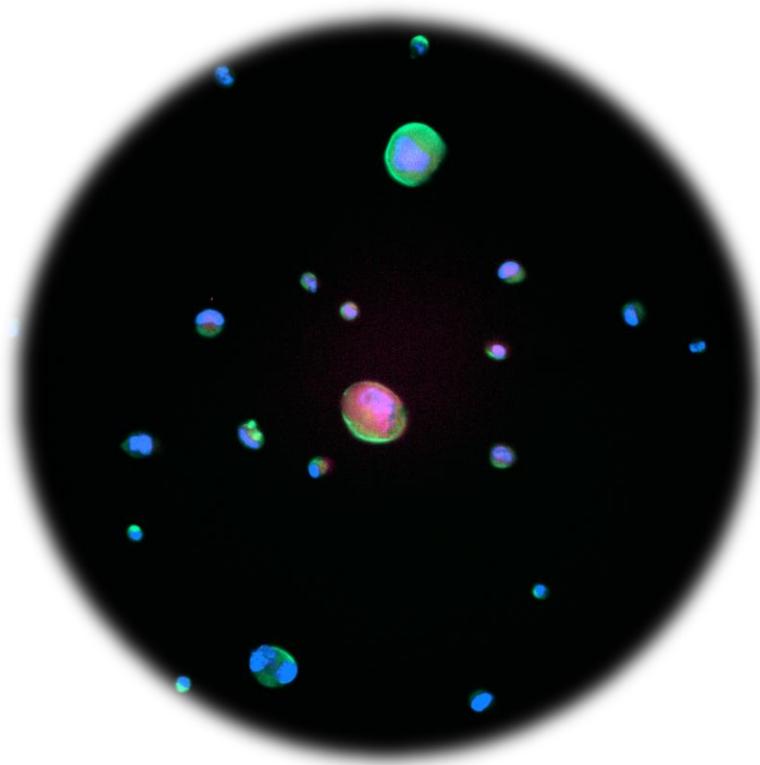


# Novel markers for detection of CTCs without EpCAM expression



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Image on front page: spiked blood with Hoechst, Vimentin, CD45, 20170317, Chaja Hudepol  
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## *Abstract*

Circulating tumour cells (CTCs) in the blood of a cancer patient can give information on the status of the cancer. Tumour cells that enter the blood stream can have different phenotypes. Epithelial cells express antigens that are mostly involved in cell adhesion and cell signalling. Mesenchymal cells on the other hand express antigens that are important for cell motility and movement. CTCs can express either one of the phenotypes, or reside in a state where antigens of both phenotypes are expressed. CTCs are currently isolated using the CELLSEARCH<sup>®</sup> system, which employs EpCAM and Cytokeratins to identify the tumour cells. However, since both antigens are characteristic for the epithelial phenotype, it could be that cells of the mesenchymal phenotype are lost.

The purpose of this research is to give an indication of the role of epithelial-mesenchymal transition in the expression of different antigens, and to identify antigens that can be used next to EpCAM and Cytokeratins in the detection of CTCs. To reach this goal, immunostaining was optimised, and the mesenchymal antigens Vimentin and Cadherin 11 were tested on cell lines with varying EpCAM expression.

It was found that there is a strong connection between Vimentin and EpCAM expression, as when one is highly expressed, the expression of the other is very minor. This is an indicator of the epithelial-mesenchymal transition. However, no link between Cytokeratins and Vimentin was found, as all cell lines that were tested expressed Cytokeratins to some extent. This could indicate that EpCAM and Vimentin are very specific markers for the epithelial and mesenchymal phenotype respectively, while Cytokeratins can be present in both phenotypes. It was also found that Vimentin is not expressed by white blood cells, which makes it a possible marker for detection of CTCs.

## *Glossary*

BC	Breast cancer
Cad11	Cadherin 11
CK	Cytokeratin (plural: CKs)
CTC	Circulating tumour cell (plural: CTCs)
EMT	Epithelial-mesenchymal transition
GaM-PE	Goat-anti-Mouse IgG-PE
GMO	Genetically modified organisms
MET	Mesenchymal-epithelial transition
NSCLC	Non-small cell lung cancer
PC	Prostate cancer
PC3-5.2	PC3- $\alpha$ Ncat 5.2
PC3-92.1	PC3- $\alpha$ Ncat 92.1 F
RT	Room temperature
WBC	White blood cell (plural: WBCs)

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# 1. Introduction

## *1.1 Cancer*

With roughly 14 million new cancer patients, and 8 million people dying from cancer each year, cancer is the largest death cause around the world [1]. Cancer is a very diverse disease. Because it is caused by, sometimes random, mutations in the genome, almost every cancer is different. This makes treatment very difficult. General treatment now exists of surgery, radiotherapy, and chemotherapy, but a lot of research is done into more tailored treatment methods. Since the presence of metastases largely decreases the chance of survival of the patient [2], looking at the cause of these metastases can result in better treatment. Incorporating circulating tumour cells (CTCs), from which metastases originate, in the detection and monitoring of cancer could be one way to allow for a more tailored and personal treatment of cancer.

## *1.2 Epithelial and Mesenchymal phenotype*

Before being able to understand the way in which CTCs come to be, it is important to understand the difference between two different phenotypes of CTCs: the epithelial and mesenchymal phenotype.

Most cells in our body are of the epithelial phenotype. They are strongly fixed in a tissue, and connect to other cells and the extracellular matrix (ECM) with membrane molecules and junctions. The strong structure that is created ensures that the cells maintain their shape, and can interact and communicate, forming a well-functioning tissue or organ. Epithelial cells often have a cuboidal shape which enables apicobasal polarity, meaning that different membrane proteins are expressed on the different sides of the cell. These cells rely heavily on their neighbours for survival [2].

Cells can however change into the mesenchymal phenotype, which means that a cell releases itself from the surrounding cells and de ECM. This comes with a loss in expression of certain proteins, and a gain of others, such as cytokeratins and vimentin respectively, as can also be seen in figure 4. Mesenchymal cells are all the cells that are not fixed in one place, and can move around the body. These are mostly blood cells and stem cells. These cells do not have strong bonds with other cells, and do not connect to a basement membrane, but rather float around in the fluids of the body or migrate through tissues using focal adhesions. Their shape varies between oval and marquis, and they have a front-back polarity. Because of their shape, these cells cover a much larger surface than an epithelial cell [3].

Normally, cells rarely go through epithelial-mesenchymal transition (EMT), this only happens in the case of damage done to a tissue, which has to be repaired. After the repair is finished, cells undergo mesenchymal-epithelial transition (MET) and become epithelial cells again. However, in cancer cells can transition more easily, which makes EMT more common [2]. What is important to note here is that in normal tissues, there is a strict difference between the epithelial and mesenchymal phenotype. A cell can either stay in the tissue and proliferate, or release from the tissue and move. In cancer, this transfer is more lenient. Resulting in a tumour cell that expresses parts of both the epithelial and mesenchymal phenotype [2], and thus resides in a hybrid state.

### 1.3 Circulating tumour cells

When a cancer patient has a malignant tumour, this tumour releases cancer cells into the bloodstream. This release of cells is due to a combination of factors. A malignant tumour can induce the growth of new blood vessels (angiogenesis) by releasing endothelial growth factors, these new blood vessels supply the tumour with enough nutrients to grow. These blood vessels are not made in a structured manner, and they vary from normal blood vessels in shape and organisation. On top of that, the vessel walls are often leaky, which allows an exchange of cells between the blood vessel and the tissue. This exchange leads to tumour cells entering the bloodstream, either by accident or on purpose [2]. The difference between entering by accident or on purpose lies mostly in the phenotype of the cell [4].

Cells that enter the bloodstream by accident are usually epithelial type cells, that have broken loose from the tissue and fall into the blood vessel. Normal cells would not survive because of their lack of cellular contact, which induces apoptosis. However, epithelial tumour cells can survive in circulation because of their cancerous properties [2] [3].

Cells with the mesenchymal phenotype often enter the bloodstream on purpose, sometimes by actively pushing themselves between the endothelial cells of the vessel wall. Since mesenchymal cells do not require cellular contact, they are more likely to survive in the bloodstream [2] [3]. Once a tumour cell enters the bloodstream it is called a CTC. If these CTCs manage to survive the force and blood pressure, and avoid the immune surveillance of the white blood cells (WBCs), they can extravasate and enter the tissue of a different organ. Here, according to the 'Seed and Soil Hypothesis', the cells can either die, enter dormancy, or proliferate and form a metastasis, depending on the hostility of the environment [2] [3].

When taking a blood sample from a cancer patient with a tumour, CTCs can be found in the blood. The presence of CTCs and metastasis is an indication of a lesser chance of recovery for the patient [2].

Once extracted, CTCs can give information about the type and the state of the cancer. Regular checks on the number and characteristics of CTCs can also indicate in an early stage if the treatment is working, and how the tumour develops [5] [6].

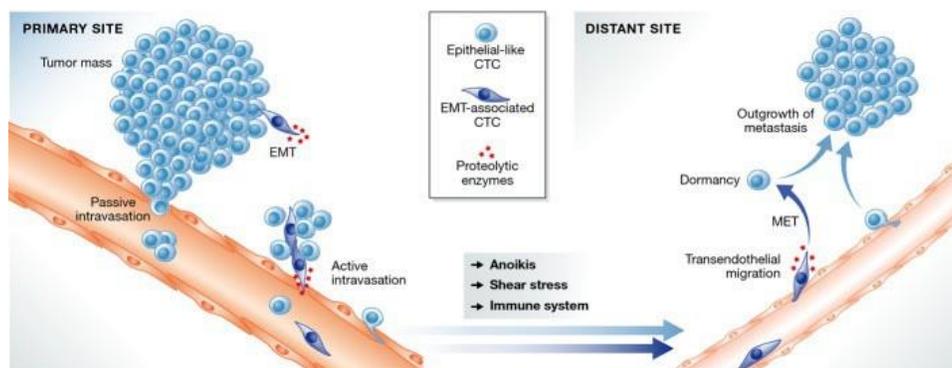


Figure 1: The process of EMT visualised [7]. Both epithelial and mesenchymal cells can leave the primary tumour site and travel to a distant site. They do this either through passive or active intravasation, depending on their phenotype. To reach a distant site, the cells have to survive the stress, evade the immune system and overcome apoptosis. At this site the cells extravasate and can either form a metastasis or enter dormancy.

