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SUMMARY OF THE DOCTORAL THESIS

**Biomolecules involved in tumor progression of cutaneous
malignant melanoma and their clinical significance**

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INTRODUCTION

Cutaneous melanoma (CM) is one of the most aggressive forms of skin cancer, characterized by high genetic heterogeneity, unpredictable evolution, resistance to therapy, increasing incidence and a mortality rate that remains high, despite the use of new molecular therapies. Globally, according to statistical projections, by 2040 the incidence of cutaneous melanoma will increase by 50% of new cases, and the number of deaths by up to 68%, if the current upward trend is maintained. The main risk factor for melanoma is exposure to ultraviolet (UV) radiation, which determines 60-70% of cases, and familial predisposition or a skin phenotype more sensitive to solar exposure may increase vulnerability to the induction of melanocyte transformation into tumor cells (Arnold M., 2023 S., Waseh, 2023). Therefore, there is a real need to evaluate the prognostic capacity of MC, knowing that early diagnosis and assessment of the risk of tumor progression, a factor that influences the increase in survival rate. Currently, studies are exploring the prognostic value of new biomolecules with a role in assessing the risk of tumor progression or predicting the response to systemic therapy, which would determine increased efficiency of disease monitoring and individualization of the therapeutic strategy.

Another particularly important aspect that justifies the need for investigation is the fact that, in advanced cutaneous melanoma, distant metastases remain the main cause of melanoma-related deaths despite to the new molecular therapies used with remarkable benefits, and the tumoral resistance to therapy that continues to determine unfavorable clinical outcomes. Survival rates have improved significantly after the approval of specific monoclonal antibodies targeting immune checkpoints, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) in 2011, and subsequently, in 2014, of antibodies specific for programmed cell death protein-1 (PD-1), in the therapy of advanced melanoma (Borst, J., 2021; Boutros, A., 2024; Gao, M., 2024). However, although ICI (immune checkpoints inhibitors) therapy has resulted in substantial increases in clinical benefit in these patients, not all tumors have regressed following immunotherapy, or clinical outcomes have not been equally durable for all patients. The percentage of patients with long-term benefit is estimated to be between 20-40% when immunotherapeutic agents are administered as monotherapy, and can increase to 60% when ICI agents are combined; however, the remaining patients treated similarly have developed resistance to treatment. Overcoming tumor resistance to systemic therapy is the goal of numerous studies, given the aggressiveness with which tumor invasion occurs in other distant organs, sometimes even

in cases where the primary melanoma tumor was excised in the initial stages of development (Machiraju, D., 2021; Thornton, J.; 2022).

Given these concerns and the current state of knowledge of factors that may influence tumor progression of cutaneous melanoma or that may contribute to increasing the capacity to assess the risk of progression, this doctoral thesis investigates biomolecules that have biomarker potential, from the category of serological biomarkers, circulating biomarkers as products of tumor tissue and tissue biomarkers, which may represent valuable sources in monitoring the evolution of the disease, from the initial stages, or in predicting the response to immunotherapy of patients with advanced cutaneous melanoma.

PURPOSE AND OBJECTIVES OF THE THESIS

The purpose of the doctoral thesis was to evaluate the expression of a set of biomolecules involved in tumor progression, aiming to highlight their variation at the circulatory or tissue level, simultaneously with the evaluation of the immune and inflammatory status in patients with cutaneous melanoma, at different stages of evolution and treatment, with significance in assessing the prognosis of the disease.

The main objectives of the thesis were focused on two directions:

1. Highlighting the relationship between the level of soluble biomarkers specific to cutaneous melanoma with the different stages of disease evolution and establishing possible correlations with tissue immunohistochemical markers, used in the diagnosis and prognosis of cutaneous melanoma. It was also aimed to characterize the systemic immune response during therapy with immune checkpoint inhibitors, anti-PD-1, by analyzing the dynamics of the main lymphocyte subpopulations and their association relationship with inflammatory or tumor markers in the circulation, analyzed in patients diagnosed with advanced cutaneous melanoma.

2. Development of an *in vitro* experimental model with the aim of evaluating the oncolytic effects of agents with anti-tumor potential, evaluated in the human melanoma cell line SK-MEL-24 and highlighting the induced changes in the processes of cell division, proliferation, apoptosis, cytokine release and expression of genes involved in cell adhesion, treatment resistance or apoptosis.

To achieve the proposed objectives, the studies were carried out in several stages, pursuing the following **specific objectives**:

➤ Quantification and evaluation of the expression of soluble proteins S100B and MIA as biomarkers of tumor progression in patients diagnosed with cutaneous melanoma and

highlighting the type of correlations established between the serum/plasma level of these proteins with the different stages of disease evolution

➤ Identification and analysis of potential correlations between circulating tumor biomarkers (S100B, MIA) and tissue markers specific to cutaneous melanoma (S100, MelanA, gp100/HMB45) as diagnostic and prognostic markers, frequently used in routine histopathology by immunohistochemical analysis (IHC).

➤ Highlighting in dynamics, the variation in the level of the main lymphocyte phenotypes (total T lymphocytes ($CD3^+$), helper T lymphocytes ($CD3^+CD4^+$), suppressor/cytotoxic T lymphocytes ($CD3^+CD8^+$), the ratio between helper T lymphocytes/cytotoxic T lymphocytes ($CD4^+/CD8^+$), receptors for total B lymphocytes ($CD19^+$) and NK cells ($CD3^+CD16^+/CD56$)) during therapy with anti-PD1 monoclonal antibodies (Nivolumab), in patients with advanced cutaneous melanoma.

➤ Exploring possible correlations between immune cell populations/subpopulations, systemic inflammatory markers (systemic immuno-inflammatory index (SII), systemic inflammatory response index (SIRI), neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR) and monocyte/lymphocyte ratio (MLR), pro-inflammatory markers (cytokines IL-6, $TNF-\alpha$) and/or tumor markers (lactate dehydrogenase (LDH), S100B protein) with significance in the evaluation and monitoring of melanoma progression during immunotherapy.

➤ Modulation and highlighting the cytolytic effects induced on the human melanoma cell line SK-MEL-24 by cytotoxic compounds - Carboplatin, Paclitaxel, immune checkpoint inhibitors - Nivolumab, Ipilimumab or natural compounds - Resveratrol and Quercetin, administered in different treatment combinations, by evaluating cell proliferation vs. dependent cellular cytotoxicity of compound.

➤ Evaluation of the modulators impact on apoptosis, cell cycle phases, cytokine release (IL-6, $TNF-\alpha$) in cell culture supernatants or the expression of genes encoding proteins involved in cell adhesion processes (intercellular adhesion molecule 1/ICAM-1), cellular resistance to treatment (mdr-1/P-glycoprotein, protectin/CD59) or apoptosis (mdm-1, p53, bcl-2).

ORIGINALITY OF THE WORK

➤ The doctoral thesis addresses various aspects of a molecular nature that contribute, through their association, to the achievement of a synthesis of information with significance in establishing the prognosis of the evolution of cutaneous melanoma.

➤ During the thesis, the variations of an original set of cellular and molecular components involved in tumor transformation and invasion are explored, whose serological or tissue level, corroborated with the stage of evolution and histopathological characteristics, can lead to an increase in the capacity to assess the risk of tumor progression, as well as to the establishment of a therapeutic conduct adapted to the characteristics of the disease for each patient.

➤ In-depth statistical analysis led to the identification of statistically significant correlations established between proteins released into the circulation as products of tumor cell activity, the innate and adaptive immune system response, simultaneously with the assessment of the inflammatory response, in the context of immune system stimulation with anti-PD-1 antibodies, aspects that reflect both tumor activity, with its aggressive potential, and the anti-tumor activity of the organism.

➤ Development of an original *in vitro* experimental model that demonstrates the cytotoxic effects exerted by a diverse panel of compounds, used in different combinations, concentrations and administration times, in order to obtain the most efficient conditions for maximum lysis effects of tumor cells in human melanoma culture, investigated using a wide range of modern techniques, optimized and adapted to the intended purpose (cell cultures, immuno-enzymatic assays, flow cytometry, proliferation and cytotoxicity assays, real-time cell analysis (RTCA), qRT-PCR).

EXPERIMENTAL PART – OWN RESEARCH

1. SOLUBLE PROTEINS S100B AND MIA AT DIFFERENT STAGES OF EVOLUTION IN CUTANEOUS MELANOMA

1.1. Materials and methods

1.1.1. Patient group and biological samples

The study retrospectively evaluated the serum level of S100 protein and the plasma level of MIA protein in a group of 176 patients, including 78 women and 98 men, with a mean age of 56.2 ± 12.08 years, registered during 2016-2022, at the Oncological Institute Prof. Dr. Alex. Trestioreanu, Bucharest, diagnosed with cutaneous melanoma in different stages of evolution. The studies were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Oncological Institute Prof. Dr. Alex. Trestioreanu, Bucharest (ethics code registered with numbers 21317/24.11.2016, 13463/26. 06. 2020).

The biological samples included in the study group were represented by peripheral blood from which serum and plasma fractions were obtained. The collection and processing of the samples was carried out depending on the clinical stage of the patient, namely: pre/postoperative, periodic control or during the therapy period (immunotherapy).

