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Clinical practice of renal cell carcinoma treatment with nivolumab

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ABSTRACT

Nivolumab alike other immune checkpoint inhibitors has been intensively developed during the last decade. Kidney cancer is among the neoplasm in treatment of which we have accumulated the most experience regarding nivolumab. As improves our understanding of the mechanisms underlying specific cellular immune response, thus improves our understanding of the place the nivolumab holds among other therapeutic options. Recent years brought development of innovative immunotherapy combinations as a method for improving immunotherapy efficacy. This review aims at providing practicing oncologists with key aspects of renal cell carcinoma treatment with nivolumab.

Key words: kidney cancer, renal cell carcinoma; nivolumab, anti-PD-1, immunotherapy; checkpoint inhibitor

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Introduction

Rare cases of spontaneous complete remissions due to potential immunological anti-tumour responses have been drawing the attention of researchers for centuries. Well-documented cases of attempts at inducing such response have been undertaken as early as in the 17th and 18th centuries. Back then, spontaneous remission was most commonly seen along with severe infection. Nineteenth century research led to the formulation of the first standardised therapies based on attenuated encapsulated bacteria [1]. However, it was only the developments in molecular biology in the second half of the 20th century that brought about the opportunity to develop a modern immunotherapeutic approach, with its rapid expansion during the last decade.

According to the Global Cancer Observatory report, in 2018 about 400 thousand new renal cell carcinoma (RCC) cases were diagnosed worldwide, with over 175 thousand RCC-related deaths [2]. The progress in RCC treatment seen in the 21st century is based on the understanding of RCC core pathomechanisms: induction of angiogenesis and deregulation of immune response (overactivation of innate inflammatory response with deficient adaptive immune response). Several new therapeutic approaches for advanced disease have been developed: inhibitors of vascular endothelial growth factor (VEGF)-dependent angiogenesis; inhibitors of mammalian target of rapamycin (mTOR) — an another protein involved in RCC pathogenesis; cytokines that induce adaptive response; and finally the immune checkpoint inhibitors — innovative particles that activate suppressed mechanism of antigen presentation and enable cytotoxic T-cell activity.

Nivolumab is one of the first immune checkpoint inhibitors. Clinical phase of its' development began in 2006 [3] and the drug gained first registration label in the 2014. Currently, as of December 2018, nivolumab is approved in Europe in six indications: melanoma, non-small cell lung cancer, renal cell carcinoma, urothelial cancer, squamous cell cancers of head and neck, and classic Hodgkin lymphoma.

The presented article aims at providing practicing oncologists with current data regarding the activity and safety profile of nivolumab in the treatment of RCC as well as valuable insights into clinical aspects of immune response in the pathophysiology of RCC.

Immune system and carcinogenesis

The immune system plays an important and multifactorial role in the aetiopathogenesis of cancer. From

Table 1. Mechanisms promoting carcinogenesis with leukocyte involvement [4]

Cancer trait	Selected responsible mechanisms
Continuous stimulation of proliferation	Direct production of growth factors (EGF, TGF, TNF, FGF, PDGF)
	Releasing growth factors bound to stroma
Resistance to antiproliferative signalling	Induction of HIF, HSP, and β-catenin production
	Decreasing expression of adhesive molecules
Evasion of cell death	Stimulation of survival-related pathways (VCAM-1)
	Chemotherapy resistance related to cathepsin concentration
Angiogenesis induction	Releasing of proangiogenic factors: VEGF, PDGF, TNF-α, Ang-2
Local invasion and metastasis	Stroma proteolysis
	Suppression of adhesive molecules functions
	Increasing endothelium permeability
	Generation of pre-metastatic niche
Immunosurveillance evasion	Depletion of cytotoxic cells
	Stimulation of suppressor Treg lymphocyte proliferation
	Promotion of mechanism leading to immunological toleration

Ang-2 — angiopoetyna-2; EGF — epithelial growth factor; FGF — fibroblast growth factor; HIF — hypoxia induced factor; HSP — heat-shock protein; PDGF — platelet-derived growth factor; TGF — transforming growth factor; TNF — tumour necrosis factor; Treg — lymphocytes T-regulatory; VCAM — vascular cell adhesion molecule; VEGF — vascular endothelial growth factor

the one side, some aspects of immune response may promote carcinogenesis, enabling survival of cancer cells in a metastatic niche or leading to inefficient protein and energy metabolism. From the other side, the immune system is the most important defence line against cancer development.

We currently know that immune cells from myeloid and lymphatic lines, both present in direct tumour microenvironment as well as distant ones, are responsible for several characteristic traits of cancer called "hallmarks of cancer". These arise through several feedback loops: stimulation of proliferation; resistance to antiproliferative signalling; evasion of apoptosis; induction of angiogenesis; local invasion and metastases formation; and escape from immunosurveillance (Table 1) [4]. Additionally, overactivation of nonspecific inflammatory response is an important driver of cachexia, one of the most common cancer complications.

Immunosurveillance and mechanisms responsible for evasion from it are among the key factors in oncology [5]. Adaptive antitumour immune response requires a difference in antigens between cancer and healthy tissue. These so-called neoantigens are released from the cancer cell and then captured and presented to the immune system by dendritic cells (DC). For proper functioning, DCs have to go through a process of activation and maturation, which enable expression of specific co-stimulatory factors required by naïve T cells (Tn). If a mature DC presents detected antigen within proper major histocompatibility complex (MHC) class I and II and with adequate co-stimulation, antigen-specific Tn are selected and activated. As a result, Tn differentiate and proliferate into cytotoxic (Tc) and memory (Tm) clones.

Activated Tc clone have to reach cancer tissue and infiltrate it. Cancer cell destruction requires Tc cell to recognize it's specific antigen, presented through class I MHC, in the absence of additional signals supressing cytotoxicity (either by cytokines or immunosuppressive cell-membrane molecules present in the tumour microenvironment).

Destroyed cancer cells release new portions of neoantigens, again detected and presented by DC, closing the cycle of immune antitumour response (Fig. 1) [6]. Each cycle iteration may promote additional clones of Tc lymphocytes active against subsequent neoantigens, potentially resulting in more and more effective response.

It is well acknowledged that evasion from immunosurveillance through disruption of at least one part of the described cycle is a nacessary condition for the cancer to develope. To achieve effective and persistent tumour control by the immune system — and therefore long-term remission — we have to facilitate closing of the cycle. This requires recognition of its weak spots and potential methods for strenghtening them.

Selected mechanisms of immunosurveillance evasion — clinical aspects

Systematic review of mechanisms used by cancer to evade immunosurveillance surpasses the limits of this manuscript. An excerpt of this complex landscape, significant in terms of the immune feedback loop described above, is presented in Table 2. Analysis of the aforementioned mechanisms at each stage of cytotoxic antitumour immune response provide practical insights
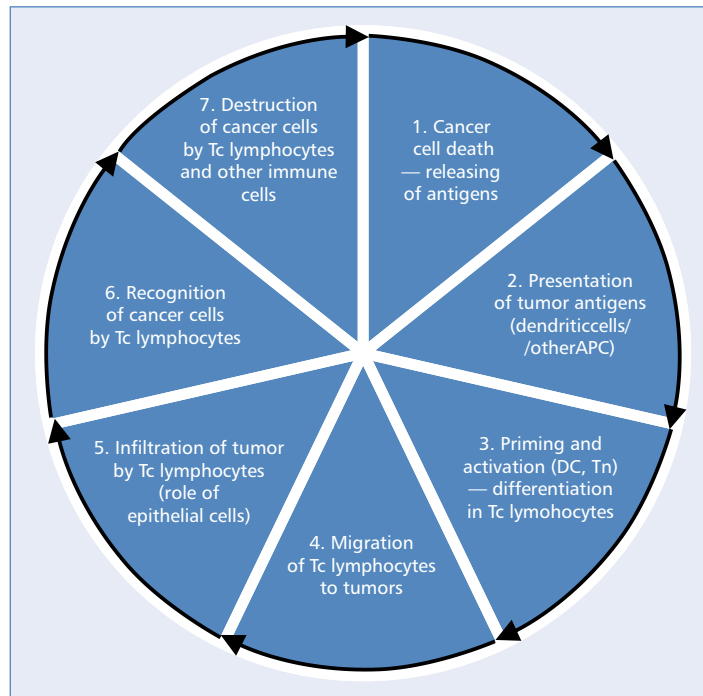
Figure 1. Cycle of immunological antineoplastic response (based on [6]). APC — antigen-presenting cells; DC — dendritic cells; Tn — lymphocytes T-naïve; Tc — lymphocytes T-cytotoxic

Table 2. Mechanisms impacting different stages of immunosurveillance [6, 11, 34, 35]

Stage	Mechanisms impairing immunosurveillance	Mechanisms promoting immunosurveillance
1. Cancer cell death — release of antigens	Low cell antigenicity (low TMB) Non-immunogenic cell death	High cell antigenicity (high TMB, mutagens) Immunogenic cell death
2. Neoantigen presentation	Immunosuppressive cytokines (IL-10; IL-4; IL-13) Low DC availability	Activating cytokines (TNF-α, IL-1, IFN-α) PRR activation (DAMPs, PAMPs)
3. Priming and Tn activation — differentiation in Tc	Suppressive co-stimulation (CTLA4:B7.1; PD-L1:PD-1; PD-L1:B7.1) Suppressive cytokines — prostaglandins Availability and variability of Tn Treg	Activating co-stimulation (CD28:B7.1; OX40:OX40L; CD27:CD70) Activating cytokines (IL-2; IL-12)
4. Migration of Tc	Chemokines engaging Treg and MDSC	Chemokines engaging Tc (CCL2; CCL3; CCL4; CCL5; CXCL9; CXCL10)
5. Tumour Tc infiltration	Angiogenesis (especially VEGF-dependent)	Adhesive particles (ICAM1, selectins)
6. Cancer cell recognition by Tc	Low cell antigenicity (low TMB) Decreased expression of MHC Low number of Tc clones	High cell antigenicity Proper TCR expression High affinity of TCR to antigen
7. Destruction of cancer cell by Tc and other immune cells	Immunosuppressive co-stimulation (PD-L1:PD-1; PD-L1:B7.1; MICA:MICB; BTLA; LAG-3) TNF-β	IFN-γ

BTLA — B- and T-lymphocyte attenuator; CCL — CC class chemokine; CTLA4 — cytotoxic lymphocyte antigen-4; CXCL — CXC class chemokine; DAMPs — damage associated molecular patterns; IFN — interferon; IL — interleukin; LAG-3 — lymphocyte-activating gene (protein product of its' transcription); MDSC — myeloid-derived suppressor cell; MHC — major histocompatibility complex; PAMPs — pathogen associated molecular patterns; PD-1 — programmed cell death 1 receptor; PD-L1 — programmed cell death 1 receptor ligand; PRR — pattern-recognizing receptor; Tc — T-cytotoxic lymphocyte; TCR — T lymphocyte receptor; TMB — tumour mutational burden; Tn — T-naïve lymphocytes; TNF — tumour necrosis factor; Treg — T-regulatory lymphocyte; VEGF — vascular endothelial growth factor

into the mechanism of action of novel therapeutic approaches. Below we describe those that are most important to understand the clinical application of immune checkpoint inhibitors.

Expression of programmed death receptor 1 (PD-1) is present mostly on mature Tc lymphocytes. Interaction of PD-1 with specific ligands, PD-L1 and PD-L2, suppresses cytotoxic activity of lymphocytes. Several signalling pathways, existing in the tumour microenvironment, induce expression of PD-1 ligands on the surface of different types of cells present in the microenvironment because the interaction between PD-1 and PD-L1/2 is a common mechanism behind immunosurveillance evasion [7, 8]. Nivolumab acts through the inhibition of this signalling. Additionally, PD-1 might play a role in the activation of Tn lymphocytes by DC, but available data suggest that this effect is not crucial for immune checkpoint inhibitor effectiveness.

Immunogenicity of cancer cells can be assessed in two categories: quantity and quality of neoantigens present in the cancer cell and the actual availability of neoantigens for DC. Tumour mutational burden (TMB), defined as the number of mutations per thousand DNA base pairs, is a rising biomarker for immunogenicity prediction. The more mutations, the more altered proteins and therefore the more neoantigens. Several reports confirm the predictive value of TMB for immunotherapy effectiveness, in terms of both overall survival and depth of response [9, 10].

The second factor defining immunogenicity of cancer cells is the actual availability of its neoantigens for DC and Tc cells. This depends on several factors, including: MHC expression; expression of neoantigens themselves; mechanisms of cancer cell death and its effective (immunogenic death) or non-effective (non-immunogenic death) neoantigen release [11].

Therapeutic influence on TMB is not yet available, but the strategy of combining immune checkpoint inhibitors with therapies aimed at increasing neoantigen expression and inducting immunogenic cancer cell death are intensively studied [12]. One of most successful approaches, often described in the literature, is the combination of immunotherapy with radiotherapy, especially valuable in oligometastatic diseases or in the presence of tumours in difficult localisations [13–15]. Practicing oncologists should be aware of numerous clinical trials evaluating the combination of immunotherapy with other drugs, including chemotherapy, and about the potentially beneficial role of palliative radiotherapy in patients receiving immune checkpoint inhibitors (these data, although arising from low numbers of cases or singular case reports, are extremely promising).

Antigen presentation by DC and recruitment of Tn lymphocytes are, besides the cytotoxic response itself, part of one of the two main phases of immune response activated by immune checkpoint inhibitors. This complex mechanism will not be fully covered, but some of its aspects have strong implications for clinical practice. Dendritic cells, in order to efficiently stimulate Tc clone proliferation, have to go properly through the activation and maturation processes. Presentation of antigens by non-activated DC fails to recruit Tn or, even worse, recruitment of Tn simultaneously with additional signalling through co-stimulatory molecules may be responsible for inducing immune tolerance.

Maturation of dendritic cells requires the coexistence of several signals. From a functionary perspective, we can divide them into two groups: cytokines and ligands of pattern-recognising receptors (PRR). PRR ligands include damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). DAMPs are mostly products of cell lysis and stroma damage: HSP, calreticulin, nucleic acids, and products of their degradation. PAMPs are mostly substances being common denominators of pathogenic microorganisms, such as evolutionary conservative parts of bacterial cell wall or viral RNA. DAMPs and PAMPs circulate in blood in the presence of tissue damage or infection and are a warning signal required for initiation of DC maturation. Lack of proper PRR stimulation dampens activation of cellular response, preventing auto-immunogenicity in normal conditions but concurrently allowing evasion of immunosurveillance during oncogenesis [16].

Gut microbiome is a significant source of PAMPs, which draws attention to the connection between gut microbiome, immunological response, and carcinogenesis. Beside its effect on DC maturation, several mechanisms of interaction between gut microbiome, and cellular and inflammatory response have been described. Numerous reports tie phenotype of commensal microbiota with effectiveness of immunotherapy — both in animal models and in humans [17–21]. Connecting negative impact of antibiotics on bacterial microflora, additional reports showed strong negative correlation between antibiotics administration and effectiveness of immune checkpoint inhibitors. Significantly worse outcomes of checkpoint inhibitor therapy (both overall survival [OS] and progression-free survival [PFS]) was shown in patients pretreated with antibiotics as compared to patients not exposed to antibiotics (Table 3). Oncologists administering immunotherapy should be aware of this connection and avoid needless antibiotics. This includes adequate differential diagnosis between infections and autoimmunological adverse events associated with immunotherapy (e. g. bacterial pneumonitis *vs.* autoimmunological pneumonitis; *Clostridium difficile* infection *vs.* autoimmunological colitis). Available data do not support attempts to modify the composition of gut microbiome in patients outside of clinical trials.

Cachexia is a multifactorial disease that includes protein and energy malnutrition in mechanisms of both

Table 3. Publication connecting antibiotics (ATB) with response to immune checkpoint inhibitors

Author, reference	Cancer type	N	Type of drug	mPFS (ATB+ *vs.* ATB–)	mOS (ATB *vs.* ATB–)
Derosa et al. 2018 [36]	mRCC	121	anti-PD-L1	1.9 *vs.* 7.4 months (p < 0.01)	17.3 *vs.* 30.6 months (p = 0.03)
Derosa et al. 2018 [36]	mNSCLC	239	anti-PD-L1	1.9 *vs.* 3.8 months (p = 0.03)	7.9 *vs.* 24.6 months (p < 0.01)
Thompson et al. 2017 [37]	mNSCLC	74	anti-PD-1	2.0 *vs.* 3.8 months (p < 0.001)	4.0 *vs.* 12.6 months (p = 0.005)
Huemer et al. 2018 [38]	mNSCLC	30	anti-PD-1	2.9 *vs.* 3.1 months (p = 0.031)	11.1 *vs.* 15.1 months (p = 0.023)
Routy et al. 2018 [20]	mRCC, mUC mNSCLC,	249	anti-PD-1; anti-PD-L1	3.5 *vs.* 4.1 months (p = 0.017)	11.5 *vs.* 20.6 months (p < 0.001)
Tinsley et al. 2018 [39]	mMM, mRCC, mNSCLC	303	anti-PD-1; anti-PD-L1	3.2 *vs.* 5.8 months (p = 0.049)	10.4 *vs.* 22.4 months (p = 0.001)

mRCC — metastatic renal cell carcinoma; mNSCLC — metastatic non-small cell lung cancer; mUC — metastatic urothelial cancer; ATB — antibiotics; mPFS — median progression-free survival; mOS — median overall survival

inadequate intake and excessive expenditure, non-specific systemic inflammation and increased catabolism. Cachexia develops in 80% of cancer cases and is the leading cause of death in nearly 20% of cancer patients. The incidence and intensity of cachexia are related to stage of disease and cancer biology. Cachexia develops commonly in patients with gastric, pancreatic, and lung cancers as well as in patients with genitourinary, lymphatic and gynaecological malignancies [22]. The presence of cachexia is a factor associated with poor prognosis.

