incomplete DNA repair. In addition, *E. coli* has been found to be associated with prostate inflammation. Mice infected with *E. coli* developed bacterial prostatitis and many deve-loped dysplastic changes; zero of the control mice developed prostate infections or inflam-mation (Elkahwaji et al. 2009).

There were studies on rectal swabs from men (rectal microbiome profiles were sequenced prior to transrectal prostate biopsy). There were significant increases in proinflammatory *Bacte-roides* and *Streptococcus* species in those diagnosed with prostate cancer (Sha et al. 2020). Inflammation may be related to neoplasia by inflicting cellular and genomic damage, trig-gering a cascade of cell repair, angiogenesis, and tissue repair on a larger level (Nakai et al. 2013). Furthermore, it has been hypo-thesized that reactive oxygen species and reactive nitrogen species are released through immune cells during times of inflammation, directly damaging cells and DNAs (De Marzo et al. 2007). This oxidative damage and cellular death is the cause of proli-ferative inflammatory atrophy, which is identified as a precursor to prostatic neoplasia and potentially adeno-carcinoma (De Marzo et al.1999).

The studies exploring the specific micro-organism and prostate cancer risk discussed above are summarized in table1 (Sha et al. 2020)

FOLIC ACID/PROBIOTIC THERAPY

What is more there were several research programmes about microbiota, folic acid meta-bolism and prostate cancer. High dietary folate intake was associated with a decre-ased risk of prostate cancer. Microbiota involved in folate production were increased in men without prostate cancer; therefore, there seems to be a difference between endo-genous folate produc-tion and folate supplementation (Liss et al. 2018). This could have implications for preven-tative medicine by encouraging men to use probiotics for natural folate production and discourage use of folate supplements.

Table 1. Studies discussed about specific gastrointestinal microbiota and prostate cancer

Study	Results	Bacteria involved
Liss et al. (2018)	Rectal swabs were taken and found an increase in *Bacteroides* and *Streptococcus* in those with prostate cancer compared to controls.	*Bacteroides, Streptococcus*
Alanee et al. (2019)	*Bacteroides* from fecal samples was highly associated with prostate cancer diagnosis.	*Bacteroides*
Golombos et al. (2018)	*Bacteroides massiliensis* was in higher relative abundance in prostate cancer cases, while *Faecalibacterium prausnitzii* and *Eubacterium rectalie* was in higher relative abundance in controls.	*B. massiliensis, F. prausnitzii, E. rectalie*
Miyake et al. (2019)	Men with more extensive prostate cancer disease (T2c-3b) had a higher rate of *Mycoplasma genitalium* infection compared to those who had benign prostate hyperplasia.	*M. genitalium*

HISTORY OF PHOTODYNAMIC THERAPY

The origin of the laser/light therapy found as an alternative treatment in medicine from ancient to contemporary time. Phototherapy trace its root back to ancient Greece, Egypt and India, however not applied for centuries (Mahmoudi et al. 2018). Ultimately, it has been rediscovered for the westen society at the outset of the 20th century by a Danish physicist, Niels Finsen. He successfully used photodynamic therapy by applying heat and light filtered through a carbon lamp for the treatment of cutaneous tubercles known as lupus vulgaris (Daniell et al.1991). The idea of necrobiosis caused by action and reaction between light and chemicals were the earliest described by Raab in Munich. He found that chemical changes in the presence of a pigment called acridine light, inducing the death of a paramecium (Raab et al.1990). Photodynamic therapy was confirmed by the Food and Drug Administration in 1999 to treat pre-cancerous skin lesions in the head and face (Lui et al.1992).

THE MECHANISM OF ACTION OF PHOTODYNAMIC THERAPY

PDT method binds the application of visible light, combined with a photosensitizer (PS) and with the oxygen (Mahmoudi et al. 2018). PDT is based on the interaction of visible light and a photosensitizer agent which under photo-activation generate short lasting cytotoxic radicals locally. After stimulation, the photosensitizer is converted from singlet to triplet state by an intersystem crossing process which, in turn, reacts with surrounding molecules to produce radical species and hydrogen peroxide, or transfers its energy to molecular oxygen to manufacture singlet oxygen. Oxygen species that are capable of eliminating target cells by oxidative stress to cell membranes and other cellular parts (Darabpour et al. 2016).

Photodynamic therapy involves the generation of ROS resulting from the interaction of photosensitizer and VIS light. However, VIS light is too weak to penetrate deep into the tissue. Also, ROS production is limited due to the hypoxic environment of tumor and the colon tissue. Furthermore, ROS production consumes most of the oxygen available to an induced hypoxic environment in the tissue, which further potentiates tumorigenesis. Talaporifin sodium (TS), a light-activated drug/photo-sensitizer, has been approved in Japan for the treatment of early-state endobronchial cancer (Wang et al. 2010). Activation of TS with a 664 nm VIS range light generates a single oxygen species, resulting in the induction of apoptotic cell death. In a Phase II trial of TS in patients with colorectal cancer and metastasis to the liver, the efficacy of the treatment depended on the number of excitation sources used to activate the drug (Kujundžić et al. 2007). This study shows that the treatment efficacy depends on the penetration of cancer tissue by the excitation light with enough photons to activate the photo-sensitizer. UCNPs with emission at UV range could be used to overcome these limitations associated with VIS light and ROS production. Deep penetrating NIR light can be used to excite the UCNPs, and the localized emission of UV can be used to kill the surrounding carcinogenic cells.

PHOTOSENSITIZERS

An optimal photosensitizer ought to have favorable structural features including physical, chemical, and biological characteristics. Many optical photosensitizers for photodynamic therapy have been tested both in vitro and in vivo. PDT photosensitizers are found in this chemical groups: porphyrins (5-aminolevulinic acid is a porphyrin precursor), chlorine and dyes such as toluidine blue O (TBO), methylene blue (MB) and Azure.

PHOTOSENSITIZERS WITH CATIONIC CHARGES

It is well known that Gram-positive bacteria can be inactivated with PDT (Sperandio et al. 2013); however Gram-negative bacteria are far more resistant to this therapy (Malik et al.1992). To overcome this limitation, besides penetrability the outer membrane with PMBN (Nitzan et al.1992) or Tris/EDTA (Bertoloni et al.1990) to allow non-cationic PS to be used, some cationic PS may also be employed.

Polycationic colours need to gain access through the outer membrane to more sensitive parts of the cell (Hamblin et al. 2002); however, the efficacy of this process depends on the size of the polycationic chain . Conjugates with eight, thirty-seven lysines and free ce6 can efficiently inactivate *Staphylococcus aureus*; but only the conjugate with thirty-seven lysines could kill *E. coli*. It is plausible that 37-lysine can interact with the outer membrane of *E. coli*, perhaps causing the loss of some LPS and rendering the remaining LPS more permeable, allowing the conjugate to penetrate. On the other hand, the 8-lysine conjugate did not provoke the same effect, which was probably due to its insufficient polycationic character (Hamblin et al. 2002).

NOVEL PHOTOSENSITIZERS FOR ANTIMICROBIAL PDT OF GRAM-NEGATIVE BACTERIA

One of the most studied groups of PS consists of porphyrin derivatives, which are described in inventions and may act as photodynamic agents, since these derivatives generate reactive oxygen forms such as singlet oxygen or oxygen free radicals when irradiated with appropriate wavelengths and in the presence of oxygen. Consequently, these compositions are suitable for curative or prophylactic treatment of several medical conditions including infections with Gram-negative cocci (e.g *Neisseria* sp.) and Gram-negative bacilli (e.g *E. coli*) (Love et al. 2011).

Another family of potent photosensitizers are the halogenated xanthenes, since they also become photoactivated upon shining visible light on the treatment site that was previously exposed to these compounds (Dees et al., 2005). These medicaments are in turn suitable for intracorporeal administration and thus were employed to achieve photodynamic therapy in human or animal tissues. In three distinct inventions, the primary component of given medicaments is a halogenated xanthene or a halogenated xanthene derivative. Furthermore, this xanthene molecule is more preferably Rose Bengal or a functional derivative of Rose Bengal (Dees et al. 2011). As it was explained before, the susceptibility of bacteria to phenothiazinium mediated PDT depends on whether the bacteria are Gram-positive or Gram-negative. New methylene blue and di methyl methylene blue, for example, were proven to be efficient at inactivating MRSA (Wainwright et al.1998). Biologically active methylene blue derivatives are also effective in deactivating a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, MRSA and fungal infections (Brown et al. 2011).

On the other hand, naturally occurring and synthetically available siderophore structures can be conjugated chemically with photoactive compounds such as chlorin to improve their penetration into bacterial cells, via microbial proteins that recognize and transport iron-loaded siderophores. In that way, PS that otherwise could not cross the cell wall and membranes can then be transported inside the bacteria (Grafe et al. 2004) allowing Gram-negative bacteria to be susceptible to this particular approach. Actually, the siderophore-transporting systems of microbes are specific to individual classes of bacteria and fungi. Due to that, siderophore-conjugates with PS are not taken up by mammalian cells, what makes them a good alternative for antimicrobial PDT, since they are not harmful to the host and are truly specific for pathogenic microbes. Then, the application of this method presents a highly efficient treatment of pathogenic Gram-positive and Gram-negative bacteria such as *P. aerugi-nosa*, *E. coli*, *Strepto-coccus pyogenes*, *S. aureus*, as well as for other antibiotic resistant microbial infections including infections that

occur in chronic wounds (Sperandio et al. 2013, Grafe et al. 2004).

Another patent describes a method and composition that utilizes Safranin O in conjunction with light irradiation (530 nm) in order to destroy microbes, especially bacteria. The Safranin O containing compound must be introduced to the treatment area and then, after a sufficient period of time, the light must be delivered to this area. This is an effective PDI approach for Gram-positive and Gram-negative bacteria, particularly good for areas surrounded by complex media such as blood serum, blood or saliva (Albrecht et al., 2005). In another study *E. coli* was only sensitive to porphyrin and light after suffering a pretreatment with toluene, which then induced susceptibility of this Gram-negative bacteria to PDT with hematoporphyrin derivative (Boye et al.,1980). In addition, positively charged (cationic) PS, including porphyrins and pthalocyanines, promote efficient inactivation of Gram-negative bacteria without the need of modifying the natural structure of the cellular envelope (Minnock et al.,1994, Merchat et al.,1996). Finally, not all microbial infections are suitable for PDT, because some infection sites may not be accessible to light (Love et al., 2007).

Several improvements are continuously made in PDT. tThe method with PS selected from toluidine blue O, methylene blue, dimethylene blue or azure blue chloride that can be employed to both hard and soft tissues was studied. Even military medical procedures are mentioned in the text, illustrating a particular utility for this therapy (Clements et al., 2010).

Hydrophilic cationic and anionic photosensitizers have been found to inactivate pathogenic bacteria. In a recent invention photosensitizers are formulated in calcium phosphate nanoparticles formulations for antibacterial PDT. These formulations were tested against *S. aureus* and Gram-negative *P. aeruginosa* demonstrating a very high percentage of killing (Gitter et al., 2011).

It has been said that certain edible or ingestible food colours are equal to or even superior to synthetic chemical photodynamic agents. They are of non-toxic nature, which definitely configures an advantage. In addition, they have the ability to be safely consumed and their breakdown is always to safe and environmental friendly products (Olson et al., 2010). By that means, an invention teaches how to treat an infected animal or decontaminate a surface, for example, by using a safe natural or synthetic food coloring agent that has photodynamic properties (Olson et al. 2010, Sperandio et al., 2013). The PS may be selected from the group of chlorophylls, carotenoids, flavenoids, quinonoids, coumarins, indigoids, curcuminoids, befalins, acthocyanins, cyanines, indocyanines, phthalocyanines, rhodamines, phenoxazines, phenothiazines, phenoselenazines, fluoresceins, porphyrins, benzoporphyrins, squaraines, corrins, croconiums, azo compounds, methine dyes, and indolenium (Sperandio et al., 2013). A novel series of PS that have advantages over other known compounds has been described. These compounds are actually meso-substituted porphyrins that have an absorption in the region of the visible spectrum, high molar extinction coefficients, and high quantum yield in singlet oxygen production (Roncucci et al., 2011). As previously mentioned, there are some limitations of porphyrin-based PDT. Among the limitations is the poor selectivity toward eukaryotic cells and the micoorganisms. In tumors, this selectivity can be enhanced by increasing the degree of hydrophobicity of the PS or by imparting amphiphilic properties to its molecule (Jori et al., 1996). Alternatively, these meso-substituted porphyrins are conjugated with a bio-organic carrier, ensuring high efficiency and selectivity against the target, i.e. Gram-negative bacteria (Roncucci et al., 2011).

BLUE LIGHT ANTIMICROBIAL PHOTOINACTIVATION

The advantages of using blue light alone to kill resistant microbes, is that the light is not as harmful to the host tissue or to the surroundings compared to UV light and moreover no added exogenous PS or dye is required (Dai et al., 2012). The most effective range is from ~390 nm to 420 nm (more accurately termed "violet" light), the next most effective range is from 450-480 nm and possible the least effective range is from 420-450 nm. Over the last five years a wide range of microbial cells, including Gram-positive bacteria, Gram-negative bacteria, mycobacteria, molds, yeasts and dermatophytes have been shown to be susceptible to blue light (Wang et al., 2017). Studies have been carried out *in vitro* using planktonic cells or biofilms, *ex vivo*, and *in vivo* using animal models (preclinical) and even in patients (clinical trials). The biological response to blue-light was firstly reported in 1881 by Charles Darwin when he

described a blue light induced phototropic response in plants (Darwin, 1881). Blue light can regulate bacterial motility, suppress biofilm formation, and subsequently potentiate light inactivation of bacteria. On the other hand, the presence of blue light may also activate or increase bacterial virulence (Hamblin et al., 2019).

The lethality of blue light for bacteria has been reported both *in vitro* and *in vivo*. Blue light can mediate a broad-spectrum antimicrobial effect on both Gram-negative and Gram-positive bacteria. While the wavelength range of 390-420 nm has been reported to be the most effective antimicrobial spectral range, both 455 nm and 470 nm have also been found to have some antimicrobial effects on some bacterial species (e.g., *S. aureus*). The mechanism of the antimicrobial effect of blue light is that blue light excites endogenous intracellular metal-free porphyrins to behave as PS as described above for the case of aPDI. This photon absorption then leads to energy transfer from the porphyrin triplet state to oxygen producing $^1O_2$ in a similar manner to PDT (Hamblin et al., 2019, Hamblin et al., 2005). Different bacteria demonstrate variable susceptibilities to blue light. Studies have reported that Gram-positive species, in general, were more susceptible to 405 nm light inactivation than Gram-negative species, which is generally consistent with the results obtained in a recent study (Murdoch et al., 2013). It has also been theorized that the differences in inactivation kinetics may be due to organism-specific differences in porphyrin levels, different individual porphyrin sub-types, or different porphyrin subcellular localizations (Maclean et al., 2009). Moreover, it has been speculated that less oxygen-tolerant bacterial species may be particularly susceptible to the effects of ROS as some microaerophilic species have been found to possess fewer key antioxidant defenses than most aerobes (Jean et al., 2004). Some studies have shown blue light to be capable of inactivating the anaerobic oral pathogens *Prevotella*, *Porphyromonas*, and *Fusobacterium*, *P. acnes* as well as microaerophilic pathogen as and *H. pylori* (Feuerstein et al., 2005).

Table 2. Studies about blue light antimicrobial action

Light Source	Radiant exposure	Bacterial species/strains	Inactivation efficacy	Ref
405-nm diode laser	20 J/cm^2	*H. pylori*	>99.9%	Hamblin et al. [2005]
380-520 nm broadband light	4 . 2-42 J/cm^2	*P. gingivalis*, *P. intermedia*, *P. nigrescens*, *P. melaninogenica*, *S. constellatus*	*P. intermedia* and *P. nigrescens*: >5 log10 at 4 . 2 J/cm^2; *P. melaninogenica*: >5 log10 at 21 J/cm^2; *P. gingivalis*:1.83 log10 at 42 J/cm^2	Soukos et al. [1998]
405 and 470 nm light	15 J/cm^2	*S. aureus*, *P. aeruginosa*	*S. aureus*: 90% at 405 nm, 62% at 470 nm; *P. aeruginosa*: 95.1% at 405 nm, 96.5% at 470 nm	Guffey and Wilborn
407-420 nm	75J/cm^2	*P. acnes*	less than 2-log10 units (99%) illuminated once; decreased by 4-log10 units (99.99%) after two illuminations and by 5-log10 units (99.999%) after three illuminations	Feuerstein et al. [2005]
405-425nm LED	110 J/cm2	*A. baumannii*	7.64-log10 CFU	Dai et al. [2011]
	118-2214 J/cm^2	*S. aureus*, *S. epidermidis*, *E. faecalis*, *S. pneumoniae*, *Corynebacterium striatum*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Serratia marcescens* *C. albicans.*	Complete inactivation (> 4-log10 CFU) in suspension was achieved in all of the isolates tested.	Dai and Gupta [2012]

MATERIAL AND METHODS

Literature on PDT for cancer treatment with the following medical subject headings search terms: colorectal cancer, prostate cancer, photodynamic therapy, clinical trials, antimicrobial photodynamic therapy and bacterial infection was reviewed. The articles were selected by their relevance to the topic.

RESULTS

PDT AND PROSTATE CANCER

As the most common treatment alternatives for localized prostate cancer, radical surgery and radiotherapy are used with a considerable morbidity. Patients with low risk and localized PC do not benefit from radical prostatectomy (Wang et al., 2019, Nelson et al., 2014). A number of alternative (focal) treatments such as high-intensity focused ultrasound, cryotherapy, and radiofrequency have been used (Gheewala et al., 2017, Golan et al, 2017). Although cancerous cells are destroyed, traditional focal treatment frequently leaves the tumor vessels intact, which can lead to recurrence of the tumor while treatment is insufficient, leaving not only the tumor parenchyma but also tumor vessels. PDT is specialized in target ablating and can prevents recurrence by reactive oxygen species such as singlet oxygen and free radicals (Wang et al., 2019, Dolmans et al., 2003). Percent negative biopsy, Gleason score, clinical stages, and PSA are tools for risk estimation in prostate cancer. Unlike radical prostatectomy and radiotherapy, it is suitable for a biopsy-based outcome after PDT (Nelson et al., 2014, Azzouzi et al., 2017).

The most important finding in recent studies was that the pooled rate of negative biopsy after PDT and decreased PSA were 55.0% and 35.0%, respectively. The control group in some studies was active surveillance and several other focal treatments (such as cryotherapy and brachytherapy), respectively (Azzouzi et al. 2017). According to the result of the randomized clinical trials, negative biopsy rate of active surveillance was less than one-third of the rate in the PDT group (Wang et al. 2019, Azzouzi et al., 2017). All the patients of the studies in this systematic review were considered having low-risk, localized PC which was well or moderately differentiated (most biopsy Gleason score was less than 6). The PSA after PDT was less than 4.0 ng/mL in the follow-up duration. In the study of high-risk PC (Nathan et al. 2002), although the PSA decreasing rate was 42.0%, the PSA after PDT was still higher than 10.0 ng/mL. This suggested that PDT was not suitable for the patients with high-risk, poorly differentiated PC. On the other hand, PDT can play an important role in patients who have recurrence after radical prostatectomy or who have failed previous definitive radiotherapy (Du et al. 2006).

The review studies show that rates of adverse events were variable but at a low level. By comparing PDT with cryotherapy, brachytherapy, and high-intensity focused ultra-sound, PDT appears to have a reasonably low rate of side effects (Wang et al. 2019), Barret et al. 2013). The most common adverse events were hematuria, erectile dysfun-ction, and dysuria. Due to the vascular target toxicity, hematuria always emerges in the duration of early posttreatment in about seven days (Kulik et al. 2014). Another notable complication was retention. There are_studies that found that retention was the most common serious adverse event in patients who underwent PDT. They thought it was associated with timing of withdrawal of the urinary catheter (Azzouzi et al. 2017). Other rare adverse events such as rectourethral fistulae and injury of seminal vesicle were almost asymptomatic and with a self-healing process, probably for extraprostatic sliding of an optical fiber (Wang et al. 2019). It is worth noting that photo toxicity is an inherent risk when using a photosensitizer (Kawczyk-Krupka et al. 2015).

The expected survival benefit of treatment for PC must be balanced against the related side effects such as erectile dysfunction and dysuria (Sachdeva 2015). Some studies found that occurrence of erectile dysfunction and incontinence during high-intensity focused ultrasound and cryotherapy was not only variable but also high (20.0-55.0%, 0.0-10.0%, 15.0-40.0%, and 1.0-10.0%, respectively)(Gómez-Veiga et al. 2014). In comparison to high-intensity focused ultrasound and cryotherapy, PDT has less effect on urinary and erectile function (Gómez-Veiga et al. 2014).Efficacy and functional outcomes of PDT were variable when using different photosensitizers. Padeliporfin and motexafin lutetium

were usually used in PC, whereas temoporfin was usually used in lung and head and neck malignancies (Dąbrowski and Arnaut, 2015). The selection of photosensitizer is important for patients if PDT is planned. Decreasing PSA was also associated with the follow-up duration. It indicated that a long-time follow-up is necessary when evaluating the changing PSA.

Table 3 PDT in prostate cancer-studies

References	PDT parameters	photosensilizer	patients	effect
Azzouzi et al (2017)	753 nm 150 mW/cm for 22 min 15 s	Padeliporfin	low-risk, localised prostate cancer (Gleason pattern 3) patiens, no previous treatment	safe, effective treatment for low-risk, localised prostate cancer
Barret et al (2013)	753 nm 150 mW/cm	Padeliporfin	Gleason 6	effective
Du at al (2006)		motexafin lutetium	Prostate adenocarcinoma	safe
Kasivisvanathan et al (2013)	753 nm 200/300 J/cm	TOOKAD	Gleason 3+3	effective
Kawczyk-Krupka et al (2015)	753 nm 150 mW/cm	palladium-based WST-09 and WST-11 photosensitisers	Prostate adenocarcinoma cells	effective

PDT AND COLORECTAL CANCERS

Recent studies have shown that photodynamic therapy (PDT) treatment has the ability to activate the tumor-specific immune responses by producing tumor-associated antigens from tumor cell residues, which afterward may be processed by APCs such as DCs and then presented to T cells (Gerosa et al. 2002). It is known that the immuno-logical memory response, which is the hallmark feature of adaptive immunities, plays crucial roles in protecting organisms from the second attack of pathogens including tumor cells (Ferlazzo et al. 2002). Upon a second encounter with the same pathogens, memory T cells can rapidly respond and mount faster and stronger immune responses than the first time the immune system response (Degli-Espsosti and Smyth, 2005). It is generally recognized that the underlying mecha-nisms of the combination therapy with ideal inhibition activities on the growth of both primary and distant tumors, as well as the immune memory protection to prevent tumor relapse, may be explained as follows.

The PDT destruction of primary tumors would generate a pool of tumor-associated antigens – TAA to trigger specific immune responses, which were then amplified by UCNP-Ce6-R837 - based PDT as the immune adjuvant, which combined with T-lymphocyte-associated protein 4 (CTLA-4) blockade would effectively induce the generation of TEM-based immune memory response to prevent tumor relapse, similar to the functions of cancer vaccines. More signify-cantly, PDT with UCNP-Ce6-R837 in combi-nation with the CTLA-4 checkpoint blockade not only showed excellent efficacy in eliminating tumors exposed to the NIR laser but also resulted in strong antitumor immunities to inhibit the growth of distant tumors left behind after PDT treatment. Furthermore, such a cancer immunotherapy strategy has a long-term immune memory function to protect treated mice from tumor cell challenge (Hayakawa and Smyth, 2006). This study presents an immune-stimu-lating UCNP-based PDT strategy in combination with CTLA-4 checkpoint blockade to effecti-vely destroy primary tumors under light exposure, to inhibit distant tumors that can hardly be reached by light, and to prevent tumor reoccurrence via the immune memory effect.

In summary, study demonstrates the great potency of integrating UCNP-based PDT with cancer immunotherapy to realize a remarkable synergistic therapeutic outcome in eliminating primary tumors, inhibiting distant tumors, and preventing tumor relapse. While immuno-therapy has become a highly promising paradigm for cancer treatment in recent years, it has long been recognized that PDT has the ability to trigger antitumor immune responses. However, conventional PDT triggered by visible light has limited penetration depth, and its generated immune responses may not be robust enough to eliminate tumors (Gang et al. 2018).

PHOTODYNAMIC THERAPY AN EMERGING TREATMENT MODALITY IN ONCOLOGY

Photodynamic therapy (PDT) is a promising method used for the control of a variety of cancers (Hu et al. 2014). PDT is a harmonized process which first requires the exposure of the cancer tissue to a photosensitizer (PS), administered either topically or intravenously, depending on the location of the targeted tissues (Portilho et al. 2013). A PS is a molecule that is taken up and localizes in the target cell and/or tissue and can only be activated by light (Wan and Lin,2014). Activation of a PS is achieved through exposure to laser irradiation at a specific wavelength. Once photons are absorbed by a PS, it is excited and stimulated from the ground state to a higher level of energy, a singlet state (Chiaviello et al. 2011). Alternatively, the molecule may convert to the triplet state through a mechanism called intersystem crossing, which results in a change in the spin of an electron. In this triplet state, the PS reacts with molecular oxygen and gives rise to free ROS that can destroy cancer tissue (Mroz et al. 2011). A major advantage of using PDT is that it achieves selective cell destruction and minimizes damage to adjacent healthy structures. PSs are taken up by all cells; however, they tend to preferentially localize in diseased tissue and remain in diseased tissue for a longer period of time due to the enhanced permeability retention (EPR) effect (Yo and Haa,2012). Consequently, it is vital to ensure PS activation only occurs once the proportion of PS in diseased tissue is greater than that present in healthy tissue (Josefsen et al. 2012). Other advantages of PDT over conventional treatment options include being a minimally invasive technique, lowering morbidity rate, ability to reserve the anatomic and functional integrity of many cells, minimal side effects, selective targeting, and no drug resistance, as well as reduced toxicity which allows for repeated treatment (Olivo et al. 2010).

SUBCELLULAR LOCALIZATION OF PHOTOSENSITIZERS (PS)

PS uptake and localization play a critical role in the effectiveness of PDT in the treatment of cancer. Subcellular localization of photosensitizers in different cellular components may induce various pathways of cell death/damage. Subcellular localization sites of PSs include the plasma membrane, lysosomes, Golgi apparatus, the nucleus and the mitochondria (Kim et al., 2014). PSs that accumulate in smaller amounts in more than one organelle (co-localization) may be used in combination to enhance the PDT efficacy of the PSs (Castano et al. 2004).

PS SOLUBILITY

Solubility also plays a role as most PSs are hydrophobic (Kim et al. 2014). Hydro-phobicity and a tendency to aggregate in aqueous environments hinder bioavailability of several PSs. Aggregation reduces increased uptake of photosensitization by the mononuclear phagocytic system (MPS) and decreased uptake by target cells as well as an increased risk of anaphylactic reactions (Sobczyński et al. 2013). Conjugation to nanoparticles can tune the water solubility and aggregation of the PCSs, without signify-cantly affecting its photophysical properties (Chernonosov et al. 2014).

PS DELIVERY AND SELECTIVITY

Abundant literature describes the use of NPs as a delivery system of drugs to increase the response to anticancer compounds (Roblero-Bartolon et al. 2015). A wide variety of organic and inorganic nano-constructs, such as liposomal, micellar, polymeric, silica and gold NPs, have been introduced to deliver high payloads of PS to desired sites, when combined with targeting processes. Advantages of using NPs include lower levels of the PSs used in treatment, increased selectivity, reduced side effects and reduced dark toxicity. In addition, peptide or antibody tags in NP systems can increase selectivity more efficiently and aid in controlling the size of the particle, which can influence better passive targeting through EPR effect and, therefore, increased cellular uptake (Mehraban et al. 2015).

ANTIBODY-MEDIATED SPECIFICITY

In an effort to increase PS accumulation specificity and reduce unwanted PDT PS and NP side effects, significant effort has been devoted towards the synthesis, and charac-terization, of bio-conjugates. Synthesis with either NPs or PSs further enhances PDT NP-PS passive drug delivery by actively and specifically targeting tumourous cells with monoclonal antibody

(mAb) conjugates. In the case of anticancer-mediated PDT, malignant cells present different types, as well as greater amounts, of many surface antigens (Abrahamse and Hamblin 2016). Antibodies against TAA (tumour-associated antigens) are easily generated, and if correctly attached to a PS drug delivery system, the PS can be directly targeted and absorbed via cell membrane endocytosis into specific tumours and therefore causes targeted cancer cell death upon PDT light activation (St Denis et al. 2013).

PS TARGETING OF CRC AND CRC STEM CELLS

Although some PSs used in PDT reveal certain tumour selectivity by the EPR effect, they can also accumulate in healthy tissues causing side effects such as phototoxic and photoallergic reactions (Varol 2015). To avoid this complication, targeted photo-dynamic therapy (TPDT) was fashioned to improve PS drug delivery to cancer tissue, and the overall specificity and efficiency of PDT was increased (Kawczyk-Krupka et al. 2015). TPDT can be divided into two mechanisms of action: passive or active targeting. Passive PDT targeting makes use of the PSs drug carrier's physicochemical factors, as well as the morphological and physiological differences between normal and tumour tissue (i.e. EPR effect) to deliver the PS to a target site (Yoo and Ha,2012). Active PDT targeting involves PS drug delivery to a specific tumour site, which is based on a molecular recognition process, using specific ligands or antibodies which bind to overexpressed cancer cell receptors (Kawczyk-Krupka et al. 2015).

Table 4. PDT in clinical trials-oncology

Photosensitizer	Clinical trial phase (ClinicalTrials.gov)		
	Phase I	Phase II	Phase III
Porfimer sodium (Photofrin)	Pancreatic cancer	Human head & neck cancer Cholangiocarcinoma	Esophageal and/or gastric cardiac cancer
Aminolevulinic acid (5-ALA)	Early stage head & neck tumors Multple basal cell carcinomas Colon cancer	Malignant gliomas Basal cell carcinoma	
Verteporfin	Brain tumors	Age – related macular degeneration	
mTHPC (Foscan)	Non-small cell lung cancer	Nasopharyngeal carcinoma Cholangiocarcinoma	
TOOKAD	Renal tumors		

ANTIBACTERIAL PDT

APPLICATION OF PHOTODYNAMIC THERAPY *IN VITRO*

The impact of with photosensitizers (XF73, XF70,CTP1) on strains of *S. aureus* resistant to methicillin (MRSA) was investigated. The findings showed that concentrations (0.005 μM) of photosensitizers, using light (13.7 J/cm^2) for 10 minutes is effective to reduce a 3log$_{10}$ (> 99.9%) of bacteria (Maisch et al. 2005). In another study the bacte-ricidal effects of the photodynamic inactivation with a porphoporphyrin photosensitizer at 624 nm wavelength with an energy density of (0.2 J/cm^2) on 40 clinical isolates of MRSA and 40 clinical isolates of MSSA isolated patients admitted to the hospital in Gdansk was evaluated. The results of the study indicated the reduction of 3log$_{10}$ in the number of bacteria (Grinholc et al. 2008). Another study evaluated the impact of PDT using photosensitizers MB (3mM) and gallium-aluminium lasers at a wavelength of 660 nm red light with an energy density and time of 35 mW, 10 J, and 285 seconds, on *S. aureus*. The findings showed the number of bacteria (4.89-6.83 log10 CFU/mL) decreased in relation to the initial concentration of bacteria (Miyabe et al. 2011). Also the effect of photodynamic therapy using a protoporphyrin 25 μM photosensitizer with laser radiation at a wavelength of 624 nm on the MRSAstrains

and MSSA strains was evaluated. The results of this study indicated that there was a reduction of 0-3 log10 and 0.2-3 log10 strains of MRSA and MSSA in the number of bacteria, respectively (Grinholc et al. 2008). Sharma assessed the effectiveness of aPDT on *S. aureus* biofilm formation. In this study, a TBO photosensitizer was used at concentrations of 10-80 μm and light source diode laser with a 640 nm wavelength. The results showed that at 40 μM concentration, biofilm was destroyed and has the least cytotoxic effect in cells (Sharma et al. 2008). Li investigated the effect of photo-dynamic therapy with 5-amino-levulinic acid (ALA) photosensitizer and a concentration of 40 mM and an optical source laser with 635 nm wavelength for activating ALA at doses (0, 100, 200, 300 J/cm2) used. The results of this study showed that ALA without exposure to light or red light does not affect bacterial biofilm. However, a significant number of cells in the biofilm was inactivated during radiation with different doses of red light in the presence of 5-aminolevulinic acid, and at the dosage of 300 J/cm2, all bacteria (99.99%) were killed (Li et al. 2013). Some scientists reported the effect of laser light on MRSA strains using TBO photosensitizer at a concentration of 50 μg/mL and a HeNe laser light with a wavelength of 632.8 nm in 1, 5, 10 minutes. The findings of the study indicated that 100% of the bacteria were killed in 15 minutes. Antibiotic resistance patterns of these strains were different before and after laser radiation, so that they were resistant to gentamicin 10 μg prior to the laser irradiation, and had an intermediate resistance to vancomycin. Moreover, after laser radiation, they became sensitive to both these antibiotics (Hajim et al. 2010). Another study showed the effect of photodynamic therapy on clinical isolates of MRSA strains and *S. aureus* strain ATCC 25923 under the conditions of using TBO photosensitizer at concentrations (80-400 μM) and Pl-ce6 at a concentration of (8 μM) and an optical source of laser 600 nm wavelength with doses (10-30 J/cm2) for 30 minutes. The results of this study showed that Pl-ce6 and TBO at the concentration (8 μM, 30 J/cm^2 – 80 μM, 30 J/cm^2) induced killing of MRSA (4log10 and 3log10) and *S. aureus* (ATCC 25923 (3log10-2log10)) (Tang et al. 2007).

For Gram negative bacteria – the photodynamic inactivation influence on *Escherichia coli* (ATCC25922) and clinical resistant strains of *E. coli* using photosensitizers of MB and toluidine blue O (TBO) was studied (Kashef et al. 2012). MB (50 μg/mL) with a laser light of red (163.8 J/cm^2) capable of reducing 53.1% and 37.6% in the number of viable *E. coli* (ATCC25922) and drug resistant *E. coli* (the initial number of bacteria was 10^4-10^5 Cfu/mL). Moreover, TBO (50 μg/mL) and a laser dose of 46.68 J/cm^2 killed 98. 2% and 83. 2% of *E. coli* (ATCC25922) and drug-resistant *E. coli*. In another study, the photodynamic efficiency on the plankton condition in *Acinetobacter baumannii* was evaluated. The results showed that the decreasing in the number of *A. baumannii* in plankton condition was 2-3log$_{10}$ reported after photodynamic inactivation with 2 photo-sensitizers of TBO and MB (Ragas et.al2010). The efficacy of photodynamic therapy on *A. baumannii* was assessed in another study – the findings showed the reducing in logarithmic growth of live cells after photodynamic inacti-vation with MB and TBO (TBO) for 5 strains of *A. baumannii* was between (1.3 log$_{10}$), (3.5-2.4 log$_{10}$), (2.9-2.2 log$_{10}$) and (2.6 log$_{10}$). Further-more, photodynamic inactivation reduced the minimum inhibitory concentrations of growth inhibitors into Azithromycin, Imipenem, Cipro-floxacin and Gentamicin antibiotics (Kashef et al. 2014). A photodynamic test of 2 photo-sensitizers of TBO and meso-Tetra(N-methyl-4-pyridyl) porphine tetra tosylate (TMP) at a concentration of 5 mg/mL on 5 strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis was performed, and the laser light killed 99.99% of bacteria (Donnelly et al. 2007).

Another study determined the efficacy of sub-lethal photodynamic therapy with the ability to form biofilm and the metabolic activity of *Enterococcus faecalis* under *in vitro* condi-tions by using indocyanine green photosen-sitizer at concentrations (2 mg/mL) and TBO and MB at a concentration of 0.2 mg/mL with a diode laser as light source for TBO and MB and indocyanine green were 635 nm-200 mW, 660 nm-150 mW, 810 nm-200 mW, respectively. The findings showed that PDT-ICG and PDT-MB and TBO-PDT sub-lethal reduces 42.8%, 22.6%, and 19.5% of biofilms, the sub-lethal dose of PDT-ICG and PDT-MB and TBO PDT reduced 98%, 94%, 82% of metabolic activity in *Enterococcus* spp., respectively (Pourhaji-bagher et al. 2016). In another study the effect of photodynamic therapy on *E. faecalis* in

biofilm formed at root infections under the laboratory conditions was investigated. The findings of the study suggest that 97% of the bacteria were reduced when they were exposed to red laser light and MB at a concentration of 25 mg/mL (Soukos et al.1998).

IN VITRO AND CLINICAL EFFECTIVENESS OF ANTIMICROBIAL PHOTODYNAMIC THERAPY (APDT)

Photodynamic therapy is effective against bacteria, viruses, fungi, and parasites but its inactivation efficiency varies according to the microorganism. In general, bacteria and viruses are more easily inactivated than fungi and parasites. Spores of bacteria and fungi, particularly endospores, and parasite eggs and cysts are more resilient to inacti-vation than the corresponding vegetative cells (Almeida et al. 2011).

BACTERIA SENSIBILITY

Gram-positive bacteria are more laser- sensitive than Gram-negative bacteria (Grinholc et al. 2008, Almeida et al. 2011). The difference in the sensitivity of the two groups is related to their different cell wall composition. Most Gram-positive bacteria have a cell wall consisting of several layers of peptidoglycans, negatively charged, which exhibit a relatively high degree of porosity. Macromolecules having a molecular weight of 30,000-60,000 (like glycopeptides and polysaccharides) can easily pass through this structure. Consequently, most photo-sensitizers (PS) can go through their membranes, since its molecular weight gene-rally is situated between 1500 to 1800 Da (Jori et al. 2006). On the contrary, Gram-negative bacteria display in the cell wall, an additional highly organized outer membrane, which is external to the peptidoglycan layer. The asym-metric nature of the outer membrane is a conse-quence of the distribution of its phospholipids, proteins, lipoproteins, and negatively charged lipopolysaccharides (Maisch et al. 2004, Sharma et al. 2011, Sperandio et al. 2013) which do not allow the passage of various molecules into its interior. However, hydrophilic molecules of 600-700 Da can diffuse through the porins (Nikaido et al.1994).Gram-positive bacteria can be efficiently inactivated by neutral and anionic PS since the diverse PS can effortlessly go through their highly permeable cell wall. Yet, these PS are not effective against Gram-negative bacteria [Hamblin et al.,75], unless they are co-administered with external membrane disrupting agents such as $CaCl_2$, EDTA, and polymyxin B, which can lead to electrostatic repulsion and destabilize the cell wall (Jori et al. 2004). Gram-negative bacteria can be directly and effectively inactivated by cationic PS since these PS are able to bind to the negatively charged components of the outer membrane and allow a more effective interaction (Hamblin et al. 2002).

As stated above, the primary difficulty of killing Gram-negative bacteria using PDT is to achieve a good penetration of the PS inside the bacterial cell wall. However, different approaches aim to eliminate this problem by, for example, creating positively charged (cationic) PS or by coupling or combining the PS with positively charged entities such as poly-L-lysine (Sperandio et al. 2013, Sahu et al. 2014), polyethyle-nimine (Tegos et al. 2006) and polymyxin B nano-peptide (PMBN) (Nitzan et al. 1992).

VIRUSES SENSIBILITY

There are several studies that suggest that lipid-enveloped viruses are more susceptible to PDT (Costa et al. 2012). It is also suggested that different types of nucleic acids viruses (DNA and RNA) present different susceptibility to PDT, but the differences between RNA and DNA viruses are not only attributed to their nucleic acid type, but also to the composition of their capsids (Costa et al. 2012).The clinical trials of aPDT application to inactivate viruses has been successful. Neutral red/proflavine was effectively used to treat herpesvirus genital infection without relevant side effects (Moore et al.1972). Porphyrins were shown to be effective against Herpes virus, the Influenza virus, and the Papillomavirus (Perlin et al.1987). aPDT is already approved to sterilize plasma. Different viruses such as Hepatitis viruses, Parvoviruses, the West Nile virus, and HIV have been effectively inactivated by methylene blue (Mohr et al. 2004).

FUNGI AND PARASITES SENSIBILITY

Since fungi and parasite cells are larger when compared to bacteria and viruses, the amount of ROS needed to kill such a larger cell is much higher than is necessary to kill a bacterial cell or

a viral particle (Demidova et al. 2005). On the other hand, the eukaryotic cell structure makes aPDT effect more difficult to work for these micro-organisms than for bacteria and viruses. Unlike bacteria and viruses, fungi and parasites are compartmented cells and, consequently, whenever the cell wall and membranes are damaged by the ROS, the PS enter its interior. Similar to bacteria, fungi also have a cell wall, which is more permeable to external substances than Gram-negatives cell wall, but less than in Gram-positives (Cabral et al. 2019). Since ROS are highly reactive and have a short lifetime, the localization of the PS into the cell is very important, since the organelles located nearby to the PS have the highest probability of being affected.

