Utilizing machine learning in analysing the anti-cancer effect of a novel immunotherapy approach

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TURUN YLIOPISTO Lääketieteellinen tiedekunta

PÄIVÖLÄ IRA: UTILIZING MACHINE LEARNING IN ANALYSING THE ANTI-CANCER EFFECT OF A NOVEL IMMUNOTHERAPY APPROACH

Rintasyöpä on yksi maailman yleisimmistä syöpätyypeistä, ja uusia syöpätapauksia ilmenee yli kaksi miljoonaa vuosittain. Rintasyöpää on perinteisesti pyritty hoitamaan kemoterapian ja vasta-ainelääkkeiden avulla, mutta immuno-onkologisten hoitojen tutkimus ja käyttö ovat lisääntyneet viimeisten vuosikymmenten saatossa. Syövän immuunihoidoissa pyritään aktivoimaan potilaan oma immuunipuolustus taistelemaan lisääntyviä syöpäsoluja vastaan. Aktiivisen immuunihoidon muoto, ICB-hoito (immune checkpoint blockade), johon tutkimuksemme keskittyy, on tapa aktivoida immuunipuolustuksen soluja taistelemaan syöpää vastaan. Uusi mahdollinen ICB-hoidon kohde on Clever-1 –reseptorimolekyyli, joka esiintyy immuunivastetta heikentävien makrofagien pinnalla. Reseptoriin kohdistuva anti-Clever-1 -vasta-aine on jo osoittanut merkittävää vastetta vaiheen I/II kliinisissä kokeissa syöpäpotilailla. Anti-Clever-1 -hoidolla pyritään kohdistamaan immuunisolut syövän kimppuun, sekä luomaan immunologisesti epäaktiivisiin syöpäkasvaimiin immuunivaste. Tässä tutkimuksessa selvitimme anti-Clever-1 -vasta-aineen molekyylibiologisia toimintamekanismeja sekä tehoa, ex vivo.

Tutkimus toteutettiin potilasnäytteillä, jotka kerättiin Turun yliopistollisessa keskussairaalassa rintasyöpäpotilailta. Potilailta kerättiin rintasyöpäkudosnäytteitä sekä verinäytteitä. Potilaiden kudosnäytteet värjättiin käyttäen immunohistokemiallisia menetelmiä. Lisäksi verinäytteiden perifeerisen veren yksitumaiset solut, joihin luetaan suurin osa ihmisen immuunisoluista, käsiteltiin anti-Clever-1 -vasta-aineella. Vasta-ainekäsitellyillä soluilla hoidettiin vastaavien potilaiden kudosnäytteitä. Tämä mahdollisti immuunisolujen toiminnan analysoinnin sekä syöpäsolujen apoptoosin kvantitatiivisen määrityksen. Analyysi tehtiin fluoresenssimikroskooppikuvista käyttäen tekoälyä hyödyntävää analyysiohjelmaa nimeltään Aivia.

Tämä uudella analyysiohjelmalla tehty tutkimus mahdollisti anti-Clever-1-vasta-aineen vaikutusten tutkimisen entistä tarkemmin ja luotettavammin. Tutkimuksessa selvisi, että anti-Clever-1-vasta-aine auttaa immuunisoluja tunkeutumaan syöpäkudokseen ja aiheuttamaan syöpäsolujen apoptoosia kudoksessa. Hyvä vaste havaittiin noin kolmellakymmenellä prosentilla potilaista.

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1. Introduction

Artificial intelligence and machine learning -based oncology are rapidly conquering the field of cancer research at the moment. The possibilities of AI are versatile since machine learning and neural networks can be used to help in cancer detection and diagnostics as well as in drug research. (1) Artificial intelligence enables the advancement of precision medicine, which is especially important in oncology since the tumor microenvironment and immunological properties are different with each patient and the response rate to current cancer treatments varies a lot. Artificial intelligence can ease drug screening ex vivo and personalized efficacy analysis that could hopefully affect positively cancer patient prognosis in the future.

One of the current cancer treatments called immunotherapy could benefit from artificial intelligence substantially: Only a small group of patients respond to the immunotherapy treatment but the clinical results are very good with selected patients. Artificial intelligence could make the selection process better to target the benefits of immunotherapy for responsive patients. (2) The immunotherapeutic target we studied in this paper is called Clever-1 (common lymphatic endothelial and vascular endothelial receptor-1), an innate immune checkpoint, which can be found on the surface of monocytes and immunosuppressive macrophage populations. Clever-1 promotes tumor growth and metastasis formation and the blockade of it induces T-cell activation and killing of the cancer cells.(3) A humanized antibody targeting Clever-1, bexmarilimab, has shown promising clinical results. (4)

In this study we investigated the effect of bexmarilimab in inducing cancer cell killing via tumor infiltrating monocytes. For this we utilized patient-derived tumor explant cultures and matched peripheral blood mononuclear cells preincubated with bexmarilimab. Cancer cell apoptosis was visualized by TUNEL staining and quantified from high-resolution images of tumor sections with artificial intelligence. Analysis of tumor sample images was done with an artificial intelligence-guided image analyzing software Aivia. The software was trained to classify different cell populations based on their fluorescence emitted in the 3D structure of the tissue. Utilizing AI in analysis enables objective and reliable analysis of the microscopic images where the software is also able to recognize non-specific staining that can be very difficult to dissociate with a human eye.

2. Review of the literature

2.1 Precision oncology

Cancer as a disease is a heterogeneous and constantly changing adversary for drug development. When a suitable treatment for the cancer patient is found, it is common for cancer cells to develop resistance. (5) Multiple genetic and phenotypic properties can affect cancer drug resistance. Precision oncology is an approach that takes into account different individual resistance factors and tries to find the most effective treatment for that patient. (6)

In practice, precision medicine is often equated with genetic and molecular markers, which have been key elements in developing specific monoclonal antibody treatments for different cancer types. Well-known examples of these genetic molecular markers used in cancer treatment are HER 2, EGFR, KRAS and the list goes on. (7) However, several studies have shown that genomic testing alone doesn't benefit most cancer patients. (5)

The field of precision oncology has focused mainly on in vivo and in vitro testing. Since these approaches have had a high failure rate and low response rates in human clinical trials, the interest has been shifted to alternative drug discovery and repurposing methods like ex vivo approaches. (8) These approaches include a variety of drug screening methods that utilize patient-derived material like cancer cells and tumor tissue or xenograft models to model the disease outside of the human body. Ex vivo approaches have not yet been used widely within the cancer research field, but in the few clinical trials, the results have been promising. Ex vivo techniques may be the final possibility for patients who have not benefited from first-line cancer therapies. (5)