Additionally, cachexia is a negative prognostic factor for immune checkpoint therapy in animal models and in human clinical trials [23, 24]. This may be due to promotion of immunosurveillance evasion through the following: induction of immunosuppression (interleukin-6 [IL-6], glucocorticosteroids, depletion of immune cells); limitation of metabolic support for highly-energetic processes associated with Tc clone activation; and increase in clearance of therapeutic monoclonal antibodies due to protein deficiency [25].

Despite common knowledge regarding the benefits of treating cachexia — mostly through adequate nutritional support [26] — we lack prospective data that allow optimisation of cachexia management in patients undergoing immune checkpoint therapy. Although promising interventions exist (e.g. non-steroidal anti-inflammatory drugs, direct and indirect IL-6 antagonists), their combination with immune checkpoint inhibitors remains a domain of research. Oncologists should be aware of decreased immunotherapy effectiveness in patients with cachexia (alternative therapies might be advised), and they should recognise that immune response requires a significant amount of energy and thus treat cachexia intensively according to current guidelines.

Nivolumab in renal cell carcinoma refractory to antiangiogenic treatment

In November 2015 nivolumab gained registration by Food and Drug Administration (FDA) in the USA and in April 2016 by the European Medicine Agency (EMA) in the indication "treatment of advanced renal cell carcinoma in patients who received prior treatment". The registration was based on the results of the phase III trial CheckMate 025 (NCT01668784) trial. This international study recruited adult patients with advanced RCC after failure of one or two lines of antiangiogenic treatment. Between October 2012 and March 2014, 821 patients were randomised to either nivolumab at a dose of 3 mg/kg body weight biweekly or everolimus in standard continuous dosing of 10 mg per day (Fig. 2) [27].

The newest, three-year update of the study results [28] showed data after a median observation time of 24 months in the nivolumab arm and 19 months in the everolimus arm (Table 4). The response rate was, respectively, 26% and 5%, although nearly 35% of patients receiving nivolumab were refractory to the treatment and had progressive disease as their best response. Typically for the immunotherapy, not all responses were seen in the first scanning, and some were obtained later. Median OS was 25.8 months in the nivolumab arm and 19.7 months in the everolimus arm (hazard ratio [HR] 0.74; p = 0.0005). Rates of two-year survival were, respectively, 52% and 42%, and rates of three-year survival were 39% and 30%, respectively. Median progression-free survival times (4.2 *vs.* 4.5 months) and median duration of response (12.3 *vs.* 12.0 months) did not differ between patients receiving nivolumab and everolimus, respectively. Durable responses were more
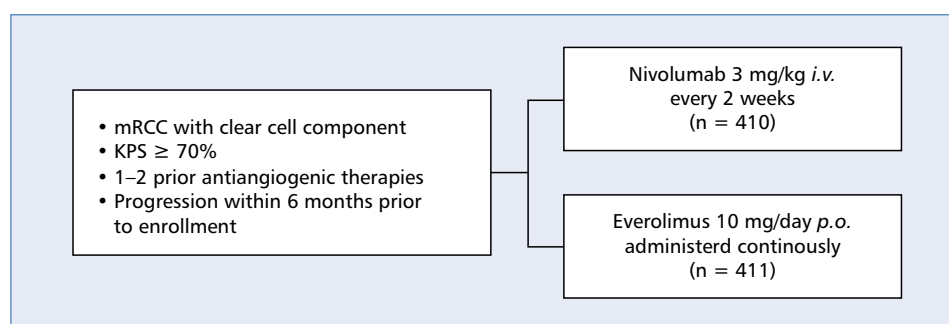
Figure 2. Scheme of CheckMate025 trial. mRCC — metastatic renal cell cancer; KPS — Karnofsky Performance Status

Table 4. Summary of CheckMate025 trial

	Nivolumab	Everolimus
ORR (CR+PR)	26%	5%
mPFS	4.2 months	4.5 months
mOS	25.8 months	19.7 months
2-year survival rate	53%	42%
3-year survival rate	39%	30%
Rate of G1–4 toxicity	80%	89%
Rate of G3–4 toxicity	21%	37%

ORR — objective response rate; CR — complete response; mOS — median overall survival; mPFS — median progression-free survival; PR — partial response

common in patients receiving nivolumab (18% *vs.* 6%). Toxicity profile was also in favour of nivolumab, with a rate of all treatment-related adverse events of 80% in the nivolumab arm and 89% in the everolimus arm with grade 3–4 adverse events present in, respectively, 21% and 37% of patients.

In the subgroup analysis [29] the benefit from nivolumab was irrespective of either MSKCC or IMDC prognostic group, number of prior treatment lines, and localisation of metastases. The only subgroup of patients with limited benefit from nivolumab were patients aged over 75 years. A trend in favour of nivolumab was seen in subgroups of patients with lung metastases and without bone or liver metastases.

Choice of optimal therapy after progression of anti-VEGF TKI — the place of nivolumab

In Poland, majority of patients with advanced RCC treated outside of clinical trials receive tyrosine kinase inhibitors (sunitinib or pazopanib) as a first-line therapy. The median progression-free survival achieved with TKI reaches 9–11 months [30, 31], and second-line treatment is inevitable in the majority of patients. In this setting options include the following: alternative TKI with a different affinity to key receptors (axitinib); mTOR inhibitor (everolimus); multi-

kinase inhibitor with additional activity against MET and AXL kinases (cabozantinib); and immune checkpoint inhibitors aimed at PD-1 (nivolumab). Only some of these options were compared head-to-head in randomised clinical trials, yet knowledge regarding different modes of action, expected efficiency, and toxicity profile allows optimisation of therapy for each patient (Table 5).

In an indirect comparison of response rates, both nivolumab and cabozantinib exhibit similar activity (25% *vs.* 5% for, respectively, nivolumab and everolimus in the CheckMate 025 trial and 17% *vs.* 3% for, respectively, cabozantinib and everolimus in the METEOR trial). However, nivolumab is associated with the highest rate of progressive disease as the best response — about 35% of cases compared with only 12% treated with cabozantinib. This suggest that nivolumab may not be an optimal choice for symptomatic patients or those in whom moderate progression may be life threatening. As mentioned previously, nivolumab might also be less active in elderly patients (> 75 years old) and in patients with cachexia [24].

Compared with everolimus, nivolumab is characterised by favourable toxicity profile and a beneficial impact on quality of life. Direct comparison of its toxicity profile with TKI is difficult due to the different methods of safety assessment used in each trial. It is well recognised that adverse event profiles differ between TKI and immune checkpoint inhibitors, and this difference can affect patients' and physicians' treatment preferences.

Table 5. Comparison of activity of drugs used in the second-line treatment of renal cell carcinoma after progression on tyrosine kinase inhibitors aimed at VEGF

	Axitinib [40]	Everolimus [27, 28]	Cabozantinib [41]	Nivolumab [27, 28]
mOS (months)	20.1	19.7	21.4	25.8
mPFS (months)	8.3	4.5	7.4	4.2
ORR (CR + PR)	19%	5%	17%	26%
CBR (CR + PR + SD)	76%	61%	87%	59%
PD as best response	17%	28%	12%	35%

CBR — clinical benefit rate; mOS — median overall survival; mPFS — median progression-free survival; ORR — objective response rate; CR — complete response; PR — partial response; SD — stable disease; PD — progressive disease

	2009	2010	2011	2012	2013	2014	2015	2016	2017
Number of patients recruited into new trials per year	136	2472	582	4862	5020	11 252	39 900	46 060	52 771
Number of new trials opened	1	6	2	13	20	58	190	329	467
Mean number of patients per trial	136	412	291	374	251	194	210	140	113
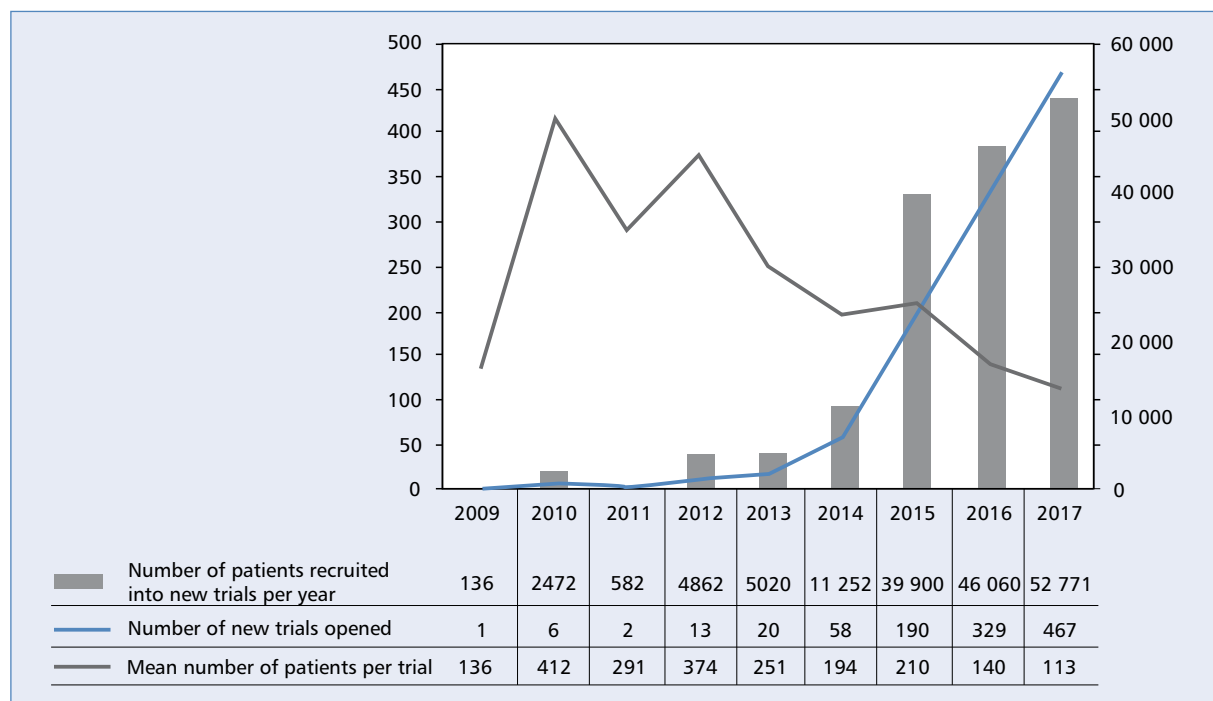
Figure 3. Trends in recruitment in trials assessing combination of immunotherapy with other therapies (based on [12])

Concluding, second-line treatment of advance RCC with nivolumab can be considered in patients: under 75 years old; optimally asymptomatic or mildly symptomatic; without critical tumour mass; without significant cachexia; and capable of withstanding autoimmunological adverse events (e.g. without contraindication to steroids).

Future of immuno-oncology — perspectives for innovative combinational therapies

Comparison of toxicity profile of anti-PD1 and anti-PD-L1 immunotherapies and their comparators within clinical trials strongly favours immunotherapy [32]. Low toxicity and increased activity, as well as potential synergy of combinational therapy, encourage research assessing combinations of both immuno-oncologic drugs and immunotherapy with other anticancer therapies and treatment modalities.

These and other factors result in growing numbers of studies dedicated to combinational therapies (Fig. 3). Between 2014 and 2017 the number of new clinical trials assessing immunotherapy combinations increased eight-fold and the number of recruited patients over four-fold [12]. As a result, a trend towards reduced population size in trials can be seen, probably due to several factors, including large numbers of innovative combinational therapies assessed in early phases on limited populations, and improved selection of patients in more advanced trials that enable sufficient statistical power with lower numbers of patients per trial.

It is difficult to predict when trial results will be published because this is affected by numerous factors: recruitment time; assessed end-points; pace of maturing data; sources of financing; and others. According to a large analysis performed by American researchers the estimated median time from recruitment closure to publication is about 47 months – nearly four years [33]. Assuming that the recruitment for trials presented in Figure 3 require 12 months on average, we may estimate that half of the trials initiated in 2014 will be published before 2020. Moreover, based on this calculation, we may anticipate results from over 500 trials assessing immunotherapy combination in the next five years.

Summary

The immunological system is a key component of both pathogenesis and treatment of RCC. Understanding of complex mechanisms that take part in activation and in effector phase of adaptive cellular immune response allows the development of more efficient therapies and leads to their effective implementation in clinical practice.

Nivolumab acts mostly through modification of the effector phase of immune response. It proved activity in several cancer types, becoming the standard of care in many. As the number of patients potentially qualifying for treatment with immune checkpoint inhibitors grows, so should the knowledge regarding their strong and weak points, subpopulations with increased or decreased treatment efficiency, and about their interactions with other therapies.

European patients with renal cell carcinoma may be treated with nivolumab after failure of at least one line of prior systemic therapy*. Nivolumab offers promising activity in terms of response rate and median overall survival, along with a favourable toxicity profile. Unfortunately, nivolumab is limited by high rates of primary resistance and decreased activity in older patients and in the presence of cachexia.

A large number of new trials assessing immunotherapy in combinations with other therapies is a consequence of encouraging results achieved with immune checkpoint monotherapy. Analysis of trends in numbers of such trials gives hope that the best in immuno-oncology is yet to come.

Conflict of interests

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Characteristics of *in vitro* model systems for ovarian cancer studies

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ABSTRACT

Nowadays, targeted therapy plays a growing role in oncological treatment. In ovarian cancer, particularly promising results are achieved with poly (ADP-ribose) polymerase (PARP) inhibitors. Recent clinical trials have shown that PARP inhibitors can result in significantly longer progression-free survival. These results encourage the search for other targeted therapies and bring hope that ovarian cancer can soon become a manageable chronic disease. The main problem in ovarian cancer research is the heterogeneity of this disease. Recent studies have shown that different histological types of ovarian cancer can originate from distinct tissues. According to the recent knowledge, "ovarian cancer" is an artificial term for distinct invasive malignancies localised within the pelvis. Genetic and immunophenotype analyses have shown that high-grade serous ovarian cancer, the most frequent histological type and the one with the worst prognosis, originates mainly from fallopian tube epithelium, while endometrioid and clear-cell cancers originate from the endometrium. For these reasons, in basic and preclinical studies on ovarian cancer, one has to carefully choose a well-defined model system, corresponding to the histological type of interest. In this article, we discuss ovarian cancer cell lines most frequently used in *in vitro* studies. Our aim is to indicate the advantages and disadvantages of different models, encompassing primary and established cell cultures, two- and three-dimensional models, etc. In particular, we would like to alert researchers to the fact that the most popular cell lines SKOV3 and A2780 do not represent a suitable model for studies on high-grade serous ovarian cancer.

Key words: ovarian cancer, *in vitro* models, ovarian cancer cell lines, 3D cell culture, high-grade serous ovarian cancer, chemoresistance

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Introduction

Ovarian cancer is characterised by high mortality. The reasons for this include late diagnosis, asymptomatic early stages of the disease, and the lack of effective tools for early diagnosis and screening.

The standard treatment of advanced ovarian cancer includes surgery as well as paclitaxel- and platinum-based chemotherapy. Most patients respond very well to the treatment, but relapse of the disease and increasing chemoresistance are problems that frequently occur. Usually, there are several relapses in the course of ovarian cancer, interwoven with disease-free periods. Patients with relapse are mainly treated with chemotherapy until the resistance develops. Recently, agents directed at specific biological targets are being introduced into the treatment of recurrent ovarian cancer. Clinical studies indicate that the survival time of patients with ovarian cancer can be significantly prolonged by drugs such as poly ADP-ribose polymerase (PARP) inhibitors (PARPi) or, to a lesser extent, the anti-angiogenic agent bevacizumab. Therefore, ovarian cancer has a chance to become a chronic disease that can be successfully controlled for years [1]. Targeted drugs, however, are currently very expensive and are therefore recommended for a limited number of indications.

In many countries, they are not reimbursed and therefore are not yet widely used in clinical practice [2].

Positive results of clinical trials with new biological drugs encourage further exploration. A significant problem in ovarian cancer research is the heterogeneity of this disease [1]. To properly design an experiment and obtain reliable results, it is crucial to specify what histological type of ovarian cancer is to be studied and to select the relevant model.

Heterogeneity of ovarian cancer

Several histological types of ovarian cancer have been distinguished; the most common are serous, endometrioid, clear-cell, and mucinous cancers. Classical theory assumed that all these tumours originate from a single layer of mesothelial epithelium covering the ovary (ovarian surface epithelium, OSE). It was assumed that the initiation of the neoplastic process occurs in OSE under the influence of cyclical stimulation by hormones, cytokines, and growth factors, secreted in the process of ovulation and tissue healing, after the oocyte release. Differentiation of the tumour in the direction of particular histological type was to be a secondary process.

