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# Evaluation of Prognostic Immune Signatures in Patients with Breast, Colorectal and Pancreatic Cancer Receiving Chemotherapy

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**Abstract.** *Background: CD97 is a member of the epidermal growth factor-seven transmembrane (EGF-TM7) receptor family and is dominantly expressed on immune cells and in a variety of malignant diseases. B7-H1 and B7-H3 are transmembrane proteins that are involved in suppression of the immune system. The aim of this study was to evaluate if these molecules are up-regulated in patients with cancer and change during chemotherapy. Materials and Methods: We analyzed cluster of differentiation (CD) protein expression levels on tumor cell lines and in blood samples of 37 patients with solid tumors at baseline and during chemotherapy; we correlated the serum levels of CD proteins with survival outcome. Results: Levels of soluble CD97 proteins were significantly elevated in all three cancer types compared to healthy controls. Patients with colorectal cancer and those with high CD97 levels had a significantly worse prognosis. Conclusion: This study showed a marked elevation of soluble CD97 expression in patients with certain cancer types and demonstrated definite changes in CD protein expression during chemotherapy in one patient with metastatic breast cancer.*

In the era of the rebirth of immunotherapy, the importance of finding a prognostic biomarker for response to new potent, but also costly drugs is needed. The role of immune response to certain types of cancer has been shown in variety of tumor entities. The ratio of infiltrating lymphocytes in breast cancer is a prognostic factor for survival (1). Kidney cancer, for example, is an immune-driven disease and spontaneous complete remissions have been reported (2). Other cases of such remission have been reported in malignant melanoma, lymphoma and neuroblastoma. The hypotheses for the mechanism of spontaneous regression are immune mediation and tumor inhibition by growth factors and cytokines (3, 4).

Cytotoxic T-lymphocytes (CTLs) can be found in the tumor core or in the tumor margin. However, infiltration of lymphocytes into the tumor is not a steady process. During the course of disease or even during treatment with immunomodulatory drugs, the proportion of tumor-infiltrating lymphocytes in the tumor tissue can markedly increase or decrease.

Various molecules are involved in the immune response to cancer. Cluster of differentiation (CD)97 is a member of the epidermal growth factor-seven transmembrane (EGF-TM7) receptor family and is dominantly expressed on immune cells, including T-cells, and in a variety of human malignant diseases. It plays a role in tumor cell invasion and influences overall prognosis, and can be a novel target or prognostic marker in different cancer types (5). CD97 and its ligand CD55 were shown to be up-regulated in pancreatic cancer and indicate an aggressive course of disease (6). Another group reported the role of CD97 in gastric cancer cell lines through the mitogen-activated protein kinases (MAPK) signaling pathway by promoting proliferation and invasion (7). Soluble forms of CD97 protein are found in body fluids during inflammatory processes (8). In patients with certain forms of cancer (*e.g.* gastric cancer), but also in chronic inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis, levels of CD97 are elevated in comparison to healthy controls (9-13).

Another important protein family that is known to mediate stimulatory and inhibitory effects on T-cell activation is the B7 family. CD274 (also known as B7-H1 or PD-L1) is widely expressed on lymphoidal and non-lymphoidal cells (endothelial and muscle cells). Furthermore, it is expressed in the tumor environment (14-16). Recently, antibodies to



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Table I. *Baseline characteristics of the patients in this study.*

| Characteristic | Value | | |
|---|---|---|---|
| Total no. of patients, n (%) | | 37 (100) | |
| Tumor type, n (%) | Breast N=15 | Colorectal N=11 | Pancreatic N=11 |
| Gender: Female, n (%) | 15 (100) | 3 (27) | 4 (36) |
| Median age (range), years | | 67 (32-87) | |
| Median no. of metastases (range) | | 1 (0-2) | |
| Stage, n (%) | | | |
| Metastatic disease | 6 (16) | 11 (100) | 10 (91) |
| Liver metastases | 4 (67) | 6 (55) | 10 (100) |
| Lung metastases | 0 | 4 (36) | 0 |
| Bone metastases | 2 (33) | 0 | 0 |
| Other | 3 (50) | 4 (36) | 1 (10) |
| Chemotherapy, n (%) | | | |
| Neoadjuvant | 9 (60) | 2 (18) | 0 |
| Palliative | 6 (40) | 10 (82) | 11 (100) |
| First-line chemotherapy regimen, n (%) | | | |
| Docetaxel | 11 (73) | 0 | 0 |
| Epirubicin/cyclophosphamide | 10 (67) | 0 | 0 |
| Trastuzumab | 3 (20) | 0 | 0 |
| Pertuzumab | 2 (13) | 0 | 0 |
| Doxorubicin, liposomal | 1 (6.7) | 0 | 0 |
| Gemcitabine/nab-paclitaxel | 0 | 0 | 3 (27.3) |
| Gemcitabine/erlotinib | 0 | 0 | 2 (18.2) |
| FOLFIRINOX | 0 | 0 | 6 (54.5) |
| FOLFOX | 0 | 7 (63.6) | 0 |
| FOLFIRI | 0 | 3 (27.3) | 0 |
| 5-Fluouracil | 2 (13) | 1 (9.1) | 0 |
| Bevacizumab | 0 | 7 (63.6) | 0 |
| Cetuximab | 0 | 4 (36.4) | 0 |