2.2 A Brief history of ex vivo drug screening

The story of ex vivo modeling started in the late 1800s when embryologist Wilhelm Roux cultured chicken embryo cells on a petri dish and developed the earliest version of ex vivo cell culture. (9) In 1960 the most popular way to culture cells was the Mosconan technique where cells were cultured in a swinging Erlenmeyer bottle. (10) Later it was understood that ex vivo analysis of cancer growth with two-dimensional models is not as realistic as with three-dimensional cancer models called spheroids (nodal cancer cell formations) or organoids (nodal cancer cell formations that have typical cancer microenvironment). (11)
Cancer cells are never the only cells in the tumor microenvironment and that is the shortage in pure cancer cell line models. A more realistic way to observe cancer growth in the human body is to culture cancer tissue derived from patients. In the late 1950s, researchers started culturing differentiated tissue slices in between a liquid and gas. A few examples can be seen in Figure 1. The challenge in this method was a necrotic region in the middle of the tissue and the tissue slices

were preserved only for 6-9 days. (12) In the 2000s researchers understood that the best way to culture patient-derived tissue is to cut the tumor into small pieces which can be cultured on a gel surface. This method is called PDEC (patient-derived explant cultures). (9)
In ex vivo drug-screening, patient derived cell or tissue cultures are used to screen treatment options for the specific cancer type. The first clinical success with ex vivo drug screening was shown with hematological malignancies in the early 2000s. One of the most important early ex vivo drug trials was done with acute myeloid leukemia patients. Ex vivo drug screening is easier with hematological malignancies since the patients' peripheral blood samples don't require surgeries or biopsies.

However, ex vivo drug screening can be done with solid tumors as well. The first ex vivo drug screening with solid tumors was done already in the late 1970s. (5) Nowadays PDEC models enable ex vivo drug screening with individual patient samples. The method is not yet in frequent clinical use but may be the future of individualized cancer treatment. (9)

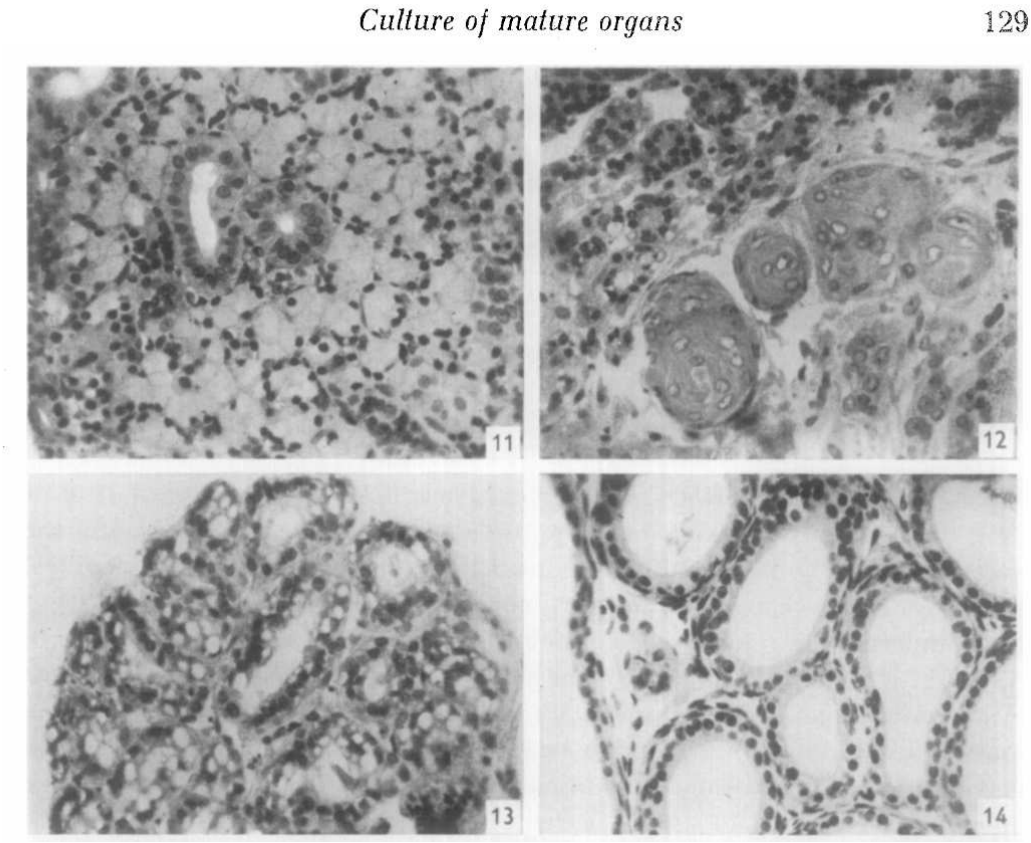

Figure 1: Early methods for tissue culturing from 1959. O.A. Trowell, The culture of mature organs in a synthetic medium,
Experimental Cell Research

2.3 Ex vivo techniques

Several clinically relevant models can be used in ex vivo drug screening in precision oncology. Some of them are presented in Figure 2 below. The patient's tumor type determines which ex vivo methods are the most suitable ones. The form and the qualities of the sample also define which methods are usable. Peripheral blood samples enable two-dimensional cell culturing easily with minimal invasion to the patient. Three-dimensional modeling however gives more realistic environment for research but requires invasive operations. There are also ethical and more often bureaucratic challenges with sample acquisition since surgical resecates are primarily used for histopathological diagnosing instead of research purposes. (5)