1.1.2. Quantitative determination of soluble S100B protein by enzyme immunoassay assay (EIA)

Quantitative determination of S100B protein in serum was performed using the CanAg S100 EIA (Enzyme Immunoassay) kit, manufactured by Fujirebio Diagnostics AB, Gothenburg, Sweden. This immuno-enzymatic method is of a non-competitive type, is carried out in two steps and is based on two monoclonal antibodies specific for different epitopes (S100A1B and S100BB) expressed by S100.

1.1.3. Quantitative determination of soluble MIA protein by enzyme-linked immunosorbent assay (ELISA)

To investigate the MIA protein, was used the photometric immunodetection kit human MIA ELISA (Enzyme-Linked Immuno-Sorbent Assay), produced by Roche Diagnostics GmbH, Mannheim, Germany, which allows the quantitative determination of human MIA protein in serum or plasma, using streptavidin-coated plates.

1.2. Results and discussion

The results were interpreted according to the stage of disease progression and were organized into 4 subgroups of patients (stage I, II, III, IV), and according to gender, into 2

other subgroups (women and men). Figure 1 graphically represents the percentages of patients who presented S100B and MIA protein values above normal limits, these being analyzed for each stage of disease progression.

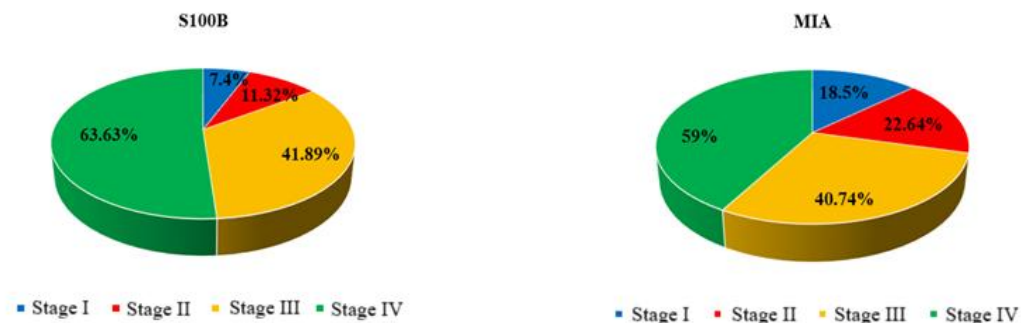


Figure 1. Percentage of patients who presented values higher than the reference value for S100B and MIA proteins, depending on the stage of disease evolution

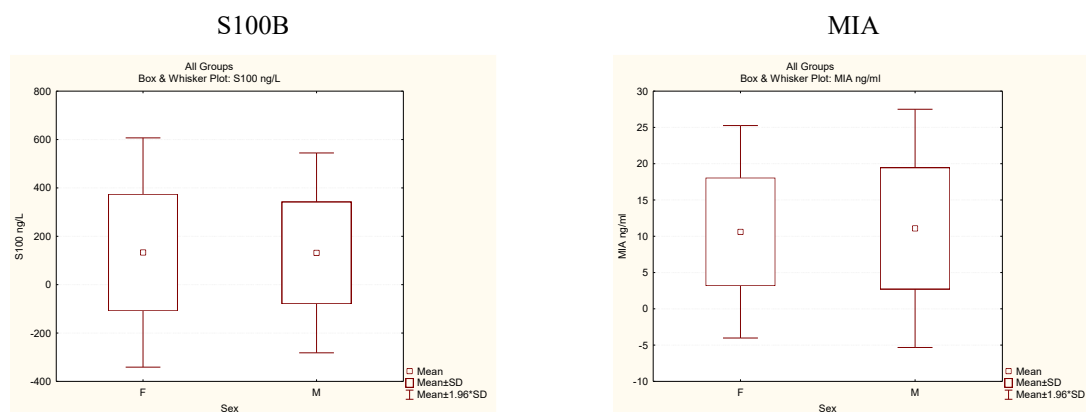


Figure 2. Graphical representation of circulating S100B and MIA protein values according to gender of the patient group

Regarding the levels of S100B and MIA protein concentrations, no significant differences were found between women and men, but a higher standard deviation was determined for S100B protein in the women's group and for MIA protein in the men's group. Also, the simultaneously increased concentrations of S100B and MIA proteins for 40.90% of stage IV patients prove and support the correlation between the blood levels of the two markers, this correlation being confirmed by the Pearson correlation coefficient r obtained for S100B vs. MIA, $r = 0.662$, $p < 0.001$.

2. EXPRESSION OF TISSUE PROTEINS S100, HMB45, MELAN A AND THEIR CORRELATION WITH SOLUBLE PROTEINS S100B AND MIA

2.1. Materials and methods

2.1.1. Patient group and biological samples

The evaluation of the cellular expression of the markers S100, MelanA, HMB45 was performed on melanoma tissue sections obtained after surgery from 76 patients who are part of the main study group (n = 176). Fragments of skin lesions were harvested from different areas of the body; trunk (n = 38), upper (n = 11) and lower limbs (n = 18), head and neck (n = 4), pelvis/hips (n = 5), for which the three melanoma-specific markers were evaluated by immunohistochemical analysis (IHC).

2.1.2. Qualitative determination of tissue proteins specific to cutaneous melanoma by immunohistochemistry (IHC) method

The IHC method is based on the detection and localization of antigens on tumor tissue sections based on the principle of specific antigen-antibody (Ag-Ab) binding. The analyzed tumor tissue was subjected to fixation, staining and microscopic visualization. Immunohistochemical analysis was performed using a set of three primary Ab (1) anti-S100 (clone: 4C4.9) (2) anti-MelanA (clone: A103) (3) anti-melanosome gp100 (clone: HMB45).

2.2. Results and discussion

The results were analyzed by structuring them into three subgroups, depending on the positive expression of the S100, MelanA and HMB45 proteins, respectively: group for each marker, group for tissue with 2 positive markers and group with 3 markers. Table 1 shows the results for the entire group of patients (n=76) and for the groups that include one tissue marker each in association with the circulating level of the two soluble proteins S100B and MIA.

Table 1. Association between circulating S100, MIA protein concentration and individual tissue expression of S100, HMB45 and/or MelanA markers

	IHC group n (%)	S100 tissue n (%)	HMB45 tissue n (%)	MelanA tissue n (%)
Number of patients	76	51	61	65
Soluble MIA				
MIA ⁽⁺⁾	31 (40,79%)	22 (43,14%)	24 (39,34%)	28 (43,07%)

MIA ⁽⁻⁾	45 (59,21%)	29 (56,86%)	37 (60,66%)	37 (58,46%)
Soluble S100B				
S100B ⁽⁺⁾	15 (19,74%)	11 (21,57%)	7 (11,48%)	12 (18,46%)
S100B ⁽⁻⁾	61 (80,26%)	40 (78,43%)	54 (88,52%)	53 (81,53%)
Soluble S100B + MIA				
S100B ⁽⁺⁾ /MIA ⁽⁺⁾	9 (11,84%)	7 (13,73%)	4 (6,56%)	8 (12,31%)
S100B ⁽⁺⁾ /MIA ⁽⁻⁾	6 (7,89%)	4 (7,84%)	3 (4,92%)	4 (6,15%)
S100B ⁽⁻⁾ /MIA ⁽⁺⁾	22 (28,95%)	15 (29,41%)	20 (32,79%)	20 (30,77%)
S100B ⁽⁻⁾ /MIA ⁽⁻⁾	39 (51,32%)	25 (49,02%)	34 (55,73%)	33 (50,77%)

MIA⁽⁺⁾ – MIA protein concentration is higher than the reference value (9.4ng/mL); MIA⁽⁻⁾ – MIA protein concentration is lower than the reference value; S100B⁽⁺⁾ – S100B protein concentration is higher than the reference value (90 ng/L); S100B⁽⁻⁾ – S100B protein concentration is lower than the reference value;

The study of melanoma-specific circulating markers, simultaneously with tissue-level evaluation, revealed that patients who had MIA protein at a circulating level with values above the normal limit showed positive tissue expression for S100 protein in 43.14% of cases; 39.34% of cases were positive for HMB45 protein and 43.07% of samples expressed MelanA protein. It was also observed that patients whose S100B and MIA values were within normal limits at the circulatory level (S100B⁽⁻⁾/MIA⁽⁻⁾) simultaneously expressed the three tissue markers in approximately 55% of melanoma tissue samples. The analysis of statistical correlations showed that the highest correlation coefficient r was obtained between HMB45 and MelanA ($r = 0.623$), but also between S100 and MelanA ($r = 0.610$), both correlations having a strong degree of statistical significance, with a p value < 0.001 .

3. VARIATION OF T, B LYMPHOCYTES AND NK CELLS IN THE DYNAMICS OF IMMUNOTHERAPY IN PATIENTS WITH ADVANCED MALIGNANT MELANOMA

3.1. Materials and methods

3.1.1. Patient group and biological samples

The study included 20 patients (13 women/7 men, mean age: 57.5 ± 12.6 years) diagnosed with malignant cutaneous melanoma, in stages III ($n = 8$) and IV ($n = 12$) and a control group of healthy volunteers ($n = 20$). Biological samples were represented by peripheral blood collected in dynamics throughout the study, from patients with advanced melanoma treated with Nivolumab. Simultaneously, complete blood count, biochemical

analyses, lymphocyte immunophenotyping analysis were determined, performed for all samples collected in dynamics (n = 104).