However, effective inactivation of fungi and parasites has already been observed (Calzavara-Pinton et al. 2012). In fact, to obtain the effective inactivation of fungi and parasites, it is necessary to adjust both PDT conditions and increase the PS concentration and the light dose (Donnelly et al. 2007). What is interesting, *Candida* spp. are effectively inactivated by aPDT, but they are not as susceptible to PDT as several prokaryotic bacteria, including *Staphylococcus aureus* or *Streptococcus mutans* (Pereira et al. 2011).It was observed that aPDT is effective for inactivating parasites, but also requires a higher PS concentration and higher light doses than those required for bacteria and viruses. aPDT with different PS have been tested for the inactivation of *Leishmania* spp. (Morgenthaler et al. 2008) and *Plasmodium falciparum* (Grellier et al.1997). Cysts of *Colpoda inflate* and eggs of helminths like *Ascaris lumbricoides* and *Taenia* spp. were also successfully photo- inactivated (Alouini, 2001).

IN VIVO STUDIES PDT IN INFECTIOUS DISEASES – PDT APPLICATIONS FOR GRAM-NEGATIVE BACTERIA

Gram-negative bacteria are responsible for many life-threatening infections in humans, especially in elderly people, and they are often innately resistant (especially *P. aerugi-nosa*) to the most commonly used antibiotics, making the search for new antibacterial drugs and alternative therapies, such as PDT, very important (Sperandio et al. 2013). The PS molecule, for instance, has to bind to the bacterial cell (Malik et al.1982), most often to the cell plasma membrane (Ehrenberg et al.1985) so the PDT killing effect can take place (Nitzan et al.1992). Gram-positive bacteria and yeasts are affected by neutral or anionic metal-free porphyrins (Malik et al.1990), while Gram-negative bacteria are not. This resistance to photosensitization by Gram-negative bacteria with anionic porphyrins was widely reported in the literature of the 1980's (Sperandio et al. 2013, Malik et al.1982, Venezio et al.1985, Nitzan et al.1987). PDT of both Gram-negative *Escherichia coli* and *P. aeruginosa* with high concentrations of hemato-porphyrin derivative (HPD) or deuteroporphyrin (DP) combined with high intensities of illumination did not result in any bacterial inactivation (Sperandio et al. 2013, Malik et al.1982, Venezio et al.1985, Nitzan et al. 1987). In addition, *E. coli* was only sensitive to porphyrin and light after suffering a pretreatment with toluene, which then induced susceptibility of this Gram-negative bacteria to PDT with hematoporphyrin derivative (Boye et al. 1980). It is only when the inner membrane of *E. coli* is exposed that porphyrin can bind to this membrane (Sperandio et al. 2013, Boye et al. 1980). Knowing this and the fact that the polycationic agent polymyxin nonapeptide can disturb and disorganize the outer-membrane structure of Gram-negative bacteria (Vaara et al. 1983), scientists were able to successfully kill *E. coli* and *P. aeruginosa* with PDT mediated by deuteroporphyrin (DP) (Nitzan et al. 1987), what represented a true advance in photodynamic inactivation of Gram-negative bacteria. Nevertheless, neither of these results (Nitzan et al.1987, Boye et al. 1980) resolved the problem of Gram-negative bacterial resistance (Malik et al. 1992].

One approach to turn Gram-negative susceptible to PDT is to pre-treat them with ethylene diamine tetraacetic acid (EDTA). It is known that Gram-negative wild-type cells treated briefly with EDTA lose up to 50% of their lipopolysaccharide into the medium and become very sensitive to hydrophobic agents (Malik et al. 1992). In fact, cationic molecules can more easily bind to the cell wall of Gram-negative bacteria, which is negatively charged due to teichoic acid residues, for example (Bourre et al. 2010). The negatively charged LPS molecules also have a strong affinity for cations such as calcium (Ca^{2+}) and magnesium (Mg^{2+}), the binding of which is required for the thermo-

dynamic stability of the outer membrane (Hancock, 1984). Again, considering the physical arrangement of the LPS layer of the Gram-negative bacteria outer membrane, treatment with low concentrations of polycations that tend to bind tightly to the highly negatively charged surface and to displace divalent cations can be effective (Malik et al.1992). As previously stated, the combined exposure to PMBN, DP and light inhibited *E. coli* and *P. aeruginosa* cell growth (Nitzan et al.1987). In addition, it was stated the disappearance of plasmid super-coiled fraction of *E. coli* when post-treated by PMBN and DP (Nir et al. 1991).Finally, a disturbance in the outer membrane of Gram-negative bacteria must occur so porphyrins and phthalocyanines can act in their inner membrane. In that way, the permeabilizing agent PMNP can disrupt the outer membrane and allow the penetration of porphyrin, consequently enabling the photosensitization of Gram-negative bacteria (Fotinos et al. 2008). In addition, through the same mechanism, EDTA treatment combined with phthalocyanines inactivate those bacteria and consist in a promising photodynamic therapy (Malik et al. 1992).

CONCLUSIONS

A photodynamic therapy-PDT is a modern, very promising modality for both scientific research and clinical treatment of oncologic and infectious diseases. The most advanced experiments were performed on tissue level in colorectal cancer cells while the most common application is in dentistry and skin diseases.

REFERENCES

Abrahamse H., Hamblin M.R., **New photosensitizers for photodynamic therapy**, In: Biochem J 2016; 473(4): 347-364.

Albrecht V., Gitter B., **Antimicrobial photodynamic therapy compound and method of use**, In: US Patent App. 10/860,296, 2005.

Almeida A., Cunha A., Faustino M.A.F., Tomé A.C., Neves M.G.P.M.S., **Photodynamic Inactivation of Microbial Pathogens: Medical and Environmental Applications**, In: Royal Society of Chemistry; Cambridge, UK: 2011. Neves MGPMS Porphyrins as antimibrobial photosensitizing agents; pp. 83-160.

Alouini Z., Jemli M., **Destruction of helminth eggs by photosensitized porphyrin,** In: J. Environ. Monit. 2001;3:548-551.

Althuis M.D., Fergenbaum J.H., Garcia-Closas M., Brinton L.A., Madigan M.P., Sherman M.E., **Etiology of hormone receptor-defined breast cancer: a systematic review of the literature,** In: Cancer Epidemiol Biomarkers Prev. 2004;13:1558-1568.

Antonic V., Stojadinovic A., Kester K.E., et al., **Significance of infectious agents in colorectal cancer development,** In: J Cancer. 2013;4(3):227-240.

Arrojado C., Pereira C., Tome J.P., Faustino M.A., Neves M.G., Tome A.C., Cavaleiro J.A., Cunha A., Calado R., Gomes N.C., et al., **Applicability of photodynamic antimicrobial chemotherapy as an alternative to inactivate fish pathogenic bacteria in aquaculture systems,** In: Photochem. Photobiol. Sci. 2011;10:1691-1700.

Arthur JC., Perez-Chanona E., Mühlbauer M., Tomkovich S., Uronis J.M., Fan T.J., et al., **Intestinal inflammation targets cancer-inducing activity of the microbiota,** In: Science. 2012;338:120-123.

Azzouzi A.R., Vincendeau S., Barret E., Cicco A., Kleinclauss F., van der Poel H.G., **Padeliporfin vascular-targeted photodynamic therapy versus active surveillance in men with low-risk prostate cancer (CLIN1001 PCM301): an open-label, phase 3, randomized controlled trial,** In: Lancet Oncol. 2017 Feb;18(2):181-191.

Barr H., MacRobert A.J., Tralau C.J., Boulos P.B., Bown S.G., **The significance of the nature of the photosensitizer for photodynamic therapy: Quantitative and biological studies in the colon,** In: Br J Cancer. 1990;62:730-735.

Barret E., Ahallal Y., Sanchez-Salas R., Galiano M., Cosset J.M., Validire P., **Morbidity of focal therapy in the treatment of localized prostate cancer,** In: Eur Urol. 2013 Apr;63(4):618-622.

Bertoloni G., Rossi F., Valduga G., Jori G., van Lier J., **Photosensitizing activity of water- and lipid-soluble phthalocyanines on *Escherichia coli,*** In: FEMS Microbiol Lett. 1990;59:149-55.

Bourre L., Giuntini F., Eggleston I.M., Mosse C.A., Macrobert A.J., Wilson M., **Effective photoinactivation of Gram-positive and Gram-negative bacterial strains using an HIV-1 Tat peptide-porphyrin conjugate,** In: Photochem Photobiol Sci. 2010;9:1613-20.

Boursi B., Mamtani R., Haynes K., Yang Y.X., **Recurrent antibiotic exposure may promote cancer formation--Another step in understanding the role of the human microbiota?,** In: Eur J Cancer, 2015;51:2655-2664.

Boye E., Moan J., **The photodynamic effect of hematoporphyrin on DNA,** In: Photochem Photobiol. 1980;31:223-8.

Broekgaarden M., Weijer R., van Gulik T.M., Hamblin M.R., Heger M., **Tumor cell survival pathways activated by photodynamic therapy: A molecular basis for pharmacological inhibition strategies,** In: Cancer Metastasis Rev. 2015;34:643-690.

Brown S.B., O'grady C.C., Griffiths J., Mellish K.J., Vernon D.I., **Biologically active methylene blue derivatives,** 2011:7915254.

Cabral J., Ag R., **Blue Light Disinfection in Hospital Infection Control: Advantages, Drawbacks, and Pitfalls. Antibiotics (Basel),** 2019;8(2):58

Calzavara-Pinton P., Rossi M.T., Sala R., Venturini M., **Photodynamic antifungal chemotherapy,** In: Photochem. Photobiol. 2012;88:512-522.

Castano A.P.D.T., Hamblin M.R., **Mechanisms in photodynamic therapy: part one - photosensitizers, photochemistry and cellular localization,** In: Photodiagn Photodyn Ther. 2004;1:279-93.

Castano A.P., Demidova T.N., Hamblin M.R., **Mechanisms in photodynamic therapy: part one – photosensitizers, photochemistry and cellular localization,** In: Photodiagnosis Photodyn Ther 2004; 1(4): 279-293.

Cheng I., Witte J.S., Jacobsen S.J., Haque R., Quinn V.P., Quesenberry C.P., et al., **Prostatitis, sexually transmitted diseases, and prostate cancer: the California men's health study,** In: PLoS One. 2010;5.

Chernonosov A.A., Ermilov E.A., Röder B., **Effect of some substituents increasing the solubility of Zn(II) and Al(III) phthalocyanines on their photophysical properties,** In: Bioinorg Chem Appl 2014; 952632-1-952632-7.

Chiaviello A., Postiglione I., Palumbo G., **Targets and mechanisms of photodynamic therapy in lung cancer cells: a brief overview,** In: Cancers 2011; 3(1): 1014-1041.

Choi C.H.R., Bakir I.A., Hart A.L., Graham T.A., **Clonal evolution of colorectal cancer in IBD,** Nat Rev Gastroenterol Hepatol. 2017;14(4):218-229.

Choudhary S., Nouri K., Elsaie M.L., **Photodynamic therapy in dermatology: a review,** In: Lasers Med Sci. 2009;24:971-80.

Chu Y.W., Wong C.H., Chu M.Y., et al., **Varibaculum cambriense infections in Hong Kong, China, 2006,** In: Emerg Infect Dis. 2009;15:1137.

Clements D.J., Pearson G.J., Williams J.A., Colles M.J., **Method for photodynamic therapy and apparatus therefor,** 2010:20100305494.

Costa L., Faustino M.A.F., Neves M.G.P.M.S., Cunha Â., Almeida A., **Photodynamic Inactivation of Mammalian Viruses and Bacteriophages,** In: Viruses. 2012;4:1034.

Costa L., Tome J.P., Neves M.G., Tome A.C., Cavaleiro J.A., Cunha A., Faustino M.A., Almeida A., **Susceptibility of non-enveloped DNA - and RNA-type viruses to photodynamic inactivation,** In: Photochem. Photobiol. Sci. 2012;11:1520-1563.

Cuevas-Ramos G., Petit CR., Marcq I., Boury M., Oswald E., Nougayrède J.P., ***Escherichia coli* induces DNA damage in vivo and triggers genomic instability in mammalian cells,** In: Proc Natl Acad Sci U S A. 2010;107:11537-11542.

Dąbrowski J.M., Arnaut L.G., **Photodynamic therapy (PDT) of cancer: from local to systemic treatment,** In: Photochem Photobiol Sci. 2015 Oct;14(10):1765-1780.

Dai T., Gupta A., Murray C.K., Vrahas M.S., Tegos G.P.,Hamblin M.R., **Blue light for infectious diseases: Propionibacterium acnes, Helicobacter pylori, and beyond?,** In: Drug Resist Updat, 2012.

Dai T., Huang Y.Y., Hamblin M.R., **Photodynamic therapy for localized infections--state of the art,** In: Photodiagnosis Photodyn Ther. 2009;6:170-88.

Daniell M.D., Hill J.S., **A history of photodynamic therapy,** In: Aust N Z J Surg. 1991;61(5):340-348.

Darabpour E., Kashef N., Mashayekhan S., **Chitosan nanoparticles enhance the efficiency of methylene blue-mediated antimicrobial photodynamic inactivation of bacterial biofilms: An *in vitro* study,** In: Photodiagnosis Photodyn Ther. 2016;14:211-217.

Darwin C., **The power of movement in plants**, In: New York, 1881.

De Marzo A.M., Marchi V.L., Epstein J.I., Nelson W.G., **Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis,** In: Am J Pathol. 1999;155:1985-1992.

De Marzo A.M., Platz E.A., Sutcliffe S., Xu J., Grönberg H., Drake C.G., et al., **Inflammation in prostate carcinogenesis,** In: Nat Rev Cancer. 2007;7:256-269. 61

Dees C.H., Scott T.C., Wachter E.A., Fisher W.G., Smolik J., **Intracorporeal medicaments for photodynamic treatment of disease,** 2005

Dees C.H., Scott T.C., Wachter E.A., Fisher W.G., Smolik J., **Intracorporeal Medicaments for Photodynamic Treatment of Disease,** 2011.

Degli-Espsosti M.A., Smyth M.J., **Close encounters of different kinds: dendritic cells and NK cells take centre stage,** In: Nat. Rev. Immunol. 2005, 5:112-124.

Dejea C.M., Fathi P., Craig J.M., et al., **Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria,** In: Science. 2018;359(6375):592-597.

Demidova T.N., Hamblin M.R., **Effect of cell-photosensitizer binding and cell density on microbial photoinactivation,** In: Antimicrob. Agents Chemother. 2005;49:2329-2335.

Dolmans D.E., Fukumura D., Jain R.K., **Photodynamic therapy for cancer,** In: Nat Rev Cancer. 2003 May;3(5):380-387.

Donnelly R.F., McCarron P.A., Tunney M.M., David Woolfson A., **Potential of photodynamic therapy in treatment of fungal infections of the mouth. Design and characterisation of a mucoadhesive patch containing toluidine blue**, In: O. J. Photochem. Photobiol. B. 2007;86:59-69.

Donnelly R.F., McCarron P.A., Cassidy C.M., Elborn J.S., Tunney M.M., **Delivery of photosensitisers and light through mucus: investigations into the potential use of photodynamic therapy for treatment of Pseudomonas aeruginosa cystic fibrosis pulmonary infection,** In: J. Control Release. 2007;117(2):217-226.

Du K.L., Mick R., Busch T.M., Zhu T.C., Finlay J.C., Yu G., **Preliminary results of interstitial motexafin lutetium-mediated PDT for prostate cancer,** In: Lasers Surg Med. 2006 Jun;38(5):427-434.

Ehrenberg B., Malik Z., Nitzan Y., **Fluorescence spectral changes of hematoporphyrin derivative upon binding to lipid vesicles, *Staphylococcus aureus* and *Escherichia coli* cells,** In: Photochem Photobiol. 1985;41:429-35.

Elkahwaji J.E., Hauke R.J., Brawner C.M., **Chronic bacterial inflammation induces prostatic intraepithelial neoplasia in mouse prostate,** In: Br J Cancer. 2009;101:1740-1748.

Shrestha E.,White J.R., Shu-Han Y., Kulac I., Ertunc O., De Marzo A.M., Yegnasubramanian S., Mangold L.A., Partin A.W., Sfanos K.S. ,**Profiling the Urinary Microbiome in Men with Positive versus Negative Biopsies for Prostate Cancer**, In: J Urol. 2018 Jan; 199(1): 161-171.

Ferlazzo G., Tsang M.L., Moretta L., Melioli G., Steinman R.M., Munz C., **Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells,** In: J. Exp. Med. 2002 195:343-351.

Feuerstein O., Ginsburg I., Dayan E., Veler D., Weiss E.I., **Mechanism of visible light phototoxicity on Porphyromonas gingivalis and Fusobacterium nucleatum,** In: Photochem Photobiol, 2005; 81, 1186-1189.

Fotinos N., Convert M., Piffaretti J.C., Gurny R., Lange N., **Effects on gram-negative and gram-positive bacteria mediated by 5-aminolevulinic Acid and 5-aminolevulinic acid derivatives,** In: Antimicrob Agents Chemother. 2008;52:1366-73.

Gandaglia G., Briganti A., Gontero P., **The role of chronic prostatic inflammation in the pathogenesis and progression of benign prostatic hyperplasia (BPH),** In: BJU Int. 2013;112:432.

Gang W., Wang J.J., Guan R., **Strategy to targeting the immune resistance and novel therapy in colorectal cancer,** In: Cancer Med. 2018;7(5):1578-1603.

Geralde M.C., Leite I.S., Inada N.M., et al., **Pneumonia treatment by photodynamic therapy with extracorporeal illumination – an experimental model.** In: Physiol Rep. 2017;5(5).

Gerosa F., Baldani-Guerra B., Nisii C., Marchesini V., Carra G., Trinchieri G., **Reciprocal activating interaction between natural killer cells and dendritic cells,** In: J. Exp. Med. 2002 195:327-333.

Gheewala T., Skwor T., Munirathinam G., **Photosensitizers in prostate cancer therapy,** In: Oncotarget. 2017 May 2;8(18):30524-30538.

Gitter B., Grafe S., Wiehe A., Albrecht V., Epple M., Schwiertz J., Ganesan K., **Calciumphosphate-based nanoparticles as carrier-systems for photodynamic therapy,** 2011:20110257586.

Golan T., Milella M., Ackerstein A., Berger R., **The changing face of clinical trials in the personalized medicine and immuno-oncology era: report from the international congress on clinical trials in Oncology & Hemato-Oncology (ICTO 2017),** In: J Exp Clin Cancer Res. 2017;36(1):192.

Gómez-Veiga F., Martínez-Breijo S., Solsona-Narbón E., Hernández C., Ciudin A., Ribal M.J., **Focal therapy for prostate cancer. Alternative treatment,** In: Actas Urol Esp. 2014 Sep;38(7):465-475.

Grafe S., Gebhardt P., Albrecht V., **Siderophore conjugates of photoactive dyes for photodynamic therapy,** 2004:20040186087.

Grellier P., Santus R., Mouray E., Agmon V., Maziere J.C., Rigomier D., Dagan A., Gatt S., Schrevel J., **Photosensitized inactivation of Plasmodium falciparum- and Babesia divergens-infected erythrocytes in whole blood by lipophilic pheophorbide derivatives,** In: Vox Sang. 1997;72:211-220.

Grinholc M., Szramka B., Kurlenda J., Graczyk A., Bielawski K.P., **Bactericidal effect of photodynamic inactivation against methicillin-resistant and methicillin-susceptible Staphylococcus aureus is strain-dependent,** In: J Photochem Photobiol B. 2008;90(1):57-63.

Hajim K.I., Salih D.S., Rassam Y.Z., **Laser light combined with a photosensitizer may eliminate methicillin-resistant strains of *Staphylococcus aureus*,** In: Lasers Med Sci. 2010;25(5):743-748.

Halaburková A., Jendželovský R., Kovaľ J., Herceg Z., Fedoročko P., Ghantous A., **Histone deacetylase inhibitors potentiate photodynamic therapy in colon cancer cells marked by chromatin-mediated epigenetic regulation of CDKN1A,** In: Clin Epigenetics. 2017;9:62.

Hamblin M.R., Abrahamse H., **Can light-based approaches overcome antimicrobial resistance?,** In: Drug Dev Res. 2019;80(1):48-67.

Hamblin M.R., O'Donnell D.A., Murthy N., Rajagopalan K., Michaud N., Sherwood M.E., **Polycationic photosensitizer conjugates: effects of chain length and Gram classification on the photodynamic inactivation of bacteria,** In: J Antimicrob Chemother, 2002;49:941-51.

Hamblin M.R., Viveiros J., Yang C., Ahmadi A., Ganz R.A., Tolkoff M.J., **Helicobacter pylori accumulates photoactive porphyrins and is killed by visible light,** In: Antimicrobial agents and chemotherapy. 2005; 49, 2822-2827.

Hancock R.E., **Alterations in outer membrane permeability,** In: Annu Rev Microbiol. 1984;38:237-64.

Hayakawa Y., Smyth M.J., **CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity,** In: J. Immunol. 2006 176:1517-1524.117.

Hu Z., Oleinick N., Hamblin M.R., **Photodynamic therapy as an emerging treatment modality for cancer and non-cancer diseases,** In: J Anal Bioanal Tech 2014; 1(2): e001.

Hunter PA., Dawson S., French G.L., Goossens H., Hawkey P.M., Kuijper E.J., **Antimicrobial-resistant pathogens in animals and man: prescribing, practices and policies,** In: J Antimicrob Chemother. 2010;65 Suppl 1:i3-i17.

Jean D., Briolat V. Reysset G., **Oxidative stress response in Clostridium perfringens**, In: Microbiology. 2004 150, 1649-1659.

Jori G., **Tumour photosensitizers: approaches to enhance the selectivity and efficiency of photodynamic therapy,** In: J Photochem Photobiol B. 1996;36:87-93.

Jori G., Brown S.B., **Photosensitized inactivation of microorganisms,** In: Photochem. Photobiol. Sci. 2004;3:403-405.

Jori G., Fabris C., Soncin M., Ferro S., Coppellotti O., Dei D., Fantetti L., Chiti G., Roncucci G., **Photodynamic therapy in the treatment of microbial infections: Basic principles and perspective applications,** In: Lasers Surg. Med. 2006;38:468-481.

Josefsen L.B., Boyle R.W., **Unique diagnostic and therapeutic roles of porphyrins and phthalocyanines in photodynamic therapy, imaging and theranostics,** In: Theranostics 2012; 2(9): 916-966.

Kang M., Martin A., **Microbiome and colorectal cancer: Unraveling host-microbiota interactions in colitis-associated colorectal cancer development,** In: Semin Immunol. 2017 Apr;32:3-13.

Kashef N., Abadi R.S.G., Djavid G.E., **Phototoxicity of phenothiazinium dyes against methicillin-resistant Staphylococcus aureus and multi-drug resistant Escherichia coli,** In: Photodiagnosis Photodyn Ther. 2012;9(1):11-15.

Kashef N., Yahyaei M., **Photodynamic inactivation decreases the minimal inhibitory concentration of antibiotics against Acinetobacter baumannii from patients with respiratory tract infections,** Laser in Medicine, 2014;11(3):13-18.

Kasivisvanathan V., Emberton M., Ahmed H.U., **Focal therapy for prostate cancer: rationale and treatment opportunities,** In: Clin Oncol (R Coll Radiol) 2013 Aug;25(8):461-473.

Kawczyk-Krupka A., Bugaj A.M., Latos W., Zaremba K., Wawrzyniec K., Sieroń A., **Photodynamic therapy in colorectal cancer treatment: The state of the art in clinical trials**, In: Photodiagn Photodyn Ther. 2015;12:545-553.

Kawczyk-Krupka A., Wawrzyniec K., Musiol S.K., Potempa M., Bugaj A.M., Sieroń A., **Treatment of localized prostate cancer using WST-09 and WST-11 mediated vascular targeted photodynamic therapy – a review,** In: Photodiagnosis Photodyn Ther. 2015 Dec;12(4):567-574.

Kim J., Santos O.A., Park J.H., **Selective photosensitizer delivery into plasma membrane for effective photodynamic therapy,** In: J Control Release 2014; 191: 98-104.

Kostic A.D., Chun E., Robertson L., **Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment,** In: Cell Host Microbe. 2013;14(2):207-215.

Kujundžić M., Vogl T., Stimac D., **A Phase II safety and effect on time to tumor progression study of intratumoral light infusion technology using talaporfin sodium in patients with metastatic colorectal cancer,** In: Journal of surgical oncology. 2007;96(6):518-524.

Kulik M., Nedelcu C., Martin F., Lebdai S., Rousselet M.C., Azzouzi A.R., **Post-treatment MRI aspects of photodynamic therapy for prostate cancer,** In: Insights Imaging. 2014 Dec;5(6):697-713.

Kwiatkowski S., Knap B., Przystupski D., Saczko J., Kędzierska E., Knap-Czop K., Kotlińska J., Michel O., Kotowski K., Kulbacka J., **Photodynamic therapy – mechanisms, photosensitizers and combinations,** In: Biomed Pharmacother. 2018;106:1098-1107.

Lampe J.W., **Dairy products and cancer,** In: J Am Coll Nutr. 2011;30(5 Suppl 1):464S-470S.

Lara-Tejero M., Galán J.E., **A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein,** In: Science. 2000;290:354-357.

Li X., Guo H., Tian Q., **Effects of 5-aminolevulinic acid-mediated photodynamic therapy on antibiotic-resistant staphylococcal biofilm: an in vitro study,** In: J Surg Res. 2013;184(2):1013-1021.

Liss M.A., White J.R., Goros M., Gelfond J., Leach R., Johnson-Pais T., **Metabolic biosynthesis pathways identified from fecal microbiome associated with prostate cancer,** In: Eur Urol. 2018;74:575-582.

Love W., Brundish D., Rhys-williams W., Feng X.D., Pugin B., **Porphyrin derivatives and their use in photodynamic therapy,** 2011:8084602.

Love W.G., Rhys-williams W., Brundish D., **Novel uses of porphyrin compounds,** 2007:20070167619.

Lui H., Anderson R.R., **Photodynamic therapy in dermatology Shedding a different light on skin disease,** In: Arch Dermatol. 1992;128(12):1631-1636.

Maclean M., MacGregor S.J., Anderson J.G. Woolsey G., **Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array,** In: Appl Environ Microbiol. 2009 75, 1932-1937.

Mahmoudi H., Bahador A., Pourhajibagher M., Alikhani M.Y., **Antimicrobial Photodynamic Therapy: An Effective Alternative Approach to Control Bacterial Infections,** In: J Lasers Med Sci. 2018;9(3):154-160.

Maisch T., Bosl C., Szeimies R.M., Lehn N., Abels C., **Photodynamic effects of novel XF porphyrin derivatives on prokaryotic and eukaryotic cells,** In: Antimicrob Agents Chemother. 2005;49(4):1542-1552.

Maisch T., Szeimies R.M., Jori G., Abels C., **Antibacterial photodynamic therapy in dermatology,** In: Photochem. Photobiol. Sci. 2004;3:907-917.

Malik Z., Hanania J., Nitzan Y., **Bactericidal effects of photoactivated porphyrins -- an alternative approach to antimicrobial drugs,** In: J Photochem Photobiol B. 1990;5:281-93.

Malik Z., Ladan H., Nitzan Y., **Photodynamic inactivation of Gram-negative bacteria: problems and possible solutions,** In: J Photochem Photobiol B. 1992;14:262-6.

Malik Z., Nitzan Y., **Effects of photoactivated hematoporphyrin derivative on bacteria and antibiotic resistance,** In: Microbios Lett. 1982;21:103-12.

Marchesi J.R., Dutilh B.E., Hall, **Towards the Human Colorectal Cancer Microbiome**, In: PLoS One. 2011;6(5):e20447-e20447.

Masood M.I., Qadir M.I., Shirazi J.H., Khan I.U., **Beneficial effects of lactic acid bacteria on human beings,** In: Crit Rev Microbiol. 2011;37:91-98.

Mehraban N., Freeman H.S., **Developments in PDT sensitizers for increased selectivity and singlet oxygen production,** In: Materials 2015; 8(7): 4421-4456.

Merchat M., Bertolini G., Giacomini P., Villanueva A., Jori G., **Meso-substituted cationic porphyrins as efficient photosensitizers of gram-positive and gram-negative bacteria,** In: J Photochem Photobiol B. 1996;32:153-7.

Minnock A., Vernon D.I., Schofield J., Griffiths J., Parish J.H., Brown S.T., **Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanine to photoinactivate both gram-negative and gram-positive bacteria,** In: J Photochem Photobiol B. 1996;32:159-64.

Miyabe M., Junqueira J.C., Costa A.C., Jorge A.O., Ribeiro M.S., Feist I.S., **Effect of photodynamic therapy on clinical isolates of *Staphylococcus* spp.,** In: Braz Oral Res. 2011;25(3):230-234.

Mohr H., Knuver-Hopf J., Gravemann U., Redecker-Klein A., Muller T.H., **West Nile virus in plasma is highly sensitive to methylene blue-light treatment,** In: Transfusion. 2004;44:886-890.

Moore C., Wallis C., Melnick J.L., Kuns M.D., **Photodynamic treatment of herpes keratitis,** Infect. Immun. 1972;5:169-171.

Morgenthaler J.B., Peters S.J., Cedeno D.L., Constantino M.H., Edwards K.A., Kamowski E.M., Passini J.C., Butkus B.E., Young A.M., Lash T.D., **Carbaporphyrin ketals as potential agents for a new photodynamic therapy treatment of leishmaniasis.** In: Bioorg. Med. Chem. 2008;16:7033-7038.

Mroz P., Yaroslavsky A., Kharkwal G.B., **Cell death pathways in photodynamic therapy of cancer,** In: Cancers 2011; 3(2): 2516-2539.

Murdoch L.E., Maclean M., Endarko E., MacGregor S.J., Anderson J.G., **Bactericidal effects of 405 nm light exposure demonstrated by inactivation of Escherichia, Salmonella, Shigella, Listeria, and Mycobacterium species in liquid suspensions and on exposed surfaces,** In: Scientific World Journal, 2012; 137805.

Musso G., Gambino R., Cassader M., **Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes,** In: Annu Rev Med. 2011;62:361-380.

Nakai Y., Nonomura N., **Inflammation and prostate carcinogenesis**, In: Int J Urol. 2013;20:150-160.

Nakonechny F., Firer M.A., Nitzan Y., Nisnevitch M., **Intracellular antimicrobial photodynamic therapy: a novel technique for efficient eradication of pathogenic bacteria,** In: Photochem Photobiol. 2010;86(6):1350-1355.

Nathan T.R., Whitelaw D.E., Chang S.C., Lees W.R., Ripley P.M., Payne H., **Photodynamic therapy for prostate cancer recurrence after radiotherapy: a phase I study.** In: J Urol. 2002 Oct;168(4 Pt 1):1427-1432.

Nelson J.B., **Perspectives on the clinical management of localized prostate cancer**, In: Asian J Androl. 2014 Jul-Aug;16(4):511-514.

Nikaido H., **Prevention of drug access to bacterial targets: Permeability barriers and active efflux,** In: Science, 1994;264:382-388.

Nir U., Ladan H., Malik Z., Nitzan Y., **In vivo effects of porphyrins on bacterial DNA,** In: J Photochem Photobiol B. 1991;11:295-306.

Nitzan Y., Gutterman M., Malik Z., Ehrenberg B., **Inactivation of gram-negative bacteria by photosensitized porphyrins,** In: Photochem Photobiol. 1992;55:89-96.

Nitzan Y., Shainberg B., Malik Z., **Photodynamic effects of deuteroporphyrin on Gram positive bacteria,** In: Curr Microbiol. 1987;15:251-8.

Nordmann P., Poirel L., Toleman M.A., Walsh T.R., **Does broad-spectrum beta-lactam resistance due to NDM-1 herald the end of the antibiotic era for treatment of infections caused by Gram-negative bacteria?,** In: J Antimicrob Chemother. 2011;66:689-92.

Olivo M., Bhuvaneswari R., Lucky S.S., **Targeted therapy of cancer using photodynamic therapy in combination with multi-faceted anti-tumor modalities,** In: Pharmaceuticals 2010; 3(5): 1507-1529.127.

Olson M.E., Allan N.D., **Natural Photodynamic Agents and their use,** 2010:20100266716.

Pearce M,M., Hilt E,E., Rosenfeld A,B., **The female urinary microbiome: a comparison of women with and without urgency urinary incontinence,** In: MBio. 2014;5:e01283.

Pereira C.A., Romeiro R.L., Costa A.C., Machado A.K., Junqueira J.C., Jorge A.O., **Susceptibility of *Candida albicans, Staphylococcus aureus*, and *Streptococcus mutans* biofilms to photodynamic inactivation: An *in vitro* study,** In: Lasers Med. Sci. 2011;26:341-348.

Perlin M., Mao J.C., Otis E.R., Shipkowitz N.L., Duff R.G., **Photodynamic inactivation of influenza and herpes viruses by hematoporphyrin,** In: Antivir. Res. 1987;7:43-51.

Plottel C.S., Blaser M.J., **Microbiome and malignancy,** In: Cell Host Microbe. 2011;10:324-335.

Portilho F.A., de Oliveira Cavalcanti C.E., Miranda-Vilela A.L., **Antitumor activity of photodynamic therapy performed with nanospheres containing zinc-phthalocyanine,** In: J Nanobiotechnology 2013; 11(1): 41.

Pourhajibagher M., Chiniforush N., Shahabi S., Ghorbanzadeh R., Bahador A., **Sub-lethal doses of photodynamic therapy affect biofilm formation ability and metabolic activity of *Enterococcus faecalis*,** In: Photodiagnosis Photodyn Ther. 2016;15:159-166.

Poutahidis T., Cappelle K., Levkovich T., Lee C.W., Doulberis M., Ge Z., **Pathogenic intestinal bacteria enhance prostate cancer development via systemic activation of immune cells in mice,** In: PLoS One. 2013;8:e73933.

Punnen S., Hardin J., Cheng I., Klein E.A., Witte J.S., **Impact of meat consumption, preparation, and mutagens on aggressive prostate cancer,** In: PLoS One. 2011;6:e27711.

Raab O., **On the effect of fluorescent substances on infusoria,** In: Z Biol. 1900;39:524-6.

Ragas X., Dai T., Tegos G.P., Agut M., Nonell S., Hamblin M.R., **Photodynamic inactivation of Acinetobacter baumannii using phenothiazinium dyes: *in vitro* and *in vivo* studies,** In: Lasers Surg Med. 2010;42(5):384-390.

Ridlon J.M., Ikegawa S., Alves J.M., Zhou B., Kobayashi A., Iida T., **Clostridium scindens: a human gut microbe with a high potential to convert glucocorticoids into androgens,** In: J Lipid Res. 2013;54:2437-2449.

Roblero-Bartolon G.V., Ramon-Gallegos E., **Use of nanoparticles (NP) in photodynamic therapy (PDT) against cancer,** In: Gac Med Mex 2015; 151: 85-98.

Roncucci G., Dei D., Giuntini F., Chiti G., Nistri D., Fantetti L., Paschetta V., Cocchi A., **Meso-substituted porphyrins**, 2011:7947674.

Routy B., Le Chatelier E., Derosa L., **Gut microbiome influences efficacy of PD-1–based immunotherapy against epithelial tumors,** In: Science. 2018;359(6371):91-97.

Sachdeva A., van der Meulen JH., Emberton M., Cathcart P.J., **Evaluating variation in use of definitive therapy and risk-adjusted prostate cancer mortality in England and the USA,** In: BMJ Open. 2015 Feb 24;5(2):e006805.

Sahu Sharma Sharma P., **Effect of poly-L-lysine-chlorin P6-mediated antimicrobial photodynamic treatment on collagen restoration in bacteria-infected wounds**, In: Photomed Laser Surg. 2014;32(1):23-29.

Sha Ni, Stefil Dixon, Mouraviev V., **The human gastrointestinal microbiota and prostate cancer development and treatment**, In: Investig Clin Urol. 2020 Feb;61(Suppl 1):S43-S50.

Shang F.M., Liu H.L., **Fusobacterium nucleatum and colorectal cancer: A review,** In: World journal of gastrointestinal oncology, 2018;10(3):71.

Sharma M., Visai L., Bragheri F., Cristiani I., Gupta P.K., Speziale P., **Toluidine blue-mediated photo-dynamic effects on staphylococcal biofilms,** In: Antimicrob Agents Chemother. 2008;52(1):299-305.

Sharma S.K., Chiang L.Y., Hamblin M.R., **Photodynamic therapy with fullerenes *in vivo*: reality or a dream?,** In: Nanomedicine (Lond) 2011;6:1813-25.

Shishkova N., Kuznetsova O., Berezov T., **Photodynamic therapy in gastroenterology,** In: J Gastrointest Cancer, 2013;44:251-259.

Siegel R.L., Miller K.D., Jemal A., **Cancer statistics, 2017,** In: CA Cancer J Clin. 2017 Jan;67(1):7-30.

Siegel R.L., Miller K.D., Jemal A., **Cancer statistics, 2019**, In: CA Cancer J Clin. 2019;69:7-34.

Sobczyński J., Tønnesen H.H., Kristensen S., **Influence of aqueous media properties on aggregation and solubility of four structurally related meso-porphyrin photosensitizers evaluated by spectrophotometric measurements,** In: Pharmazie 2013; 68(2): 100-109.

Soncin M., Fabris C., Busetti A., Dei D., Nistri D., Roncucci G., **Approaches to selectivity in the Zn(II)-phthalocyanine-photosensitized inactivation of wild-type and antibiotic-resistant *Staphylococcus aureus,*** In: Photochem Photobiol Sci. 2002;1:815-9.

Sonn G.A., Aronson W., Litwin M.S., **Impact of diet on prostate cancer: a review,** In: Prostate Cancer Prostatic Dis. 2005;8:304-310.

Soukos N.S., Ximenez-Fyvie L.A., Hamblin M.R., Socransky S.S., Hasan T., **Targeted antimicrobial photochemotherapy,** In: Antimicrob Agents Chemother. 1998;42(10):2595-2601.

Sperandio F.F., Huang Y.Y., Hamblin M.R., **Antimicrobial Photodynamic Therapy to Kill Gram-negative Bacteria,** In: Recent Pat Antiinfect Drug Discov. 2013 Aug;8(2):108-20.

St Denis T.G., Dai T., Hamblin M.R., **Killing bacterial spores with blue light: when innate resistance meets the power of light,** In: Photochem Photobiol 2013; 89(1): 2-4.

Tang H.M., Hamblin M.R., Yow C.M., **A comparative in vitro photoinactivation study of clinical isolates of multidrug-resistant pathogens,** In: J Infect Chemother. 2007;13(2):87-91.

Tegos G.P., Anbe M., Yang C., Demidova T.N., Satti M., Mroz P., **Protease-stable polycationic photosensitizer conjugates between polyethyleneimine and chlorin(e6) for broad-spectrum antimicrobial photoinactivation,** In: Antimicrob Agents Chemother. 2006;50:1402-10.

The Global Cancer Observatory. Cancer fact sheets, In: Lyon: World Health Organization; 2019.

Thursby E., Juge N., **Introduction to the human gut microbiota,** In: Biochem J. 2017;474(11):1823-1836.

Tjalsma H., Boleij A., Marchesi J.R., Dutilh B.E., **A bacterial driver-passenger model for colorectal cancer: Beyond the usual suspects,** In: Nat Rev Microbiol. 2012;10(8):575-582.

Tulstrup M.V., Christensen E.G., Carvalho V., Linninge C., Ahrné S., Højberg O., **Antibiotic treatment affects intestinal permeability and gut microbial composition in Wistar rats dependent on antibiotic class,** In: PLoS One. 2015;10:e0144854.

Vaara M., Vaara T., **Polycations sensitize enteric bacteria to antibiotics,** In: Antimicrob Agents Chemother. 1983;24:107-13.

Varol M., **An alternative treatment modality of diseases using photodynamic therapy with a wide range biological targeting possibility,** In: J Biol 2015; 3: 21-25.

Venezio F.R., DiVincenzo C., Sherman R., Reichman M., Origitano T.C., Thompson K., **Bactericidal effects of photoradiation therapy with hematoporphyrin derivative,** In: J Infect Dis. 1985;151:166-9.

Wainwright M., Phoenix D.A., Laycock S.L., Wareing D.R., Wright P.A., **Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of *Staphylococcus aureus,*** In: FEMS Microbiol Lett. 1998;160:177-81.

Wan M.T., Lin J.Y., **Current evidence and applications of photodynamic therapy in dermatology,** In: Clin Cosmet Investig Dermatol 2014; 7(1): 145-163.

Wang L., Yang H., Li B., **Photodynamic therapy for prostate cancer: a systematic review and meta-analysis,** In: Prostate Int. 2019;7(3):83-90.

Wang S., Bromley E., Xu L., Chen J.C., Keltner L., **Talaporfin sodium. Expert opinion on pharmacotherapy,** 2010;11(1):133-140.

Wang Y., Murray C.K., Hamblin M.R., Hooper D.C., Dai T., **Antimicrobial blue light inactivation of pathogenic microbes: State of the art,** In: Drug Resist Updat. 2017 33-35, 1-22.

Windahl T., Andersson S.O., Lofgren L., **Photodynamic therapy of localized prostatic cancer,** In: Lancet. 1990 Nov 3;336(8723):1139.