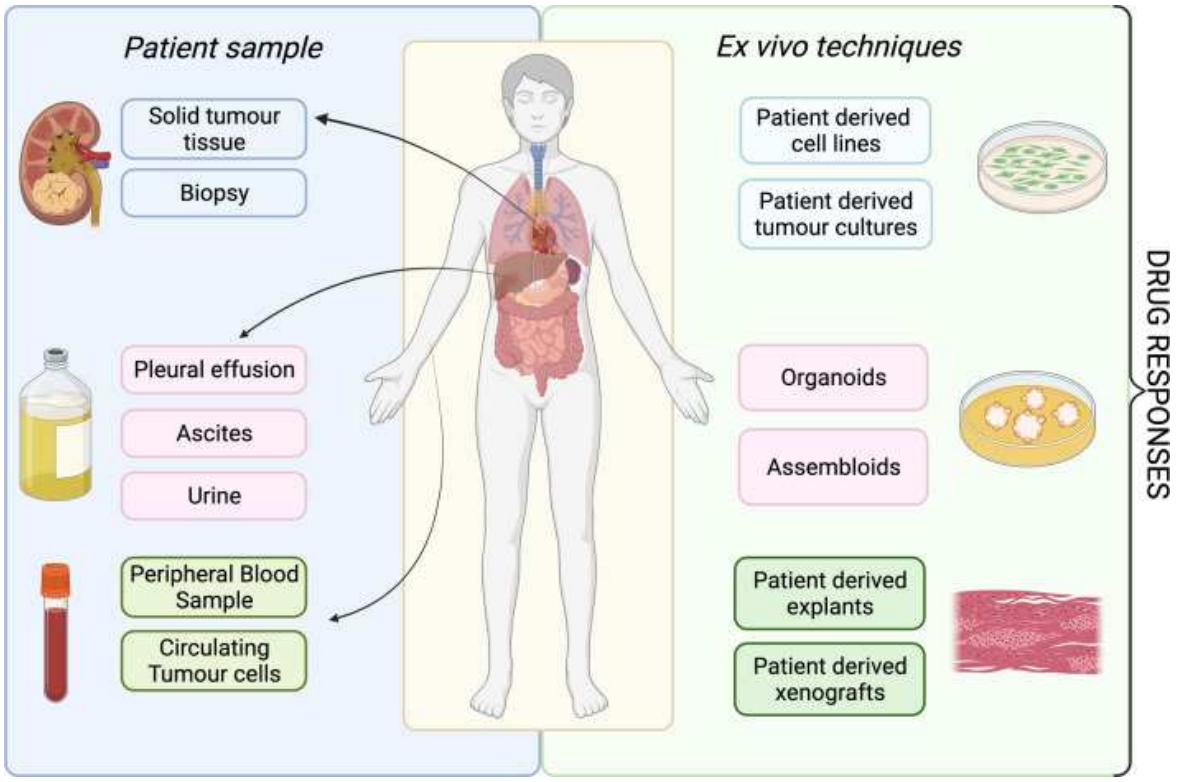

Figure 2: Ex vivo techniques, Williams et all: *Precision oncology using ex vivo technology: a step towards individualised cancer care? (5)*

2.3.1 Patient-derived cell lines

Patient-derived cell lines (PDCLs) are specific cell lines derived from the primary tumor tissue. They enable diverse drug screening which has been done for example with HPV-negative head and neck squamous carcinoma cells by Lepikhova et al. (13) In this study, 45 different cell lines were screened with 220 anticancer drugs and the data provided multiple new genomic variations associated with drug resistance. Another PDCL study made by Kim et al. used 96 malignant effusions from 77 lung adenocarcinoma patients to create 23 cell lines. (14) With these PDCLs, the group could present that the drug screenings ex vivo reflected patient drug response in a clinical

setting. These findings suggest that PDCLs could be used in precision oncology by clinicians when selecting the most effective treatments for each patient.

2.3.2 Patient-derived tumor cultures

Another way for ex vivo drug screening is patient-derived tumor cultures which use multiple cell populations, not only cancer cells. The cell suspension can be from solid tumor or interstitial fluid like ascites or pleural fluid. This method has been used with rare cancer types and three research groups have reported using this technique in direct patient treatment. (4) One of the case studies done for direct patient treatment was done by Mäkelä et all. (15) In this study, they reported the first use of ex vivo drug screening in combination with Next-generation Sequencing to find a suitable treatment for a patient, suffering from epithelial-myoepithelial carcinoma, who could not benefit from surgery, radio- or chemotherapy.

2.3.3 Organoids & Assembloids

Organoids are organ-like 3D cell structures that model essential features of an original organ in vivo. Organoids can be derived from a patient's healthy or cancer tissue. They can be used to screen anti-cancer drugs more reliably since they are able to mimic the tumor structure and microenvironment ex vivo and give more clinically relevant results in drug screening. (16) Usui et al. studied drug concentrations and resistance with colorectal carcinoid (CRC) organoids by studying drug toxicity in 2D cell culture compared to the CRC organoid. An interesting finding was that cell death in organoids correlated with the drug concentration which enables drug dosage studies that can't be done as reliably using 2D cancer models. (17)

Organoids are formed with one cell type, but in the tumor site, there are always different types of cells working together. Organoids that are formed from multiple different cell types are called assemblies and they can produce even more realistic information about cancer and the tumor microenvironment. (5)

Organoids have properties that are especially practical in precision medicine and drug screening. Organoids have been used in panels that offer an efficient way to find sensitive cancer therapies for individual patients. Researchers are currently eager to develop this method to fit the demand of clinical use. (16) Currently the biggest challenge with organoids is the lack of blood supply, which leads to growth limitation and center necrosis due the lack of nutrients and oxygen. (5)

2.3.4 Patient-derived explants & xenografts

Patient-derived explants are surgical resecates or biopsies from the patient's tumor which can be cultured to maintain viability for drug screening. (18) There are multiple ways to culture patient-derived explants and some of them include the patients' serum to promote tumor growth. (5) Patient-derived xenografts are fresh tumor pieces but instead of culturing, they are planted into

immunodeficient mice. (19) The xenografts offer even more realistic environment for research, but the challenge is to find enough suitable tumor material for explants or xenografts, because mainly surgical resecates or biopsies are taken into histopathological analysis. The small amount of tissue is also limiting the number of drugs that can be screened with these methods at the moment. The xenograft generation process is also costly and slow taking up to 4-8 weeks. One limitation is also the large demand for animal testing. Patient-derived explants or xenografts have not been in use with patients yet but they would be a realistic way to mimic the tumor microenvironment for drug screening purposes. (5)

2.4 Artificial intelligence developing cancer research

Artificial intelligence (AI) is currently revolutionizing cancer research and its applications in oncology are limitless. Artificial intelligence is a broad term used to describe software that mimics human cognition. Machine learning and deep learning are two significant subclasses of AI. Machine learning enables software to learn and recognize patterns in data and use that knowledge to make decisions on new data. Deep learning instead creates artificial neural networks that are interconnected in multiple ways. (20)

One of the first ways AI was used in oncology was cancer detection from patient samples. In 2015-2016 a group of researchers hosted a competition to develop an algorithm to detect lymph node metastases from histopathologically stained samples. The results of the best AI algorithms were surprising: they were able to detect lymph node metastases from test images more accurately than a panel of 11 pathologists. (21) AI could also provide better methods for cancer diagnosis and staging: In a study of Liu et al an AI based algorithm was used to detect breast cancer lymph node metastasis, which are a key element of breast cancer staging. (22) The algorithm called Lymph Node Assistant, LYNA achieved higher sensitivity compared to pathologists.