### 1.3.1 Detection of CTCs

Detection and isolation of CTCs is rather difficult, since there are as little as 1 to 10 CTCs per ml of blood. In a clinical setting, these CTCs are currently detected and isolated using CELLSEARCH<sup>®</sup>. This is the only FDA approved technique for clinical use.

This machine binds an anti-EpCAM antibody to the CTC. Connected to the anti EpCAM is a ferrofluid which enables isolation of CTCs using a magnetic field. After isolation, the EpCAM positive cells are stained with anti-Cytokeratin and CD45 to distinguish between blood cells and CTCs [8].

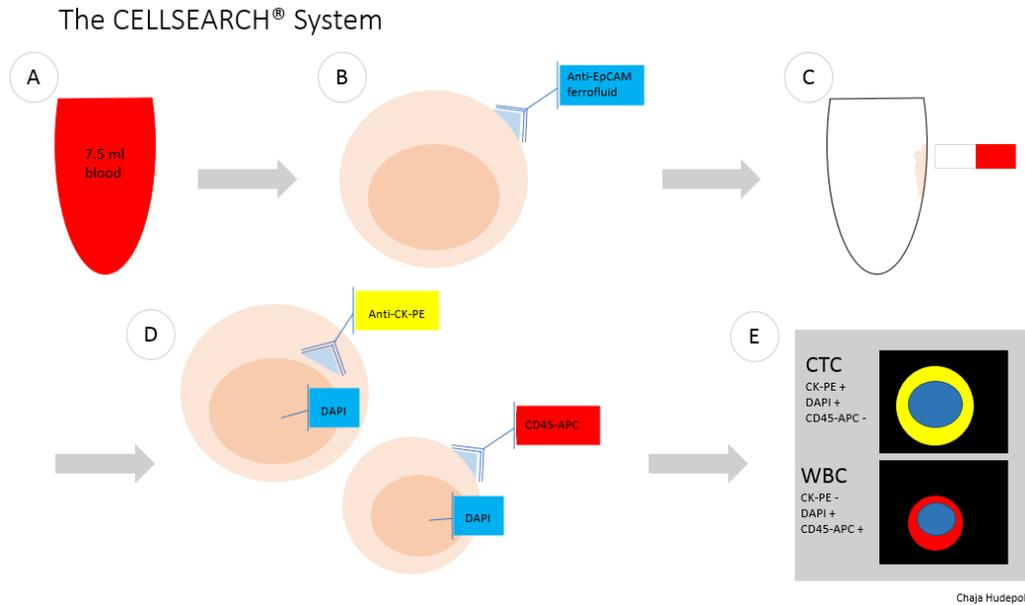


Figure 2: The CELLSEARCH<sup>®</sup> system. (A) 7.5 ml of blood is taken from a patient. (B) CTCs are enriched with an anti-EpCAM-ferrofluid labeling by the Autoprep system, (C) a magnetic field is used for separation. (D) Isolated cells are stained with DAPI (blue), Anti-CK-PE (yellow), and CD45-APC (red). (E) Cells are analyzed on their expression of different markers. CTCs are identified as expressing both DAPI and CK, WBCs are identified as expressing DAPI and CD45.

EpCAM is a protein that plays an important role in the adhesion of different epithelial cells [9], and is specific for the epithelial phenotype. However, during the process of epithelial to mesenchymal transition, cells can lose the expression of epithelial proteins, such as EpCAM and Cytokeratins, and gain expression of mesenchymal proteins, such as Vimentin and Cadherin 11. Therefore, mesenchymal CTCs that do not necessarily express EpCAM, are currently not detected using CellSearch.

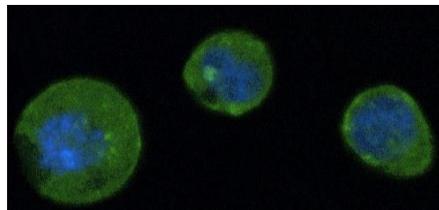


Figure 3: LNCaP cells (prostate cancer cell line) stained with EpCAM-FITC (green, 2 $\mu$ g/ml) and Hoechst 33342 (blue, 4  $\mu$ g/ml) magnification 400x (40x 0.6NA Nikon).

## 1.4 Antigens

In figure 4, one can see the process of EMT visualised. The epithelial cell has its distinct cuboidal shape and has markers such as EpCAM, E-Cadherin, and Cytokeratins. The mesenchymal phenotype on the right is characterised by a round shape, and markers such as N-cadherin, Cadherin 11, and Vimentin. Because in cancer the process of EMT is more lenient, there is an intermediate phenotype possible, where cells express some epithelial and some mesenchymal markers. In this research the focus will lie on the expression of EpCAM, Cytokeratins, Vimentin, and Cadherin 11, as a means to identify the phenotype of the cell.

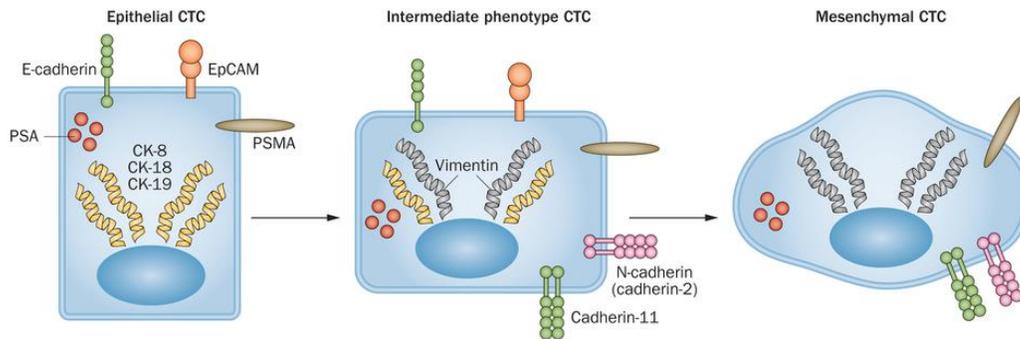


Figure 4: The transition from epithelial to mesenchymal phenotype visualized. Epithelial markers, such as EpCAM and CKs are lost, while mesenchymal markers, such as Vimentin and Cad11 are gained during the transition [10].

### 1.4.1 Cadherin 11

Cadherins are a kind of type 1 trans-membrane protein that plays an important role in cell-cell adhesion. Cadherin stands for ‘calcium dependant adhesion’, meaning that all cadherins bind using Calcium. Cadherin 11 (Cad11) is a type 2 cadherin [11] which is mostly found in osteoblasts, and weakly expressed in lung, brain, and testicular tissue [12] [13]. The expression of Cad11 is associated with mesenchymal tissue formation [14] and control in the differentiation of these into osteoblasts. It may also function in cell sorting, migration, and alignment during the maturation of osteoblasts [12]. The Cad11 complex can also play a role in contacting the cell-matrix, particularly in leading extensions of the cell. Such a complex could facilitate the ability of a motile cell to interact with its surroundings, which could include both matrix proteins, as well as other mesenchymal cells [15].

It has been found that cells that undergo an epithelial to mesenchymal transition (EMT) begin to express Cad11 as they invade the surrounding tissue to form new structures [15]. This implies that Cad11 plays an important role in the formation of metastasis. As Cad11 is mostly found in osteoblasts, cancers that develop this protein also show most metastasis in or around bone tissue [12] [16]. Cadherin 11 has been found in a couple of different cancer cell lines, such as PC3 [12], MDA-MB-157, MDA-MB-231, BT549, HS578T, and MCF-7ADR [15]. PC3 is a prostate cancer cell line, the others are breast cancer cell lines. In prostate cancer, it has also been shown that Cad11 specifically causes the large number of bone metastases [12]. The breast cancer cell lines mentioned are also shown to cause extremely invasive tumours, which led Chu et al. to conclude that Cad11 is a specific marker for the most invasive cancer cell lines [12].

### 1.4.2 Vimentin

Vimentin is an intermediate filament of type III, which has a diameter of approximately 10nm. Next to intermediate filaments, there are two other types of filaments, namely Actin filaments and microtubules. All filaments play their own role in maintaining the structure and shape of the cell [17]. All filaments, including Vimentin, are found inside the cytoplasm of the cells. Since Vimentin is attached to the nucleus, mitochondria and cell membrane, it can induce changes in cell shape and structure [18]. It has also been shown that Vimentin plays an important role in EMT and MET; when Vimentin expression is lost, cells start expressing epithelial traits, such as Cytokeratins, whereas when Vimentin is expressed, cells change into the mesenchymal state [19]. This also makes Vimentin an important mesenchymal antigen.

In cancer, Vimentin plays a role in the prevention of autophagy, and in regulating cellular motility. Additionally, by influencing the 14-3-3 proteins, Vimentin can modulate intracellular signalling and cell cycle control pathways [20].

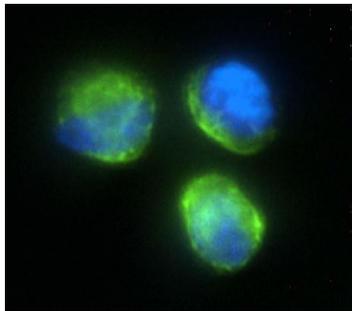


Figure 5: PC3 (prostate cancer) cells stained with Vim-FITC (green, 2 $\mu$ g/ml) and hoechst 33342 (blue, 4 $\mu$ g/ml) magnification 400x (40x 0.6NA Nikon).

### 1.4.3 Cytokeratins

Cytokeratins (CKs) are also a class of intermediate filaments, and part of the cytoskeleton. CKs are specific for epithelial cells, and different types of cancer express a different spectrum of CKs. This trait can be used to identify different carcinomas, or find the origin of a metastasis. There are about 20 different CKs known, of which CKs 1-8 are acidic, and CKs 9,10,12-14,16-20 are basic or neutral [21]. CKs are a very common marker used to detect tumour cells either in metastasis or in blood. It is unclear whether CKs also influence the invasiveness or motility of the tumour cell.

