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The Impact of Immune Interaction on the Metastatic Infiltration of Colorectal Carcinoma to Lymph Nodes

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Abstract. *Background/Aim: Tumour-infiltrating lymphocytes (TILs) and Granzyme B play crucial roles in immune reactions against colorectal carcinoma (CRCa). The inhibitor of Granzyme B is Serpin B9. The aim of this study was to evaluate the effect of immunohistological parameters of TILs on the prognosis of CRCa and presence of lymph node metastasis. Patients and Methods: A total of 152 patients who underwent surgery for CRCa were analyzed, including 63 patients in cohort stage II, according to the Union for International Cancer Control (UICC), and 89 patients in cohort UICC stage III. The TIL pattern was classified as peritumoural (PTL), intratumoural (ITL), intrastromal (ISL) or Crohn-like, and immunohistological staining of TIL and cancer cells was also performed. Results: A significantly higher density of $CD8^+$ and $CD4^+$ TILs was observed in the UICC II group, and significantly higher densities of $CD4^+$ TILs were observed in the UICC II group in all distinguished patterns. In the same cohort, higher numbers of $CD57^+$ cells and $FoxP3^+$ TILs and Granzyme B levels were observed. In cohort UICC III, there was a higher density of ISL, PTL $CD8^+$, $CD25^+$ TILs and cancer cells showed staining for Serpin B9. CD57, Granzyme B and CD8 were demonstrated as positive prognostic factors of overall survival, and CD57 and $CD4^+$ TILs were demonstrated as positive prognostic factors of recurrence. Conclusion: TILs and CD57 are promising prognostic factors of CRCa. The association of Serpin B9 with lymph node metastasis reveals a potential mechanism for tumour resistance to immune reaction.*

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Colorectal carcinoma (CRCa) is a serious health and social issue, reflecting the high incidence of this malignant diagnosis, particularly in Europe (*i.e.*, over 40 patients per 100,000 individuals in Slovakia, Hungary, Denmark or the Netherlands (1)). The current treatment of CRCa respects the staging according to the Union Internationale Contre le Cancer (UICC), which deals with the TNM classification and other risk factors, such as positive resection margin, bowel obstruction or perforation, high-grade and mucinous components of histological findings, less than 13 examined regional lymph nodes, and lymphatic, endovascular or perineural infiltration. These risk factors do not reflect immune interactions between tumours and macro-organisms (2). Tumour-infiltrating lymphocytes (TILs) are markers of these immune interactions.

Some CRCa patients experience early recurrency after radical surgical therapy, even when a good prognosis is expected according the current histopathological parameters. These patients would benefit from a more intense dispenserisation of adjuvant oncological treatments.

Published studies have revealed the presence, pattern and immunohistochemical and functional parameters of TILs as relevant prognostic factors of CRCa (3-5). Nevertheless, the relation of TILs and regional lymph node infiltration as an important prognostic factor is not deeply understood.

Immunobiology of colorectal carcinoma. Cancer progression can be described from the point of view of immune interactions as a three-step process: elimination, equilibrium and escape (6). The elimination phase involves functional immunosurveillance, and the equilibrium phase involves changes in the immunophenotype of cancer cells in response to immune responses. These changes lead to escape and progression (7).

Natural killers. Natural killer (NK) cells play a crucial role in native and specific immunity. These cells identify and destroy infected somatic cells or malignant transformed cells (8). NK cells do not need any previous stimulation. Another function of

these cells is to support the differentiation of B and T lymphocytes and the maturation of dendritic cells (DCs). Common markers of NKs are CD57 and CD56. In 1997, Coca demonstrated that the presence of tumour-infiltrating $CD57^+$ cells is a positive prognostic factor after the radical surgical treatment of colorectal carcinoma in patients without adjuvant oncological therapy (9). Subsequently, Menon et al. confirmed these results, showing that tumour infiltration by $CD56^+$ $CD57^+$ cells is a positive prognostic factor of the disease-free interval (DFI) (3). Liska published similar findings, confirming that $CD57^+$ tumour-infiltrating lymphocytes are positively correlated with significantly longer DFI and overall survival (OS) (10). Phenotype analysis of the activating receptors of NK cells in the peripheral blood of colorectal carcinoma patients has revealed that the expression of these receptors is significantly decreased compared with healthy controls. The low expression of perforin in NK cells in peripheral blood was also associated with a higher grade of colorectal carcinoma (11).