Diagnostics could also be made non-invasively with images like CT or MRI scans, possibly avoiding the need for surgery: In a study by Jiang et al, a neural network called the Peritoneal Metastasis network was able to detect clinically occult peritoneal metastases from CT images, which enabled early detection of these metastases and helped to accomplish personalized treatment for these cancer patients without unnecessary surgical operations. (23) In precision medicine, early detection is the key to effective treatments. Early cancer detection can be done with simple blood tests since AI can spot more effectively biomolecules like circulating tumor DNA from the blood and make predictions about the disease (20). Researchers from Johns Hopkins University School of Medicine developed a blood test called CancerSEEK which can detect the eight most common cancer types and predict these cancer types from circulating tumor DNA and protein biomarkers. What is remarkable is that this method is also the only screening test currently available for five of these cancers. The AI algorithm in this study was trained to take into account the ctDNA, protein biomarkers, and the gender of the patient. (24)

Another aspect of utilizing AI in cancer research is drug target detection and efficacy. AI has been used to create new drug structures with suitable molecule structures in silico for target sites. AI can also be very useful for finding new therapeutic uses for existing drugs. This method called drug repurposing has turned out to be a safe and cost-effective way to create cancer medications. (20) For example, Gottlieb et al created a neural network called PREDICT which has been used to detect new indications for known medications and for novel products by integrating similarities of diseases and drugs. One known identification by PREDICT is the repurposed use of progesterone in renal cell carcinoma. (25) The concept of precision medicine relies on drug screening, and AI is an efficient helper in that process. A field of cancer treatment that relies a lot on drug screening is the immune checkpoint inhibitor therapies. These therapies have low response rates, but they work exceptionally well with selected patients (20). Johann et et al. created a neural network that predicts immunotherapy response from melanoma patients. The algorithm detects prognostically important features from histopathological melanoma tissue samples. (26) AI algorithms can be beneficial in analyzing therapy response early, which can be vital for a patient's prognosis. A machine learning model called CURATE.AI has been used to monitor therapy responses and adjust drug dosages with small patient groups. (27) These kinds of AI approaches could be the enablers of personalized cancer treatments and precision medicine if the technologies can be adapted to clinical work.

3. Materials & methods

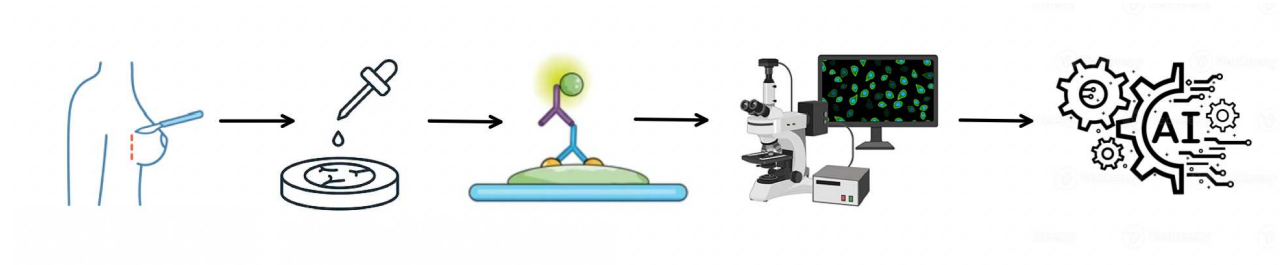

Figure 3: Materials and methods as images

The precise details about tissue staining and imaging can be found in the thesis of Arno Ylitalo: *Clever-1 in cancer – Assessing the efficacy of a novel immunotherapy approach in patient-derived explant cultures.* (28) This paper will focus more on the use of AI in image analysis and statistical methods.

3.1 Tissue sample collection

The study was made using breast tissue samples, which were collected from naive breast cancer patients during breast ablation surgery at Turku University Hospital. We also attained blood samples from the same breast cancer patients. We extracted the peripheral blood mononuclear cells (PBMC) from these samples using centrifugation and the Ficoll-Paque® method. The PBMCs have round nuclei and include heterogenous lymphocytes like T-cells, B-cells and NK-cells, monocytes, and dendritic cells. Finally, the tissue samples and PBMC samples were preserved frozen in OCT blocks and serum supplemented with 10% DMSO, respectively.

3.2 Tumor ex vivo treatment, staining & imaging

The PBMCs were stained using Carboxyfluorescein diacetate succinimidyl ester. The staining was done to be able to track PBMCs in the breast tissue later. The count of cells was checked before and after staining and the dead cells were identified by Trypan Blue staining and excluded from the cell counts. Lastly, one million cells were treated with anti-Clever-1 antibody (FP1305 from Faron Pharmaceuticals), one million cells with Human IgG4 antibody (Ultra-LEAF™ Purified by Biolegend), and one million cells were left with no antibody treatment to be used as a negative control.

The second phase was to coculture tumor samples with stained PBMCs:s. The tumor samples were thawed and suspended in a solution containing DME medium, fetal calf serum, and P/S solution. The tumor pieces were cut into three pieces and set in wells. The tissue samples were introduced to each patient's PBMCs with FP1305 antibody, IgG4 antibody, and negative control cells. Finally, the tissue samples were incubated for 22 to 26 hours and frozen with O.C.T.tm compound (Tissue-TekR).

The tissue samples were stained using an antibody solution containing: Pan Cytokeratin A488 1/200 (Thermo Fisher Scientific), CD8 A555 1/100, and FP1305 A647 10µg/ml antibody (Faron Pharmaceuticals) or IgG4 A647 10µg/ml (Ultra-LEAFTM Purified by Biolegend) for control samples. Anti-cytokeratin staining was done to be able to see epithelial tumor cells. To find the apoptotic cells in mammary adenocarcinoma tissue TUNEL staining was done according to the manual provided by the manufacturer. Nuclear Hoechst 33342 (Thermo Fisher Scientific) staining was done to detect all cells in tissue samples.

The final step before analysis was imaging. Treated tissue samples and control samples were imaged with two different types of spinning disk confocal microscopes with four laser lines detecting 405µm, 488µm, 561µm and 640µm channels. The imaging of untreated sections was done with a Carl Zeiss LSM780 laser scanning confocal microscope using three 1 µm interval and 2x2 810µmx810µm area. The treated tissue samples were imaged with an area of 3x3 tiles (2048 × 2048 pixel) and four 1µm stacks. The clearest sections of the tissue were selected for the imaging.