3.1.2. Quantitative determination of immune system cells by flow cytometry (immunophenotyping)

The experiments to evaluate the expression of lymphocyte receptors were performed using the BD Stimulitest TMIMK PLUS kit (Becton Dickinson & Co., BD Biosciences, San Jose, USA). This kit uses the direct immunofluorescence method by labeling with two fluorochromes, fluorescein isothiocyanate (FITC) and phycoerythrin (PE), necessary for the identification of lymphocyte populations and subpopulations in peripheral blood and allowed the determination of the percentage of the three populations: T lymphocytes (CD3⁺), B lymphocytes (CD19⁺) and NK cells (CD16⁺/CD56⁺).

3.2. Results and discussion

The T lymphocyte subsets, B lymphocytes and NK cells were determined for each patient for a minimum of 3 cycles of Nivolumab administration. Figure 3 shows the proportion (%) of patients who recorded an increasing, stationary or decreasing trend in values, determined during Nivolumab therapy and monitoring of lymphocyte immunophenotypes, for each immunological parameter.

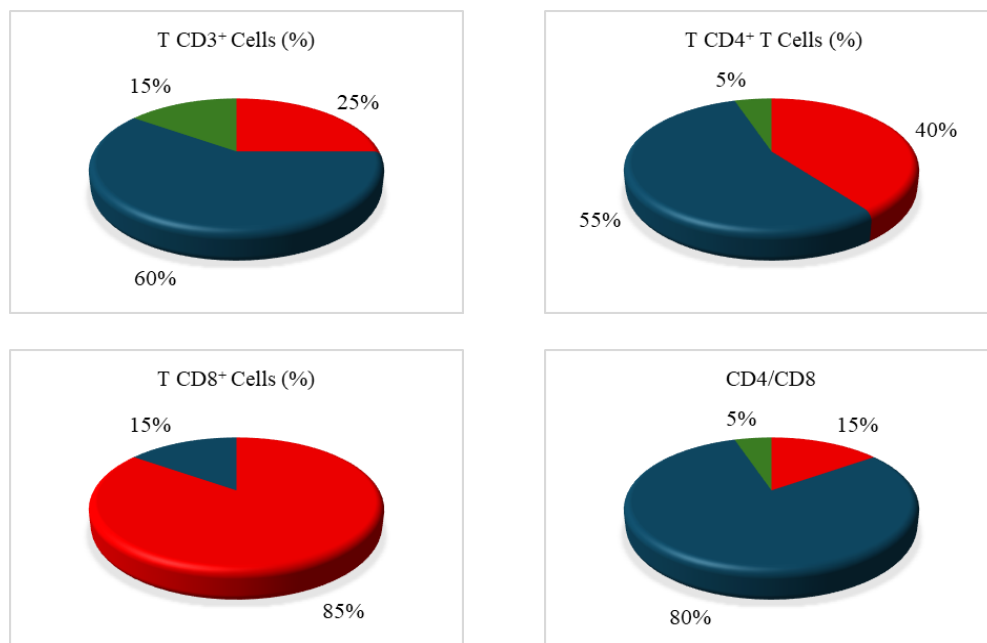


Figure 4. Heatmap correlogram for the analysis of correlation coefficients r between immune cellular components analyzed in patients with advanced melanoma treated with Nivolumab

From the analysis of the results, the negative correlation between NK cells and CD19⁺ B lymphocytes was observed, this is supported by the results obtained by the analysis of the variation trend, which showed a decrease in the percentage of B lymphocytes present in the circulation, with the mention that the variation remained within normal limits for all patients, while the percentage of NK cells showed an increasing trend during the treatment with Nivolumab. Also, the percentage of cytotoxic CD8⁺ T lymphocytes and NK cells increased moderately during the immunotherapy with Nivolumab, and 30% and 15% of these patients, respectively, showed values higher than the upper normal limit for the two parameters analyzed. The maintenance of B lymphocytes within normal limits suggests the stability of the immune response in terms of anti-tumor action through the production of Ac or the antigen presentation function and are associated with a good prognosis of the disease evolution.

4. STUDY OF CORRELATIONS BETWEEN LYMPHOCYTE POPULATIONS, INFLAMMATORY MARKERS AND CIRCULATING TUMOR MARKERS

4.1. Materials and methods

4.1.1. Patient group and biological samples

The group of patients analyzed was represented by the same group of patients diagnosed with melanoma in stages III and IV ($n = 20$) treated with nivolumab, for whom immune cell phenotype analysis was performed, from the total samples ($n = 104$) were selected samples collected at 3-month intervals.

4.1.2. Determination of systemic inflammatory markers (NLR, MLR, PLR, SII, SIRI)

The following ratios were calculated based on data provided by the complete blood count from peripheral blood samples: neutrophil/lymphocyte ratio (NLR), monocyte/lymphocyte ratio (MLR), platelet/lymphocyte ratio (PLR), systemic immune-inflammatory index (SII), systemic inflammatory response index (SIRI).

4.1.3. Quantitative determination of pro-inflammatory cytokines IL-6, TNF- α and serum tumor markers S100B and LDH

Detection of serum levels of IL-6 and TNF- α cytokines was performed using the Human IL-6 and Human TNF-alpha Uncoated ELISA immunoenzymatic kits, produced by Invitrogen, Bender MedSystems GmbH, Vienna, Austria. The concentrations of S100B proteins and lactate dehydrogenase (LDH) were determined from the same samples.

4.2. Results and discussion

The analysis of correlations between immune cell populations and inflammatory indices revealed the following associations: moderate, negative correlation between NLR ratio and CD3⁺ T lymphocytes, CD4⁺ T lymphocytes and CD19⁺ B lymphocytes; moderate, negative correlation between SIRI and SII indices with CD19⁺ B lymphocytes; strong, positive correlation between SIRI and SII; strong, positive correlation between SIRI/SII with NLR, MLR and PLR. Figure 5 presents graphs for four of the correlations highlighted following the statistical analysis.

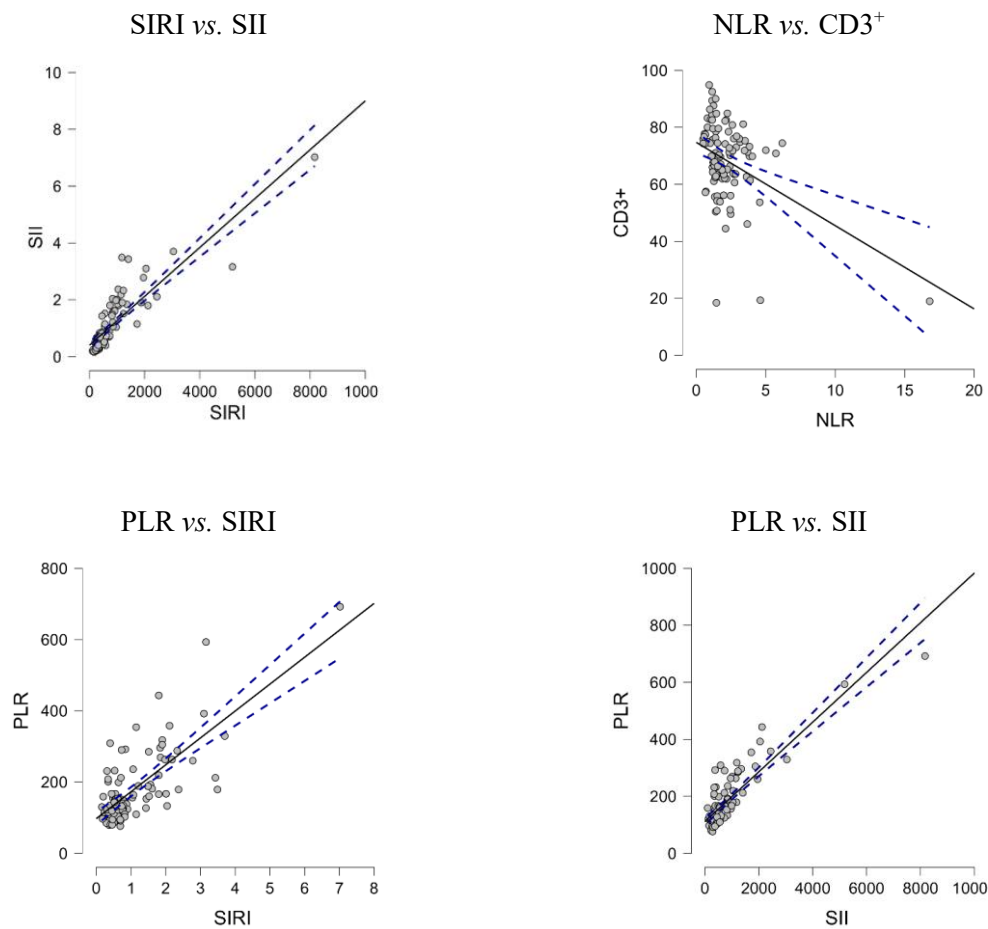


Figura 5. The correlations established between inflammatory factors and populations/subpopulations of immune cells analyzed (— linear regression curve; --- equality line).