Yoo J.O., Ha K.S., **New insights into the mechanisms for photodynamic therapy-induced cancer cell death,** In: Int Rev Cell Mol Biol 2012; 295: 139-174.

Zhu T.C., Finlay J.C., Hahn S.M., **Determination of the distribution of light, optical properties, drug concentration, and tissue oxygenation *in-vivo* in human prostate during motexafin lutetium-mediated photodynamic therapy,** In: J Photochem Photobiol B. 2005 Jun 1;79(3):231-241.

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

Application of *Clostridium* spp. and their toxins/enzymes in treatment of oncologic and other pathologies

***Zygmunt Gofron, Monika Kabała, Gajane Martirosian**

Śląski Uniwersytet Medyczny w Katowicach, Wydział Nauk Medycznych w Kato-wicach, ul. Medyków 18, 40-752 Katowice

***Corresponding author: zygmuntgofron@gmail.com, Zygmunt Gofron, Katedra i Zakład**

Mikrobiologii Lekarskiej WNMK SUM, ul. Medyków 18, 40-752 Katowice

ABSTRACT

Clostridium is a genus of Gram-positive bacteria including several significant human pathogens. Bacteria are obligate anaerobes capable of producing spores. Bacterial toxins are responsible, among others for botulism, tetanus, gas gangrene and gastrointestinal tract infections. However, after years it turned out that bacteria of the *Clostridium* genus, their toxins and enzymes may be used in modern medicine. Botulinum toxin nowadays is used commercially for several medical and cosmetic purposes. Collagenase *Clostridium histolyticum* (CCH) is an enzyme, that dismantles collagen. CCH is used as for the treatment of connective tissue diseases such as Dupuytren's contracture and Peyronie's disease. Bacteria from *Clostridium* genus and their toxins and enzymes found also application in oncology. In 1813, Vautier reported regression of cancer in patients with gangrene, caused by *Clostridium perfringens*. Hypoxia is a pathophysiological feature in the majority of solid tumours. Research show also that it is possible to use *Clostridium* spp. for biotechnology purposes in oncology. A recombinant produced C-terminus of the *C. perfringens* enterotoxin was conjugated to gold Nanoparticles to produce a C-CPE-AuNP complex, which can be later used for killing tumor cells with laser. Similarily the *C. butyricum* strain has been successfully used in the therapy of colorectal cancer in the mouse model. It can reduce the expression of inflammatory cytokines, which are vital in carcinogenesis process. Also *C. novyi* – NT with iron oxide found application in modern imaging of solid tumors in Magnetic Resonance Imaging (MRI). Also *C. novyi*- NT spores can be successfully labeled with iron oxide nanoparticles for MR imaging. Bacteria of the genus *Clostridium* have been an important clinical problem for centuries. In this review we will discuss the application of *Clostridium spp*. and their toxins/enzymes in medicine, especially in treatment of oncologic and other pathologies in the light of medical literature, as well as our own experiences.

INTRODUCTION

Clostridium is a species of Gram-positive obligate anaerobes, capable of producing spores. The bacterial reservoir is soil and digestive system of human and animals.The bacterial malignancy factors are toxins, produced most often in the intestinal lumen, wounds or soft tissues. Bacterial toxins are responsible, among others for botulism (*C. botulinum*), tetanus (*C. tetani*), gas gangrene and gastrointestinal tract infections (*C. perfringens*) and symptomatic *Clostridioides difficile* infections (CDI), which can cause symptoms such as abdominal pain, diarrhea and fever. This is also the most common cause of antibiotic associated diarrhea. Clostridial infection may be lethal. In this review we will discuss the application of *Clostridium spp*. and their toxins/ enzymes in medicine, especially in treatment of oncologic and other pathologies in the light of medical literature.

HISTORY: WHAT WE KNOW ABOUT DEVELOPMENT CLOSTRIDIAL INFECTIONS?

Through ages clostridial infections causing gas gangrene were serious treat for surgeons. But it wasn't until the first half of the 20th century that William Welch discovered the etiological factor of gas gangrene. Welch was an American physician, pathologist and bacteriologist. He was precursor of modern medicine named one of the "Big Four" founding professors at the Johns Hopkins Hospital and also the founder of the Johns Hopkins School of Hygiene and Public Health, the first School of Public Health in the USA. Welch's research was principally in bacteriology, and he is the discoverer of the microorganism that causes gas gangrene. It was named *Clostridium welchii* in recognition of that fact, but now the microorganism is known as *Clostridium perfringens.*

C. botulinum was first recognized and isolated in 1895 by Emile van Ermengem from home – made ham associated with a botulism outbreak. The isolate was originally named *Bacillus botulinus*, after the Latin word for sausage, *botulus* ("sausage poisoning" was a common problem in XVIII/XIX century Europe most likely caused by botulism toxin).

First descriptions of tetanus in Hippocrates' Aphorisms dated to IV century BCE. In 1889, *C. tetani* was isolated from a patient by Kitasato Shibasaburō. Moreover he later proved that the disease is caused by toxins produced by bacteria.

C. histolyiticum was first isolated in 1916 by Weinberg and Séguin. They discovered bacterial cultures may cause extensive local

tissue destruction, splitting of the skin and sometimes autoamputation in human body. In 1923 Bergey, Harrison, et al. reclassified it as *Clostridium histolyticum*.

Clostridium butyricum is uncommonly reported as a human pathogen and is widely used as a probiotic in Asia (particularly in Japan and China). First strains were isolated in the 1930s in Japan from soil. For over 60 years *C. butyricum* was used as a probiotic in Japanese hospitals, especially during an antibiotic therapy with strong antibiotics (eg III generation cephalosporins or Levofloxacin).

Clostridioides (Clostridium difficile was first isolated from the stool of a healthy infant by Hall and O'Toole in 1935. It was not recognized as a pathogen until 1978, however, that George and colleagues associated *C. difficile* with human disease and discovered that *C. difficile* was the microorganism responsible for the majority of cases of anti-biotic-associated diarrhea in the developed countries. The species was transferred from the genus *Clostridium* to *Clostridioides* in 2016, thus giving it the binomial *Clostri-dioides difficile*. This new name reflects the differences in taxonomy between this species and members of the genus *Clostridium*.

MEDICAL APPLICATION OF *CLOSTRIDIUM* SPP. AND THEIR TOXINS/ENZYMES

However, after years it turned out that bacteria of the genus *Clostridium*, their toxins and enzymes are used not only in cosmetic purposes but in modern medicine as well. We currently use clostridial toxins and enzymes, but also biotechnologically modified microorganisms for clinical purposes.

Botulinum toxin (BTX), produced by the bacterium *Clostridium botulinum* and related species, is a neurotoxic polypeptide protein. It prevents the release of the neuro-transmitter acetylcholine from axon endings at the neuromuscular junction and thus causes flaccid paralysis in mechanism of localized reduction of muscle activity by inhibiting acetylcholine release at the neuromuscular junction. In 2004, the US Food and Drug Administration approved its application in the treatment of various medical conditions, such as facial wrinkles, strabismus, cervical dystonia, blepharospasm, and hyperhidrosis. Since then crowds of patients could take advantage of this method. The toxin nowadays is more widely used both for medical and cosmetic purposes. In neurology it is used for treatment of disease's connected with muscle spasticity such as head and neck (dystonia), vocal cords and jaw spasm. Dystonia is a condition characterized by involuntary muscle contraction in one or more regions. It may be idiopatic or secondary to other neurologic conditions. Many patients complains the pain accompanying the disease. Current medical strategies consist of oral treatment, behavioral modification of lifestyle, exercises and BTX injection, where intermittent injections were found superior to the above treatment (Grazzi, 2014). It is not only effective and well- tolerated treatment but also BTX reduces concomitant pain.

Also chronic migraine (CM) reductant for other treatment methods is an indication for botulinum use. CM affects 1,4-2,2 % of the population (Diener, 2004). In the treatment protocols NSAIDs and triptans play the greatest role. Recent studies show that Onabotulinum toxin A is a safe, well-tolerated and effective headache prophylactic treatment for CM (Blumenfeld, 2010). The mechanism of action is still not well recognized although it is suggested that toxin inhibits the release of the neuro-transmitters responsible for neural inflammation (Aoki, 2005). Recent studies show encouraging results as the Onabotulinum toxin A seems to be efficient especially in patients who do not tolerate oral preventive drugs (Grazi, 2014).

Strabismus is either intermittent or persistent deviation of ocular alignment. It may have many underlying causes among which abnormal anatomical development of extraocular muscles, impaired neurological input to extraocular muscles, uncorrected refractive error or hereditary factors are the most common ones. There are various treatments associated with strabismus eg. orthoptic exercises and ophthalmic surgery. BTX use in strabismus is modern alternative treatment in this indication. BTX injection temporarily paralyses the extraocular muscle and results in a changed ocular alignment that persists over time (usually after 2-3 months) (Bunting, 2013). Once a muscle is paralysed, opposing muscles take on a greater movement force and the eye position changes allowing the visual axes to move into a straighter eye alignment (Bunting, 2013)

Important is the fact that BTX found also application in the urology field. As it is effective treatment for overactive smooth muscles,

overactive bladder (OAB) reductant for behavioral therapy and oral medications (alfa-blockers, beta-3-agonist) may be treated with it. For many patients with urinary incontinence on the basis of OAB the BTX injection therapy may be the only solution as injected directly to bladder detrusor causes muscle relaxation, weakens the muscle and brings temporary relief (generally up to 6). Side effects may include urinary tract infections and urinary retention that may require catherization. Despite this treatment method is find safe and effective. Also lower urinary tract symptoms (LUTS) associated with benign prostatic hyperplasia (BPH) may be treated in minimally invasive therapy with BTX injections directly to prostate during cystoscopy. This method may be recommended for patients who do not will or cannot undergo surgical procedures (Transurethral resection or enucleation of prostate), due to comorbidity. Intraprostatic injections induces smooth muscle relaxation and atrophy of the prostate gland which result in diminishing LUTS (Chuang, 2006).

Last but not least BTX is used for prevention of facial wrinkles and for excessive sweating therapy in cosmetology and broadly understood anti-aging medicine. Wrinkles are the effect of hyperfunction of muscular action of facial muscles. The BTX mechanism of action makes it an ideal agent in prevention of facial wrinkles (Blitzer, 1997). BTX injections decrease facial muscles activity thus smooth the facial skin and diminishes facial lines. Sweat glands activity is regulated via parasympathetic synapses, thus Ach release may be blocked by BTX, drastically reducing sweat production. Hyperhidrosis results from overactivity of sweats glands based on central dysregulation of autonomic neural system. If topical (stronger antiperspirants, aluminum hydroxide) or systemic (anticholinergics) remedies fail to help, BTX is highly effective therapy (Kreyden, 1997). Injections must be applied to each armpit or hand depending on affected area, resulting in 3-6 months period of time free of the symptoms (Naumann, 1998)

Collagenase C*lostridium histolyticum* (CCH) on the other hand, is an enzyme produced by the bacterium *C. histolyticum* that dismantles collagen. It is used as for the treatment of connective tissue diseases such as Dupuytren's contracture (DC), a condition where the fingers bend towards the palm and cannot be fully straightened, and Peyronie's disease (PD), a connective tissue disorder caused by the growth of fibrous plaques in the soft tissue of the penis.

Dupuytren's disease is nonmalignant fibromatosis of the fibrous skeleton of the hand manifesting in a progressive flexion contracture of the finger. It is more common in developed countries especially among older people (Langer, 2017). Alcohol abuse, diabetes and vibration disease take part in disease etiology. Although surgical methods has been used for many years it was the development of CCH that modernized disease treatment. Surgical treatment is characterized by large number of side effects and undesirable events such as poor healing of the wound (especially in diabetic patients), infections, paresthesia and neurovascular injuries (Denkler, 2010). The effectiveness and safety of CCH use in DC was confirmed in two placebo controlled trials Collagenase Option for the Reduction of Dupuytren's I and II (CORD I and CORD II) (Keller, 2017). Following researches confirmed effectiveness and safety of the procedure with no serious side effects (Rohit, 2019).

As mentioned above PD is a connective tissue disease that affects tunica albuginea of the penis characterized by the plaques deforming penis (because of the penile curvature). Although many treatment options has been described (oral treatment, surgery, Extracorporeal Shock Wave Therapy – ESWT), CCH is the most up to date therapy that represents the most promising advance in the treatment of PD in long time (Jordan, 2014). Effectively used to successfully treat Dupuytren's contracture found an application in PD after a while. Patient are given a series of intralesional injections associated with traction therapy with vacuum devices. Overall effects of the treatment are very good despite the fact that patients with significant curvature may not achieve functionally satisfactory results and may need to undergo surgery (Ralph, 2010).

But Clostridium group bacteria found application also in oncology. Historically it was Vautier who reported in 1813 regression of cancer in patients with gangrene, and in such individuals, the tumour was found to be infected with *Clostridium spp.* (Wei, 2007). Later, it was shown that the causative agent was the bacterium *Clostridium perfringens.* Hypoxia is a pathophysiological feature in the majority of solid tumours. Hypoxic areas in poorly vascularized tumours are main barriers to successful cancer therapy. The blood vessels in tumours are structurally and functionally

abnormal, resulting in unsettled blood supply. The hypoxic microenvironment in solid cancers is ideal for multiplication of anaerobic bacteria. *Clostridium spp*. was shown to cause tumour regression in a rodent model (Roberts, 2014). However, in subsequent clinical studies in human populations, significant therapeutic effect was not demonstrated. Research show also that it is possible to use clostridium for biotechnology purposes in oncology. A recombinant produced C-terminus of the *C. perfringens* enterotoxin was conjugated to gold Nanoparticles to produce a C-CPE-AuNP complex (Becker, 2018). By binding to claudins, the C- CPE should allow to target the AuNPs onto the claudin expressing tumor cells for a subsequent cell killing by application of the gold nanoparticle-mediated laser perforation technique. Furthermore cells without claudin expression (not showing cancer morphology) were spared in treatment. Observations show that C-CPE can be used to functionalize gold nanoparticles in order to specifically and efficiently kill a broad spectrum of claudin expressing tumor cells. The area of oncology treatment using microbiology techniques may require further researches.

The *C. butyricum* strain has been successfully used in the therapy of colorectal cancer (CRC) in the mouse model. Among other, inflammations it is one of the CRC risk factor. Toll Like Receptors (TLRs) are significantly upregulated in intestinal epithelial cells in colitis. The activation of TLRs pathways activates the transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and participates in intestinal tumorogenesis. Chen *et al.* demonstrated that *C. butyricum* can reduce the expression of Toll Like Receptor 4 and NF-κB. In addition, *C. butyricum* can reduce Th17 cell populations, which are vital in carcinogenesis process although its role is not yet understood in spleen. Moreover, the role of *C. butyricum* as an antitumour factor through induction of apoptosis, inhibition of tumour growth and improvement of immune response was described.

Noteworthy is the fact that bacterium is widely used as a probiotic in Asia (particularly in Japan and China), especially during an antibiotic therapy with strong antibiotics (eg III generation cephalosporins or Levofloxacin). Simultaneously prevalence of CRC in Asian countries are much lower than in Western countries (Wong, 2019). Of course etiology of CRC is complex yet in the light of the up to date research, further clinical trials on human model are required.

Clostridium novyi strain NT is bioengineered strain that lacks pathogenic toxins. Due to their affinity to oxygen-depleted parts it can selectively colonize hypoxic regions (eg solid tumors). Labeling *C. novyi* NT with iron oxide found application in modern imaging of solid tumors in Magnetic Resonance Imaging (MRI). *C. novyi* strain NT spores can be successfully labeled with iron oxide nanoparticles for MR imaging in animal model. Noteworthy is the fact that in many animal models intravenous infusion of the spores resulted in tumor size reduction and increased necrosis in histologic preparations. This leaves a chance for the above strain to be used in targeted oncologic therapy based on genetically modified microorganism (GMO) in the future.

Also, the role of *C. difficile* in the wider application of fecal microbiota transplantation (transfer) (FMT) cannot be ignored in the above study. It is the *Clostridioides* infections resistant to conservative treatment that gave impact to the use of this method among others in oncological patients. The oncological patients often have weakened immunity as a result of the specific treatment and the underlying diseases required prolonged antibiotic therapy, which may result in the development of CDI.

DISCUSSION/CONCLUSION

Bacteria of the genus Clostridium have been an important clinical problem for centuries. The eighteenth and nineteenth centuries brought the discovery of etiological factors of the *Clostridium spp*. as pathogens. Even nowadays *Clostridioides difficile* and *C. perfringens* are a real challenge for the health care of many countries. However, over time, we've learned to use *Clostridium spp*. for commercial purposes, including cosmetology and medicine. This mainly applies to the use of botulinum in neurology and anti aging medicine. Also Collagenase *Clostridium histolyticum* (CCH) in the treatment of connective tissue diseases including Peyronie's and Dupuytren's disease therapy. Opportunities for their use in biotechnology and oncological treatment are opening up and the first discoveries often related to animal models encourage further research in this direction.

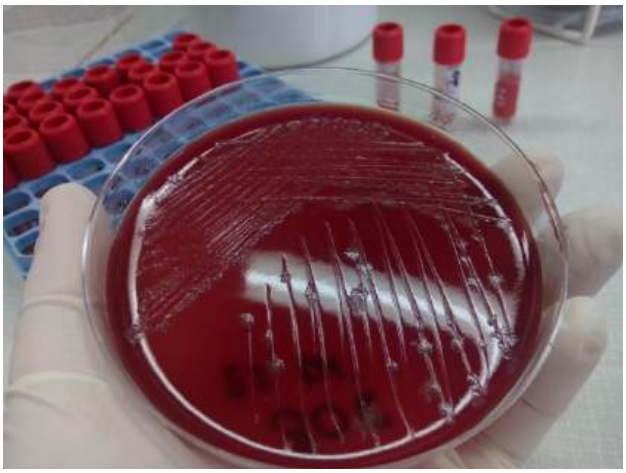

Figure 1. Picture of *Clostridium* sp. colony (photograph taken by M. Kabała)

REFERENCES

Aoki K.R., **Review of a proposed mechanisms for the antinociceptive action of botulinum toxin type A**, In: Neurotoxicology 2005; 26: 785-793.

Aoki K.R., **Evidence for antinociceptive activity of botulinum toxin type A in pain management**, In: Headache 2005; 43: 9-15.

Arora R., Angermann P., Aspalter P., Binter A., Deml Ch., Danninger R., et al., **Prospective observation of Clostridium histolyticum collagenase for the treatment of Dupuytren's disease in 788 patients: the Austrian register**, In: Arch Orthop Trauma Surg. 2019; 139: 1315-1321.

Becker A., Leskau M., Schlingmann-Molina B., Hohmeier S.C., Alnajjar S., et al., **Functionalization of goldnanoparticles by the *Clostridium perfringens* enterotoxin C-terminus for tumor cell ablation using the gold nanoparticle-mediated laser perforation technique**, In: Scientific Reports 2018; 8: 1-15.

Blitzer A., Binder W.J., Aviv J.E., Keen M.S., Brin M.F., **The management of hyperfunctional facial lines with botulinum toxin**, In: Arch Otolaryngol Head Neck Surg 1997; 123: 389-392.

Blumenfeld A., Silberstein S., Dodick D., Aurora S., Turkel C., Binder W., **Method of injection of Onabotulinum toxin A for chronic migraine: a safe, well-tolerated and effective treatment paradigm based on the PREEMPT clinical program**, In: Headache 2010; 50: 1406-1418.

Bunting H.J., Dwason E.L., Lee J.P., Adams G.G., **Role of inferior rectus botulinum toxin injection in vertical strabismus resulting from orbital pathology**, In: Strabismus. 2013; 21(3): 165-8.

Chen D., Jin D., Huang S., Wu J., Xu M., et al., **Clostridium butyricum, a butyrate-producing probiotic, inhibits intestinal tumor development through modulating Wnt signaling and gut microbiota**, In: Cancer Lett. 2020; 469: 456-467.

Chuang Y.C., Chiang P.H., Huang C.C., Yoshimura N., Chancellor M.B., **Botulinum toxin type A improves benign prostatic hyperplasia symptoms in patients with small prostates**, In: Urology 2005; 66: 775-779.

Chuang Y.C., Chiang P.H., Yoshimura N., De Miguel F., Chancellor M.B., **Sustained beneficial effects of intraprostatic botulinum toxin type A on lower urinary tract symptoms and quality of life in men with benign prostatic hyperplasia**, In: BJU Int. 2006; 98: 1033-1037.

Crouch E.R., **Use of botulinum toxin in strabismus**, In: Current Opinion in Ophthalmology 2006;17(5):435-40.

Denkler K., **Surgical complications associated with fasciectomy for dupuytren's disease: a 20-year review of the English literature**, In: Eplasty 2010; 27: 10-15.

Diener H.C., Limmroth V., **Medication-overuse headache: a worldwide problem**, In: Lancet Neurol. 2004; 3: 475-483.

Fagien S., **Botox for the treatment of dynamic and hyperkinetic facial lines and furrows: Adjunctive use in facial aesthetic surgery**, In: Plast Reconstr Surg 1999; 103: 701-713.

Hahn P., **Epidemiology of Dupuytren's disease**, In: Orthopade 2017; 46: 298-302.

Jordan G.H., Carson C.C., Lipshultz L.I., **Minimally invasive treatment of Peyronie'sdisease: evidence-based progress**, In: BJU Int 2014; 114: 14-24.

Keller M., Arora R., Schmiedle G., Kastenberger T., **Treatment of Dupuytren's disease with collagenase Clostridium histolyticum**, In: Orthopade 2017; 46: 321-327.

Kuo H.C., **Prostate botulinum A toxin injection – An alternative treatment for benign prostatic obstruction in poor surgical candidates**, In: Urology 2005; 65: 670-674.

Langer M.F., Grunert J., Unglaub F., Wieskotter B., Oeckenpohler S., **The fibrous skeleton of the hand: Changes with Dupuytren's contracture**, In: Orthopade 2017; 46: 303-314.

Grazzi L., Usai S., Bragg D.C., Sharma N., **Botulinum toxin A: a new option for treatment of chronic migraine with medication overuse**, Update on treatments for dystonia. In: Neurol Neurosci Rep. 2014; 14: 454.

McVary K.T., Roehrborn C.G., Chartier-Kastler E., Efros M., Bugarin D., Chen R., **A multicenter, randomized, double-blind, placebo controlled study of BoNT-A 200 U to treat lower urinary tract symptoms in men with benign prostatic hyperplasia**, In: J. Urol. 2014; 192: 150-156.

Naumann M., Bergmann I., Hofmann U., Hamm H., Reiners K., **Botulinum toxin for focal hyperhidrosis: Technical considerations and improvements in application (letter)**, In: Br J Dermatol 1998; 139: 1123-1124.

Ralph D., Gonzalez- Cadavid N., Mirone V., **The management of Peyronie'sdisease: evidence-based 2010 guidelines,** In: J Sex Med 2010; 7: 2359-2374.

Roberts N.J., Zhang L., Janko F., Collins A., Bai R.Y., Staedtke V., et al., **Intratumoral injection of *Clostridium* novyi-NT spores induces antitumor responses,** In: Sci. Transl Med. 2014; 13: 249.

Rodrigo J.J., Niebauer J.J., Brown R.L., Doyle J.R., **Treatment of Dupuytren's contracture. Long-term results after fasciotomy and fascial excision**, In: J Bone Joint Surg Am 1976; 58: 380-387.

Wei M.Q., Mengesha A., Good D., Anné J., **Bacterial targeted tumour therapy – dawn of a new era**, In: Cancer Lett 2008; 259: 16-27.

Wong M., Ding H., Wang J., Chan P., Huang J., **Prevalence and risk factors of colorectal cancer in Asia**, In: Intest Res. 2019, 17(3): 317-329.

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

Computational analysis of AP-2γ role in bladder cancer

Damian Kołat[1#*], Żaneta Kałuzińska[1#], Elżbieta Płuciennik[1]

[1] Department of Molecular Carcinogenesis, Medical University of Lodz, 90-752 Lodz, Poland

[#] These authors contributed equally to the work

[*] Corresponding author: Damian Kołat, Department of Molecular Carcinogenesis, Medical University of Lodz, e-mail: damian.kolat@stud.umed.lodz.pl

ABSTRACT

AP-2γ protein is a member of AP-2 transcription factors family, which participate in early developmental processes. Its oncogenic function has been confirmed in few cancers (e.g. breast, skin or ovarian) whereas the role in bladder cancer (BLCA) is not well understood. Nowadays, disruptions in pathways such as FGFR3/RAS or TP53/RB1 are the best known, yet new genes that would allow a better understanding of this heterogeneous tumor are being researched. The current literature indicates the promoting effect of AP-2γ encoding gene (*TFAP2C*) on distant recurrence or tumor growth. Similar relationship, according to which increase in *TFAP2C* expression significantly worsen prognostic endpoints, was observed by performing GEPIA on bladder cancer cohort. Using UALCAN platform, we noticed *TFAP2C* expression increase in bladder cancer compared to normal tissue. To assess the role of this gene in BLCA, GSEA analysis was performed regarding the AP-2γ targets on *TFAP2C* "high" and "low" groups. Beside interesting patterns related to groups distinction by genes whose expression profile is specific to BLCA, we found other curious results which we implemented for DAVID bioinformatic resources. Using functional annotation, we described selected gene sets having highest statistical significance. For *TFAP2C* high group we observed descriptors of many signaling pathways (e.g. PI3K-AKT, MAPK, ERBB) but also processes e.g. loss of adhesion, regulation of apoptosis, proliferation or cell cycle. For *TFAP2C* low group we matched the processes guided by miRNA pathways, verifying the literature data of AP-2γ interaction with RNA molecules. Within such, descriptions of processes revealed implication in cell differentiation, positive cell-to-cell communication or transcriptional misregulation in cancer. Conclusively, our analyzes outline the distinct consequences of various *TFAP2C* levels, simultaneously indicating the need for deeper reflection on the subject of *TFAP2C* participation in the BLCA.

INTRODUCTION

BLADDER CANCER

EPIDEMIOLOGY

Bladder Carcinoma (BLCA) is the tumor whose incidence has been decreasing over recent years. It is more common in men and is concurrently the fourth cancer in the frequency of occurrence among them (Cassell, 2019). However, cancer-related deaths are stable in men, whereas slightly reduced over the last years in women. According to the American Cancer Society, estimates for the year 2020 concerns about 81,400 new cases of cancer within which 17,980 as fatal (American Cancer Society, 2020). Regarding the newest Polish statistics, in 2017 the incidence of new bladder cancer cases exceeded 5,770 among men (Wojciechowska, 2017).

CLASSIFICATION

Using information from the https://www.cancer.gov website, the bladder cancer can be divided into transitional and squamous cell carcinomas, or adenocarcinoma. These three types are established depending on which cells lining the bladder are transfor-ming into cancerous. Apart from the classical histological division, along with the development of techniques in a branch of biochemistry, it is also possible to distinguish molecular types. University of North Carolina (UNC) demarcates luminal or basal bladder cancer. In contrast, three groups (basal, luminal and TP53-like) are dis-tinguished by MD Anderson Cancer Center (MDA) and five groups by The Cancer Genome Atlas (TCGA) consortium (Inamura, 2018). The last two are in agreement since the main three subtypes defined by TCGA correspond to the one summarized by MDA, concisely characterized in the following subsections. Finally, the latest type of distinguishment appears to be the Lund classification system (Aine, 2015) which considers e.g. different transcription factors, indicating their importance in such heterogeneous disease. Since the prediction of targeted treatment response is determined by molecular subtypes (Aine, 2015), it is reasonable to collate the current state of knowledge about the molecular background along with a more thorough consideration of the aspect of transcription factors for a broader view of alterations at the cellular level.

Basal bladder cancer

The first main molecular subtype of urothelial carcinoma is characterized by a resemblance to cancer stem cells (CSCs) and possessing expression of biomarkers typical for epithelial-to-mesenchymal transition (EMT). Human xenografts metastasize more widely and take advantage of the EMT-dependent mechanism more profusely compared to luminal type (McConkey, 2016a). Expression signature in this tumor consists of Annexin-1, cluster of differentiation 49 (*CD49*), epidermal growth factor receptor (*EGFR*) and Cyclin B1 proteins upregulation (Dadhania, 2016). Nevertheless, better sensitivity to neoadjuvant chemotherapy (NAC), in contrast to luminal type, was confirmed in the literature (McConkey, 2016b).

Luminal bladder cancer

In this type of tumor, there is a similarity to normal urothelium (both intermediate and superficial layers) (Dadhania, 2016). Luminal bladder cancer does not appear to be strongly EMT-dependent seeing that it may cooperate with cancer-associated fibro-blasts (CAFs). Changes in expression concern enrichment of GATA-binding protein 3 (*GATA3*), human epidermal growth factor Receptor 2 (*HER2*), Src kinase or E-Cadherin proteins and mutations of fibroblast growth factor receptor 3 (*FGFR3*), cyclin dependent kinase inhibitor 1A (*CDKN1A*), tuberous sclerosis 1 (*TSC1*) and E74-like ETS transcription factor 3 (*ELF3*) genes (Cancer Genome Atlas Research Network, 2014).

Neuronal bladder cancer

Very few cases (5%) refer to tumors that do not express differentiation biomarkers of either luminal or basal types. They are discriminated by higher proliferation, abundant expression of neuronal and neuroendocrine genes and numerous Tumor Protein p53 (*TP53)* or Retino-blastoma protein 1 (*RB1)* mutations. Com-pletely diverse treatment recommendations go concurrently with different patient survival, being a much worse in that case compared to any luminal or basal variant (Inamura, 2018).

MOLECULAR BACKGROUND OF BLADDER CANCER

A multistep process of carcinogenesis can be divided into three basic stages – initiation, promotion and progression (Said, 2013). Subsequent tumor invasion and epithelial-to-mesenchymal transition which are processes of metastasis are embraced in the final phases of progression, briefly visualized in Figure 1. There are a few groups of factors that can give rise to bladder cancer – environmental and molecular factors or epigenetic and genetic changes within tumor suppressors can be distin-guished (Shin, 2017). In terms of the first group, smoking and exposure to chemical compounds such as aromatic amines, fungicides (Letasiova, 2012), N-nitroso compounds (Catsburg, 2014) or arsenic (Jankovic, 2007) may contribute to the development of bladder cancer, although this is not necessarily the case (Czerniak, 2016). Genetic changes include six critical regions on different chromosomes, in sequence: chromo-some 3 (q22-q24), 5 (q22-q31), 9 (q21-q22), 10 (q26), 13 (q14), and 17 (p13) (Majewski, 2008), with a loss of heterozygosity (LOH) event at the long arm of chromosome 9 considered as one of the very first observed phenomena in bladder cancer (Chow, 2000).

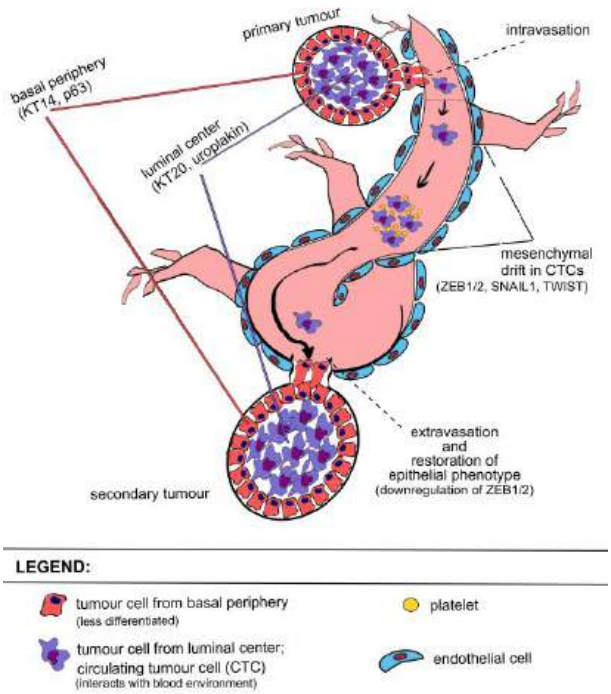

Figure 1. EMT process during tumor expansion (based on (Czerniak, 2016))

Within the epigenetic changes, a group of genes established as forerunners (FRs) plays an important role in the enforcement of field effect which refers to genomic alterations throughout whole bladder mucosa caused by chemical carcinogens (Czerniak, 2016). At least five of such genes have been mapped in the immediate closeness of the *RB1* gene on the thirteenth chromosome in one of the above critical regions – 13q14. This includes G protein-coupled receptor 38 (*GPR38*) which is an alias to motilin receptor (*MLNR*) (McConkey, 2010), lysophosphatidic acid receptor 6 (*LPAR6/P2RY5*), integral membrane protein 2B (*ITM2B*), ADP-ribosylation factor-like protein 11 (*ARL11*), and calcium binding protein 39 like (*CAB39L*) (Czerniak, 2016). The summary of regions' localization is exhibited graphically (Figure 2). FR genes are thought to be novel tumor suppressors that are interrupted during tumor development even before more common anti-oncogenes (McConkey, 2010). Using the example of the *RB1* gene, the forerunners' positioning can take place at a short distance or even within the gene e.g. *CHC1L* (Chromosome condensation 1-like) and *ITM2B* or *P2RY5*, respectively (Majewski, 2008). Using methylation and mutation analysis, Czerniak et al. showed that the FRs are more frequent methylated than they undergo mutations (Czerniak, 2016). Representatives of this group have various functions. For example, *ITM2B* shows similarity to B-cell lymphoma 2 (BCL-2) proteins subfamily containing single BH3 domain (Bcl-2 homology 3 domain), while *P2RY5* is included in G protein-coupled receptors that is responsible for binding lysophosphatidic acid (LPA) – lipid signaling molecule (Fleischer, 2004; Pasternack, 2008). Lee et al. proved that the restoration of the proper functioning of these genes resulted in the induction of tumor cell death via apoptosis and the inhibition of the cell cycle (Lee, 2007). Furthermore, the participation of *LPAR6* and *CAB39L* have been confirmed in the expansion of cells derived from urothelium, which resulted in the development of luminal and basal cancer, respectively (Czerniak, 2016).

Depending on the origin cell that initiates the development of bladder cancer, there are two main pathways of tumor progression – luminal papillary that is characterized by superficiality and basal nonpapillary which leads to direct muscle-invasive manner (McConkey, 2010). These two ways of cancer development correspond to the alleged dual-track concept which was invented in the seventies of the XIX century based on histology and clinical data of bladder (Koss, 1974). At present, the molecular insight separating the two aforementioned pathways has been already carried out.

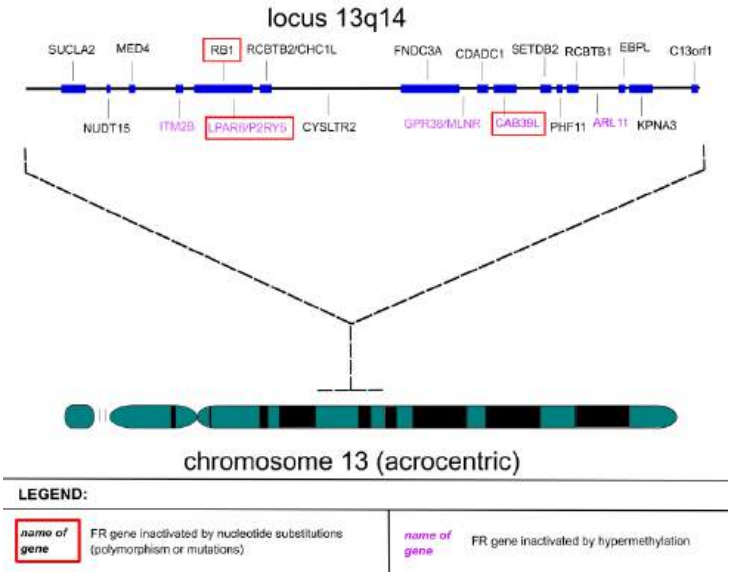

Figure 2. Genomic location of selected forerunner genes (based on (Czerniak, 2016; Majewski, 2008))

Superficial papillary tumor

The onset of this type is associated with hyperplastic changes in urothelium known as low-grade intraurothelial neoplasia (or dysplasia) and concerns 80% of bladder tumor cases. Based on grading they possess low malignancy as this grow with a non-invasive propensity and superficially, thus there is less possibility for metastasis, although 10-15% of tumors could progress into invasive phenotype. Likewise, the phenomenon of relapse is very common in this subtype (Majewski, 2008; McConkey, 2010). As mentioned earlier, various forerunner genes are implicated in diverse subtypes of bladder cancer, therefore *LPAR6* is thought to have an impact on the luminal pathway and is involved in the initiation of field effect which is an inseparable part of urothelial carcinogenesis and drives cell expansion (Czerniak, 2016).

Based on epidemiological studies it is known that chemical induction includes right majority of bladder cancers (at least in the West) (Letasiova, 2012). Triggered inter-mediate uropro-genitor cells were confirmed to be sensitive to one of the nitroso compounds – N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) which could lead to afterward DNA alterations, same as tobacco carcinogens (Czerniak, 2016). One of the modifications is aforementioned allelic loss located in chromosome 9, which concerns inactivation of p16 and IFNα (interferon α) in their loci on 9p21 and 9p22 regions, respect-tively. As a result, this could lead to disruption of cell cycle regulation or apoptosis which subsequently influence the cancer progression (McConkey, 2010). Proteins associated with luminal papillary carcinoma include forkhead box A1/estrogen receptor 1 (FOXA1/ESR1) interacting pathway, GATA3 or tripartite motif containing 24 (TRIM24) transcription factors. Subsequently formed low-grade papillary tumor may progress to high-grade invasive cancer owing to various structural alterations or copy-number variations (CNVs) (Czerniak, 2016).

Invasive non-papillary tumor

In comparison to the superficial papillary tumor, the solid non-papillary one is derived from high-grade intraurothelial neoplasia (also termed carcinoma in situ – CIS) and principally concerns patients with no previous history. Aggressive invasion of basement membrane and highly metastatic phenotype result in lymph nodes and distant sites occupation (McConkey, 2010). Similar to the above, FR genes are also involved in triggering field effect and cell expansion, however in case of basal uro-progenitor cells the further development can be dependent on *CAB39L* (Czerniak, 2016). Proteins included in pathways that drive progression of basal nonpapillary track are p63, EGFR, signal transducer and activator of trans-cription 3 (STAT3), or hypoxia-inducible factor 1-alpha (HIF-1α), and what is suggested in other research – participation of PTEN/PI3K/AKT/mTOR pathway (phosphatase and tensin homolog; phosphate-dylinositol-4,5-bisphosphate 3-kinase; serine/threonine kinase 1; mammalian target of rapamycin) is thought to affect muscle-invasive phenotype (Knowles, 2009). Deve-

lopment dependent on PTEN inactivation may also require loss of p53 (or p53 pathway malfunction connected with loss of p21) which promotes in situ carcinoma (Puzio-Kuter, 2009; Stein, 1998). Interestingly, *FGFR3* mutations are found more than twice frequent in superficial papillary tumors, but still occur in invasive nonpapillary cancers in almost 20% (McConkey, 2010). A summary of the development of bladder cancer based on the dual-track concept and depending on the different uniprogenitor cells is presented in figure 3.