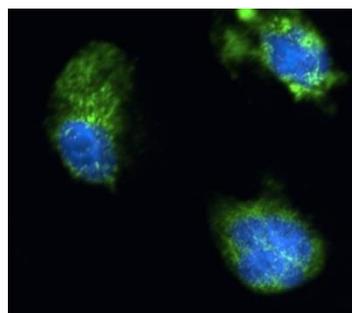


Figure 6: MCF-7 (breast cancer) cells stained with C11-FITC (green, 5 $\mu$ g/ml) and Hoechst 33342 (blue, 4 $\mu$ g/ml) magnification 400x (40x 0.6NA Nikon).

### 1.5 Aim of this research

Because CTCs are very rare in the blood of a cancer patient, it would be ideal to be able to isolate all of them. Since CTCs are now isolated using EpCAM, the EpCAM negative CTCs are lost. Therefore, the goal of this research is to optimise immunostaining and characterize EpCAM positive and negative cell lines on the presence of epithelial and mesenchymal antigens to determine their EMT status, and possibly identify alternative markers for the CellSearch system. This would also show if there is indeed a relation between the epithelial and mesenchymal phenotype, which would indicate the role of EMT and MET in the detection of CTCs.

For this purpose, six cell lines were chosen: two prostate cancer, two lung cancer and two breast cancer cell lines. Of each cancer, one cell line has a high EpCAM expression and one has a low or negative EpCAM expression.

These cell lines were tested for their expression of three proteins: Vimentin, Cadherin 11, and Cytokeratin numbers 4,5,6,8,10,13,18. The first two of these are proteins characterizing the mesenchymal phenotype, while the last is an indication of the epithelial phenotype, which is already in use in the CellSearch system. These three proteins, together with the known EpCAM expression should give an indication of the EMT status.

Table 1: Showing the expression of different antigens, as was found in literature.

		Epithelial phenotype		Mesenchymal phenotype	
		EpCAM	Cytokeratins	Vimentin	Cadherin 11
PC	LNCaP	+ [22]	+ [22]	- [22]	- [12]
	PC3	- [23]	+ [24]	+	+ [25]
NSCLC	NCI-H1650	++ [23]	+ [26]	+ [27]	?
	NCI-H1563	- [23]	+ - [23]	+ [28]	?
BC	MCF7	+ [23]	+ [29]	- [30]	- [15]
	MDA-MB-231	- + [23]	++ [29]	+ [15]	++ [15]
control	WBCs	- [23]	- [23]	?	?

## 2. Methods

Since all data collected per cell line had to be comparable, the protocol for staining the cells was standardized as much as possible. Therefore, all cells were fixed immediately after harvesting, and were used within a week after fixation. For all stains using antigens inside the cells, cells were permeabilised, this included Vimentin and CKs. However, to avoid unspecific staining, cells stained for surface antigens were not permeabilised.

### 2.1 *Culturing cell lines*

To ensure optimal conditions for the cells in culture, they were checked upon twice a week. During the check was looked at the colour of the medium, the density of the cell growth in the flask, and the shape and attachment of the cells. the medium was replaced twice a week. If overgrowth in the flask reached full covering, cells were harvested and sown in a new flask with a density of 5K (5000 cells per square cm). The cell medium was stored in the fridge, and transferred to 50ml bottles for usage, this to ensure the sterility of the large bottle of medium. For refreshment of the medium, 5ml of medium was used for a T25 flask, and 15ml for a T75 flask. To transfer the cells, PBS (Sigma, Cat: P-5368, Lot: SLBQ7495V) was used to wash the cells, followed by the addition of 1 or 3 ml of 0.05% Trypsin-EDTA (Gibco, Cat: 25300-062, Lot: 1754037) to release the cells from the flask for transfer. The cells with Trypsin were incubated for 5 minutes at 37 degrees, followed by the addition of 2 or 6 ml of medium. The suspension was then transferred to a tube and the concentration of cells was counted using the Luna Cell Count. After counting, a new flask was filled with medium, and the appropriate amount of cell suspension was added.

The cells that were not used for culture were fixed immediately after harvest. First the suspension was centrifuged and the supernatant aspirated. Then the cells were resuspended in 1ml of a 1% Formaldehyde solution (Sigma, Cat: 533998-500ml, Batch: 08996DM), and incubated at room temperature for 10 minutes. The suspension was then again centrifuged and aspirated, followed by the addition of 2ml of PBS/BSA1% (Sigma, Cat: A7906-100g, Lot: SLBT0167). The fixated cells were stored at 4°C. Cells were stored for a maximum of one week after fixation.

#### 2.1.1 PC3

For this research, both two modified lines of PC3, one highly positive and the other very negative for Cad11, and the normal PC3 line were cultured.

PC3 cells are epithelial and originate from prostate cancer, but were derived from a metastasis in the hip bone. With 62 chromosomes, the cells are nearly triploid [31]. The cells were grown in RPMI-1640 (Lonza, Cat: BE12-702F, Lot: 6MB165) with 10% FBS (Sigma, Cat: F7524, Lot: 124M3337) [32] at 37 degrees in the CO<sub>2</sub> incubator.

The genetically modified cell lines are PC3 $\alpha$ N cat 5.2 and PC3 $\alpha$ N cat 92.1F, which will be referred to as PC3-5.2 and PC3-92.1 respectively. These cell lines were modified for their Cad11 expression. PC3-5.2 has a very low Cad11 expression, while the expression on PC3-92.1 is very high. These cells were grown in RPMI-1640 with 10% FBS and 1% L-glutamine (Lonza, Cat: BE17-605E, Lot: 5MB108). To each 50 ml of medium, 200  $\mu$ l of Geneticin (Gibco, Cat: 10131-035, Lot: 1852838) was added. Because these cells are genetically modified organisms (GMO), it was not possible to use live cells for experiments. Therefore, the cells were fixed for ten in minutes in the flow hood using a 1% formaldehyde solution in PBS before continuation of the staining protocol.

### 2.1.2 LNCaP

LNCaP is a prostate cancer cell line with epithelial morphology. Cells were derived from a lymph node metastasis [22]. They were grown at 37 degrees in the CO<sub>2</sub> incubator in RPMI1640 with 10%FBS and 1%Penstrep (Lot: 4MB132).

### 2.1.3 NCI-H1650

NCI-H1650 is a lung cancer cell line with epithelial morphology and very high EpCAM expression. The cells were taken from a metastasis in the lung. The cells were grown in the CO<sub>2</sub> incubator at 37 degrees in RPMI-1640 with 10% FBS and 1% penstrep.

### 2.1.4 NCI-H1563

This is a very EpCAM negative lung cancer cell line. It was taken from an adenocarcinoma in the lung. Cells were cultured in RPMI1640 with 10%FBS and 1% penstrep, and grown at 37 degrees in the CO<sub>2</sub> incubator.

### 2.1.5 MCF-7

MCF-7 is a breast cancer cell line that was derived from a metastasis in the pleura. The cells naturally grow in small cultures, and express an epithelial phenotype. Cells are cultured in DMEM (Lonza, Cat: BE12-604F, Lot: 6MB175) with 10%FBS and 1% penstrep, in the CO<sub>2</sub> incubator at 37 degrees.

### 2.1.6 MDA-MB-231

This is the second breast cancer cell line. It was also derived from a metastasis in the pleura. Cells are of the epithelial type and grow in DMEM with 10%FBS and 1% penstrep, and grew in the CO<sub>2</sub> incubator at 37 degrees.

## 2.2 *Blood lysis*

To obtain white blood cells, whole EDTA blood was lysed. During lysis, the red blood cells are destroyed, and only the WBCs remain. This is done using an Ammonium lysis buffer. This buffer contained 830mg NH<sub>4</sub>CL, 100mg KHCO<sub>3</sub>, and 3.9mg EDTA, dissolved in 100ml MilliQ. The blood was diluted 1:20 in this buffer, and incubated on ice for twenty minutes. It was then spun off for five minutes at 500xg and aspirated, washed twice with 2ml PBS/AC, containing 100ml PBS, 50mg BSA, and 390mg Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>. Afterwards the WBCs were fixed with a 1% Formaldehyde solution, and suspended in PBS/BSA1%. These WBCs could then be used for testing.

### *2.3 Immunofluorescent Staining*

Since both CKs and Vimentin are inside the cell, cells have to be permeabilised before staining. For CK staining, the C11 (MCBP, Lot: D339) antibody blend was used, which stains CKs 4,5,6,8,10,13,18. This blend was combined with FITC and had a concentration of 1mg/ml, and was always diluted 1:10 in PBS/BSA1% before usage. For Vimentin, the Vimentin-FITC antibody from eBioscience was used. This product contains Vimentin clone V9 (eBioscience, Cat: 11-9897-80, Lot: 4298312), at a concentration of 0,5mg/ml. For the staining this was diluted 1:10 with PBS/BSA1% to give a concentration of 50µg/ml.

Two versions of anti-Cad11 were used in this research, the first concerned clone 16.a.c.5, which was donated by Nijmegen. The second version concerned anti-Cad11 from Biolegend (Cat: 368702, Lot: B206555, clone 16G5). Both versions are unconjugated and require the use of a secondary antibody.

For this purpose, Goat-anti-mouse IgG-PE (SantaCruz Biotechnology, cat: SC-3738, Lot: D0612) was chosen. This antibody has a concentration of 200µg/ml, but was diluted 1:10 before usage. From now on this antibody will be referred to as GaM-PE.

For tests using WBCs, the CD45 antibody was used to distinguish WBCs from CTCs, as the antigen is only found on WBCs. Two versions of this antibody were used in the tests. The CD45-PerCP antibody (Aczon, Cat: CD45-GMF000S, Lot: 1405OCS-3648) has a concentration of 0.176mg/ml, while CD45-APC (MCBP) has a concentration of 30µg/ml.

For immunofluorescent staining after fixation, cells were permeabilised using 250µl PBS/Saponin 0.05% solution (Sigma, Cat: 84510-100g, Lot: BCBD149V) for 10 minutes at room temperature (RT), and afterwards spun down for 5 minutes at 500xg and aspirated. The cells were then resuspended in a total volume of 100µl of PBS/BSA1%/Saponin0.05%, together with the antibodies, and incubated for 10 minutes at RT. The concentrations for the antibodies were 2µg/ml for Vimentin, 1µg/ml for Cytokeratins, 5 µg/ml for Cadherin 11, and 2µg/ml for CD45. After incubation, the cells were again spun down and aspirated.