In 1999, Dubeau challenged the above scenario and proposed that most cases of ovarian cancer originate from epithelia lining structures originating from Müller's ducts, i.e. the cervix, uterus, and fallopian tubes [3]. Over time, experimental evidence was gathered to support the Dubeau theory. Substantial immunophenotypical, genetic, and molecular differences between individual histological types of ovarian cancer have been demonstrated. For example, the majority of serous cancers have common features with the Müllerian epithelium lining the fallopian tubes, e.g. expression of HOXA and PAX8 proteins. Expression of these proteins is not observed in OSE. It is currently assumed that the majority of high-grade serous ovarian cancer (HGSOC) cases originate from malignant epithelial cells of the fallopian tube, which are re-implanted on the ovary and/or peritoneum surface. This is explained by the rapid spread of HGSOC. In turn, low-grade serous ovarian cancers (LGSOC) are derived from ovarian inclusion cysts and are of diverse origin. Some of these cysts arise due to OSE invagination, and some as a result of implantation of the fallopian tube epithelium. Under the influence of the local microenvironment, the cancer process may initiate in the cyst. Endometriotic foci, e.g. the fragments of the endometrium that have migrated up the fallopian tube and nested on the surface of the ovary, are considered as precursors of endometrioid and clear-cell cancers. This is supported by, among others, the protective effect of tubal ligation, which reduces the risk of development of

these histological types, due to blocking of the migration path of their precursors. Mucinous tumours have many morphologic features in common with gastrointestinal cancers and glandular cervical cells. Their origin is still unexplained [4, 5].

Individual histological types of ovarian cancer also differ in molecular profile. HGSOC is characterised by a high percentage of *TP53* gene mutations (over 95% of cases) and a loss of *BRCA1* or *BRCA2* gene function. There are no mutations of other genes in HGSOC, while the high variability of DNA copy number in the entire genome is characteristic (copy number variation, CNV). LGSOC are characterised by the presence of *BRAF* or *KRAS* gene mutations. Endometrioid and clear-cell cancers show instability of microsatellite sequences and *PIK3CA* and *PTEN* gene mutations. In clear-cell carcinoma, *ARID1A* gene mutations additionally occur, and in the endometrioid cancer — *CTNNB1* gene mutations. Mutations of the *KRAS* gene are characteristic for the mucinous type [5, 6].

In 2004, Shih and Kurman proposed a new classification, dividing ovarian cancers into two types (Table 1). Type II includes HGSOC; sometimes poorly differentiated, clear-cell carcinomas are also included. Type II cancers are usually diagnosed in stage III or IV according to FIGO and have a very poor prognosis. They account for about 75% of all cases. Type I consist of the remaining histological types. Their diagnosis is made in earlier stages, and the prognosis is favourable [7].

In conclusion, the knowledge accumulated over the last dozen or so years has redefined the understanding of the disease traditionally known as ovarian cancer. Many data indicate that it is an artificial term that includes various pelvic neoplasms that have separate histogenesis, other mutational trajectories, and a diverse clinical picture. The knowledge about the heterogeneity of ovarian cancers should therefore be taken into account in both clinical practice and research design.

In vitro models used in ovarian cancer research

The main model in basic and preclinical studies on ovarian cancer are cell lines maintained as *in vitro* culture. Cell lines can be stabilised (capable of an infinite number of *in vitro* divisions) or primary, i.e. freshly taken from the body. The most common objects of research are cancer cells, normal precursor cells of a given cancer, and stem cells.

The limitation of the cellular culture model is the loss of tissue histology, lack of endocrine, paracrine, and nerve signalling, and lack of gradients of nutrients and other substances found in the living organism. However, the enormous advantages, including ease to propagate

Table 1. Classification of ovarian cancers based on clinical and molecular features [7]

Type I (25% of cases)	Type II (75% of cases)
Low-grade serous ovarian cancer (LGSOC) Clear-cell cancer Endometrioid cancer Mixed cancer Mucinous cancer	High-grade serous ovarian cancer (HGSOC)
Derived from precursor lesions (borderline tumours, endometriosis)	Derived from serous tubal intraepithelial cancer (STIC)
Somatic mutations in *KRAS*, *BRAF*, *PTEN*, *PIK3CA*, *CCTNB1*, *ARID1A*, *PPP2R1A* genes	95% of cases have *TP53* gene mutation 40–50% of cases have inactivated *BRCA* genes
Slow growth	Fast growth and high aggressiveness
Limited to the ovary	Rarely limited to the ovary
Diagnosed mostly at I and II clinical stage according to FIGO	Diagnosed mostly at III and IV clinical stage according to FIGO
Low sensitivity to chemotherapy	High sensitivity to chemotherapy
Rare relapses	Frequent relapses
Favourable prognosis (80% of 5-year survival)	Unfavourable prognosis (10% of 5-year survival)

cells, stability, and reproducibility of the model, determine its popularity and irreplaceable role.

Primary cell lines

Short-term cultures of freshly *ex-vivo* harvested cells are a valuable model, especially when correlation of the results of *in vitro* studies with clinical data is possible. However, they have many limitations, such as the need for every-time preparation of cells from biological material, slow *in vitro* growth, and limited viability. The reproducibility of this model is low due to the fact that the cells come from a different donor and from another cancer each time. In addition, the clonal selection of cells progresses during the culture, and their initial composition changes.

In cancer research, the primary cultures of epithelium considered to be the precursor tissue of a given cancer are often used as a control. In ovarian cancer, ovarian epithelial cells — OSE — have been used for this purpose for a long time. Because the theory that some HGSOC originate from the fallopian tubal epithelium has been recognised, it seems reasonable to use these epithelial cells as a control. However, studies that only use OSE are still being published. An even greater mistake is the use of whole ovarian fragments containing stromal elements and germinal cells. According to current knowledge, the original tissues for various ovarian cancers are: fallopian tubal epithelium, ovarian epithelium, endometrium, and endometriosis, and possibly the intestinal epithelium or epithelium covering the peritoneum. Examples of primary and immortalised control epithelia are presented in Table 2.

The normal cells can be maintained in *in vitro* culture for 6–8 weeks. Over time, the loss of specific markers, followed by apoptosis or the aging process (senescence), is observed. Senescent cells are huge, have numerous vacuoles, and stop dividing.

Primary cultures of ovarian cancer cells can be derived from solid tumours or peritoneal fluid. In the first case, the preparation starts with the mechanical dissociation of the tissue and the enzymatic digestion of extracellular matrix proteins. In case of peritoneal fluid, the initial elimination of erythrocytes is indicated, e.g. by density gradient centrifugation. From primary culture, fibroblasts can be eliminated through so-called differential trypsinisation — fibroblasts are detached from the surface of culture vessel after about two minutes of reaction with trypsin, and tumour cells show stronger adhesion. Primary cultures of ovarian cancer cells are relatively easy to derive compared to other cancers — their advantage is high viability, strong adhesion to the surface, and rapid cell division. In case of obtaining material from patients after chemotherapy, cell viability may be limited and their *in vitro* growth slowed down. It should also be remembered that these cells do not represent all cell clones present in the tumour before the start of therapy due to selection processes [18].

Primary cultures of tumour cells often die after about 2–3 months of *in vitro* maintenance. In some cases, stable cell lines can be established. In our practice, with the material from the peritoneal fluid from eight patients, we managed to derive one stable tumour line [19]. Recently, Ince et al. developed a special culture (growth) medium (Ovarian Carcinoma Modified Ince, OCMI) for the derivation of stable lines of ovarian cancer cells. This medium is based on commercially

Table 2. Examples of primary and immortalised control epithelium considered as ovarian cancer precursor tissue

Line name	Source	Type	Modification	Evaluated markers	References
FT33-shp53-R24C	FT	I	Retroviral transfer of hTERT, p53, CDK4R24C or shRNA	CK-7, PAX8	Creative Bioarray [8]
HFTEC	FT	P	–	CK-8/18, CK-14, CK-19	Life Line Cell Technology [9]
HOSEpiC	OSE	P	–	CK-14, CK-18, CK-19	ScienCell Research Laboratories [10]
Human Primary Ovarian Surface Epithelial Cells	OSE	P	–	CD326, E-cadherin	ABM [11]
HOSE1, HOSE2	OSE	I	Lentiviral transfer of hTERT, Cdk4, or cyclin D1	–	Sasaki et al., 2009 [12]
iFTSEC283	FT	I	–	–	Gjyshi et al., 2018 [13]
IOSE-29, IOSE-80	OSE	I	Simian virus 40	α-, β-, γ-catenin, CA125, E-cadherin, F-actina, pan-cytokeratin	Auersperg et al., 1999 [14]
IOSE-C9, IOSE-C10, IOSE-C21	OSE	I	Retroviral transfer of hTERT	CK-7, CK-8, CK-14, CK-16, CK-18 CK-19, CA125, E-cadherin	Li et al., 2007 [15]
NOSE4, NOSE11, NOSE19L3	OSE	P	–	AE1/AE3, CA125, CK-7, factor VIII EpCAM, E-cadherin, FSP	Lawrenson et al., 2009 [16]
OE-E6/E7	FT	I	Retroviral transfer of E6/E7 HPV16	CK-19	Lee et al., 2001 [17]

Source: FT — fallopian tube epithelium, OSE — ovarian surface epithelium; *type*: I — immortalised cell line, P — primary cell line

available WIT-T (Cellaria) medium, dedicated to human breast epithelial cell culture, contains the addition of serum and epidermal growth factor (EGF), insulin, hydrocortisone, cholera toxin, and for cells derived from endometrioid or mucinous cancer also 17-β-oestradiol (variant OCMIe). According to Ince et al., the OCMI medium allows stable lines of ovarian cancer to be obtained in 95% of cases [20].

Stable cell lines

Established cell lines are the most commonly used models in cancer research. Their application has contributed to significant progress in the understanding of cancer biology. Cell line can be regarded as stable when it has been passaged *in vitro* at least 60 times, has a stable genetic profile, proliferates well, is viable, and can be kept in culture without problems. In databases such as The Cancer Cell Line Encyclopedia (CCLE) or The Cancer Genome Atlas (TCGA), over 1000 different tumour cell lines, including several dozen ovarian cancer lines, have been catalogued so far.

Stable tumour cell lines fairly accurately reflect the spectrum of genetic changes in primary tumours. How-

ever, the immortalisation process and long-term *in vitro* culturing can affect their molecular profile. Cell lines made available today by professional repositories have a defined genetic profile; the most frequently used for this purpose is the analysis of the length of selected repetitive DNA sequences (short tandem repeats, STR). Research laboratories should verify the STR profile of their cell lines every few years and in case of non-compliance should acquire a new cell tranche from an authorised repository. More and more often, the editors of scientific journals require the source of cells to be stated and do not accept the use of cells with an unverified genetic profile.

Many commonly used cell lines were established several decades ago. In some cases, only modern molecular analyses have detected mistakes in their classification. Probably some lines were incorrectly classified initially or they were exchanged with others. Such situations were even detected in the NCI-60 panel, comprising 60 cell lines established in the National Cancer Institute at Bethesda, commonly used for preclinical studies of new drugs [21]. An example is the MDA-MB-435 line, considered for many years as a breast cancer line. Based on the evaluation of the gene

expression profile [22], karyotype analysis, comparative genomic hybridisation (CGH), and single nucleotide polymorphisms (SNP) analysis [23], this line was found identical to the M14 melanoma cell line. The discussion about the origin of both these lines is still ongoing [24]. Another breast cancer line, known as MDA-N, was found, based on molecular analysis, to be identical to the MDA-MB-435 line. In turn, the MCF-7/ADR-RES line, described as an Adriamycin (doxorubicin)-resistant variant of the MCF-7 breast cancer line, is probably a variant of the ovarian cancer line OVCAR8 [25]. It also turned out that many classic cell lines are contaminated with admixture of HeLa cervical cancer cells — the first-ever stabilised cancer cell line [26, 27]. The best option while designing the experiments is to opt out of such uncertain cell models.

Cellular models for ovarian cancer research

In the case of ovarian cancer research, it is particularly important to precisely specify the origin of cell lines, because individual histological types in fact represent distinct disease entities. Unfortunately, awareness of this phenomenon is not universal. Moreover, many of the commonly used ovarian cancer cell lines have an unclear histological origin — either indefinite from the beginning or challenged today during in-depth analyses.

In 2013, a study by Anglesio et al. was published, in which attention was drawn to the need to re-classify available ovarian cancer cell models in terms of their histological origin [28]. Another two publications, by Domcke et al. [29] and Beaufort et al. [30], aimed at organising knowledge about available cellular models and sensitising researchers to the problem of their histological origin, by analysing a panel of dozens of ovarian cancer cell lines. Despite the exhaustive molecular, morphological and genetic analyses, the origins of many lines have still not been precisely determined.

As described in part entitled "heterogeneity of ovarian cancer", the most common type of ovarian cancer is high-grade serous cancer (HGSOC), which also has the worst prognosis. Therefore, it should be expected that this type of histology is the main subject of basic and preclinical research. Analysis of publications indexed in the PubMed database indicates that SKOV3, A2780, OVCAR3, CAOV3, and IGROV1 lines are the most frequently quoted ones; however, among them, there is no good HGSOC model [29].

The SKOV3 line is usually considered as a model of serous cancer. However, in the original publication, presenting its derivation, it has only been briefly described as "an adenocarcinoma cell line derived from an ascitic fluid of ovarian cancer patient" [31]. In turn, the A2780 line was described as being derived from endometrioid adenocarcinoma [32], and further studies confirm this classification.

Domcke et al. used the data from public repositories — CCLE and TCGA — to compare gene expression profiles in cell lines and postoperative ovarian cancer samples. Additionally, taking into account the genetic profiles of cells (presence of specific mutations and variability of the copy number of DNA — copy number variation, CNV), the authors proposed a ranking of 47 cell lines in terms of their suitability as HGSOC models. SKOV3 and A2780 cell lines received the "unlikely HGSOC" label (do not correspond to HGSOC) [29]. The cells of these lines do not have the main features of HGSOC, such as high levels of CNV and the presence of mutations in *TP53* and *BRCA1* or *BRCA2* genes. Instead, they have mutations in non-HGSOC genes, such as *ARID1A* (characteristic for clear-cell and endometrioid cancer) and *PIK3CA* (associated with clear-cell cancer).

Anglesio et al. also explicitly question the suitability of the SKOV3 and A2780 lines as HGSOC models [28]. Beaufort et al. classify the SKOV3 and A2780 lines as derived from clear-cell or endometrioid cancer ("putative histology: endometrioid/clear-cell") [30]. Shaw et al. have shown that tumours that develop from SKOV3 cells after administration to nude mice have a clear cell morphology with accumulation of glycogen in the cytoplasm (microscopic image of "light" cells) [33]. A similar image was observed in three-dimensional culture (3D) of SKOV3 cells [34].

In conclusion, recent studies have confirmed that A2780 cells are derived from endometrioid cancer and have shown that SKOV3 cells are most likely to represent clear-cell cancer. The classification of SKOV3 cells is still not completely unambiguous, as some authors report the presence of *TP53* mutation, which is a typical feature of serous cancer.

OVCAR3 is the third most cited ovarian cancer line. It was obtained from ascitic fluid from a patient with recurrent ovarian cancer diagnosed as "poorly differentiated papillary adenocarcinoma" [32]. Both OVCAR3 and CAOV3 have the *TP53* gene mutation; however, according to Domcke et al., in terms of other features, they differ from the HGSOC characteristics [29]. Other researchers recognise that OVCAR3 cells are likely to represent HGSOC [30, 34].

The IGROV1 line, the last of the five most frequently cited, shows a hypermutator phenotype, and in hierarchical clustering, based on the gene expression profile, it is located away from ovarian lines, and close to cell lines originating from lung, liver, stomach, and small intestine tumours [29]. In the analysis of Beaufort et al. IGROV1 cells were assigned with the label "mixed histology" [30]. So, it is a line that is difficult to classify, and therefore it is better to give up on it when designing the research, in favour of other, more reliable models.

OAW42 and ES2 cell lines are less frequently used in ovarian cancer research, and their histological origin is

also unclear. The ES2 line is sold by the ATCC (American Type Culture Collection) as a model of clear-cell cancer; however, the histology of the primary tumour is not described in the source article [35]. Based on molecular features, Beaufort et al. concluded that ES2 cells correspond to clear-cell carcinoma [30], whereas Angelsio et al. question this histological type based on *in vivo* studies: in ES2 cell tumours they did not observe cells with a light, glycogen-rich cytoplasm [28]. In turn, Domcke et al. classify ES2 cells as "possibly HGSOC" [29].

The OAW42 line is described in the source publication as derived from serous ovarian cancer. Modern studies mostly confirm the serous type [30, 36] but not the high-grade type. In a publication by Domcke et al. this line received the label "unlikely HGSOC" [29]. Lee et al. assessed that the architecture of structures created by OAW42 cells in 3D culture corresponds to well-differentiated (G1) serous cancer [34]. However, the presence of mutations in *ARID1A* and *PIK3CA* genes (characteristic of endometrioid and clear-cell cancers) raises some doubts [29, 30]. So, this is another uncertain model of ovarian cancer, which should be replaced with recently introduced cell lines of certain origin.

As shown above, there is no reliable HGSOC model among the most commonly used ovarian cancer lines: two lines are derived from serous cancer, but not necessarily of high-grade type (OVCAR3 and CAOV3), the SKOV3 line is probably derived from clear-cell cancer and the A2780 line originates from endometrioid cancer; IGROV1 may be derived from another organ, two models have an uncertain histological origin (ES2 and OAW42).

It would be reasonable to give up the use of lines of unclear origin, but paradoxically they are still widely used. There may be several reasons for this phenomenon. Some researchers are probably not aware of the problem. Sometimes technical aspects dictate the popularity of a given line — high cell viability, low culture requirements, fast divisions, etc. A certain argument for using these lines is also the fact that they are well characterised and have extensive literature documentation to which research results could be related.

Instability of cellular models

Another problem may be the instability of cell lines in *in vitro* culture. Many lines have been in use since the 1970s and 1980s. In various laboratories around the world, and even in different repositories — under one name — there are different clones of the same cell line.