CD274 and its ligand were approved for treatment of various solid tumors such as renal cell cancer, lung cancer and melanoma (17-19). Other tumor types are under investigation in clinical trials. Surrogate markers for response to these treatments are still unknown. Moreover, no valid test has yet been developed as a standard for testing this protein neither on endothelial cells nor on infiltrating lymphocytes. Lacking these tests, patients will be treated with checkpoint inhibitors independently of expression of CD274 and its ligand.

The levels of expression of CD274 and its ligand have so far not clearly been associated with response to treatment. This could be explained by the fact that CD274 expression varies through the course of cancer treatment. Powderly and colleagues showed that tumors that did not express CD274 at baseline gained CD274 expression during the course of treatment (20). Soluble forms of this molecule are detected in human serum. Elevated expression of soluble CD274 in patients with cancer is correlated with cancer metastasis and poor clinical outcome (21). Another important member of the B7 family is the CD276 (B7-H3) molecule. A high expression of CD276 is correlated with worse prognosis in gastric cancer through regulation of Interferon gamma (IFNγ) (22-24). CD276 positively regulates interleukin 10 (IL10) and signals through inhibition of IL2. Progression of prostate cancer is correlated with high levels of CD276 (24-26). Soluble forms of CD276 are inhibited by the presence of matrix metalloproteinase (27). In several auto-immune diseases (*e.g.* multiple sclerosis), higher levels of CD276 were detected (28).

The aim of this study was to describe the levels of soluble CD proteins (CD97, CD274, CD276) in patients with advanced or metastatic breast, colon or pancreatic cancer at baseline before the start of cytotoxic treatment and during the course of their chemotherapy. Our results suggest that these proteins might serve as readily-measureable prognostic or predictive markers in patients with malignant diseases.

## Materials and Methods

Between November 2012 and June 2016, we analyzed blood samples of 37 patients with advanced pancreatic (n=11), colon (n=11) or breast cancer (n=15) at baseline and during the course of their chemotherapy treatment. We correlated the serum levels of CD proteins (CD97, CD274 and CD276) with response to chemotherapy and overall survival. Negative controls were used from 30 healthy volunteers.

*Human serum samples*. Blood samples were collected at baseline within 4 weeks of chemotherapy start and monthly during treatment

with approval of the St. Josef Hospital (Vienna, Austria) Institutional Review Board (EK no. 02/2011) and informed consent was obtained from all patients. In order to obtain sera, blood was placed at 37˚C for 1 h. The blood clot obtained was then spun at 500 × *g* for 20 min and the serum removed from the upper layer.

The clinical parameters of the patients analyzed in this study are presented in Table I. The tumor marker cancer antigen 15-3 (CA15-3) was analyzed in lithium-heparin plasma at our central laboratory by electrochemical luminescence immunoassay.

Blood from healthy controls was obtained from the Biobank-Project (EK Nr. 559/2005) of the Department of Rheumatology (Medical University of Vienna) with ethical approval from the Ethics Committees of the Medical University of Vienna (EK no. 559/2005).

Octaplas®, a solvent/detergent-treated plasma preparation pooled from several thousand donors, was obtained from Octapharma GmbH (Vienna, Austria) and used as an additional source for determining the average amount of soluble cell surface receptors in human plasma of healthy donors.

*Monoclonal antibodies*. The following monoclonal antibodies (mAbs) used in this study for ELISA were established and produced at our laboratory: CD97 mAbs VIM3C and 8-154, CD274 mAbs 5-496 and 1-550, and CD276 mAbs 13I-241 and 7-517 were used as capture and detection mAbs, respectively. All mAbs used for capture were biotinylated.