3.3 AI analysis & statistical methods

The Analysis of the microscope images was done with an artificial intelligence -guided image analysis software Aivia (AiviaWeb version 2.0). The algorithm was educated to classify different cell populations based on their fluorescence emitted in the 3D structure of the tissue. The teaching was done using two representative microscopic pictures where reliable examples of apoptotic and alive cells could be found. The algorithm was then perfected to detect only the desired cell types from the nonspecific staining.

Image assays were done from the tissue images of a randomised group of nine breast ablation surgery patients from Turku University hospital. The purpose of the first assays was to detect apoptotic cells from the tumor tissue samples. The assays were done with tumor tissue treated with PBMC:s, which had been treated with Clever-1 antibody and IgG4 antibody. Assays were also done with control PBMC:s without antibody treatment. The algorithm was taught to detect all cells from the microscopic images and divide them in two classes: apoptotic and live. The detection based to the Hoecst and Tunel staining: Hoecst staining detected all cells in the tissue samples and the Tunel staining detected apoptotic cells. The algorithm was taught to recognise the difference between the emitted fluorescence between these stainings. The advantage of the algorithm was the possibility to analyse samples in three dimensions since the imaging was done in multiple layers. All the images for the apoptosis assay were analysed with the same algorithm and with the same threshold values. The software gave also data about the classes: cell count, cell volume, surface area, volume ratio, and class confidence. Cells with class confidence over 70% were counted as reliable so the cells with lower confidence rate were discarded from further analysis. Also the detected particles with too small volume (<0,0001µm^3) for a cell were discarded from the further analysis.

Another assay was done from the tissue images from the same breast ablation surgery patients

from Turku University Hospital. The purpose of the second assay was to study the amount of the treated peripheral blood mononuclear cells infiltrated inside the tumor tissue. In the tumor site patients' immune cells such as PBMC:s are allured by cytokines to infiltrate into the cancer tissue. The idea of the anti-Clever-1 treatment is to induce that immunologic response and increase cancer cell apoptosis by activating immune responses in the tumor site.

The second assay was done with the same samples as the first one, but the algorithm was changed to detect PBMCs infiltrated into the cancer tissue. Green CSFE staining was used to detect all PBMC cells. The second algorithm gave the count of PBMC:s in the samples as well as the volume, volume ratio and surface area of the cells. All the images for this assay were analysed with the same algorithm and the same treshold values. All the detected particles with too small volume (<0,0001µm³) to be PBMC:s were discarded from further analysis.

3.4 Statistical methods

Table 1 summarizes the results of image analysis with the Aivia algorithm. The mean and the standard deviation of each image analysis can be seen in the Table 1.

66 microscope images were analyzed overall, including 19 with FP1305, 18 with IgG4 control, 7 of untreated PBMC controls and finally an additional 6 images of control tumors not treated with PBMCs.

We compared the values in the anti-Clever-1 –treated PBMC assay with the results from the control-antibody (IgG4) –assay and calculated the p-values presented in the Table 1. We employed a two sample t-test to compare the means of the anti-Clever-1-treated and IgG4-treated cell groups to see if there is a statistically significant difference between the means of the groups.

4. Results

4.1 Anti-Clever-1 treated PBMC migration into explants

The CFSE staining visualizes Anti-Clever-1 and IgG4 treated PBMC:s with green color in Figure 7. Hoechst staining stains all cells in the tissue with blue color. Since other tumor consisting cells than treated PBMC:s are not stained with the bright green CFSE staining, they can be seen in blue. The Anti-Clever-1 treatment has induced more PBMC migration into the tumor tissue in some patients compared to the control antibody IgG4. The patients that were responsive to the treatment with more PBMC migration can be seen in Figure B. Figure 8 shows a count of CFSE-positive PBMCs:s in cancer tissue samples. Patients are shown with different colors. The left value is the control antibody IgG4, and FP1305 is the anti-Clever-1 antibody. Five of the nine patients indicate a response to treatment which means more migrated PBMCs in the tumor site compared to the control antibody.