Beaufort et al. compared two variants of SKOV3 and A2780 cells — from the European Collection of Authenticated Cell Lines (ECACC) repository and from the academic laboratory where they have been propagated for years. Most analyses yielded the same results

for both variants, but differences were also observed, e.g. regarding sensitivity to docetaxel and paclitaxel in the case of the A2780 line, and sensitivity to paclitaxel, carboplatin, doxorubicin, and gemcitabine in the case of SKOV3. Two variants of SKOV3 also differed regarding the presence of mutations in the *HRAS* and *APC* genes, and the level of EpCAM protein expression. In contrast, the A2780 variants differed in the *BRCA2* gene mutation.

Another example of such differences is *TP53* gene mutation in the SKOV3 line. In the publication by Beaufort et al. two methods of mutation detection were used: deep sequencing of selected amplicons and exon sequencing using the Supported Oligo Ligation Detection (SOLiD) method. In the SKOV3 line the frameshift mutation was detected only by the deep sequencing method (c.del267C) [30]. The presence of this mutation in SKOV3 cells has been previously described by the Ikediobi team [37]. In our studies we detected this mutation in SKOV3 cells from the American collection (ATCC) using Sanger's sequencing [19]. In a publication by Elias et al. [38] the presence of an (unspecified) mutation/deletion of the frameshift in a cisplatin-resistant variant of SKOV3-cis line was mentioned. In turn, Anglesio et al. did not observe *TP53* mutation in SKOV3 cells [28], which was referred to in the publication by Ince et al. [20]. Domcke et al. referred to data from the CCLE encyclopedia, which also does not record *TP53* mutation in the SKOV3 line [29].

The described discrepancies may be the result of many years of culturing; the SKOV3 line has been in use since 1973, and currently there are many different clones around the world. Another reason may be the use of different mutation detection methods by different authors. In addition, many authors refer to the results of other authors' research and do not verify them experimentally.

Another example of differences in established cell models is WT1 marker expression. Ince et al. did not observe the expression of WT1 in the A2780 line [20], while in our experiment we detected single WT1-positive cells. The differences also concern the expression of EpCAM in the OVCAR3 line — Domcke et al. obtained a negative result [29], while we observed a moderate reaction in all cells [19]. The results of our detection of CD44 marker expression were also different in comparison with the results of Beaufort et al. [30] for three cell lines: SKOV3, OVCAR3, and OAW42 [19].

It is worth noting, however, that three types of cell morphology (epithelial, spindle, and round type) described by Beaufort et al. [30] are probably a fairly stable feature and characteristic of various cell lines. In our studies we had identical observations: SKOV3, OAW42, and OVCAR3 cells showed epithelial morphology, ES2 had the shape described as spindle, and

A2780 — round. Beaufort et al. showed a significant correlation between cell morphology and the origin of the cell line — 14 out of 19 lines with epithelial morphology were from ascitic fluid. In addition, cells with epithelial morphology were more often derived from serous cancer (83%) compared to round (33%) and spindle cells (56%). The morphological type also correlated with the treatment with platinum derivatives — 10 out of 14 patients from whom epithelial cells were derived had previously received chemotherapy based on platinum compounds [30]. The OVPA8 line we derived shows epithelial morphology and has the characteristics attributed to this morphological type — it originated from ascitic fluid, from a patient with serous ovarian cancer, who had been previously treated with platinum derivatives [19].

Cellular models of high-grade serous ovarian cancer (HGSOC)

As can be seen from the above-mentioned data, a large portion of ovarian cancer research was performed on cell lines that do not correspond to HGSOC. This is due to two reasons: firstly, until recently, there was no knowledge about the fundamental differences between the histological types of ovarian cancer, and secondly, there is a lack of well-defined HGSOC cell models. The practical aspects are probably not without significance: cell lines that have low culture requirements, a short division cycle, good growth of tumours after inoculation *in vivo*, etc. are more willingly chosen. This may partly explain the huge popularity of the SKOV3 line.

According to Domcke et al., the best HGSOC models are the relatively unknown KURAMOCHI and OVSAHO cells [29]. These two lines and JHOS4 (also recommended by Domcke) were extensively tested by Elias et al. [38], who confirmed the features of HGSOC. It transpired, however, that the cells of these lines are characterised by certain limitations, such as poor growth in immunodeficient mice (SCID), especially in the case of JHOS4 lines [38].

As mentioned above, Ince et al. have developed an OCMI culture medium that enables the efficient establishment of ovarian cancer cell lines. Five new cell lines were derived using OCMI: OCI-P5x, OCI-U1a, OCI-P8p, OCI-P2a, and FCI-P2p, from patients with confirmed HGSOC [20]. These lines are provided by the Sylvester Comprehensive Centre Life Tumour Culture Core at the UM Miller School of Medicine in Miami. The first publications in which these lines were used are already available [39, 40]; however, there are still no publications directly devoted to HGSOC.

The OVPA8 line, recently derived by our team, is another HGSOC model that will soon be made available through the ECACC (accession no 19061601 and

19061602). This line has important practical advantages, such as relatively fast growth (doubling time — 44 hours) and resistance to unfavourable culture conditions, such as high confluence or old culture medium [19].

Models for studies on molecular background of chemotherapy resistance

Chemotherapy plays an important role in the treatment of advanced ovarian cancer. In most cases, the response to treatment is very good; primary tumour chemoresistance is a rare phenomenon. However, the resistance in relapse is a problem.

Studies on the molecular basis of the acquired chemoresistance are based on cell lines that have different sensitivity to cytotoxic drugs. Many cell lines derived from peritoneal fluid from patients with ascites are resistant to platinum compounds and other drugs. At that time, clonal selection has already took place and only cells tolerating high concentration of drugs survive; for example, cisplatin-resistant KURAMOCHI or OVPA8 cells; in the latter the IC50 for cisplatin is $16.23\ \mu$M [19].

Lines derived from primary tumours tend to be susceptible to cytotoxic compounds and can be used to obtain a cell variant that is resistant to the study drug. An example is the A2780 cell line derived from the tumour before the start of chemotherapy. The cells of this line are sensitive to cisplatin and paclitaxel. However, numerous variants have been developed that have resistance to these and other drugs (e.g. topotecan, doxorubicin, or auranofin). The IGROV1 line also comes from an untreated patient and is susceptible to cisplatin, and it has numerous drug-resistant variants created in laboratories. The SKOV3 line comes from a patient who has been treated with thiotepa, and these cells are sensitive to platinum derivatives. Numerous cell variants resistant to cisplatin, carboplatin, etoposide, paclitaxel, vinblastine, or vincristine have been derived for research purposes (Table 3).

Classical chemoresistance mechanisms include drug clearance by ABC transporters, glutathione detoxification, intensification of pro-survival signalling, efficient repair of DNA damage, and suppression of apoptosis in cancer cells. More recent studies indicate that ovarian cancer has many more complex mechanisms responsible for the development of chemoresistance. Other important factors include the presence of cancer-associated fibroblasts (CAF) [68, 69], changes in the protein composition of the extracellular matrix [70, 71], epithelial-mesenchymal transition (EMT) [72], the presence of stem cells [73], as well as epigenetic mechanisms [74–76]. Susceptibility to chemotherapy and prognosis may also be associated with a specific gene expression profile in the tumour, although the results of genomic testing are not consistent [77–79].

Table 3. Ovarian cancer cell lines used for research on drug resistance mechanisms

Primary line	Origin	Sampling time	Chemotherapy	Cellular variants	Sensitivity	Resistance
A2780	T	P	N		CP, P	
				A2780^{AF-R} (Landini, 2017) [41]		Aura
				A2780^{CP} (Behrens, 1987) [42]; A2780^{CIS} (Masuda, 1988) [43]; A2780^{CR1}, A2780^{CR2} (Januchowski, 2014) [44]; A2780^{C30}, A2780^{C200}, A2780^{CP70} (Sak, 2015) [45]; A2780^{C12} (Sun, 2018) [46]		CP
				A2780^{ADR} (ECACC) [47]; A2780^{DR1}, A2780^{DR2} (Januchowski, 2014) [44]		D
				A2780^{PTX} (Han, 2013) [48]; A2780^{PR1}, A2780^{PR2} (Januchowski, 2014) [44], A2780^{TR}, A2780^{PTX10} (Sak, 2015) [45]		P
				A2780^{W1TR1}, A2780^{W1TR2} (Januchowski, 2014) [44]		Topo
COLO-704	A	R	l.d.			
				COLO-704^{rCDDP1000} (RCCLC) [49]		CP
ES2	T	P	N			
				ES2^{PR20} (Jazaeri, 2013) [50], ES2^{C12} (Sun, 2018) [46]		CP
				ES2^{TR160} (Ho, 2018) [51]		P
IGROV1	T	P	N		CP	
				IGROV1$^{Pt0.5}$, IGROV1^{Pt1} (Perego, 1996) [52], IGROV1^{CP} (Stewart, 2006) [53], IGROV1^{CDDP} (Stordal, 2012) [54]		CP
				IGROV1^{OHP} (Benedetti, 2008) [55]		OP
				IGROV1^{MX3} (Maliepaard, 1999) [56]		MK
				IGROV1^{T8} (Maliepaard, 1999) [56]		Topo
KURAMOCHI	A	R	CP			CP
OAW28 (41M)	A	R	l.d.			
				41M^{cisR} (Judson, 2012) [57]		CP
OAW42	A	R	CP			
				OAW42^{A} (Redmond, 1993) [58]		CP, D, EP, TP, WB, WK
OV90	A	l.d.	l.d.			
				OV90^{C-A}, OV90^{C-D} (Sherman-Baust, 2011) [59]		CP
				OV90^{D-6}, OV90^{D-7} (Sherman-Baust, 2011) [59]		D
				OV90^{P-3}, OV90^{P-7} (Sherman-Baust, 2011) [59]		P
OVCAR3	A	R	CF, CP, D			CP, P
				OVCAR3^{DDP} (Liu, 2017) [60]		CP
OVCAR4	A	R	CP			CP
PEO1	A	R	CP			
				PEO1^{CDDP} (Macleod, 2005) [61]		CP
SKOV3	A	R	T			
				SKOV3^{ip1} (Yu, 1993) [62]	P	
				SKOV3^{CDDP-P} (Yan, 2007) [63], SKOV3^{PR25} (Jazaeri, 2013) [50]		CP
				SKOV3^{VP} (Kubota, 1994) [64]		EP
				SKOV3^{CBP} (Li, 2004) [65]		MK
				SKOV3$^{Taxol-P}$ (Yan, 2007) [63], SKOV3^{TR} (Lee, 2015) [66]		P
				SK VCR$^{0.015}$, SK VCR$^{0.1}$, SK VCR$^{0.25}$, SK VCR$^{2.0}$ (Bradley, 1989) [67]		WK
OVPA8	A	R	CP, KP, P		P	CP

Origin: A — ascites, T — tumour; *sampling time*: P — primary disease, R — relapsed disease, l.d. — lack of data; *cytotoxic agents:* Aura — auranofin, CF — cyclophosphamide, CP — cisplatin, D — doxorubicin, EP — etoposide, KP — carboplatin, MK — mitoxantrone, OP — oxaliplatin, P — paclitaxel, T — thiotepa, Topo — topotecan, TP — tenoposide, WB — vinblastine, WK — vincristine, N — untreated

Cancer stem cells

The theory of cancer stem-like cells (CSLC) assumes the existence of a specific population of cells with the ability of self-renewal and differentiation towards all tumour cell populations. These cells have an increased clonogenic potential and the ability to form spheroids *in vitro* and potential for tumour development (tumorigenicity) *in vivo*. It is postulated that in ovarian cancer, tumour stem cells are responsible for primary tumour development, as well as intraperitoneal dissemination of the disease, its recurrence, and chemoresistance (reviewed in: [80, 81]).

Attempts to isolate ovarian cancer stem cells from a tumour or cell culture are based on the detection of specific markers, assessment of functional features, and clonogenic potential as well as tumorigenicity. Among the proposed CSLC markers are proteins typical for embryonic stem cells, such as NANOG, OCT4, NESTIN, ABCG2, or BMI1 and surface markers CD133, CD117, CD44, CD24, and EpCAM. Some authors indicate aldehyde dehydrogenase (ALDH1) activity as typical for CSLC [82]. Another functional feature attributed to CSLC is the activity of ABC transporters, which allows the removal of cytotoxic compounds and other substances. This feature is used for selection by flow cytometry (Fluorescence-Activated Cell Sorting, FACS). The cells that efficiently remove the Hoechst 33342 dye create in the FACS cytogram a so-called side population (SP). Some studies have confirmed that SP cells have greater tumorigenicity.

Unfortunately, no studies so far have allowed the identification of a reliable set of markers to isolate ovarian cancer stem cells. Many studies indicate that the phenotype of these cells can be very different. The most frequently proposed markers are CD133, CD44, CD24, and CD117 in combination with ALDH1 activity. It is accepted that CSLCs comprise a small percentage of primary tumour cells. Paradoxically, the expression of CD44 or CD24 is observed in a very large percentage of tumour cells. This may be the effect of phenotype plasticity of tumour cells that undergo epithelial-mesenchymal transition (EMT) and further changes towards the undifferentiated/stem-like phenotype [83]. Probably not all cells expressing these markers have functional features of CSLC. However, it has been shown that high expression of CD133, CD117, CD44, or CD24 may correlate with adverse clinical and pathological features (e.g. poor histological differentiation, higher clinical stage, chemoresistance, or shorter survival time).

The theory of tumour stem cells has important implications in therapy: it is postulated that CSLC can survive chemotherapy and give rise to relapse. Targeting cancerous stem cells can therefore be an attractive therapeutic option [18]. For many CSLC markers, inhibitors have already been developed, which are currently tested in preclinical studies (extensive review by Klemb et al. 2018 [81]).

Mesenchymal stem cells

In many cancers, the presence of mesenchymal stem cells (MSCs) is detected. In ovarian cancer, these cells probably come from visceral fat [84, 85].

The studies of Klopp's team have shown that stem cells of visceral adipose tissue isolated from the omentum stimulate ovarian cancer cells in *in vitro* experiments — they stimulate their proliferation, migration rate, and chemo- and radio resistance [84]. Buckahnovic's team observed that cancer-associated mesenchymal stem cells (CA-MSC) isolated from the tumour stimulate proliferation, expression of stemness markers, and increased chemoresistance of ovarian cancer cells in vitro [86]. In the opinion of these researchers, CA-MSC present in ovarian cancer come from the omentum. Normal MSCs migrate to the tumour and are converted to CA-MSC under the influence of the local microenvironment, including factors secreted by tumour cells and hypoxia. CA-MSCs have altered the expression pattern of over 1000 genes compared to normal MSCs from adipose tissue. Coffman showed that the origin of MSC is crucial for interactions with cancer cells. MSCs from the bone marrow stimulate the proliferation of breast cancer cells, but not ovarian cancer cells. However, ovarian cancer cells respond to MSC stimulation from visceral fat (omentum). Conversely, the breast tumour microenvironment leads to the transformation of MSCs from the bone marrow into CA-MSC but does not exert such an effect on MSC from adipose tissue. These processes may be associated with tissue-specific metastasising of breast cancer to the bone, and ovarian cancer to the peritoneum [85]. Perhaps this also explains the discrepancies in the literature; on one occasion the stimulating effect of MSC on cancer cells was observed, and at other times an inhibiting effect (reviewed by Klopp et al. [87]). Different effects could be related to the type of MSC used.

Three-dimensional cellular models (3D)

The previously discussed *in vitro* models relate to cell culture in a monolayer (two-dimensional culture, 2D), which is technically more convenient and easier, but this differs greatly from the physiological conditions in the tumour. The main limitation of this model is the lack of a typical microenvironment. This is particularly important when testing new drugs; that is why the results obtained in the 2D model are often not confirmed in further *in vivo* studies [88, 89].

A partial solution to this problem comes in the form of three-dimensional (3D) *in vitro* models. They fill the

gap between two-dimensional cell culture and animal models. On the one hand, the 3D models allow partial simulation of environmental features *in vivo*, and on the other hand, they offer the majority of the advantages of traditional cell culture.

3D models are constructed by creating the conditions for cell growth that promote the formation of so-called spheroids (3D structures) or by implanting cells into three-dimensional scaffolds, composed of extracellular matrix proteins or synthetic biomaterials [89, 90].

Lee et al. used poly(2-hydroxyethylmethacrylate) (polyHEMA) coated plates for induction of spheroid formation and obtained 3D cultures for 31 different ovarian cancer lines. Comparison of biological and molecular features of cells in 2D and 3D cultures as well as in the form of mouse xenografts showed that 3D models to a greater extent reflect the tumour characteristics. Cells in 3D culture were characterised by slower proliferation and greater chemoresistance. There were also differences in the expression of selected biomarkers: in the 3D model, higher expression of E-cadherin and β-catenin and lower expression of vimentin were observed, as compare to 2D culture. Only 30% of the tested lines expressed WT1, CA125, and PAX8, with CA125 and PAX8 having increased expression and WT1 reduced in 3D cultures [34].

Heredia-Soto et al. evaluated ovarian cancer spheroids produced by cells of 16 different lines. Spheroids with a diameter of 400 μm allowed the diffusion level of nutrients and oxygen characteristic for a tumour depth of 100 μm to be obtained. Tests on this model could be carried out for up to 14 days without producing excessive areas of necrosis. Cytotoxicity tests showed a higher tolerance of cells to platinum-based treatment in 3D than in 2D models [91].