Simultaneously with the dynamic evaluation of inflammatory factors during immunotherapy, the analysis of the variation in the release of the two pro-inflammatory cytokines involved in tumor progression, IL-6 and TNF- α , was also performed on the same 3 serum samples collected from the same patient, selected at 3-month intervals T1-T3. In the dynamics of IL-6 cytokine release, the concentrations decreased with the increase in the number of treatment cycles, from sample T1 to sample T3, suggesting a positive influence of the immune response stimulation therapy, which leads to the decrease of pro-inflammatory factors favoring tumor growth and invasion (Figure 6). The variation in the level of TNF- α protein concentrations in the circulation during immunotherapy was insignificant both between the 3 collection times, but also compared to the control group (Figure 7).

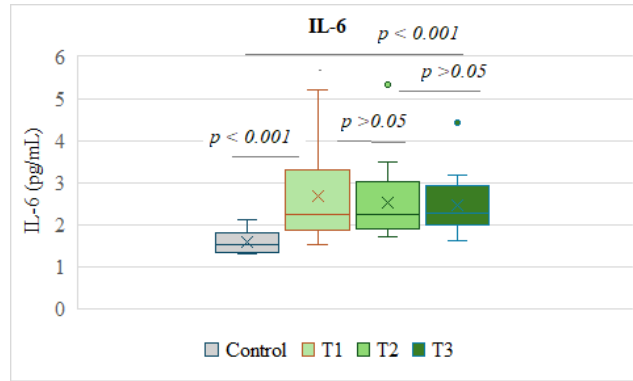


Figure 6. Dynamics of variation in the level of the circulating IL-6 cytokine, during Nivolumab therapy, for 3 collection points (T1, T2, T3) at an interval of 3 months.

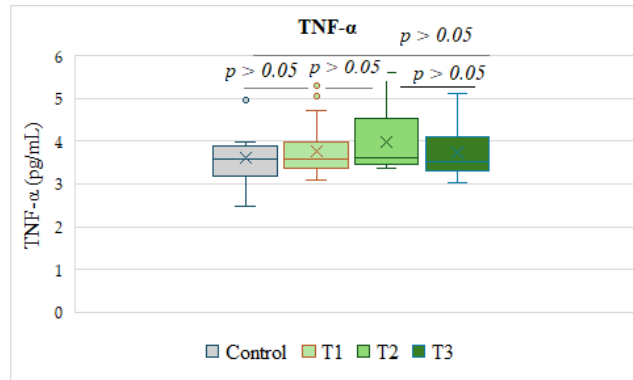


Figure 7. Dynamics of variation in circulating TNF- α cytokine levels during Nivolumab therapy for 3 collection points (T1, T2, T3) at a 3-month interval.

The analysis of correlations between systemic inflammatory markers and soluble tumor markers studied revealed that the serum level of the LDH enzyme was directly correlated with the S100 protein ($r = 0.483$, $p < 0.01$) and the IL-6 cytokine ($r = 0.600$, $p < 0.001$), supporting the involvement of the three serum components in favoring the tumor

progression process, taking into account the role of each biomolecule, but also in resistance to immunotherapy, confirming the value of predictive/prognostic factors of the disease evolution (Figure 8). The TNF- α level did not correlate with any of the two inflammatory markers, and IL-6 established a weak positive correlation with SIRI ($r = 0.297$, $p < 0.01$), but not with the SII index.

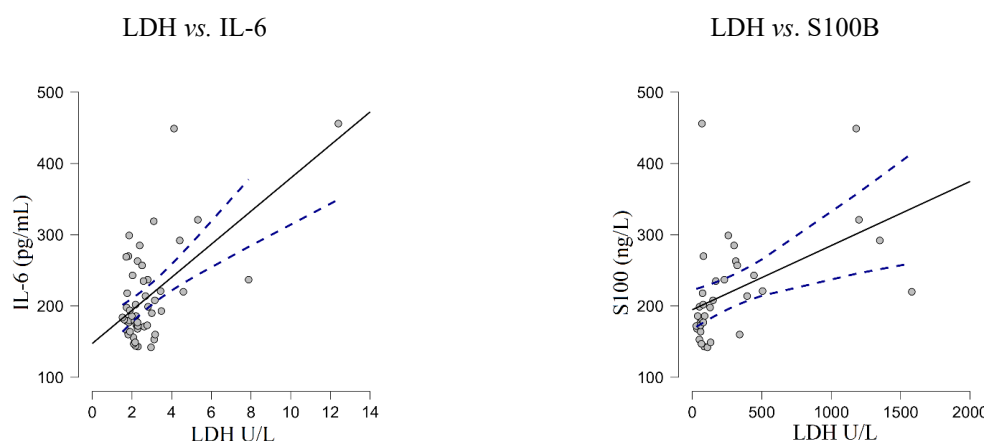


Figure 8. Analysis of correlations between LDH vs. IL-6; LDH vs. S100B; (— linear regression curve; --- equality line).

5. EFFECTS OF SOME MODULATORY AGENTS ON THE HUMAN MELANOMA CELL LINE SK-MEL-24

5.1. Materials and methods

5.1.1. Development of the *in vitro* experimental model

- *In vitro experimental model* was performed on the SK-MEL-24 human melanoma cell line, adherent, with stellate morphology, developed from metastatic tissue from the excised lymph node of a Caucasian patient (male, 67 years old) diagnosed with malignant melanoma American Type Culture Collection (ATCC® HTB-71™, Manassas, VA, USA).
- *SK-MEL-24 melanoma cell line* was cultured in DMEM:F12 medium with 10% SFB, 2 mM L-Gln and a mixture of antibiotics (penicillin/streptomycin), using 25 or 75 cm² culture plates, followed by 24 h/ 37°C incubation in an atmosphere with 5% CO₂ and 95% humidity.

- **Treatments:** After approximately 24 h of culture, when cell confluence is approximately 70% - 80%, the cells were treated for 24 - 48h with different concentrations of oncolytic compounds (Carboplatin, CPt, Paclitaxel, Pxl), monoclonal antibodies (Nivolumab, Niv; Ipilimumab, Ipi) and natural compounds (resveratrolm, Rsv; Quercetin, Qct), administered both individually and in combinations.

5.1.2. Cell viability assessment

It was performed by the colorimetric method (MTS) using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit, (Promega, Madison, WI, USA) which allows the identification of metabolically active cells that have the ability to reduce the MTS compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] to the formazan product. After the incubation period, the colored reaction product was quantified spectrophotometrically by measuring the absorbance at wavelength $\lambda = 492$ nm on a Dynex ELISA plate reader (DYNEX Technologies—MRS, Chantilly, VA, USA). The viability of treated cells was expressed as a percentage in relation to untreated cells (NT), the latter being considered control cells with 100% viability.

The calculation formula used was the following:

Cell viability (%) = $100 \times (T - B) / (C - B)$, where T represents the optical density of treated cells, C is the optical density of untreated cells (control), and B is the optical density of the culture medium without cells (blank).

Cell lysis was expressed based on the cell viability results, using the formula:

Cell lysis (%) = 100% - cell viability (%).

Experiments were performed in triplicate ($n = 3$) and the results were expressed as mean $\pm$ standard deviation (SD), possible non-specific reactions between MTS and the studied compounds were eliminated by measuring the absorbance in the absence of cells and subtracted from the final analysis.

5.1.3. Real-time cell analysis method (RTCA)

The RTCA technique is based on the use of the xCELLigence (DP) system (ACEA Biociences, USA) and the technology of electronic cell sensor arrays, which allows real-time monitoring of several cellular activities, such as cell proliferation vs. cytotoxicity, adhesion, invasion or cell migration. Any change in cell morphology or adhesion determines the alteration of the cell index (CI), which represents a quantitative measure of the number of cells in a well.

5.1.4. Analysis of apoptosis and cell cycle progression

Apoptosis was assessed by the double-label detection method (Annexin V-FITC Apoptosis Detection Kit, Becton Dickinson (BD) Biosciences, Mountain View, CA, USA) with Annexin-FITC and PI; cell cycle phase modulation was achieved by labeling cells with PI (FxCycle PI/Rnase solution, Thermo Fisher Scientific, Invitrogen Life Technologies Corporation, Hillsboro, OR, USA), both of which were followed by data acquisition by flow cytometry using the BD FACS Canto II equipment and Diva 6.2 software installed on the flow cytometer. Apoptotic and necrotic events were assessed with Diva 6.2 software, and nuclear DNA content and cell cycle phases were estimated using ModFITLT 2.3 software (Verity Software House, Topsham, ME, USA).

5.1.5. Determination of IL-6 and TNF- α cytokine release

It was performed by the ELISA immunoenzymatic method and allowed to highlight the impact of single or combined treatments on the secretion of the two cytokines, compared to untreated cells. The quantification of these proteins was performed using the human IL-6 and TNF- α uncoated ELISA kits (Invitrogen, Bender MedSystems GmbH, Vienna, Austria).

5.1.6. Gene expression evaluation by qRT-PCR technique

Gene expression was assessed by the TaqMan method for a series of genes encoding proteins involved in biological processes that favor the growth and development of melanoma cells, or resistance to antitumor therapy, namely TP53, ABCB1, ICAM1, CD59, BCL2, MDM1, using the TaqMan fluorescence methodology (qRT-PCR). The following were used: RNeasy Mini kit (Qiagen, Hilden, Germany) for RNA purification; High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) for reverse transcription of RNA into cDNA; TaqMan – probes for the analyzed target genes. The PCR amplification reaction was performed using 20 ng of total cDNA in a reaction with a total volume of 25 μ L, using the ChromoFour Real Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). All experiments were performed in triplicate. The results were normalized relative to the GAPDH gene and calculated using the $2^{-\Delta Ct/2-\Delta\Delta Ct}$ method.