In the case that a secondary antibody was used, the cells were washed using 100 µl of PBS/BSA1%, and incubated 10 minutes at RT, spun down and aspirated. Then the cells were resuspended in a total volume of 100 µl of PBS/BSA1% with 5 µg/ml GaM-PE, and incubated 10 minutes at RT.

The cells were then spun down and aspirated, and resuspended in 500µl PBS/BSA, with Hoechst to an end concentration of 4µg/ml. After another 10 minutes of incubation at RT the cells were stored at 4°C or on ice for analysis. One difference in this protocol was for the staining with Cadherin 11. Since this is a membrane protein, no permeabilization was done to minimise a-specific staining.

## *2.4 Conjugating antibodies to FITC-fluorochrome*

To avoid the need for a secondary antibody, the anti-Cad11 from Nijmegen was also conjugated using FITC as a fluorochrome. For this, a Zebbar spin desalting column was used (Thermo Fischer). Before use, the column was opened at the top and the bottom, and placed in a collection tube. One side was marked to maintain the same position in the centrifuge. The column was then centrifuged for 2 minutes at 1000xg, and the storage solution was discarded. Afterwards the column was washed three times using 1ml of a sodium bicarbonate buffer (210 mg NaHCO<sub>3</sub> in 250ml MilliQ), spun down, and the solution discarded.

The column was then placed in a new collection tube, and 500 µl of the antibody was pipetted on the column. The column was then again spun down, and the solution was collected in the tube.

Then a solution of 20mM fluorochrome in anhydrous DMSO was prepared. 1ml of the antibody was collected in an Eppendorf tube, to which a 20 times molar excess of the fluorochrome-DMSO solution was added. This solution was suspended and stored for 2 hours at 4°C, wrapped in aluminium foil.

A new Zebbar column was prepared like the first one, after which it was placed in a new collection tube. The antibody-fluorochrome solution was then slowly pipetted on the column and spun down. The product was transferred to an Eppendorf tube and stored at 4°C.

## *2.5 Analysis of cells*

For analysis of the samples, both the flowcytometer (BDFACS aria cellsorter) and the fluorescent microscope (Nikon Eclipse E400) were used. For the flowcytometer, the variables were optimised for each test, while with the microscope, the variables were kept as constant as possible.

The flowcytometer takes the sample, which consists of a suspension with cells stained with a fluorescent label, and mixes this sample with the sheath fluid. This dilution flows through a nozzle that forms droplets, with each droplet containing one cell. These droplets pass through a laser beam, and the way in which the light is scattered tells something about the size (forward scatter) and complexity (side scatter) of the object inside the droplet. Additionally, the fluorescent channels detect the light sent by the excited fluorescent label.

The sensitivity of all these detectors can be adjusted to enable optimal detection and distinction between background and results. During the tests, the voltages, which can enhance or decrease a signal, were kept constant per test, but varied between tests. Therefore, the flowcytometer results that have different voltages can not be compared.

The programme FACSDIVA was used for analysis of the data. During analysis, the gates for the fluorescent labels were set at the border of the background that was found in the negative controls. An exception was made when the result was hardly distinguishable from the background, in this case the background was also put within the gate, and during analysis was mostly looked at a change in the mean of the result.

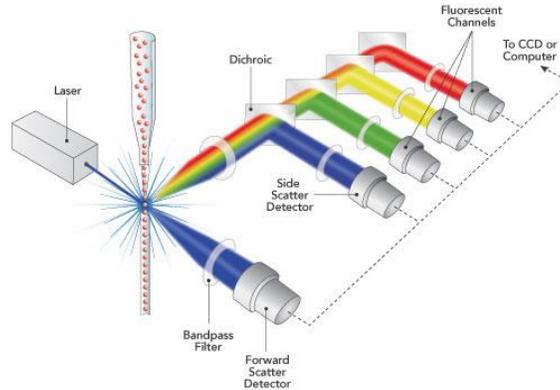


Figure 7: The working of the flowcytometer. Light is scattered by the events in the droplets, and is detected in different detectors [33].

The fluorescent microscope (Nikon Eclipse E400) works with a laser that is filtered through an excitation filter. This filter allows only one colour of the laser to pass to the sample. Here this light excites the fluorescent labels on the cells, which emit another wavelength of light. This light is then detected by a camera (Hamamatsu C8484-03G or C11440-4U2). The image given by the camera could be visualised in the program Hokawo (Hamamatsu Photonics), which allowed for a change in shutter time and LUT values. These values were kept as constant as possible for all tests, to allow comparison between test results. Images that were obtained were also merged in Hokawo, while a scalebar was added using the program ICY [34]. Objectives that were mostly used for images were 100x and 400x magnification (10x 0.3NA Nikon, 40x 0.6NA Nikon), and reflection cubes were DAPI, FITC, PE, and APC.

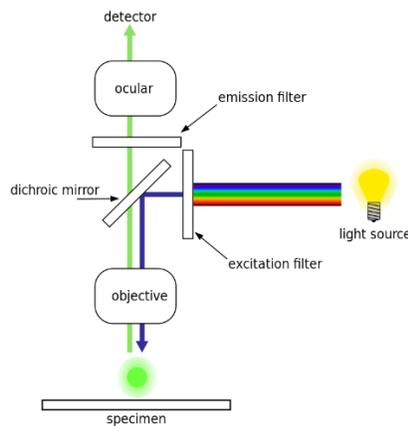


Figure 8: The working of a fluorescent microscope. Laser light is filtered and sent to the specimen, which sends a different wavelength of light back to the observer [35].

### 3. Results

The aim of the research was to test EpCAM positive and negative cell lines for the expression of epithelial and mesenchymal markers. Below the findings of this research are shown.

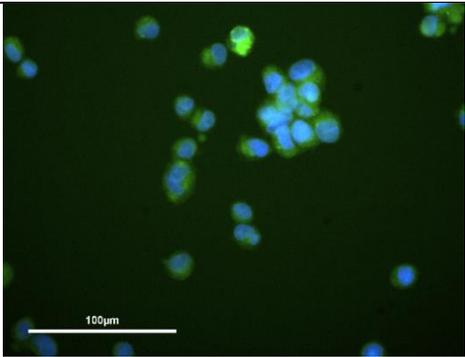
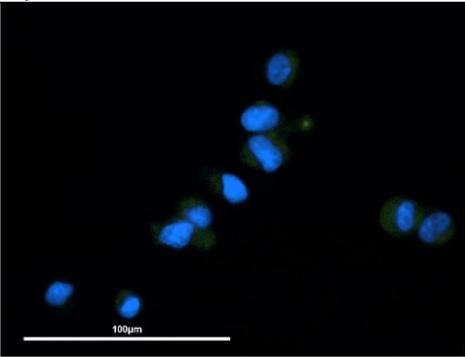
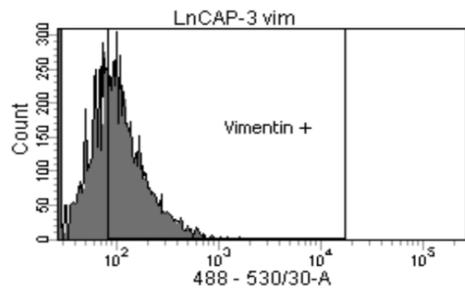
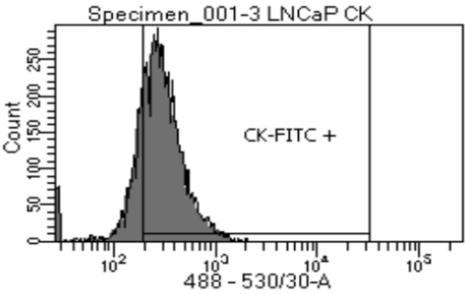
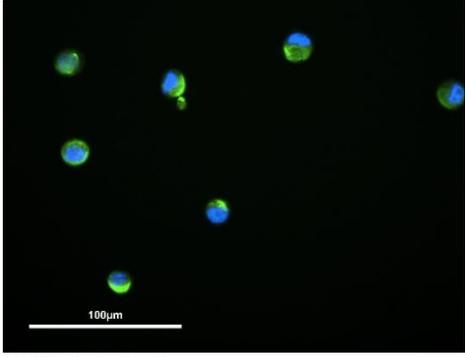
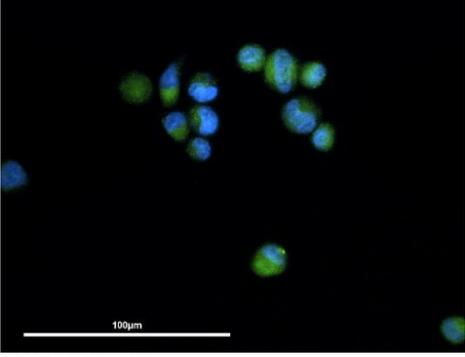
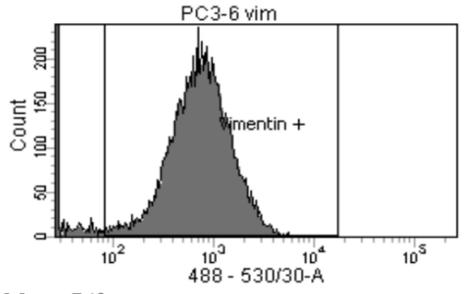
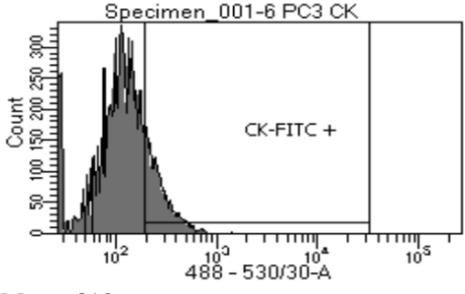
#### 3.1 *Vimentin and Cytokeratins in cell lines*