T lymphocytes. T lymphocytes represent the primary component of antibody-dependent cellular cytotoxicity, which maintains elimination and equilibrium in cancer progression. There are two basic features: type of T cell receptor (TCR) and expression of CD4 or CD8. The most frequent TCR comprises α and β chains. These αβ TCR lymphocytes have CD4 or CD8 coreceptors. The CD4 molecule is the coreceptor of main histocompatibility complex II (MHC II), and CD8 is the coreceptor of MHC I. Most $CD8^+$ lymphocytes are precursors of cytotoxic memory T cells, and most $CD4^+$ cells are precursors of helper T (Th) cells (12). Th cells can be divided into two groups, Th1 and Th2, according to cytokine production. Th1 supports and maintains cytotoxic immune reactions by producing interferon γ and interleukin 2 (IL-2). Another type of helper T cells, Th17, produces IL-17, and RORγ is a marker of Th17 cells (13).

Cytotoxic T lymphocytes. Jass showed that infiltration along the invasive margins of CRCa is an independent prognostic factor of CRCa (14). Not only the numbers but also the patterns of TILs were revealed as significant prognostic factors of CRCa. Ropponen reported that Crohn-like reactions and TILs in invasive margins are positive prognostic factors of OS DFI (15). The strongest prognostic value of OS was demonstrated for $CD8^+$ intratumoural (intra-epithelial) T lymphocytes without any prognostic value of intrastromal $CD8^+$ T lymphocytes (16-19). In addition, memory T lymphocytes were demonstrated as positive significant prognostic factors for DFI and OS, particularly in the presence of cytotoxic T cells (5, 20).

Helper T lymphocytes. Colorectal cancer-infiltrating Th1 lymphocytes are positive prognostic factors, and Th17 cells are negative prognostic factors. The significant impact of Th2 on CRCa prognosis has not been demonstrated (21). Yoshida showed that a high RORγ/TCR ratio in TILs is associated with significantly shorter overall survival and the metastasis of lymph nodes (13). Subsequently, Li reported that increased numbers of Th17 cells are intratumourally and peritumourally correlated with higher stages of CRCa (22).

Regulatory T lymphocytes. Regulatory T lymphocytes (Tregs) are characterised as T cells with immunosuppressive effects. Natural regulatory $FoxP3^+$ T lymphocytes are the most frequent types of regulatory T lymphocytes in peripheral blood. Physiologically, natural regulatory T cells maintain immune tolerance to somatic cells (12, 23). Another type of immunosuppressive T lymphocyte is induced Tregs. These cells are derived from T helper cells. Th3 lymphocytes are also $CD4^+$ immunosuppressing T cells that also act as immunosuppressive T lymphocytes in the intestinal mucosa and protect against immune reactions to alimentary antigens. In addition, $CD8^+$ immunoregulatory lymphocytes have also been detected in the micro-environment of solid tumours and CRCa. These lymphocytes have been defined as $CD8^+$ CD28- cells (24, 25). Frisulo showed evidence of the presence of immunosuppressing $CD8^+$ $CD25^+$ $FoxP3^+$ T lymphocytes in peripheral blood (26), and Chaput revealed the presence of immunosuppressive $CD8^+$ $FoxP3^+$ TILs in CRCa (27).

The increased numbers of Treg (*i.e.* $FoxP3^+$ cells) exert effective immune responses against tumour cells (28). Terme demonstrated that increased numbers of peritumoural $FoxP3^+$ T cells are associated with a higher stage of CRCa and shorter overall survival (29). A meta-analysis of the published results of 10 studies revealed immunosuppressing TILs as negative prognostic factors in six cases. The remaining studies showed no significant influence (30).

Granzyme B. Granzyme B is a serine protease stored in cytotoxic granules (31). Granzyme B is produced in NK cells and T lymphocytes. Under special conditions, Granzyme B is produced in $CD4^+$ lymphocytes, mast cells, macrophages, neutrophils, basophils, DCs and regulatory lymphocytes. Granzyme B is also produced in non-immune cells, such as smooth muscle cells, chondrocytes, keratinocytes and type II pneumocytes (32).

Serpin B9. Serpin B9 is a human endogenous inhibitor of Granzyme B. The physiological function of Serpin B9 is to protect against the effects of cytotoxic granule leakage. Studies have shown that Serpin B9 is secreted from endothelial cells, smooth muscle cells and hepatocytes. Immunoprivileged cells, *i.e.*, trophoblast cells, Sertoli cells, granulosa cells and cells of the eye lens, also produce this inhibitor. The proposed function of Serpin B9 is protection against cytotoxic immunity (33). In case of malignancies, Serpin B9 is produced by melanomas, breast cell carcinoma and cervical cancer. In addition, CRCa cells also produce Serpin B9 (34).