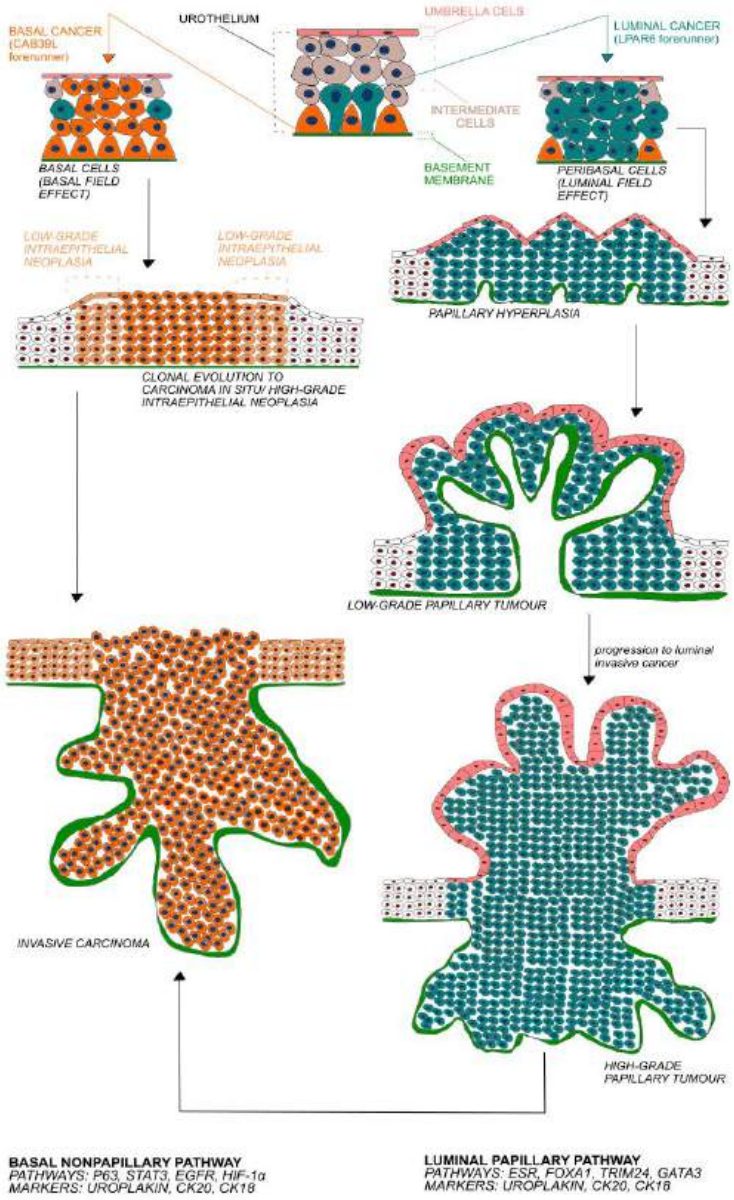

Figure 3. Molecular development of basal and luminal subtypes of bladder cancer (based on (Czerniak, 2016))

TRANSCRIPTION FACTORS (TFS)

Scientific reports indicate that transcription factors shape the expression profile of specific bladder cancer molecular subtype among which examples most frequently given are Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ), Retinoid X Receptor alpha (RXR-α), Forkhead Box M1 (FOXM1), FOXA1, GATA3, and STAT3 (Choi, 2014; Eriksson, 2015; Rebouissou, 2014). Expression of both cell adhesion molecules (CAMs) and matrix metalloproteinases (MMPs) – a groups implycated in steps of tumor invasion – is also thought to be regulated through specific transcription factors. In the case of E-cadherin (representative of CAMs) regulation during cancer development, participation of Snail, Slug

and SMAD Interacting Protein 1 (SIP1) factors were confirmed to suppress its expression (Makrilia, 2009). Secondly, matrix metalloproteinases are known to be regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), STATs, Erythroblast Transformation-Specific (ETS) or Activator Protein 1 (AP-1) and Activating enhancer-binding Protein 2 (AP-2) transcription factor families (Fanjul-Fernandez, 2010; Overall, 2002; Yan, 2007). Representative of the last family of the above – AP-2γ (encoded by *TFAP2C* gene) was assessed as a transcription factor whose overexpression correlates with the distant recurrence or tumor growth (Yamashita, 2019). Such tendency was further supported by our preliminary analysis on the bladder cancer TCGA cohort, which showed a statistically significant relationship where the increase in *TFAP2C* expression worsened prognostic endpoints (disease-free survival (DFS), overall survival (OS)). This prompted us to dwell on this issue in the hope of finding another representative transcription factor whose designation could potentially assist molecular classification.

AP-2 FAMILY OF TRANSCRIPTION FACTORS

Classification of the entire TFs network is based on occurrence of ten superclasses which varies in terms of structural properties and sequence similarity (Ehsani, 2016; Yamashita, 2019). One of the three most-extensive superclasses called Basic Domains contains the AP-2 family which belongs to the basic Helix-Span-Helix (bHSH) class. All genera of bHSH are capable to distinguish particular G/C-rich motifs e.g. CCCCAGGC (Mitchell, 1987), GCCN3/4GGG or GCCN3/4GGC (Mohibullah, 1999). Binding region resembles those of the other classes within Basic Domains superclass, both Basic leucine zipper factors (bZIP) and Basic helix-loop-helix factors (bHLH), whereas domain loop length discriminates bHLH class from bHSH (longer in the second class) (Bolander, 2004). Characteristic regions of bHSH members (thereby AP-2 family factors) are shown in figure 4. N-terminal site of AP-2 members comprises transactivation domain, whilst DNA-binding domain (together with internal dimerization domain (Kannan, 1999)) are located from the C-terminal space (Eckert, 2005). The proper functioning of individual domains is ensured by structural motifs – Proline/Glutamine-rich, basic α-helix and HSH, respectively. The last two, if separated, are still able to link two members of AP-2 family, nonetheless DNA-binding functio-nality is disrupted (Williams, 1991).

This is important because AP-2 factors function as homodimers or heterodimers, consequently the ability to co-interact is essential. All five AP-2 family representatives: α, β, γ, δ and ε (Orlic-Milacic, 2016) are critical for gene expression regulation along with apoptosis or cell cycle control during proper early developmental stages (Hilger-Eversheim, 2000). Initial location of AP-2 factors is nucleus and their activity can be modulated by regulation of subcellular localization (Pellikainen, 2004), DNA-binding capability (Mazina, 2001), transactivation potential (Aqeilan, 2004) or degradation (Li, 2006) – those are possible through post-translational phosphorylation (Garcia, 1999), sumoylation (Zhong, 2003), reduction/oxidation reactions (Huang, 1998), or via interactions with other proteins (Eckert, 2005). Despite the participation of whole family in normal development, their overexpression has already been observed in distinct tumors (Hoei-Hansen, 2004; Jager, 2005; Pellikainen, 2004).

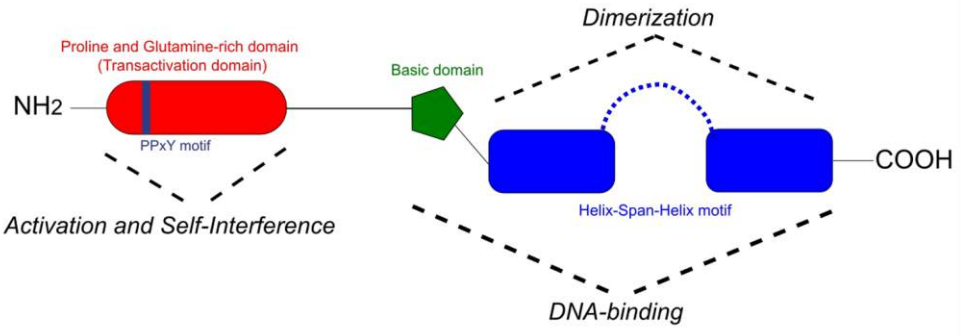

Figure 4. Specific domains of AP-2 family (based on (Eckert, 2005; Kannan, 1999; LiCalsi, 2000))

TFAP2C gene

Gene encoding AP-2γ is localized on the twentieth chromosome (cytological location: 20q13.31) on the plus strand and its overall size is 9,982 of bases with seven exons within. This AP-2 family member encodes transcription factor that recognizes specific sequence SCCTSRGGS (S = G/C, R = A/G) (Woodfield, 2010) and is implicated in the development of eyes, limbs, face or neural tube through genes activation. In accordance with the NCBI Reference Sequence Database, only one mRNA variant undergoes transcription from this gene (accession number: NM_003222.3). Graphical presentation of *TFAP2C* gene and additional information are presented in figure 5.

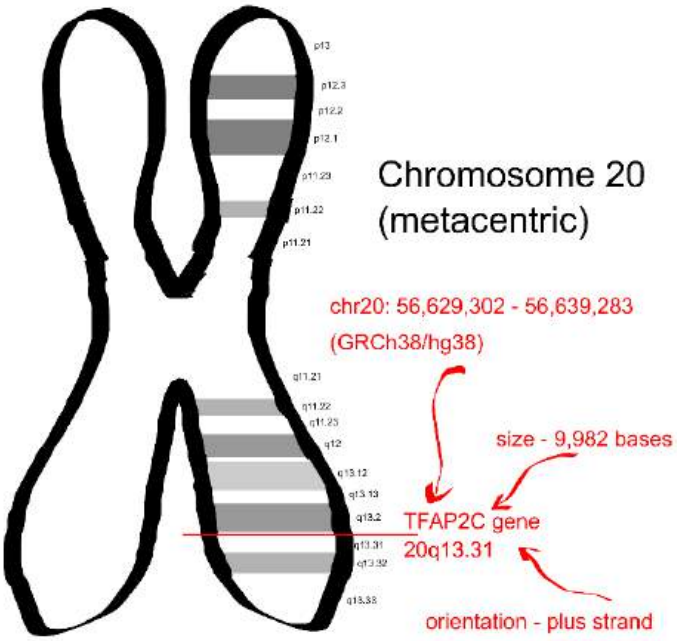

Figure 5. Localization of TFAP2C gene (based on GeneCards database)

AP-2γ protein

Properties of AP-2γ protein e.g. dimerization, sequence recognition or influence on cellular and viral enhancers – all resembles entire AP-2 family characteristics described above. UniProt KnowledgeBase determines that there is only one isoform in addition to the canonical sequence (identifiers: Q92754-2 and Q92754-1, respectively). AP-2γ activity can be modulated via post-translational modifications (PTMs) however only one location is experimentally acknowledged and has their cause-and-effect relation-ship confirmed i.e. sumoylation at lysine 10 that leads to inhibition of activity (Eloranta, 2002). An interesting issue is a way AP-2γ functionality is considered – predominantly it is perceived as oncogene yet some cases are suggesting its tumor suppressor capa-bilities (Kolat, 2019). Still, literature data predominantly indicate the oncogenic nature of this factor, which confirms the reliability of our preliminary analyzes. The behavior of this factor has been reliably studied on the example of interaction with WW Domain Containing Oxidoreductase (*WWOX*) suppressor, during which AP-2γ proliferation-promoting activity is inhibited (Aqeilan, 2004).

Despite the enormity of available knowledge describing affected signaling pathways such as FGFR3/RAS, TP53/RB1 or PI3K/AKT/mTOR, the heterogeneity of bladder cancer inclines a more thorough understanding of this cancer by identifying potentially useful genes. By initial insight of databases, we noticed significant differences e.g. in the level of *TFAP2C* in normal tissue compared to cancerous or the effect of different expression of this gene on the survival of oncological patients. Therefore, we aimed our research to perform *in silico* analysis for determination of AP-2γ role in bladder cancer.

MATERIALS AND METHODS

GEPIA (Gene Expression Profiling Interactive Analysis, gepia2.cancer-pku.cn) was used for analyzing prognostic endpoints on the BLCA cohort depending on *TFAP2C* level.

UALCAN (http://ualcan.path.uab.edu) was used to analyze the expression level of *TFAP2C* gene, its prognostic value (according to median values of genes) and correlation with clinic-pathological parameters. This portal performs analyses of cancer OMICS data (TCGA and MET500) (Chandrashekar, 2017).

cBioPortal for Cancer Genomics (https://www.cbioportal.org) was applied to analyze CNVs of *TFAP2C* and correlation with another genes.

TCGA (http://cancergenome.nih.gov) was used to extract clinical data of 412 BLCA cases (status of May 2, 2020) together with their mRNAseq profiling (level 3 RNASeqV2, RSEM normalized). For further analyses, we excluded non-tumor type of samples (according to TCGA Barcode, acquired from https://docs.gdc.cancer.gov) which resulted in total data of 408 patients.

GSEA (Gene Set Enrichment Analysis, http://genepattern.broadinstitute.org/gp) was conducted using entire database of molecular signatures (eight major collections acquired from http://software.broadinstitute.org/gsea/msigdb/index.jsp). 5175 genes being targets for AP-2γ were taken into account for enrichment analysis. Targets for transcription factor were combined using three databases (excluding duplicates): Gene Transcription Regulation Database (GTRD, version 19.10 (Yevshin, 2019; Yevshin, 2017)), TRANScription FACtor database (TRANSFAC, version 2019.2) and Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRRUST, version v2). Defined phenotypes ("TFAP2C high" or "TFAP2C low") were established by the median value of AP-2γ and restricted to extreme patients to show larger differences between groups. Functional analysis was performed using the tTest metric with a weighted statistic to score hits/misses and permutation type concerning phenotype.

DAVID (Database for Annotation, Visualization and Integrated Discovery) Bio-informatics Resources (Huang, 2009) (version 6.8, https://david.ncifcrf.gov) were used to annotate genes to processes they regulate, their molecular function, localization or implicated signaling pathways. All annotation databases were included during analyses.

RESULTS

DIFFERENCES OF GENE EXPRESSION DEPENDING ON TISSUE TYPE, TUMOR STAGE, HISTOLOGY, MOLECULAR SUBTYPES, NODAL METASTASIS AND MUTATION STATUS

To understand the role of AP-2γ transcription factor, at the beginning we compared their mRNA level in cancer tissue and normal tissue in patients using UALCAN tool. We observed statistically significant higher expression of *TFAP2C* in cancer compared to normal bladder tissue (fig. 6A). We also analyzed how the level of its expression changed depending on clinical data. We observed increasement of *TFAP2C* expression in II-IV bladder cancer stages (with the highest noticed in stage IV) compared to normal tissue (fig. 6B). Moreover, higher mRNA level of *TFAP2C* was connected with mutation in *TP53* gene in bladder cancer (fig. 6C). The analyses of *TFAP2C* expression level in different histological and molecular subtypes also shows some significant relationships compared to normal tissue (fig. 6D and E). Finally, mRNA level of *TFAP2C* increases during metastasis to lymph nodes (fig. 6F).

DEPENDENCE OF PROGNOSTIC ENDPOINTS ON THE LEVEL OF *TFAP2C* GENE

To investigate the connection between *TFAP2C* expression and survival prognosis, we used Kaplan-Meier plots form GEPIA repository. Using median cutoff of overall group and 95% confidence interval, we observed that high *TFAP2C* expression correlates with unfavorable prognosis i.e. DFS and OS (HR = 1.6, $p = 0.0069$; HR = 1.4, $p = 0.024$, respectively) (fig. 7).

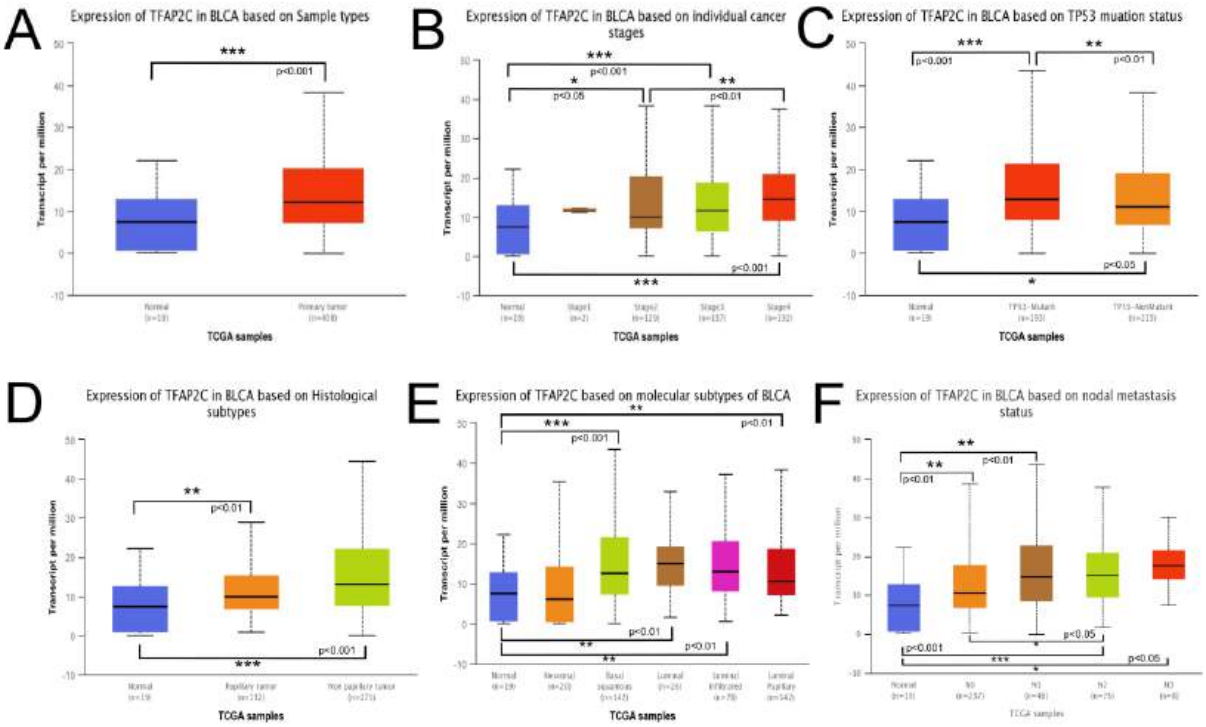

Figure 6. Variability of TFAP2C mRNA expression levels for selected UALCAN characteristics.
(A) Sample/tissue type. (B) Cancer stages. (C) TP53 mutation status. (D) Histological subtypes.
(E) Molecular subtypes. (F) Nodal metastasis status

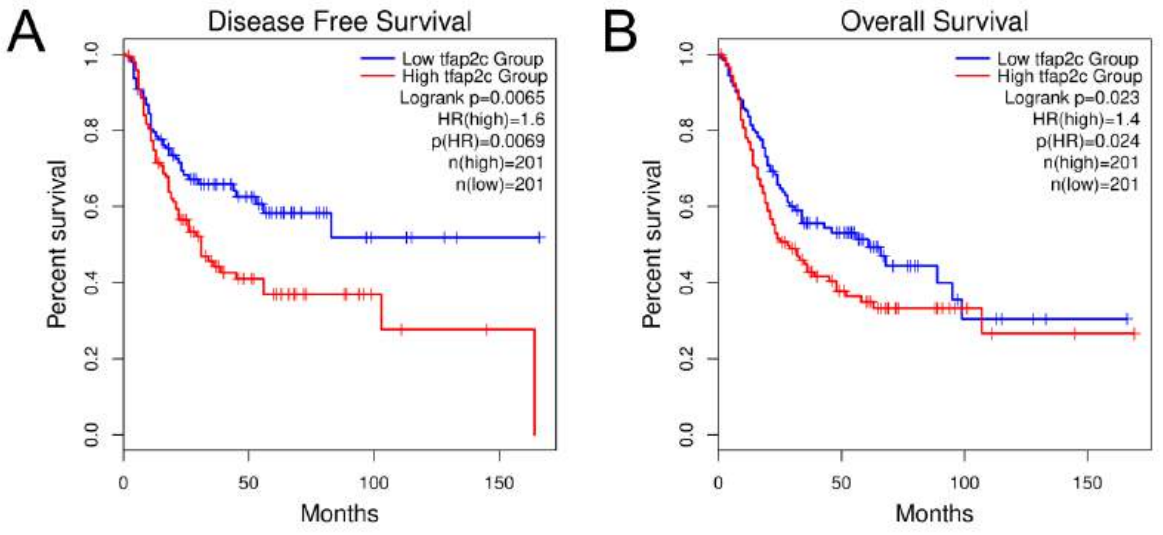

Figure 7. Prognostic endpoints analysis according to mRNA expression of TFAP2C gene.
(A) Disease-Free Survival. (B) Overall Survival

COPY-NUMBER VARIATIONS AND CO-EXPRESSION ANALYSIS

Subsequently, we examined whether the changes in *TFAP2C* expression may result from copy number alternations using bladder cancer patients' data (TCGA, Firehose Legacy) via cBioPortal database (fig. 8).

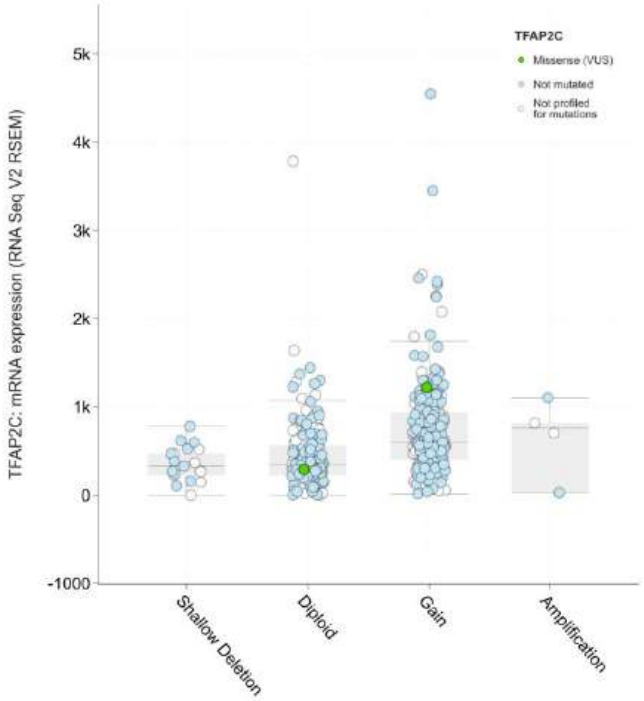

Figure 8. Putative copy-number alterations of TFAP2C gene in the BLCA cohort

Mutations were observed only in "diploid" and "gain" of *TFAP2C* gene. There was not noticed gene amplification, so we can conclude that changes in *TFAP2C* expression in bladder cancer are not due to the copy number alternations.

Using RNAseq of 413 BLCA patients from cBioPortal (Z-score threshold = 0) we aimed to identify genes correlating with *TFAP2C* expression and the processes they are implicated in. Co-expression analysis showed that *TFAP2C* positively correlates with 99 genes (Spearman's rank correlation coefficient $\geq$ +0.3) and negatively with 19 genes (Spearman's rank correlation coefficient $\leq$ -0.3). Ontological classification of selected genes was presented in figure 9.

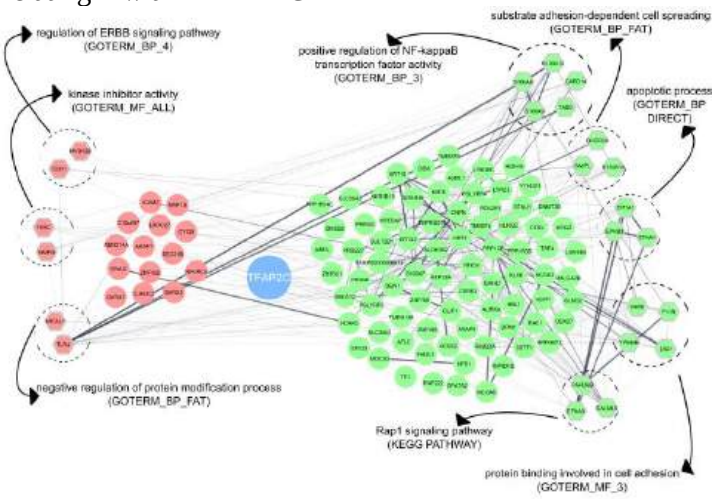

Figure 9. Genes negatively (red) and positively (green) co-expressed with TFAP2C according to cBioPortal with additional selected ontology classification

GENE SET ENRICHMENT ANALYSIS

Important gene sets established through GSEA concerned biological signatures of BIOCARTA canonical pathways, chemical & genetic perturbations (CGP), gene ontology (GO): biological processes (BP), hallmarks or regulatory target gene sets. Collectively, 10 gene sets enriched in "TFAP2C high" were significant at FDR < 0.25 while 3 gene sets in "TFAP2C low" significant at p < 0.01. Exemplary heatmaps for both phenotypes are presented in Figure 10.

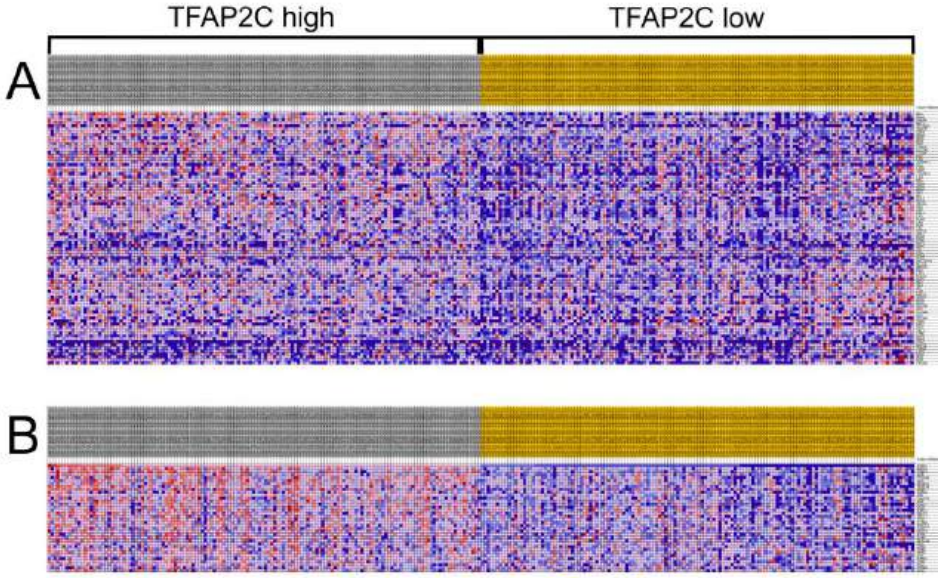

Figure 10. Heatmaps generated from GSEA analysis (only core enrichment genes). (A) LINDGREN_BLADDER_CANCER_CLUSTER_3_UP gene set. (B) NIKOLSKY_ BREAST_CANCER_20Q12_Q13_AMPLICON gene set

FUNCTIONAL ANNOTATION CLUSTERING

Afterwards, each gene set (only core enrichment genes) was implemented into DAVID for further data mining. Detailed subdivision and obtained gene-annotation enrichments from GSEA gene sets can be found in table 1.

Table 1. Gene-annotation enrichment analysis of selected gene sets. Gene sets were chosen with the highest possible significance for each phenotype (FDR < 0.25 for "TFAP2C high"; p < 0.01 for "TFAP2C low")

Phenotype	MSigDB collection	Selected gene set	Selected annotation database	Description
TFAP2C high	C2:CGP	NIKOLSKY_BREAST_CANCER_20Q12_Q13_AMPLICON	GO:BP_DIRECT	Regulation of cell proliferation
		LINDGREN_BLADDER_CANCER_CLUSTER_3_UP	KEGG	PI3K-Akt signaling pathway
		HOLLERN_EMT_BREAST_TUMOR_DN	GO:BP_DIRECT	Negative regulation of cell-cell adhesion
		LANDIS_BREAST_CANCER_PROGRESSION_UP	GO:BP_ALL	Positive regulation of cellular process
		WHITFIELD_CELL_CYCLE_M_G1	GO:BP_DIRECT	Intermediate filament organization

		OUELLET_OVARIAN_CANCER_INVASIVE_VS_LMP_UP	KEGG	Hippo signaling pathway
		BIDUS_METASTASIS_UP	KEGG	MAPK signaling pathway
	C2:CP Reactome	REACTOME_TRANSCRIP-TIONAL REGULATION_BY_THE_AP_2_TFAP2_FAMILY_OF_TRANS-CRIPTION_FACTORS	GO:BP_DIRECT	Negative regulation of apoptotic process
	C2:BP Biocarta	BIOCARTA_HER2_PATHWAY	KEGG	ERBB signaling pathway
	Hallmarks	HALLMARK_G2M_CHECKPOINT	GO:BP_DIRECT	G2/M transition of mitotic cell cycle
TFAP2C low	C3:MIR	MIR4265	KEGG	Transcriptional misregulation in cancer
		MIR4296	GO:BP_DIRECT	Cell differentiation
		MIR4713_3P	GO:BP_ALL	Positive regulation of cell communication

DISCUSSION

BLCA is a tumor whose frequency has decreased in recent years. The classification takes into account the division into histological or molecular subtypes, with the latter considering e.g. categorization by the transcription factors which may indicate not only the expression profile but also the potential treatment type resulting from the available actionable targets. Because the identification of both new molecules involved in signaling pathways and TFs guiding their expression is crucial, we focused on investigating the transcription factor whose upregulation significantly worsened the prognostic endpoints of bladder cancer patients. Using available literature, there was a noticeable trend indicating the oncogenic nature of *TFAP2C* in various cancers. While the role of AP2γ-encoding gene in lung cancer was ambiguous (Chang, 2017; Kang, 2017; Kim, 2016), the data for breast cancer (Gee, 2009; Williams, 2009), testicular carcinoma (Hoei-Hansen, 2004), primary ovarian tumors (Odegaard, 2006), melanoma (Lal, 2013) or neuroblastoma (Gao, 2014) are consistent indicating association of *TFAP2C* with e.g. increased proliferation, tumor growth, cell cycle progression but also treatment resistance or poorer prognosis. Analysis of the putative copy-number alteration in relevant cohorts using cBioPortal (TCGA Firehose Legacy studies for breast, testis, ovary, skin; Broad, Nature 2015 for neuroblastoma) indicated that the observed *TFAP2C* effect on the above processes and prognosis is not due to gene mutations. The same conclusions could be drawn from the BLCA analysis (TCGA Firehose Legacy study), which encouraged the selection of most strongly co-expressed genes (correlation less than -0.3 or more than +0.3) that resulted in the extraction of 118 genes further analyzed ontologically. Within such, additional gene ontology indicated regulation of pathways (ERBB, Rap1), apoptosis or adhesion-dependent cell spreading. With the use of UALCAN web resource we performed expression analysis in terms of BLCA characteristics such as cancer stages, nodal metastasis, *TP53* mutation status and histological subtypes or molecular subtypes, collectively indicating that *TFAP2C* expression increases with stage or metastasis status and possesses highest level in luminal subtype (molecularly) or non-papillary subtype (histologically). Additionally, its expression is higher in tumors with positive p53 mutational status.

Lastly, there was statistically significant difference between *TFAP2C* expression in normal tissue compared to cancerous (in favor of the latter). To understand what molecular alterations occur with different levels of *TFAP2C* in bladder cancer patients, a gene set enrichment analysis was performed on the BLCA cohort subdivided into groups with high or low *TFAP2C* expression through the context of AP-2γ targets. Collected data included gene sets related to canonical pathways, genetic perturbations, biological processes, hallmarks or regulatory target gene sets. Subsequently, functional annotation enrichment analysis was performed on genes that have contributed the most. Considering the group with high level of *TFAP2C* gene expression, contribution to numerous signaling pathways (MAPK, ERBB, Hippo, PI3K/Akt) regulation was proposed along with e.g. negative impact on apoptotic process or cellular adhesion. Contrastingly, in "TFAP2C low" group the enriched gene sets were associated with regulatory targets guided by microRNA (miRNA) which has a reference to the literature because many interactions of AP-2 family members with long non-coding RNA (lncRNA) or miRNA have been proven (Kolat, 2019). Nevertheless, gene ontology of the most significant gene sets for "low" phenotype revealed functional annotation connected with positive cell communication or cell differentiation, which indicates the alleged one absolute difference between the "high" and "low" groups associated with intercellular communication – in the first group, intercellular commu-nication is supposedly limited by loss of adhesion, while in the second group the increase in intercellular communication was noted. We speculate that such state may result from different levels of *TFAP2C*, which in the case of a decrease in its expression directly impacts on miRNA network and allows the regulation of processes that are not possible in the case of *TFAP2C* high expression.

SHORT CONCLUSION

To conclude, computational analysis of AP-2γ role showed that "TFAP2C high" group is more associated with phenomena or signaling pathways that are perceived negatively when excessively occur at molecular level. Instead, results for the "TFAP2C low" group indicate the regulation of cellular processes via a wide range of miRNA transcripts, which concurs with the literature data of the present yet minorly explored network of interaction between AP-2γ and miRNA or lncRNA molecules. We believe that further research towards profound exploration of regulated processes not only on the AP2-RNA axis but with investigating other regulators of the protective phenotype, will allow a deeper understanding of the scenario that occur with low *TFAP2C* expression in bladder cancer patients.

REFERENCES

Aine M., Eriksson P., Liedberg F., Hoglund M., Sjodahl G., **On molecular classification of bladder cancer: Out of one, many**, In: Eur Urol. 2015; 68(6):921-3.

American Cancer Society, **Cancer Statistics Center,** In: American Cancer Society, 2020; Accessed 21 May 2020, http://cancerstatisticscenter.cancer.org/.

Aqeilan R.I., Palamarchuk A., Weigel R.J., Herrero J.J., Pekarsky Y., Croce C.M., **Physical and functional interactions between the wwox tumor suppressor protein and the ap-2gamma transcription factor**, In: Cancer Res. 2004; 64(22):8256-61.

Bolander F.F., **Molecular Endocrinology 3rd Edition**, 2004; In: Academic Press; ISBN: 9780121112325.

Cancer Genome Atlas Research Network, **Comprehensive molecular characterization of urothelial bladder carcinoma,** In: Nature, 2014; 507(7492):315-22.

Cassell A., Yunusa B., Jalloh M., Mbodji M.M., Diallo A., Ndoye M., et al., **Non-muscle invasive bladder cancer: A review of the current trend in africa**, In: World J Oncol. 2019; 10(3):123-131.

Catsburg C.E., Gago-Dominguez M., Yuan J.M., Castelao J.E., Cortessis V.K., Pike M.C., et al., **Dietary sources of n-nitroso compounds and bladder cancer risk: Findings from the los angeles bladder cancer study**, In: Int J Cancer, 2014; 134(1):125-35.

Chandrashekar D. S., Bashel B., Balasubramanya S. A. H., Creighton C. J., Ponce-Rodriguez I., Chakravarthi B., et al. **Ualcan: A portal for facilitating tumor subgroup gene expression and survival analyses**. In: Neoplasia, 2017; 19(8):649-658.

Chang T.H., Tsai M.F., Gow C.H., Wu S.G., Liu Y.N., Chang Y.L., et al., **Upregulation of microrna-137 expression by slug promotes tumor invasion and metastasis of non-small cell lung cancer cells through suppression of tfap2c**, In: Cancer Lett. 2017; 402:190-202.

Choi W., Porten S., Kim S., Willis D., Plimack E.R., Hoffman-Censits J., et al., **Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy**, In: Cancer Cell, 2014; 25(2):152-65.

Chow N.H., Cairns P., Eisenberger C.F., Schoenberg M.P., Taylor D.C., Epstein J.I., et al., **Papillary urothelial hyperplasia is a clonal precursor to papillary transitional cell bladder cancer**, In: Int J Cancer. 2000; 89(6):514-8.

Czerniak B., Dinney C., McConkey D., **Origins of bladder cancer**, In: Annu Rev Pathol. 2016; 11:149-74.

Dadhania V., Zhang M., Zhang L., Bondaruk J., Majewski T., Siefker-Radtke A., et al., **Meta-analysis of the luminal and basal subtypes of bladder cancer and the identification of signature immunohistochemical markers for clinical use**, In: EBioMedicine, 2016; 12:105-117.

Eckert D., Buhl S., Weber S., Jager R., Schorle H., **The ap-2 family of transcription factors**, In: Genome Biol. 2005; 6(13):246.

Ehsani R., Bahrami S., Drablos F., **Feature-based classification of human transcription factors into hypothetical sub-classes related to regulatory function**, In: BMC Bioinformatics, 2016; 17(1):459.

Eloranta J.J., Hurst H.C., **Transcription factor ap-2 interacts with the sumo-conjugating enzyme ubc9 and is sumolated *in vivo***, In: J Biol Chem. 2002; 277(34):30798-804.

Eriksson P., Aine M., Veerla S., Liedberg F., Sjodahl G.Hoglund M., **Molecular subtypes of urothelial carcinoma are defined by specific gene regulatory systems**, In: BMC Med Genomics, 2015; 8:25.

Fanjul-Fernandez M., Folgueras A.R., Cabrera S., Lopez-Otin C., **Matrix metalloproteinases: Evolution, gene regulation and functional analysis in mouse models**, In: Biochim Biophys Acta, 2010; 1803(1):3-19.

Fleischer A., Rebollo A., **Induction of p53-independent apoptosis by the bh3-only protein itm2bs**, In: FEBS Lett. 2004; 557(1-3):283-7.

Gao S.L., Wang L.Z., Liu H.Y., Liu D.L., Xie L.M., Zhang Z.W., **Mir-200a inhibits tumor proliferation by targeting ap-2gamma in neuroblastoma cells**, In: Asian Pac J Cancer Prev. 2014; 15(11):4671-6.

Garcia M.A., Campillos M., Marina A., Valdivieso F., Vazquez J., **Transcription factor ap-2 activity is modulated by protein kinase a-mediated phosphorylation**, In: FEBS Lett. 1999; 444(1):27-31.

Gee J.M., Eloranta J.J., Ibbitt J.C., Robertson J.F., Ellis I.O., Williams T., et al., **Overexpression of tfap2c in invasive breast cancer correlates with a poorer response to anti-hormone therapy and reduced patient survival**, In: J Pathol. 2009; 217(1):32-41.

Hilger-Eversheim K., Moser M., Schorle H., Buettner R., **Regulatory roles of ap-2 transcription factors in vertebrate development, apoptosis and cell-cycle control**, In: Gene. 2000; 260(1-2):1-12.

Hoei-Hansen C.E., Nielsen J.E., Almstrup K., Sonne S.B., Graem N., Skakkebaek N.E., et al., **Transcription factor ap-2gamma is a developmentally regulated marker of testicular carcinoma in situ and germ cell tumors**, In: Clin Cancer Res. 2004; 10(24):8521-30.

Huang da W., Sherman B.T., Lempicki R.A., **Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists**, In: Nucleic Acids Res. 2009; 37(1):1-13.

Huang Y., Domann F.E., **Redox modulation of ap-2 DNA binding activity *in vitro***, In: Biochem Biophys Res Commun. 1998; 249(2):307-12.

Inamura K., **Bladder cancer: New insights into its molecular pathology**, In: Cancers (Basel). 2018; 10(4).

Jager R., Friedrichs N., Heim I., Buttner R., Schorle H., **Dual role of ap-2gamma in erbb-2-induced mammary tumorigenesis**, In: Breast Cancer Res Treat. 2005; 90(3):273-80.

Jankovic S., Radosavljevic V., **Risk factors for bladder cancer**, In: Tumori. 2007; 93(1):4-12.

Kang J., Kim W., Lee S., Kwon D., Chun J., Son B., et al., **Tfap2c promotes lung tumorigenesis and aggressiveness through mir-183- and mir-33a-mediated cell cycle regulation**, In: Oncogene. 2017; 36(11):1585-1596.

Kannan P., Tainsky M.A., **Coactivator pc4 mediates ap-2 transcriptional activity and suppresses ras-induced transformation dependent on ap-2 transcriptional interference**, In: Mol Cell Biol. 1999; 19(1):899-908.

Kim W., Kim E., Lee S., Kim D., Chun J., Park K.H., et al., **Tfap2c-mediated upregulation of tgfbr1 promotes lung tumorigenesis and epithelial-mesenchymal transition**, In: Exp Mol Med. 2016; 48(11):e273.

Knowles M.A., Platt F.M., Ross R.L., Hurst C.D., **Phosphatidylinositol 3-kinase (pi3k) pathway activation in bladder cancer**, In: Cancer Metastasis Rev. 2009; 28(3-4):305-16.

Kolat D., Kaluzinska Z., Bednarek A.K., Pluciennik E., **The biological characteristics of transcription factors ap-2alpha and ap-2gamma and their importance in various types of cancers**, In: Biosci Rep. 2019; 39(3).

Koss L.G., Tiamson E.M., Robbins M.A., **Mapping cancerous and precancerous bladder changes. A study of the urothelium in ten surgically removed bladders**, In: JAMA. 1974; 227(3):281-6.

Lal G., Contreras P.G., Kulak M., Woodfield G., Bair T., Domann F.E., et al., **Human melanoma cells over-express extracellular matrix 1 (ecm1) which is regulated by tfap2c**, In: PLoS One. 2013; 8(9):e73953.

Lee S., Jeong J., Majewski T., Scherer S.E., Kim M.S., Tuziak T., et al., **Forerunner genes contiguous to rb1 contribute to the development of in situ neoplasia**, In: Proc Natl Acad Sci U S A. 2007; 104(34):13732-7.

Letasiova S., Medve'ova A., Sovcikova A., Dusinska M., Volkovova K., Mosoiu C., et al., **Bladder cancer, a review of the environmental risk factors**, In: Environ Health. 2012; 11 Suppl 1:S11.

Li M., Wang Y., Hung M.C., Kannan P., **Inefficient proteasomal-degradation pathway stabilizes ap-2alpha and activates her-2/neu gene in breast cancer,**. In: Int J Cancer, 2006; 118(4):802-11.

LiCalsi C., Christophe S., Steger D.J., Buescher M., Fischer W.Mellon P.L., **Ap-2 family members regulate basal and camp-induced expression of human chorionic gonadotropin**, In: Nucleic Acids Res. 2000; 28(4):1036-43.

Majewski T., Lee S., Jeong J., Yoon D.S., Kram A., Kim M.S., et al., **Understanding the development of human bladder cancer by using a whole-organ genomic mapping strategy,** In: Lab Invest. 2008; 88(7):694-721.

Makrilia N., Kollias A., Manolopoulos L., Syrigos K., **Cell adhesion molecules: Role and clinical significance in cancer**, In: Cancer Invest. 2009; 27(10):1023-37.

Mazina O.M., Phillips M.A., Williams T., Vines C.A., Cherr G.N., Rice R.H., **Redistribution of transcription factor ap-2alpha in differentiating cultured human epidermal cells**, In: J Invest Dermatol. 2001; 117(4):864-70.

McConkey D.J., Choi W., Ochoa A., Dinney C.P.N., **Intrinsic subtypes and bladder cancer metastasis**, In: Asian J Urol. 2016a; 3(4):260-267.

McConkey D.J., Choi W., Shen Y., Lee I.L., Porten S., Matin S.F., et al., **A prognostic gene expression signature in the molecular classification of chemotherapy-naive urothelial cancer is predictive of clinical outcomes from neoadjuvant chemotherapy: A phase 2 trial of dose-dense methotrexate, vinblastine, doxorubicin, and cisplatin with bevacizumab in urothelial cancer,**. In: Eur Urol. 2016b; 69(5):855-62.