5.1.7. Statistical analysis was performed using GraphPad Prism version 10.3 (GraphPad Software Inc., San Diego, CA, USA). Results were expressed as mean $\pm$ standard deviation (SD), using Student t-test and ANOVA analyses. *P* values < 0.05 were considered statistically significant. Correlation analysis was performed by calculating the Pearson correlation coefficient (*r*), and correlations were classified according to *r* values as follows:

$r \leq \pm 0.29$, weak correlation, $\pm 0.3 \leq r \leq \pm 0.49$, moderate correlation, $\pm 0.5 \leq r \leq \pm 1$ strong correlation.

5.2. Results and discussion

➤ *Evaluation of treatment-induced cellular cytotoxicity on SK-MEL-24 melanoma cells*

The effects of each compound on cell viability were analyzed and it was found that the induced effects varied in a dose- and time-dependent manner. The viability of human melanoma SK-MEL-24 tumor cells was reduced following treatment at 48h compared to the viability of tumor cells treated at 24h with the same compounds (Figure 9).

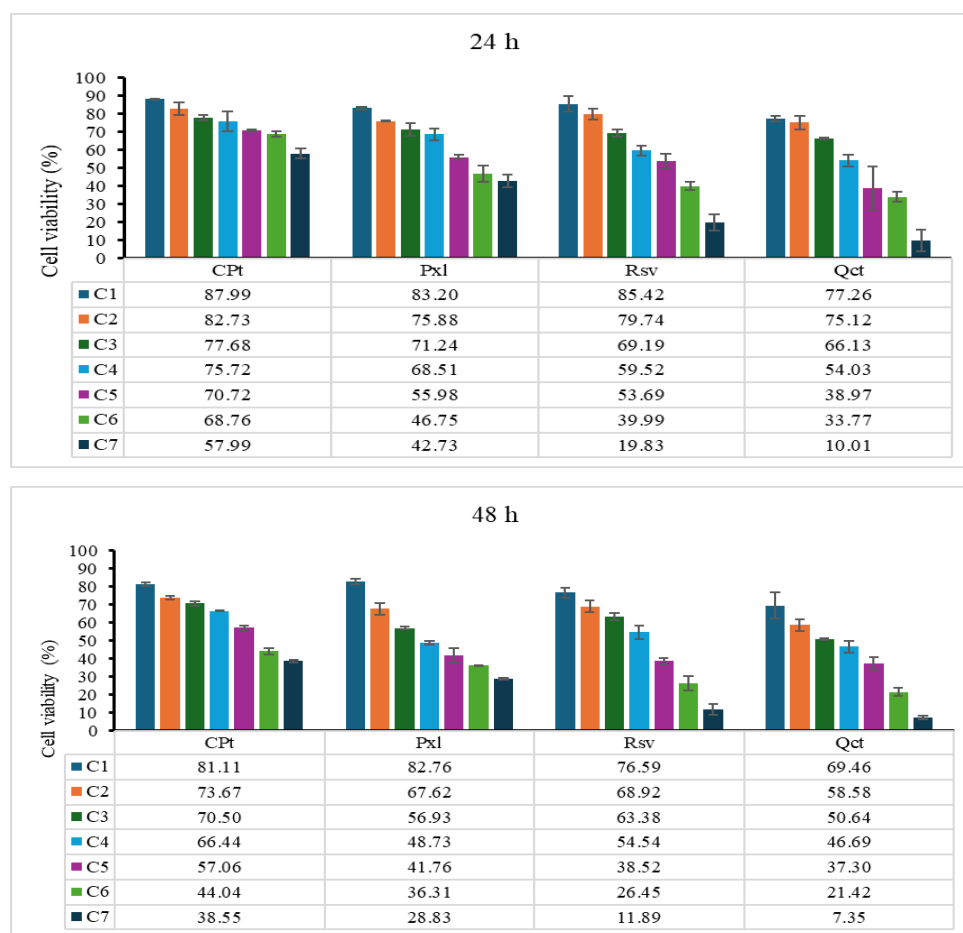


Figure 9. Viability of SK-MEL-24 melanoma cells, treated for 24 and 48 h with increasing concentrations of Cpt, Rsv, Qct and Pxl

Administration of the compounds for 48 h increased the anti-proliferative effects of these compounds compared to 24 h treatments. Based on the results obtained from the MTS test, expressed as percentages of cell viability and accompanied by SD values ($\pm$),

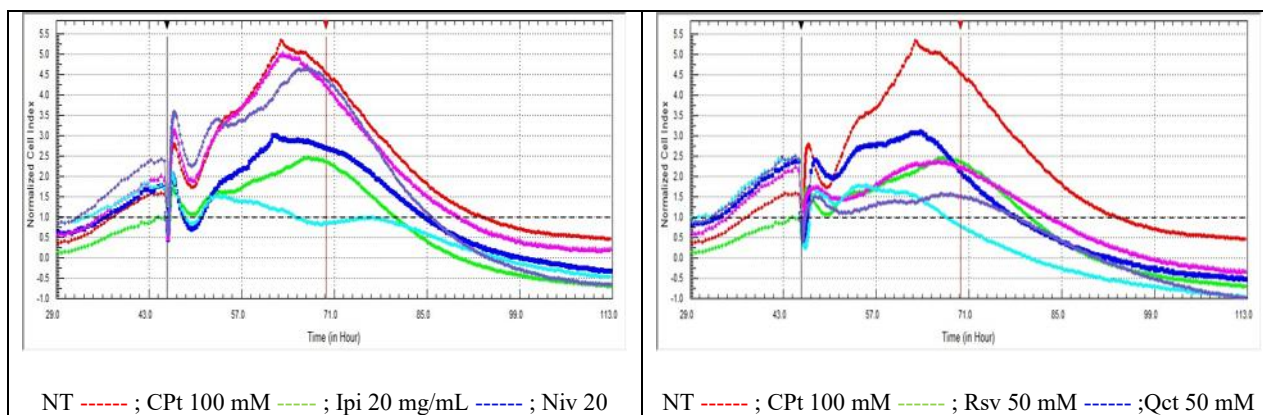
IC₅₀ and IC₇₅ values were calculated for each compound tested on the human melanoma line SK-MEL-24 (Table 2). IC₅₀ and IC₇₅ values represent the concentrations of each compound required to inhibit cell growth by 50% and 75%, respectively, presented as mean $\pm$ SD:

Table 2. IC₅₀ and IC₇₅ values that induce lysis of SK-MEL-24 cells

Compound	IC ₅₀		IC ₇₅	
	24 h	48 h	24 h	48 h
CPt	251,94 $\pm$ 23,24	114,16 $\pm$ 0,28	451,52 $\pm$ 53,31	237,79 $\pm$ 2,31
Pxl	2,56 $\pm$ 0,04	1,31 $\pm$ 0,02	5,21 $\pm$ 0,20	3,69 $\pm$ 0,09
Rsv	83,82 $\pm$ 1,00	52,19 $\pm$ 0,46	166,64 $\pm$ 7,06	133,17 $\pm$ 1,36
Qct	58,97 $\pm$ 3,33	26,83 $\pm$ 5,62	136,67 $\pm$ 0,10	115,84 $\pm$ 2,61

➤ ***Real-time analysis of melanoma cell activity, under the action of individual or combined treatments***

Following the analysis of the effects of the administration of cytostatic agents, CPt or Pxl as mono-treatment on melanoma cells, a significant reduction in the cell index was found, demonstrating the strong inhibition of cell proliferation, this inhibition being directly associated with the cytotoxic effects induced by these compounds. Combined treatments, such as CPt with Ipi, Rsv or Qct, showed a decrease in CI compared to single treatments, suggesting an increase in the cytotoxic effect compared to the effects of individual treatments. SK-MEL-24 cells were treated when the cell index (CI) reached the value of 1. The black horizontal dotted line represents the reference line for normalized CI = 1.0. In figure 10 the viability curves of the treated cells are represented and compared with those recorded for untreated cells (NT).



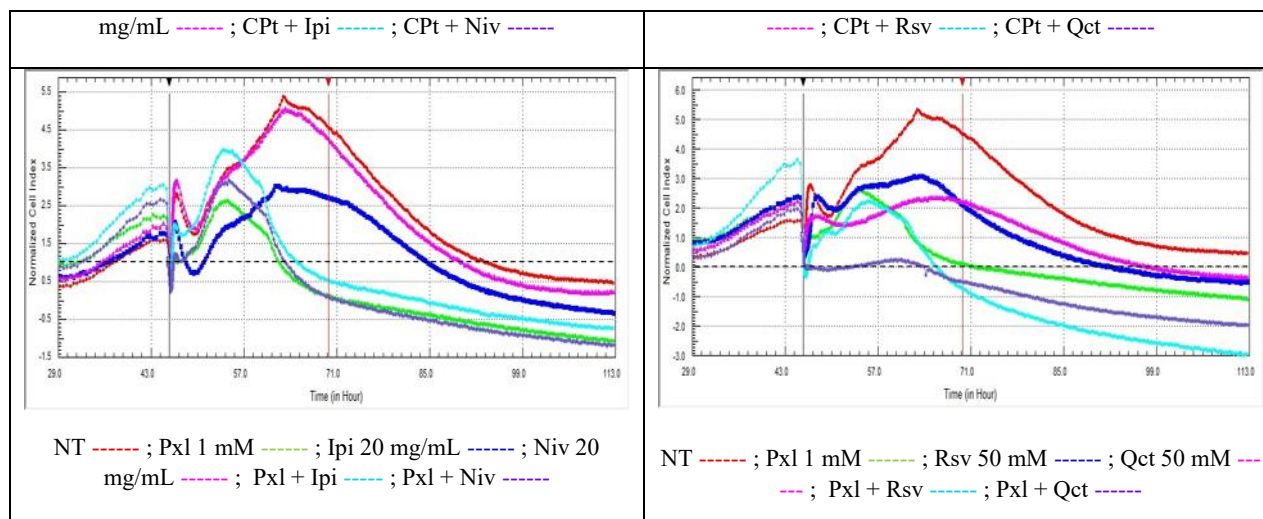


Figure 10. Real-time monitoring of cytolytic effects, mediated by compounds administered individually or in combinations on human melanoma SK-MEL-24 cells.