The unique feature of ovarian cancer is metastasis in the form of so-called implants to the peritoneum and the omentum. Omentum is a fat-rich visceral fold that covers the abdominal organs. The surface of the omentum and the peritoneum is covered by a single layer of mesothelial cells, placed on the basal membrane, made of collagen type I and IV, fibronectin, vitronectin, and laminin. The tissue stroma contains fibroblasts, immune cells, endothelial cells, and extracellular matrix proteins. Ovarian cancer implants can be initiated either by single tumour cells or by spherical cellular aggregates 30–200 μm in size, which are exfoliated from primary tumour. These structures circulate in the peritoneal fluid and can adhere to the peritoneal epithelium. The exact composition and functional features of ovarian cancer spheroids in the intraperitoneal metastasising process have not yet been recognised [91].

Barbolina et al. developed a three-dimensional model of intraperitoneal metastases in which ovarian cancer cells were cultured in 3D gels made of type I collagen. Integrin signalling initiated by collagen binding has been shown to express early growth response 1 (EGR1) transcription factor, which induces the expression of metallothionein-matrix metallopeptidase (MT1-MMP), promoting proteolytic collagen degradation and invasion of the peritoneum [92, 93]. A similar model was used by Loessner et al. to study the interaction between ovarian cancer cells and the extracellular matrix and mechanisms of drug resistance. OV-MZ-6 and SKOV3 cells were introduced into synthetic polyethylene glycol hydrogels with the possibility of modulating biophysical features (such as stiffness) and biochemical parameters (integrin binding sites, protease activity level). These gels are stable for up to 28 days, allowing for longer experiments. It was shown that the proliferation of cells in the 3D environment was dependent on the proteolytic remodelling of the extracellular matrix. Ovarian cancer cells in 3D culture showed a higher survival rate after paclitaxel treatment than did cells in 2D culture [94].

Muranen et al. used a 3D model to analyse the mechanisms of acquiring resistance of ovarian and breast cancer cells to treatment with a small molecule inhibitor of the PI3K/mTOR pathway — BEZ235. Spheroids formed on the rBM matrix (reconstituted basement membrane) and without rBM participation were tested. Cells anchored in the rBM matrix were more resistant to BEZ235; cells lacking contact with rBM were more likely to undergo apoptosis. In cells attached to rBM, a higher expression of many pro-survival signalling proteins was observed, which may explain their better adaptive response and higher resistance to the inhibitor [95].

Models of 3D cultures are also used to obtain organotypic cultures, consisting of mixed cell populations (e.g. adipocytes, macrophages, endothelial cells), which better reflects tumour physiology. These models may be created using established or primary cell lines [96]. In organotypic models, it is advisable to use labelled, e.g. fluorescent, cells. This enables visualisation as well as separation and testing of pure cell populations, e.g. for changes in mRNA, miRNA, and/or specific proteins expression level [90, 96].

Very interesting observations have been made in a mixed culture of ovarian cancer cells with primary human adipocytes containing fluorescently labelled lipids. In this system, lipid transfer from adipocytes to tumour cells was observed, which led to accelerated proliferation of cancer cells, both *in vitro* and after administration to mice. The presence of adipocytes also stimulated the migration rate and invasion of cancer cells. These results suggest that the fatty acids provided by the adipocytes may be a source of energy for ovarian cancer cells [97]. In our opinion, this phenomenon may also be related to observations indicating a poorer ovarian cancer prognosis in women with obesity [98].

An attempt was made to create a 3D model that recapitulate the carcinogenesis of fallopian tube epithelial cells. Studies by Lawrenson et al. were based on a spheroid model of primary human fallopian tube secretory epithelial cells (FTSEC), isolated from the fallopian tubes immediately after surgery. Formation of spheroids was induced by FTSEC culture on polyHEMA-coated surface. The spheroids consisted of a cylindrical layer of epithelial cells surrounding the hyaline core, resembling the extracellular matrix of the fallopian tube. The spheroids persisted in culture for 30–60 days. Gene expression profile analysis revealed changes in the expression of over 1000 genes in cells grown in the 3D model, compared to classical cultures. These were mainly genes involved in the DNA replication process and cell cycle control [99]. Using a similar technique, this team received a 3D model of immortalised, transformed primary epithelial cells covering the ovary. The described models are suitable for testing the mechanisms of carcinogenesis and origin of HGSOC, as well as for screening for potential new drugs [96].

3D culture systems, although technically more complex, are gaining more and more importance as they allow to obtain conditions closer to the real ones. The results of experiments conducted simultaneously in 2D and 3D cultures confirm a much better resemblance of ovarian cancer biology in three-dimensional cultures. The limitation of the 3D model is the lack of functional vascularisation and the lack of cells mediating the adaptive immune response [88]. Other limitations include low availability of primary cells and the short lifespan of 3D cultures [96].

Conclusions

Ovarian cancer cell lines are a convenient model for *in vitro* studies. Cell culture is relatively cheap and simple, and the molecular features of the tumour cells in the culture fairly accurately reflect the initial tumour profile. Using stable cell lines, the reproducibility of the model between different laboratories and individual experiments is relatively high.

The limitations of the cell model are the lack of typical histology of tissues, the lack of functional vascularisation, and cells mediating the adaptive immune response. There are also no endocrine, paracrine, and nerve signalling and gradients of various substances found in the living organism.

A partial solution to the above problems is three-dimensional (3D) culture in the presence of extracellular matrix proteins or bio-similar polymers, and mixed cell cultures of tumour cells with other cellular components of the tumour (organotypic culture).

Unfortunately, in the case of many popular ovarian cancer cell lines, the problem is their incomplete characteristics, found in the original articles describing their establishment. Despite the great efforts made in recent years, it has not been possible to fully verify the histological origin of several commonly used cell lines.

Moreover, among the most commonly used ovarian cancer cell lines there is not a certain HGSOC model, instead there is one line that may even come from another organ (IGROV1), two models with uncertain histology (ES2 and OAW42), and two models of serous cancer, but not necessarily high-grade (OVCAR3 and CAOV3). The most frequently used SKOV3 line is probably derived from clear-cell carcinoma, the second most popular A2780 corresponds to endometrioid cancer.

As the most reliable models of high-grade serous ovarian cancer, the two almost unknown lines KURAMOCHI and OVSAHO are currently recommended, as well as the OVCAR4 line, used more often in research [29]. Ince et al. describe five cell lines derived by them (OCI-P5x, OCI-U1a, OCI-P8p, OCI-P2a, and FCI-P2p) as having the HGSOC phenotype [20]; however, these lines have been used very rarely in science, so far. Also, the OVPA8 line derived by our team comes from a patient with histologically confirmed HGSOC, and all our molecular and genetic tests confirm this phenotype. This line will soon be made available by the ECACC (accession no 19061601 and 19061602).

When choosing a model for ovarian cancer research it is necessary to pay attention to its specificity — both advantages and limitations. There is no perfect or universal model — the best possible model should be adjusted for assumed research goals.

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Differential diagnosis of autoimmune pituitary failure and pituitary macroadenoma during nivolumab therapy in an NSCLC patient — a case report

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ABSTRACT

We report a case of patient with non-small-cell lung cancer with expression of PD-L1 molecule on 1% of cancer cells, who was treated with chemotherapy, radiotherapy, and, during disease progression, with nivolumab immunotherapy. In the course of immunotherapy our patient developed symptoms of multi-axis hypopituitarism. Pituitary macroadenoma was diagnosed. In differential diagnosis, autoimmune inflammation of the pituitary gland in the course of nivolumab therapy was considered. After pituitary failure symptoms resolved, the immunotherapy was continued, with two-year remission of the disease.

Key words: non-small-cell lung cancer, immunotherapy, nivolumab, hypopituitarism

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Introduction

Cancer cells have developed mechanisms that allow them to defend against the host immune system. One of the signalling pathways used by cancer cells in this purpose is the interaction between PD-1 (programmed death 1) and PD-L1 (programmed death ligand 1) molecules. Through stimulation of the PD-1 receptor on the surface of lymphocytes their anti-cancer activity toward tumour cells expressing PD-L1 molecule is inhibited. A similar mechanism is the binding of B7-1 or B7-2 molecules on the surface of antigen-presenting cells (APCs) with the CTLA4 (cytotoxic T-lymphocyte-associated antigen 4) molecule on lymphocyte, which also leads to lymphocyte anergy. Due to the discovery of these associations, immune checkpoint inhibitors in the form of anti-PD1 monoclonal antibodies (nivolumab, pembrolizumab), anti-PD-L1 (atezolizumab, durvalumab, avelumab), and anti-CTLA4 (ipilimumab, tremelimu-

mab) were developed. By blocking these control points, it is possible to activate the destruction of cytotoxic T lymphocytes and cancer cells [1–3].

Development of immunotherapy allowed the extension of the survival time in patients with cancer, including non-small-cell lung cancer (NSCLC) [4]. Immunotherapy is used both in monotherapy and in combination therapy, not only in lung cancer, but also in the treatment of melanoma, kidney cancer, bladder cancer, and others [5]. The mechanism of action of immunological drugs, in addition to the intended therapeutic effects, also causes a number of adverse reactions, including autoimmune and inflammatory reactions defined as immune-related adverse events (irAEs). Skin complications, and gastrointestinal as well as liver and endocrine symptoms are most often observed [6–8]. Endocrinopathies are a group of complications that are usually irreversible [9]. They are mainly connected with the pituitary, thyroid, and adrenal glands [10].

The exact mechanism that causes endocrinopathy is still not fully understood.

Hypopituitarism is the second most frequent endocrine disorder in patients receiving immunotherapy [11, 12]. Among patients with the above-mentioned pathology, a number of cases with enlargement of the pituitary gland revealed in imaging studies have been described. This applies mostly to patients treated with ipilimumab [13]. *Hypopituitarism* was accompanied both by features of enlarged gland and its normal picture with no changes within it. However, tumour metastases to the pituitary gland are extremely rare. Usually they refer to patients with breast and lung cancer [14]. Their symptomatology results mainly from the pressure on surrounding brain structures.

Case report

An 80-year-old woman, a long-time tobacco smoker, was subjected in 2004 to right hand side mastectomy due to breast cancer with subsequent adjuvant chemotherapy. In 2016 a follow-up evaluation was performed for possible recurrence of breast cancer, which showed elevated levels of cancer markers. In chest computed tomography (CT) a tumour in segment 10 of the left lung and lymph node package in left pulmonary hilus (Fig. 1) were visualised.

During bronchoscopy endobronchial biopsy was performed, and in the sections from the left lung tumour non-small-cell cancer not otherwise specified (NOS) was diagnosed. In molecular studies neither mutations in the *EGFR* gene (the most common mutations in exons 18-21 were examined with use of real-time PCR) nor abnormal ALK protein (immunohistochemistry with D5F3 antibody clone) were detected, and the expression of PD-L1 was positive only on 1% of cells cancer (immunohistochemistry with SP263 antibody clone). In March 2016 the patient was qualified for chemotherapy with cisplatin and gemcitabine. Due to grade 4 haematological toxicity, which occurred after the first chemotherapy cycle, treatment was permanently discontinued. In May 2016 the patient underwent chest radiotherapy with a total dose of 20 Gy. In August 2016 positron emission tomography (PET) combined with CT (PET-CT) was performed, which showed the disease progression in the form of osteosclerotic metastases in thoracic and lumbar vertebrae. The patient used radiotherapy of the spine with a dose of 20 Gy and subsequently, in December 2016, was qualified for treatment with nivolumab at a dose of 3 mg/kg of body weight every two weeks within a dedicated extended access program (EAP).

After two months of treatment, stabilisation of lesions in the lungs was observed, followed by partial remission (PR). Immunotherapy was well tolerated for 11 months. In November 2017, the 22nd nivolumab treatment cycle began. Then the patient reported

Figure 1. Computer tomography image from January 2016

Figure 2. Magnetic resonance image of the head suggesting pituitary tumour of macroadenoma character with dimensions 26 × 16 × 14 mm (October 2017)

difficulties in reading, weakness, worse well-being, and weight loss. Hyperkalaemia and hyponatraemia have been reported in laboratory studies. In a previous magnetic resonance examination of the head (October 2017) in the area of the sella turcica and suprasellar cisterns, a tumour filling the entire sella turcica and suprasellar cistern with dimensions of 26 × 16 × 14 mm was revealed. This lesion showed heterogeneous contrast enhancement. The image suggested the presence of pituitary tumour with the features of macroadenoma (Fig. 2).
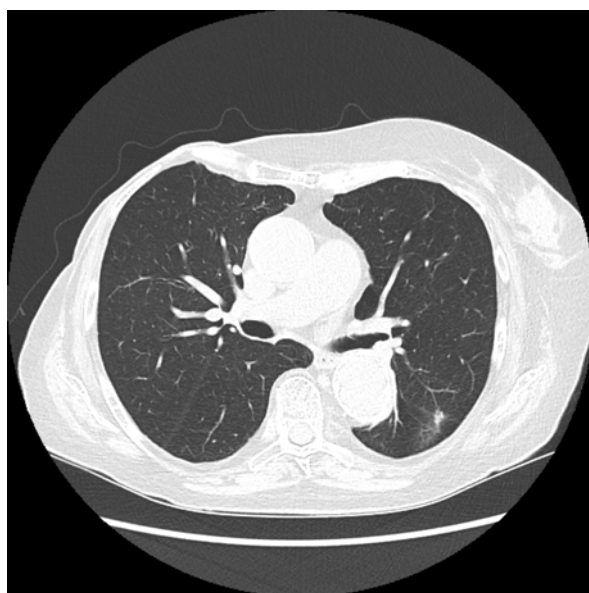
Figure 3. Partial remission of the disease in March 2018

Figure 4. Control magnetic resonance examination of the head showing regression of pituitary tumour to dimensions 16 × 10 × 15 mm (April 2018)

Laboratory tests showed decreased gonadotropins (LH < 0.10 mIU/mL, FSH 0.11 mIU/mL) and growth hormone (0.579 ng/mL) levels, elevated prolactin concentration (48.50 ng/mL), and reduced thyrotropin (0.205 mIU/L), free triiodothyronine (1.50 pg/mL), and free thyroxine (0.96 ng/dL) levels. The concentration of adrenocorticotropic hormone (ACTH) was 1.15 pg/mL, while cortisol was 9.7 µg/dL. Based on available results, hypopituitarism in the hypothalamic–pituitary–adrenal axis with accompanying secondary hypothyroidism and secondary adrenal insufficiency were diagnosed. Due to the patient's condition, the 23rd administration of nivolumab was postponed. After endocrinology consultation, levothyroxine treatment was used in a substitution dose — initially 12.5 µg for three days, followed by 25 µg and hydrocortisone in a daily dose of 40 mg. After finding an inadequate substitution of L-thyroxine, the dose was increased to 50 µg and autoimmune thyroid disease was excluded. Then, the hydrocortisone doses were reduced to 20 mg per day. After stabilisation of the patient's general condition and improvement of the results of laboratory tests, it was decided in January 2018 to continue the immunotherapy and the 23rd dose of nivolumab was administered. The interval in the use of nivolumab was 1.5 months. Resolution of endocrine symptoms during a long break in immunotherapy suggested an autoimmune background of pituitary gland inflammation. Chest CT performed in March 2018 showed a further regression of lesions in the lungs (Fig. 3).

Follow-up brain NMR performed in April 2018 showed the regression of lesion in the sella turcica area, mainly at the level of the pituitary stalk (infundibulum), up to 16 × 10 × 15 mm. The lesion in
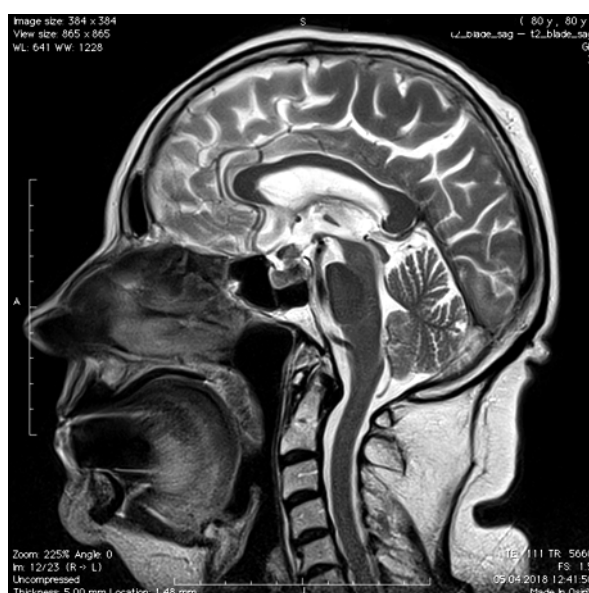
CNS showed a heterogeneous contrast enhancement, and its character was not entirely clear, considering the regression of its dimensions as well as its oncological history and the use of immunotherapy (Fig. 4). In July 2018, the patient was admitted in order to administer the 35th dose of nivolumab; however, the treatment was postponed due to herpes zoster diagnosis. During the next hospitalisation, despite visible infected, residual herpes zoster-related skin lesions, it was decided that nivolumab would be given.

The patient is still being treated with nivolumab (for 23 months, the last hospitalisation was in September 2018). In the last control chest CT performed in September 2018, there was continuous regression of cancer lesions in the left lung (Fig. 5). In addition, the patient continues to receive hydrocortisone in a dose 20 mg daily and levothyroxine 50 µg daily for the treatment of endocrine complications during nivolumab therapy.

Literature review

The phase III CheckMate 057 and CheckMate 017 clinical trials compared the effectiveness of nivolumab and docetaxel in the second-line treatment in NSCLC patients. Among the immune-related endocrinopathies no pituitary complication was found [15].