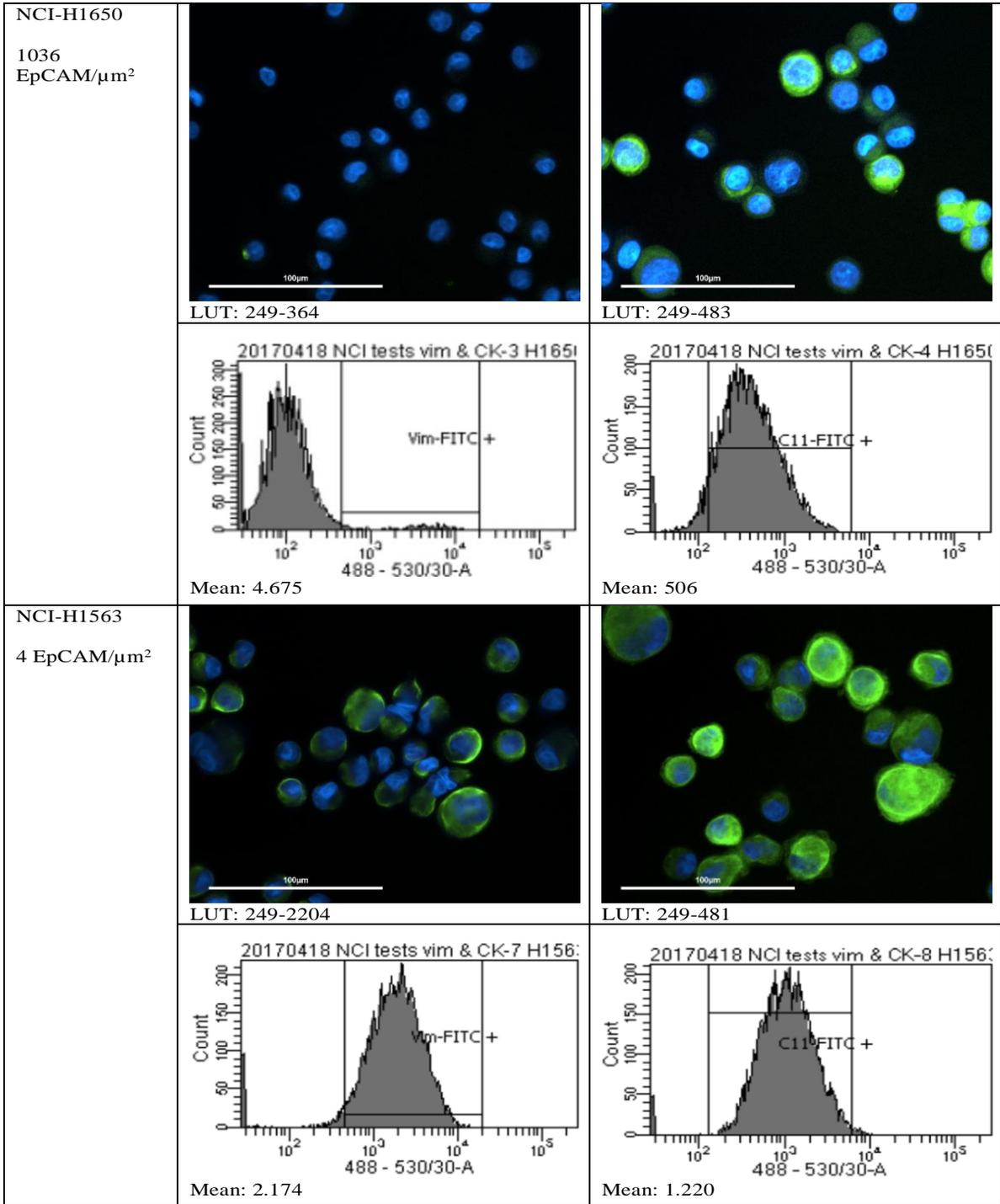
Vimentin and CKs were titrated on PC3 (EpCAM low) and MCF-7 (EpCAM high) respectively. From these titrations, the concentrations of 2µg/ml for Vimentin and 1µg/ml for CKs were chosen to work with in further tests. Optimal concentration was chosen based on the mean of the signal, the increase in this mean with increased concentration, and the possibility to distinguish the signal from the background.

Table 2: Results of the titration tests using several concentrations of antibody for detection. Green indicates chosen concentrations. Numbers noted are mean values of signal as derived from flowcytometry analysis. Vimentin was tested on PC3. Cytokeratin was tested on MCF7.

	1µg/ml	2µg/ml	4µg/ml	5µg/ml
Vimentin (PC3)	3.123	5.293	9.008	--
Cytokeratins (MCF7)	10.670	22.371	--	57.503

After determining the optimal concentration for Vimentin and CKs, these antibodies were tested on EpCAM- and EpCAM+ cell lines. The results are depicted in Figure 9 on the following pages. It shows that LnCAP, PC3, NCI-H1563, and MDA-MB-231 have a high Vimentin expression, while in NCI-H1650 and MCF7 the expression is much less. For Cytokeratins, both NCI's and MDA-MB-231 are very positive, but the other cell lines also show some expression.

	Vimentin	Cytokeratins
LNCaP 764 EpCAM/ $\mu\text{m}^2$	 <p>LUT: 249-2205</p>	 <p>LUT: 250-481</p>
	 <p>LnCAP-3 vim</p> <p>Count</p> <p>Vimentin +</p> <p>488 - 530/30-A</p> <p>Mean: 127</p>	 <p>Specimen_001-3 LNCaP CK</p> <p>Count</p> <p>CK-FITC +</p> <p>488 - 530/30-A</p> <p>Mean: 375</p>
PC3 11 EpCAM/ $\mu\text{m}^2$	 <p>LUT: 249-2205</p>	 <p>LUT: 250-483</p>
	 <p>PC3-6 vim</p> <p>Count</p> <p>Vimentin +</p> <p>488 - 530/30-A</p> <p>Mean: 768</p>	 <p>Specimen_001-6 PC3 CK</p> <p>Count</p> <p>CK-FITC +</p> <p>488 - 530/30-A</p> <p>Mean: 312</p>



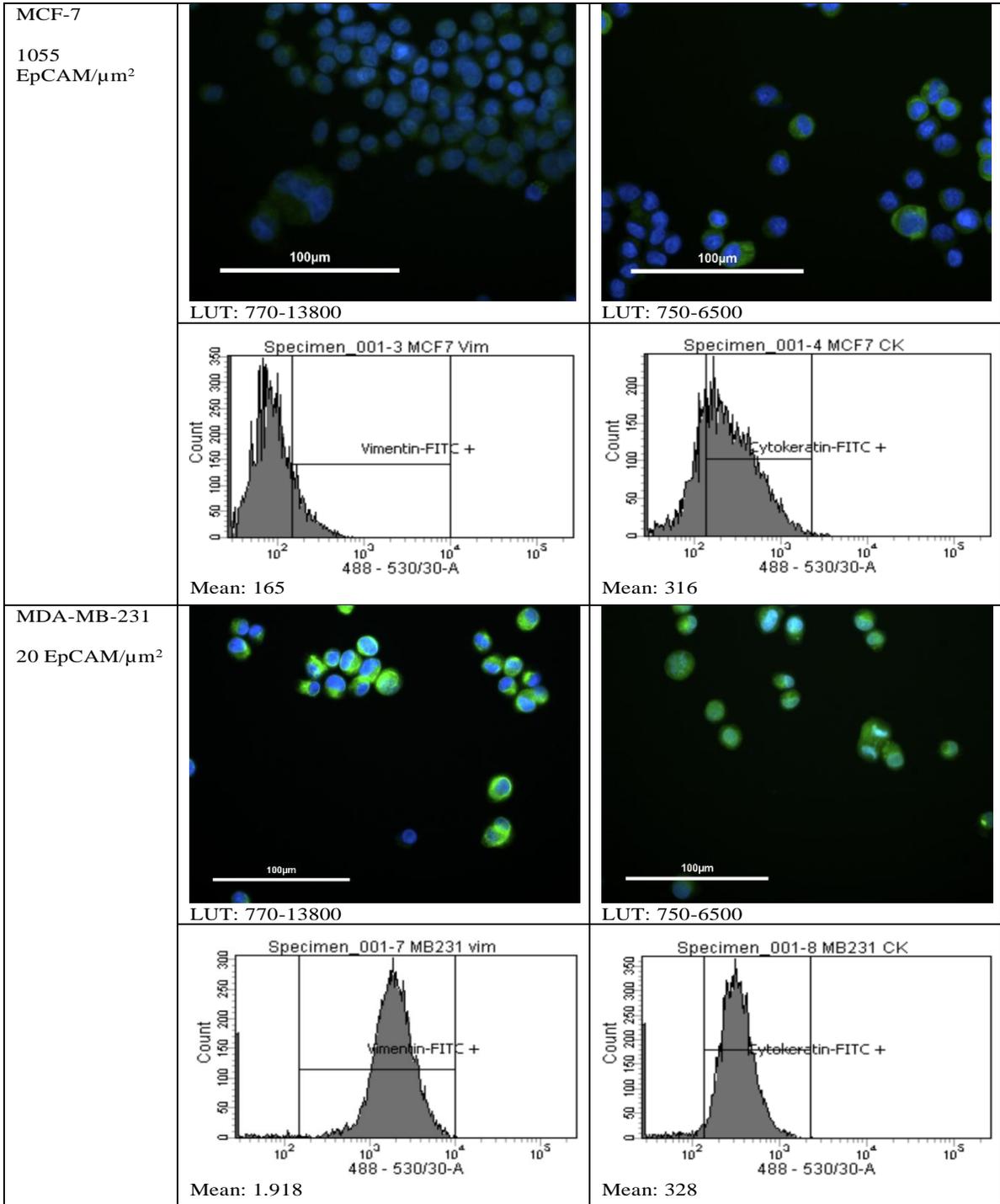


Figure 9: Microscopic images and flowcytometer results of all tests on cell lines. The nucleus is stained with Hoechst 33342 (blue, 4 $\mu\text{g}/\text{ml}$ ), and Vimentin (2 $\mu\text{g}/\text{ml}$ ) and CK (1 $\mu\text{g}/\text{ml}$ ) were labelled with FITC (green). The integration time for FITC was 2 seconds, and that of Hoechst was 12ms. All images were taken using the 400x magnification (40x 0.6NA Nikon). LUT values for MCF7 and MDA-MB-231 differ from the rest due to the use of a different camera (Hamamatsu C11440-4U2, instead of C8484-03G). Flowcytometry gates were set at the edge of the background noise, so all signal that is left of the gate can be seen as noise. The mean of the data is given below each graph. The number of EpCAM antigens per  $\mu\text{m}^2$  is noted underneath each cell line.

