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The Effects of Treatment on Peripheral Blood Immune Cell Profile in Pancreatic Ductal Adenocarcinoma (PDAC)

SKAISTE TULYTE[1,2], DAINIUS CHARACIEJUS[3], REDA MATUZEVICIENE[4], AUSRA JANIULIONIENE[4], MANTAS RADZEVICIUS[4], TADAS ZVIRBLIS[5,6] and AUDRIUS SILEIKIS[7]

[1]*Hematology, Oncology and Transfusion Medicine Center, Vilnius University Hospital Santaros Klinikos, Vilnius, Lithuania;*
[2]*Institute of Clinical Medicine, Faculty of Medicine, Vilnius University, Vilnius, Lithuania;*
[3]*Department of Pathology, Forensic Medicine and Pharmacology, Faculty of Medicine, Vilnius University, Vilnius, Lithuania;*
[4]*Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine, Faculty of Medicine, Vilnius University, Vilnius, Lithuania*
[5]*Department of Mechanical and Material Engineering, Vilnius Gediminas Technical University, Vilnius, Lithuania;*
[6]*Institute of Biomedical sciences, Department of Human and Medical Genetics, Vilnius University, Vilnius, Lithuania;*
[7]*Clinic of Gastroenterology, Nephrourology and Surgery, Institute of Clinical Medicine, Faculty of Medicine, Vilnius University, Vilnius, Lithuania*

Abstract. *Background/Aim: This study evaluated whether circulating lymphocytes, assessed by flow cytometry, is a prognostic biomarker in pancreatic ductal adenocarcinoma (PDAC). Patients and Methods: We studied T cell subsets in blood samples from a cohort of 41 patients diagnosed with PDAC. Patients underwent surgery of the primary site and adjuvant chemotherapy or were treated with 1st line chemotherapy (mFOLFIRINOX regimen or gemcitabine alone). The changes in T cell subpopulations during treatment were evaluated at the initial diagnosis before surgery, and after 2 and 4 months. Friedman test was used for statistical analysis. Results: A decline in CD19+ B lymphocytes, natural killer (NK) cells CD3-CD56+CD16+, and T regulatory cells CD4+FOXP3+ during treatment was observed. NKT-like cells CD3+CD56+ and cytotoxic T cells CD3+CD8+ tended to increase after two months and decrease after that. Conclusion: Statistically significant changes in lymphocyte counts in peripheral blood were detected in patients with PDAC during treatment.*

Correspondence to: Skaiste Tulyte, MD, Santariskiu 2, LT-08661, Vilnius, Lithuania. Tel: +37 061480726, e-mail: skaiste.tulyte@santa.lt

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Surgery remains the only primary treatment method of cure for patients with pancreatic ductal adenocarcinoma (PDAC). For neoadjuvant and adjuvant purposes, the mFOLFIRINOX (Oxaliplatin, Irinotecan, Leucovorin, 5-Fluorouracil) chemotherapy regimen is usually used. The mFOLFIRINOX regimen and gemcitabine alone or in combination are indicated for patients with unresectable or metastatic disease. Immunotherapy has long been considered ineffective in PDAC, but some drugs of this class have now been tested in phase III studies (1). Today, studies of the immune system, both in the tumor microenvironment and peripheral blood, remain relevant and may be used for predictive purposes in the future.

Previous investigations have shown that infiltration by cytotoxic CD3+CD8+ lymphocytes is associated with increased overall survival (OS) and disease-free survival (DFS) (2). However, in the tumor microenvironment, effector CD8+ T cells, which can cooperate with pancreatic cancer cells, could be suppressed (3). Although natural killer (NK) cells have been associated with anticancer cytotoxicity, their effects vary across tumors, with some authors associating them with recurrence-free survival in patients with PDAC (4). Increased levels of T regulatory cells have been observed during various periods of PDAC development (5).

Evaluation of circulating T lymphocyte subsets has been the subject of research in human cancer for years. Surgical resection of the pancreas may positively influence the immune system (6). It was suggested recently that minimally invasive resection is associated with increased numbers of circulating regulatory CD4+CD25+CD127low T-cells and reduced CD3+CD4+ T-cell population response (7). Chemotherapy has been shown to both stimulate and inhibit the immune system in the tumor microenvironment (8). Gemcitabine alone appears to activate cellular immunity without altering the proportions of B- or T-cell populations (9). FOLFIRINOX regimen may influence the increase of effector T cells and reduction of suppressor cells in the neoadjuvant setting (10) and various changes in advanced disease (11).