Faje et al. reported a group of 17 patients with hypopituitarism, out of 154 melanoma patients treated with ipilimumab. In all patients, enlargement of the pituitary gland was revealed in brain NMR. The most frequently

Figure 5. Control computed tomography of the chest (September 2018). Persistent regression of neoplastic lesion in left lung

reported symptoms were headache and fatigue. In laboratory tests, hyponatraemia, features of hypothyroidism (decreased ft4 and TSH level in the lower normal range), and secondary adrenal insufficiency (decreased cortisol and ACTH levels) were observed. LH and FSH were also in the lower normal range. In eight patients with brain metastases, radiotherapy of the central nervous system was performed before diagnosis of pituitary pathology. During follow-up, after substitution treatment with prednisone, a relatively fast regression of the enlarged pituitary gland was observed. The authors suggest that persistent enlargement of the pituitary gland after treatment initiation, without visible regression, argues in favour of another process, e.g. metastatic. However, the resolution of changes in the pituitary gland after substitution treatment results rather from the autoimmune process associated with immunotherapy [13].

Kastrisiou et al. published a case of a patient treated with nivolumab due to lung adenocarcinoma, in whom pituitary hypoplasia occurred, but without enlargement of the gland. After administration of 11 cycles of nivolumab, the patient presented symptoms such as dizziness, gait disturbances, cachexia, and confusion. In the CT scan of the head, metastatic lesions were excluded. Based on laboratory tests, hypothyroidism was diagnosed. Treatment with levothyroxine and liothyronine was used; additionally, it was decided to discontinue nivolumab therapy. After six weeks of treatment, TSH levels normalised; however, the patient's general condition deteriorated — fatigue, loss of appetite, joint stiffness, nausea, and abdominal pain intensified. Physical

examination revealed exfoliative keratolysis, dehydration, and low blood pressure. The symptoms indicated adrenal insufficiency, and laboratory tests showed low cortisol and ACTH plasma levels. In the next imaging of the head, no metastatic lesions and enlargement of the pituitary gland were observed, the concentration of all hormones normalised under the influence of substitution treatment, and resolution of previously seen symptoms was observed. The adrenocortical insufficiency induced by hypopituitarism as a complication of anti-PD-1 antibody treatment was hypothesised [16].

In a publication by Kitajima et al. two cases of patients treated with nivolumab due to melanoma and with isolated ACTH deficiency were presented. The first case, a male patient, at the 13th administration of nivolumab reported malaise with low levels of cortisol and ACTH and no changes in brain NMR. In the next case, after 13 cycles of therapy nivolumab was changed to ipilimumab due to occurrence of lung metastases. After the second administration of ipilimumab, fever, asthaenia, and dizziness were observed. The laboratory tests indicated a decrease in ACTH and cortisol concentrations and a normal magnetic resonance image of the head [17].

Okano et al. described the case of a male patient treated with nivolumab due to melanoma, who presented symptoms of hypopituitarism with visible moderate enlargement of the gland in an imaging test [18].

In 2017, Mengoli et al. published a paper describing the first case of a female patient with lung adenocarcinoma with *ALK* gene rearrangement and with known metastasis to the pituitary gland. Initially the patient was admitted to the neurology department due to vision disorders and polydipsia. Magnetic resonance of the head revealed a change in the pituitary gland area. Initially, it was thought that it was a pituitary adenoma pressing on the optic nerve. A partial resection was performed showing the rearrangement of *ALK* gene in tumour cells. The chest CT scans and pathomorphological examination revealed an adenocarcinoma of the left lung. Chemotherapy was initiated; however, after the progression of the lesion in the pituitary gland immunotherapy (without the intended effect) and radiotherapy were introduced, leading to regression of the lesion. Stabilisation of the disease was obtained only after the introduction of crizotinib [19].

In the present case, features of hypopituitarism were found during treatment with anti-PD-1 antibody — nivolumab. In magnetic resonance imaging of the head, enlargement of the pituitary gland was detected, possibly corresponding to macroadenoma. After the implementation of hormonal substitution treatment, a control head NMR was performed, showing regression of pituitary lesion. On the basis of the available literature, the most likely mechanism responsible for

the development of the pathology described above and its regression is hypothyroidism and autoimmune inflammation of pituitary gland during immunotherapy. Radiological differentiation included the presence of adenoma and metastatic lesions. Pituitary adenomas most often exhibit different characteristics of endocrine disorders, while metastatic lesions usually have no hormonal activity and are extremely rare. Also, a quite fast regression during substitution treatment contradicts the possible concept of adenoma or metastatic lesion.

Therefore, the syndrome of hormonal and structural disorders related to the pituitary gland described by the authors should be associated with the adverse effect of immunotherapy with nivolumab. It should be noted that this is one of the few descriptions in which hormonal disorders were accompanied by an enlarged pituitary gland. The presented case report is also further proof of the possibility of obtaining a response to treatment with nivolumab in patients with very low PD-L1 expression on tumour cells.

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Skeletal muscle metastasis from oesophageal adenocarcinoma — case report and literature review

ABSTRACT

The most common sites of metastases from oesophageal carcinoma are lymph nodes, liver, lungs, and bones. Metastases to skeletal muscles are very rare and are characterised by extremely poor prognosis. A 62-year-old man with advanced oesophageal adenocarcinoma underwent chemoradiotherapy. More than six months after the primary diagnosis, the patient presented with distant solitary metastasis to the skeletal muscle of the left lower leg. He complained of severe pain and swelling of the left lower leg. Radiological and pathological examination confirmed metastatic character of the lesion. The patient was qualified for radiotherapy.

Metastases to skeletal muscle are very rare, and no guidelines have been established for the treatment for these patients. It seems that chemotherapy and radiotherapy can be considered as the best treatment modality for these patients.

Key words: oesophageal carcinoma, skeletal muscle metastasis, oesophageal adenocarcinoma

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Introduction

According to the International Agency for Research of Cancer (IARC), oesophageal cancer is currently the eighth most common cancer worldwide. In 2018, there were approximately 572,000 newly diagnosed patients, which accounted for 3.4% of all cancers, and approximately 509,000 patients died (5.3% of all cancer-related death) [1]. Oesophageal tumours are characterised by poor prognosis, and the five-year survival rate is around 10% [2]. Metastases of oesophageal cancer are most often located in the lymph nodes, liver, lungs, and bones. Skeletal muscles are a very rare location of metastases, and until now no guidelines for their treatment have been developed.

Case report

A 62-year-old man, with the history of obesity and nicotinism, was seen in the Oncologic Surgery Outpatient Clinic of The Maria Sklodowska-Curie Institute — Oncology Center in Warsaw in July 2018 with diagnosis of oesophageal cancer. Previously the patient repeatedly reported to the primary care physician due to gastroesophageal reflux. He was referred for endoscopic examination of the upper gastrointestinal tract in July 2018. An ulcerative infiltration of the middle-lower part of the oesophagus was found. The infiltration was also visualised in computed tomography (CT) of the chest. Histopathological examination of specimens collected during gastroscopy confirmed the diagnosis of poorly differentiated (grade 3) oesophageal adenocarcinoma. The imaging examinations: ultrasound (US) of the abdominal cavity, CT of the abdominal cavity and pelvis, and positron emission tomography (PET), revealed the infiltration of the thoracic oesophagus, spreading over 88 mm, from the level of the trachea bifurcation to the supracardiac area. The infiltration formed a conglomerate with the pathological lymph nodes of the subcarinal area on the left side, with the largest transverse dimension 76 mm × 40 mm. The features of mediastinal lymph node involvement — group 2L, 7, 8 (cT3 cN2/3 cM1) were demonstrated. The patient case was discussed at a multidisciplinary meeting in July 2018. The patient

was qualified to induction chemotherapy with response evaluation following the second cycle, followed by radical chemoradiotherapy and possible surgical treatment. Induction chemotherapy according to the FLOT scheme (docetaxel + oxaliplatin + 5-fluorouracil + calcium folinate) was initiated in August 2018. The patient received 100% of the planned dose with moderately good tolerance of treatment. After two FLOT cycles, the patient underwent PET examination (August 2018) and partial regression was observed. The patient was qualified for a definitive chemoradiotherapy (50 Gy in 25 fractions of 2 Gy in combination with chemotherapy according to the FOLFOX scheme — oxaliplatin + 5-fluorouracil + calcium folinate, every 14 days). Treatment was continued from September to December 2018. Due to grade G1 haematological toxicity and worsening of treatment tolerance, the dose of chemotherapy was reduced. Six chemotherapy cycles were given (two cycles with full doses of drugs and four cycles with reduced doses) comprising the full planned dose of radiation. After the completion of combination treatment (January 2019), the patient came to the Oncological Outpatient Clinic due to the appearance of tumour-like lesion of the left crus, which was accompanied by severe lower limb pain. A magnetic resonance (MR) examination (January 2019) revealed an abnormal, heterogenous solid mass with dimensions of 143 mm × 25 mm × 17 mm, located medially to tibia, approximately in the middle of tibial shaft, destroying the cortical layer of the bone asymptomatically (Fig. 1). In addition, there was evidence of oedema in the flexor digitorum longus muscle. The infiltration into the neurovascular bundle of the posterior tibial artery was excluded. Bone scintigraphy (February 2019) did not reveal other pathological changes. A core needle
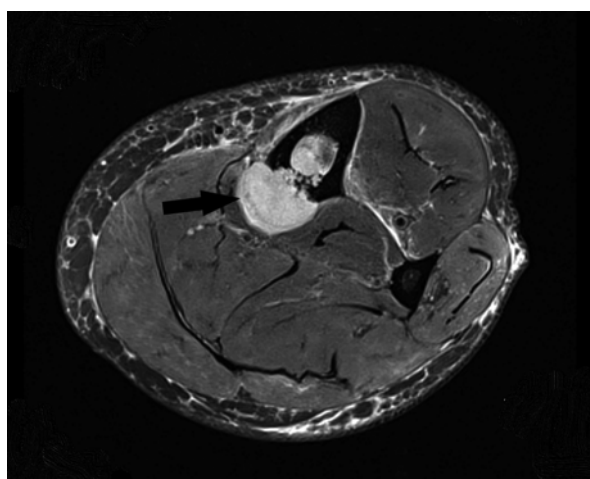
Figure 1. Axial magnetic resonance image of the left lower leg. The metastasis of the oesophageal adenocarcinoma to the flexor digitorum longus muscle with infiltration of the tibial bone

biopsy of the tumour lesion in the flexor digitorum longus muscle was performed. Histopathological examination confirmed poorly differentiated G3 adenocarcinoma. In an immunohistochemical study, tumour cells expressed CKAE1/AE3 and CDX2 (in some cancer cells). The staining for CK7, CK20, chromogranin A, CD56, and CK5/6 was negative. The patient was qualified for radiotherapy for the area of a single metastasis to the flexor digitorum longus muscle and the left tibia (with a total dose of 35 Gy, in five fractions of 7 Gy). Treatment was carried out from February 14 to March 4, 2019. The lower limb pain was reduced. In order to prevent bone fractures, treatment with denosumab was initiated (March 2019). Currently, the patient is under continuous follow-up.

Discussion

Oesophageal cancer metastases are most commonly located in lymph nodes, lungs, pleura, liver, stomach, peritoneum, kidneys, adrenal glands, bones, and brain [3]. Metastases to skeletal muscles are very rare and usually locate in the muscles of the lower limb. The reason for the rare occurrence of oesophageal cancer metastases in this location is not fully understood. Some authors suggest that it may be associated with rich blood supply, systolic activity, frequent pH changes, and the production of lactic acid in skeletal muscles, which inhibit the development of cancer cells in this area [4].

A systematic literature review was carried out using PubMed. There were 25 articles in which clinical cases of patients with oesophageal cancer metastases in skeletal muscles were presented (Table 1). Most patients had metastases in skeletal muscles of the lower limb — thigh and lower leg. The most common clinical signs accompanying the diagnosis were pain and the presence of palpable tumour-like lesions. In our patient, metastasis was found in the left crus muscle and was diagnosed very shortly after completion of combination therapy due to primary oesophageal adenocarcinoma. The patient reported severe pain in the lower limb, intensifying during movement.

Diagnosis of skeletal muscle metastasis is difficult, and it is often misdiagnosed in physical examination and imaging examinations as sarcoma or other soft tissue pathologies [4]. Ultrasound is used to differentiate solid and cystic lesions [5], while PET is considered to be a more specific study than computed tomography in imaging lesions in skeletal muscles.

Wu et al. and Sohda et al. used a PET/CT imaging technique to diagnose metastasis of oesophageal adenocarcinoma to skeletal muscle. In the presented patient, a PET study performed in August 2018 showed no pathologies in the skeletal system. The lesion was visualised by magnetic resonance and finally confirmed by histopathological and immunohistochemical examination.

Table 1. Publications addressing oesophageal carcinoma patients with metastases to the scelatal muscles

Number	Author	Age	Gender	Metastases location
1.	Schultz et al. (1986)	ND	ND	Gluteus minimus muscle
2.	Miura et al. (1998)	58	M	Left shoulder
3.	Pretorius et al. (2000)	62	M	Right vastus lateralis muscle
	Pretorius et al. (2000)	ND	ND	ND
4.	Rehman et al. (2002)	71	M	Right thigh
5.	Lekse et al. (2003)	78	F	Inferior rectus eyeball muscle
6.	Wu et al. (2005)	67	M	Right gluteus minimus muscle
7.	Koike et al. (2005)	ND	ND	Deltoid muscle
8.	Heffernan et al. (2006)	67	F	Right infraspinatus muscle
9.	Hayata et al. (2009)	61	F	Gluteus maximus muscle
	Hayata et al. (2009)	58	M	Right forearm
10.	Norris et al. (2009)	58	M	Right iliacus muscle
11.	Hsieh et al. (2011)	58	M	Left psoas muscle
12.	Uygur et al. (2011)	62	F	Right temporal muscle
13.	Lu et al. (2012)	71	M	Left erector spinae
14.	Cincibuch et al. (2012)	64	M	Left quadriceps muscle
	Cincibuch et al. (2012)	76	M	Right gluteus minimus muscle
	Cincibuch et al. (2012)	57	M	Right subscapularis muscle
	Cincibuch et al. (2012)	42	M	Iliacus muscle
	Cincibuch et al. (2012)	60	M	Numerous metastases (including the gluteus maximus muscle)
15.	Matsutani et al. (2013)	72	M	Left triceps muscle
16.	Leuzzi et al. (2013)	65	M	Right paraspinal muscles
17.	Sohda et al. (2014)	49	M	Left thigh
18.	Maruzen et al. (2015)	45	M	Left thigh
19.	Domínguez et al. (2015)	53	M	Left gluteus medius muscle
20.	Thumallapally et al. (2016)	73	M	Left rectus eyeball muscle
21.	Azadeh et al. (2016)	65	M	Right iliacus muscle
22.	Fujimoto et al. (2017)	77	M	Left forearm
23.	Saito et al. (2017)	56	M	Left shoulder
24.	Mendiola et al. (2018)	61	M	Left iliopsoas muscle
25.	Abiad et al. (2019)	19 patients treated from 1997 to 2017 with metastases to skeletal muscle		

M — male; F — female; ND — no data

El Abiad et al. analysed 1341 patients treated for oesophageal cancer. Only 25 of them had distant metastases to soft tissues (skeletal muscles and/or subcutaneous tissue). The average age at diagnosis of metastasis was 64 years, and the average time from the diagnosis of primary oesophageal cancer until the metastasis was diagnosed was 9.6 months. In the presented patient it was 62 years and less than seven months, respectively.

El Abiad et al. showed that the incidence of soft tissue metastases was related to the histological subtype of oesophageal cancer. Adenocarcinoma far more frequently spreads to skeletal muscles and subcutaneous tissue (85%) than squamous cell carcinoma of the oesophagus (15%).

To date, no harmonised guidelines have been developed for the treatment of patients with oesophageal cancer metastases to skeletal muscles. Each patient

should be treated individually. When planning treatment, the clinical stage of primary disease, the patient's performance status and general condition, as well as prognostic factors should be considered [6].

Based on literature review, it can be seen that the majority of patients were treated with chemotherapy. In other patients, radiotherapy and/or surgical resection of the metastasis was used. It seems that patients with single metastasis in skeletal muscle should be resected or irradiated, whereas patients with multiple metastases should receive chemotherapy [6–8].

Regardless of the treatment used, patients with primary oesophageal cancer with distant metastases have a poor prognosis. The five-year survival rate is estimated at around 5% [4]. Diagnosis of metastases in skeletal muscle is usually associated with terminal stage of the disease and very poor prognosis [9]. The average survival time from the diagnosis of metastasis is 7.5–9 months [6, 8, 10, 12]. For comparison, the average survival time from the diagnosis of primary stage IV oesophageal cancer is 13 months [6].

Summary

Metastases of oesophageal adenocarcinoma to the skeletal muscles are rare, and so far no guidelines for their treatment have been developed. The occurrence of even a single, isolated metastasis in this area is associated with the terminal stage of the disease and is characterised by extremely poor prognosis. Due to the limited treatment options and the risk of complications, it seems that the use of chemo- or radiotherapy in these patients can bring significant benefits.

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Uncontrolled reactivation of EBV infection in a 26-year-old woman

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ABSTRACT

This report describes the case of a 26-year-old woman, who was admitted to oncological centre with symptoms of lymphoma, but final diagnosis indicated CAEBV virus infection. The patient had never been treated for chronic diseases before, and lymphoma was suspected due to: clinical symptoms (neck lymphadenopathy, febrile conditions), and imaging and endoscopic ultrasound examinations (CT, EUS). During the examinations in the oncological centre, lymphoma diagnosis was turned down and CAEBV infection was recognised. Despite the treatment applied in accordance with global standards, the patient developed multi-organ failure, which led to her death.

