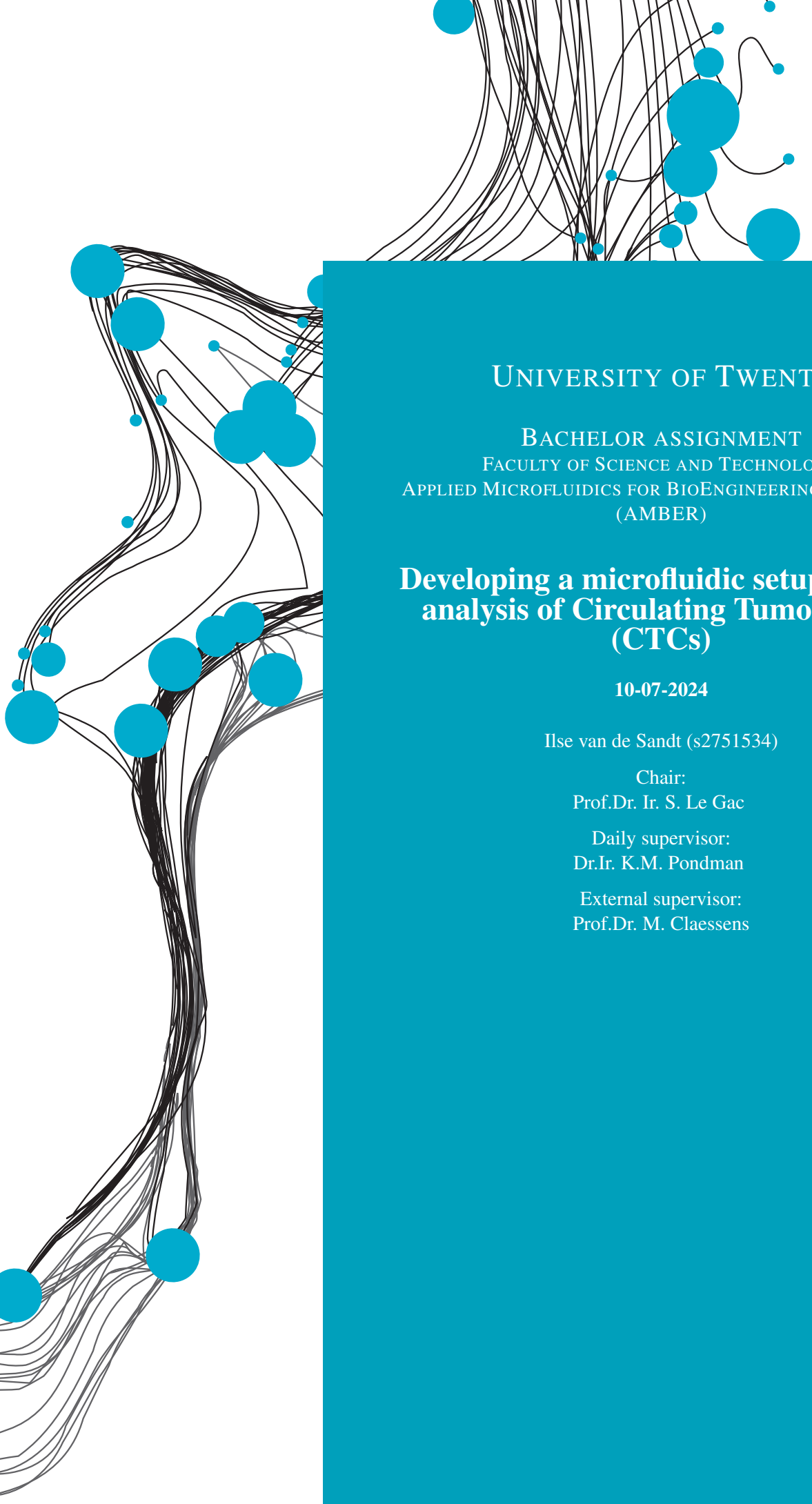

UNIVERSITY OF TWENTE

BACHELOR ASSIGNMENT
FACULTY OF SCIENCE AND TECHNOLOGY
APPLIED MICROFLUIDICS FOR BIOENGINEERING RESEARCH
(AMBER)

Developing a microfluidic setup for the analysis of Circulating Tumor Cells (CTCs)