*Enzyme linked immunosorbent assay (ELISA)*. Soluble CD97, CD274 or CD276 were detected and measured by a sandwich ELISA. Flat-bottomed, 96-well ELISA plates (Corning Life Sciences, Tewskbury, MA, USA) were coated with 100 µl of capture mAbs (5 µg/ml) overnight at 4˚C. The plate was washed twice with a phosphate-buffered saline (PBS)-Tween 0,5% solution. Blocking was performed by adding 200 µl of a 2% PBS-bovine serum albumin solution to each well and incubating again overnight at 4˚C. After washing with PBS-Tween, 100 µl of plasma or serum was added and incubated for 4 h at 4˚C. Recombinant CD97, CD274 and CD276 proteins from R&D Systems Inc. (Minneapolis, MN, USA) were used as positive controls to generate a standard curve. The plate was then washed three times with PBS-Tween. Afterwards, the respective biotinylated detection mAbs directed against CD97, CD274 and CD276 were added (5 µg/ml) and the plates incubated for a further 2 h at 4˚C. After washing with PBS-Tween three times, 50 µl of streptavidin-alkaline phosphatase (1:1000, from Jackson Immunoresearch, Newmarket, UK) was added per well and plates incubated for 1 h at room temperature. Unbound streptavidin-alkaline phosphatase was then removed by washing three times. Substrate buffer containing diethanolamine was added to analyze the bound streptavidin-alkaline phosphatase molecules colorimetrically using an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA).

*Flow cytometric analysis*. The following cell lines were analyzed in this study: HCT116 colon carcinoma cells, MCF7 breast cancer cells and Capan-1 pancreatic cancer cells, which were all kindly provided by Walter Berger (Institute of Cancer Research, Medical University of Vienna). Oregon Green® 488-conjugated goat anti-mouse IgG Ab (Life Technologies, Carlsbad, CA, USA) was used as second step reagent to detect primary CD97, CD274 and CD276 mAbs. Flow cytometric analyses were performed using FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

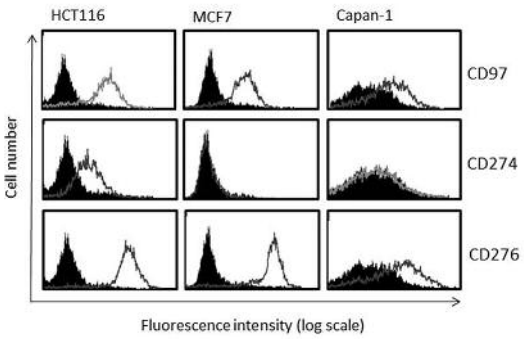


Figure 1. *Cell surface expression of cluster of differentiation 97 (CD97), CD274 and CD276 on colon carcinoma (HCT116), breast cancer (MCF7) and pancreatic cancer (Capan-1) cells was analyzed by flow cytometry and results are presented as overlay histograms. Open histogram (grey line) demonstrates reactivity of the indicated mAbs, reactivity of isotype control is also shown (filled histograms). Gate was set on the live cell population (not shown). Data shown are representative of two independent experiments.*

*Statistical analysis*. Data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) applying Mann–Whitney *U*-test or Kruskal–Wallis test with Dunn's post test for multiple comparisons. *p*-Values were accepted as being significant at less than 0.05. For correlations of metric variables, the Spearman correlation coefficient was used. Overall survival data were analyzed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Survival curves were created using Kaplan–Meier curves. Groups were compared using the log-rank-test with p-values being significant at less than 0.05.

## Results

CD97, CD274 and CD276 are widely expressed cell surface receptors on immune cells but also epithelial cells and on their malignant counterparts. In order to analyze the cell-surface expression of these receptors on tumor cells, we analyzed HCT116 colon carcinoma cells, MCF7 breast cancer cells and Capan-1 pancreatic cancer cells. The results presented in Figure 1 demonstrate that CD97 was strongly expressed on the surface of all three tumor cell lines tested. In contrast, CD274 was only found on HCT116 cells, being absent from MCF7 cells and Capan-1 cells. High levels of CD274 expression were detected on all three cells lines. This profile supports the conclusion that CD97 is a broadly expressed cell surface marker on tumor cells.

The measurement of soluble CD97 molecule showed high expression in patients with all tumor subtypes in comparison to controls (negative control and healthy individuals). In the analysis of the subgroup of cancer entities, we found a significant elevation of CD97 at baseline in 70% of patients before chemotherapy in breast ($p$=0.003), colon ($p$=0.0171) and pancreatic cancer