➤ **Evaluation of gene expression associated with the biological processes studied**

Figure 11 graphically presents the results of gene expression modulation, in which the increase in CD59 gene expression is observed for each compound administered individually. The strongest gene modulation effect was obtained on the MDM1 gene, which following the administration of Rsv 50 μ M recorded a 66.24-fold increase, while Qct 50 μ M determined a 679.25-fold increase in MDM1 gene expression compared to the control.

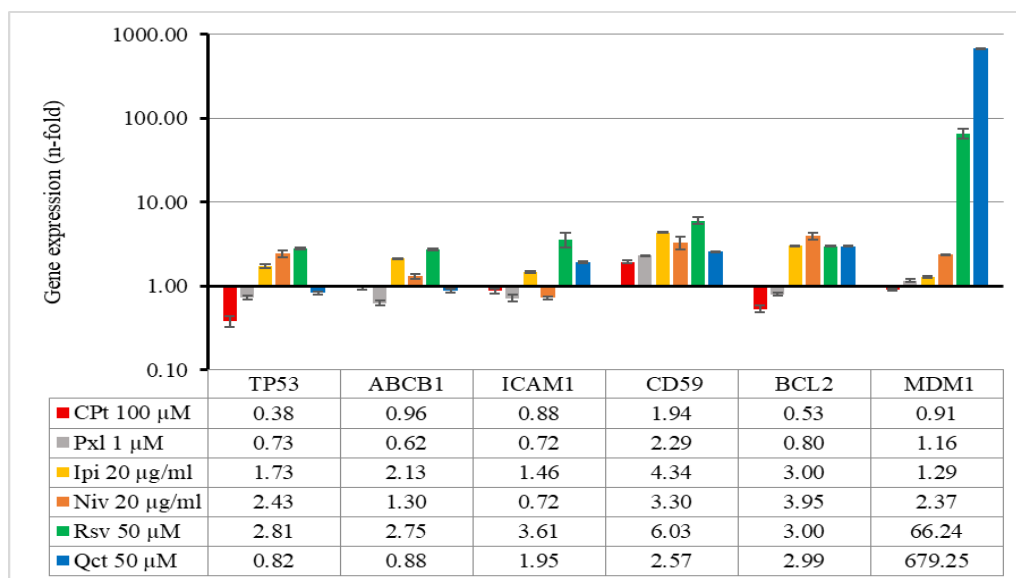


Figure 11. Real-time PCR analysis of the modulation of TP53, BCL-2, MDM-1, ICAM-1, ABCB1 and CD59 gene expression, following single treatments with the chemotherapeutic agents included in the study of SK-MEL-24 melanoma cells

Combined treatments with CPt, administered to melanoma cells, determined an increase in gene expression for TP53, ABCB1, CD59, BCL-2 and MDM1, but a significant increase in gene expression was exerted on the MDM1 gene by the combination of CPt and Ipi, with a 372.99-fold increase.

Also, the combination of CPt and Niv induced an increase in the expression of the TP53, ABCB1, CD59, MDM1 genes, but also a reduction in the expression of the BCL2 gene. The combination of CPt with Rsv led to gene expression modification effects, increasing, for ABCB1 (2.31-fold), ICAM1 (2.71-fold) and CD59 (1.86-fold), and in the case of the combination with Qct, increases in gene expression were observed for ICAM1 (2.10-fold) and MDM1 (2.37-fold).

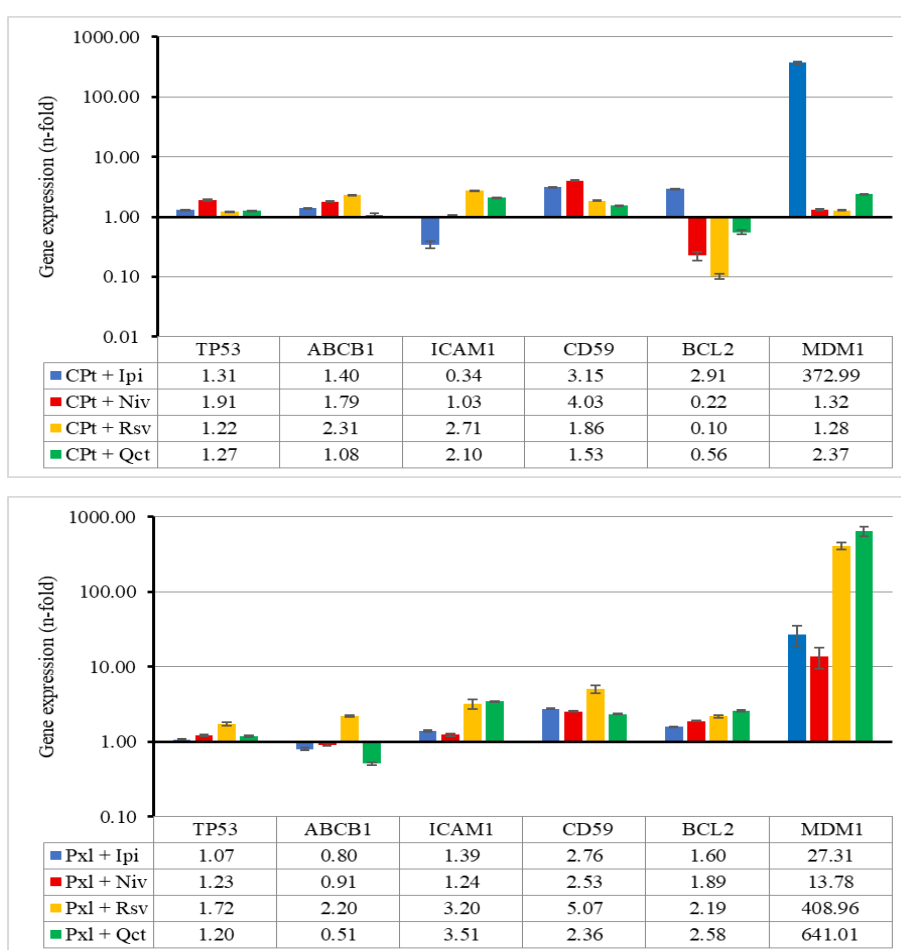


Figure 12. Real-time PCR analysis of the modulation of TP53, BCL-2, MDM-1, ICAM-1, ABCB1 and CD59 gene expression following treatment of SK-MEL-24 melanoma cells with combinations of CPt with mAb or natural compounds

Also, following combined treatments with Pxl, it was found that the strongest effect was that of increasing MDM1 gene expression under the modulating action of

combinations of Pxl + Ipi (27.31 times), Pxl + Niv (13.78 fold), Pxl + Rsv (408.96 fold) or Pxl + Qct (641.01 fold).

➤ ***Evaluation of apoptosis modulation in SK-MEL-24 melanoma cells***

SK-MEL-24 melanoma cells cultured for 24 h were treated with the studied compounds, at different concentrations. The results revealed an increase in the induction of early apoptosis following 24 h treatments with a single compound or combinations of the tested compounds, compared to untreated cells (Table 3). Significant effects were revealed in the case of treatments in which CPt was associated with the natural bioactive compounds Rsv or Qct, reaching values of apoptotic cells of up to 10.7% and, respectively, 12.5% of the treated melanoma cells. Also, treatments in which Pxl was associated with Rsv or Qct led to the induction of early apoptosis in 8.9% and 12.9% of the cells, respectively.