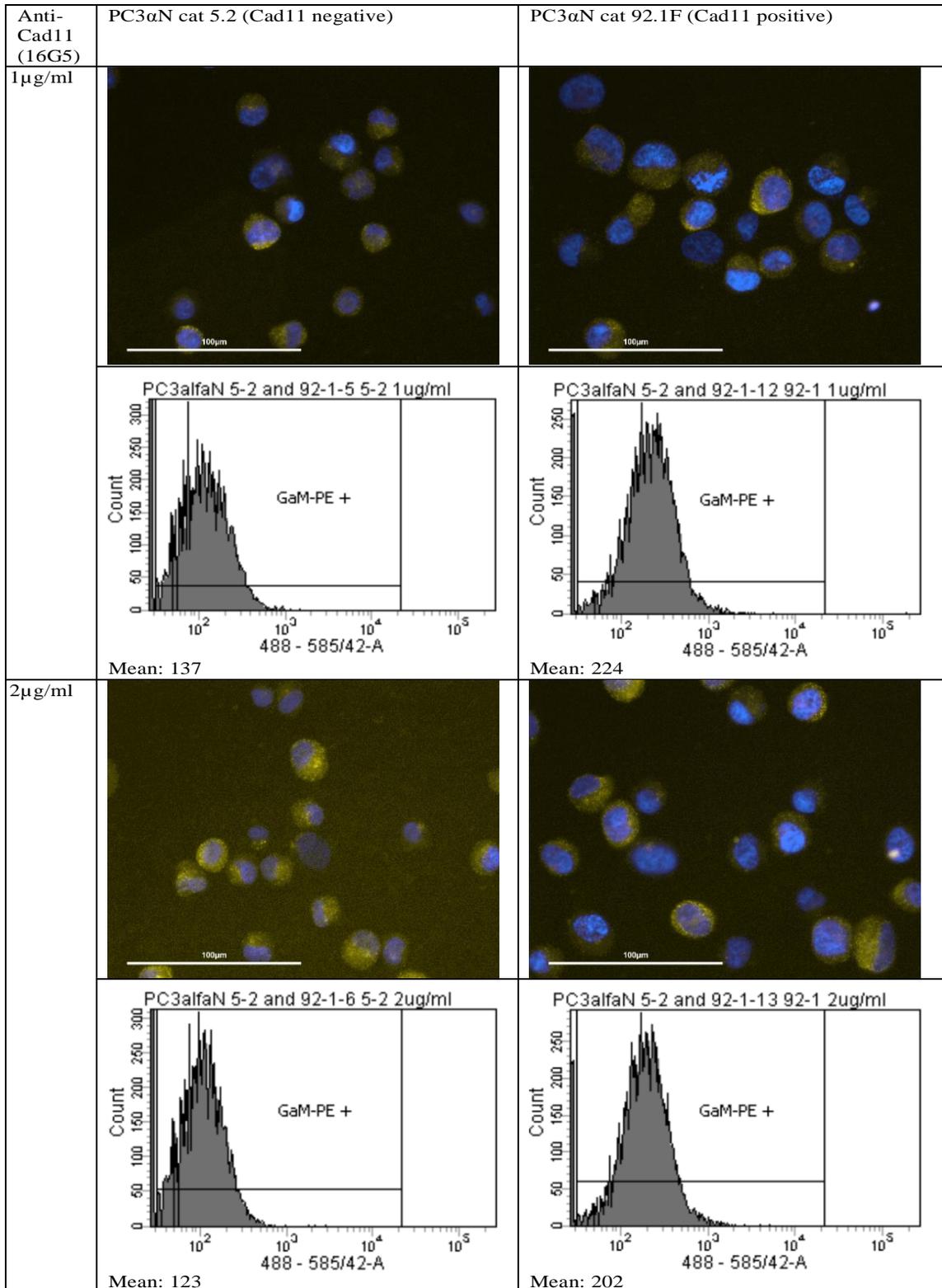
### 3.2 Development of Cadherin 11 staining

During this research, two clones of the Cad11 antibody were tested for their optimal concentration, as the intention was to use Cad11 staining parallel to Vimentin and CKs in the cell line tests. Clone 16.a.c.5 of anti-Cad11 was donated to the MCBP group by the university of Nijmegen. This clone was conjugated with FITC at the MCBP lab. The first titration with this conjugated antibody done on PC3-5.2 and PC3-92.1, did not show a difference between signal and background. A follow-up test was done, which compared the conjugated and unconjugated antibody, using GaM-PE as a secondary antibody in both samples. Figure 10 shows that also in this experiment there is no difference between the background noise and expected signal.

Therefore, anti-Cad11 was commercially purchased (clone 16G5, Biolegend). Since this Cad11 was unconjugated, GaM-PE was used as a secondary antibody. Figure 11 shows the microscopic images and the flowcytometry plots for both cell lines at different concentrations of the antibodies. It is visible that with an increase in concentration, the difference between the means of the negative and positive cell line increases slightly. However, the mean values of both cell lines stay relatively constant with increasing concentrations.

Anti-Cad11 (16.a.c.5)	FITC expression	PE expression
Cad11-FITC	<p>20170421 Cad11 2ndAB-3 92-1 Cad11-fit 488 - 530/30-A Mean: 199</p>	<p>20170421 Cad11 2ndAB-3 92-1 Cad11-fit 488 - 585/42-A Mean: 228</p>
Cad11-FITC GaM-PE	<p>20170421 Cad11 2ndAB-6 92-1 Cad11-Fit 488 - 530/30-A Mean: 219</p>	<p>20170421 Cad11 2ndAB-6 92-1 Cad11-Fit 488 - 585/42-A Mean: 266</p>
Cad11 GaM-PE	<p>20170421 Cad11 2ndAB-5 92-1 Cad11 Gal 488 - 530/30-A Mean: 208</p>	<p>20170421 Cad11 2ndAB-5 92-1 Cad11 Gal 488 - 585/42-A Mean: 258</p>

Figure 10: Flowcytometry results of Cad11 (16.a.c.5) testing with secondary antibody (GaM-PE). First row shows the results with only conjugated Cad11-FITC, second row shows staining with Cad11-FITC and GaM-PE, third row shows staining with unconjugated Cad11, with GaM-PE. Left column shows FITC expression, right column shows PE expression. Mean is noted underneath every graph. Voltages were kept constant over all tests.



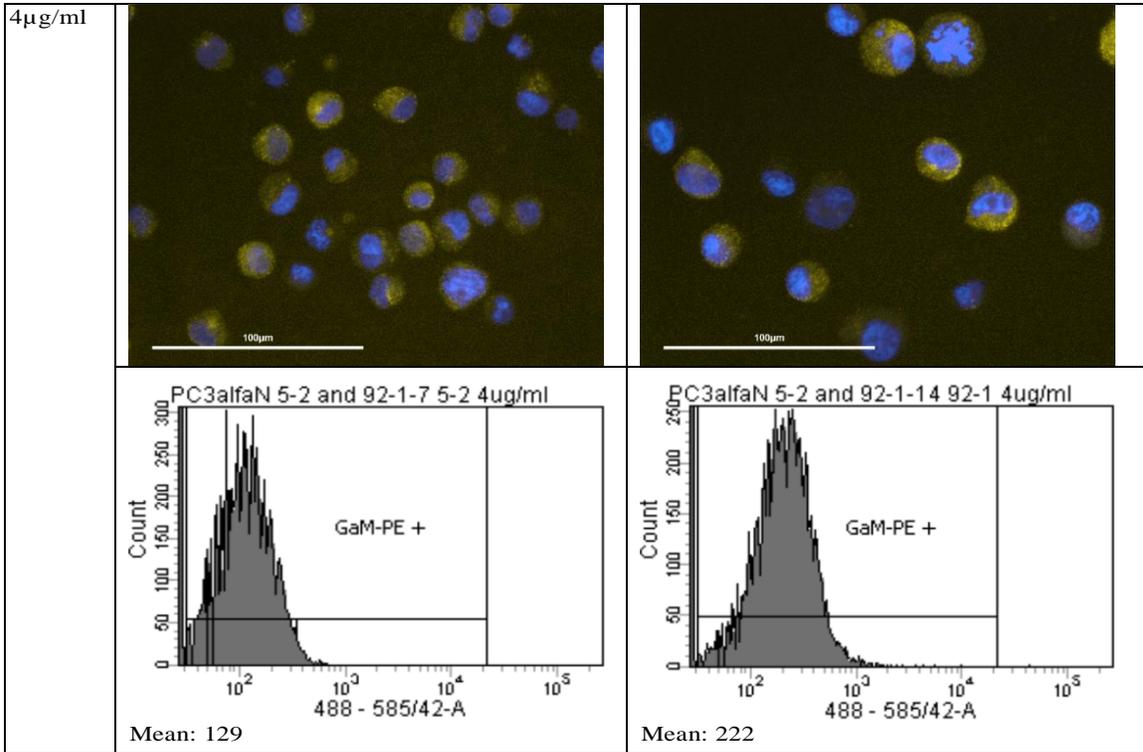


Figure 11: Testing of the commercial Cadherin 11 clone. Tested on cell lines with high (PC3αNcat92.1F) and low (PC3αNcat5.2) Cad11 expression. The nucleus is stained with Hoechst 33342 (blue, 4μg/ml) and the Cad11 is shown by GaM-PE (yellow). Magnification 400x (40x 0.6NA Nikon). LUT values for PE are: 2seconds, 208-465, and are constant for all images. The mean signal intensity of the flowcytometry plots is noted beneath each graph.

### 3.3 Vimentin and Cadherin 11 tests on blood

To determine if either of the antibodies would bind a-specifically to the WBCs, two tests were performed on blood; one Vimentin titration and one test of Cad11 from Biologend.

For the Vimentin tests, cells were stained with Hoechst, Vimentin, and CD45-PerCP. However, the concentration of the last, which was 1 $\mu$ g/ml, was too low to make this antibody detectable. It is visible in the flowcytometry results shown in figure 12 that Vimentin did not stain all cells that were stained with Hoechst. In panel B the cells are stained with Hoechst (blue), and three populations can be distinguished (bottom left, top middle, and right). The population on the bottom left is a combination of lymphocytes and monocytes. The population on the top, going almost horizontally, are the granulocytes. The last population, on the right, shows the cancer cells spiked in the blood. Panel C shows only the cells that are stained with Vimentin, it shows only the PC3 cells that were spiked in blood, the other two populations are not present.