Aim. The effect of immunohistological parameters of TILs on the lymph node metastasis of CRCa was evaluated based on a comparison between groups of patients with or without lymph node metastasis. Patients with a high risk of short OS and a short DFI after radical surgical therapy were examined in the present study.

Patients and Methods

A total of 152 patients (94 males, 58 females) who underwent radical surgical therapy due to CRCa stage UICC II or UICC III were included in the present retrospective study. These individuals underwent surgical treatment in the Department of Surgery at the Teaching Hospital and Medical School in Pilsen between 2006-2012. Patients with Crohn's disease, ulcerous colitis, familiar adenomatosis, a small number of examined lymph nodes and malignancy duplicity in anamnesis were excluded from the present study. Patients presenting ileus, tumour rupture or acute bleeding were also excluded. The follow-up of patients occurred during standard dispenserisation. Authors evaluated OS, DFI, localisation of recurrence (local, peritoneal and distant), localisation of tumour (right colon, left colon, and rectum), the highest level of CRP and leukocytosis in the first postoperative week, 30-day postoperative morbidity according to Clavien-Dindo (35), regimen and modality of oncological treatment.

Histological and immunohistochemical examination. Three different localities of each tumour were examined. Tissue samples for light microscopy were fixed in 4% formaldehyde and embedded in paraffin. Five-micrometre-thick sections were cut from the tissue blocks and stained with haematoxylin-eosin. Staging, lymph node metastasis and grade of tumour were examined according to the guidelines of the WHO 2000 Disease stage was evaluated according to the guidelines of the UICC 2009 (2). The presence or absence of endovascular infiltration (VE), endolyphatic (LI) and perineural infiltration (PI) of colorectal carcinoma were also evaluated. The number of TILs was quantitavely evaluated. To assess the pattern of TILs, intratumoural (ITL), intrastromal (ISL), peritumoural (PTL) and Crohn like reactions were distinguished. The layout of TILs within the patterns was semiquantitatively assesed as none (0), mild (1), middle (2) and severe (3) infiltration. For immunohistochemical (IHC) studies, the following primary antibodies were used: anti-CD4 (clone SP35, ready-to-use Ventana, Rocklin, CA, USA), anti-CD8 (clone C8/144B, 1:50, Dako, Glostrup, Denmark), anti-FoxP3 (clone mAbcam 450, 1:20, Abcam, United Kingdom, Cambridge), anti-CD25 (clone 4c9 ready-to-use, Ventana, Rocklin, CA, USA) anti-CD57 (clone NK1, ready-to-use; Ventana, Rocklin, CA, USA), anti-Granzyme B (clone 11F1, 1:30, Leica Biosystems, USA, Buffalo Grove), anti-CD56 (clone 11B6, 1:400, Leica Biosystems, USA, Buffalo Grove), and anti-Serpin B9 (clone PI9-17,1:100, Abcam, United Kingdom, Cambridge). Microwave pretreatment was used in all cases. The primary antibodies were visualised using a supersensitive streptavidin-biotin-peroxidase complex (Biogenex, San Ramon, CA, USA). The appropriate positive and negative control slides were employed. The number of positive cells was evaluated in five high-power microscope fields (400× magnification), and the results are expressed as the number of immunopositive cells per high-power microscope field.

Statistical analysis. Differences in parameters between UICC II and UICC III, rectum and colon groups, right/left colon, IHC parameters and VE, LI, and PI were analysed using the Mann-Whitney *U*-test. Results were visualised using box plots. The relation between type of recurrence and IHC parameters was evaluated using Kruskal-Wallis and ANOVA. DFI and OS were determined using the Kaplan-Meier method. For comparison of the DFI and OS between specified groups, the Gehan-Wilcoxon test was used. The influence of prognostic factors was analysed using the Cox model. The analysis was performed using STATISTICA software (version 12, Cz, StatSoft, Inc. 2013, Zlicin, Czech Republic).

Results

Description of the cohorts and follow up. A total of 42 men and 21 women were included in the UICC II group, and 52 men and 37 women were included in the UICC III (with lymph node metastasis) group. The average age of the men in the UICC II group was 69.00 years (median 70.20, min. 65.00, max. 86.56), and the average age of women in the same cohort was 71.54 years (median 72.53, min. 49.59, max. 84.79). The average age of the men in UICC III group was 65.77 years (median 65.12, min. 47.48, max. 87.70), and average age of women in the same cohort was 69.20 years (median 68.79, min. 39.09, max. 84.79).