McConkey D.J., Lee S., Choi W., Tran M., Majewski T., Lee S., et al., **Molecular genetics of bladder cancer: Emerging mechanisms of tumor initiation and progression**, In: Urol Oncol. 2010; 28(4):429-40.

Mitchell P.J., Wang C., Tjian R., **Positive and negative regulation of transcription in vitro: Enhancer-binding protein ap-2 is inhibited by sv40 t antigen**, In: Cell. 1987; 50(6):847-61.

Mohibullah N., Donner A., Ippolito J.A., Williams T., **Selex and missing phosphate contact analyses reveal flexibility within the ap-2[alpha] protein: DNA binding complex**, In: Nucleic Acids Res. 1999; 27(13):2760-9.

Odegaard E., Staff A.C., Kaern J., Florenes V.A., Kopolovic J., Trope C.G., et al., **The ap-2gamma transcription factor is upregulated in advanced-stage ovarian carcinoma**, In: Gynecol Oncol. 2006; 100(3):462-8.

Orlic-Milacic M., **Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors Current neurology and neuroscience reports**, In: U.S. National Library of Medicine, 2016; Accessed 21 May 2020 <https://www.ncbi.nlm.nih.gov/biosystems/1427839/>.

Overall C.M., Lopez-Otin C., **Strategies for mmp inhibition in cancer: Innovations for the post-trial era**, In: Nat Rev Cancer. 2002; 2(9):657-72.

Pasternack S.M., von Kugelgen I., Al Aboud K., Lee Y.A., Ruschendorf F., Voss K., et al., **G protein-coupled receptor p2y5 and its ligand lpa are involved in maintenance of human hair growth**, In: Nat Genet. 2008; 40(3):329-34.

Pellikainen J., Naukkarinen A., Ropponen K., Rummukainen J., Kataja V., Kellokoski J., et al., **Expression of her2 and its association with ap-2 in breast cancer**, In: Eur J Cancer. 2004; 40(10):1485-95.

Puzio-Kuter A.M., Castillo-Martin M., Kinkade C.W., Wang X., Shen T.H., Matos T., et al., **Inactivation of p53 and pten promotes invasive bladder cancer**, In: Genes Dev. 2009; 23(6):675-80.

Rebouissou S., Bernard-Pierrot I., de Reynies A., Lepage M.L., Krucker C., Chapeaublanc E., et al., **Egfr as a potential therapeutic target for a subset of muscle-invasive bladder cancers presenting a basal-like phenotype**, In: Sci Transl Med. 2014; 6(244):244ra91.

Said N., Frierson H.F., Sanchez-Carbayo M., Brekken R.A., Theodorescu D.,**Loss of sparc in bladder cancer enhances carcinogenesis and progression**, In: J Clin Invest. 2013; 123(2):751-66.

Shin J.H., Lim J.S., Jeon B.H., **Chapter 3 - Pathophysiology of Bladder Cancer** In: Ku Hyeon J. **Bladder Cancer**, 2017; In: Academic Press; ISBN: 0-12809-940-2.

Stein J.P., Ginsberg D.A., Grossfeld G.D., Chatterjee S.J., Esrig D., Dickinson M.G., et al., **Effect of p21waf1/cip1 expression on tumor progression in bladder cancer**, In: J Natl Cancer Inst. 1998; 90(14):1072-9.

Williams C.M., Scibetta A.G., Friedrich J.K., Canosa M., Berlato C., Moss C.H., et al., **Ap-2gamma promotes proliferation in breast tumour cells by direct repression of the cdkn1a gene**, In: EMBO J. 2009; 28(22):3591-601.

Williams T., Tjian R., **Characterization of a dimerization motif in ap-2 and its function in heterologous DNA-binding proteins**, In: Science. 1991; 251(4997):1067-71.

Wojciechowska U., Didkowska J., **Zachorowania i zgony na nowotwory złośliwe w Polsce,** In: Krajowy Rejestr Nowotworów, 2017; Accessed 21 May 2020, http://onkologia.org.pl/.

Woodfield G.W., Chen Y., Bair T.B., Domann F.E., Weigel R.J., **Identification of primary gene targets of tfap2c in hormone responsive breast carcinoma cells**, In: Genes Chromosomes Cancer, 2010; 49(10):948-62.

Yamashita H., Kawasawa Y.I., Shuman L., Zheng Z., Tran T., Walter V., et al., **Repression of transcription factor ap-2 alpha by ppargamma reveals a novel transcriptional circuit in basal-squamous bladder cancer**, In: Oncogenesis. 2019; 8(12):69.

Yan C., Boyd D.D., **Regulation of matrix metalloproteinase gene expression**, In: J Cell Physiol. 2007; 211(1):19-26.

Yevshin I., Sharipov R., Kolmykov S., Kondrakhin Y., Kolpakov F., **Gtrd: A database on gene transcription regulation-2019 update**, In: Nucleic Acids Res. 2019; 47(D1):D100-D105.

Yevshin I., Sharipov R., Valeev T., Kel A., Kolpakov F., **Gtrd: A database of transcription factor binding sites identified by chip-seq experiments**, In: Nucleic Acids Res. 2017; 45(D1):D61-D67.

Zhong L., Wang Y., Kannan P., Tainsky M.A., **Functional characterization of the interacting domains of the positive coactivator pc4 with the transcription factor ap-2alpha**, In: Gene. 2003; 320:155-64.

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

Relationship between redox status in blood of patients with chronic myeloid leukemia (CML) and values of selected inflammatory markers – preliminary studies

Maria Drzewicka[1*], Maria Możdżeń[2], Iwona Urbanowicz[3], Beata Celuch[4], Ewa Mędraś[5], Tomasz Wróbel[5]

[1] Department of Food Science and Dietetis, Wroclaw Medical University, Borowska 211, 50-556 Wrocław, Poland

[2]Student Scientific Club Department of Food Science and Dietetics, Wroclaw Medical University, Poland

[3]Department of Medical Laboratory Diagnostics, Division of Clinical Chemistry and Laboratory Hematology, Wroclaw Medical University, Borowska 211A, 50-556 Wrocław, Poland

[4]Analytical Laboratory of the University Clinical Hospital, Wroclaw Medical University, Borowska 213, 50-556 Wrocław, Poland

[5]Department and Clinic of Haematology, Blood Neoplasms, and Bone Marrow Transplantation, Wroclaw Medical University, Wybrzeże Pasteura 4, 50-367 Wrocław, Poland

[*]Corresponding author: Maria Drzewicka, Department of Food Science and Dietetics, Wroclaw Medical University, Borowska 211, 50-556 Wrocław, Poland, e-mail: maria.drzewicka@wp.pl

ABSTRACT

The results of several experimental and clinical studies confirm the relationship between chronic inflammation and the concurrent oxidative stress as well as the development of many cancers.

The aim of this study was to assess the relationship between the redox status in the blood serum of the patients with chronic myeloid leukemia (CML) and the hs-CRP concentration as well as the values of the calculated markers: neutrophil-lymphocyte ratio (NLR), lymphocyte-monocyte ratio (LMR) and platelet-lymphocyte ratio (PLR).

Blood serum, obtained from 35 subjects (16 women and 19 men, aged 27-86) the CML, the patients of the Clinic of Hematology, Blood Neoplasms and Bone Marrow Transplantation of Medical University in Wroclaw, was used in this investigation. All the patients suffered from a chronic phase of the disease.

In the patients' serum the malondialdehyde (MDA) concentration as the end product of lipid peroxidation was determined using the TBARS method. A degree of DPPH radical reduction was measured in order to estimate the total antioxidative capacity of the deproteinized serum (TAC). Additionally, the serum hs-CRP concentration was also determined. Based on the results of the patients' blood count tests, the markers for NLR, LMR and PLR were calculated.

The serum MDA concentration in women was 7.72 μM/L and in men 6.92 μM/L. The TAC value of women was 251.55 mM Trolox/L whereas of men it was 244.67 mM Trolox eqvivalents/L. Statistically significant correlations were observed between the markers: PLR, NLR, LMR, including the negative correlations between the PLR and LMR as well as NLR and LMR, and the positive correlation between the NLR and PLR. However, no statistically significant correlation was found between MDA, TAC and hs-CRP levels and PLR, NLR, LMR values.

INTRODUCTION

CML is the disease entity that is caused by a clonal proliferation of multipotent bone-marrow stem cells resulting from the occurrence of the BCR/ABL gene mutation localized in Philadelphia chromosome (Ph) (Żołnierowicz, 2010; Jabbour, 2018). Chromosome Ph is an aberration consisting in the translocation between chromosomes 9 and 22, which results in mutant BCR/ABL proteins with tyrosine kinase activity (Druker, 2006).

Exposure to ionizing radiation has been proved to be an etiological factor of CML. It has been suggested that reactive oxygen species (ROS) take part in CLM patho-physiology (Ahmad, 2008 a).

At the moment tyrosine kinase inhibitors (TKI) are used in a standard CML therapy as they make it possible to get a complete cytogenetic response in a significant percentage of patients. However, increasingly frequent cases of resistance to imatinib treatment are observed, which is attributed to the significant role of ROS (Koptyra, 2006; Ammar, 2020). It has been established that BCR/ABL kinase induces the ROS growth due to the activation of the PI-3K/mTOR, kinase pathway, which in consequence results in the increase of proliferative activity of cancerous cell and the inhibition of apoptosis. (Kim, 2005; Sattler, 2000).

ROS are natural products of cellular oxygen metabolism (Ścibior-Bentkowska, 2009).

Oxidative stress is an imbalance resulting from overproduction or accumulation of ROS due to the insufficiency of antioxidant systems or dysfunction of antioxidant enzymes. The role of oxidative stress has been the subject of numerous studies for the last decades (Reuter, 2010; Sosa, 2013; Toyokuni, 2016; Valko, 2007; Udensi, 2014).

When the ROS level increases significantly it may result in the damage of nucleic acids, proteins and other cell parts, multisystem dysfunctions as well as mutations (Reuter, 2010). Apart from ROS, the concentration of endogenic antioxidants, that is the antioxidant capacity, has a significant impact of the oxidative stress induction. When a person is healthy, endogenic antioxidants play a protective role against the increase of ROS level and the cells with the balanced redox status are less susceptible to the genetic material damage (Sosa, 2013).

Inflammatory states favour formation of a large number of ROS in cells; therefore, they increase the oxidative stress intensity and stress – induced DNA mutations. ROS may contribute to abnormal gene expressions and dysfunction of signaling pathways and as a result of the progressive cell damage may cause cancerous transformation. (Hole, 2011). On the other hand the state of oxidative stress in the early stages of tumour growth may be the consequence of the inflammatory state which accompanies cancerous disease (Reuter 2010).

There are some widely known methods used to determine the oxidative stress level (Nielsen, 1997; Singh, 2009,). These methods are based on the measurement of ROS and antioxidant concentration in blood (Al-Gayyar, 2007). Malondialdehyde (MDA), the stable end product of lipid oxidation is adopted to be the marker of unfavourable effects of ROS activity (Ahmad, 2010). Looking at different methods determining antioxidant concentration in blood serum, DPPH assay turned out to be useful, cheap and simple to perform. Thanks to this method, it is possible to measure the total antioxidant capacity of the deproteinised blood serum (TAC) (Chrzczanowicz, 2008; Kedare, 2011).

Markers such as C-reactive protein is used to assess the inflammatory state in the organism (Cavicchia, 2009). The determination of hs-CPR value in the blood serum using an ultrasensitive method finds its application in diagnostics and monitoring of the inflammatory states (Bassuk, 2004). The C-reactive protein level may indicate the activity of carcinoma cells as well as the host's response to the presence of tumour (Stasik, 2008).

The inflammatory state markers: NLR (neutrophil-to-lymphocyte ratio), LMR (lymphocyte-to-monocyte ratio) or PLR (platelet-to-lymphocyte ratio) are widely inve-stigated with regard to their prognostic suitability for cancerous disease. (Wójcik, 2016; Forget, 2017; Yang, 2018) These markers reflect the systemic inflammatory response (SIR) which is appropriate in the prognostic assessment of the patients with numerous tumours (Ying, 2014). The advantages of this solution are the low cost of research and the fact that the marker levels can be easily determined only on the basis of the complete blood count (Sylman 2018).

Taking into consideration the fact that the oxidative stress, the chronic inflammatory state and the incidence of cancerous diseases are closely linked, it can be assumed that the all-in observation of the markers of the oxidative stress and the inflammatory state during the therapy may be helpful in the assessment of the disease advancement. The observation could also provide valuable information leading to a more targeted patient care (Reuter 2010).

The aim of this study was to assess the relationship between the redox status in the blood serum of the patients with chronic myeloid leukemia (CML) and the hs-CRP concentration as well as the values of the calculated markers: NLR, LMR and PLR.

MATERIALS AND METHODS

PATIENTS

The study was conducted in a group of 35 subjects (16 women and 19 men) with chronic myeloid leukemia (CML), at the median age of 57.5 years (in the age range: 27-86). The patients were from the Clinic of Hematology, Blood Neoplasms and Bone Marrow Transplantation of Medical University in Wroclaw. The diagnosis was made based on the standard clinic-hematological and cytogenetic criteria. The studies involved patients in the chronic phase of the disease treated with tyrosine kinase inhibitors.

All detailed information about the CML patients (the clinical stage of disease, the body mass index – BMI, smoking status, and the level of education) is presented in table 1. The research was approved by the Ethics Board of Wroclaw Medical University (No KB 172/2018) and all the patients provided their written consent for volunteering in the research.

BLOOD SAMPLE COLLECTION AND PREPARATION

The blood samples were taken from the patients' antecubital vein under fasting conditions. Hematological parameters including white blood cell count (WBCs) and the types (neutrophil, lymphocyte and monocyte) and platelets were determined by a hematological analyser (Sysmex XN2000, TOA Medical Electronics, Japan). NLR (the neutrophil-to-lymphocyte ratio) was calculated by dividing the absolute neutrophil count by the absolute lymphocyte count. The same calculation method was applied for other markers, that is, PLR (platelet-lymphocyte ratio) and LMR (lymphocyte-monocyte ratio).

The sample tubes were centrifuged for ten minutes at 3500 rpm to obtain a clear serum. The serum was separated and then stored at -80°C prior to the analysis.

DETERMINATION OF HS-CRP CONCENTRATION

The hs-CRP protein concentration was determined in the patients' blood serum using the immunoturbidimetric method, consisting in measurement of the changes in light dispersion and transmission of the reaction product of CRP protein molecules with anti-human CRP antibodies. The hs-CRP concentration measurement was carried out in the Kornelab 7.2.1 (Thermo Fisher Scientific, USA) automatic analyser.

DETERMINATION OF MALONDIALDEHYDE (MDA) CONCENTRATION

Serum malondialdehyde concentration (MDA) was evaluated with the spectro-photometric method using a Spectronic GENESYS 6 UV-visible spectrophotometer (Thermo Electron Corporation, USA); the measurement was based on the reaction between MDA and thiobarbituric acid (TBA) and the extraction of the product: 1-butanol. The absorbance of the pink supernatant was measured at λ=535 nm and the results were calculated using the molar coefficient, and expressed in μM of MDA/L of serum (Ahmad, 2008 b).

MEASUREMENT OF TOTAL ANTIOXIDANT STATUS (TAC)

The total antioxidant capacity (TAC) in serum was measured making use of the DPPH assay described by Chrzczanowicz (Chrzczanowicz, 2008). The serum samples were deproteinized with the use of the equal volume of acetonitryle. DPPH radical has an unpaired valence electron in its structure, therefore, when it reacts with antioxidants, which are electron donors or hydrogen radicals, 1.1-diphenyl-2-picrylhydrazine with a pale purple colour is formed. TAC was measured spectrophotometrically at λ = 517 nm using a Spectronic GENESYS 6 UV-visible spectrophotometer (Thermo Electron Corporation, USA). The results were expressed in mM Trolox equivalents/L serum.

STATISTICAL ANALYSIS

The data were analysed using Statistica Stat Soft 13.1. The nonparametric Mann–Whitney U test was used for group comparisons, and the Spearman correlation analysis was performed for the measurements of the relationship between MDA concentration and TAC value in female and male blood serum as well as NLR, LMR PLR levels and TAC value, hs-CRP value and MDA concentration.

For all statistical procedures, the significance level was set at $p < 0.05$.

RESULTS

Table 1 presents the characteristics of the examined women and men with CML.

Table 1. Characteristics of CLM patients with respect to gender

Parameter	Women (n=16)	Men (n=19)
Age, median (range) [years]	57 (28-86)	58 (27-79)
BMI, n (%) [kg/m^2]		
18,5-24.9	5 (31.2)	6 (31.6)
≥25	11 (68.8)	13 (68.4)
Clinical stage of disease (%)	chronic phase (100)	chronic phase (100)
Treatment, n (%):		
Imatinib	11 (68)	14 (73.7)
Nilotinib	3 (18.7)	3 (15.8)
Dasatinib	2 (12.5)	2 (10.5)
Smoking status, n (%):		
never smoker	15 (93.7)	13 (68.4)
current smoker	0	4 (21.1)
previous smoker	1 (6.3)	2 (10.5)
Educational level [%]: primary		
secondary	0	5.3
vocational	68.8	36.8
university	12.5	26.3
	18.7	31.6

BMI – body mass index

The median age of women and men was similar, in both groups the patients over 60 years of age constituted approximately 45%. The BMI was over 25 in approximately 68% of the examined group, which indicates obesity of a large number of the individuals. All the examined patients suffered from the chronic phase of CML. 68% women and 73% men were treated with imatinib. A small number of patients in the tested groups (5 females and 5 males) were treated with the second-generation kinase inhibitor (nilotinib and dasatinib). Majority of the patients were non-smokers – only 3 individuals of the examined groups admitted to smoking. Taking into account education there were twice as many females having secondary education whereas twice as many males had higher education.

Table 2 shows the results of the oxidative stress parameters (MDA, TAC), hs-CRP concentration as well as inflammatory state markers calculated from the results of the hematological parameters of the white blood cell system.

Table 2. Results of the redox state parameters, hs-CRP concentration in CLM patients' blood serum and calculated NLR, LMR and PLR markers

Parameter	Women (n=16)	Men (n=19)	p value
MDA [μM/L]			NS*
median	7.72	6.92	
(range)	5.28-10.41	5.62-10.33	
TAC [mM Trolox equiv./L]			NS
median	251.55	244.67	
(range)	28.0-493.43	31.33-426.29	
hs-CRP [mg/L]			NS
median	2.18	2.14	
(range)	(0.23-10.69	(0,12-9.28)	
NLR			NS
median	2.26	1.54	
(range)	(0.90-5.97)	(0.09-4.97)	
LMR			NS
median	2.96	3.28	
(range)	(1.29-9.50)	(1.25-23.32)	
PLR			NS
median	125.81	106.49	
(range)	(67.29-244.53)	(3.02-188.00)	

NS* – not statistically significant; NLR – neutrophil-lymphocyte ratio; LMR – lymphocyte-monocyte ratio; PLR – platelet-lymphocyte ratio; MDA – malondialdehyde concentration; TAC – total antioxidant capacity

During the examination, it was observed that MDA and TAC concentrations were slightly higher in the blood serum of the female CLM patients if compared to the male. However, the differences were not statistically significant. The median of hs-CRP concentration in blood serum of both female and male patients did not exceed the reference values set up for this marker (hs-CRP < 3 mg/L). No significant differences were observed between the two gender groups.

The results of white blood cell parameters – neutrophils, lymphocytes and monocytes as well as platelet count measured during the patient's blood count were used to determine NLR, PLR and LMR markers. The differences between the groups of females and males with CML were not statistically significant in the values of NLR, PLR and MLR markers.

Table 3 presents statistically significant correlations between NLR, PLR and LMR markers.

Table 3. Statistically significant correlations between NLR, PLR and LMR markers of CLM patients with respect to gender

Variables	R	p-value
Women n=16		
PLR vs LMR	-0,618	0,011
NLR vs PLR	0,624	0,010
NLR vs LMR	-0,568	0,022
Men n=19		
PLR vs LMR	-0,411	0,081
NLR vs PLR	0,711	0,0007
NLR vs LMR	-0,491	0,033

n-number of subjects; R-correlation coefficient

High correlations between the values of PLR and LMR (negative), NLR and PLR (positive) as well as NLR and LMR (negative) were found in the groups of the examined women. On the other hand, in the group of the male patients the relationships between PLR a LMR and NLR a LMR (negative) were average whereas between NLR and PLR (positive) they were very high (R = 0.711).

Table 4 shows correlations between MDA, TAC, hs-CRP levels and PLR, NLR, LMR values.

Table 4. Correlations between NLR, PLR and LMR markers and MDA, TAC and hs-CRP concentration of CLM patients with respect to gender

Variables	PLR R p-value	NLR R p-value	LMR R p-value	MDA R p-value	TAC R p-value
Women n=16					
hs-CRP	-0,150 0,579	0,415 0,110	0,106 0,696	0,221 0,411	0,041 0,880
MDA	0,116 0,668	0,249 0,353	0,091 0,737	-	0,121 0,656
TAC	0,259 0,333	0,106 0,696	-0,124 0,649	0,121 0,656	-
Men n=19					
hs-CRP	0,063 0,797	-0,007 0,977	0,153 0,533	0,088 0,721	0,161 0,509
MDA	-0,049 0,842	-0,125 0,611	0,086 0,726	-	0,151 0,538
TAC	0,049 0,842	-0,002 0,994	0,200 0,412	0,151 0,538	-

n – number of subjects; R – correlation coefficient

There was no statistically significant correlation between the PLR, NLR, LMR markers and the concentration of MDA, TAC and hs-CRP in both the female and male groups.

DISCUSSION

There is a lot of evidence of ROS participation in modifications of cellular reactions and there are implications that oxidative stress may be a significant pathophysiological factor of various types of leukemia, due to the initiation of lipid peroxidation and DNA damage (Devi, 2000, Oltra, 2001, Al-Gayyar 2007, Ciarcia, 2010). It is suggested that patients with leukemia may experience oxidative stress due to a greater number of mature and immature myeloid cells and the cell-related unknown factors (Rajeshwari, 2013).

BCR-ABL kinase supposedly stimulates ROS, which causes oxidative DNA damage and genome instability, which in consequence makes an intracellular environment more susceptible to mutations and may lead to disease progression. This fact suggests that ROS may play a significant role in resistance to treatment, which in turn may cause CLM progression (Ahmad, 2010).

In the available literature, few studies deal with the redox status in blood of patients suffering from different types of leukemia.

In this study the concentration of the lipid peroxidation products was measured and antioxidant ability to neutralise ROS was determined in order to assess fully redox status in CML patient's blood serum.

The mechanism of the formation of a great number of lipid peroxidation products may be based on the increased ROS production by the mature or immature myeloid cells, which leads to the oxidative stress (Gutowicz, 2011). Formed lipoperoxyl radicals can be regrouped in the cyclisation reaction to endoperoxides with the stable end peroxidation product – MDA with mutagenic and carcinogenic activity (Valko, 2007).

The examinations conducted so far, have shown that high oxidative stress occurs in CML and other types of leukemia (Petrola, 2012, Ahmad, 2010). According to Petrola (Petrola, 2012), MDA concentration reflects the range of lipid peroxidation and modulates gene expression connected with tumour promotion. The studies conducted by Ahmad (Ahmad, 2008 a, Ahmad, 2008 b, Ahmad, 2010) show that the application of drugs from different groups (imatinib, hydroxycarbamite) and different derivatives

within a certain group (I and Il generation kinase inhibitors) had a great impact on the MDA level in the CLM patients' blood. The CML phase was of importance as well, the considerably higher MDA concentration was found in the acceleration phase if compared with the chronic one. Nielsen et al. (Nielsen, 1997), based on determining the level of MDA in the blood of healthy Danes of both sexes aged 20-79, proposed reference values of this indicator in the range 0.36-1.24 μMm/L. In this study, both women and men had MDA serum levels approximately 6 times higher, which may reflect the degree of oxidative stress associated with the disease (Tab.2).

The available literature lacks information about determination of the total oxidation capacity (TAC) with the use of method of DPPH radical reduction in CML patients' blood serum. Mazor et al. (Mazor, 2008) observed that DPPH concentration in blood serum of children suffering from ALL was significantly lower if compared with the control group. It clearly indicated that the capacity of low-molecular-weight anti-oxidants to scavenger ROS was depleted.

The DPPH assay is based on the measurement of the scavenging capacity of ROS in different biological material. In this study the method, adapted by Chrzczanowicz et al. (Chrzczanowicz, 2002), was applied to determine the complete oxidation capacity (TAC) of deproteinised blood serum. The DPPH measurement method used in the serum was only reflecting the content of low molecular-weight-antioxidants. Hence, there could be no significant correlation between MDA and DPPH levels in patients' blood (Tab.4). Various factors, such as the structure of the molecules of these low-molecular-weight antioxidants, as well as the impact of the disease itself and therapy, could have an effect on the changes in the concentration of these components and their antioxidant activity.

CRP as an acute phase protein is a very sensitive marker of the inflammatory state (Bassuk, 2004). It is believed that the hs-CRP test, which is cheap and easy to use due to a low diagnostic sensitivity of many tumour markers, may provide useful infor-mation to assess the prognosis in patients and the choice of a therapy (Stasik, 2008).

The median value of in this investigation did not exceed the reference values both in female and male groups, which similarly as for other markers can be the consequence of the fact that all the patients were in the chronic phase of the disease and revealed a positive response to the performed therapy (Tab.2).

NLR, PLR or LMR markers can provide valuable prognostic information about different tumors. Studies on the prognostic role of these markers in various cancer diseases have been published (Li, 2018, Sun, 2018, Zhou, 2014, Yang, 2018, Li, 2017, Ying, 2018, Feng, 2018).

In literature there are no unequivocal reference ranges for the values of NLR, PLR and LMR markers that could be used to compare the obtained results. Lee et al. (Lee, 2018) conducted a retrospective study in which the values of NLR, PLR and LMR markers were calculated in 12000 healthy adults from the South Korean population in the age range 19-70 years. The conducted investigation showed that the mean values of NLR for all age groups was 1.65, and the values for males and females were 1.63 and 1.66, respectively. The average LMR value was 5.31 (for males and females – 5.05 and 5.60, respectively) whereas the mean PLR value was 132.40 (for males and females 122.73 and 142.60, respectively). The values of the NLR, LMR and PLR markers in the CML patients in this investigation were similar to these dates. It is difficult to compare these values with the cut off points put forward by the South Korean researchers due to inter-individual variability, racial and population differences. Statistically significant correlations between PLR, NLR and LMR markers in both women and men, found in this study, may result from the specificity of the disease, as well as from the inter-dependence of individual components of the white blood cell system (tab. 3).

This study revealed no statistically significant differences in MDA, DPPH, hs-CRP concentrations and the values of NLR, PLR and MLR markers between the groups of females and males with CML (tab. 4). The lack of correlation between the above markers could be caused by the applied therapy, which stabilized the patients' condition.

LIMITATIONS OF THE STUDY

There are limitations to this study. These were preliminary and pilot studies. The number of patients was not too high with a wide age range. Moreover, the studies were carried out only with the participation of patients in the chronic phase of the disease, treated with drugs from the group of tyrosine kinase inhibitors, which could have influenced the obtained results. Another limitation of the study was the determination of a few selected markers of inflammation and redox status. In further studies, the range of the determined redox status indicators should be increased.

SHORT CONCLUSION

The study found that statistically significant correlations occur between individual markers of the inflammatory state: PLR, NLR, LMR, including negative correlations between the PLR and LMR as well as NLR and LMR markers and the positive correlation between NLR and PLR markers. It speaks for the interdependence of the components of the white blood cell system and may suggest the inflammatory state in progress. No correlation was found between the mentioned markers and the MDA, TAC and hs-CRP concentration. Further investigations are necessary to determine relationships between the oxidative stress level and the concomitant inflammatory state and the response of the CLM patients' antioxidative system. The investigation should involve a bigger number of participants and should focus on the determination of values of a wider marker range for the redox status and the inflammatory state.

REFERENCES

Ahmad R., Tripathi A.K., Tripathi P., Singh R., Singh S., Singh R.K., **Oxidative stress and antioxidant status in patients with chronic myeloid leukemia,** Indian J Clin Biochem. 2008 a; 23(4):328-333.

Ahmad R., Tripathi A.K., Tripathi P., Singh R., Singh S., Singh R.K., **Studies on lipid peroxidation and non-enzymatic antioxidant status as indices of oxidative stress in patients with chronic myeloid leukaemia,** Singapure Medical Journal. 2010; 51(2):110-114.

Ahmad R., Tripathi A.K., Tripathi P., Singh S., Singh R., Singh R.K., **Malondialdehyde and protein carbonyl as biomarkers for oxidative stress and disease progression in patients with chronic myeloid leukemia,** Vivo, 2008 b; 22(4):525-528.

Al-Gayyar M.M.H., Eissa L.A., Rabie A.M., El-Gayar A.M., **Measurements of oxidative stress status and antioxidant activity in chronic leukaemia patients,** J Pharm .Pharmacol. 2007; 59:409-417.

Ammar M., Mahmoud L.B., Medhaffar M., Hanen Ghozzi H., Sahnoun Z., Hakim A., et al., **Relationship of oxidative stress in the resistance to imatinib in Tunisian patients with chronic myeloid leukemia: A retrospective study,** J Clin Lab Anal. 2020; 34:e23050, https ://doi.org/10.1002/jcla.23050

Bassuk S.S., Rifai N., Ridker P.M., **High-sensitivity C-reactive protein: clinical importance,** Curr Probl Cardiol 2004; 29:439-493.

Cavicchia P.P. Steck S.E., Hurley T.G., Hussey J.R., Ma Y., Ira S. Ockene I.S., **A new dietary inflammatory index predicts interval changes in serum high-sensitivity C-reactive protein,** J. Nutr. 2009; 139:2365-2372.

Chrzczanowicz J., Gawron A., Zwolińska A., de Graft-Johnson J., Krajewski W., Krol M., et al., **Simple method for detrmining human serum 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavering activity - possible application in clinical studies on dietary antioxidants,** Clin Chem Lab Med. 2008; 46(3):342-349.

Ciarcia R., D'Angelo D., Pacilio C., Pagnini D., Galdiero M., Fiorito F., et al., **Dysregulated calcium homeostasis and oxidative stress in chronic myeloid leukemia (CML) cells**, J Cell Physiol. 2010; 224(2):443-53.

Devi G.S., Prasad M.H., Saraswathi I., Raghu D., Rao D.N., Reddy P.P., **Free radicals antioxidant enzymes and lipid peroxidation in different types of leukemias**, Clin Chim Acta. 2000; 293(1-2):53-62.

Druker B.J., Guilhot F., O'Brien S.G., Gathmann I., Kantarjian H., Gattermann N., et al., **Five-Year Follow-up of Patients Receiving Imatinib for Chronic Myeloid Leukemia,** N Engl J Med. 2006; 355:2408-2417.

Feng J.-F., Huang Y., Zhao Q., Chen Q.-X., **Clinical significance of preoperative neutrophil lymphocyte ratio versus platelet lymphocyte ratio in patients with small cell carcinoma of the esophagus,** The Scientific World Journal, 2013, DOI.org/10.1155/2013/504365.

Forget P., Khalifa C., Defour J.P., Latinne D., Van Pel M.C., De Kock M., **What is the normal value of the neutrophil-to-lymphocyte ratio?,** BMC Res Notes. 2017; 10:12, DOI 10.1186/s13104-016-2335-5

Hole P.S., Darley R.L., Tonks A., **Do reactive oxygen species play a role in myeloid leukemias?,** Blood. 2011; 117(22):5816-5826.

Jabbour E., Kantarjian H., **Chronic myeloid leukemia: 2018 update on diagnosis, therapy, and monitoring**, Am J Hematol. 2018; 93:442-459.

Kedare S.B., Singh R.P., **Genesis and development of DPPH method of antioxidant assay,** JFST. 2011; 48(4):412-422.

Kim H.J., Chu S.C., Gramlich J.L., Pride Y.B., Babendreier E., Chauhan D., et al., **Activation of the PI3K/mTOR patway by BCR-ABL contributes to increased production of reactive oxygen species,** Blood, 2005; 105:1717-1723.

Koptyra M., Falinski R., Nowicki M.O., Stoklosa T., Majsterek I., Nieborowska-Skorska M., et al., **BCR/ABL kinase induces self-mutagenesis via reactive oxygen species to encode imatinib resistance,** Blood. 2006; 108(1):319-327.

Lee J.S., Kim N.Y., Na S.H., Youn Y.H., Shin C.S., **Reference values of neutrophil-lymphocyte ratio, lymphocyte-monocyte ratio, platelet-lymphocyte ratio, and mean platelet volume in healthy adults in South Korea**, Medicine. 2018; 97:26(e11138).

Li W., Tao L., Zhang L., Xiu D., **Prognostic role of lymphocyte to monocyte ratio for patients with pancreatic cancer: a systematic review and meta-analysis,** OncoTargets Ther. 2017; 10:3391-3397.

Li X., Dai D., Chen B., Tang H., Xie X., Wei W., **The value of neutrophil-to-lymphocyte ratio for response and prognostic effect of neoadjuvant chemotherapy in solid tumors: A systematic review and meta-analysis,** J Cancer, 2018; 9(5):861-871.

Mazor D., Abucoider A., Meyerstein N., Kapelushnik J., **Antioxidant status in pediatric acute lymphocytic leukemia (ALL) and solid tumors: The impact of oxidative stress,** Pediatr Blood Cancer, 2008; 51:613-615.

Nielsen F., Mikkelsen B.B., Nielsen J.B., Andersen H.R., Grandjean P., **Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors,** Clin Chem.1997; 43:71209-1214.

Oltra A.M., Carbonell F., Tormos C., Iradi A., Guillermo T., Saez G.T., **Antioxidant enzyme activities and the production of MDA and 8-oxo-dg in chronic lymphocytic leukemia**, Free Radic Biol Med. 2001; 30(11):1286-92.

Petrola M.J., Montenegro de Castro A.J. da Silva Pitombeira M.H., Cavalcante Barbosa M., de Souza Quixadá A.T., Barroso Duarte F.B., **Serum concentrations of nitrite and malondialdehyde as markers of oxidative stress in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors,** Rev Bras Hematol Hemoter. 2012; 34(5):352-5.

Rajeshwari U., Shobha I., Raghunatha R., Andallu B., **Oxidative stress and antioxidant status in acute and chronic myeloid leukemia patients,** Open Journal of Blood Diseases, 2013; 3(8):17-22.

Reuter S., Gupta S.C., Chaturvedi M.M., Aggarwal B.B., **Oxidative stress, inflammation, and cancer: How are they linked?,** Free Radic Biol Med. 2010; 49:1603-1616.

Sattler M., Verma S., Shrikhande G., Byrne C.H., Pride Y.B., Winkler T., et al., **The BRR/ABL tyrosine kinase induces production of reactive oxygen species in hematopoietic cells,** J Biol Chem. 2000; 275:24273-24278.

Singh R.K., Tripathi A.K., Tripathi P., Singh S., Singh R., Ahmad R., **Studies on biomarkers for oxidative stress in patients with chronic myeloid leukaemia,** Hematol Oncol Stem Cell. Ther. 2009; 2(1):285-288.

Sosa V., Moliné T., Somoza R., Paciucci R., Kondoh H., Leonart M.E., **Oxidative stress and cancer: An overview,** Aging Res Rev. 2013; 12:376-390.

Stasik Z., Skotnicki P., Nowak-Sadzikowska J., Kulpa J.K., **Białko C-reaktywne u chorych na nowotwory złośliwe,** J Oncol. 2008; 58(5):441-446.

Sun Y., Zhang L., **The clinical use of pretreatment NLR, PLR, and LMR in patients with esophageal squamous cell carcinoma: evidence from a meta-analysis,** Cancer Manag Res. 2018; 10:6167-6179.

Ścibior-Bentkowska D., Czeczot H., **Komórki nowotworowe a stres oksydacyjny,** Postepy Hig Med Dosw. 2009; 63:58-72.

Toyokuni S., **Oxidative stress as an iceberg in carcinogenesis and cancer biology**, Arch Biochem Biophys. 2016; 595:46-49.

Udensi K., Tchounwou P.B., **Duall effect of oxidative stress on leukemia cancer induction and treatment,** J Exp Clin Cancer Res. 2014; 33:106.

Valko M., Leibfritz D., Moncol J., Cronin M.T.D., Mazur M., Telser J., **Free radicals and antioxidants in normal physiological functions and human disease,** Int J Biochem Cell Biol. 2007; 39:44-84.

Wójcik E., Stasik Z., Rychlik U., Tarapacz J., Kulpa J.K., Brandys K., **Wskaźniki NLR i PLR w ocenie rokowania chorych na nie drobnokomórkowego raka płuc**, Diagnostyka Laboratoryjna, 2016; 52(1):13-20.

Yang Y., Jiang J., Yang H., Wu Z., Xiao Z., Xiang B., **The lymphocyte-to-monocyte ratio is a superior predictor of overall survival compared to established biomarkers in HCC patients undergoing liver resection,** Sci Rep. 2018; 8:2535, DOI:10.1038/s41598-018-20199-2.

Ying H.Q., Deng Q.W., He B.S., Pan Y.Q., Wang F., Sun H.L.,et al., **The prognostic value of preoperative NLR, d-NLR, PLR and LMR for predicting clinical outcome in surgical colorectal cancer patients,** Med Oncol. 2014; 31:305, DOI 10.1007/s12032-014-0305-0.

Zhou X., Du Y., Huang Z., Xu J., Qiu T., Wang J., et al., **Prognostic value of PLR in various cancers: a metaanalysis,** PloS One. 2014; 9(6):e101119.

Żołnierowicz J., Kawiak J., Hoser G., **Patogeneza przewlekłej białaczki szpikowej- od genu do terapii celowanej,** Hematologia 2010; 1(3):195-218.

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

A short guide on the selection of melanocytes and melanoma cells' isolation procedures for cancer research

Anna Sobiepanek[1,*], Patrycja D. Kowalska[1,2], Marta Soszyńska[3], Tomasz Kobiela[1], Aneta Ścieżyńska[3,4,*]

***corresponding authors: aneta.sciezynska@wum.edu.pl and asobiepanek@ch.pw.edu.pl**

[1] Laboratory of Biomolecular Interactions Studies, Chair of Drug and Cosmetics Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

[2] Polish Stem Cells Bank, Warsaw, Poland

[3] Department of Histology and Embryology, Medical University of Warsaw, Warsaw, Poland

[4] Department of Regenerative Medicine, Military Institute of Hygiene and Epidemiology, Warsaw, Poland

ABSTRACT

The skin contains about 5% of melanin-producing cells called melanocytes. The analysis of melanocytes is of greatest interest for multiple fields of dermatology, especially those concerning the detailed molecular mechanism of melanogenesis and melanin transfer to keratinocytes. An effective and simple method aimed at the culture of melanocytes is also essential for the investigation regarding melanoma development, the deadliest skin cancer. The main purpose in the current melanoma research is focused on the better understanding of the metastasis process, which can lead to the development of new techniques concerning early diagnosis as well as an effective treatment. Because differences and similarities in cell properties can be observed simultaneously between the clinical samples, gaining general knowledge about the melanoma phenomenon, particularly in case of the unusual tumors, is a priority.

Unfortunately, due to the low percentage of melanocytes in the skin, the establishment of a pure melanocyte culture (without fibroblast and keratinocyte contamination) is difficult. For this reason, access to larger amounts of patient-derived cell models and established cell lines should be assessed by laboratories which are focused on the isolation of the cells of interest themselves. Here, we present an overview of the traditional as well as innovative procedures of isolation and culture of melanocytes and melanoma cells, which will be useful for the improvement of the existing laboratory protocols, as well as it may inspire novel laboratories to enlarge the spectrum of their scientific interest in melanoma research.

Keywords: melanocytes, melanoma, cell isolation, procedures

INTRODUCTION

Cancer remains as one of the biggest threats to human wellbeing, therefore novel diagnostic tools and procedures for its detection should be developed. The establishment of a quick but trustworthy test for the identification of cancer biomarkers in the human organism is intensively conducted by various scientists (Sobiepanek and Kobiela 2018). The availability of many different cell lines (from normal to the cancerous types being at different stages of progression) is of great importance for the researches performed *in vitro*. One of the main scientific objectives is focused on the better understanding of the metastasis process, what is indispensable for further development of novel techniques aimed at early diagnosis and effective treatment *in vivo*. To help with this issue and at the same time to better comprehend the metastasis process as well as to develop an effective treatment, access to larger numbers of melanoma models like primary cells, cell lines, mouse models and computer simulations (theoretical models) is essential. Since each model has its limitation (e.g. the *in vitro* models lack responsiveness from the immune system), application of several models at the same time of the study may help in a detailed understanding of cancer or its response to the proposed treatment (Madhunapantula et al. 2019). On the other hand, some cases of cancer may be unique (e.g. unusual or rare mutation, very aggressive tumor cells), thus cell isolation would be beneficial for further research and highly recommended. Furthermore, the isolation of the normal and cancerous cells from the same patient would be of tremendous value to widen the knowledge about tumor development and changes occurring between both types of cells. In this article, our interest will be focused on melanocytes and melanoma cells.