Key words: CAEBV, lymphoma, allo-HSCT

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Introduction

The Epstein-Barr virus (EBV) is a herpes virus that can cause acute and chronic infections; however, the first infection is usually asymptomatic. The virus primarily attacks B lymphocytes, in which it begins a latent (hidden) infection (in the form of an episome), lasting lifelong [1]. Reactivation can lead to monoclonal, uncontrolled proliferation. The virus also has oncogenic potential and is responsible for the endemic form of lymphoma in Equatorial Africa (Burkitt's lymphoma) and nasopharyngeal cancer. Humans are the only reservoir, and the source of infection is a sick or infected person. The infection occurs through contact with saliva, but it is also possible through blood transfusions, and transplantation of haematopoietic cells or solid organs [2, 3].

The incidence of uncontrolled reactivation is rare; only a few cases of the above syndrome are described in the literature.

Herein the case of a patient hospitalised in Department of *Cancer* and *Cardio-Oncology Diagnostics* of the Cancer Centre in Warsaw is presented.

Case report

On March 10, 2016 a 26-year-old woman was urgently admitted to our Department of *Cancer* and *Cardio-Oncology Diagnostics* and Palliative Medicine Clinic for diagnostics of lymphopoietic malignancy. From January 2016 the patient was diagnosed in various hospitals due to neck lymphadenopathy and febrile states. A computed tomography (CT) examination was performed in which a tumour-like lesion of the pancreas and retroperitoneal lymphadenopathy were described. The endoscopic ultrasound (EUS) evaluation revealed a lesion in the pancreatic body (suspected lymphoma infiltration, non-diagnostic result of previously performed biopsy). NK/T cell nasal type lymphoma was suspected in histopathological neck lymph node examination.

At admission the patient was in a good general condition (performance status ECOG 0). In the physical examination, attention was paid to a hard, painless infiltration within the right parotid and enlarged nuchal and neck lymph nodes.

Abnormalities in the laboratory tests included normocytic anaemia, leukopenia, elevated liver enzymes,
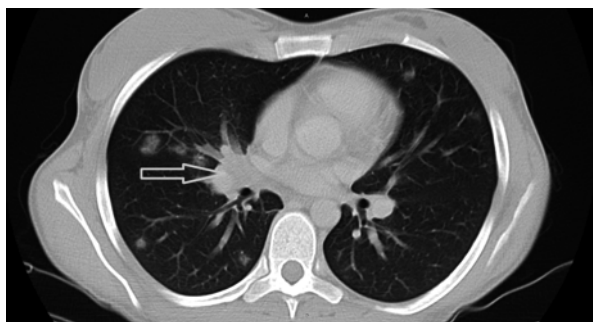
Figure 1. Computed tomography (CT) of the chest. Numerous focuses in the lung. Enlarged lymph nodes of the pulmonary hila
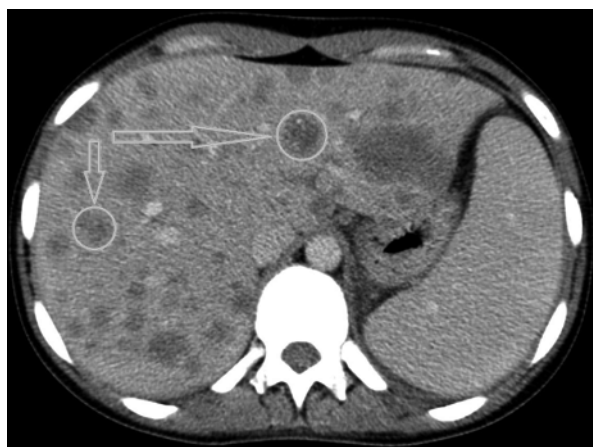
Figure 2. CT scan of the abdominal cavity. Enlargement of the liver and spleen. Numerous centres of reduced density in the whole liver

Figure 3. CT scan of the abdominal cavity. Enlarged lymph nodes of the liver hilum

elevated D-dimer levels, and abnormalities in the coagulation system.

Computed tomography of the neck and chest (11/03/2016) showed heterogeneous nodal infiltration in the area of the right mandibular angle and branch, bilateral, numerous, poorly-separated mottled densities in lung parenchyma, nodal lesions in a right pulmonary hilum with suspected disintegration (Fig. 1), hepatosplenomegaly, numerous hypodense focal lesions in the liver, irregular hypodense area in the left kidney cortex — suspicion of infiltration, and, in addition, numerous lymph nodes of borderline size (Fig. 2, Fig. 3).

Due to the rare histopathological diagnosis and atypical course of the disease, the specimen from biopsy was consulted in Department of Pathology and the presence of neoplastic disease was not confirmed. *Lymphadenitis reactiva* associated with reactivation of EBV was diagnosed. The material provided was not eligible for testing to assess the presence of EBV RNA (EBV encoded RNA, EBER) by means of the FISH method.

Due to the suspicion of EBV infection, blood was collected for antibody testing. The serum level of beta-2-microglobulin was 9.04 mg/L (0.70–1.80) (Table 1).

On March 14, 2016, in order to perform flow cytometry, a fine-needle aspiration biopsy of the nodal lesion in the right parotid region was carried out under ultrasound (US) control. The result indicated an active EBV infection with associated lymphadenopathy. The predominance of CD4+/HLA DR+ lymphocytes over CD8+/HLA DR+ indicated no T-cell conversion, which may indicate the infection progression. In the trepanobiopsy, changes characteristic for bone marrow image in the course of EBV infection were found.

Histopathological examination of the material sampled during Tru-Cut biopsy from liver confirmed active hepatitis with extensive EBV-induced necrosis. Immunohistochemical reactions showed that EBV infected only large (blastic) T lymphocytes. The whole picture corresponded to a chronic, active EBV infection (CAEBV — chronic, active EBV) accompanied by a high titre of antibodies against EBV antigens, which corresponds to its active replication. The EBER result was positive in T-cell lymphoid cells.

Based on analysis of examinations performed, lymphoma was excluded (Table 2). CAEBV treatment was implemented in accordance with current guidelines together with the antibacterial and antifungal drugs (Table 3).

Laboratory tests showed stabilisation of morphological and biochemical parameters.

Continuation of the treatment resulted in the resolution of febrile conditions, reduction of infiltrative lesions within the right parotid, and a decrease in viraemia (15/03 — 21,150,115 copies/mL, 25/03 — 9,512,940 copies/mL).

On 26/03/2016 there was a sudden deterioration of the general condition of the patient with dyspnea,

Table 1. Differentiation of disease entities that may mask CAEBV

Feature	Lymphoma	CMV	EBV	Solid tumour in the generalised phase
Lymphadenopathy	+	+	+	Possible
Splenomegaly	+	+/–	+/–	–
Hepatomegaly	+	+/–	10–15% of patients	In the case of metastatic lesions
Febrile states	+	+	+	–
Weight loss	+	+/–	+/–	+/–
Pharyngitis and tonsillitis	–	+/–	+	–
Skin rash	Primary cutaneous lymphoma	–	5% of patients	Paraneoplastic syndrome
Changes in complete blood counts	+	+	+	With bone marrow infiltrations
Serological tests	–	Specific antibodies	Specific antibodies, EBV DNA/RNA	–
Increased inflammation parameters	+/–	+	+	–
Hepatitis	–	+	20–90% of patients	–
Changes in the bone marrow image (trepanobiopsy)	Characteristic for underlying disease	–	Characteristic image in CAEBV	With bone marrow infiltrations
Histopathological image of peripheral organ biopsy	Characteristic for underlying disease	Reactive	Reactive	Characteristic for underlying disease
LDH	Elevated	Could be elevated	Could be elevated	Normal
Flow cytometry	Characteristic for underlying disease	Not performed routinely	Not performed routinely, characteristic image in CAEBV	Not performed routinely, little usefulness
Imaging examinations	Organ infiltration changes	Not performed routinely	Organ infiltration changes in CAEBV	Solid tumour, metastatic lesions

CMV — cytomegalovirus; EBV — Epstein-Barr virus; LDH — lactate dehydrogenase

Table 2. Differentiation of CAEBV and lymphomas [3–5]

Feature	CAEBV	Lymphoma
General symptoms (febrile states, lymphadenopathy, hepatosplenomegaly, asthaenia, weight loss)	Present	Present
Abnormalities in complete blood counts	Non-specific lesions (in 98% of cases leukocytosis with a lymphocyte percentage > 50%, atypical lymphocytes in the history)	Depending on the type of lymphoma: increased leukocytosis (less frequently leukopaaenia), thrombocytopaenia, anaemia
Serological tests	Positive	Negative
Histopathological examination/flow cytometry	Characteristic for EBV infection	Characteristic for a given type of lymphoma
Indicators of inflammation	Elevated	+/–
Liver parameters	Hepatitis 20–90%	Elevated LDH level
Treatment	See the table 3	Immunochemotherapy depending on the type of lymphoma

EBV — Epstein-Barr virus; LDH — lactate dehydrogenase

Table 3. Treatment of EBV infection [3, 6, 7]

Treatment	Infectious mononucleosis	CAEBV
Symptomatic treatment	— Rest, avoiding injuries and effort, — Antipyretic drugs — Corticosteroids (for upper airway obstruction, anaemia, autoimmune thrombocytopaenia, rash with involvement of mucous membranes after penicillin)	— Ineffective
Causative treatment	— Not recommended — Ganciclovir or acyclovir for consideration in the lymphoproliferative syndrome — Immunological reconstruction with secondary immunodeficiency (reduction of doses of immunosuppressive drugs)	— Bone marrow transplantation as the most effective method — Antiviral drugs (ganciclovir, acyclovir, vidarabine) — Immunostimulatory drugs (IL-2, interferon alpha and gamma) — Immunosuppressants (corticosteroids, cyclosporine A, immunoglobulins) — Chemotherapy — Corticosteroids with etoposide (an inhibitor of topoisomerase II necessary for EBV replication)

EBV — Epstein-Barr virus; IL-2 — interleukin 2

jaundice, and features of haemorrhagic diathesis. In additional blood tests, pancytopenia occurred, bilirubin and transaminases level increased, and acute renal failure and electrolyte abnormalities were observed. The patient was transferred to the Intensive Care Unit (ICU), where further deterioration of the general condition was observed. Despite the intensive treatment implemented, no improvement was achieved and the patient died. In the autopsy multi-organ failure following CAEBV was indicated as the immediate cause of death.

Discussion

Chronic active EBV disease (CAEBV) is a lymphoproliferative disorder characterised by clearly elevated levels of anti-EBV or EBV DNA in the blood and EBV RNA or protein in the lymphocytes in the tissues. The disease was described for the first time by Virelizier et al. in 1978 [8].

The clinical picture of CAEBV mainly includes: fever, hepatomegaly, splenomegaly, lymphadenopathy, rash, hypersensitivity to mosquito bites, diarrhoea, urethritis, abnormal transaminase activity, thrombocytopaenia, and anaemia.

Rarer forms of CAEBV include: pancytopaenia, CNS involvement, intracranial calcifications, inflammation of the salivary glands, sinusitis, and oral mucosal ulcerations [9, 10].

Life-threatening complications in the course of the disease are: haemophagocytic syndrome, malignant lymphoma, disseminated intravascular coagulation (DIC), hepatic failure, gastrointestinal ulcer perforation, coronary artery aneurysms, myocarditis, interstitial pneumonia, and leukaemia [11].

CAEBV diagnostic criteria include:
— clinical manifestation (depends on which cell line is predominantly infected with EBV: T lymphocytes (worse prognosis) — fever, anaemia, lymphadenopathy, hepatomegaly, high titre of anti-EBV antibodies (Ab); NK lymphocytes (better prognosis) — mononuclear lymphocytosis, hypersensitivity to mosquito bites, high IgE titre);
— EBV viraemia;
— extremely high titre of IgG antibodies against capsid antigen (anti-VCA);
— absence of antibodies against nuclear antigens (anti-EBNA) [10].

The presence of EBV in CAEBV is also detected in CD4+ T cells, CD8+ T cells, and NK cells. CAEBV T-cell type is associated with an increased risk of coronary artery anomalies, and CAEBV NK cells type with hypersensitivity to insect bites and high titre of IgE antibodies [12].

The five-year survival in the CAEBV syndrome is 50–80% [13, 14].

The CAEBV treatment strategy consists of three steps:
1. stabilisation (immunochemotherapy);
2. cytoreduction (multi-drug chemotherapy);
3. reconstruction (allogenic haematopoietic stem cell transplantation — HSCT).

In the first stage, the treatment assumes the use of prednisolone 0.5–2 mg/kg/day 7 days a week, cyclosporine A 3 mg/kg × 2/day 7 days a week and etoposide 150 mg/m^2/day 1 day a week.

In the second stage, in cases presented in the literature, CHOP (vincristine 1.5 mg/m^2, maximum 2 mg day 1, cyclophosphamide 750 mg/m^2 day 1, pirarubicin 25 mg/m^2 day 1 and 2, prednisolone 50 mg/m^2 day 1–5) or ESCAP (etoposide 250 mg/m^2 day 1, cytosine ara-

Table 4. PTLD-EBV management [17, 18]

Procedure	EBV-DNA-aemia/pre-emptive therapy	PTLD-EBV
Rituximab	+	+
Reduction of immunosuppression	+	+
EBV-CTL	+	+
DLI	+	+
Chemotherapy	–	+
Antiviral drugs	–	–

EBV-CTL — human cytotoxic T lymphocytes against EBV-infected cells; DLI — donor lymphocytes infusion

binoside 1.5 g/m^2 2 times on days 1–5, L-asparaginase 6000 U/m^2/day on days 5–9, methylprednisolone 62.5 mg/m^2 2 times daily on days 1–5, prednisolone 30 mg/m^2 on days 6–9) were used [6].

The treatment of choice in these patients is bone marrow transplantation. Patients are at high risk of complications related to transplantation due to multi-organ failure that accompanies infection. In Japanese works, dozens of cases of such successful treatment have been presented.

The benefits of antiviral drugs (acyclovir, ganciclovir), vidarabine, interferon alpha, or interleukin 2 have not been demonstrated so far, although they may be useful in some cases of CAEBV [15]. Etoposide, corticosteroids, and cyclosporin A are reserved for patients with advanced EBV syndrome, but no clear benefits have been demonstrated. They can also be used to reduce the clinical symptoms associated with CAEBV [7].

Autologous LAK cells (interleukin-2-activated lymphocytes), EBV-specific cytotoxic T lymphocytes, and lymphocytes from identical HLA sublines are successfully used in the treatment of solid organ transplant recipients in whom EBV-dependent posttransplant lymphoproliferative disorders (EBV-PTLD) are a constant problem due to the continuous increase in the number of transplantations performed. The incidence of EBV-PLTD after allo-HSCT is 3.2% [16]. PLTD are heterologous lymphoproliferative disorders that develop after transplantation of haematopoietic cells or solid organs as a result of T-lymphocyte suppression. The diagnosis requires the presence of two of the three following factors:

1. biopsy and histological evaluation or flow cytometry for the presence of CD 19+ and CD 20+ antigens;
2. monoclonal or oligoclonal cell populations with virus markers;
3. presence of EBV in cells (DNA, RNA, or EBV protein).

The management strategy in these patients includes the following points:

1. prophylaxis of EBV-DNA-aemia reactivation in a seropositive patient with no symptoms of infection and without EBV-DNA-aemia;
2. therapy preceding the onset of EBV disease in individuals with present EBV-DNA-aemia disease without disease symptoms;
3. treatment of confirmed or probable EBV disease [17, 18] (Table 4).

In patients after haematopoietic stem cell transplantation, EBV therapy strategies include B-cell mass reductions, anti-CD20+ monoclonal antibodies (rituximab), and T-cell immunotherapy (donor lymphocyte infusion — DLI and cytotoxic T-EBV-CTL lymphocytes) [19].

It should be emphasised that antiviral therapy has no effect on the reduction of EBV-infected lymphocyte B cells and is of no clinical significance in the treatment of overt PTLD-EBV.

Conclusions

Chronic active EBV disease (CAEBV) is a rare systemic disease with a poor prognosis, with a mortality of app. 40%. It mainly affects Asian regions, causing the proliferation of T or NK cells in immunocompetent individuals. Due to the wide spectrum of symptoms, establishing the final diagnosis can be very difficult [20].

Acknowledgments

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New opportunities in the treatment of patients with *BRAF V600E* mutated colorectal cancer

The introduction of multidrug schedules incorporating targeted agents has significantly improved the prognosis of patients with metastatic colorectal cancer, with a median overall survival (OS) surpassing three years in several clinical trials. This improvement of prognosis in the general population unveiled a poor prognosis associated with the presence *of BRAF V600E* mutation, with a median OS of only 12 months. Based on the success of BRAF inhibitors in *BRAF V600E* mutated melanoma, several attempts to utilise those drugs in patients with *BRAF V600E* mutated colorectal cancer were undertaken, but the activity of monotherapy with anti-BRAF agents was disappointing. However, the improvement in understanding the molecular effects of *V600E* mutations and mechanisms behind secondary resistance to BRAF inhibitors through MEK signalling pathway enabled the utilisation of BRAF V600E as a molecular target even in patients with metastatic colorectal cancer. The results of the BEACON study, which evaluated multiagent BRAF V600E inhibition in colorectal cancer, suggest a major shift in treatment strategy: introduction of the first multidrug schedule without classic cytotoxic drugs in colorectal cancer.