The UICC II group included 27 patients with rectal carcinoma and 36 patients with colon carcinoma. The UICC III group included 40 patients with rectal carcinoma and 49 patients with colon carcinoma.

Patients with rectal cancer were significantly older then the patients with colon tumours (p-Value=0.000369).

The three-year DFI was 82.14% in the UICC II group and 71.4% in the UICC III group. The three-year OS was 90.51% in the UICC II group and 86.6% in the UICC III group. The five-year OS was 80.85% in the UICC II group and 78.45% in the UICC III group. There were too many censours after the fourth year of follow-up to evaluate the five-year DFI. There was no significant difference in DFI and OS between the UICC groups and no significant difference in DFI and OS between groups of patients with rectal cancer and colon cancer.

Comparison between UICC II and UICC III cohorts. A significant difference in age was observed between UICC II and UICC III. Patients in the UICC III group were older (p-Value=0.016345).

Quantitative analysis revealed that the density of $CD8^+$ TILs was significantly increased in the UICC II group, regardless of localisation (p-Value<0.000001, Figure 1A). In the UICC II cohort, a significantly higher number of $CD4^+$ TILs was observed, regardless of localisation (p-Value<0.000001, Figure 1B). Comparison of the results of CD57 staining revealed higher numbers of $CD57^+$ cells in the UICC II cohort (p-Value<0.000001, Figure 1C). Quantitative analysis also revealed significantly higher densities of