Table 3. Apoptotic effects induced by individual or combined treatments

Treatment administrated	Apoptotic events (%) ($\pm$ SD)		
	Early apoptosis	Late apoptosis	Total events
NT	3.2 $\pm$ 0.87	3.2 $\pm$ 0.54	6.4 $\pm$ 0.08
CPt (100 μ M)	11.4 $\pm$ 0.08	3.9 $\pm$ 0.87	15.3 $\pm$ 1.23
Pxl (1 μ M)	13.5 $\pm$ 0.40	6.6 $\pm$ 0.73	20.1 $\pm$ 0.79
Ipi (20 μ g/mL)	6.3 $\pm$ 0.32	2.1 $\pm$ 0.12	8.4 $\pm$ 1.86
Niv (20 μ g/mL)	8.5 $\pm$ 1.87	4.3 $\pm$ 0.84	12.8 $\pm$ 0.09
Rsv (50 μ M)	5.5 $\pm$ 0.96	6.8 $\pm$ 0.09	12.3 $\pm$ 0.51
Qct (50 μ M)	13.5 $\pm$ 0.94	8.9 $\pm$ 0.04	22.4 $\pm$ 0.89
Pxl + Ipi	5.2 $\pm$ 0.05	8.8 $\pm$ 0.32	14 $\pm$ 0.45
Pxl + Niv	9.7 $\pm$ 0.04	4.6 $\pm$ 0.71	14.3 $\pm$ 0.54
Pxl + Rsv	8.9 $\pm$ 0.73	13.2 $\pm$ 1.74	22.1 $\pm$ 2.24
Pxl + Qct	12.9 $\pm$ 1.91	10.8 $\pm$ 1.43	23.7 $\pm$ 0.96
CPt + Ipi	3.5 $\pm$ 0.54	4.6 $\pm$ 1.32	8.1 $\pm$ 0.56
CPt + Niv	3.5 $\pm$ 0.92	2.2 $\pm$ 0.04	5.7 $\pm$ 0.40
CPt + Rsv	10.7 $\pm$ 1.59	4.7 $\pm$ 0.42	15.4 $\pm$ 1.43
CPt + Qct	12.5 $\pm$ 1.34	8.8 $\pm$ 1.63	21.3 $\pm$ 1.54

➤ **Evaluation of cell cycle modulation in SK-MEL-24 cells**

Cell cultures treated singly or in combination showed decreased cellular distribution in S phase compared to control cells. Individual treatments with Rsv or Qct induced the highest decrease in nuclei (%) in S phase, up to 3.22% and 0.76%, respectively; and in G0/G1 phase the strongest effect of decreasing the number of nuclei was induced by Pxl, alone or combined with Ipi or Niv (Figure 13).

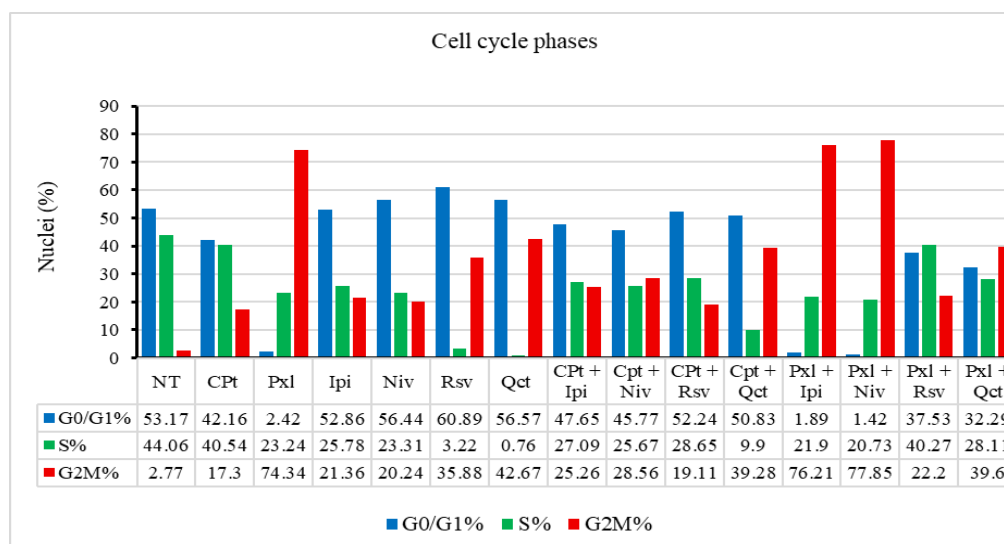


Figure 13. Modulation of cell cycle phases (G0/G1, S and G2M) of human melanoma SK-MEL-24 cells, treated with antineoplastic compounds administered individually or in combinations

The combined treatments showed a significant decrease in S-phase nuclei for the combination of CPt and Qct, up to 9.9%, while the combinations of CPt with Ipi, with Niv and Rsv induced S-phase values below 30%, of 27.09%, 25.67% and 28.65%, respectively, similar to the individual treatments. Similar effects were observed for the combined treatments of Pxl with Ipi (21.09%), Niv (20.73%) and Qct (28.11%) (Figure 13).

Statistical analysis of possible correlations between cell cycle phases and gene expression of the gene set mentioned in the previous section, highlighted a strong positive correlation between the expression of the gene involved in ABCB1 therapy resistance and the G0/G1 phase ($r = 0.528$, $p < 0.05$) and a negative correlation with the proliferation index ($PI = S+G2M$) ($r = -0.528$, $p < 0.05$) (Figure 14).

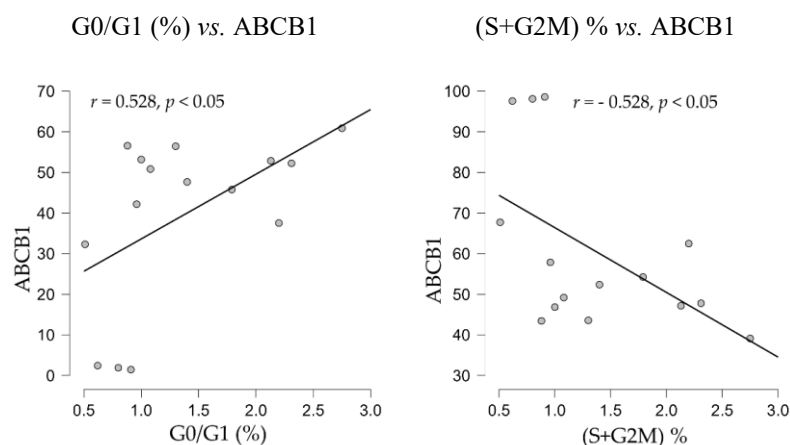


Figure 14. Statistical correlations between cell cycle phases and ABCB1 gene expression

➤ **Evaluation of IL-6 and TNF- α cytokine release modulation in SK-MEL-24 cells**

The results showed that the combined treatments, compared to the individual ones, induced stronger effects of modulation of IL-6 production.

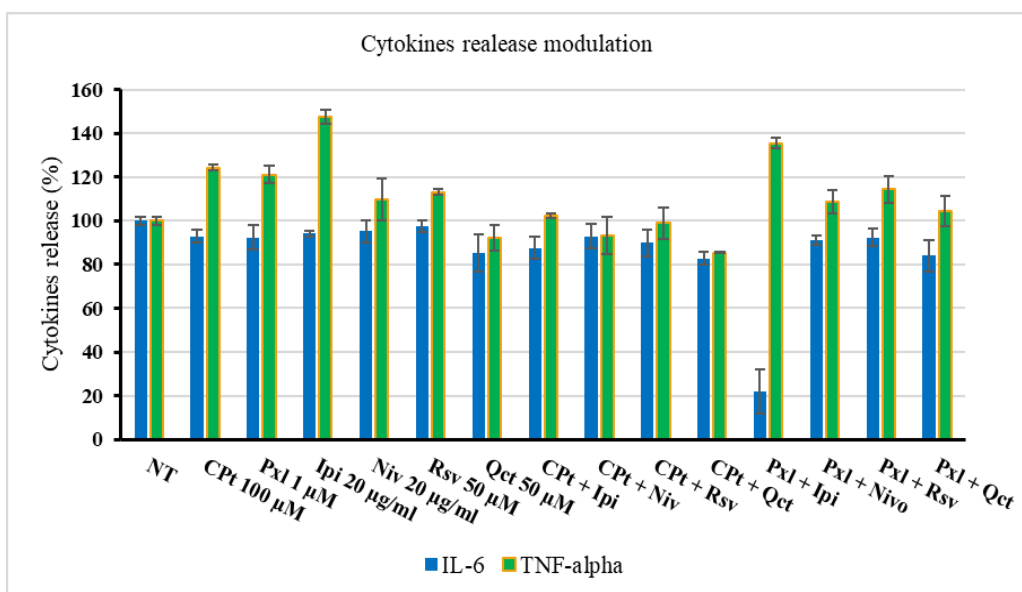


Figure 15. Evaluation of the release of IL-6 and TNF- α cytokines in the supernatant of SK-MEL-24 culture cells, treated with compounds administered individually or in combinations

Thus, the combinations of Cpt or Pxl with Qct determined a reduction in release of 82.84% and 84.02%; also, the combination of Pxl and Ipi determined a decrease of 78.11% (IL-6 release of 21.89%) as an anti-inflammatory reaction. Regarding the modulation of the release of the cytokine TNF- α , a significant effect of stimulating the release of TNF- α

was recorded under the action of the monoclonal antibody Ipi, administered individually or in combination with Pxl, of up to 147.65% and 135.67%, respectively, although the latter in combination with the other compounds, determined a reduction in the release of TNF- α , compared to the single treatments (Figure 15).

These results were also analyzed in terms of various statistical correlations with the results obtained in the evaluation of the modulation of cell cycle progression under the action of anti-tumor agents administered to cells in culture. Thus, it was found that the distribution of nuclei in the G2M phase strongly negatively correlates with the level of IL-6 released in the supernatant of cultured melanoma cells (Figure 16).