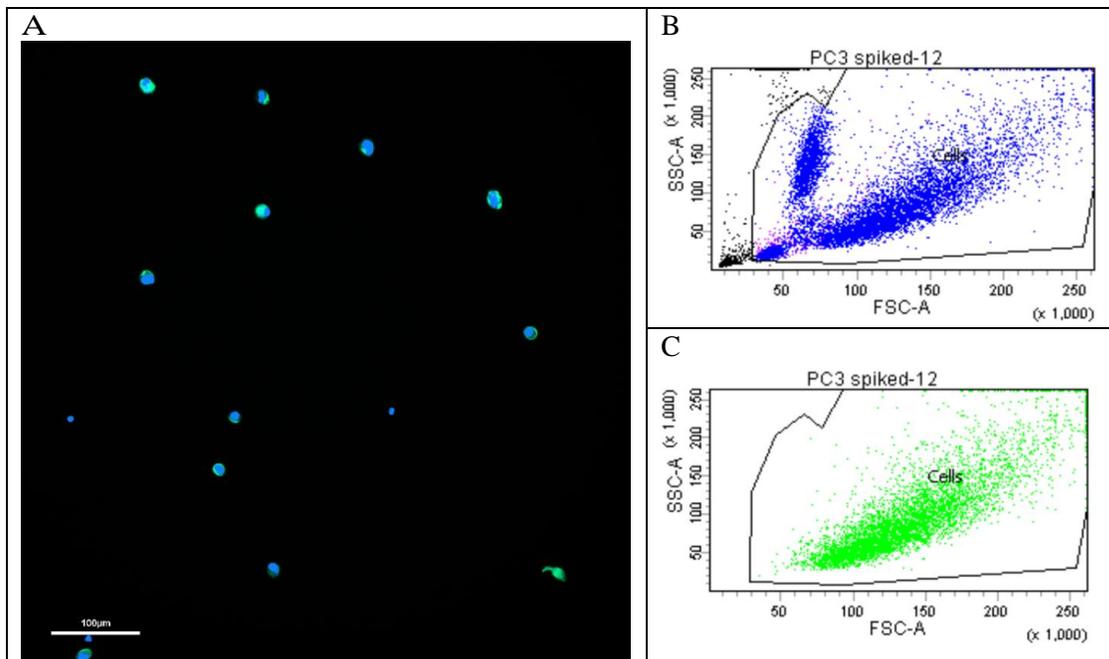


Figure 12: Microscopic and flowcytometry results from spiked blood staining with Hoechst 33342 (Blue, 4 $\mu$ g/ml) and Vimentin (Green, 2 $\mu$ g/ml), cell line used for spiking was PC3. Microscopic image (panel A) was taken using 100x magnification (10x 0.3NA Nikon). Panel B shows all cells that were stained with Hoechst. Panel C shows all cells that were stained with Hoechst and Vimentin.

The Cad11 expression of white blood cells was also tested, using blood and PC3-92.1 cells for spiking. A concentration of 5 $\mu$ g/ml of Cad11 and GaM-PE was used, and CD45-APC was added to a concentration of 2 $\mu$ g/ml. In figure 13 Panel A, PE signal (yellow) is coming from both the PC3-92.1 cells (large arrow), and the WBCs (small arrow). This is confirmed by the corresponding flow cytometry plot in panel B, that shows that both PC3-92.1 cells and granulocytes were stained with PE. In panel C, which shows the flowcytometry results of a sample only containing GaM-PE, and no primary antibody, there is a lot binding between the GaM-PE and the cells directly. This indicates that GaM-PE bound a-specifically to the PC3-92.1 cells and the granulocytes in the blood.

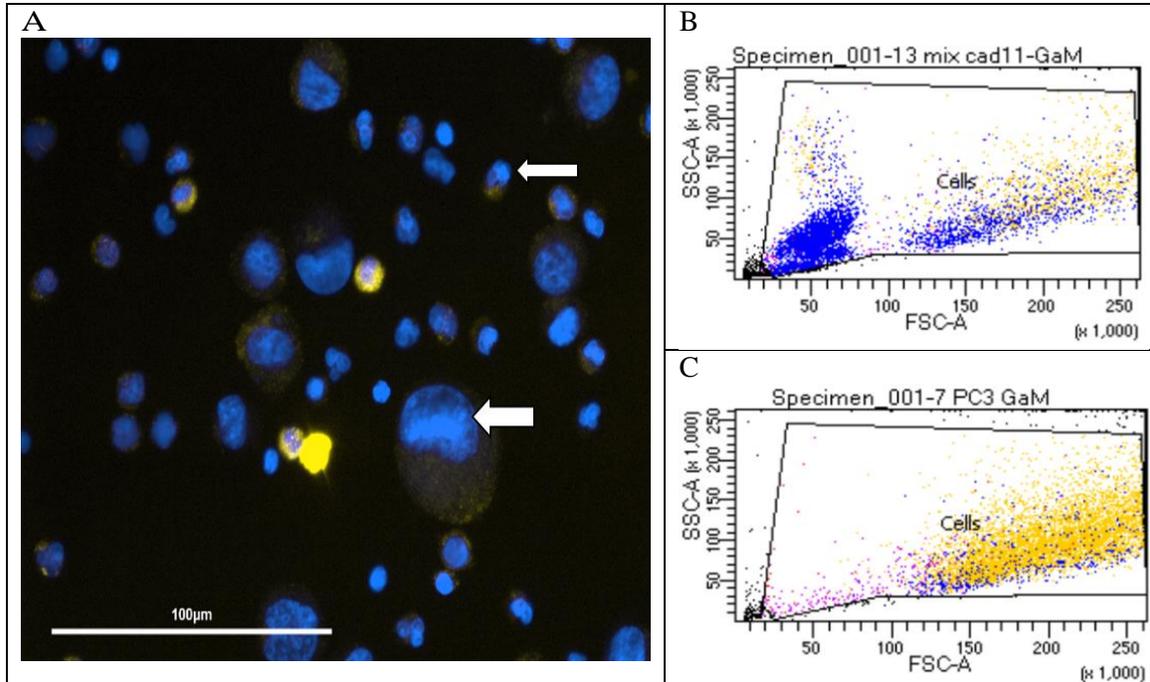


Figure 13: Microscopic (A) and flowcytometer (B,C) results from spiked blood staining with Hoechst 33342 (Blue, 4µg/ml) and Cad11, with GaM-PE (Yellow, 5µg/ml), cell line used for spiking was PC3-92.1. Microscopic image was taken using 400x magnification (40x 0.6NA Nikon). Panel A shows PC3-92.1 cells (large arrow) and WBCs (small arrow) stained with GaM-PE. Panel B corresponds with this image. Panel C shows PC3-92.1 cells stained with only the secondary antibody, GaM-PE.

### 3.4 Expression of EMT markers in cell lines

In Table 3, all the results of this research are summarised. The vimentin Expression strongly corresponds with the EpCAM expression, as when one is positive, the other is negative. The colour green indicates that the results of the test corresponded with literature. The colour red indicates that this was not the case.

Table 3: The expression of proteins according to literature, and the results found in this research. Green indicates that the result is as was expected. Red indicates that the results differed from literature.

		Epithelial phenotype		Mesenchymal phenotype	
		EpCAM	Cytokeratins	Vimentin	Cadherin 11
PC	LNCaP	+	+ -	-	-
	PC3	-	+ -	++	+
NSCLC	NCI-H1650	++	+	-	?
	NCI-H1563	-	+ -	+	?
BC	MCF7	+	+ -	-	-
	MDA-MB-231	- +	++	++	++
control	WBCs	-	-	-	?

## 4. Discussion

The goal of this research was to optimise immunostaining and characterize EpCAM positive and negative cell lines on the presence of epithelial and mesenchymal antigens to determine their EMT status. This was done by comparing the expression of epithelial antigens (EpCAM, Cytokeratins) and a mesenchymal antigen (Vimentin).

The intention was to use Cadherin-11 as a mesenchymal antibody, next to Vimentin. An unconjugated version of Cad11, donated to the lab by a research group in Nijmegen, was chosen for this purpose. The antibody was conjugated to FITC in the MCBP lab, and was used in a titration on PC3-5.2 and PC3-92.1. However, during this test, no signal was detected. A possible explanation could be that the FITC had bound to the active sites of the antibody, making it unable to bind to the antigens. Another possible cause of the problem might be that the PC3 cell lines had mutated and lost their Cad11 expression. Both hypotheses were tested by comparing the unconjugated and conjugated Cad11 in combination with GaM-PE, and by using MDA-MB-231 next to PC3-5.2 and PC3-92.1. MDA-MB-231 was chosen because of its high Cad11 expression found in literature. Unfortunately, this test still showed no clear difference in signal between the negative and positive samples. This led to the conclusion that the antibody itself must be malfunctioning.

Therefore, a new, unconjugated clone of anti-Cadherin 11 was purchased. This clone was tested on both GMO PC3 cell lines, using GaM-PE as a secondary antibody. In this titration, some difference between the negative controls and positive controls was found, as could be seen in figure 11, and it was decided to continue with a concentration of 5µg/ml for spiking in blood.

From the testing of Cad11 with GaM-PE on spiked blood, it became clear that GaM-PE not only bound a-specifically, it also bound directly to both the PC3-92.1 cells, and the granulocytes in the blood. This led to the conclusion that the GaM-PE antibody from SantraCruzBiotech was malfunctioning, and no further conclusions on the Cad11 antibodies could be drawn from this test or the titrations done previously with GaM-PE.