Patients and Methods

We originally included 41 patients who presented with early, advanced, or metastatic PDAC between 2018 and 2019 in the Vilnius University Hospital Santaros Klinikos. Patients with early disease stage were treated only with surgery (stage I) or received adjuvant chemotherapy (stage II, III). Patients with advanced or metastatic disease underwent chemotherapy alone. According to local standards, gemcitabine monotherapy and mFOLFIRINOX regimen were chosen in both adjuvant and metastatic settings. Pancreaticoduodenectomy and resection of the pancreas were acceptable surgical techniques. Gemcitabine was dosed at 1,000 mg/m^2 IV over 30 min; weeks 1 through 8: Weekly administration for the first seven weeks, followed by one week of rest; after week 8: Weekly administration on days 1, 8, and 15 of each 28-day cycle. mFOLFIRINOX regimen consisted of oxaliplatin 65 mg/m^2 IV over 3 h on day 1, irinotecan 150 mg/m^2 IV over 90 min on day 1, Leucovorin (l-LV) 200 mg/m^2 IV over 2 h on day 1, 5-Fluorouracil 2.4 g/m^2 for 46 h continuous infusion and was administered every two weeks. The Vilnius regional bioethics committee approved the study before its initiation in 2017 (158200-17-935-437). Informed consent was obtained and documented from each participant.

Peripheral blood samples were collected before any treatment at diagnosis, two months, and four months after treatment initiation. In total, 121 samples were collected and evaluated. Patient samples were prepared for the eight color cytometric analysis using the Lysed whole blood technique and monoclonal antibodies as surface markers. To obtain absolute cell counts for sample staining and incubation, we used Lyse No Wash technique with TruCount tubes (Becton Dickinson, San Jose, CA, USA). Monoclonal antibodies against the following proteins were used: CD45-V450, CD19-V500, CD3-PerCP, CD4-APC-H7, CD8-PE-Cy7, CD25-FITC, CD127-PE, cFoxP3-APC, CD16-PE, CD56-APC.

Stained samples were acquired on the FACSCanto II flow cytometer (Becton Dickinson), and data were analyzed by FACS Diva version 8.0.2. software (Becton Dickinson). We used the sequential gating technique and biexponential dot plots to reveal desired cell population hierarchies for data display.

Descriptive statistics such as frequency (%) and mean (standard deviation) were calculated for qualitative and quantitative data, respectively. Friedman's one-way repeated measure analysis of rank variance was used to evaluate parameter differences between different time points. A two-tailed *p*-value less than 0.05 was considered to be significant. Statistical analysis was performed using Statistical Analysis System (SAS) package version 9.2, Cary, NC, USA.

Table I. *Summary of clinical characteristics of all 41 patients.*

Characteristics	Value
Cohort size	41
Demographics	
Female (no., %)	25 (61%)
Male (no., %)	16 (39%)
Median age at diagnosis (years)	63.0 (SD 11.84)
Localisation of tumor	
Head	30 (73%)
Body	2 (5%)
Tail	9 (22%)
Tumour stage	
I	1 (2.4%)
II	5 (12.2%)
III	17 (41.5%)
IV	18 (43.9%)
Lymph node status	
N0	11 (26.8%)
N+	30 (73.2%)
Distant metastasis	
M0	32 (78%)
M1	9 (22%)
Tumour grade	
Grade 1	2 (4.9%)
Grade 2	20 (48.8%)
Grade 3	16 (39%)
Indeterminate grade	1 (2.4%)
Not adenocarcinoma histologies	1 anaplastic, 2 indeterminate, 1 acinar, 1 adenosquamous
Type of treatment	
Surgery	18 (43.9%)
Adjuvant chemotherapy	15 (36.6%)
Chemotherapy 1st line	23 (56.1%)
Chemotherapy regimen	
mFOLFIRINOX	13 (34.2%)
Gemcitabine	25 (65.8%)