The BEACON study, published on 20th September 2019 in the "New England Journal" of Medicine by Kopetz et al. [1], was a randomised, open-label, phase III trial that compared the combination of encorafenib (BRAF inhibitor) and cetuximab (anti-EGFR antibody) with or without addition of binimetinib (MEK inhibitor), with a standard second-line chemotherapy: FOLFIRI with the addition of cetuximab. The study included patients with *BRAF V600E* mutated colorectal cancer, who failed one or two lines of therapy. The trial's primary endpoint was overall survival in patients receiving triplet-therapy as compared to standard therapy, with an additional primary endpoint that compared the response rates (RR). Major secondary endpoints included a comparison of OS in patients receiving doublet therapy compared to a standard arm, and a comparison of progression-free survival (PFS) between arms. From 1677 screened patients, 665 patients underwent randomisation in 1:1:1 ratio to all arms of the trial. After a median follow-up time of 7.8 months, the trial met its primary endpoint. Median

OS in patients receiving encorafenib, binimetinib, and cetuximab was 9.0 months (95% confidence interval [CI] 8.0–11.4) as compared to only 5.4 months (95% CI 4.8–6.6) in patients receiving standard therapy, with a hazard ratio (HR) of 0.52 (95% CI 0.39–0.70; $p < 0.001$). The second primary endpoint — RR — was also significantly improved in patients receiving triplet therapy (26%; 95% CI 18–35) in comparison with the control arm (2%; 95% 0–7) ($p < 0.001$). The achieved results were consistent among most analysed subgroups, with a reduced benefit from triplet therapy in the North America region. Survival was also improved in patients receiving encorafenib with cetuximab, with a median OS of 8.4 months (95% CI 7.5–11.0) and HR of 0.60 (95% CI 0.45–0.79; $p < 0.001$), when compared to the control arm. A comparison of triplet therapy with doublet therapy, although not prefigured in the protocol, showed a trend for OS improvement with triplet therapy (HR 0.79; 95% CI 0.59–1.06). Analysis of PFS also demonstrated benefit of both triplet (4.3 months; 95% CI 4.1–5.2) and doublet (4.2 months; 95% CI 3.7–5.4) therapy versus the standard arm (1.5 months; 95% CI 1.5–1.7). As expected, the toxicity profile differed significantly between study arms, with a moderate increase of skin and gastrointestinal toxicities with triplet therapy. The rate of grade 3 or higher adverse events was 58% in patients receiving triplet therapy, 50% in patients receiving doublet therapy, and 61% in patients receiving standard chemotherapy. The rate of adverse events that led to treatment discontinuation was, respectively, 7%, 8%, and 11%. Additionally, no difference in the rate of adverse events that led to death was seen.

Significant OS benefit achieved in the BEACON study with both triplet and doublet targeted therapy can be considered as a major breakthrough in the treatment of patients with *BRAF V600E* mutated colorectal cancer. This is the first trial dedicated to patients with colorectal cancer that showed significant clinical benefit associated with combining molecularly targeted agents with acceptable toxicity profile. Based on these results, a combination of encorafenib with cetuximab with the addition of binimetinib should be considered the new standard of care in the second-line

treatment of patients with *BRAF V600E* mutated colorectal cancer. Nevertheless, attention should be paid to the significant patient selection, because the trial included fewer than 40% of screened patients, which mirrors the exceptionally poor prognosis among this group of patients. Traditionally, as is the case of most modern targeted therapies or immunotherapeutic agents, the single most important factor limiting wide implementation of this strategy is the cost of a therapy that utilises not just two, but three molecularly targeted agents. Cost-effectiveness assessment of triplet therapy may lead to disappointing conclusions, especially when including the only slightly inferior results achieved with doublet therapy.

Nivolumab and ipilimumab as a first-line treatment of patients with non-small cell lung cancer

The introduction of immune check-point inhibitors (CPSs) is probably the single greatest achievement of the last decade in the systemic treatment of solid tumours. We must be aware that modern immunotherapy is not a universal solution in all solid tumours, but it has significantly revolutionised the treatment of several cancer types, including melanoma, renal cell carcinoma, and lung cancer. The benefit of CPIs in the treatment of patients with lung cancer, although numerically lower when compared to gains in melanoma or renal cell carcinoma, has its greatest impact on modern oncology practice due to the higher prevalence of lung cancer. Considering the actual guidelines, it seems that all patients with non-small cell lung cancer, excluding patients with present activating mutations (*EGFR/ROS/ALK/BRAF*), should receive CPIs in the first-line treatment — either as a monotherapy (in cases with PD-L1 > 50%) or as a combination with chemotherapy (in cases with PD-L1 < 50%). The number of phase III trials assessing CPIS with initial or complete data, which were published within the last two years might be intimidating, and we can even assume that the current standard will become at least partially obsolete in the near future. The role of platinum-based chemotherapy doublets, the long-time standard of care in the first-line treatment of non-small cell lung cancer, is now limited and may even become marginalised. This scenario is becoming a reality with the recent publication of a trial assessing the combination of nivolumab with ipilimumab in the first-line treatment of non-small cell lung cancer, which expands the chemotherapy-free approach also into the population with PD-L1 expression below 50%.

The results of the aforementioned trial were published on 28th September 2019 in the "New England Journal of Medicine" by Hellmann et al. [2]. The CheckMate 227 trial was a randomised, open-label, phase III trial that compared nivolumab alone or in combination with either ipilimumab or chemotherapy with a standard platinum-doublet chemotherapy. Patients recruited into the trial had non-small cell lung cancer, either squamous carcinoma or adenocarcinoma, without activating mutations in the *EGFR* gene or the presence of *ALK*

gene fusions. Depending on the central assessment of PD-L1 status (either > 1% or < 1%), patients were randomised in 1:1:1 ratio to a combination of nivolumab with ipilimumab, nivolumab alone, or standard chemotherapy (if PD-L1 expression was > 1%) or to a combination of nivolumab with ipilimumab, a combination of nivolumab with chemotherapy, or standard chemotherapy (if PD-L1 expression was < 1%). The primary endpoint was overall survival in patients with PD-L1 expression > 1% compared between a combination of nivolumab and ipilimumab and standard chemotherapy (results of the second primary endpoint — a comparison of PFS in patients with tumour mutational burden [TMB] equal to or higher than 10 mutations per MB were published previously [3]). Patients assigned to the combination of nivolumab and ipilimumab received nivolumab at a dose of 3 mg/kg body weight every two weeks along with ipilimumab 1 mg/kg body weight every six weeks. Patients assigned to the nivolumab monotherapy received nivolumab at a fixed dose of 240 mg biweekly, and patients assigned to the combination of nivolumab with chemotherapy received nivolumab at a fixed-dose of 360 mg every three weeks along with standard platinum-based doublet chemotherapy every three weeks (up to four cycles of chemotherapy). The control arm for both PD-L1 > 1% and < 1% received standard treatment of platinum-based chemotherapy (patients with adenocarcinoma: up to four cycles of cisplatin or carboplatin with pemetrexed with an option of continuing maintenance pemetrexed; patients with squamous carcinoma: up to four cycles of cisplatin or carboplatin with gemcitabine). The trial included 1189 patients with a confirmed PD-L1 expression of > 1% and 550 patients with PD-L1 expression of < 1%. After a median follow-up time of 29.3 months, the trial met its primary endpoint in patients with PD-L1 expression > 1%: median OS in patients receiving nivolumab and ipilimumab reached 17.1 months (95% CI 15.0–20.1) as compared to 14.9 months (95% CI 12.7–16.7) in patients receiving chemotherapy (p = 0.007). HR for death was 0.79 (97.2% CI 0.65–0.96) with a commentary that this should be interpreted along with analysis of

survival curves: initially favouring chemotherapy, then crossing and subsequentially favouring nivolumab and ipilimumab. Benefit from double immune blockade was confirmed in most subgroups, with the exception of patients with liver metastases and those without history of smoking. The response rate was 35.9% in patients receiving nivolumab and ipilimumab and 30% in patients receiving chemotherapy, with a median duration of response of, respectively, 23.2 months (95% CI 15.2–32.2) and 6.2 months (95% CI 5.6–7.4). Benefit from a combination of nivolumab with ipilimumab was also observed in the population with PD-L1 expression < 1% (pre-planned descriptive analysis): median OS was 17.2 months (95% CI 12.7–22.0) in patients receiving immunotherapy combination as compared to 12.2 months (95% CI 9.2–14.3) in patients receiving chemotherapy, with an HR of 0.62 (95% CI 0.48–0.78). Similar results were seen in combined analysis of PD-L1 > 1% and PD-L1 < 1% populations: median OS was 17.2 months (95% CI 15.2–19.9) and 13.9 months (95% 12.2–15.1), respectively. Comparison of nivolumab and ipilimumab with nivolumab monotherapy showed numerically better results achieved with the combination in terms of two-year survival rate and median duration of response, both in PD-L1 > 1% and PD-L1 > 50% populations. Better results in terms of two-year survival rate and median duration of response were seen also with immunotherapy combination as compared to combination of nivolumab and chemotherapy in the population with PD-L1 expression < 1%. In a detailed analysis neither PD-L1 expression, TMB status, nor their combination allowed selection of patients who could derive greater benefit from a combination of nivolumab and ipilimumab. Despite the previously shown correlation between TMB and median PFS [3], this has not translated into OS benefit. In terms of safety, the rate of grade 3 or greater adverse events was similar between the nivolumab-ipilimumab arm and the chemotherapy arm (32.8% and 36.0%, respectively). However, both severe adverse events (24.5% *vs.* 13.9%) and adverse events that led to treatment discontinuation (18.1% *vs.* 9.1%) were more common in patients receiving nivolumab with ipilimumab. No significant difference in rates of adverse events leading to death was seen (1.4% in the combination immunotherapy group *versus* 1.1% in the chemotherapy group). The published results did not include data regarding quality of life.

The results of CheckMate 227 have a meaningful impact on the role of immunotherapy in the first-line treatment of patients with non-small cell lung cancer and justify skipping chemotherapy in the majority of patients. However, it seems that the benefit from combined immunotherapy might be reduced in patients with PD-L1 expression 1–49%. The benefit seen in the whole population with PD-L1 expression > 1% is mostly driven by exceptional results obtained in patients with expression > 50%. Similar effects have also been reported in immunotherapy trials in different types of cancer. In practice, the patients with PD-L1 expression within 1–49% might be the best candidates for the combination of immunotherapy and chemotherapy. Other factors limiting the benefit from combined immunotherapy are the presence of liver metastases and lack of smoking history, which may provide guidance regarding treatment individualisation. Additionally, CheckMate 227 provided important, albeit negative, results assessing TMB as a predictive marker for immunotherapy. The idea that a higher number of genetic mutations increases the variety of presented neoantigens, promoting induction of immune response, does not translate into OS benefit. Unfortunately, none of the biomarkers reported in CheckMate 227 allow selection of a population with greater benefit from combined immunotherapy. Considering the remarkably high costs of such treatment, the lack of an adequate biomarker undermines wide implementation of combination immunotherapy into clinical practice. The growing potential of immunotherapy in the first-line treatment of patients with non-small cell lung cancer is revolutionising both patient treatment and the functioning of health care systems. The greatest challenge in the upcoming years, especially in chronically underfinanced systems, will be optimisation of treatment to achieve the best results with an acceptable immunotherapy-associated financial burden.

The next PARP inhibitors proved to be effective in the treatment of patients with ovarian cancer

Treatment of patients with ovarian cancer is a significant clinical challenge, despite its relative sensitivity to platinum-based chemotherapy, which can induce long-lasting responses. Unfortunately, the majority of patients achieving partial or complete response will eventually relapse with a reduced probability of re-inducing response. One of the strategies evaluated in this setting is inhibition of PARP, the enzyme responsible for the repair of single-strain DNA breaks. Inhibition of PARP activity leads to the accumulation of single-strain DNA breaks, which subsequently generates double-strain DNA breaks leading to cancer cell death. PARP inhibitors exhibit particular activity in the presence of other DNA repair dysfunctions, such as *BRCA1/2* mutations or the presence of other mechanism of homologous repair deficiency (HDR). PARP inhibi-

tion, initially developed as a salvage treatment, is useful also as a maintenance treatment after first-line therapy. Several different PARP inhibitors have proved effective as a salvage treatment (olaparib, rucaparib, veliparib), but until recently only olaparib has proved its role in the maintenance strategy. Now the situation is changing because the next two phase III trials evaluating PARP either as part of induction and maintenance treatment or just as maintenance treatment have been published.

The VELIA/GOG-3005 study, the results of which were published by Coleman et al. [4] on 28th September 2019 in the "New England Journal of Medicine", evaluated veliparib used as an addition to standard first-line induction chemotherapy (carboplatin and paclitaxel) and continued as a maintenance treatment. This randomised, double-blinded, phase III trial included chemotherapy-naive patients with stage III or IV (according to International Federation of Gynaecology and Obstetrics; FIGO) high-grade serous carcinoma of the ovary, fallopian tube, or peritoneum, irrespective of BRCA1/2 and HDR status. Veliparib was used orally at a dose of 150 mg twice daily during chemotherapy and then 300 mg twice daily with a possible escalation to 400 mg twice daily as a maintenance treatment. Patients were randomised in a 1:1:1 ratio to either veliparib used during both induction and maintenance phases, veliparib used during induction phase and placebo during maintenance phase, or to placebo used both during induction and maintenance phases (control arm). The primary endpoint was the comparison of PFS between patients receiving veliparib in the induction and maintenance phase with the control arm, evaluated hierarchically first in patients with BRCA mutations, then in patients with confirmed HDR, and finally in the intention-to-treat (ITT) population. One of the key secondary endpoints was overall survival. Altogether, 1140 patients were recruited into the trial, among whom 298 (26%) had BRCA mutations (19% germinal mutations and 7% somatic tumour mutations) and 627 (55%) had confirmed HDR status. After a median follow-up of 28 months, the trial met its primary endpoint in all hierarchically analysed groups. In patients with BRCA mutations the median PFS reached 34.7 months in the veliparib arm compared to 22.0 months in the placebo arm (HR for progression or death 0.44; 95% 0.28–0.68; p < 0.001). In HDR patients the PFS reached, respectively, 31.9 months as compared to 20.5 months (HR 0.57; 95% CI 0.43–0.76; p < 0.001), and in ITT analysis 23.5 vs. 17.3 months (HR 0.68; 95% CI 0.56–0.83; p < 0.001). The achieved effect was seen in most of the analysed subgroups with the exception of patients with macroscopically non-radical cytoreduction — no benefit from veliparib was seen in this group. Patients without confirmed HDR also gained less benefit from veliparib when compared to patients with either HDR or BRCA mutations. Due

to data immaturity, OS analysis was impossible. In the safety analysis a modest increase of adverse events was seen in patients receiving veliparib in the induction and maintenance phases (88% vs. 77% in the placebo arm), although this did not reduce the chemotherapy intensity. The most common veliparib-related adverse event was thrombocytopaenia. Among patients receiving veliparib one case of myelodysplastic syndrome and one case of acute myeloid leukaemia were seen, which is comparable to the risk described with other PARP inhibitors. Quality-of-life comparison showed no difference between study arms.

Results of the second trial were published by González-Martín et al. [5] also on 28th September 2019 in the "New England Journal of Medicine". The PRIMA/ENGOT-OV26/GOG-3012 trial was a randomised, double-blinded, phase III trial that compared niraparib, a novel PARP inhibitor, with placebo in patients with FIGO stadium III–IV ovarian, fallopian tube, or peritoneal cancers, who achieved response (CR or PR) after induction chemotherapy. Patients were randomised in a 2:1 ratio to either neratinib (administered at a dose of 300 mg once daily, although on-trial amendment allowed reduction of initial dose to 200 mg per day in patients under 77 kg body weight or with thrombocytopaenia below 150,000 platelets per cubic millimetre) within 12 weeks after finishing induction chemotherapy. The primary endpoint was PFS assessed hierarchically in the population with positive HDR status and then in the overall population. Key secondary endpoints included overall survival. The trial included 733 patients, and after a median follow-up time of 13.8 months it met its primary endpoint. In patients positive for HDR the median PFS reached 21.9 months in the niraparib group and 10.4 months in the placebo group (HR for progression or death 0.43; 95% CI 0.31–0.59; p < 0.001). In the overall population the difference was 13.8 months in the intervention arm as compared to 9.2 months in the control arm (HR 0.62; 95% CI 0.50–0.76; p < 0.001). This effect was maintained in all analysed subgroups. At the point of analysis OS data were immature (about 11% of events), but per-protocol OS analysis was undertaken. The rate of two-year survival was 84% in the niraparib arm and 77% in the placebo arm (HR 0.61; 95% CI 0.27–1.39; not statistically significant). The most common adverse events associated with niraparib were anaemia, thrombocytopaenia, and neutropaenia. The rate of grade 3 or worse adverse events was 70.5% in patients receiving niraparib and 18.9% in patients receiving placebo. Dose reductions were required in 70.9% of patients receiving niraparib and 8.2% of patients receiving placebo, with a discontinuation rate due to adverse events of, respectively, 12% and 2.5%. Quality-of-life comparison showed no difference between study arms.

The two presented trials broaden the availability of PARP inhibitors for patients with ovarian cancer, confirming the potential of PARP inhibition also at the early phase of treatment. The differences between specific compounds and in trial designs limits direct comparison, but also enables individualisation of treatment according to risk-factor profile, patient performance, and preferences. From this perspective, diversity is highly desirable.

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