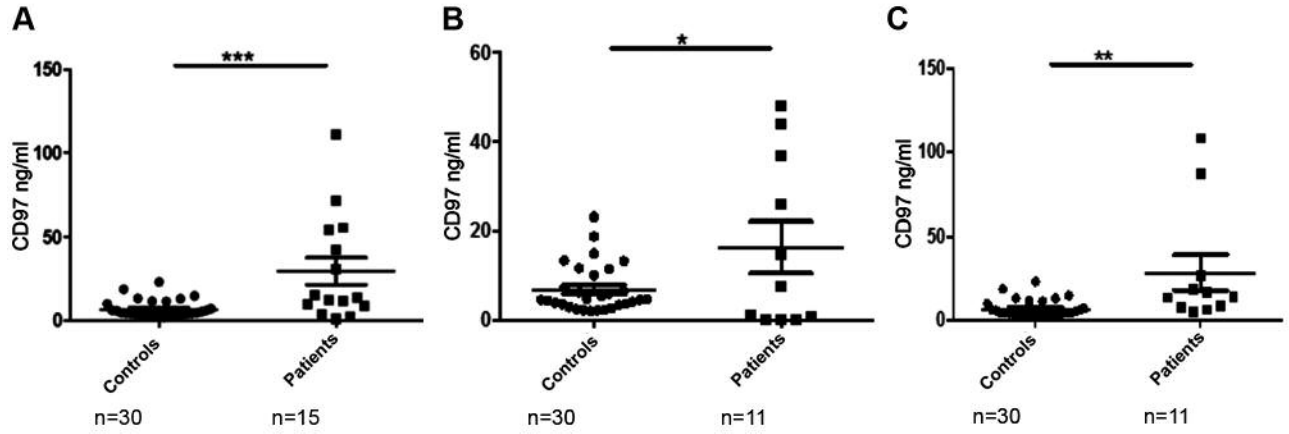


Figure 2. *Levels of cluster of differentiation 97 (CD97) at baseline in patients with breast (A), colon (B) and pancreatic (C) cancer compared to healthy controls were analyzed via ELISA. Results are displayed as mean values±SEM. Statistical analyses were performed with an unpaired two tailed t-test. p-values: *p<0.05; **p<0.01; ***p<0.001. The level of the soluble form of CD97 was statistically significantly higher in comparison to control values for all three tumor entities.*

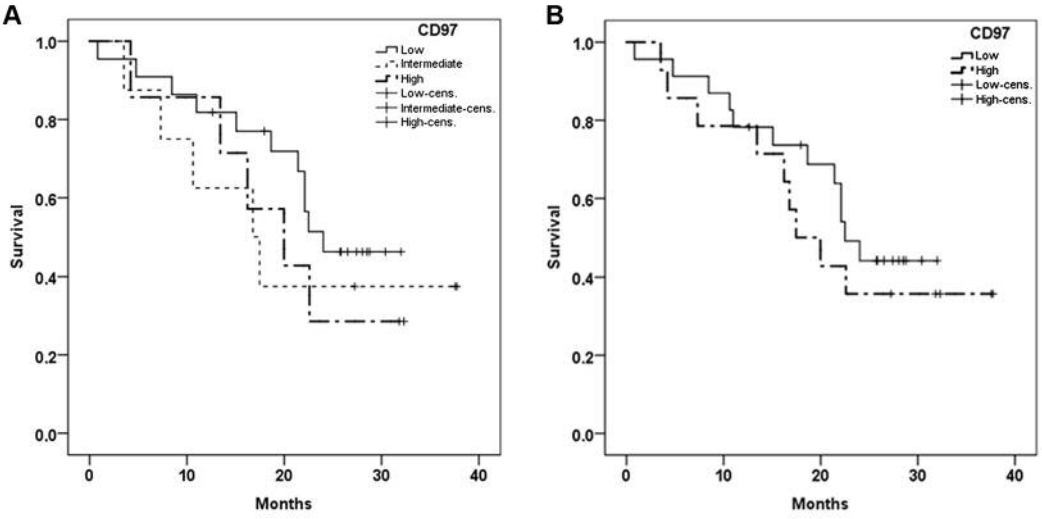


Figure 3. *Overall survival in patients with low (<20 ng/ml) vs. medium (20-50 ng/ml) vs. high (>50 ng/ml) serum cluster of differentiation 97 (CD97) expression (median of 24 vs. 16.8 vs. 20 months, p=0.553) (A) and low (≤25 ng/ml) vs. high (>25 ng/ml) CD97 expression (22.5 vs. 17.5 months, p=0.465).*

($p$=0.0018) (Figure 2). Highest levels were found in patients with breast and pancreatic cancer, with up to a six-fold increase of baseline soluble CD97 compared to healthy controls. Moreover, we found a difference in the level of CD97 in male and female patients. The CD97 molecule was significantly more highly expressed in female patients (mean value 57.93 ng/ml) when compared with male patients (mean value 35.63 ng/ml). In women, the soluble form was present in 80% of cases, while in male patients it was detected in 60% of cases.