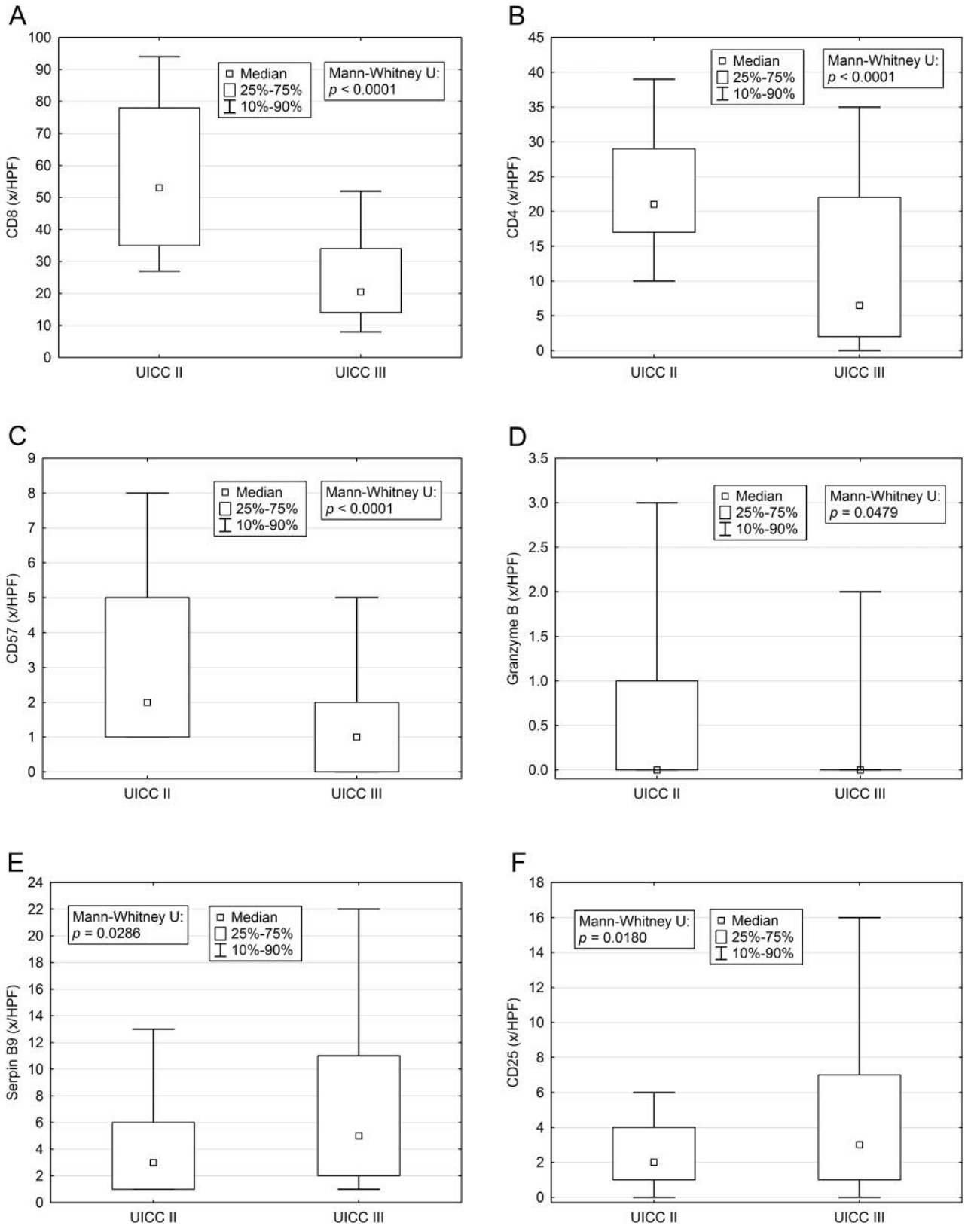

Figure 1. *Continued*

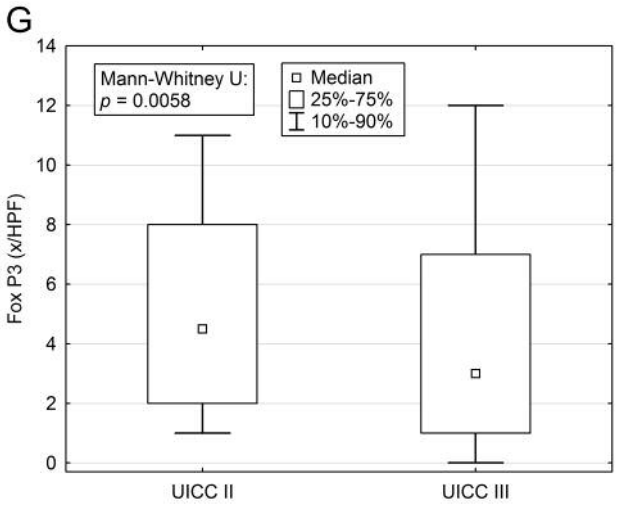

Figure 1. *Comparison of IHC parameters between UICC II and UICC III cohorts – quantitative analysis. A: Density of CD8+ TILs in the UICC II and III groups (p-Value<0.000001). B: Density of CD4+ TILs in UICC II and III cohorts (p-Value<0.000001). C: Quantitative analysis of the density of CD57+ in the UICC II and III groups (p-Value<0.000001). D: Quantitative analysis of the densities of Granzyme B-stained lymphocytes in the study groups (p-Value=0.047917). E: Quantitative analysis of the densities of cancer cells stained for Serpin B9 (p-Value=0.028638). F: Quantitative analysis of the density of CD25+ TILs in the UICC II and III groups (p-Value=0.017952). G: Quantitative analysis of the density of FoxP3 in the UICC II and III groups (p-Value=0.005763).*

lymphocytes stained for Granzyme B in the UICC II group (*p*-Value=0.047917, Figure 1D). Significantly increased densities of cancer cells stained for Serpin B9 were observed in the UICC III group with lymh node metastasis (*p*-Value=0.028638, Figure 1E). Comparison between the study groups revealed increased $CD25^+$ lymphocytes in the UICC III group (*p*-Value=0.017952, Figure 1F). Elevated densities of $FoxP3^+$ lymphocytes were observed in the UICC II group and evaluated quantitatively (*p*-Value=0.005763, Figure 1G).

Semiquantitative analysis revealed higher densities of $CD8^+$ ISL were observed in the UICC III group (*p*-Value=0.006073, Figure 2A). Higher densities of $CD8^+$ PTL were proven in the UICC III cohort, evaluated semiquantitatively (*p*-Value=0.023275, Figure 2B). Semiquantitative analysis also revealed increased densities of $CD4^+$ TILs in the UICC II cohort in all analysed patterns group (ITL, ISL, PTL and Crohn-like, Figure 2C, D, E and F) in comparison with UICC III.

There was no significant difference in leukocytosis and serum levels of CRP, CD56 and $CD8^+$ ITL between the analysed cohorts.

Immunohistochemical parameters, focusing on localisation in the right or left colon were also analysed, but no significant differences were observed bettween cohorts. However, semiquantitative analysis revealed that higher densities of $CD8^+$ ITL were observed in the colon compared with the rectum (ITL 0-3 *vs*. 4, *p*-Value=0.01706).