Results

The median age in the selected cohort was 63.0±11,84 years; 15 (36.6%) individuals underwent surgery and adjuvant chemotherapy, and three (7.5%) surgery alone for early PDAC (Table I). Twenty-three (56.1%) individuals were treated with chemotherapy alone for advanced or metastatic disease. Thirteen individuals (34,2%) received the FOLFIRINOX regimen, and 25 (65.8%) gemcitabine alone in the adjuvant or metastatic setting. Most patients in our cohort had aggressive disease (G2, G3). The results of

Table II. *Changes in parameters over time.*

Parameters	Visit 1 Mean (SD)	Visit 2 Mean (SD)	Visit 3 Mean (SD)	*p*-Value
CA19-9 9 (kU/l)	1047.06 (2,234.99)	848.39 (2,173.91)	1,081.53 (2,772.04)	0.002
WBC ($\times 10^9$/l)	7.47 (2.51)	6.85 (2.97)	6.04 (2.8)	0.012
LYM (%)	29.53 (9.2)	33.53 (11.7)	34.23 (12.36)	0.058
LYM ($\times 10^9$/l)	2.1 (0.79)	2.05 (0.61)	1.90 (0.87)	0.011
CD19 (cells/μl)	201 (99.66)	190.09 (103.01)	185.09 (164.23)	0.012
CD3+CD56+ (cells/μl)	122.86 (103.96)	152.95 (111.77)	128.52 (90.33)	0.012
CD8+CD57+ (cells/μl)	137.28 (126.42)	153.51 (103.55)	155.41 (145.49)	0.157
CD3+CD57+ (cells/μl)	226.87 (195.93)	248.51 (159.77)	245.01 (201.56)	0.303
CD3+ (cells/μl)	1,576.27 (639.49)	1,584.43 (515.35)	1,493.97 (664.5)	0.303
CD3+CD4+ (cells/μl)	1,033.09 (480.23)	1,039.24 (419.32)	949.995 (432.02)	0.310
CD3+CD8+ (cells/μl)	432.09 (211.53)	498.31 (208.78)	482.56 (317.15)	0.005
CD3+CD4-CD8- (cells/μl)	73.44 (100.63)	68.26 (96.68)	62.41 (90.53)	0.070
CD3-CD56+CD16+ (cells/μl)	195.6 (176.34)	143.878 (118.04)	103.25 (93.76)	<0.001
CD3-CD56+CD16- (cells/μl)	82.28 (83.11)	67.9 (74.09)	70.4 (89.85)	0.109
CD4+CD25+CD127+/– (cells/μl)	74.89 (36.65)	64.63 (24)	65.315 (39.96)	0.249
CD4+FOXP3+ (cells/μl)	44.67 (30.7)	30.98 (17.63)	30.656 (21.27)	0.004
CD8+CD25+CD127+/– (cells/μl)	0.86 (1.54)	0.8 (1.61)	0.93 (1.67)	0.219
CD8+FOXP3+ (cells/μl)	0.18 (0.26)	0.3 (0.65)	0.3 (0.54)	0.637

tumor marker CA 19-9, white blood cells, lymphocyte count, and an analysis across subpopulations are shown in Table II.

The decline in CD19+ B lymphocytes [from 201.0 (99.66) to 190.09 (103.01) and 185.09 (164.23) cells/μl $p=0.012$], NK cells CD3-CD56+CD16+ [from 195.6 (176.34) to 143.88 (118.04) and 103.25 (93.76) cells/μl $p<0.001$], T regulatory cells CD4+FOXP3+ [from 44.67 (30.69) to 30.983 (17.63) and 30.656 (21.27) cells/μl, $p=0.004$] was observed at two and four months of follow up. Natural killer T (NKT)-like cells CD3+CD56+ [from 122.85 (103.96) to 152.95 (111.77) and 128.52 (90.33) cells/μl, $p=0.012$], cytotoxic T cells CD3+CD8+ [from 432.09 (211.53) to 498.31 (208.78) and 482.56 (317.15) cells/μl, $p=0.005$] tended to increase after two months and decrease thereafter. Statistically significant changes in parameters are shown in Figure 1.

Discussion

Circulating T and B lymphocytes remain the subject of research in solid tumors. The impact of various treatment modalities on circulating subsets in different cancer types during the novel therapy era intrigues investigators.