We analyzed the outcome of patients with high expression of CD97 (≥25 ng/ml) concerning overall survival. Patients were followed-up for a median of 22 months (range=1-38 months) from the start of chemotherapy and collection of baseline blood samples. The median overall survival of patients with all tumor types with low CD97 (<25 ng/ml) at baseline was 22.5 months (95% confidence interval=19.7-25.3 months) and was 17.5 months (95% confidence interval=11.6-23.3 months) for the high level group (≥25 ng/ml), although not statistically significantly different ($p$=0.465) (Figure 3). In

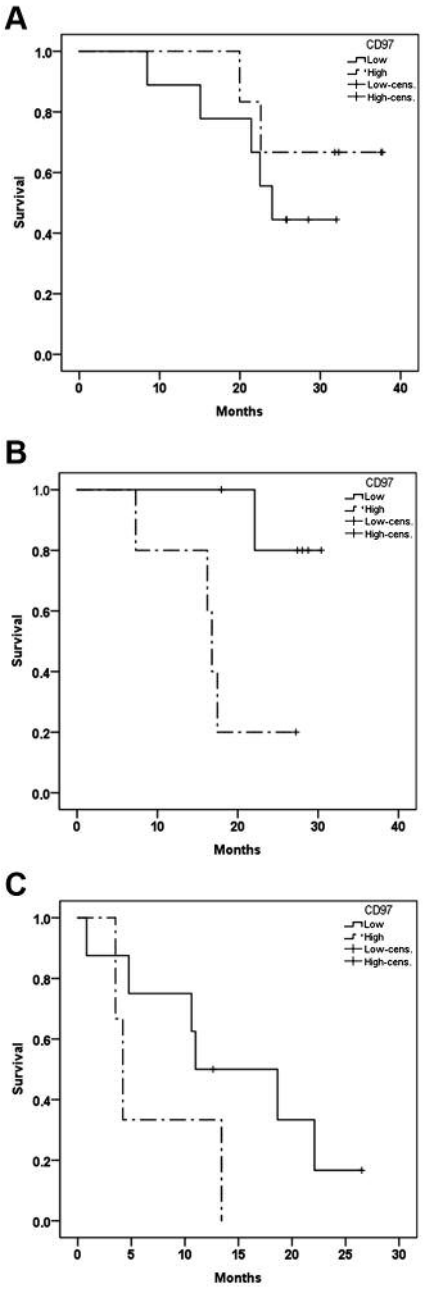


Figure 4. *Overall survival in patients with high and low cluster of differentiation 97 (CD97) expression at baseline categorized by tumor entity: breast cancer (24.01 months vs. not reached, respectively, p=0.4) (A), pancreatic cancer (4.2 vs. 11 months, respectively, p=0.15) (B) and colorectal cancer (not reached vs. 16.8 months, p=0.019).*

the small subgroup of patients with colorectal cancer, those with low CD97 levels (n=6) survived significantly longer than those with high expression (n=5) ($p$=0.019) (Figure 4). There was a trend towards better survival in patients with pancreatic cancer with low CD97 levels *vs*. those with a high level (4.2 *vs*. 11 months; $p$=0.15). In patients with breast cancer, no survival difference was found between the groups.

CD97 levels showed a varying course in patients during chemotherapy. We collected blood samples and clinical data of one representative patient over a time period of 15 months during different treatment schedules. In 13 samples from this patient with breast cancer, the rise and fall of CA15-3 correlated graphically with the radiographic and clinical response to chemotherapy, although not statistically significant ($p$=0.302, Spearman rho=0.311) (Figure 5).

Since CD97 is a protein involved in inflammation and infection, we compared c-reactive protein (CRP) levels during chemotherapy with the levels of CD97. There was no correlation between the variation of CD97 and CRP levels, nor the presence of a clinical infection.

CD274 was not significantly elevated in patients at baseline compared to healthy controls. On the contrary, levels of soluble CD276 were significantly elevated for all three tumor types at baseline (Figure 6). Highest levels of soluble CD274 were observed in patients with breast cancer. In a few samples, extremely high levels of CD274 were detected.

CD276 expression was mostly negative in our cancer patient samples, but a few samples with very high levels even during the course of chemotherapy were observed. For example, in one patient with breast cancer, the CD276 level was extremely low at baseline, whereas during the first cycles of chemotherapy, the level of soluble CD276 increased 50-fold during the remainder of treatment (Figure 7). In a few patients with pancreatic cancer, high levels of CD276 measured at random time points showed great variations during the course of treatment with no causal explanation (for example change in therapy). These high levels became undetectable in the follow-up samples.