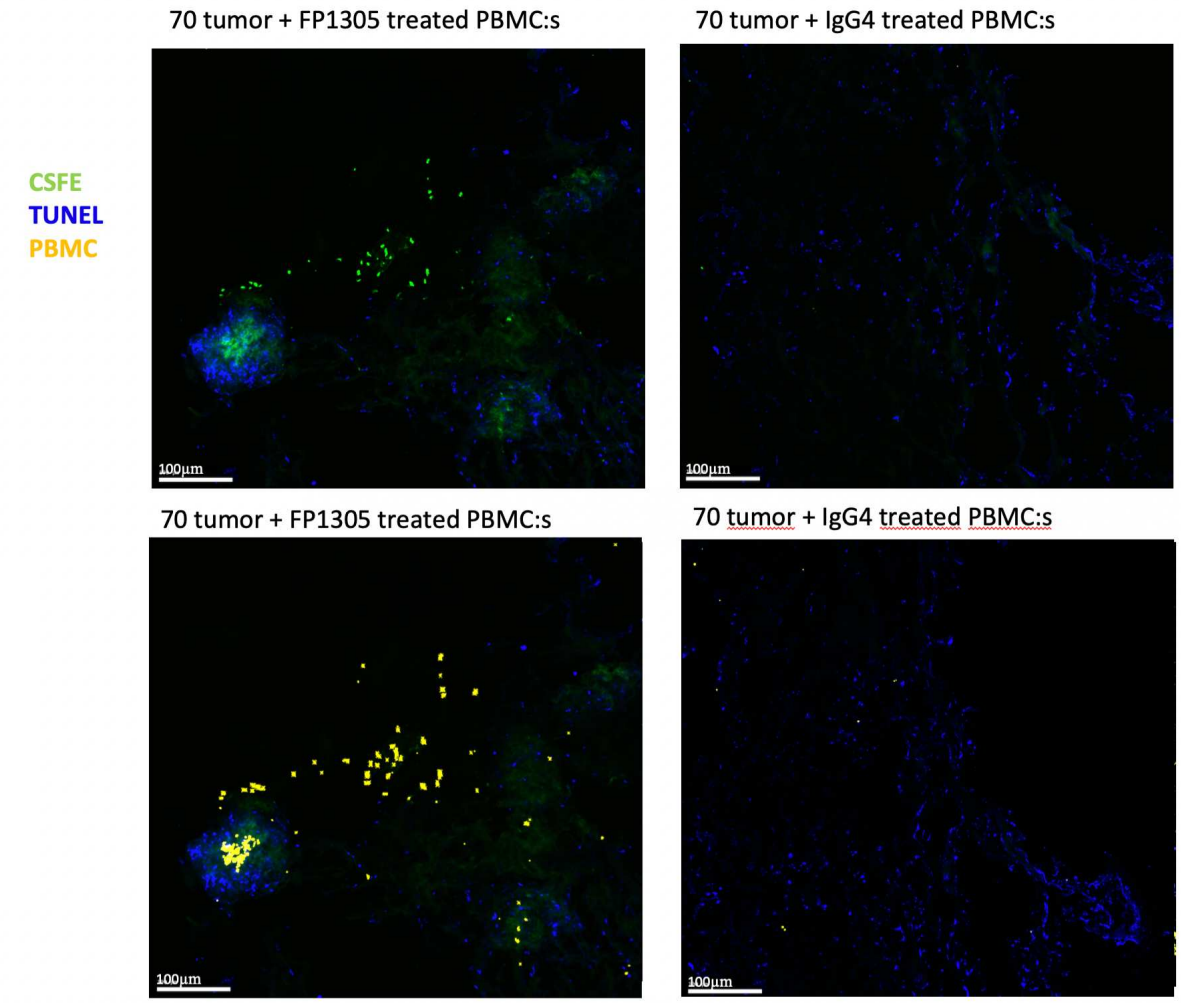

Figure 7: Anti-CLEVER-1 treated tissue compared to control antibody treated tissue. CFSE stained PBMCs are shown in green in the upper images. Aivia has recognized PBMCs in the lower images.

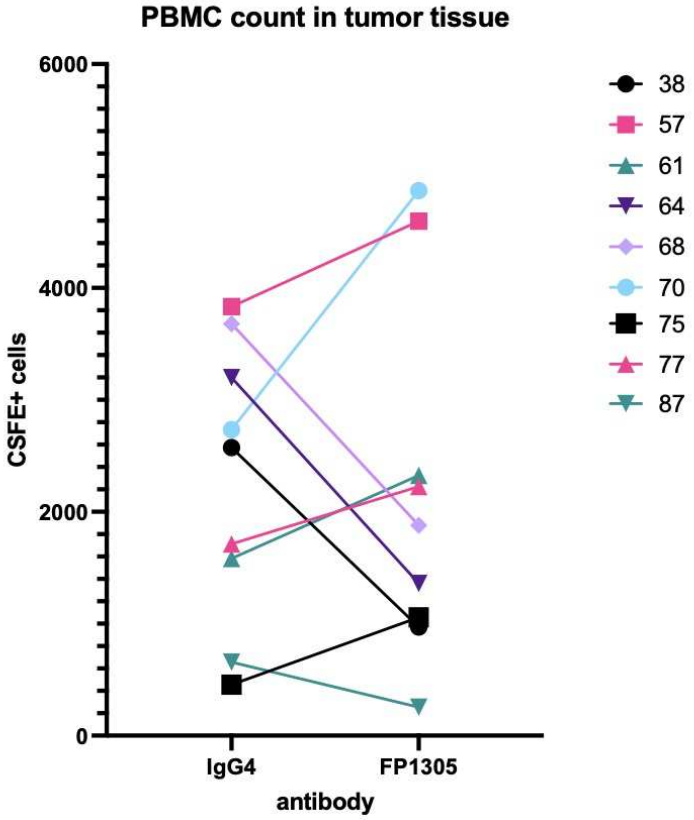

Figure 8: PBMC count in tumor tissue after treatment by patient number and antibody.

4.2 Apoptosis induced by Anti-Clever-1 treated PBMC:s

The TUNEL staining shows apoptotic cells as pink, which can be seen in Figure 4. The algorithm of Aivia has also identified apoptotic cells and they can be seen as yellow in Figure 5. The non-apoptotic cells can be seen in blue by the Hoechst stain in both comparisons. Anti-Clever-1 treated PBMC:s have induced more apoptosis in comparison with control antibody IgG4 with three patients that have responded to the treatment. The responders can be seen in Figure 6. Figures X and Y are visualizing the difference between apoptotic cell counts in a responder and a non-responder. Figure Z shows a percentage of apoptotic cells in cancer tissue samples. Patients are shown with different colors. The left value is the control antibody IgG4 and FP1305 is the anti-Clever-1 antibody. Three of the nine patients indicate a response to treatment that correlates with previous studies and preliminary results from a phase I/II clinical trial with advanced solid tumors. Additionally, three patients (numbers 57, 70, and 75) demonstrated a correlation between the higher count of PBMCs in the tumor tissue and the percentage of apoptosis.