Prognostic factors. Evaluation of the studied parameters revealed statistically significant differences in the following independent prognostic factors of OS, regardless of UICC stage and localisation of CRCa:

Positive prognostic factors of OS: Granzyme B, semiquantitatively (*p*-Value=0.012300). Increase of one unit diminishes the risk of death by 36.2% (95% confidence interval 5.75-61.51%).

CD57, semiquantitatively (*p*-Value=0.000400). Increase of one unit decreases the risk of death by 30.62% (95% confidence interval 9.31-46.93%).

$CD8^+$ TIL, quantitatively (*p*-Value=0.004900). Increase of one unit decreases the risk of death by 1.97% (95% confidence interval 0.34-3.57%).

Negative prognostic factors of OS: Leukocytosis in postoperative weeks (*p*-Value=0.029600). Increase of one unit increases the risk of death by 7.95% (95% confidence interval 1.71-14.57%).

CRP in postoperative weeks, quantitatively (*p*-Value=0.000900). Increase of 10 units increases the risk of death by 6.91% (95% confidence interval 3.26-10.70%). Evaluation of the studied parameters revealed statistically significant differences in the following independent prognostic factors of DFI, regardless of UICC stage and localisation of CRCa:

Positive prognostic factors of DFI: CD57, semiquantitatively (*p*-Value=0.021000). Increase of one unit decreases the risk of recurrence by 13.12% (95% confidence interval 0.25-24.71%).

$CD4^+$ ITL, semiquantitatively (*p*-Value=0.025000). The course is described as a Kaplan-Meier function (Figure 3). The hazard ratio of relapse is 2.16 without any $CD4^+$ ITL (95% confidence interval (0,911092-5,147486).

Authors did not prove any studied parameters as a negative prognostic factor of DFI.

Analysis of the corelation between immunohistochemical parameters and VE, LI, PI showed the benefit of the increased infiltration of $CD8^+$ TILs. These results were associated with a lower appearence of VE (Figure 4, *p*-Value=0.003818).

Discussion

Comparison between groups. Higher densities of $CD4^+$ and $CD8^+$ lymphocytes (measured quantitatively) were observed in patients without metastasis in regional lymph nodes, consistent with published articles in which TILs showed a positive influence on DFI and OS (4). In the UICC II group, significantly higher semiquantitatively measured densities were observed in intrastromal, intratumoural, peritumoural and Crohn-like patterns. However, semiquantitative analysis