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Abstract

With one in eight women developing breast cancer during their lifetime, it is clear that research in this area is essential. Of the patients who die from this disease, metastasis is the cause in 90% of the cases. Circulation of tumor cells in the bloodstream is a key aspect of the metastatic cascade and secondary tumor formation. In this study, a microfluidic recirculation setup was developed to gain a better understanding of the effect of shear stresses on circulating tumor cells (CTCs) in breast cancer metastasis. This setup mimics the conditions in the blood flow by creation of a stable, controlled, continuous and unidirectional flow through a microfluidic chip. Thereby inducing physiologically relevant shear stresses. The system was validated with THP-1 monocytes by circulating them for 4 hours through the system with a shear stress of 20 $dyne/cm^2$. After circulation presto blue and live/dead assays showed that the viability of the THP-1 was not affected by the shear stress. Following validation, breast cancer cell lines MCF-7 and MDA-MB-231 were circulated in the system for 2 hours with a shear stress of 10 $dyne/cm^2$. Cell viability assays demonstrated that for both cell lines the viability decreased due to circulation induced shear stresses. To investigate the change in protein expression, related to epithelial to mesenchymal transitions (EMT), due to shear stresses, immunostaining was performed. It was found that protein expression remained the same after circulation. Lastly, quantitative polymerase chain reaction (qPCR) was performed to investigate the change in gene expression, related to EMT, induced by shear stress from the circulation. It was found that the gene expression changed to a more epithelial phenotype. Changes in gene expression were bigger for the invasive MDA-MB-231 cell line than for the non-invasive MCF-7 cell line. These results show that a microfluidic setup that successfully circulates cells was build and thereby makes the analyzation of these CTCs possible.

Contents

Chapter 1

Introduction

Breast cancer is one of the most prevalent forms of cancer worldwide, with one in eight women developing breast cancer in her lifetime. This type of cancer is characterised by the abnormal and uncontrolled proliferation of breast cancer cells that have undergone genetic mutations. [1] The survival rate of breast cancer depends on the type and stage at which it is diagnosed. The five-year survival rate* for all types and stages combined is 91%. However, this number is significantly lower for patients with distant metastasis, at 31%. [2] In fact, metastasis is responsible for 90% of the cancer-related deaths. [1]

Metastatic cancers have such high mortality rates, because they are often unresponsive to existing treatments. This could be due to genetic changes, making them resistant to therapies. Another reason is that the metastatic tumor is growing in a different organ, thus having a different microenvironment and therefore responding different to treatments. Surgeries are also not an option since the tumor cells are spread throughout the body. Furthermore, the mechanism of cancer metastasis is not yet fully understood, making it difficult to design new treatment options. [3, 4]

In metastasis, cancer cells leave the primary tumor and travel through the blood or lymph vessels to another part in the body, called the secondary site, where a new tumor is formed. [1] The metastatic cascade consists of five steps: local invasion, intravasation, circulation, extravasation and colonization. This study is focused on the circulation of circulating tumor cells (CTCs) in the bloodstream. In this step, the tumor cells are called CTCs and are affected by shear stresses from the blood flow. Given that these shear stresses are absent in the original tumor environment, it is interesting that the CTCs are able to survive in these conditions. [5] However, the chances hereof are low with less than 0.01% of CTCs ultimately developing into metastases. [6]

Studies have shown that CTCs react to these shear stresses by changing their phenotype in a process called epithelial to mesenchymal transitions (EMT). [5, 7] It is hypothesized that this also affects their ability to survive circulation and invade distant tissues. During EMT cancer cells change from epithelial to mesenchymal phenotype. The cells aquire more mesenchymal features like reduced cell adhesion and increased motility, giving them the ability to invade, resist stress and disseminate. [5, 7]

To be able to investigate the effect of shear stress on the viability and EMT of CTCs, a system is needed that can mimic the conditions in the blood circulation. Therefore, this study focuses on developing such a microfluidic setup. The setup will be constructed, validated with THP-1 monocytes and tested and analyzed in terms of their viability and EMT with breast cancer cell lines MCF-7 and MDA-MB-231. It is hypothesised that CTC viability decreases due to circulation. The more mesenchymal cells, like MDA-MB-231, are hypothesized to be better equiped to survive. Furthermore, EMT can be observed through the decreased expression of cell adhesion proteins and the increased expression of structural proteins.