Table 3 shows the results of the tests with Vimentin and CKs, with respect to literature. In most cases, the test results corresponded with the found literature. The only cases in which literature and test were not similar, was with the testing of PC3 and LNCaP with CKs and with NCI-H1650 with Vimentin. In the case of the latter, expression should have been positive but was found to be negative. The reason for this difference is unclear, as it could be caused by factors such as the number of transfers of the cell line, or mutations in the cell line itself. This can only be determined by repeating the test. In the case of PC3 and LNCaP with CKs, the microscopic image and flowcytometry results seemed contradicting. For PC3, the flowcytometer results are very minor, but using the microscope the CKs are clearly visible. For LNCaP, this is the other way around. This difference could be caused by the cells, as the cells that were used were not completely fresh, and the microscopic images were taken on the day of the test, while the samples were analysed in the flowcytometer three days later. It could be also that the microscope shows unspecific binding which decreased over the days after, or that the antibody solution had expired.

From the tests done on blood, it became clear that Vimentin is not present in WBCs, as the difference between the WBC populations and the PC3 population is clearly visible. This means that Vimentin can be used as a possible maker, next to EpCAM and CKs to identify CTCs.

## 5. Conclusion

The goal of this research was firstly to optimise immunostaining, and secondly to characterize the status in the process of EMT for EpCAM positive and negative cell lines. This was done based on the presence of epithelial and mesenchymal antigens. These would indicate the relation between the epithelial and mesenchymal phenotype, which would show the role that EMT and MET can play in the detection of CTCs.

Immunostaining was optimised by defining the optimal concentrations of the antibodies Vimentin and Cytokeratins, and by defining incubation times that suit both CTCs and WBCs. From the tests that were done, it can be concluded that there likely is a connection between EpCAM and Vimentin expression, as there is a constant tendency that when EpCAM expression is low, Vimentin expression is high, and vice versa. A similar link between Vimentin and Cytokeratin expression was however not clearly found, as almost all cell lines were found to express Cytokeratins to some extent. Thus, this research does give a strong indication of the role that EMT plays in the expression of different proteins, and that there are indeed cells that can reside in an intermediate state, expressing both epithelial and mesenchymal antigens. However, no conclusion can be drawn on the relation between Vimentin and Cytokeratins as indicator of the cell phenotype. This could indicate that EpCAM and Vimentin are very specific markers for the Epithelial and mesenchymal phenotype respectively, while Cytokeratins can be present in both phenotypes.

## 6. Recommendations

Firstly, for continuation of the tests with Cad11, it is advisable to test the antibody with a secondary antibody on PC3-92.1, PC3-5.2, and MDA-MB-231. Of these cell lines, both PC3-92.1 and MDA-MB-231 should have a high Cad11 expression, whereas PC3-5.2 should have a low expression. In this test one could check if the problem of low signal lies indeed in the faulty GaM-PE used in this research, in both clones of the Cad11 antibody, or in the modified PC3 cell lines. From this point on, one could continue to either conjugate the Cad11 antibody and test its functioning directly, or continue testing with a secondary antibody. If from these tests it turns out that one of the two Cad11 antibodies functions well, a titration could be done, followed by a test on blood. If this test then again yields positive results, meaning that the Cad11 does not bind to the WBCs, one could continue with testing the antibody on different cell lines to see if there is a correlation between the expression of Cad11, Vimentin, and EpCAM.

Furthermore, one might opt to redo the tests done in this research. Since all tests were only done once, factors such as transfer number, expiration of the antibody solution, or human error could have affected the outcome of this research.

Additionally, in this research the focus lied on prostate, lung, and breast cancer cell lines. To broaden the scope of this research, cell lines from colon cancer could be added. Colo-320 (with an EpCAM density of  $5/\mu\text{m}^2$ ) and SW480 (with a density of  $6075 \text{ EpCAM}/\mu\text{m}^2$ ) would be a good starting point. If consistent results are found, tests could also be done on blood samples from patients, to see if more CTCs can be found using the additional markers.

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## 8. Appendix

### *8.1 Appendix a: Cell culture protocol*

- Withdraw medium from the T flask with a 1 ml pipette (vacuum pump).
- Wash the cell layer thoroughly with 5ml or 15ml pre-warmed PBS
  - Withdraw PBS from the T flask with a 1 ml pipette (vacuum pump).
- Add trypsin solution to the culture vessel (1ml for T25 or 3ml for T75)
  - Incubate the cells with the trypsin at 37°C for 3 - 10 minutes until cells have detached.
- Add serum-containing medium to inactivate the trypsin.
  - Disperse the cells by repeated pipetting over the culture vessel surface.
  - Cell clumps can be reduced by pipetting onto the bottom of the T-flask. Try to avoid frothing (bubble formation) as this might disrupt the cells.
- Transfer the single cell suspension to a sterile tube
- Pipet 12µl of cell suspension into Lunar cell counter counting chamber
  - Calculate the amount of cell suspension that is needed in order to seed a new T-flask
  - For example 2T75's with 5000 cells/cm<sup>2</sup> , cell suspension 1.3x10<sup>6</sup> cells/ml
- Add fresh medium to the new T-flask (5ml for T25, 15ml for T75), then add the calculated amount of cells suspension.
- Write name, date and cell type and passage number (and experimental treatment) on the flask. Use the designated area on the side of the flask, so cultures can be easily and quickly identified to avoid long opening of incubator doors (and resulting CO<sub>2</sub> and temperature drop).

For fixation in cell culture lab:

- Centrifuge the cells in the tube (e.g. 300 rpm for 4 minutes at room temperature)
- Withdraw the medium, do not disturb the pellet.
- Resuspend the pellet in 1ml 1% formaldehyde in PBS
- Incubate 10 minutes at RT
- Centrifuge for 5 minutes with 500 xg
- Aspirate the supernatant using vacuum pump
- Add PBS 1x/BSA 1% to starting volume
- Store at 4 degrees Celsius

## *8.2 Appendix b: Red blood cell lysis protocol*

### Reagents:

- Lysis solution: 155mM ammoniumchloride, 10mM kaliumcarbonate, 0,1mM Na<sub>2</sub>EDTA, pH 7,25
- PBS AC: 1x PBS with 13,3mM Trisodiumcitrate and 0,5% Bovine serum albumin(BSA)
- EDTA and CSB blood

### Protocol lysing RBCs:

- Dilute the blood 20x with the lysis solution. Note the starting volume.
- Incubate for 20 minutes on ice.
- Centrifuge for 5 minutes with 300 xg
- Aspirate the supernatant
- Resuspend with PBS-AC solution in twice the original volume
- Centrifuge for 5 minutes with 300 xg
- Aspirate the supernatant
- Add PBS-AC in the starting volume
- Centrifuge for 5 minutes with 300 xg
- Aspirate the supernatant
- Add PBS1x/BSA1% to starting volume
- Store at 4 degrees Celsius

### 8.3 Appendix c: Cell staining protocol

#### Materials:

- Hoechst 33342 diluted in MQ (1mg/ml)
- Antibodies

#### Method:

- Spin down for 5 minutes, 500xg and aspirate supernatant
- *Fixation of cells*
  - Resuspend cell pellet in 1% formaldehyde 1ml
  - Incubate for 10 minutes at room temperature
  - Spin down for 5 minutes at 500xg and aspirate supernatant
- Add 4ml PBS 1x/BSA 1%
- Divide samples (250.000-500.000 cells per sample)
- Spin down for 5 minutes, 500xg and aspirate supernatant
- *Permeabilization of cells*
  - Resuspend cell pellet in 250µl PBS/saponin 0.05%
  - Incubate for 10 minutes at room temperature
  - Spin down for 5 minutes at 500xg and aspirate supernatant
- Add PBS 1x/BSA 1% (/ saponin 0.05%) + staining reagents to a total volume of 100µl
- Incubate for 10 minutes at RT
- Spin down for 5 minutes, 500xg and aspirate supernatant
- *Adding secondary antibody*
  - Resuspend cell pellet in 100µl PBS1x/BSA1%
  - Incubate 10 minutes at RT
  - Spin down for 5 minutes, 500xg and aspirate supernatant
  - Add PBS 1x/BSA 1% (/ saponin 0.05%) + secondary antibody to a total volume of 100µl
  - Incubate for 10 minutes at RT
  - Spin down for 5 minutes, 500xg and aspirate supernatant
- Add 500 µl PBS 1x /BSA 1% and Hoechst to an end concentration of 4µg/ml
- Incubate for 10 minutes at room temperature
- Store at 4 degrees Celsius
- For flowcytometry: take 300 µl of the stained cell suspension and if needed add 200 µl PBS/BSA 1%, shortly vortex directly before use at a low speed.
  - Clean FACS and check calibration
  - Take negative control at low flow rate 1 and set lasers so that all negative signal (=background) is below  $10^2$  or any reasonable point far enough and distinguishable from positive samples.
  - Record, then take out sample, vortex next sample and press next tube, etc.

## 8.4 Appendix d: Antibody conjugation protocol

### Materials:

- Unconjugated antibody (4°C)
- Anhydrous DMSO
- Zebbar spin desalting columns, 2ml (200-700µl samples)
- Fluorochrome
- Sodium bicarbonate buffer (0.1M, pH 8.4) (210mg NaHCO<sub>3</sub> in 250ml MilliQ)
- Collection tubes
- Storage buffer (PBS/BSA1% filtered with 0.02% sodium azide)
- PBS

### Method:

- Preperation of Zebbar column for antibody rebuffering
  - Twist bottom and loosen cap of the column and place in collection tube
  - Mark one side
  - Centrifure at 1000xg for 2 minutes, discard solution
  - Add 1ml sodium bicarbonate buffer, centrifuge, discard solution
    - Repeat 3x
- Preperation of antibody
  - Place Zebbar column in new collection tube
  - Pipette 500µl of antibody on column
  - Centrifuge, collect sample
- Covalent conjugation
  - Prepare a 20mM stock solution of fluorochrome in anhydrous DMSO
  - Add 20-fold molar excess of fluorochrome-DMSO per ml of antibody in an Eppendorf tube
  - Suspend, vortex, wrap in aluminium foil
  - Incubate 2 hours at 4°C
- Preperation of Zebbar column for removing unreacted fluorochrome
  - Twist bottom and loosen cap of the column and place in collection tube
  - Mark one side
  - Centrifure at 1000xg for 2 minutes, discard solution
  - Add 1ml sodium bicarbonate buffer, centrifuge, discard solution
    - Repeat 3x
- Rebuffering and storage
  - Place Zebbar column in new collection tube
  - Pipette conjugated antibody on column
  - Centrifuge, collect sample
  - Store sample in new vial at 4°C