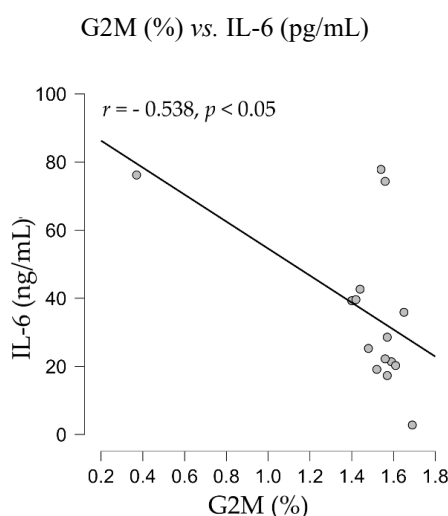


Figure 16. Correlation between cell cycle phases and cytokine release

GENERAL CONCLUSIONS

Cutaneous melanoma (CM) is the most aggressive form of skin cancer. The aggressive, unpredictable nature and genotypic and phenotypic heterogeneity of melanoma represent real challenges in monitoring and establishing the prognosis of the disease evolution. The evaluation of prognostic factors by using molecular tumor markers, both at the time of diagnosis, based on clinical and histopathological characteristics, and during systemic therapies or to monitor the risk of disease recurrence, is recommended by international clinical practice guidelines for diagnosis, treatment and monitoring. The identification of the prognostic potential of molecular and cellular components involved in the development of biological processes that favor tumor progression is urgently needed,

given the continuously increasing incidence and the low survival rate reported for patients with advanced melanoma.

During the doctoral thesis, the following studies were conducted:

1. A retrospective study, which included 176 patients (78 women and 98 men), diagnosed with cutaneous melanoma, at different stages of disease progression, for whom the circulating concentrations of S100B and MIA proteins were determined. In addition, for a number of 76 patients within the group of patients above, the tissue expression of melanoma tissue-specific markers was evaluated, namely: S100, MelanA and HMB45. Statistical analysis of the results allowed the formulation of the following conclusions:

- the level of soluble S100B and MIA expression increases with the stage of disease progression, the proteins being overexpressed in patients with multiple distant metastases, supporting a positive correlation of the concentration level with tumor burden, results that are also confirmed by other research studies;
- the analysis of the correlation coefficient r revealed a direct, highly significant correlation between the two proteins in patients in stages III and IV, which underlines the risk of tumor progression. In stages I and II, no statistical correlation was established between the two proteins.
- the simultaneous investigation of the S100B and MIA proteins demonstrated that in stage IV of evolution, 81.81% of patients presented values above the normal limit for at least one of these proteins, and 40.90% had both proteins with concentrations higher than the normal limit, which demonstrates the usefulness of the simultaneous evaluation of the two markers in determining the risk of melanoma progression.
- in the initial stages, I and II, at least one of the two proteins showed abnormal values for 22.22% and, respectively, 31.98%, percentages that support the prognostic value of soluble S100B and MIA proteins since the initial stages.
- analysis of potential correlations between the tissue expression of S100, HMB45, MelanA proteins and the circulating level of S100B and MIA proteins, showed that the positive tissue expression of the three biomarkers correlates with the increased blood levels of S100B and MIA proteins in stages III and IV.
- after excision of melanoma tissue, it was found that 11.53% of patients who had the three tissue markers expressed intensely positively continued to present elevated levels of S100B protein concentration, indicating possible tumor invasion or the presence of occult tumors and the need for frequent monitoring, even if clinical signs are not detectable.

- The results obtained from this study were disseminated by their publication in the Journal of Personalized Medicine (Bolovan L.M., 2023).

2. A prospective study, in which the immune and inflammatory response was monitored in dynamics for 20 patients diagnosed with advanced MC, in stages III and IV, eligible for anti-PD-1 mAb therapy (Nivolumab). For these patients, the quantifiable variation of populations/subpopulations of T lymphocytes, B lymphocytes and NK cells was monitored during the treatment period, simultaneously with the analysis of inflammatory indicators, calculated based on data obtained for cells of the innate immune system (neutrophils, platelets, monocytes) and the adaptive immune system (lymphocytes), as well as for the pro-inflammatory cytokines IL-6 and TNF- α . Given the prospective nature of this study, the results are preliminary and led to the following conclusions:

- patients with therapeutic benefits during the study recorded a progressive increase in the number of CD16+56+ NK cells and CD8+ cytotoxic T lymphocytes
- the group of patients with tumor progression during therapy showed a tendency to increase the percentage of NK cells in 50% of cases.
- CD19+ B lymphocytes showed a tendency to decrease with the increase in the number of treatment cycles, a tendency that remained within normal limits
- the percentage of CD19+ B lymphocytes correlated negatively with the percentage of NK cells; thus, the decrease in the percentage of B lymphocytes is compensated by the increase in the percentage of NK cells, with the increase in the period of therapy administration.
- the CD4+/CD8+ ratio was below the minimum normal value for a percentage of 35% of patients at the time of study initiation, and 75% of them had an unfavorable evolution. This biomarker has exploratory value, and literature data do not show a uniform evolution of the CD4+/CD8+ ratio.
- statistical correlation analysis showed that low levels of CD19+ B lymphocytes were associated with increased values of SIRI and SII indices, which suggests an unfavorable response to therapy and the presence of systemic inflammation, which contributes to the decrease in survival rate
- the positive, highly significant correlation between SIRI and SII suggests, in the case of high values, a reduced response to immune therapy and an unfavorable prognosis.

- NK cells did not establish any association relationship with the analyzed inflammatory biomarkers, which may lead to support of its value as an independent prognostic factor.
- IL-6 cytokine release was reduced during Nivolumab therapy, which demonstrates an increase in the effectiveness of therapy by decreasing the pro-inflammatory action exerted by IL-6 when it is above normal limits. TNF- α was maintained within normal limits, suggesting a favorable prognosis of the response to therapy.
- the variation in the expression of lymphocyte populations during Nivolumab therapy for patients with CD may represent an important source of therapeutic guidance in the event of the installation of cellular immunosuppression or resistance to treatment, but the results obtained are relative, and therefore investigations on larger groups of patients are necessary.
- the inflammatory markers SIRI, SII, NLR, PLR, MLR integrate cells of the innate and adaptive immune system, and the correlations between these parameters could reflect the acute immune response, oriented towards stimulating the inflammatory process as a process that facilitates tumor progression and resistance to immunotherapy of advanced cutaneous melanoma.
- The results obtained from this study were disseminated by their publication in the journal Biomedicines (Bolovan L.M., 2025).

3. An *in vitro* experimental study, which was carried out on the human melanoma cell line SK-MEL-24, on which the oncolytic potential of a panel of compounds with anti-tumor action, such as CPt, Pxl, Niv, Ipi, Rsv or Qct, was tested, highlighting the impact of these treatments on the processes of cell division, proliferation, apoptosis and the molecular changes induced at the level of some genes involved in tumor progression. The results led to the following conclusions:

- the induced cellular cytotoxicity varied in a dose-dependent manner and the duration of administration of the compounds, so that the effects on melanoma cells in culture were greater after their administration for 48 h compared to the effects obtained after administration for 24 h.
- for the cytostatic compounds Pxl and CPt, after individual administration, it was found that Pxl induced a greater decrease in melanoma cell viability compared to CPt, for all concentrations.

- for the bioactive compounds Qct and Rsv, administered individually, it was observed that the strongest effect of reducing viability was induced by Qct, but both determined a decrease in viability up to 80% in the treated cells, even at the lowest concentrations applied.
- individual treatments with mAb Ipi and Niv revealed similar effects to each other.
- following the combined treatments, it was revealed that Pxl, applied either together with Ipi or Niv, or with Rsv or Qct, determined the greatest decrease in CI in the RTCA test, which suggests a strong action of inhibiting cell proliferation.
- the association between the cytostatic Pxl with the bioactive compounds Rsv or Qct induced the most apoptotic events in SK-MEL-24 melanoma cells, and following the administration of individual treatments, the strongest apoptotic effects were induced by the treatment with Pxl or Qct.
- Pxl, alone or combined with Ipi or Niv, induced the strongest effect of decreasing the number of nuclei in G0/G1, concomitantly with the increase in the percentage of cells in the G2M phase.
- the effects induced by combinations of the cytostatic agents CPt or Pxl with mAb Ipi or Niv confirmed that the association between the DNA damage capacity of cytotoxic agents is potentiated by the presence of mAb, which, although they do not have direct cytotoxic effects on tumor cells, contribute to the enhancement of the cytostatic effect by in vitro induction of non-specific cellular stress on tumor cell culture, causing a reduction in the percentage of cells in the S phase, reflecting the decrease in DNA replication and the arrest of cell proliferation.
- the effects exerted by CPt, Pxl, Qct, Rsv, Ipi and Niv, administered individually or in combination, on the expression of the TP53, BCL-2, MDM-1, ICAM-1, ABCB1, CD59 genes, particularly influenced the induction of MDM1 gene overexpression. Thus, the expression of this gene increased significantly following the application of the following single or combined treatments: Qct (679.25 times), Pxl + Qct (641.01 times), Pxl + Rsv (408.96 times) or CPt + Ipi (372.99 times). These data demonstrate that MDM1 overexpression induces an increase in mitotic stress, stimulating the expression of the p53 protein and the initiation of the apoptosis process.
 - The results obtained from this study were disseminated by their publication in the journal Applied Sciences (Bolovan L.M., 2025).

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DISSEMINATION OF RESULTS

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