Melanocytes are the cells of neuroectodermal origin, which can be found in the skin, brain, eyes, ears, lungs, heart, hair and in some mucous membranes. Their broad spectrum of functions depends on their location in the human body, e.g. in the brain melanocytes may be involved in the process of detoxification; in the heart they play a role in the reduction of free radicals and anti- inflammatory processes; while in the skin,

hair and eyes melanocytes provide protection against the ultraviolet radiation due to melanin synthesis during melanogenesis. In the epidermis melanocytes constitute only 5% of all types of the skin cells, which contributes to about 1,200 melanocytes per mm^2 of the skin (independently of the human race), thus isolation and culture of the pure melanocytes may be of great challenge for many scientists (Marczyńska and Przybyło 2013, Wikramanayake et al. 2014, Rzepka et al. 2016, Johansen 2017).

Melanoma arises from melanocytes as a result of mutation/s and subsequent uncontrolled cell proliferation. Among skin cancers, melanoma represents the most malignant type, with the highest rate of mortality. According to the World Health Organization, nearly 288,000 of melanoma cases were diagnosed in 2018 worldwide and the occurrence of this disease in 2025 may be even greater and reach approximately 340,000 cases per year (nearly 20% more cases). The majority of the melanoma cells sustain their ability to melanin synthesis; thus, they form pigmented melanoma – typically brown or black. In rare cases with disrupted melanin synthesis, amelanotic melanoma may also occur. Many different clinical subtypes of melanoma have been described, but the most frequent are: acral lentiginous melanoma (ALM), nodular melanoma (NM), lentigo maligna melanoma (LMM) and superficial spreading melanoma (SSM). On the other hand, the development of melanoma consists of six steps: (i) the common acquired melanocytic nevus, (ii) a melanocytic nevus with lentiginous melanocytic hyperplasia, (iii) a melanocytic nevus with aberrant diffe-rentiation and melanocytic nuclear atypia, (iv) the radial growth phase (RGP) of the primary melanoma, (v) the vertical growth phase (VGP) of the primary melanoma and (vi) metastatic melanoma (MM) (Forman et al. 2008, Dummer et al. 2016, Elder 2016, Sobiepanek et al. 2017).

Although, there are many commercially available cell lines of melanocytes and melanoma cells, the possibility of cell isolation directly from individuals allow for a comprehensive analysis of cell function and differentiation, as well as cance-rogenesis. The establishment of pure cultures of melanocytes and/or melanoma cells is indispensable for the researchers focused on the analysis of the *in vitro* relationship between melanocytes and keratinocytes (De Luca et al. 1988), mechanism of melanin production and its biosynthesis (Bao et al. 2015, Knapp and Iden 2020), melanocytes implementation in the 3D skin equivalents (Gledhill et al. 2015), as well as for the diagnostic procedure aimed at the differentiation melanomas from melanocytes *in vitro* (Fujiwara et al. 2019).

STATE OF ART

The skin is the largest organ of the human body, composed of the epidermis and the dermis built by progenitors and variously differentiated cells (De Falco et al. 2014). The main component of the outermost epidermal layer are keratinocytes. Keratinocytes undergo the differentiation process during which they maturate into corneocytes. These non-nucleated flattened and cornified cells, together with ceramides, cholesterol and free fatty acids form water impermeable layer located at the surface of the skin called *stratum corneum* (Sobiepanek et al. 2019). Other components of the epidermis are: the Langerhans cells (dendritic cells of the immune system of the integument), the Merkel cells (cells of the neural origin) and melanocytes. Melanocytes are located in the stratum basalis, the deepest layer of the epidermis located directly above the basement membrane, which separates the epidermis from the dermis (Sobiepanek et al. 2020). They are interspersed among the keratinocytes at a ratio of 1:30 depending on the body site (De Falco et al. 2014, Dehdashtian et al. 2018, Graham et al. 2019). The concept of a melanocyte interacting with a specified group of keratinocytes, creating the Epi-dermal Melanin Unit (EMU), was first proposed by Fitzpatrick and Breathnach in 1963. However, there are votes in favor to include the Langerhans cells in the physiological functional unit, which would be known as the keratinocyte-Langerhans-melanocyte (KLM) unit (Nordlund 2007).

In order to maintain the homeostasis *in vivo*, melanocytes require the presence of keratinocytes to which they bind through adhesion molecules called E-cadherins. The downregulation of their expression breaks the control of keratinocytes over melanocytes. In general, melanocytes have a small proliferative potential and rarely divide, therefore melanocyte overactivity leads to pathological conditions (Santiago-Walker et al. 2009, Marczyńska and Przybyło 2013). Moreover, changes in the cadherins expression pattern, from the E-cadherin into the

N-cadherin is observed during the epithelial-mesenchymal transition (EMT) that leads to the malignant transformation. The cadherin-switch allows for the mobility of melanocytes and for their interaction with fibroblasts, endothelial cells or themselves. This also leads to the penetration of the deeper layer of the dermis and to the further cell expansion through the blood and lymphatic vessels (Santiago-Walker et al. 2009, Marczyńska and Przybyło 2013).

Although, the molecular bases of melanoma progression are still the subject of an intensive research, it is associated with mutations in genes responsible for the proli-feration and apoptosis, as well as with epigenetic changes, loss of adhe-sion ability or production of the autocrine growth factors, which disturbs the signal trans-duction pathway in melanocytes. (Pokrywka and Lityńska 2012). The National Cancer Institute distinguishes four types of the patient-derived models (PDMs) for cancer research: the *in vitro* patient-derived tumor cell cultures (PDCs) and cancer-associated fibroblasts (CAFs), the *ex vivo* patient-derived organoids (PDOrg), as well as the *in vivo* patient-derived xenografts (PDXs). Several PDMs of melanoma and skin cancers are commercially available (6 cases of PDCs) or undergoing quality control like the Next-Generation Sequencing (NGS) and tumor-igenicity verification (10 developed PDCs) (Evrard et al. 2019). The PDCs may reflect the characteristic features of the patient's tumor cells and can be used to deepen the molecular validation process e.g. gene expression profiling or immunohistochemical staining with the selected biomarkers. On the other hand, with the use of the optimal technique for melanocytes and melanoma cell isolation and culture, the variety of the available cell lines can be signifi-cantly increased (Bleijs et al. 2019). Therefore, here we summarize the spectrum of techniques used for melanocytes and melanoma cell isolation and culture described in literature and commonly used in laboratories. We believe this will be helpful for the improvement of the existing protocols or an inspiration for novel scientists who intend to perform their first expe-riments on melanocytes and melanoma cells.

SEARCH STRATEGY AND SELECTION CRITERIA

Literature analysis has been performed with the use of the PubMed database and the search term combinations of "melanocytes – melanoma – cell isolation – techniques – cell culture *in vitro*". For further analysis we have chosen articles in which melanocytes and/or melanoma cell isolation methods from human or animal tissues samples were well described. Moreover, during hand search in Google Scholar, some additional articles were also investigated for a better comprehension of the subject.

REVIEW AND DISCUSSION

For the *in vitro* research purpose, commercially available cell lines are frequently selected. Since 1978 over 350 human melanoma cell lines have been established and characterized in the Mela-noma Research Center as well as in the Wistar Institute, which initiated major advances in melanoma research. These different cell lines, from each tumor progression stage (tab. 1), may be purchased from companies like ATCC, Biocompare, European Collection of Cell Cultures (ECACC), JCRB Cell Bank, Rockland Immunochemicals Inc. and Merck (formerly Sigma Aldrich). The establish-ment of a mela-noma cell line is difficult and the success rate often depends on the stage of cancer pro-gression. For example, the RGP melanoma cell lines are rarely developed, mainly due to the small specimen size available for isolation. On the other hand, the success rate for VGP melanomas culture ranges from 30-70% and also depends on the size of the lesion obtained during surgery. The highest success rate (75-80%) for the cell line establishment is observed for metastatic melanomas, in which the most common sources of samples are the lymph node metastases and the least common cutaneous nodules (Hsu et al. 1999). Despite the great diversity in melanoma cell lines which origin-nated from the different stages of melanoma progression, the establishment of the cell line models containing specific mutations, for exam-ple, in *BRAF* or *NRAS* genes, is also of great importance for scientific purposes (Schadendorf et al. 2018). An extremely valuable study can also be performed on the multiple types of cell lines isolated directly from the tumor and healthy samples of one patient. This model allows for the comparison between closely related cells that differ in morphology or in their invasive potential. Some of the related mela-noma cell lines are presented in table 2.

Table 1. Examples of commercially available cell lines from different progression stages of melanoma

RGP site	VGP site	Lymph node MM	Solid tumor MM
SBC12 WM1552C WM1862 WM35	WM75 WM115 WM278 WM278 WM793 WM902B WM983A	A2058 GAK Hs 294T Ma-Mel-15 MeWo SK-MEL-1 WM266-4	451Lu A375 C32 CHL-1 Colo 794 G-361 WM1026

Table 2. Examples of the multiple cell lines developed from the same patient (Hsu et al. 1999)

Vertical Growth Phase	Metastatic Melanoma
WM75	WM373
WM115	WM165-1, WM165-2, WM239A, WM239B, WM266-1, WM266-2, WM266-3, WM266-4
WM278	WM1617
WM740V	WM858, WM873-1, WM873-2, WM873-3

In case of melanocytes, the commercially available cell lines are limited to two types of cells offered from each company: neonatal or adult skin. However, there is also a choice between cells obtained from lightly, moderately or darkly pigmented donors. Some of the established and commercially available melanocytes are listed in table 3. These cell lines are frequently used in cancer research studies. Unfortunately, the comparison of cells obtained from different donors may lead to a misinterpretation of the obtained results and mask the true mechanisms which melanoma pathogenesis undergoes. Due to this reason, the isolation of the cancerous and normal cells from the same patient becomes increasingly important.

Table 3. Examples of the commercially available melanocytes

Company	Melanocytes and their source
ATCC	Primary Epidermal Melanocytes; Normal, Human, Neonatal (HEMn) Primary Epidermal Melanocytes; Normal, Human, Adult (HEMa)
Cell Applications, INC. (distributor MERCK)	Human Epidermal Melanocytes: HEM, adult Human Epidermal Melanocytes: HEM, neonatal
Gibco™ Thermofisher Scientific	Human Epidermal Melanocytes, adult, lightly pigmented donor, (HEMa-LP) Human Epidermal Melanocytes, neonatal, lightly pigmented donor, (HEMn-LP) Human Epidermal Melanocytes, neonatal, moderately pigmented donor, (HEMn-MP) Human Epidermal Melanocytes, neonatal, darkly pigmented donor, (HEMn-DP)
PromoCell	Normal Human Epidermal Melanocytes (NHEM) Normal Human Epidermal Melanocytes 2 (NHEM 2)
Ximbio	Immortalised Human Melanocyte [PIG1] Cell Line

Ameri Research Inc. showed in 2016 that the total cost of the necessary instruments, consumables and reagents required for cell culture and isolation reached about 3.4 billion dollars and by the year of 2024 this cost may increase to even 11.5 billion dollars, what highlights the importance of the research using cell culture techniques (Ameri Research Inc.

webpage). In general, the isolation procedure consists of a few stages. First, (i) the tissue should be collected during surgery, then the sample should be stored in a dedicated transport media (ii), next the sample should be washed (iii), fragmented (iv) and digested (v) with the use of specific enzymes (e.g. collagenase, dispase, hyaluronidase, liberase or trypsin). After cell separation (vi), features of the isolated cells should be tested and confirmed by molecular methods. The most important and frequently used protocols implemented for melanocytes and melanoma cell isolation are summarized in table 4 and table 5.

Table 4. The described conditions for melanocyte isolation. The used abbreviations are: BBE – bovine brain extract, bFGF – basic fibroblast growth factor, BPE – bovine pituitary extract, CT – cholera toxin, EDTA – ethylenediaminetetraacetic acid, EGF – epidermal growth factor, FBS – fetal bovine serum, FCS – fetal calf serum, HBSS – Hanks Balanced Salt Solution, IBMX – isobutylmethyl xanthine, TPA – 12 tetradecanoylphorbol 13-acetate (otherwise known as PMA, phorbol 12-myristate 13-acetate)

The method of epidermis separation from the dermis (tissue origin)	The method of receiving a single cell suspension for seeding	The used culture medium	Reference
The tissue was incubated in 0.25% trypsin in PBS for 30 min at 37°C, next the epidermis was mechanically separated from the dermis (human)	A single-cell suspension was obtained by gentle agitation in the medium	McCoy's 5A medium with 10% CS, 1 nM CT and 0.33 µM IBMX	Tsuji and Karasek 1983
The tissue was incubated in Eagle's minimal essential medium without calcium (MEMS) with 0.25% trypsin, 200 U/ml penicillin, 100 µg/ml streptomycin and 100 ng/ml TPA at 4°C overnight (human)	The tissue was shaken vigorously in the melanocyte growth medium and the supernatant with cells was collected	Ham's F-10 medium with 10% Nu-serum, 2% CS, penicillin, streptomycin, 48 nM TPA, 2.5 nM CT and 0.1 mM IBMX	Halaban and Alfano 1984
The skin biopsy was minced and digested in 0.05% trypsin/0.01% EDTA at 37°C for 3 h (human)	The cells were collected from the received supernatant	Dulbecco-Vogt Eagle's and Ham's F12 medium (3:1) with 5% FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 0.18 mM adenine, 0.4 mg/ml hydrocortisone, 1 nM CT, 10 ng/ml EGF, 20 pM triiodothyronine, 10 ng/ml PMA, 50 IU/ml BBE and 50 IU/ml penicillin/ streptomycin	De Luca et al. 1988
The tissue was cut into pieces and incubated in Eagle's minimum essential medium (EMEM) with 0.5% collagenase Type V 5% FBS for 1-2 h at 37°C (human)	The cell suspension was obtained by treating the tissue with 0.05% trypsin and 0.53 mM EDTA for 5-10 min at 37°C	EMEM with 10% FBS, 0.2 µg/m1 CT, 50 nM PMA, 0.05 mg/ml gentamicin, 2.5 µg/ml fungizone and keratinocyte serum-free medium	Tobin et al. 1995
The tissue was cut into pieces and digested in 2 mg/ml dispase at 4°C, mechanical separation of the epidermal tissue was performed with forceps (human)	The tissue was incubated in 0.25% trypsin at 37°C for 5 to 10 min	MCDB153 with 10 µg/ml bovine insulin, 10 µg/ml transferrin, 2.8 µg/ml hydrocortisone, 2 mM glutamine, 10 U/ml nystatin, 1 ng/ml vitamin E, 10 nM PMA, 100 ng/ml CT, 100 µg/ml bovine hypothalamic extract and 2% FCS chelex-treated	Goodall et al. 1994
The epidermis was separated from the dermis after an overnight incubation in 0.25% trypsin at 4°C (human)	undescribed	Medium 199 with 10 ng/ml EGF, 1 nM triiodothyronine, 10 µg/ml transferrin, 10 µg/ml insulin, 1.4 µM hydrocortisone, 1 nM CT, 10 ng/ml bFGF and 5-10% FBS	Hara et al. 1996
The tissue was cut into small pieces and incubated in 0.25% trypsin for 12 h at 4°C, and next the epidermis was mechanically separated from the dermis (sheep)	A single-cell suspension was derived by vigorous pipetting in 10% FCS from the epidermis	Melanocyte basal medium with 0.05 µg/ml amphotericin B, 1 ng/ml bFGF, 13 mg/ml BPE, 50 µg/ml gentamicin, 0.5 µg/ml hydrocortisone, 10 ng/ml PMA, 5 µg/ml bovine insulin, 1% FCS, CT	Sanchez Hanke et al. 2005
The tissue was digested with 0.2-0.4% dispase for 1-18 h depending on the thickness of the dermis (human)	The tissue was digested with 0.05% trypsin-EDTA	Melanocyte medium (254-CF)	Ghosh et al. 2008

The tissue was minced into 0.2×0.5 cm pieces, digested in 0.25% trypsin solution or in 0.05% trypsin in 0.02% EDTA at 4°C for 20 h, next the tissue was incubated at 37°C for 2 h, the mechanical separation of the epidermal tissue was performed with forceps (alpaca)	The epidermal tissue was digested in 0.25% trypsin in 0.02% EDTA for 8 min at 37°C and vigorous pipetting in Melanocyte Basal Medium + 10% CS and filtration through a 200- pore steel sifter with a pore diameter of 76 μm	Melanocyte basal medium supplemented with 0.2 μg/ml CT, 0.05 mg/ml gentamicin, 2.5 μg/ml fungizone, 50 μg/ml BPE, 0.5 μg/ml hydrocortisone, 1 ng/ml bFGF, 5 μg/ml insulin and 10 ng/ml TPA	Bai et al. 2010
The tissue was cut into small stripes and digested in 1.07 U/ml Dispase II in HBSS at 4°C overnight, next the mechanical separation of the epidermal tissue was performed with forceps (fox)	Mechanical blowing was performed and cell suspension was obtained with a cell sieve (200 meshes)	Keratinocyte serum free medium (K-SFM) with 10% FBS and 1% penicillin- streptomycin, EGF and BPE (for 3 days); next the full medium was supplemented with PMA (for 7-10 days)	Bao et al. 2015
The subcutaneous adipose tissue was manually removed from the dermis with a scalpel, the remaining tissue was cut into small pieces and incubated in 2.4 U/ml dispase in PBS for 1.5 h, next the epidermis was mechanically separated from the dermis (human)	The tissue was treated with 0.25% trypsin and 0.53 mM EDTA for 10 min at 37°C and next the suspension was filtered through a 200 m filter	Medium 254 supplemented with a human melanocyte growth supplement (HMGS)	Zhang et al. 2017
The mechanical removal of the subcutaneous tissue, mincing and digestion with trypsin (human)	The cells were collected from the received supernatant	DermaLife medium	Fujiwara et al. 2019
The tissue was incubated in RPMI with 10%FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 nM sodium pyruvate, 10 nM non-essential amino acids and 5 mg/ml Dispase II at 4°C overnight (mouse)	The tissue incubated in TrypLE for 20 min at room temperature	RPMI-1640 with 200 nM TPA and 200 pM CT	Knapp and Iden 2020

Table 5. The described conditions for melanoma isolation. The used abbreviations are: CT – cholera toxin, FBS – fetal bovine serum, FCS – fetal calf serum, G418 – geneticin, PMA – phorbol 12- myristate 13-acetate

Applied method of melanoma cell isolation (tissue origin)	The used culture medium	Reference
The tissue was digested with dispase (1:2) in RMPI for 3 h at 37°C, and next digested with collagenase (1:100) in Tris-buffered saline containing $CaCl_2$ for 2 h at 37°C, the cell suspension was filtrated through 70- μm nylon strainers (horse)	RPMI-1640 with 10% FCS, 5 mM glutamine, 1 mM sodium pyruvate, 10,000 U/ml penicillin and 10 mg/ml streptomycin	Chapman et al. 2009
The tissue was cut into small pieces and digested with 235 U/ml collagenase and 850 U/ml hyaluronidase in RPMI+1%FBS for 2 h at 37°C, and next the suspension was filtered through 70- and 40- μm strainers (human)	RPMI-1640 with 10% FBS	Luo et al. 2013
The tissue was digested with 60 μg/ml liberase Blendzyme TM mix in Medium 199 for 1 h at 37°C and filtered through 70 μm nylon mesh (human)	Medium 199	Boiko 2013
The tissue was digested with 200 U/ml collagenase type IV for 20 min at 37°C, and next incubated with 0.05% trypsin-0.5 mM EDTA as well as 100 U/ml DNase solution for 2 h at 37°C. The suspension was filtered through a 40 μm strainer (human)	80% MCDB153 and 20% L15 supplemented with 2% FBS, 5 μg/ml insulin and 1.68 mM $CaCl_2$	Slipicevic et al. 2014
The tissue was digested with trypsin and collagenase (human)	Ham F12 medium with 10 ng/ml CT, 100 nM PMA, 100 μg/ml G418 and 50 μg/ml gentamicin	Weidmann et al. 2017
The tissue was mechanically disaggregated and next digested with 2 mg/ml collagenase type II for 2 h at 37°C, the suspension was filtered with a 70 μm nylon strainer (dog)	RPMI-1640 Glutamax growth medium with 10% FCS and 100 U/ml penicillin and 0.1 mg/ml streptomycin	Segaoula et al. 2018
The tissue was mechanically disaggregated (human)	DMEM with 10% FBS, 2 mM glutamine and a 1% penicillin/ streptomycin mixture	Heitzer et al. 2019
The mechanical removal of the subcutaneous tissue, mincing and digestion with trypsin (human)	DermaLife medium	Fujiwara et al. 2019

The study in which the cells are isolated from the tissue can begin as soon as the consent of the local Bioethics Committee for these experiments is acquired. The researcher may proceed with the following steps of the isolation procedure and adapt it to his/her needs or capabilities:

a) tissue collection during the surgery and its transport

The first two steps of the process require tissue collection after surgery (after a written consent is signed by the patient) and its transport to the destinated laboratory. The most common sources of normal melanocytes are newborn foreskin or adult skin removed for cosmetic as well as health reasons (Halaban 2005). On the other hand, the lymph nodes are the most frequent source of melanoma cells in comparison with cutaneous tissue. Typically the tissue is stored in a medium containing a mixture of an antibiotic-antimycotic solution (e.g. 400 U/ml penicillin, 400 μg/ml streptomycin or 10 μg/ml Amphotericin B) and subsequently transported to the laboratory within 24-48 hours while stored at 4°C. Prolongation of the transport time may affect the quality of the tissue sample, and in consequence, the success rate of the subsequent cell isolation and culture (McLeod and Mason 1995, Ghosh et al. 2008, Bai et al. 2010).

b) tissue preparation for cell isolation and the disaggregation step

Large tissue fragments should be rinsed with an antiseptic solution (e.g. 2.5 mg/ml iodine solution, 70% ethanol) for 5-10 minutes. If the tissue fragments are very small, medium with 10-times greater concentration of an antibiotic solution should be used for quick rinsing only (rinsing with the antiseptic solution, like in case of the large tissue fragments, will fix the cells). Subsequently, the tissue should be washed with a Phosphate-Buffered Saline (PBS) or Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} or Mg^{2+} ions for 5-10 minutes. The subcutaneous fat should be removed from the tissue with the use of a scalpel and the tissue should be cut into smaller pieces. The size of the tissue fragment recommended for the enzymatic digestion is 1 cm x 1 cm, while for the mechanical disaggregation 1 mm x 1 mm. Furthermore, the incubation of the tissue fragments with a buffer containing ethylenediaminetetraacetic acid (EDTA; 1-50 mM depending on the cell type) may decrease the adhesion of naturally adherent cells and allow to obtain a single cell suspension (McLeod and Mason 1995, Ghosh et al. 2008, Godwin et al. 2014, Heitzer et al. 2019, Reichard and Asosingh 2019).

In order to separate the epidermis from the dermis, the enzymatic digestion (mainly with dispase and/or collagenase; for up to 2 hours at 37°C or 24 hours at 4°C) is frequently implemented. After separation of the layers, melanocytes can be found within the basal layer of the epidermis. To obtain cell suspension, the epidermis should be shortly washed with PBS or DPBS and incubated with a typsin/EDTA solution or a TrypLE reagent (up to 30 minutes at 37°C). Next, the enzyme should be inactivated and cell suspension should be filtered through a cell strainer, typically nylon or polyethylenterephthalat (PET) meshes (McLeod and Mason 1995, Halaban 2005, Godwin et al. 2014). The mesh size should be adjusted to the cell type; in case of melanocytes and melanoma cells the mesh size should be approximately 200 μm and 70 μm, respectively (Bai et al. 2010, Boiko 2013). Before cell seeding or specific cell separation, suspension after filtration should be centrifuged at 1000 g for 5-10 minutes. Next, the pellet with cells should be resuspended in the appropriate medium and cells may be counted before approaching the next step (Bai et al. 2010, Zhang et al. 2017).

Another approach in order to obtain cell culture is to attach the tissue fragment to the culture dish, the so-called skin explants method, however it is rarely used (Tobin et al. 1995).

c) the separation of different cell types and cell culture

Once cell suspension is assessed, the desired method of cell separation may be implemented without the cell culture. Cells coupled with fluorochromes or magnetic particles, may be separated by the use of the flow cytometry analysis: the fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), respectively (Hu et al. 2016). For this purpose, the presence of particular biomarkers expressed on the cell surface should be known to the investigators. For example, in order to sort cells of melanotic origin, a CD117 marker (the transmembrane tyrosine kinase growth factor receptor) is frequently used (Fujiwara et al. 2019). Other biomarkers specific for the detection of melanoma cells are CD45, CD31,

CD2, CD3, Glycophorin A, EpCAM (Epithelial Cell Adhesion Molecule) (Boiko 2013). After sorting, the obtained melanocytes or melanoma cells may be cultured or used directly for the planned experiments.

Cells may be seeded into 6-well plates (usually at a density of 1×10^5 cells per well) in a complete growth medium. For melanocytes and melanoma cell cultures, many different commercially available media may be used like DMEM (Dulbecco's Modified Eagle Medium), RPMI-1640 (Roswell Park Memorial Institute Medium), Ham's F10, Ham's F12, Medium 199 or MCDB153, almost all supplemented with fetal bovine serum (FBS, 1-10%) or fetal calf serum (FCS, 1-10%) and an antibiotic mixture (see Tables 4 and 5). In some cases, the ready-to-use media were applied like DermaLife (for both cell types) or Medium 254 supplemented with HMGS (in case of melanocytes only). For the stimulation of melanocyte growth *in vitro* four mitogens may be added to the growth medium: TPA (12-O-tetradecanoylphorbol 13-acetate; otherwise known as phorbol 12-myristate 13-acetate, PMA), cholera toxin (CT), endothelin 1 (ET1) and stem cell factor (SCF) (Bai et al. 2010, Godwin et al. 2014). Cholera toxin decreases the growth of cells typically stimulated by serum added to the medium. Therefore, in the presence of CT, the growth of fibroblasts, the most frequent contaminant of the melanocytes culture, is signify-cantly reduced. However, in the case of metastatic melanoma cultures, the addition of the CT will influence their morphology. On the other hand, fibroblasts can proliferate in the TPA-supplemented medium even in the presence of CT, that is why a selective combination of the growth factors is required to minimalize the proliferation of the contaminant cells and stimulate the desired cells (Halaban 2005).

In some cases, the co-culture may be separated due to the differential trypsin digestion as it is noted in case of keratinocytes-melanocytes cell culture (Ścieżyńska et al. 2019). The application of low concentrated trypsin (e.g. 0.05%) for a short time (up to 5 minutes) allows to collect only melanocytes or melanoma cells present in the culture. In order to detach keratinocytes, a longer time of incubation (10-15 minutes) with trypsin is needed (Fujiwara et al. 2019). Unfortunately, the separation of melanocytes from fibroblasts causes more difficulties (a similar trypsinization time) and requires cell incubation with a selective agent like geneticin (G418). It is an aminoglycoside antibiotic, which eliminates the quickly dividing cells (in this case fibroblasts) from the mixed cell culture by blocking the polypeptide synthesis. The effective concentration of geneticin is described usually as 100 μg/ml which should be added to the cell culture in the full growth medium for 2-3 days (Halaban and Alfano 1984, Zhang et al. 2017). Next, pure cell cultures may undergo experiments which confirm their melanotic origin.

d) confirmation of the cells' origin

To confirm the origin of the cell, specific biomarkers characteristic for each cell type should be selected. In this step the biomarkers must be expressed by the cells, in contrast to the cell sorting methods where the biomarkers had to be present on the cell surface. For this reason, the real-time polymerase chain reaction (qPCR) as well as immunohistochemical (IHC) or immunofluorescent (IF) cell staining methods are typically used. The selection of genes and proteins is strictly determined due to the possible false positive results. If cells express melanocyte markers (genes *MLANA*, *MITF*, *DCT*, *TYR;* positive staining of the S100 protein family or of the HMB45-antigen), they are qualified as cells of melanocytic origin (Li et al. 2012, Weinstein et al. 2014). In case of the accompanying cells, their presence in the culture can be confirmed by biomarkers like the cytokeratin family for keratinocytes (Guo and Jahoda 2009) and the collagen family for fibroblasts (Nissen 2019).

Another frequently used approach is based on the detection of tyrosinase activity in the cultured cells of melanocytic origin, a specific biochemical marker of these cells. The DOPA assay allows not only to confirm the purity of the melanocyte/melanoma culture but also the phenotypic characteristics of the cells. To determine the ratio between melanocytes and keratinocytes in co-culture experiments, cells must be grown on coverslips, then fixed, permeabilized, subjected to the reaction with L-DOPA and scored for positivity (brown stained cells) under the optical microscope (De Luca 1988, Ghosh et al. 2008, Bai et al. 2010).

The presence of mycoplasma contamination in the established cell lines should be checked before cell banking or further cell culturing. A frequently applied method to detect most

species of mycoplasma (*M. fermentans, M. hyorhinis, M. arginini, M. orale, M. salivarium, M. hominis, M. pulmonis, M. arthridtidis, M. bovis, M. pneumoniae, M. pirum, M. capricolum*) is PCR due to its sensitivity and specificity. The second approved method is DNA staining with DAPI (4,6-diamidino-2-phenylindole-dihydrochloride), Hoechst 33258 or Hoechst 33342. Although it is an easy and rapid test, the interpretation of results in some cases can be difficult and require experience (Nikfarjam and Farzaneh 2012).

e) cell banking

As soon as the origin and purity of the isolated cells is confirmed, they should be frozen before direct experiments are performed. This step is important due to the fact that cells have a limited lifespan in culture (Hayflick 1965). Melanocytes derived from newborn foreskin can proliferate up to 18 passages in culture, whereas cells derived from adult skin only up to 5 passages. Some components of the growth medium may also prolong the lifespan of these cells before the signs of senescence appear (e.g. bFGF, HGF/SF and ETl) (Halaban 2005). As a consequence, only cells at a low number of passages are suitable for functional studies and the sooner the cells will be frozen, the more experiments can be performed with their use (Dell'Anna and Cario-André 2019).

Cells may be stored in liquid nitrogen for a long period of time and may be sub-sequently, easily transported in dry ice. The recommended concentration of dimethyl sulfoxide (DMSO) in the freezing medium (full growth medium) is 7.5% (v/v), while cell density should be in the range of $0.5\text{-}1\times10^6$ cells/ml (Godwin et al. 2014).

SHORT CONCLUSION

Although the isolation of cells is time-consuming, it is highly recommended in case of unique cell types or cells with low commercial access (e.g. melanocytes). Primary cells allow for a better understanding of cell interactions, their function and changes occurring during cell differentiation or tumor development. The isolation of melano-cytes and melanoma cells may be performed according to several protocols. The basic steps include: tissue collection during surgery, securing the sample in proper conditions for their transport, preparation of the tissue for subsequent fragmentation, digestion, cell separation, cell culture and confirmation of the cell origin. Each step may be adjusted to the researchers needs based on the described conditions.

ACKNOWLEDGEMENT

This work was supported by the Warsaw University of Technology, the Medical University of Warsaw and the National Science Centre (Poland) – grants no. 2016/23/N/NZ5/02588 and 2017/27/N/ST4/01389. Additionally, AS would like to thank the National Science Centre (Poland) for the financial resources from the doctoral scholarship ETIUDA 6 (grant no. 2018/28/T/ST4/00511).

ORCID

Anna Sobiepanek https://orcid.org/0000-0003-3186-7582

Patrycja D. Kowalska https://orcid.org/0000-0003-0635-7214

Marta Soszyńska

Tomasz Kobiela https://orcid.org/0000-0001-7155-659X

Aneta Ścieżyńska https://orcid.org/0000-0003-0438-2678

REFERENCES

Bai R., Sen A., Yu Z., Yang G., Wang H., Fan R., Lv L., Lee K.-B., Smith G.W., Dong C**., Validation of Methods for Isolation and Culture of Alpaca Melanocytes: A Novel Tool for *In vitro* Studies of Mechanisms Controlling Coat Color**, Asian Australas. J. Anim. Sci 23, 2010, 430-436.

Bao J., Wang L., Wang G., Liu X., Yang F., **Isolation and Culture of Melanocytes from the Arctic Fox (Alopex Lagopus)**, Italian Journal of Animal Science 14, 2015, 4005.

Bleijs M., Wetering M., Clevers H., Drost J., **Xenograft and organoid model systems in cancer research**, EMBO J 38, 2019.

Boiko A.D., **Isolation of Melanoma Tumor-Initiating Cells from Surgical Tissues**, in: Has, C., Sitaru, C. (Eds.), Molecular Dermatology, Methods in Molecular Biology. Humana Press, Totowa, NJ, 2013, 253-259.

Chapman S.W.K., Metzger N., Grest P., Feige K., von Rechenberg B., Auer J.A., Hottiger M.O., **Isolation, establishment, and characterization of ex vivo equine melanoma cell cultures**, In Vitro Cell.Dev.Biol.-Animal 45, 2009, 152-162.

De Falco M., Pisano M.M., De Luca A., **Embryology and Anatomy of the Skin**, in: Baldi, A., Pasquali, P., Spugnini, E.P. (Eds.), Skin Cancer: A Practical Approach, Current Clinical Pathology, Springer, New York, NY, 2014, 1-15.

De Luca M., D'Anna F., Bondanza S., Franzi A.T., Cancedda R., **Human epithelial cells induce human melanocyte growth in vitro but only skin keratinocytes regulate its proper differentiation in the absence of dermis**, The Journal of Cell Biology 107, 1988, 1919-1926.

Dehdashtian A., Stringer T.P., Warren A.J., Mu E.W., Amirlak B., Shahabi L., **Anatomy and Physiology of the Skin**, in: Riker, A.I. (Ed.), Melanoma: A Modern Multidisciplinary Approach, Springer International Publishing, Cham, 2018, 15-26.

Dell'Anna M.L., Cario-André M., ***In Vitro* Study of Vitiligo**, in: Picardo, M., Taïeb, A. (Eds.), Vitiligo, Springer International Publishing, Cham, 2019, 225-236.

Dummer R., Siano M., Hunger R.E., Lindenblatt N., Braun R., Michielin O., Mihic-Probst D., von Moos R., Najafi Y., Guckenberger M., Arnold A., **The updated Swiss guidelines 2016 for the treatment and follow-up of cutaneous melanoma,** Swiss Med Wkly 146, 2016, w14279.

Elder D.E., **Melanoma progression**, Pathology 48, 2016, 147-154.

Evrard Y.A., Alcoser S.Y., et al., **Comparison of PDX, PDC, and PDOrg models from the National Cancer Institute's Patient-Derived Models Repository (PDMR)**, 2019.

Forman S.B., Ferringer T.C., Peckham S.J., Dalton S.R., Sasaki G.T., Libow L.F., Elston D.M., **Is superficial spreading melanoma still the most common form of malignant melanoma?** Journal of the American Academy of Dermatology 58, 2008, 1013-1020.

Fujiwara S., Nagai H., Jimbo H., Jimbo N., Tanaka T., Inoie M., Nishigori C., **Gene Expression and Methylation Analysis in Melanomas and Melanocytes From the Same Patient: Loss of NPM2 Expression Is a Potential Immunohistochemical Marker for Melanoma**, Front. Oncol. 8, 2019, 675.

Ghosh D., Shenoy S., Kuchroo P., **Cultured Melanocytes: From Skin Biopsy to Transplantation**, Cell Transplant 17, 2008, 351-360.

Gledhill K., Guo Z., Umegaki-Arao N., Higgins C.A., Itoh M., Christiano A.M., **Melanin Transfer in Human 3D Skin Equivalents Generated Exclusively from Induced Pluripotent Stem Cells**, PLoS ONE 10, 2015, e0136713.

Godwin L.S., Castle J.T., Kohli J.S., Goff P.S., Cairney C.J., Keith W.N., Sviderskaya E.V., Bennett D.C., **Isolation, Culture, and Transfection of Melanocytes**, Current Protocols in Cell Biology 63, 2014.

Goodall T., Buffey J.A., Rennie I.G., Benson M., Parsons M.A., Faulkner M.K., MacNeil S., **Effect of melanocyte stimulating hormone on human cultured choroidal melanocytes, uveal melanoma cells, and retinal epithelial cells**, Invest. Ophthalmol. Vis. Sci. 35, 1994, 826-837.

Graham H.K., Eckersley A., Ozols M., Mellody K.T., Sherratt M.J., **Human Skin: Composition, Structure and Visualisation Methods**, in: Limbert, G. (Ed.), Skin Biophysics: From Experimental Characterisation to Advanced Modelling, Studies in Mechanobiology, Tissue Engineering and Biomaterials, Springer International Publishing, Cham, 2019, 1-18.

Guo A., Jahoda C.A.B., **An improved method of human keratinocyte culture from skin explants: cell expansion is linked to markers of activated progenitor cells**, Experimental Dermatology 18, 2009, 720-726.

Halaban R., **Culture of Melanocytes from Normal, Benign and Malignant Lesions**, in: Pfragner, R., Freshney, R.I. (Eds.), Culture of Human Tumor Cells, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2005, 289-318.

Halaban R., Alfano F.D., **Selective elimination of fibroblasts from cultures of normal human melanocytes**, In Vitro 20, 1984, 447-450.

Hara M., Toyoda M., Yaar M., Bhawan J., Avila E.M., Penner I.R., Gilchrest B.A**., Innervation of melanocytes in human skin**, The Journal of Experimental Medicine 184, 1996, 1385-1395.

Hayflick L., **The limited in vitro lifetime of human diploid cell strains**, Experimental Cell Research 37, 1965, 614-636.

Heitzer E., Groenewoud A., Meditz K., Lohberger B., Liegl-Atzwanger B., Prokesch A., Kashofer K., Behrens D., Haybaeck J., Kolb-Lenz D., Koefeler H., Riedl S., Schaider H., Fischer C., Snaar-Jagalska B.E., de'Jong D., Szuhai K., Zweytick D., Rinner B., **Human melanoma brain metastases cell line MUG-Mel1, isolated clones and their detailed characterization**, Sci Rep 9, 2019, 4096.

Hsu M.-Y., Elder D.E., Herlyn M., **Melanoma: The Wistar Melanoma (WM) Cell Lines**, in: Masters, J.R.W., Palsson, B. (Eds.), Human Cell Culture: Cancer Cell Lines Part 1, Human Cell Culture, Springer Netherlands, Dordrecht, 1999, 259-274.

Hu P., Zhang W., Xin H., Deng G., **Single Cell Isolation and Analysis**, Front. Cell Dev. Biol. 4, 2016.

Johansen C., **Generation and Culturing of Primary Human Keratinocytes from Adult Skin**, Journal of Visualized Experiments 130: 56863, 2017.

Knapp S.K., Iden S., **Melanocyte differentiation and epidermal pigmentation is regulated by polarity proteins (preprint)**, Cell Biology, 2020.

Li L., Fukunaga-Kalabis M., Herlyn M., **Isolation and Cultivation of Dermal Stem Cells that Differentiate into Functional Epidermal Melanocytes**, in: Mitry, R.R., Hughes, R.D. (Eds.), Human Cell Culture Protocols, Methods in Molecular Biology, Humana Press, Totowa, NJ, 2012, 15-29.

Luo Y., Nguyen N., Fujita M., **Isolation of Human Melanoma Stem Cells Using ALDH as a Marker**, Current Protocols in Stem Cell Biology 26, 2013.

Madhunapantula S.R.V., Patel T.N., Annageldiyev C., Sharma A., **Animal (mouse) models of melanoma**, in: Animal Models in Cancer Drug Discovery, Academic Press, 2019, 401-417.

Marczyńska D., Przybyło M., **Melanocyty – komórki barwnikowe o wielu obliczach**, Kosmos 62, 2013, 491-499.

McLeod S.D., Mason R.S., **Isolation of enriched human melanocyte cultures from fetal, newborn and adult skin**, Methods Cell Sci 17, 1995, 187-193.

Nikfarjam L., Farzaneh P., **Prevention and Detection of Mycoplasma Contamination in Cell Culture**, Cell Journal 13, 4, 2012, 203-12.

Nissen N.I., Karsdal M., Willumsen N., **Collagens and Cancer associated fibroblasts in the reactive stroma and its relation to Cancer biology**, J Exp Clin Cancer Res 38, 2019, 115.

Nordlund J.J., **The Melanocyte and the Epidermal Melanin Unit: An Expanded Concept**, Dermatologic Clinics 25, 3, 2007, 271-81.

Pokrywka M., Lityńska A., **Celując w czerniaka**, Targeting the melanoma 39, 2012.

Reichard A., Asosingh K., **Best Practices for Preparing a Single Cell Suspension from Solid Tissues for Flow Cytometry**, Cytometry 95, 2019, 219-226.

Rzepka Z., Buszman E., Beberok A., Wrześniok D., **Od tyrozyny do melaniny: ścieżki sygnalizacyjne i czynniki regulujące melanogenezę**, Postepy Hig Med Dosw 70, 2016, 695-708.

Sanchez Hanke M., Kief S., Leuwer R., Koch U., Moll I., Brandner J.M., **In vitro Isolation and Cell Culture of Vestibular Inner Ear Melanocytes**, Audiol Neurotol 10, 2005, 191-200.

Santiago-Walker A., Li L., Haass N.K., Herlyn M., **Melanocytes: From Morphology to Application**, Skin Pharmacol Physiol 22, 2009, 114-121.