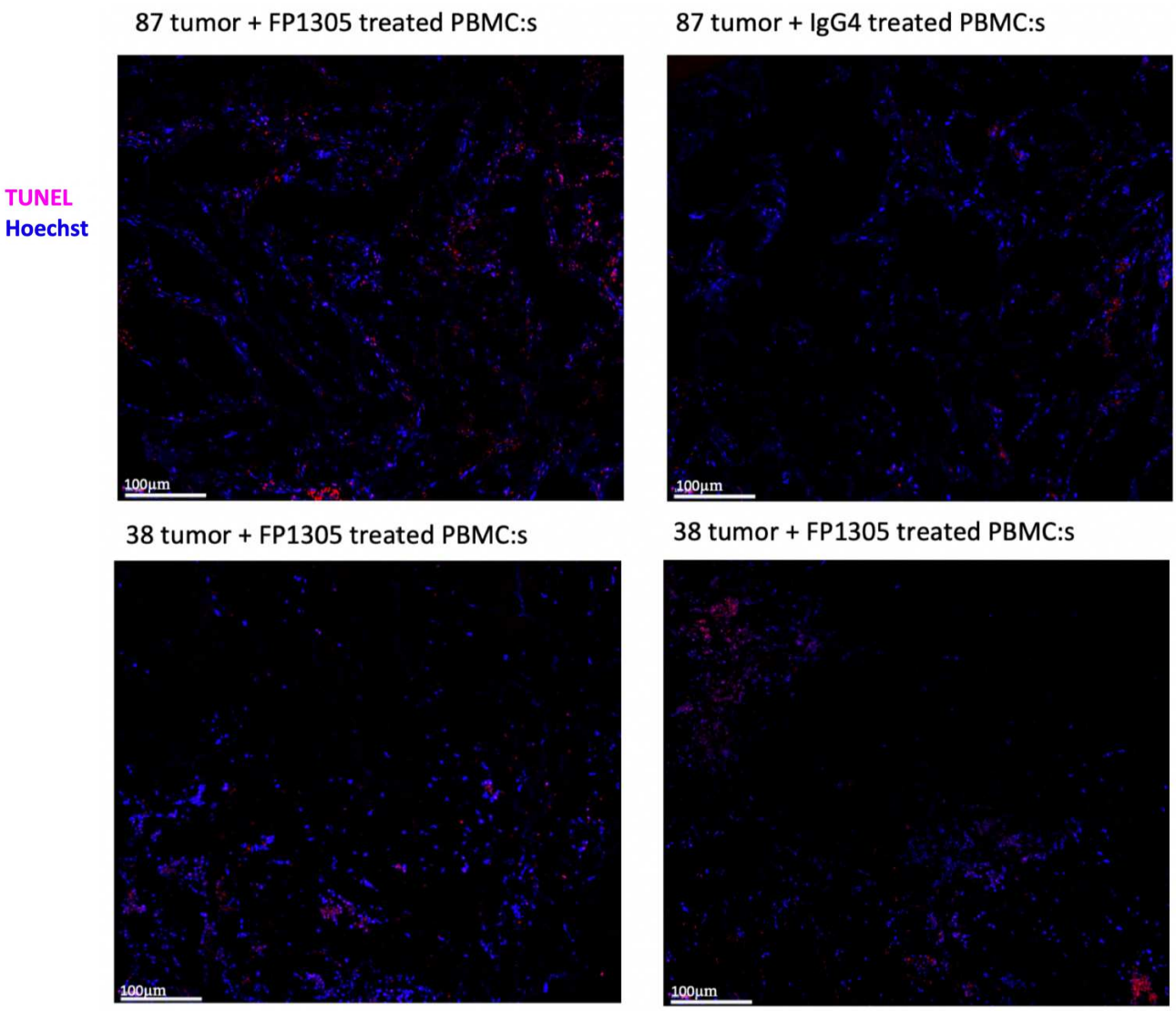

Figure 4: TUNEL binding (pink) in responder 87 and non-responder 38. Anti-Clever-1 treated (FP1305) tissue compared to the control antibody (IgG4).

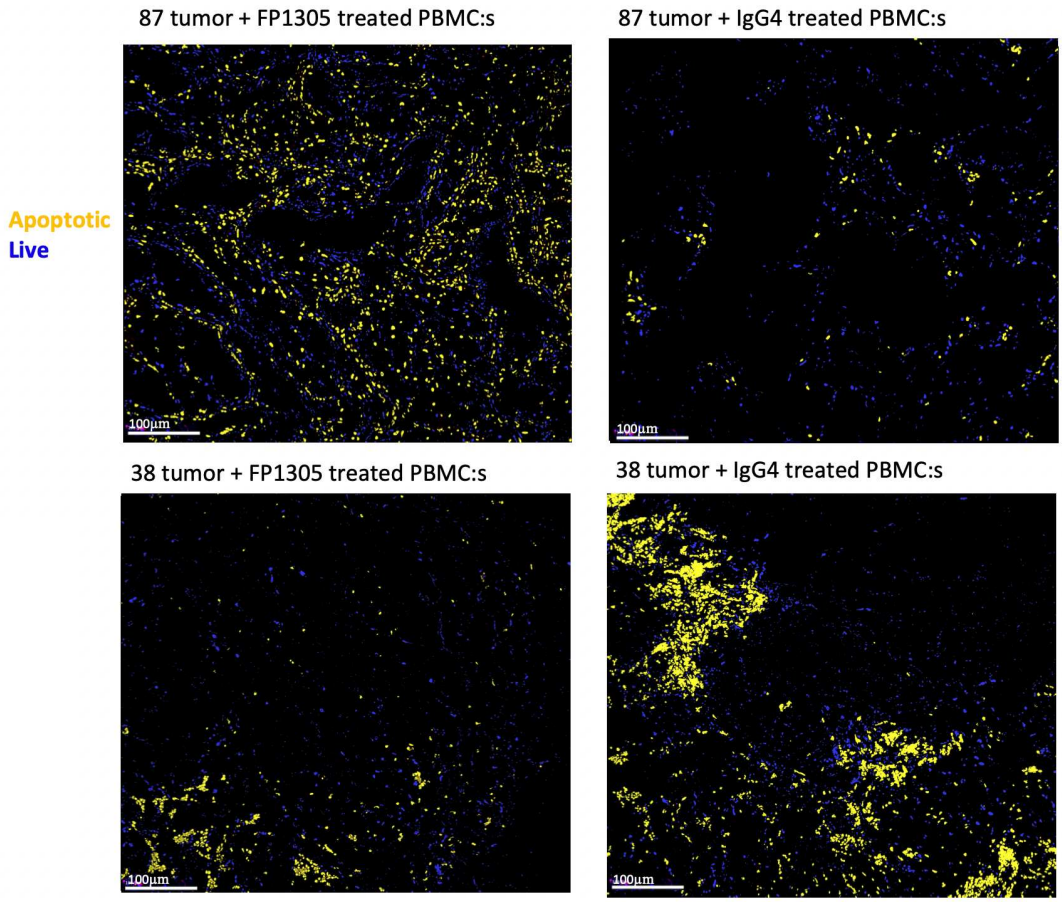

Figure 5: AIVIA has classified cancer cells as apoptotic or live. The anti-Clever-1 treated image and control antibody-treated image have been compared to from a responder 87 and non-responder 38.

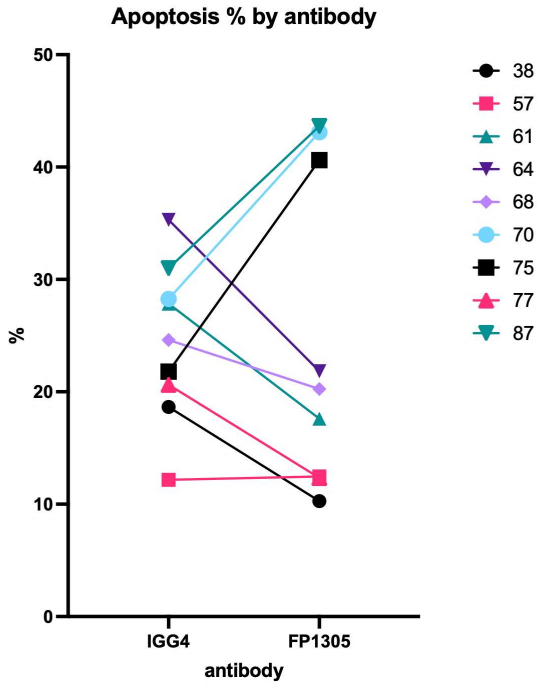

Figure 6: Apoptotic cells in tumor tissue after treatment by patient and antibody.