## Discussion

In the 1980s, immunotherapy was the promising therapeutic for various cancer types, and the hallmark treatment for renal cell cancer, IFNα and IL2 being the only available treatments for more than a decade for this disease. Despite the high toxicity of high-dose IL2 (29), complete remissions and durable cure can be achieved with this treatment in selected patients. At the beginning of 2000, anti-angiogenic drugs became the rising stars in oncological treatment. With bevacizumab as a vascular endothelial growth factor (VEGF) antibody, the era of anti-angiogenesis began (30). In the following years, tyrosine kinase inhibitors that target VEGF were licensed, followed by multi-kinase inhibitors directed against different targets of tumor cell growth and neo-angiogenesis. These drugs have improved the prognosis of various cancer types significantly, but curing cancer is still an unresolved issue even when combining classic chemotherapy with targeted agents.

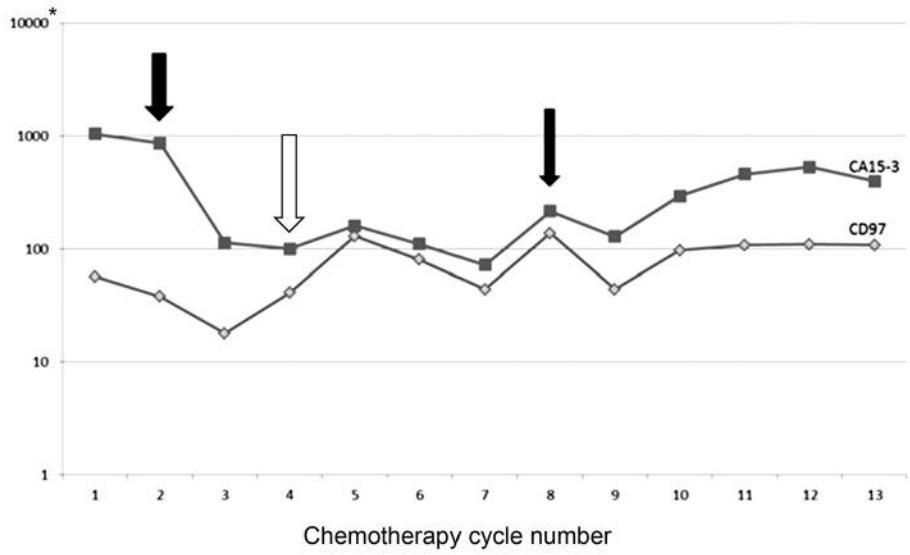


Figure 5. *Carcinoma-antigen 15-3 (CA15-3) and cluster of differentiation 97 (CD97) levels at baseline and measured every 4 weeks during chemotherapy in a representative breast cancer patient. The white arrow marks radiographic response, the black arrow progressive disease according to Radiographic Evaluation Criteria. For Solid Tumors. *Results of CA15-3 (U/ml) and CD97 (ng/ml) were drawn on a logarithmic scale to integrate both variables with different units in one figure.*

Tumor vaccines have also not shown any improvement in either the metastatic setting or in the adjuvant treatment (31).

Last year the horizon re-opened for immunotherapy when results for check-point inhibitors were published. The first candidate tumor melanoma, like kidney cancer, was known to be a tumor that is likely to respond to immunotherapy (32). The combination of Ipilimumab and the CD274 inhibitor Nivolumab is standard of care for patients with wild-type BRAF (rapidly accelerated fibrosarcoma B protein) metastatic melanoma (33). The mechanism of action is the activation of T-lymphocytes that attack the tumor and lead to regression of the tumor and metastases.

The proteins we studied in our patient series (CD97, CD274 and CD276) are proteins that are related to T-cell activation, increased invasiveness of the tumor cells, cell adhesion and migration.

CD97 is the most broadly expressed member of the EGF-TM7 family and has three ligands: CD55, a negative regulator of the complement cascade; integrins; and chondroitin sulfate. CD97 plays an important role in in adhesion and migration of normal immune cells but also in the invasion of human cancer cells. Steinert et al. described an association in colorectal cancer between high levels of CD97 and lymphatic invasion and poor clinical stage (12). Another report was published on rectal cancer correlating tumor recurrence and metastatic disease with high levels of CD97 (34). We showed have that baseline levels of CD97 in patients with metastatic colorectal cancer were significantly elevated compared to healthy patients and had an impact on survival. In the literature, high CD97 levels at baseline in colorectal cancer were associated with a shorter overall survival.

The interesting fact is that CD97 levels showed great variability during the course of chemotherapy. In one patient we showed that at different time points (once monthly) during treatment CD97 levels were relatively randomly elevated or decreased.