Tumor-infiltrating B lymphocytes are thought to play a role in PDAC initiation and further development (12). In general, B cells demonstrate pro-tumorigenic, even immunosuppressive effects, but in the lymphoid structures, their function changes into tumor inhibitory and immunostimulatory (13). Tumor-infiltrating B cells are found in different solid tumors (*e.g.*, breast and ovarian cancer) and may predict improved survival (12). Cancer patients seem to have lower circulating B cells, as shown in non-small cell lung cancer research (14). Higher number of circulating CD19+ B cells in patients with nasopharyngeal carcinoma revealed a better 5-year OS prognosis. It was an independent prognostic factor for OS, PFS, and DMFS in that setting (15). In preclinical PDAC models, tumor-infiltrating B cells contribute to tumor progression (12). Furthermore, B cells may represent pro-fibrotic features through the secretion of factors that stimulate collagen production in PDAC patients (16). Antineoplastic treatment seems to have a reductive effect on circulating B cells. The numbers of CD19+ B cells decreased after radiation treatment in patients with esophageal cancer (17) and patients with limited-stage small-cell lung cancer (18). Nevertheless, data concerning circulating B cells and PDAC treatment are limited. Our results show a significant decrease in absolute numbers of CD19+ B cells after two and four months of treatment.

The role of NK cells in solid tumors and even PDAC is much more perceptible. NK cells have the ability of anti-tumoral cytotoxicity (19). High levels of NK cells in peripheral blood and TME are associated with better survival results in resectable PDAC patients (20). The prognostic significance of circulating NK cells may be controversial as different investigators demonstrate poorer OS results in patients with advanced PDAC (19, 21). The effect of surgery on NK cells in PDAC patients remains unclear (22). In a preclinical mouse model, resection and adjuvant chemotherapy