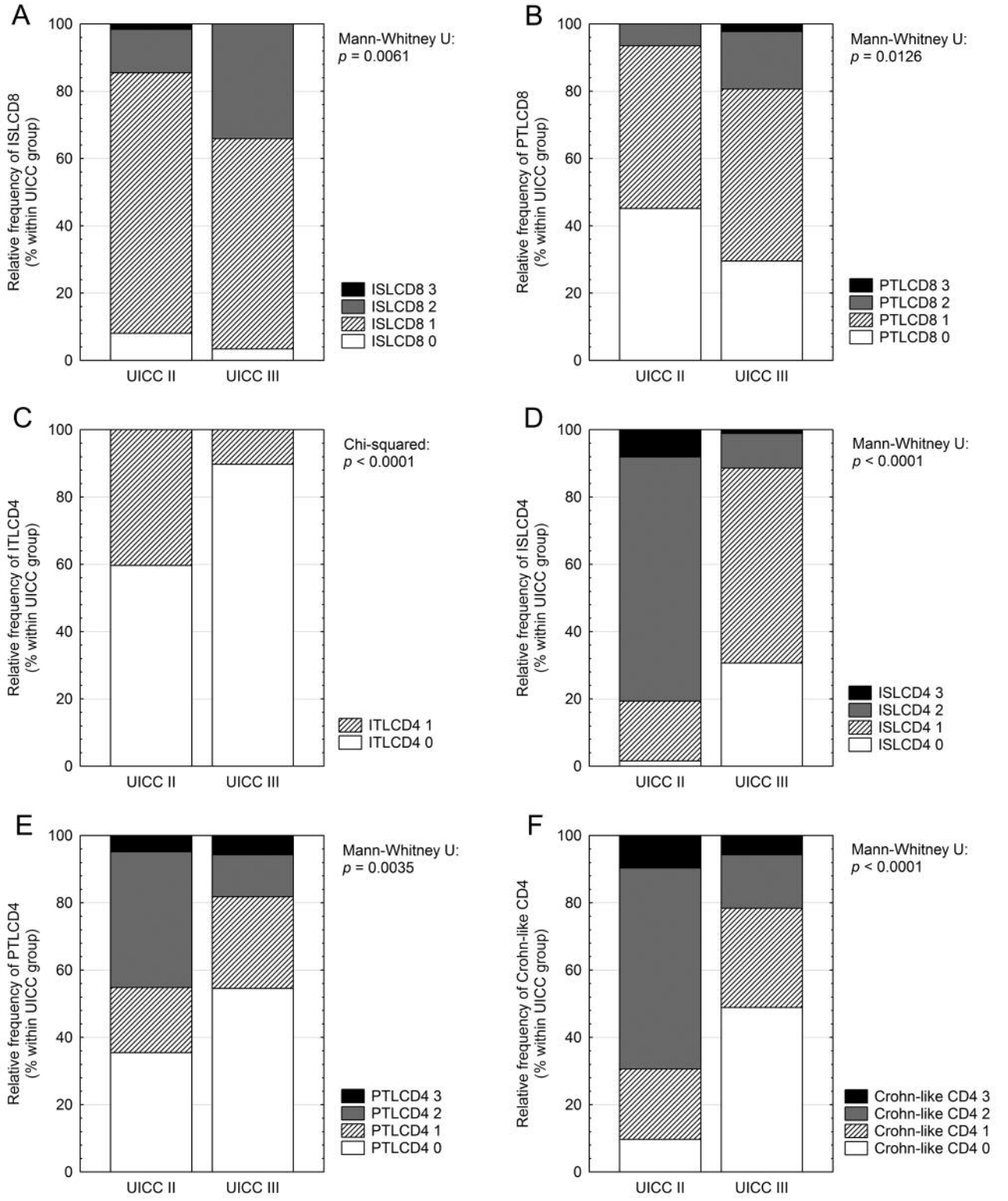

Figure 2. *Comparison of TIL between UICC II and UICC III cohorts – semiquantitative analysis. A: Density of CD8⁺ ISL in the UICC II and III groups, semiquantitative analysis (p-Value=0.006073). B: Densities of CD8⁺ PTL in the UICC II and III groups, semiquantitative analysis (p-Value=0.023275). C: Semiquantitative analysis of CD4⁺ ITL in the UICC II and III groups (p-Value<0.000001). D: Semiquantitative analysis of CD4⁺ ISL in the UICC II and III groups (p-Value<0.000001). E: Semiquantitative analysis of CD4⁺ PTL in the UICC II and III groups (p-Value=0.003518). F: Semiquantitave analysis of Crohn-like CD4⁺ in the UICC II and III groups (p-Value<0.000001).*