Schadendorf D., van Akkooi A.C.J., Berking C., Griewank K.G., Gutzmer R., Hauschild A., Stang A., Roesch A., Ugurel S., **Melanoma**, Lancet 392, 2018, 971–984.

Scolyer R.A., Long G.V., Thompson J.F., **Evolving concepts in melanoma classification and their relevance to multidisciplinary melanoma patient care**, Molecular Oncology 5, 2011, 124-136.

Segaoula Z., Primot A., Lepretre F., Hedan B., Bouchaert E., Minier K., Marescaux L., Serres F., Galiègue-Zouitina S., André C., Quesnel B., Thuru X., Tierny D., **Isolation and characterization of two canine melanoma cell lines: new models for comparative oncology**, BMC Cancer 18, 2018, 1219.

Slipicevic A., Somasundaram R., Sproesser K., Herlyn M., **Isolation of Melanoma Cell Subpopulations Using Negative Selection**, in: Thurin, M., Marincola, F.M. (Eds.), Molecular Diagnostics for Melanoma, Methods in Molecular Biology, Humana Press, Totowa, NJ, 2014, 501-512.

Sobiepanek A., Baran J., Milner-Krawczyk M., Kobiela T., **Different Types of Surface modification used for Improving the Adhesion and Interactions of Skin Cells**, OAJBS 2, 2020, 275-278.

Sobiepanek A., Galus R., Kobiela T., **Application of the tape stripping method in the research on the skin condition and its diseases**, Review and Research on Cancer Treatment 5, 2019, 4-14.

Sobiepanek A., Kobiela T., **Application of biosensors in cancer research**, Review and Research on Cancer Treatment 4, 2018, 4-12.

Sobiepanek A., Milner-Krawczyk M., Lekka M., Kobiela T., **AFM and QCM-D as tools for the distinction of melanoma cells with a different metastatic potential**, Biosensors and Bioelectronics, 93, 2017, 274-281.

Ścieżyńska A., Nogowska A., Sikorska M., Konys J., Karpińska A., Komorowski M., Ołdak M., Malejczyk J., **Isolation and culture of human primary keratinocytes – a methods review**, Exp Dermatol, 2019, 1-6.

Tobin D.J., Colen S.R., Bystryn J.-C., **Isolation and Long-Term Culture of Human Hair-Follicle Melanocytes**, Journal of Investigative Dermatology 104, 1995, 86-89.

Tsuji T., Karasek M., **A Procedure for the Isolation of Primary Cultures of Melanocytes from Newborn and Adult Human Skin**, Journal of Investigative Dermatology 81, 1983, 179-180.

Weidmann C., Pomerleau J., Trudel-Vandal L., Landreville S., **Differential responses of choroidal melanocytes and uveal melanoma cells to low oxygen conditions,** Mol. Vis. 23, 2017, 103-115.

Weinstein D., Leininger J., Hamby C., Safai B., **Diagnostic and prognostic biomarkers in melanoma,** J Clin Aesthet Dermatol 7, 2014, 13-24.

Wikramanayake T.C., Stojadinovic O., Tomic-Canic M., **Epidermal Differentiation in Barrier Maintenance and Wound Healing**, Advances in Wound Care, 2014, 3: 272-80.

Zhang R., Liu Q., Cheng S., Zhu J., **The Clonal Growth Behavior of Melanocytes Derived From Melanocyte Stem Cells in Glabrous Skin**, JDC 1, 2017.

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

Implementation of Geneticin in the *in vitro* cell culture and *in vivo* studies

Anna Sobiepanek[1,*], Patrycja D. Kowalska[1,2], Marta Soszyńska[3], Aneta Ścieżyńska[3,4,*]

***corresponding authors: aneta.sciezynska@wum.edu.pl and asobiepanek@ch.pw.edu.pl**

[1]Laboratory of Biomolecular Interactions Studies, Chair of Drug and Cosmetics Biotechnology, Faculty of Chemistry, Warsaw University of Technology, Warsaw, Poland

[2]Polish Stem Cells Bank, Warsaw, Poland

[3]Department of Histology and Embryology, Medical University of Warsaw, Warsaw, Poland

[4]Department of Regenerative Medicine, Military Institute of Hygiene and Epidemiology, Warsaw, Poland

ABSTRACT

Geneticin, widely known as G418, is an aminoglycoside antibiotic produced by a bacteria *Micro-monospora rhodorangea*. Geneticin blocks the polypeptide synthesis in both prokaryotic and eukaryotic cells by inhibiting the elongation step. In consequence, rapidly dividing cells are affected more than those with a small proliferation rate. Therefore, geneticin is frequently used in molecular biology as a selective agent for mammalian cells with a different time of population doublings and during cell transfection. The dose of the G418 that is used for the selection of the transfected eukaryotic cells depends on many factors like: the type of the cells and organism from which they were isolated, as well as the growth conditions and the selected culture media. Which is why, the concentration of this antibiotic should be selected for each cell line individually.

Here, we present an overview regarding the spectrum of applications, in which geneticin can be imple-mented as a general selective agent in normal and cancerous cell culture studies.

Keywords: Geneticin G418, cells' selection, cell culture, cancer

INTRODUCTION

In 1974 a new aminoglycoside produced as the major component by a new species of a gram-positive and spore-forming bacteria *Micromonospora rhodorangea* was introduced to the public and named Geneticin. The production of geneticin was carried out by the submerged fermentation in a soybean-dextrin medium, and next subsequent adsorption from the fermentation broth by an ion-exchange procedure (Wagman et al. 1974).

Geneticin is composed of three rings functionalized with ammonium, hydroxyl and methyl groups, thus its structure is similar to gentamicin (especially gentamicin B1 and gentamicin A – other aminoglycoside antibiotics known since 1944; Aubrecht et al. 2011), therefore it may be called as gentamicin G or by a well-known abbreviation G418 (Vicens and Westhof 2003). The lyophilized base is a white powder soluble in water or methanol, whereas sulfate is soluble only in water. A typical stock solution is of a 50 mg/ml concentration (Wagman et al. 1974).

Although with some limitations geneticin retains a high biological and pharmaceutical compound of interest. Geneticin blocks the polypeptide synthesis due to the irreversible binding to the 70S and 80S cell ribosomes and inhibition of the protein elongation. In consequence, geneticin presents broad toxicity against bacteria, yeast, protozoa, helminths and mammalian cells. Resistance to G418 is conferred by the bacterial gene for aminoglycoside-3'-phosphotransferase (APH(3')) that can be expressed in eukaryotic cells (Vicens and Westhof 2003, Prokhorova et al. 2017).

SEARCH STRATEGY AND SELECTION CRITERIA

For the literature analysis performed with the use of the PubMed and Google Scholar databases, the combination of the search terms "geneticin – G418 – cell culture – transfection – cell selection" has been implemented. As the selection criteria we have chosen articles in which geneticin was used as a means for the cell selection or treatment purposes. Moreover, during hand-search some additional articles were also investigated for a better comprehension of the subject.

STATE OF ART

Geneticin presents a strong influence on all types of cells from prokaryotic to the eukaryotic, especially for those with a high proliferation rate. G418 mode of action mainly results in the inhibition of protein synthesis, the activation of phosphati-dylinositol phospholipase C (which leads to the release of GPI-anchored proteins), as well as the increase of dihydroxyacetone phosphate acyltransferase and the peroxisomal β-oxidation activity (Jin et al. 2004). Some of these functions, however, may be reduced. For example, the irreversible binding of G418 to ribosomes may be compro-mised by the bacterial aminoglycoside phosphotransferases APH(3')II and APH(3')I encoded by the genes on transposons Tn5 and Tn601 (903), respec-tively. As a con-sequence, cells transfected with genes of neomycin resistance (neo) from transposon Tn5 or Tn601 gain resistance to G418 and grow in media supplemented with this antibiotic. The selection strategy should be applied individually for various types of cells, as geneticin's effective concentration differs according to the growth medium, culture conditions as well as metabolic rate of the cells (Davies and Jimenez 1980, Scholar 2007).

Response to G418 depends on cell metabolism, i.e. some cell lines or clones are able to better tolerate a metabolic load than others. Moreover, geneticin may differently influence cell growth and their proliferation rate depending on the composition of the culture media (serum, glutamine and insulin concentration). As an example, the addition of G418 may cause the change of the flux of glucose from the lactate production towards either the TCA cycle to provide energy or other biosynthetic pathways (Yallop and Svendsen 2001). Further action of geneticin on cells can include caspase-3-dependent apoptosis (initiated by at least two pathways: cytochrome c release and endoplasmic reticulum (ER) stress) with cell shrinkage and nuclear fragmentation (Jin et al. 2004).

REVIEW AND DISCUSSION

In general, the use of geneticin may be implemented in case of one of these three applications: (i) for selecting stable clones after transfection, (ii) to eliminate the quickly dividing cells from the mixed cell culture and (iii) as a potential treatment for several diseases. Each application requires an individual analysis regarding the optimal concentration of G418, time of treatment, as well as the application of the culture medium and other conditions.

G418 FOR THE SELECTION OF STABLE TRANSFECTANTS

Transfection is a process of introducing exogenous genetic material in the form of DNA, RNA, messenger RNA (mRNA), small interfering RNA (siRNA), microRNA (miRNA) and short hairpin RNA (shRNA) into mammalian cells, that is widely used in the study of genes and protein expression. Two types of transfection can be obtained depending on the applied methodology: transient (chemical or physical) and stable (biological and physical). In principle the physical method is based on the direct injection of the genetic material, biolistic particle delivery, electroporation or laserfection; whereas the biological method is connected with the virus-mediated gene transfer (the so-called transduction). On the other hand, the chemical method requires the usage of cationic polymers, cationic lipids or calcium phosphate. The selected approach depends on the cell type and the object (gene or protein) of the experiment (Sheikh et al 2017).

The usability of geneticin as a selective agent after transfection has been known since 1980, because only positively transfected cells and resistant to geneticin are able to grow in culture media supplemented with this antibiotic (Davies and Jimenez 1980). Typically, 24 or 48 hours after cell transfection the old medium is replaced by the full growth medium with the appropriate G418 concentration and the incubation is carried out though 1-4 weeks with the G418-medium exchange every 3-4 days. Articles describing various protocols of the cell selection methods after transfection were gathered in Table 1, e.g. the physical method – electroporation (Fountain et al. 1988), biological – with virus (Belsham et al. 2004) and chemical – with Lipofectamine 2000 reagent (Yang et al. 2019). Due to the fact, that the optimal dose of G418 for the selection of transfected eukaryotic cells depends on numerous factors (e.g. cell type, culture medium, growth conditions and the cells' metabolic rate), the most suitable approach is to self-determine the sensitivity of each cell line to geneticin before performing the transfection (Tab. 2). Several concentrations of G418 ranging from

100 μg/ml to up to even 2 mg/ml should be tested. The treatment should be carried out for at least 48 hours. Next, the cytotoxic effect of G418 on cells can be checked with the MTT test (the conversion of tetrazolium salt into formazan by living cells and calculation of the half maximal inhibitory concentration – IC_{50}) (Sehati et al. 2019, Sadeghiyeh et al. 2019). If the antibiotic concentration is too low or the cell seeding density is too high, the cells can escape the selection. Thus, the selected drug concentration should be approximately 25-50% higher than the minimal concentration required to kill 100% of the cells within 7-14 days (Mally et al. 1992). Another matter to discuss is the growth medium used for the cell culture. Some medium components (e.g. serum, glutamine, insulin) may significantly influence the effect of geneticin (tab. 3), which is why the experiment aimed at the selection of a proper G418 concentration should be performed in the medium appropriate for the study (Yallop and Svendsen 2001).

Table 1. Sample conditions for cell transfection and next clone selection with geneticin (***shortcuts: Biol.TM – biological transfection method, Chem.TM – chemical transfection method, Phys.TM – physical transfection method; CF- calf serum, HS – horse serum)

Cell type used for transfection (cell line)	Growth medium	Transfection method (TM)	Clone selection with G418 (concentration and time)	Reference
Human skin fibroblasts (HSF)	EMEM+15% FBS	Phys.TM: electroporation	400 μg/ml for 4 weeks	Fountain et al. 1988
Spontaneously immortalized human skin keratinocytes (HaCaT)	EMEM+10% FBS, 100 U/ml penicillin and 50 μg/ml streptomycin	Chem.TM: the Ca^{2+}-phosphate precipitation method	800 μg/ml	Boukamp et al. 1990
Mouse murine melanoma (HFH18)	DMEM+5% CS	Phys.TM: electroporation	1000 μg/ml for 10-14 days	Armstrong et al. 1994
Human primary lung cancer cell lines	RPMI+10% FBS	Chem.TM: DMRIE-C reagent	800 μg/ml for 12-21 days	Tomizawa et al. 2001
Human cervical cancer (HeLa); Human melanoma (A375, WM9)	(not mentioned)	Phys.TM: electroporation	50 μg/ml (WM9), 500 μg/ml (HeLa, A375)	Leaman et al. 2002
Human melanoma (A2058)	DMEM+10% FCS, 2 mM glutamine, 10 mM HEPES, penicillin-streptomycin (100 IU/ml, 100 μg/ml)	Chem.TM: Lipofectamine	400 μg/ml	Sounni et al. 2002
Primary fetal mouse hypothalamus cells	DMEM+10% FBS, 10% HS, 1% penicillin-streptomycin and 20 mM D-glucose	Biol.TM: virus	400-600 μg/ml for 2-3 weeks	Belsham et al. 2004
Human colon cancer (Lovo)	RPMI+10% FBS, 100 μg/ml streptomycin and 100 μg/ml penicillin	Chem.TM: LipofectAMINE2000	1000 μg/ml for 4 weeks	Wang et al. 2007
Human melanoma (SK28, 1205Lu)	RPMI+10% FCS and antibiotics	Chem.TM: FuGENE reagent	700 μg/ml for 3 weeks	Alexaki et al. 2010
Rat epithelial cells (Fischer rat thyroid line 5; FRTL-5)	Coon's modified F-12 medium + 5% CS and 6- hormone mixture	Chem.TM: FuGENE reagent	for 2 weeks	Di Palma et al. 2013

Human melanoma (A375)	DMEM+10% FCS, 15 mM HEPES, 2 mM glutamine, 100 U/ml penicillin and streptomycin	Chem.TM: Lipofectamine 2000	500 mg/ml for 2 weeks	Wang et al. 2014
Human neuroblastoma (SH-SY5Y)	DMEM+10% FCS, 100 µg/ml penicillin-streptomycin and 1% Glutamax	Chem.TM: Lipofectin reagent	500 µg/ml for 4 weeks	Pirou et al. 2017
Human skin cancer (A431), human melanoma (SK-MEL-28)	(A431) RPMI+10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin; (SK-MEL-28) MEM+10%FBS, 1 mM sodium pyruvate, 1× non-essential amino acid, 100 U/ml penicillin and 100 µg/ml streptomycin	Chem.TM: Lipofectamine® 3000 reagent	600 µg/ml for 2 weeks	Lee et al. 2018
Mouse inner medullary collecting duct 3 (IMCD3)	DMEM-F12+10% FBS and 1% penicillin-streptomycin-kanamycin cocktail	Chem.TM: Lipofectamine® 3000 reagent	800 ng/µl for 5-6 days	Mirvis et al. 2019
Human lung cancer (A549)	RPMI+10% FBS	Chem.TM: jetPEI® solution	50 mg/ml for 2 weeks	Sadeghiyeh et al. 2019
Human melanoma (A375)	RPMI+10% FBS	Chem.TM: jetPEI® solution	460 µg/ml for 72 h	Sehati et al. 2019
Human melanoma (A375, SK-MEL-28)	DMEM+10% FBS	Chem.TM: Lipofectamine 2000 reagent	500 µg/ml for 4 weeks	Yang et al. 2019

Table 2. Sample of the self-determined IC_{50} concentration of geneticin for the used cell lines

Cell type (cell line)	Growth medium	Selection of G418 concentration	IC_{50}	Reference
melanoma (A375)	RPMI+10% FBS	MTT assay (100, 200, 400, 600, 800, 1000, 1200 µg/ml) for 72 h	460 µg/ml	Sehati et al. 2019
lung cancer (A549)	RPMI+10% FBS	MTT assay (100, 200, 400, 600, 800, 1000, 1200 µg/ml)	494.1 µg/ml	Sadeghiyeh et al. 2019

Table 3. The IC_{50} concentrations of geneticin used after cell transfection in different growth media (TOKU-e Company, information materials)

Cell type (cell line)	Growth medium	IC_{50}
colon cancer (Caco-2)	DMEM	500 µg/ml
	MEM	1000 µg/ml
hamster, Chinese ovary (CHO-K1)	DMEM	600 µg/ml
	DMEM + F12 medium	150 µg/ml
	EMEM	500 µg/ml
	Ham's F-12 nutrient mixture	1000 µg/ml
cervical cancer (HeLa)	DMEM	1000 µg/ml
	DMEM + F12 medium	600 µg/ml
	EMEM	400 µg/ml

G418 FOR ELIMINATING CELLS FROM MIXED CULTURE

Except for cells transfected with the neomycin resistance gene, other type of cells naturally resistant to geneticin are melanocytes. These slowly dividing cells (origi-nating from the neural crest) are present in the epidermis and are responsible for the production of melanin – the UV photon-absorbing pigment (Sobiepanek and Kobiela 2020). Their mitosis *in vivo* has been rarely observed, but during the melanocytes culture *in vitro* the doubling time varies from 48 hours to up to even 10 days depending on the growth conditions (De Luca et al. 1988, Hoerter et al. 2012). These cells are important for the research connected with their maturation, migration, melanin production as well as cell transformation to melanoma, the deadliest type of skin cancer (Satyamoorthy and Herlyn 2002, Sobiepanek et al. 2017).

During melanocyte isolation from skin mainly two types of contaminant cells may occur: keratinocytes (other components of the epidermis) and fibroblasts (components of the dermis) (Halaban and Alfano 1984, Sobiepanek et al. 2020). Keratinocytes can be easily separated either from melanocytes as well as from fibroblasts based on differential trypsin digestion (Ścieżyńska et al. 2019), however, separation of melano-cytes and fibroblasts causes a lot of difficulties. The first attempts were made with the addition of cholera toxin (CT) or 12-O-Tetradecanoylphorbol-13-acetate (TPA) to the culture medium, but fibroblasts remained insensitive to these components (Halaban 2005). In 1984 Halaban and Alfano showed that the antibiotic G418 at the concentration of 100 μg/ml applied for 2 days can help in receiving a pure culture of normal melanocytes. From that time, geneticin has been used frequently for these purposes; especially due to the fact that this method requires minimal efforts to obtain a good success rate (Tomonobu et al. 2019). The elimination of fibroblasts, however, may not be immediate and may require some time to observe the expected effect. Moreover, the exposure of melanocyte-fibroblast coculture to G418 has to be repeated in some cases in order to entirely eliminate fibroblasts (Halaban 2005). A few examples of the implementation of geneticin prior to fibroblast eradication from melanocyte culture are gathered in table 4. Similar approach can be included for the elimination of fibroblasts from melanoma cell culture (Chapman et al. 2009).

Table 4. Examples of the G418 usage on cell coculture to eliminate fibroblasts and receive pure melanocytes culture (***shortcuts: IBMX – isobutylmethyl xanthine, EGF – epidermal growth factor, BPE – bovine pituitary extract)

Origin of cells	Growth medium	G418 dose and incubation time	Research focus	Reference
Human neonatal foreskin	Ham F-10 medium + 10% Nu-serum, 2% FCS, penicillin-streptomycin, 48 nM TPA, 2.5 nM CT and 0.1 mM IBMX	100 μg/ml for 48 h	to establish a pure human melanocyte culture *in vitro*	Halaban and Alfano 1984
Human skin from biopsy	Dulbecco-Vogt Eagle's and Ham's F12 media (3:1) + 5% FCS, 5 μg/ml insulin, 5 μg/ml transferrin, 0.18 mM adenine, 0.4 mg/ml hydrocortisone, 1 nM CT, 20 pM triiodothyronine, 10 ng/ml EGF, 10 ng/ml PMA, 50 μg/ml BBE and 50 IU/ml penicillin/streptomycin	100 μg/ml for 2-4 days	to gain knowledge of the dependencies between melanocytes and keratinocytes cultured *in vitro*	De Luca et al. 1988
Human eyes	Ham's F12 medium + 10% FBS, 100 nM TPA, 0.1 mM IBMX and 10 ng/ml CT	100 μg/ml for 3-7 days	to establish isolation and cultivation methods of human uveal melano-cytes	Hu et al. 1993
Human scalp	EMEM + 10% FBS, 0.2 μg/ml CT, 50 nM PMA, 0.05 mg/ml gentamicin, 2.5 μg/ml fungizone and keratinocyte serum-free medium	(details not mentioned)	what is the role of amelanotic hair-follicle melanocytes in hair growth and diseases	Tobin et al. 1995
Hanwoo cattle skin from muzzle	DMEM + 2.5% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1 % non-essential amino acids, 2.5 ng/ml EGF, 25 μg/ml BPE, 10 ng/ml TPA, 100 U/ml penicillin, 100 μg/ml streptomycin	100 μg/ml for 3-4 days	investiga-ting melanin production and gene expression (MC1R, Tyr, Tyrp-1, Tyrp-2)	Amna et al. 2012
Human neonatal foreskin	Clonetics MGM-4 Melanocyte Growth Media-4 with 100 nM endothelin-3 (EDN3)	150 μg/ml for 48 h	investiga-ting melanin production, transfer and uptake	Gledhill et al. 2015

G418 FOR POTENTIAL TREATMENT OF VARIOUS DISEASES

While being an antibiotic, geneticin may be used in medicine as an antiparasitic agent, but with some limitations due to its toxicity to human ear and kidney (Vincens and Westhof 2003). The *in vivo* research of Aubrecht et al. performed on mice (with the lethal dose of 153 mg/kg) confirmed that the primary target organs of G418 toxicity are kidney and liver. This was shown by the serum biochemistry analysis (a significant increase in the parameters: blood urea nitrogen (BUN) and creatinine (CRE) – markers of the kidney functionality) and histopathological data (present signs of mild nephrotoxicity) (Aubrecht et al. 2011). In case of the *in vitro* study of normal rate kidney cells treated with 400 μg/ml of G418 for 3 days, the cells presented obvious apoptotic features: cell rounding and shrinkage as well as nuclear condensation and fragmentation. Apoptosis was activated in those cells by the caspase- 3 pathway (Jin et al. 2004). In general, G418 acts through different mechanisms depending on the number of positive charges on the antibiotic, its concentration and the organ where the toxicity develops. Known mechanisms also include: the production of hydroxyl radicals by geneticin/ metal ion complexes and the disturbance of membrane functionalities by the interaction with phospholipids and the inhibition of the phospholipases activity (Vincens and Westhof 2003).

Furthermore, in case of patients with cancer, the treatment of the occurring infection may cause a risk to the patient. An example was shown during the *in vitro* study of the human breast cancer cell (MCF-7) treatment with geneticin (10 μg/ml), which allowed the cancer cell survival (by suppressing apoptosis) despite all glucose consumption from the medium under hypoxia (1% O_2). At the same time the untreated cells after glucose consumption died. As it is well-known, in solid tumors there are regions where oxygen supply is very low (hypoxic conditions), thus the addition of G418 to treat the infection may cause a side effect of the cancer cells' resistance as well as the limitation of the action of the drugs dedicated to cancer treatment (Lee et al. 2002).

On the other hand, the anti-apoptotic activity of geneticin may be used against perinatal hypoxic-ischemic (H-I) brain injury to cause a neuroprotective effect. The *in vivo* study was performed on an animal model of Sprague-Dawley rat pups and an intraperitoneal injection of 0.1 μg/kg G418 was made before and after the rats were placed in the hypoxic chamber for a 2.5-hour period (92% N_2 and 8% O_2). In case of mice with H-I brain injury and treated with G418, a decreased number of apoptotic cells was observed as well as a decrease of the Bax/Bcl-2 expression ratio and a decrease of caspase-3 activity (Ju et al. 2008).

Administration of geneticin has proven helpful also in the treatment of patients suffering from several genetic disorders (Vincens and Westhof 2003). A genetic abnormality may be a discrete mutation not influencing our organism or of a major significance (e.g. insertion or deletion), which in consequence leads to genetic diseases. Some disorders may be inherited, while others occur randomly or because of some environmental influence. Genetic disorders are caused by the nonsense or frameshift mutation, which induces the premature termination codons (PTCs) (Miller and Pearce 2014). The most common human genetic disease is cancer, where the appearance of a PTC in a tumor suppressor gene results in the loss of the protein or the synthesis of a truncated protein unable to inhibit cell proliferation or to promote apoptosis. The known examples are mutations in *p53* and *APC* suppressor genes present in 50% of human cancers. The first application of G418 in disease-causing nonsense mutation was in 1985 (Nudelmann et al. 2010). Aminoglycosides (such as geneticin and gentamicin) induce the readthrough of PTCs by binding to ribosomes, which restores the synthesis of a full-length functional protein in the cultured *in vitro* mammalian cells and *in vivo* animal models (Floquet et al. 2011, Bidou et al. 2017). An example is the human cancer cell line containing a PTC (the non-small-cell lung cancer cell line, H1299; p53-null), for which high levels of the readthrough were obtained in the presence of G418 during the *in vitro* study. Also, the viability of cancer cells with the nonsense-mutated *p53* gene was significantly decreased after the aminoglycoside treatment (Floquet et al. 2011).

About 12% of human genetic disorders involve the PTCs (Kuschal et al. 2013), where various aminoglycosides have presented a therapeutic potential for the treatment of cystic fibrosis, Duchenne muscular dystrophy, dystrophic epidermolysis bullosa, the Werner syndrome as well as the Hurler syndrome (Heier and DiDonato 2009, Prokhorova et al. 2017). Recent

development also emphasizes the use of translational read-through inducing drugs as a strategy for treating nonsense mutation-based on retinal disorders (Nagel-Wolfrum et al. 2014, Samanta et al. 2019). Another thera-peutical focus of G418 is on the spinal muscular atrophy (SMA) disease, where it demonstrated an ability to induce the readthrough of the SMN target and to increase the SMN protein level. Moreover, geneticin in the dose of 14 mg/kg administered by the intraperitoneal injection into a SMA mouse increased its motor functions during the *in vivo* experiment (Heier and DiDonato 2009). In general, this antibiotic and the derivatives of aminoglycoside antibiotics offer a rational basis for developing new personalized strategies of treatment for various diseases.

SHORT CONCLUSION

Undeniably, geneticin is a very useful antibiotic with a wide range of application possibilities. For cell biology it allows to eliminate the slowly dividing cells from the culture. In melanocyte-fibroblast coculture the concentration of 100 μg/ml of G418 applied for 2 days can help in receiving a pure culture of normal melano-cytes. In case of molecular biology, it helps in selecting only those cells which underwent trans-fection. The applied concentration of geneticin has to be determined experimentally by using the cells of interest exposed to the treatment of broad G418 concentrations in an appropriate medium and via the MTT assay. The selected drug concentration should be approximately 25-50% higher than the minimal concentration required to kill all the cells within 7-14 days after transection. Concerning the medical field geneticin can be used against various diseases but when taking proper safety precautions. So far, it has been utilized as an antiparasitic agent, neuroprotective compound in the (H-I) brain injury or it has been used to treat patients with genetic disorders caused by nonsense or frameshift mutations.

ACKNOWLEDGEMENT

This work was supported by the Warsaw University of Technology, the Medical University of Warsaw and the National Science Centre (Poland) – grants no. 2016/23/N/NZ5/02588 and 2017/27/N/ST4/01389.

ORCID

Anna Sobiepanek https://orcid.org/0000-0003-3186-7582

Patrycja D. Kowalska https://orcid.org/0000-0003-0635-7214

Marta Soszyńska

Aneta Ścieżyńska https://orcid.org/0000-0003-0438-2678

REFERENCES

Alexaki V.-I., Javelaud D., Van Kempen L.C.L., Mohammad K.S., Dennler S., Luciani F., Hoek K.S., Juàrez P., Goydos J.S., Fournier P.J., Sibon C., Bertolotto C., Verrecchia F., Saule S., Delmas V., Ballotti R., Larue L., Saiag P., Guise T.A., Mauviel A., **GLI2-Mediated Melanoma Invasion and Metastasis,** JNCI: Journal of the National Cancer Institute 102, 2010, 1148-1159.

Amna T., Park K.M., Cho I.-K., Choi T.J., Lee S.S., Seo K.-S., Hwang I., **Substantial Effect of Melanin Influencing Factors on In vitro Melanogenesis in Muzzle Melanocytes of Differently Colored Hanwoo,** Asian Australas. J. Anim. Sci 25, 2012, 1029-1037.

Armstrong C.A., Murray N., Kennedy M., Koppula S.V., Tara D., Ansel J.C., **Melanoma-Derived Interleukin 6 Inhibits In Vivo Melanoma Growth.** Journal of Investigative Dermatology 102, 1994, 278-284.

Aubrecht J., Goad M.E.P., Czopik A.K., Lerner C.P., Johnson K.A., Simpson E.M., Schiestl R.H., **A high G418-resistant *neo*^{R} transgenic mouse and mouse embryonic fibroblast (MEF) feeder layers for cytotoxicity and gene targeting *in vivo* and *in vitro,*** Drug and Chemical Toxicology 34, 2011, 433-439.

Belsham D.D., Cai F., Cui H., Smukler S.R., Salapatek A.M.F., Shkreta L., **Generation of a Phenotypic Array of Hypothalamic Neuronal Cell Models to Study Complex Neuroendocrine Disorders**, Endocrinology 145, 2004, 393-400.

Bidou L., Bugaud O., Belakhov V., Baasov T., Namy O., **Characterization of new-generation aminoglycoside promoting premature termination codon readthrough in cancer cells,** RNA Biology 14, 2017, 378-388.

Boukamp P., Stanbridge E.J., Foo D.Y., Cerutti P.A., Fusenig N.E., **c-Ha-ras oncogene expression in immortalized human keratinocytes (HaCaT) alters growth potential in vivo but lacks correlation with malignancy,** Cancer Res. 50, 1990, 2840-2847.

Chapman S.W.K., Metzger N., Grest P., Feige K., von Rechenberg B., Auer J.A., Hottiger M.O., **Isolation, establishment, and characterization of *ex vivo* equine melanoma cell cultures,** In Vitro Cell.Dev.Biol.-Animal 45, 2009, 152-162.

Davies J., Jimenez A., **A New Selective Agent for Eukaryotic Cloning Vectors,** The American Journal of Tropical Medicine and Hygiene 29, 1980, 1089-1092.

De Luca M., D'Anna F., Bondanza S., Franzi A.T., Cancedda R., **Human epithelial cells induce human melanocyte growth in vitro but only skin keratinocytes regulate its proper differentiation in the absence of dermis,** The Journal of Cell Biology 107, 1988, 1919-1926.

Di Palma T., Filippone M.G., Pierantoni G.M., Fusco A., Soddu S., Zannini M., **Pax8 has a critical role in epithelial cell survival and proliferation,** Cell Death Dis 4, 2013, e729-e729.

Floquet C., Deforges J., Rousset J.-P., Bidou L., **Rescue of non-sense mutated p53 tumor suppressor gene by aminoglycosides,** Nucleic Acids Research 39, 2011, 3350-3362.

Fountain J.W., Lockwood W.K., Collins F.S., **Transfection of primary human skin fibroblasts by electroporation**, Gene 68, 1988, 167-172.

Gledhill K., Guo Z., Umegaki-Arao N., Higgins C.A., Itoh M., Christiano A.M., **Melanin Transfer in Human 3D Skin Equivalents Generated Exclusively from Induced Pluripotent Stem Cells,** PLoS ONE 10, 2015, e0136713.

Halaban R., **Culture of Melanocytes from Normal, Benign and Malignant Lesions,** in: Pfragner R., Freshney R.I. (Eds.), **Culture of Human Tumor Cells,** John Wiley & Sons, Inc., Hoboken, NJ, USA, 2005, 289-318.

Halaban R., Alfano F.D., **Selective elimination of fibroblasts from cultures of normal human melanocytes,** In Vitro 20, 1984, 447-450.

Heier C.R., DiDonato C.J., **Translational readthrough by the aminoglycoside geneticin (G418) modulates SMN stability *in vitro* and improves motor function in SMA mice *in vivo*,** Human Molecular Genetics 18, 2009, 1310-1322.

Hoerter J.D., Bradley P., Casillas A., Chambers D., Denholm C., Johnson K., Weiswasser B., **Extrafollicular Dermal Melanocyte Stem Cells and Melanoma,** Stem Cells International 2012, 1-10.

Hu D.N., McCormick S.A., Ritch R., Pelton-Henrion K., **Studies of human uveal melanocytes in vitro: isolation, purification and cultivation of human uveal melanocytes,** Invest. Ophthalmol. Vis. Sci. 34, 1993, 2210-2219.

Jin Q.H., Zhao B., Zhang X.J., **Cytochrome c release and endoplasmic reticulum stress are involved in caspase-dependent apoptosis induced by G418,** CMLS, Cell. Mol. Life Sci. 61, 2004.

Ju M., Lee H.J., Lee S.J., Seo E.S., Park H.J., Lee K.Y., Lee G.H., Choi E.J., Kim J.K., Lee J.W., Chung H.L., Kim W.T., **Neuroprotective effects of geneticin (G418) via apoptosis in perinatal hypoxic-ischemic brain injury,** Korean J Pediatr 51, 2008, 170.

Kuschal C., DiGiovanna J.J., Khan S.G., Gatti R.A., Kraemer K.H., **Repair of UV photolesions in xeroderma pigmentosum group C cells induced by translational readthrough of premature termination codons,** Proceedings of the National Academy of Sciences 110, 2013, 19483-19488.

Leaman D.W., Chawla-Sarkar M., Vyas K., Reheman M., Tamai K., Toji S., Borden E.C., **Identification of X-linked Inhibitor of Apoptosis-associated Factor-1 as an Interferon-stimulated Gene That Augments TRAIL Apo2L-induced Apoptosis,** J. Biol. Chem. 277, 2002, 28504-28511.

Lee T.S., Park S.H., Lee J.W., Lim S.H., **The Effect of Geneticin on the Survival of a Human Breast Cancer Cells under Hypoxic Condition,** J Korean Breast Cancer Soc 5, 2002, 279.

Lee Y.S., Lee C.H., Bae J.T., Nam K.T., Moon D.B., Hwang O.K., Choi J.S., Kim T.H., Jun H.O., Jung Y.S., Hwang D.Y., Han S.-B., Yoon D.Y., Hong J.T., **Inhibition of skin carcinogenesis by suppression of NF-κB dependent ITGAV and TIMP-1 expression in IL-32γ overexpressed condition,** J Exp Clin Cancer Res 37, 2018, 293.

Mally M.I., McKnight M.E., Glassy M.C., **Protocols of Electroporation and Electrofusion for Producing Human Hybridomas**, in: Chang D.C., Chassy B.M., Saunders J.A., Sowers A.E. (Eds.) **Guide to Electroporation and Electrofusion**, Elsevier, 1992, 507-522.

Miller J.N., Pearce D.A., **Nonsense-mediated decay in genetic disease: Friend or foe?,** Mutation Research/Reviews in Mutation Research 762, 2014, 52-64.

Mirvis M., Siemers K.A., Nelson W.J., Stearns T.P., **Primary cilium loss in mammalian cells occurs predominantly by whole-cilium shedding,** PLoS Biol 17, e3000381, 2019.

Nagel-Wolfrum K., Möller F., Penner I., Wolfrum U., **Translational read-through as an alternative approach for ocular gene therapy of retinal dystrophies caused by in-frame nonsense mutations,** Vis Neurosci 31, 2014, 309-316.

Nudelman I., Glikin D., Smolkin B., Hainrichson M., Belakhov V., Baasov T., **Repairing faulty genes by aminoglycosides: Development of new derivatives of geneticin (G418) with enhanced suppression of diseases-causing nonsense mutations,** Bioorganic & Medicinal Chemistry 18, 2010, 3735-3746.

Pirou C., Montazer-Torbati F., Jah N., Delmas E., Lasbleiz C., Mignotte B., Renaud F., **FGF1 protects neuroblastoma SH-SY5Y cells from p53-dependent apoptosis through an intracrine pathway regulated by FGF1 phosphorylation,** Cell Death Dis 8, 2017, e3023-e3023.

Prokhorova I., Altman R.B., Djumagulov M., Shrestha J.P., Urzhumtsev A., Ferguson A., Chang C.-W.T., Yusupov M., Blanchard S.C., Yusupova G., **Aminoglycoside interactions and impacts on the eukaryotic ribosome,** Proc Natl Acad Sci USA 114, 2017, E10899-E10908.

Sadeghiyeh N., Sehati N., Mansoori B., Mohammadi A., Shanehbandi D., Khaze V., Baradaran B., **MicroRNA-145 replacement effect on growth and migration inhibition in lung cancer cell line,** Biomedicine & Pharmacotherapy 111, 2019, 460-467.

Samanta A., Stingl K., Kohl S., Ries J., Linnert J., Nagel-Wolfrum K., **Ataluren for the Treatment of Usher Syndrome 2A Caused by Nonsense Mutations,** IJMS 20, 2019, 6274.

Satyamoorthy K., Herlyn M., **Melanoma: Biology**, in: Encyclopedia of Cancer, Elsevier, 2002, 131-139.

Scholar E., **Geneticin**, in: XPharm: The Comprehensive Pharmacology Reference, Elsevier, 2007, 1-2.

Sehati N., Sadeghie N., Mansoori B., Mohammadi A., Shanehbandi D., Baradaran B., **MicroRNA-330 inhibits growth and migration of melanoma A375 cells: *In vitro* study,** J Cell Biochem 121, 2020, 458-467.

Sheikh S., Coutts A.S., La Thangue N.B., **Transfection**, in: Basic Science Methods for Clinical Researchers, Elsevier, 2017, 191-209.

Sobiepanek A., Baran J., Milner-Krawczyk M., Kobiela T., **Different Types of Surface modification used for Improving the Adhesion and Interactions of Skin Cells,** OAJBS 2, 2020, 275-278.

Sobiepanek A., Kobiela T., **The study of the skin cells viscoelastic changes using QCM-D measurement**, In: Rasooly A., Ossandon M., Baker H., **Bioengineering Technologies. Methods in Molecular Biology**, Humana Press, 2020.

Sobiepanek A., Milner-Krawczyk M., Lekka M., Kobiela T., **AFM and QCM-D as tools for the distinction of melanoma cells with a different metastatic potential,** Biosensors and Bioelectronics 93, 2017, 274-281.

Sounni N.E., Baramova E.N., Munaut C., Maquoi E., Frankenne F., Foidart J.-M., Noël, A., **Expression of membrane type 1 matrix metalloproteinase (MT1-MMP) in A2058 melanoma cells is associated with MMP-2 activation and increased tumor growth and vascularization: MT1-MMP Promotes Tumor Growth and Angiogenesis.** Int. J. Cancer 98, 2002, 23-28.

Ścieżyńska A., Nogowska A., Sikorska M., Konys J., Karpińska A., Komorowski M., Ołdak M., Malejczyk J., **Isolation and culture of human primary keratinocytes – a methods review**. Exp Dermatol, 2019, 1-6.

Tobin D.J., Colen S.R., Bystryn J.-C., **Isolation and Long-Term Culture of Human Hair-Follicle Melanocytes,** Journal of Investigative Dermatology 104, 1995, 86-89.

Tomizawa Y., Sekido Y., Kondo M., Gao B., Yokota J., Roche J., Drabkin H., Lerman M.I., Gazdar A.F., Minna J.D., **Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene SEMA3B.** Proceedings of the National Academy of Sciences 98, 2001, 13954-13959.

Tomonobu N., Kinoshita R., Sumardika I.W., Chen Y., Inoue Y., Yamauchi A., Yamamoto K., Murata H., Sakaguchi M., **Convenient methodology for extraction and subsequent selective propagation of mouse melanocytes in culture from adult mouse skin tissue.** Biochemistry and Biophysics Reports 18, 2019, 100619.

Vicens Q., Westhof E., **Crystal Structure of Geneticin Bound to a Bacterial 16S Ribosomal RNA A Site Oligonucleotide,** Journal of Molecular Biology 326, 2003, 1175-1188.

Wagman G.H., Testa R.T., Marquez J.A., Weinstein M.J., **Antibiotic G-418, a new Micromonospora-produced aminoglycoside with activity against protozoa and helminths: fermentation, isolation, and preliminary characterization,** Antimicrob. Agents Chemother. 6, 1974, 144-149.

Wang J., Yang Y., Xia H.H.X., Gu Q., Lin M.C.M., Jiang B., Peng Y., Li G., An X., Zhang Y., Zhuang Z., Zhang Z., Kung H.F., Wong B.C.Y., **Suppression of FHL2 Expression Induces Cell Differentiation and Inhibits Gastric and Colon Carcinogenesis,** Gastroenterology 132, 2007, 1066-1076.

Wang Y., Tu Y., Lu J., Tao J., Li Y., **c-FLIPp43 induces activation of the nuclear factor-κB signaling pathway in a dose-dependent manner in the A375 melanoma cell line,** Molecular Medicine Reports 10, 2014, 1438-1442.