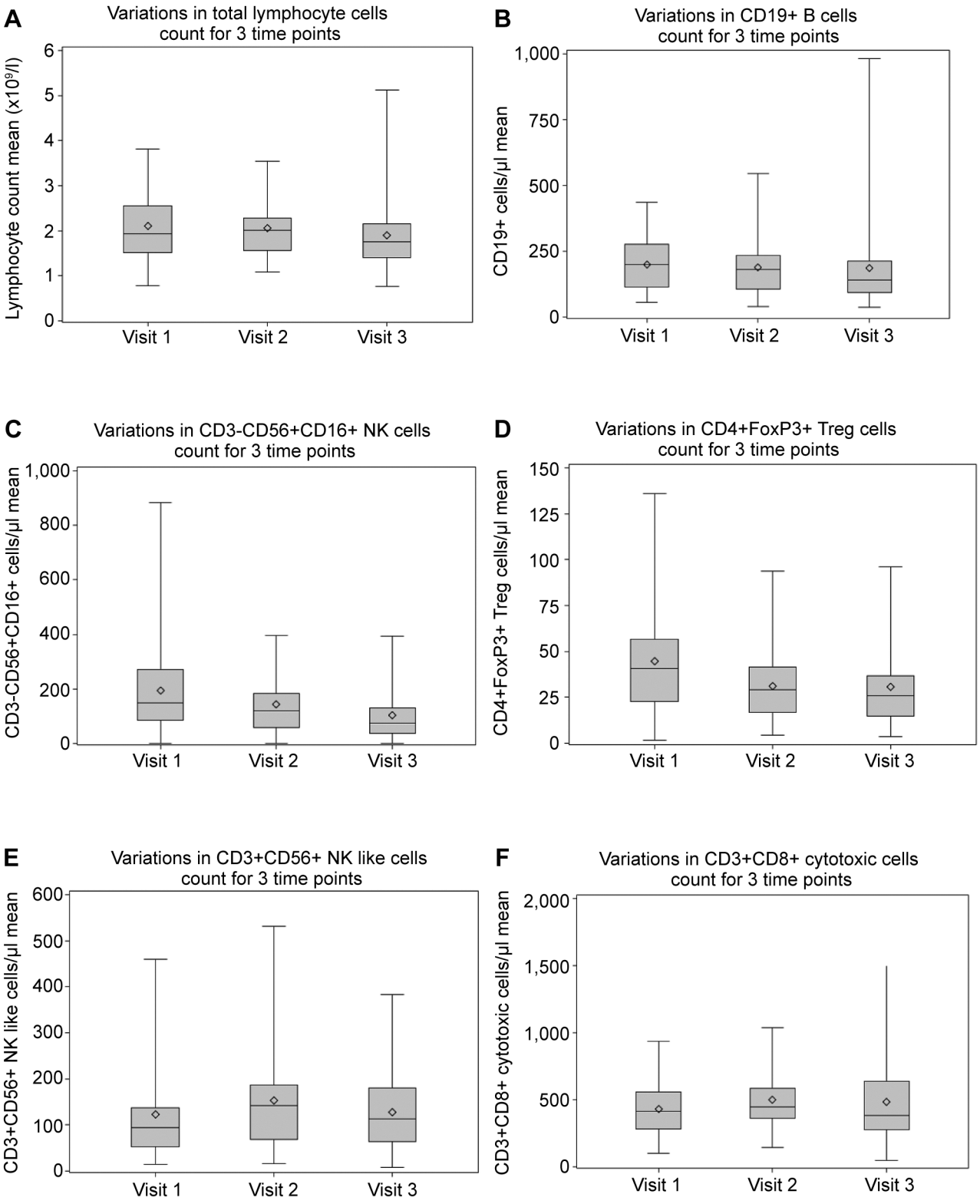

Figure 1. *Variations in peripheral blood cells absolute counts mean values at three time points. A) Variations in total lymphocyte cells count at three-time points. B) Variations in CD19+ B cells count for 3-time points. C) Variations in CD3-CD56+CD16+ NK cells count for three-time points. D) Variations in CD4+FoxP3+ Treg cells count for three-time points. E) Variations in CD3+CD56+ NK-like cells count for three-time points. F) Variations in CD3+CD8+ cytotoxic cells count for three-time points.*

with gemcitabine activated NK cells and prevented recurrence (23). Gemcitabine alone supposedly has a positive impact on circulating NK cells (24), and this effect of the chemotherapeutic agent is confirmed in other cancer types, *e.g.*, lung cancer (25). The impact of a novel FOLFIRINOX regimen is not described yet. Our results revealed the negative effect of treatment on circulating NK CD3-CD56+CD16+ cells. NK CD3+CD56+ cells varied over time.

Some studies confirmed that Tregs are increased in the peripheral blood and the tumor microenvironment in PDAC patients (26), which may lead to a poorer prognosis (27). In later investigations, Tregs were discovered to play a role in fibroblast function (28). Chemotherapy containing cisplatin/gemcitabine may decrease CD4+CD25+FOXP3+ levels, as was shown in nonsmall-cell lung cancer patients (29). FOLFIRINOX regimen in PDAC patients tends to reduce T regulatory (FoxP3+) cell density (30). Our study confirms a reduction of CD4+FOXP3+ during treatment.

Cytotoxic T cells play a crucial role in the PDAC microenvironment, and the increased number of those cells determines better survival results. The effect of conventional chemotherapy with gemcitabine on the number of CD3+CD8+ cells is not clear (31). Oxaliplatinum combinations increased the number of cytotoxic T cells in TME in gastric cancer (32). Our findings were also controversial.

The role of NKT-like cells has recently been highlighted in cancer studies of other localizations. The number of NKT-like cells was increased in HCC patients following stereotactic body radiotherapy, and higher levels were associated with a favorable prognosis (33). Properties of NKT-like cells, such as PD-L1 expression, are impaired in HCC cells and could be used as a potential target for immunotherapy (34). The number of NKT-like cells increased in patients with CRC treated with surgery alone (35), and the percentage of CD16+ NKT-like cells in the samples collected before surgery correlated with shorter disease-free survival (36). In gastric cancer tumor samples, low numbers of CD3+CD56+ NKT-like cells indicated poor survival (37). Unfortunately, such data for NKT-like cells are not described for PDAC. In our study, their levels changed significantly throughout treatment.

Limitations of our study include its small cohort, single-institution design, different treatment strategies and regimens, and indefinite impact of the results on PFS and OS.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

All the Authors have contributed significantly to the concept design of this manuscript and the work leading to the final manuscript. All Authors have reviewed the article and agreed with its content.

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