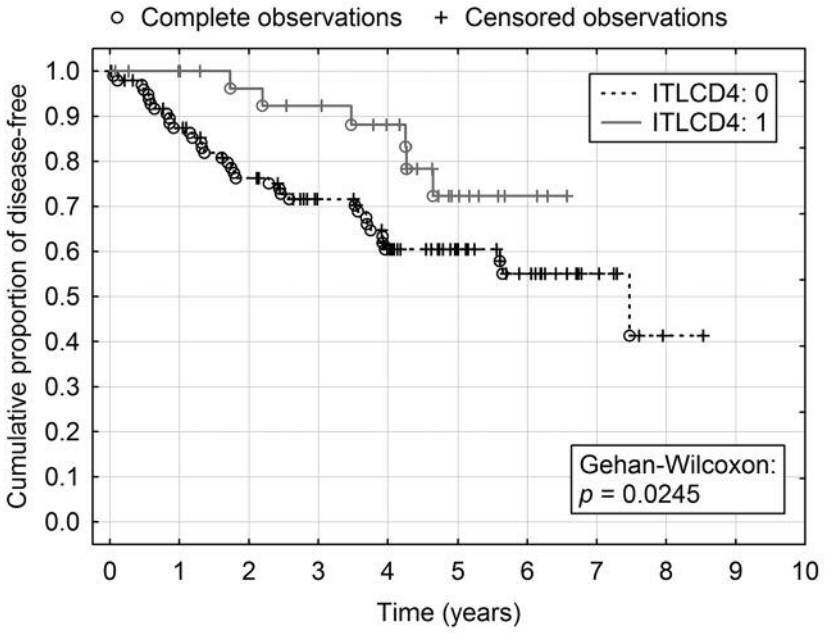

Figure 3. *DFI of patients with and without CD4+ ITL (p-Value=0.025000).*

of $CD8^+$ TILs in specific patterns showed significantly higher densities intrastromaly and peritumouraly in the UICC III group with lymph node metastasis. This controversy could reflect the limits of semiquantitative assessment. In the UICC II group, significantly higher densities of TIL stained for Granzyme B were observed, thereby indicating increased local cytotoxic activity. This result is consistent with a previous publication by Galon (5). Evaluation of the role of Tregs did not show clear results. Higher numbers of $FoxP3^+$ lymphocytes were observed in the UICC II group, whereas higher densities of $CD25^+$ lymphocytes were observed in the UICC III group. This result could reflect the fact that not all Tregs have Foxp3 molecules on their cell surfaces (23). There was no difference in the immunohistochemical parameters of the right and left colon. This result contradicts the findings of previous articles showing higher numbers of TIL in the right colon (36). Notably, those studies also included rectal localisation. This explanation supports the significantly increased presence of $CD8^+$ TIL in colonic tumours compared with rectal localisation in the present study.

Prognostic factors. The strongest independent positive prognostic factor of OS and DFI was observed using $CD57^+$ cells. This pool primarily contains mature NK cells and a highly differentiated oligoclonal subpopulation of $CD8^+$ T lymphocytes (37). The density of $CD57^+$ cells was also higher in patients without metastasis in lymph nodes. Similar results have been published in previous studies (9, 10, 27). A low density of CD 57^+ tumour-infiltrating lymphocytes is a promising prognostic factor for adjuvant chemotherapy for patients after radical surgical therapy due to CRCa. Another significant positive prognostic factor is the increased density of cancer cells stained for Granzyme B. This serine protease is produced in NK and T lymphocytes during cytotoxic reactions. The observed positive influence of Granzyme B and $CD8^+$ TIL on prognosis is consistent with previous published studies (5, 38, 39). However, there was no strong significant correlation between long overall survival and intratumoural (intra-epithelial) TIL, in contrast with the findings of Ohtani (16). In the present study, a protective influence of CD8 TIL on endovascular infiltration was consistent with previous findings (5).

The correlation between VELIPI and CD56-positive tumour cells was also assessed in the present study, without any significant results. CD56 was assessed because of its association with increased perineural infiltration in rectal carcinoma (40). Interestingly, an increased density of tumour cells positive for Serpin B9 staining was observed in the UICC III cohort. Taken together, these findings suggest that Serpin B9 produced by cells of CRCa support metastasis to regional lymph nodes. Thus, studies evaluating the expression of Serpin B9 in analogical cohorts of patients are needed.

Higher serum levels of CRP and leukocytosis in the postoperative period were observed as negative prognostic factors of OS. There was no correlation between

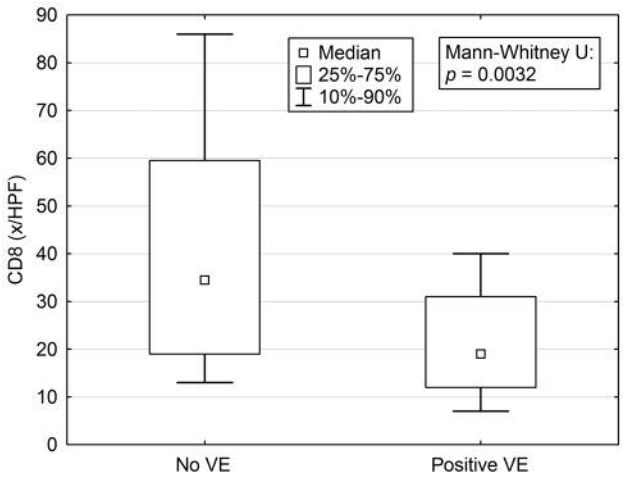

Figure 4. *CD8+ TIL is associated with lower appearence of VE (p-Value=0.003818).*

complications and recurrence, leading to speculation concerning the correlation of systemic inflammatory response with general performance status.

Conclusion

The results of the present study demonstrated the important role of local immune reaction in the regulation of CRCa progression into lymph node metastasis. This fact is particularly clear in the case of Granzyme B. However, TIL is a well-described prognostic factor of CRCa, and some specific markers could improve the selection of patients with increased risk of reccurence. These patients would benefit from the indication of adjuvant oncological treatment and intensive form of dispenserisation, particularly when stage UICC II CRCa is diagnosed. The most promising prognostic factor based on the presented results is CD57. Comparison of the cohorts with or without metastatic affliction of regional lymph nodes revealed an association between increased density of Serpin B9-positive cancer cells and lymph node metastasis as a novel factor in the mechanisms of CRCa progression.

Conflicts of Interest

None of the Authors have any conflict of interest to disclose regarding this study.

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