Yallop C.A., Svendsen I., **The effects of G418 on the growth and metabolism of recombinant mammalian cell lines**, Cytotechnology 35, 2001, 101-114.

Yang X., Jiang J., Zhang C., Li Y., **Baicalein restrains proliferation, migration, and invasion of human malignant melanoma cells by down-regulating colon cancer associated transcript-1,** Braz J Med Biol Res 52, 2019, e8934.

Review and Research on Cancer Treatment
Volume 6, Issue 1 (2020)

Personalized Medicine: from molecular methods to targeted therapy in cancer

Patrycja Grosman-Dziewiszek*, Adam Szeląg

***Corresponding author: Department of Pharmacology, Wroclaw Medical University,ul. Jana Mikulicza-Radeckiego 2, e-mail: patrycja.grosman-dziewiszek@umed.wroc.pl**

ABSTRACT

Personalised medicine, according to the Advisory group for the H2020 Health, refers to a medical model using characterisation of individual phenotypes and genotypes for tailoring the right therapeutic strategy for the right person at the right time, and/or to determine the predisposition to disease and/or to deliver timely and targeted prevention. The development of genomic technologies methods leads to the identification of multiple mutations in a large variety of cancers. The data based on molecular technologies like molecular profiling, DNA, RNA expression methods, and also immunohistochemistry and proteomics aims to identify and evaluate molecular targets that may be candidates for drug discovery. In general the mechanism of targeted cancer therapies focuses on blocking growth, progression and spread of cancer by interfering with molecular targets that are involved in this process.

Small molecules like tyrosine-kinase inhibitors and serine/threonine kinase inhibitors or monoclonal antibodies are the examples of anticancer targeted therapy. The application of personalized medicine is still a work in progress. The development of targeted therapies makes cancer treatment more effective and reduce the cytotoxic effect of non-cancer cells.

In this review, the methods of identification targeted molecules like zebrafish cancer model and molecular profiling will be presented. This review will focus on the existing and future technologies that could improve the development of targeted therapies for treatment of resistant cancer in individual patients. Specifically, it will concentrate on reviewing the examples of current successful oncologic therapies include BRAF inhibitors (vemurafenib), RET inhibitors (sorafenib), and epidermal growth factor.

INTRODUCTION

WHO reported that cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths, or one in six deaths, in 2018. Lung, prostate, colo-rectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervical and thyroid cancer are the most common among women (WHO). Conventional treatment of cancer are hormonal and cytotoxic therapies.

The poor clinical outcome for most cancer types is caused by a diverse array of factors, including late diagnosis, tumor heterogeneity, metastasis, lack of targeted treatment options and resistance to therapy, tumor recurrence, and a failure to translate preclinical breakthroughs into meaningful patient benefit (Doll, 2019). Targeted cancer therapies are expected to be more effective than conventional treatment.

Personalized medicine refers to medical model using characterisation of individual phenotypes and genotypes (e.g. molecular profiling, medical imaging, lifestyle data) for tailoring the right therapeutic strategy for the right person at the right time. Moreover personalized medicine allowed to determine the predisposition to disease. Personalized medicine relates to the concept of patient-centred care.

Gene expression profiling and genome-wide sequencing have played significant roles in knowing a tumor's molecular sequence and allowed for creation of targeted therapies. Targeted therapy is one of the most developing therapy for cancer. Molecular targeted therapy blocks the growth of cancer cells by interfering with specific targeted mole-cules involved in carcinogenesis and tumor growth.

Targeted cancer therapies may be more therapeutically beneficial for many cancer types, including lung, colorectal, breast, lymphoma and leukemia. Moreover, recent advances made it possible to analyze and tailor treatments to an individual patient's tumor. The main types of targeted cancer therapies are monoclonal antibodies, small molecule inhibitors and immunotoxins (Baudino, 2015).

Latest advances in molecular methods such as next-generation sequencing (NGS), including DNA sequencing, RNA sequencing, whole-exome sequencing, copy number variation analysis, and DNA methylation arrays, have increased our understanding of cancer biology, and leads to the development of a new comprehensive molecular cancer classification (Sicklick, 2019).

In 1984, EGFR, as the first receptor, was associated with an oncogene, v-ERBB, which was known to induce sarcomas and leukemias in chickens (Downward, 1984).

The first clinical trial of molecularly targeted drugs for the off-label treatment of heavily

pretreated metastatic cancer was the SHIVA trial. The molecular profile of each patient's tumour was established with a mandatory biopsy of a metastatic tumour and large-scale genomic testing. Only patients with a molecular alteration in one of three molecular pathways (hormone receptor, PI3K/AKT/mTOR, RAF/ MEK) were included. Patients were matched to one of ten regimens including 11 available mole-cularly targeted agents (erlotinib, lapatinib plus trastuzumab, sorafenib, imatinib, dasatinib, vemurafenib, everolimus, abiraterone, letrozole, tamoxifen). The results show that use of mole-cularly targeted agents outside their indications does not improve progression-free survival compared with treatment at physician's choice in heavily pretreated patients with cancer (Le Tourneu, 2015).

In the same time combination of everolimus with trastuzumab plus paclitaxel as first-line treatment for patients with HER2-positive advanced breast cancer (BOLERO-1: A Phase 3, Randomised, Double-Blind, Multicentre Trial) shown that progression-free survival was not significantly different between groups in the full analysis population (Hurvitz, 2015).

Disease progression in patients with HER2-positive breast cancer receiving trastuzumab might be associated with activation of the PI3K/Akt/mTOR intracellular signalling path-way. The addition of the mTOR inhibitor everolimus to trastuzumab might restore sensi-tivity to trastuzumab. In a randomised, double-blind, placebo-controlled phase 3 trial of evero-limus for women with trastuzumab-resistant, HER2-positive advanced breast cancer (BOLERO-3), the addition of everolimus to trastuzumab plus vinorelbine significantly pro-longs progression-free survival (PFS) in patients with trastuzumab-resistant and taxane-pretreated, HER2-positive, advanced breast cancer (Andre, 2015).

SEARCH STRATEGY AND SELECTION CRITERIA

In development of targeted therapies the first step is identification of marker and the next step is understanding the role of marker (prognostic, predictive).

In marker selection the following methods are developed**:**

GENOMICS

Understanding the molecular characteristics at a genomic level is critical to develop new treatment strategies. The identification of indi-vidual targetable alteration with a genomic methods might predict a therapeutic response to immune-checkpoint inhibitors or identify can-cer-specific proteins. Based on that, persona-lized anticancer vaccines are designed. Clinical applications of cancer genomics include moni-to-ring treatment responses and characterizing mechanisms of resistance. Traditional appro-aches to the genetic characterization of clinical oncology specimens include cytogenetic ana-lysis, fluorescence in situ hybridization (FISH), and molecular studies of single genes. These methodologies are complementary to each other and generate information of diagnostic and prognostic relevance. Next-generation sequen-cing (NGS) allows rapid analysis of multiple genes for clinically actionable somatic variants (Cottrell, 2014; Al.-Kateb, 2015). The appli-cation of massively parallel or next-generation sequencing (NGS) to large-scale cancer geno-mics discovery projects has revealed new information about the underlying genomic drivers of cancer development and progression across multiple anatomical locations. NGS and various analytical approaches are now being introduced into clinical practice to better inform the clinical care of patients with cancer (Berger, 2018). The application of NGS technologies to the characterization of human tumours has provided unprecedented opportunities to under-stand the biological basis of different cancer types, develop targeted therapies and interve-nets, discover genomic biomarkers of drug response and resistance, and to guide clinical decision-making regarding the treatment of patients (Garraway, 2013; Hyman, 2017). Increased levels of precision are being achieved in the clinical care by including cancer geno-mics in diagnostic medicine.

Therapeutic aplications of DNA sequencing was evaluated in I-PREDICT clinical study. This cross-institutional prospective study used tumor DNA sequencing and timely recommendations for individualized treatment with combination therapies. Administration of customized multi-drug regimens was feasible, with 49% of consented patients receiving personalized treatment. Targeting of a larger fraction of iden-tified molecular alterations, yielding a higher 'matching score', was correlated with signifi-

cantly improved disease control rates, as well as longer progression-free and overall survival rates, compared to targeting of fewer somatic alterations (Sicklick, 2019).

Whole-genome, whole-exome, and whole-transcriptome sequencing pro vide the opportunity for discovery the full spectrum of oncogenicalterations in cancer tumours (12, Caldow Pilgrim, 2013). Cancer precision medicine in the clinical practice mainly focuses on the role of liquid biopsy, particularly circulating tumor DNA, as a potential tool for cancer screening, selection of an appropriate drugs, surveillance of minimal residual diseases, and early detection of recurrence (Low, 2019).

The development of NGS approaches in clinical laboratories need guidelines to ensure that NGS testing to direct patient care is performed to the same rigorous standards as other clinical tests focused on the analysis of nucleic acids, such as DNA sequence analysis by Sanger methodlogy, DNA copy number analysis by microarray analysis, and detection of chromosome aberrations by interphase FISH [8]. The guidelines for clinical NGS analysis (both the technical and bioinformatics components) was publi-shed by The College of American Pathologists (CAP), the U.S. Centers for Disease Control and Prevention (CDC) and the New York State Department of Health (Cottrell, 2013; El-Khoueiry, 2018). Several organizations formalized guidelines under which clinical NGS can be performed (Cottrell, 2013). The quality and use of molecular tests in medicine routine practice are regulated by Implementation of Guidelines on PG and PK, Good Genomic Practices, Guidelines on genomic BM and drugs co-development, PG methodology in PhVG ICH E18 genomic samples and data handling (Garcia, 2017).

The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC) have surpassed the 1000 Genomes Project by sequencing thousands of tumors across different cancer types. Comprehensive genetic profiling of tumor samples has uncovered novel oncogenes and tumor suppressor genes by comparing their mutation frequencies with the background mutation rate, by detecting mutation profiles with significant bias toward certain mutation types (Becquemont, 2009).

Methodology implications for drug clinical development of Next Generation Sequencing (NGS) for clinical use are analysis of a panel of genes, analysis of whole exome or genome and large unbiased sequencing.

PROTEOMICS, PHOSPHOPROTEOMICS, PROTEOGENOMICS

Analysis of the expressed proteins in a tumor and their modification states reveals if and how DNA mutations are translated to the functional level. Proteomic changes including posttranslational modifications are essential steps of oncogenesis. Proteomics technology has only recently become comparable in depth and accuracy to RNAseq and allow the analysis of formalin-fixed and paraffin-embedded biobank tissues, on both the proteome and phosphoproteome levels. Mass spectrometry-based proteomic studies is technology for large-scale and unbiased proteomic analyses. Proteomic with genomic and clinical data generate a personalized panomics profile for each patient for better treatment decisions (Doll, 2019) modification states reveals if and how these mutations are translated to the functional level.

Phosphoproteomics measures the identity and quantity of tens of thousands of phosphorylation sites, serving as an ultimate read-out of the activity of kinases to detect aberrant kinase activities and altered signaling pathways, which are the most important alterations in oncogenic transformation. Drugs targeting mitogen-activated protein kinase 1 (MAPK), PI3K, serine/threonine-protein kinase B-raf (BRAF), vascular endothelial growth factor (VEGF), ALK, EGFR inhibit their targets directly at the protein level – and not at the gene level (Yaffe, 2013).

Cancer proteogenomics promises new insights into cancer biology and treatment efficacy by integrating genomics, transcriptomics and protein profiling including modifications by mass spectrometry (Satpathy, 2020).

PHARMACOGENOMICS

Pharmacogenomics is the study of the role of the genome in drug response and allows to optimize drug therapy, based on the patients genotype, to ensure maximum efficiency with minimal adverse effects (Bequenmont, 2009). In cancer treatment, pharmacogenomics tests are used to identify which patients are most likely to respond to certain cancer drugs. Innovative

tumor profiling methodologies are utilized to elucidate the pharmacogenomic landscape of tumor cells in order to support the molecularly guided delivery of therapeutics. Personalized medicine in oncology link the data of genomic, transcriptomic, and proteomic analysis of tumor samples to aid in therapy decision. In a typical screening of such type, multiple genes and proteins impli-cated in tumor initiation, progression, and drug resistance are analyzed in tumor biopsies.

The success of the technology has already been demonstrated for various combinations of altered biomarkers and therapeutic molecules, such as epidermal growth factor receptor (EGFR) expression and EGFR tyrosine kinase inhibitors/antibodies, or the expression of programmed death ligand 1 (PD-L1) with anti-PD1 and anti-PD-L1 therapies (Astras, 2020).

MOLECULAR IMAGING

Medical imaging plays a central role in clinical oncology. The future of such imaging is molecularly targeted imaging agents. Molecular imaging differs from conventional anatomical imaging in that imaging probes are utilized to visualize target molecules-of-interest. Molecular imaging plays an important role in oncology and personalized medicine by allowing earlier diagnosis, assessing early response to treatment and by predicting treatment response. Molecular imaging has an impact on drug development by streamlining preclinical and clinical tests for new drug candidates. Molecular imaging allows not only localization of a tumor in the body but also allows imaging of the expression and activity of specific molecules (protein kinase) as well as biological processes like angiogenesis, hypoxia and apoptosis. This processes influence on tumor behavior and response to treatment (McDermott, 2016).

Several markers related to angiogenesis including VEGF/VEGFR, amb3 integrins, hypoxia-inducible factor-1 or MMPs can be targeted for single-photon emission computed tomography (SPECT)/PET angiogenesis imaging. Preclinical trials on probes currently used for imaging the VEGF and VEGFRs pathways, such as antibodies against VEGF and radiolabeled VEGF-A, have shown promising data for further implementation in clinical practice (Fukumura, 2007). In other clinical study a method for conjugating a therapeutic antibody to a molecular magnetic resonance imaging was investigated. This study concluded that cet-PEG-dexSPION nanoparticle could be a promising nanomedicine for therapeutic targeting of EGFR-expressing tumor cells. The therapeutic antibody cetuximab and non-invasive monitoring methods improved treatment efficacy (Tseng, 2015).

There are many advantages associated with the ability to measure receptor expression by imaging: its' non-invasiveness, the ability to assess sites, which are difficult to sample and avoids sampling error from biopsies when receptor expression is hetero-geneous. Tumor receptor imaging can measure the therapeutic target expression and could be used to direct patient selection for targeted therapy (Mc Dermott, 2016).

IMMMUNOHISTOCHEMISTRY

The use of immunohistochemistry (IHC) for the determination of carcinoma biomarkers is a well-established and powerful technique. Immunohistochemisty is readily available in pathology laboratories, is easy to perform, assess and can provide clinically meaningful results in relatively short time (Thunninsen, 2017). There is a significant correlation between the IHC marker expression and disease progression and the prognosis of drug effects (Barbalan, 2018).

Multiplex immunohistochemistry allows the demonstration of multiple protein antigens in individual histological sections of formalin-fixed paraffin-embedded tumors or other types of tissue. Well-designed and optimized immunohistochemistry assays maximize the information available from limited tissues and demonstrating the histo-anatomical relationships among key cell types which express the included biomarkers (Steele, 2018).

The American Society of Clinical Oncology and the College of American Pathologists develop a guideline to improve the accuracy of immunohistochemical (IHC) estrogen receptor (ER) and progesterone receptor (PgR) testing in breast cancer and the utility of these receptors as predictive markers (Hammond, 2010).

ZEBRAFISH CANCER MODEL

The zebrafish (*Danio rerio*) has been established as one of the most important model organisms for cancer research. This model is particularly suitable for live cell imaging and high-throughput drug screening. The zebrafish represents a powerful platform for cancer research in the development of target therapies. The zebrafish cancer model was improved for drug discovery and toxicological and phenoltypical screening (Bootorabi, 2017).

The zebrafish is ideal for large-scale screening approaches and allows both chemical and genetic screening to identify genes and pathways underlying diseases, as well as phenotypic screening for the discovery of new drugs (Zhao, 2015). The compounds, drugs or small molecules, can be added directly to the water environment of the zebrafish (MacRae, 2015). Zebrafish allow unrivalled *in vivo* imaging of cellular behaviour thanks to optical clarity and a range of tissue specific transgenic lines (Brown, 2017). The noninvasive high-resolution imaging methods in transparent zebrafish embryos visualize cancer progression and reciprocal interaction with stroma in a living organism (Chen, 2017).

The zebrafish cancer models are link between *in vitro* cell culture and *in vivo* mammalian models for a rapid pre-clinical drug development. Moreover, given the high genetic and physiological similarities with humans, zebrafish can be used for anticancer drug screening. Transplanted human cancer cells are able to respond to zebrafish cytokines, modulate the zebrafish microenvironment, and take advantage of the zebrafish stroma during cancer progression. In addition to genetic and molecular studies, zebrafish model is also ideal for large-scale chemical screens to identify small molecules that influence different aspects of hematopoiesis (Gore, 2018).

A transgenic zebrafish melanoma model based on the zebrafish mitfa promoter coupled with the human oncogenic HRASG12V (Le, 2013). Transgenic zebrafish embryos developed melanocyte hyperplasia with the induction of RAS-RAF-MEK-ERK and RAS-PI3K-AKT-mTOR signaling pathways. The zebrafish model was useful for the screening of compounds directed against mitogen-activated protein kinases, extracellular signal-regulated kinases (MEK/ERK) and PI3K/mTORi pathways (Rapamycin), alone or in combination (Thomas, 2012). Clinical trials using rapamycin analogs combined with MEKi or PIK3K/mTORi are currently underway (Bootorabi, 2017).

The zebrafish model has been recently used to identify key molecules in skin cancer, which includes melanoma and squamous cell carcinoma (SCC), as well as compounds for SCC target therapy (Shin, 2016; Jun, 2011). Transgenic mitf-BRAFV600E; p53/zebrafish embryos have been created for the evaluation of early transcriptional activity within melanoma pathogenesis and to provide a model for chemical genetic screening in the context of melanoma therapy (White, 2011).

INNOVATIONS IN CLINICAL TRIAL DESIGN

New trial design uses gentic profiles to highlight biomarker differeces. In recent years, the therapeutic management of selected patients with cancer based on patient's mecha-nisms of tumorigenesis, DNA profiling using next-generation sequencing, proteomic and RNA analysis, and immune mechanisms after bioinformatic analysis is essential to optimize patient's treatment (Fountzilas, 2018).

The traditional, large phase II and phase III adjuvant trial models need to be replaced with smaller, shorter, and more focused trias that need to be more efficient and adaptive in order to quickly assess the efficacy of new agents. The shift from the traditional multiphase trial model to an increase in phase II adjuvant and neoadjuvant trials in earlier-stage disease incorporating surrogate endpoints for long-term survival enables better efficacy of therapeutic agents in shorter time frazes (Wulfkuhle, 2017).

The National Cancer Institute–Molecular Analysis for Therapy Choice (NCI-MATCH) trial is a study that relies on genomic assays to screen and enroll patients with relapsed or refractory cancer after standard treatments. The analytical validation processes for the next-generation sequencing (NGS) assay that was tailored for regulatory compliant was used in the trial. Thousands of patients who have relapsed or refractory solid tumors and lymphomas after standard systemic treatment was recruited and screened. The patients were assign

to a treatment appropriately matched to their cancer genotype. Analytical validation involved testing cells and tumor tissues of multiple types in an effort to determine assay performance over a wide range of tumor specimens. The NCI-MATCH trial will provide an opportunity for cancer patients to be matched to treatments targeted to specific molecular defects based on the genomic analysis of their tumors (Lih, 2017).

The FOCUS4 (Molecular selection of therapy in colorectal cancer: a molecularly stratified randomized controlled trial program) trial evaluates patients with advanced, metastatic colorectal cancer whose disease is stable or responds to first-line chemo-therapy, who are assigned to one of five sub-studies for randomization to a targeted agent (vs. control) based on tumor biomarkers (Kaplan, 2013).

In "N-of-1" trial, the determination of the optimal treatment for each patient based on tumor characteristics were performer. In a modified "Nof-1" study design, the anti-tumor activity of anticancer agents was matched to patients' genotype. Patients were treated according to their molecular profiling. The progression-free survival (PFS) was longer with the targeted treatment compared to PFS associated with their previous systemic treatment. In this study, tumor whole-genome sequencing and RNA expression analysis identified suggested targets for anticancer therapy in 13 tumor types (Von Hoff, 2010).

The use of vemurafenib and trametinib in BRAF V600E-mutated melanoma patients has led to substantial survival improvements (Sosman, 2012). Targeting EGFR mutations and the EML4-ALK fusion product in lung cancer with erlotinib and crizotinib, respectively, has led to remarkably improved outcomes (Shaw, 2013). Targeting the PI3K/AKT/mTOR pathway with cognate inhibitors used in combination (but not as single agents) resulted in stable disease for greater than 6 months and partial response rates of up to 45% in individuals with PIK3CA mutations (Janku, 2014)

REVIEW

TREATMENT OPTIONS

PI3K/AKT/MTOR PATHWAY GENES

Mutations in PIK3CA activate the AKT/mTOR pathway and have been described in breast, colon, gastrin, brain and biliary tract cancers (Holcombe, 2015). Somatic muta-tions are less common in biliary tract cancers; PTEN and PIK3CA mutations were observed in about 1 and 12-14% of GBCA, respectively (Ross, 2015). The tumors with these mutations are sensitive to PI3K specific inhibitors currently under investigation, as well as mTOR inhibitors, such as everolimus, temsorolimus, and rapamycin (Sicklicki, 2016).

HER2

The earliest targeted therapies block growth signals like trastuzumab (Herceptin), gefitinib (Iressa), imatinib (Gleevec), and cetuximab (Erbitux). Over the past 2 decades, there has been an extraordinary progress in the regimens developed for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast and stomach cancer. Trastuzumab, pertuzumab, lapatinib, and ado-trastuzumab emtansine (T-DM1) are commonly recommended anti-HER2 target agents by the U.S. Food and Drug Admi-nistration (FDA) (Wang, 2019). Studies on the HER2 gene develop pharmacological anti-HER2 agents to inhibit this pathway. In 1998 trastuzumab became a therapeutic for breast cancer patients with HER2 overexpression (Balduzzi, 2015) Trastuzumab is a monoclonal IgG1 class humanized murine antibody, binding the ECD of HER2 transmembrane receptor (Pinto, 2013). The mechanism of action is binding to the the HER2 receptor and inhibit signal transduction pathways and angiogenesis. Moreover, trastuzumab is cell-cycle agrest, apoptosis and DNA rep air induction agent (Sakai, 2018).

Trastuzumab was the first target approved specifically for early stage HER2-positive breast cancer in combination with cytotoxic agents, such as taxane, after completion of doxorubicin therapy (Ferretti, 2006).

PHILADELHIA CHROMOSOME

The identification of Philadelphia chromosome [t(9;22)] led to the discovery of imatinib mesylate. Imatinib was approved by the FDA in 2002 for the treatment of newly diagnosed Philadelphia chromosome positive chronic myeloid leukemia (Druker, 2001).

BRAF PROTEIN MUTATION

The cell growth signaling protein BRAF is present in an altered form (BRAF V600E) in many melanomas. Vemurafenib targets this mutant form of the BRAF protein and is approved to treat patients with inoperable or metastatic melanoma that contains this altered BRAF protein (Janku, 2014).

MUC 16

MUC16 (CA125) has been extensively used as a biomarker for ovarian cancer, and its expression has been associated with disease progression. MUC 16 plays role in fundamental processes, including protection of the epithelium and human carcinogenesis. The expression of mucins in resting, normal polarized cells is intricately controlled, with expression restricted on the apical membranes of exposed epithelia. Loss of cell polarity during carcinogenesis lead to mucins expression all over the cell surface. The cell become available to interact with several growth factor receptors, that are typically restricted to the basolateral surface, and modulate their downstream signaling in various cancers (Joshi, 2016).

MUC16 overexpression has been observed in several human malignancies, including ovarian, pancreatic, breast, and lung (Haridas, 2011). Moreover, MUC 16 and its ligands are potential targets for therapeutic intervention using monoclonal antibodies and immunotherapy (Aithal, 2018).

Current clinical study: Cyclophosphamide Followed by Intravenous and Intraperitoneal Infusion of Autologous T Cells Genetically Engineered to Secrete IL-12 and to Target the MUC16ecto Antigen in Patients With Recurrent MUC16ecto+ Solid Tumors is two phases trial. Screening phase to determine MUC16ecto tumor expression in ovarian, primary peritoneal or fallopian tube carcinoma. In intervention phase patients are eligible to receive the CAR+ T cells, the frozen leukapheresis product will be thawed and used to generate the 4H11-28z/fIL-12/EGFRt+ genetically-modified T cells to evidence ist safety and anti-tumor activity (Clinicaltrial.gov).

CURRENT CLINICAL TRIALS

To share the data on cancer medicine the new network was created. The Oncology Data Network (ODN) is a fully cooperative, collaborative data-sharing European network providing near real-time information on cancer medicine usage at scale. Data on cancer medicine use are collated through technology-enabled automation direct from participating hospitals' existing systems and translates from diverse sources into a common language enabling direct comparability via an automated regimen mapping algorithm (Kerr, 2020).

Integration of genomic data with drug screening from personalized in vitro and in vivo cancer models guide precision cancer care and fuel next generation research. The development of a robust precision cancer care platform, which integrates whole exome sequencing (WES) with a living biobank enables high throughput drug screens on patient-derived tumor organoids. 56 tumor-derived organoid cultures, and 19 patient-derived xenograft (PDX) models have been established from the 769 patients enrolled in an IRB approved clinical trial. To exetend the genomics for better identification therapeutic options for the majority of patients with advanced disease, high throughput drug screening effective strategies were used. Analysis of tumor derived cells from four cases, two uterine malignancies and two colon cancers, identified effective drugs and drug combinations that were subsequently validated using 3D cultures and PDX models. This clinical trial creates the platform to provide personalized therapeutic options for individual patients and promotes the discovery of novel therapeutic approaches (Pauli, 2017).

Next-generation sequencing (NGS) of circulating tumor DNA (ctDNA) is not yet routinely implemented in the setting of a phase I clinical trials. To supports blood-based genomic profiling a new molecular profiling program TARGET was designed. The primary aim is to match patients with a broad range of advanced cancers to early phase clinical trials on the basis of analysis of both somatic mutations and copy number alterations (CNA) across a 641 cancer-associated-gene panel in a single ctDNA assay. For the first 100 TARGET patients, ctDNA data showed good concordance with matched tumor and results were turned round within a clinically acceptable timeframe for Molecular Tumor Board (MTB) review. When a 2.5% variant allele frequency (VAF) threshold was applied, actionable mutations were identified in 41 of 100 patients, and 11 of these patients

received a matched therapy. These data support the application of ctDNA in this early phase clinical trial. Genomic profiling of contemporaneous tumor material enhances patient verification to novel therapies and provides a practical template for bringing routinely applied blood-based analyses to the clinic (Rothwell, 2019).

The ESCAT trial defines clinical evidence-based criteria to prioritise genomic altera-tions as markers to select patients for targeted therapies. This classification system aims to offer a common language in cancer medicine and drug development. The Euro-pean Society for Medical Oncology (ESMO) Translational Research and Precision Medicine Working Group (TR and PM WG) propose a classification system for molecular aberrations as clinical targets. ESCAT defines six levels of clinical evidence for molecular targets according to the implications for patient management: 1. targets ready for implementation in routine clinical decisions, 2. investigational targets that likely define a patient population that benefits from a targeted drug but additional data are needed; 3. clinical benefit previously demonstrated in other tumour types or for similar molecular target, 4. preclinical evidence of actionability, 5. evidence supporting co-targeting approaches and the last – lack of evidence for actionability (Mateo, 2018).

DISCUSSION AND SHORT CONCLUSION

The promising model is to combine targeted therapy with other therapeutic strategies like chemotherapy, radiation, and immunotherapy to determine how they may combine to exert more efficacious therapeutic effects and improve the outcomes of cancer patients.

Historically, cancer has been studied, and therapeutic agents have been evaluated based on organ site, clinical staging, and histology. The development of molecular profiling methods has expanded knowledge of cancer at the molecular level. Numerous cancer subtypes are being described based on biomarker expression and genetic mutations rather than traditional classifications of cancer. The development of new molecular methods promotes the discovery of novel therapeutic approaches that can be assessed in clinical trias. Moreover, provides personalized therapeutic options for individual patients where standard clinical options have been exhausted.

REFERENCES:

Aithal A., Rauth S., Kshirsagar P., Shah A., Lakshmanan I., Junker W.M., et al., **MUC16 as a novel target for cancer therapy**, In: Expert Opin Ther Targets, 2018; 22(8):675-686.

Al-Kateb H., Nguyen T.T., Steger-May K., Pfeifer J.D., **Identification of major factors associated with failed clinical molecular oncology testing performed by next generation sequencing (NGS)**, In: Mol Oncol. 2015; 9(9):1737-1743.

Astras G., Papagiannopoulos C.I., Kyritsis K.A., Markitani C., Vizirianakis I.S., **Pharmacogenomic Testing to Guide Personalized Cancer Medicine Decisions in Private Oncology Practice: A Case Study**, In: Front Oncol. 2020; 10:521.

Balduzzi S., Mantarro S., Guarneri V., Tagliabue L., Pistotti V., Moja L., et al., **Trastuzumab-containing regimens for metastatic breast cancer**, In: Cochrane Database Syst Rev. 2014; 2014(6):CD006242.

Bărbălan A., Nicolaescu A.C., Măgăran A.V., Mercuţ R., Bălăşoiu M., Băncescu G., et al., **Immunohistochemistry predictive markers for primary colorectal cancer tumors: where are we and where are we going?,** In: Rom J Morphol Embryol. 2018; 59(1):29-42.

Becquemont L., **Pharmacogenomics of adverse drug reactions: practical applications and perspectives**, In: Pharmacogenomics, 2009; 10 (6): 961-9.

Bootorabi F., Manouchehri H., Changizi R., Barker H., Palazzo E., Saltariet A., et al., **Zebrafish as a Model Organism for the Development of Drugs for Skin Cancer**, In: Int J Mol Sci. 2017; 18(7):1550.

Brown H.K., Schiavone K., Tazzyman S., Heymann D., Chico T.J., **Zebrafish xenograft models of cancer and metastasis for drug discovery**, In: Expert Opin Drug Discov. 2017; 12(4):379-389.

Caldow Pilgrim C.H., Groeschl R.T., Quebbeman E.J., Gamblin T.C., **Recent advances in systemic therapies and radiotherapy for gallbladder cancer**, Sin: urgical Oncology. 2013; 22(1):61-67, doi: 10.1016/j.suronc.2012.12.001.

Chen L., Groenewoud A., Tulotta C., Zoni E., Kruithof-de Julio M., van der Horst G., et al., **A zebrafish xenograft model for studying human cancer stem cells in distant metastasis and therapy response**, In: Methods Cell Biol. 2017; 138:471-496.

Doll S., Gnad F., Mann M., **The Case for Proteomics and Phospho-Proteomics in Personalized Cancer Medicine**, In: PROTEOMICS - Clinical Applications, 2019; 13(2, SI): 1800113.

Druker B.J., Talpaz M., Resta D.J., Peng B., Buchdunger E., Ford J.M., et al., **Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia**, In: N Engl J Med. 2001; 344(14):1031-7.

El-Khoueiry A.B., Rankin C., Siegel A.B., Iqbal S., Gong I.Y., Micetich K.C., et al., **S0941: a phase 2 SWOG study of sorafenib and erlotinib in patients with advanced gallbladder carcinoma or cholangiocarcinoma**, In: British Journal of Cancer. 2014; 110(4):882-887.

Ferretti G., Papaldo P., Fabi A., Carlini P., Felici A., Cognetti F., **Adjuvant trastuzumab with docetaxel or vinorelbine for HER-2-positive breast cancer**, In: Oncologist. 2006; 11(7):853-854.

Fountzilas E., Tsimberidou A.M., **Overview of precision oncology trials: challenges and opportunities**, In: Expert Rev Clin Pharmacol. 2018; 11(8):797-804.

Fukumura D., Jain R.K., **Tumor microvasculature and microenvironment: targets for anti-angiogenesis and normalization**, In: Microvasc Res 2007; 74:72-84.

Garcia E.P., Minkovsky A., Jia Y., Ducar M. D., Shivdasani P., Gong X., et al., **Validation of OncoPanel: A Targeted Next-Generation Sequencing Assay for the Detection of Somatic Variants in Cancer**, In: Arch Pathol Lab Med. 2017; 141(6):751-758.

Garraway L.A., Lander E.S., **Lessons from the cancer genome**, In: Cell. 2013; 153(1):17-37.

Gore A.V., Pillay L.M., Venero Galanternik M., Weinstein B.M., **The zebrafish: A fintastic model for hematopoietic development and disease**, In: Wiley Interdiscip Rev Dev Biol. 2018; 7(3):e312.

Hammond M.E., Hayes D.F., Dowsett M., Allred D.C.,Hagerty K. L., Badveet S., et al., **American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer,** In: Arch Pathol Lab Med. 2010; 134(7):e48-e72.

Haridas D., Chakraborty S., Ponnusamy M.P., Lakshmanan I., Rachagani S., Eric Cruzet E., et al**., Pathobiological implications of MUC16 expression in pancreatic cancer,** In: PLoS One 2011; 6(10):e26839.

Holcombe R.F., Xiu J., Pishvaian M.J., Millis S.Z., Gatalica Z., Reddy S.K., et al., **Tumor profiling of biliary tract carcinomas to reveal distinct molecular alterations and potential therapeutic target**, In: Journal of Clinical Oncology, 2015; 33(suppl 3) 2015 abstr 285.

Janku F., Hong D.S., Fu S., Piha-Paul S.A., Naing A., Falchook G.S., et al., **Assessing PIK3CA and PTEN in early-phase trials with PI3K/AKT/mTOR inhibitors**, In: Cell Reports. 2014; 6(2):377-387.

Joshi S., Kumar S., Choudhury A., Lakshmanan I., Rachagani S., Cruz E., et al., **Altered Mucins (MUC) trafficking in benign and malignant conditions**, In: Oncotarget 2014; 5(17):7272-84

Jung D.W., Kim J., Che Z.M., Oh E.S., Kim G., Eom S.H., Im S.H., Ha H.H., Chang Y.T., Williams D.R., **A triazine compound S06 inhibits proinvasive crosstalk between carcinoma cells and stromal fibroblasts via binding to heat shock protein 90**, In: Chem. Biol. 2011;18:1581-1590.

Kaplan R., Maughan T., Crook A., Fisher D., Wilson R., Brown L., et al., **Evaluating many treatments and biomarkers in oncology: a new design**, In: J Clin Oncol. 2013; 31(36):4562-8.

Kerr D., Arnold D., Blay J.Y., Buske C., Carrato A., Gerritsen, W., et al., **The Oncology Data Network (ODN): A Collaborative European Data-Sharing Platform to Inform Cancer Care**, In: Oncologist, 2020; 25(1):e1-e4.

Le X., Pugach E.K., Hettmer S., Storer N.Y., Liu J., Wills A.A., et al., **A novel chemical screening strategy in zebrafish identifies common pathways in embryogenesis and rhabdomyosarcoma development**, In: Development. 2013;140:2354-2364.

Lih C. J., Harrington R. D., Sims D. J., Harper K. N., Bouk C. H., Datta V., et al., **Analytical Validation of the Next-Generation Sequencing Assay for a Nationwide Signal-Finding Clinical Trial: Molecular Analysis for Therapy Choice Clinical Trial**, In: The Journal of molecular diagnostics: JMD 2017; 19(2), 313-327.

MacRae C.A., Peterson R.T., **Zebrafish as tools for drug discovery**, Nat. Rev. Drug. Discov. 2015; 14:721-731.

Mateo J., Chakravarty D., Dienstmann R., Jezdic S., Gonzalez-Perez A., Lopez-Bigas N., et al., **A framework to rank genomic alterations as targets for cancer precision medicine: the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT)**, In: Ann Oncol. 2018; 29(9):1895-1902.

McDermott S., Kilcoyne A., **Molecular imaging-its current role in cancer**, In: QJM. 2016; 109(5):295-299.

Pauli C., Hopkins B.D., Prandi D., Shaw R., Fedrizzi T., Sboner A., et al., **Personalized *In Vitro* and *In Vivo* Cancer Models to Guide Precision Medicine,** In: Cancer Discov. 2017; 7(5):462-477.

Pinto A. C., Ades F., de Azambuja E., Piccart-Gebhart M., **Trastuzumab for patients with HER2 positive breast cancer: delivery, duration and combination therapies**, In: Breast 22. 2013;(Suppl. 2), S152-S155.

Ross J.S., Wang K., Virgil D., Catenacci T., Chmielecki J., Ali S.M., et al., **Comprehensive genomic profiling of biliary tract cancers to reveal tumor-specific differences and genomic alterations**, In: Journal of Clinical Oncology, 2015;33(suppl 3) 2015 abstr 231.

Rothwell D.G., Ayub M., Cook N., Thistlethwaite F., Carter L., Dean E., et al., **Utility of ctDNA to support patient selection for early phase clinical trials: the TARGET study**, In: Nat Med. 2019; 25(5):738-743.

Sakai H., Tsurutani J., Iwasa T., Komoike Y., Sakai K., Nishio K., et al., **HER2 genomic amplification in circulating tumor DNA and estrogen receptor positivity predict primary resistance to trastuzumab emtansine (T-DM1) in patients with HER2-positive metastatic breast cancer**, In: Breast Cancer 25. 2018; 605-613.

Satpathy S., Jaehnig E.J., Krug K., Kim B. J., Saltzman A. B., Chan D. W., et al., **Microscaled proteogenomic methods for precision oncology**, In: Nat Commun. 2020; 11(1):532.

Shaw A.T., Kim D.W., Nakagawa K., Seto T., Crino L., Ahn M.J., et al., **Crizotinib versus chemotherapy in advanced ALK-positive lung cancer**, In: The New England Journal of Medicine. 2013; 368(25):2385-2394.

Shin Y.S., Cha H.Y., Lee B.S., Kang S.U., Hwang H.S., Kwon H.C., et al., **Anti-cancer Effect of Luminacin, a Marine Microbial Extract, in Head and Neck Squamous Cell Carcinoma Progression via Autophagic Cell Death**, In: Cancer Res. Treat. 2016; 48:738-752.

Sicklick J.K., Fanta P.T., Shimabukuro K., Kurzrock R., **Genomics of gallbladder cancer: the case for biomarker-driven clinical trial design**, In: Cancer Metastasis Rev. 2016; 35(2):263-275.

Low S.K., Nakamura Y., **The road map of cancer precision medicine with the innovation of advanced cancer detection technology and personalized immunotherapy**, In: Japanese Journal of Clinical Oncology, 2019; 49(7):596-603.

Sosman J.A., Kim K.B., Schuchter L., Gonzalez R., Pavlick A.C., Weber J.S., et al., **Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib**, In: The New England Journal of Medicine, 2012; 366(8):707-714.

Steele K.E., Brown C., **Multiplex Immunohistochemistry for Image Analysis of Tertiary Lymphoid Structures in Cancer**, In: Methods Mol Biol. 2018; 1845:87-98.

Thomas H.E., Mercer C.A., Carnevalli L.S., Park J., Andersen J.B., Conner E.A., et al., **mTOR Inhibitors Synergize on Regression, Reversal of Gene Expression, and Autophagy in Hepatocellular Carcinoma**, In: Sci. Transl. Med. 2012; 4:139ra84.

Thunnissen E., Allen T.C., Adam J., Aisner D.L., Beasley M. B., Borczuk A.C. et al., **Immunohistochemistry of Pulmonary Biomarkers: A Perspective From Members of the Pulmonary Pathology Society**, In: Arch Pathol Lab Med. 2018; 142(3):408-419.

Tseng S.H., Chou M.Y., Chu I.M., **Cetuximab-conjugated iron oxide nanoparticles for cancer imaging and therapy**, In: Int J Nanomed. 2015; 10:3663-85.

Von Hoff D.D., Stephenson J.J., Rosen P., Loesch D.M., Borad M.J., Anthony S., et al., **Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers**, In: J Clin Oncol. 2010; 28(33):4877-83.

Wang J., Xu B., **Targeted therapeutic options and future perspectives for HER2-positive breast cancer**, In: Sig Transduct Target Ther. 2019; 4: 34.

White R.M., Cech J., Ratanasirintrawoot S., Lin C.Y., Rahl P.B., Burke C.J., et al., **DHODH modulates transcriptional elongation in the neural crest and melanoma**, In: Nature. 2011; 471:518-522.

https://www.who.int/news-room/fact-sheets/detail/cancer 1.06.2020.

Wulfkuhle J.D., Spira A., Edmiston K.H., Petricoin E.F., **3rd. Innovations in Clinical Trial Design in the Era of Molecular Profiling**, In: Methods Mol Biol. 2017; 1606:19-36.

Yan M., Schwaederle M., Arguello D., Millis S.Z., Gatalica Z., Kurzrock R., **HER2 expression status in diverse cancers: review of results from 37,992 patients**, In: Cancer and Metastasis Reviews, 2015; 34(1):157-164.

Zhao S., Huang J., Ye J., **A fresh look at zebrafish from the perspective of cancer research**, In: J. Exp. Clin. Cancer Res. 2015; 34:80.

Index of authors