One explanation for the variation of CD97 level could be the fact that the activation of T-cells is a regulated process. In urothelial cancer, CD274 expression for example was measured on tumor cells and T-lymphocytes before and during treatment with antibody to CD274 or its ligand. Observations showed that patients that had negative expression on their tumor cells at baseline gained CD274 expression during treatment, which was measured on the re-biopsy specimen (20). Therefore, markers that represent activation of T-lymphocytes are not expressed in a steady state. These protein levels show great variation during the course of disease, making it difficult to correlate expression of these proteins with response to therapy as a prognostic marker. In our patient samples, chemotherapy most likely influenced the levels of soluble CD97. There was no correlation found between the level of CD97, clinical infection or elevated CRP level. Infection could be one reason for activation of CD97 through up-regulation of the ligand CD55. We also did not find a significant correlation between tumor response nor tumor marker response in colorectal or pancreatic cancer. Tumor progression as defined by response evaluation criteria in solid

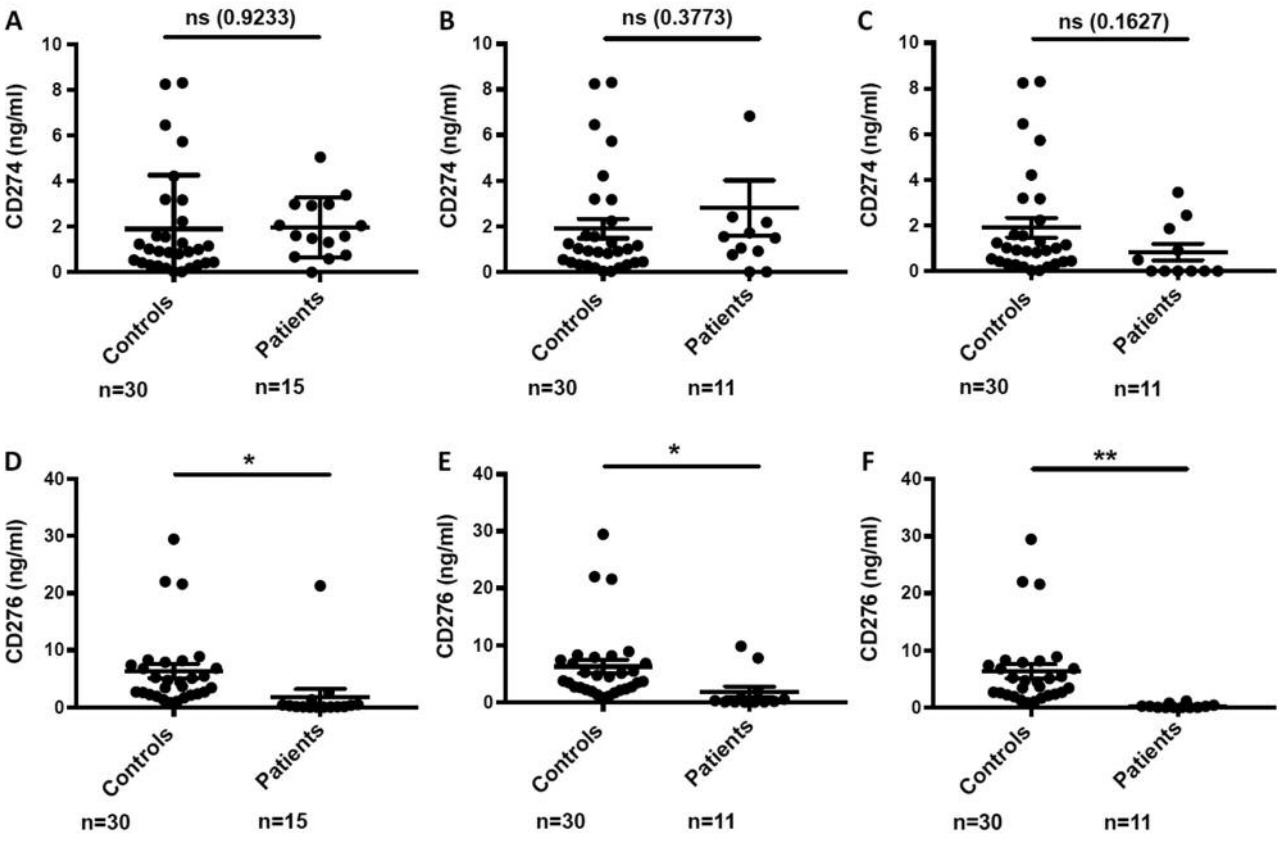


Figure 6. *Levels of cluster of differentiation 274 (CD274) in 30 samples collected at baseline from untreated patients with breast (A), colon (B) and pancreatic (C) cancer compared to healthy controls were analyzed via ELISA. Results are displayed as mean values±SEM. Statistical analyses were performed with an unpaired two tailed t-test. p-values: *p<0.05; **p<0.01; ***p<0.001. The level of the soluble form of CD274 was not markedly elevated in comparison to that for healthy controls. Soluble CD276 was statistically significantly higher in comparison to control values in all three tumor entities, breast (D), colon (E) and pancreatic cancer (F).*