	Total n=9	Anti-Clever-1-treated n=9	IgG4-antibody control n=9	Untreated PBMC control n=7	Tumor negative control n=6	Clever-1-treated n=5	p-value
Total cell value mean (sd)	11429 (5614)	11760 (6420)	10990 (6334)	6613 (5271)	8200 (1126)	4689 (3819)	0.02-0.03
Tunel stain value, mean (sd)	2154 (1493)	2167 (1860)	2082 (1347)	773 (836)	2065 (1203)	682 (658)	>0.05
CFSE+ PBMCs, mean (sd)	2759 (2310)	2589 (2665)	2901 (1948)	24 (n=1)			>0.05

Table 1: Summary of the results of image analysis with the Aivia algorithm. The mean and the standard deviation of each image analysis can be seen in the table. P-values presented compare the values in the anti-Clever-1 –treated PBMC assay with the results from the control-antibody (IgG4) –assay. The calculation was made using a two sample t-test.

5. Discussion

During the last decade, artificial intelligence-guided algorithms and neural networks have found their place in the world of cancer research and precision medicine. The potential of AI in cancer research is diverse since the possible ways to use AI extend from cancer drug research to diagnosing, clinical treatments, and prevention. There are already several research groups that have used the help of artificial intelligence successfully in different sectors of cancer research and the number of ongoing studies is increasing constantly. The limitations of artificial intelligence are the huge demand for data and resources that the development of a reliable AI algorithm needs. In a clinical setting, the data collection takes time and brings challenges with the bureaucracy. In this study, we utilized artificial intelligence to analyse the anti-cancer effect of a novel immunotherapy approach. The AI software gave us fascinating data about the tissue samples, but the teaching of the algorithm was quite time-consuming.

Immunotherapy has revolutionized cancer treatments and it is a hot topic amongst different cancer research groups around the world. Immunotherapies can be divided into passive and active immunotherapies and this study focuses on active immunotherapy, specifically a method called immune checkpoint blocking, which targets the key regulators of the immune system aiming to establish anti-tumor immune responses in the tumor site. In this study, our focus was on blocking the Clever-1 receptor. The main purpose of this study was to understand the effect of anti-Clever-1 treatment, which has already shown promising results in clinical studies with cancer patients. A novel aspect of this study was the utilization of machine learning in image analysis, resulting in more accurate and reliable results compared to the previous analyses on ex vivo assays.

The first way to demonstrate the biological effect of anti-Clever-1 treatment in the tumor microenvironment was the ex vivo apoptosis assay. The purpose of this assay was to image apoptosis induced by anti-Clever-1 treated PBMC:s. PBMCs or peripheral blood mononuclear cells include lymphocytes like T-cells, B-cells and NK-cells, monocytes, and dendritic cells. In this study, we were especially interested in the monocytes, which turn into macrophages at the tumor site. Normally the tumor microenvironment has M2-type macrophages which promote tumor growth and reduce inflammation. The Anti-Clever-1 treatment by blocking the Clever-1 receptors on the surface of the M2 macrophages can turn those macrophages into M1-type macrophages with tumor suppression and immunostimulation properties. In this assay, we imaged these tumor suppression properties by detecting the apoptosis induced by anti-Clever-1 treated PBMC:s. Patients' PBMCs:s were treated with anti-Clever-1 antibody and these cells were cocultured with the cancer tissue samples. The percentage of apoptosis was then analyzed with an artificial intelligence algorithm called Aivia, which was taught to recognize apoptotic cells from immunofluorescent microscopic pictures of the tissue. The amount of apoptosis induced by anti-Clever-1 treated PBMCs was compared to multiple controls. The control groups included tissue cocultured with PBMC:s treated with a control human IgG4, tissue cocultured with untreated PBMC:s, and tissue cultured without PBMC:s. Three of the nine patients indicated a response to

anti-Clever-1 treatment compared to the control group. The approximately 30% response rate correlates with previous studies with human tissue and Clever-1 blockade like the work of Virtakoivu et al in phase I/II clinical trial of anti-Clever-1 treatment. (3)

Another way to demonstrate the biological effect of anti-Clever-1 treatment in the tumor microenvironment was the PBMC migration analysis. The patients' PBMCs were stained with CFSE staining and treated with an anti-Clever-1 antibody or the IgG4 control antibody. The goal was to demonstrate the patients' own PBMC migration into the tumor tissue. The PBMCs are a crucial part of interactions of the tumor microenvironment and the cellular properties of the tumor. The understanding of the cell composition of the tumor microenvironment is key to developing effective personalized cancer treatments. The migration of PBMCs affects the tumor microenvironment significantly and turns anti-inflamed cold tumors into inflamed hot tumors that are easier to target with immunotherapies. Monocytes are effective carriers for antibodies to the tumor site. The antibody binds to the monocyte in the vascular system and is then carried to the target tissue. This process induces a great immunologic reaction in the tumor site and increases the infiltration of other lymphatic cells in the tissue. The purpose of this assay was to find out if the anti-Clever-1 treatment helps PBMCs to infiltrate into the tumor site which enables their tumor-suppressive and inflammation-inducing properties. Five of the nine patients showed a response to the anti-Clever-1 treatment in this assay, which possibly indicates that the anti-Clever-1 treated PBMCs were able to infiltrate better into the tumor tissue compared to the control antibody with these patients and could be a solution for some patients with challenging cold tumors.

In conclusion, this study provided novel information about the anti-cancer effect of anti-Clever-1 treatment in the tumor microenvironment by demonstrating the apoptosis and the increased tumor infiltration of immune cells induced by anti-Clever-1 treated PBMC:s. In this study, we had a similar response rate in comparison to the other studies with human cancer tissue with anti-Clever-1 treatment and some of the responders had a higher amount of apoptosis as well as migrated PBMC:s in the tumor site. Two patients showed a correlation between a high amount of PBMC:s infiltrated in the tissue and a high apoptosis percentage, but the result was not statistically significant. The biggest limitation of this study was the small study cohort since the patient material from Turku University Hospital included nine patients. Further studies will be needed to achieve statistical significance, but we were able to prove successfully that some patients' responses to the anti-Clever-1 treatment can be seen in an ex vivo assay in addition to the clinical trials. An interesting prospect for future studies would be the possible correlation between the ex vivo response and the response in a clinical trial. This way an ex vivo assay could be used to determine possible treatment decisions for cancer patients in the future. Another exciting aspect for future research would be exploring the factors that differentiate responders from non-responding patients and the properties that affect the effectiveness of cancer immunotherapy.

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