tumors (RECIST) criteria on imaging or rise of tumor markers is a process that is typically slower than that for the markers that are measured. Therefore, the rapid variation of the CD97 levels cannot be correlated with the clinical parameters of progression. However, baseline levels of CD97 are prognostic for overall survival, and therefore pre-define the aggressiveness of the disease. Furthermore, chemotherapy affects certain clusters of cancer cells that express the membranous form of the CD97 molecule.

Tumor markers also represent the tumor burden in a patient. In breast cancer, the tumor marker CA15-3 (also known as MUC-1 or CD227) has a sensitivity of >90% in detecting and monitoring metastatic disease (35). We showed a correlation between CD97 level, clinical response and the tumor marker response (percentage of rise and fall) in one patient with breast cancer with a long follow-up and regular blood samples. Nevertheless, when correlating all levels at specific time points, the correlation was not statistically significant. There have been previous reports that tumor marker progression and radiographic progression are not simultaneous processes. Nicolini and colleagues showed that progression as shown by tumor marker CA15-3 in patients with breast cancer was significantly earlier than radiographic progression (36). This is probably also the case when comparing CD97 and CA15-3 levels at different time points. We saw a trend for an equal rise and fall of both CD97 and CA15-3 during chemotherapy, but no significant correlation when measured at the same time point. So far, there have been no reports of a correlation of CD97 expression and tumor marker CA15-3 response in the literature. Mlecnik and colleagues mentioned CD97 as a tumor marker in their review about immunosurveillance, but did not find any correlation between levels of expression and disease outcome (37). Davies and colleagues suggested that the soluble form of CD97 can be used as a potential tumor marker (38).

CD 274 and CD276 were found at very low levels in all three cancer types. Only in 10% and 7%, respectively, of patients were levels significantly elevated. Interestingly,

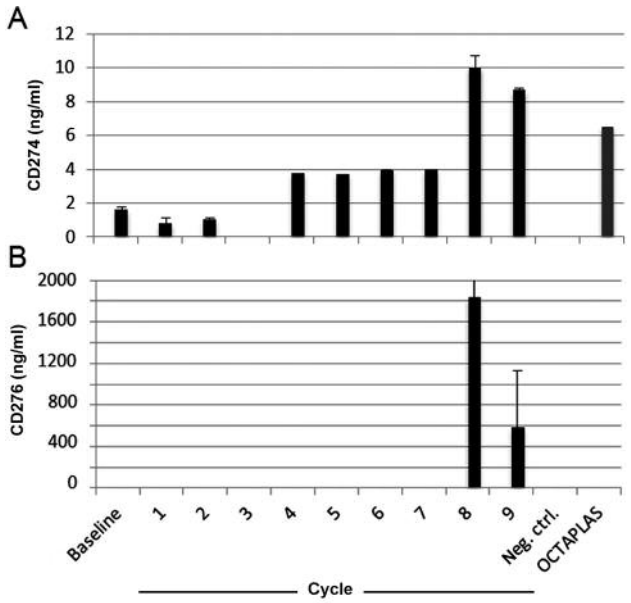


Figure 7. *Cluster of differentiation 274 (CD274) (A) and CD276 (B) in a representative patient with metastatic breast cancer at baseline and during cycles 1-9 of chemotherapy (monthly blood tests). Expression levels of the two forms were unpredictably elevated at different time points. No correlation was found between CD274 and CD276 levels with response to treatment or tumor marker levels of CA15-3. CD276 levels were very low at baseline and during the first cycles of chemotherapy. A marked rise of CD276 (>1,800-fold) was observed during cycle 8 to 10 of chemotherapy.*

samples collected during chemotherapy showed an extreme increase of these proteins, but also a decrease in levels of healthy controls in follow-up samples. Chen and colleagues showed that the level of the soluble form of B7H family proteins increases with age (39). Patients with renal cell cancer also have a higher expression of CD274 (40). In the specific tumor entities studied herein (colon, breast and pancreatic cancer), we only found CD276 as one of the B7H family proteins significantly expressed at baseline. But there was no relevant decrease or increase during chemotherapy treatment, as we showed for CD97.

CD97 is a candidate protein that warrants further investigation in clinical research as a potential tumor marker in breast, colorectal and pancreatic cancer. Soluble CD97 is significantly associated with worse prognosis but is not related to clinical infection and inflammation in patients with cancer.

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