* Percentage of people that are still alive five years after diagnosis

Chapter 2

Theoretical background

2.1 Breast cancer

2.1.1 General

As previously stated, breast cancer is one of the most common cancers worldwide with more than 2.26 million cases in women in 2020 [8]. This type of cancer is caused by genetic mutations which results in the abnormal and uncontrollable growth of breast cells. It's origin lays in the lobules, tubes or connective tissue of the breast [4]. Three different types of breast cancer can be divided based on their molecular subtype. These are: luminal types (estrogen receptor (ER) positive) , human epidermal growth factor receptor 2 (Her2) positive, and triple negative breast cancer (TNBC). The subtypes differ in treatment options and metastatic potential. For the luminal and Her2+ subtypes, specific treatment options are available. However, for the TNBC no specific therapy is available. Moreover, TNBC has the highest chance of metastasis and is therefore considered the most aggressive subtype. [5]

2.1.2 Metastatic process

During metasasis cancer cells leave the primary tumor and travel trough the blood or lymph vessels to another part in the body, called the secondary site, where a new tumor is formed. The most common metastatic sites of breast cancer are bone, brain, lung, liver and lymph nodes, but this is also dependent on the type. The formation of a metastatic site is a multi-step process, which is also called the metastatic cascade. These steps include: invasion, intravasation, circulation, extravasation and colonization, as shown in figure 2.1. [5, 9]

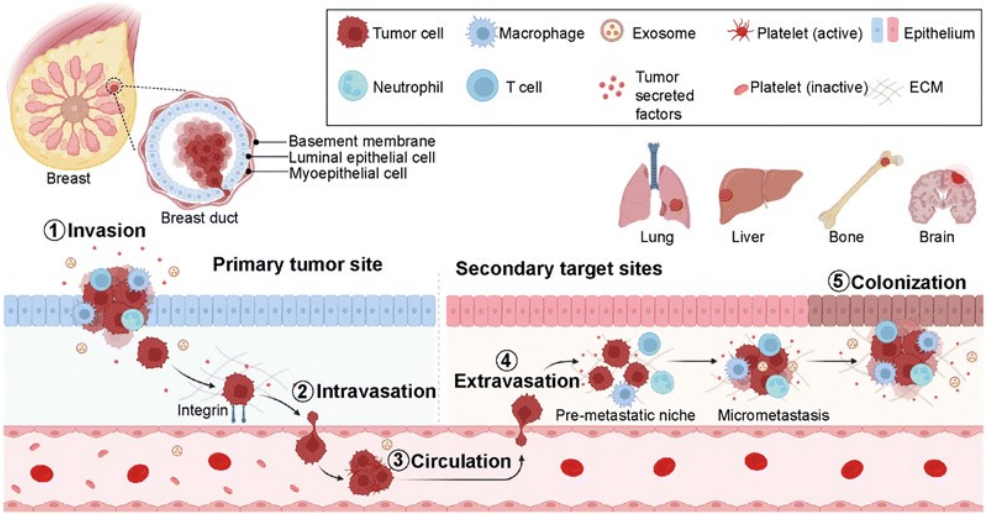

Figure 2.1: Overwiew of the metastatic cascade, consisting of local invastion, intravasation, circulation, extravasation and colonization. [9]

Invasion
During local invasion tumor cells undergo gene mutations, which are triggered by epigenetic changes. Epithelial to mesenchymal transitions play an import role in this step. During EMT cancer cells change from epithelial to mesenchymal phenotype. In this process, cell-cell junctions and cell-matrix attachments are destroyed, which results in a decrease of cell adhesion of the cancer cells. Furthermore, the cytoskeleton is remodeled to enhance motility. This gives them the ability to invade, resist stress and disseminate. Together, these changes result in the release of tumor cells from the original tumor. [5, 10, 11]

Intravasation
After the tumor cells leave the primary tumor they need to cross the endothelial barrier to enter a blood or lymph vessel and thereby enter the circulation. This is called intravasation. Intravasation depends on the tumor cells themselves, other cells in the tumor microenvironment, proteases, signalling molecules and environmental conditions. Together they allow the tumor cell to enter the circulation. [12]

Circulation
Once the cancer cell enters the circulation it is called a circulating tumor cell (CTC). These CTC's are exposed to new challenges. The first is anoikis, which is a form of programmed cell death caused by detachment from the extracellular matrix (ECM). Furthermore, the cancer cells get attacked by immune cells. Lastly, the cells are exposed to shear forces from the lymph or blood flow. To survive anoikis, breast cancer cells activate multiple signalling pathways. Breast cancer cells can also form aggregates with platelets, which protects them from shear stress. The aggregates also allow macrophages to bind and thereby prevent attack by the immune system. [5, 7]

Extravasation
After circulation, CTCs attach to the endothelium of the lymph or blood vessel and enter the secondary tissue. This is called extravasation. The tissue where the cancer cells will move into has different characteristics than the original tumor microenvironment. Therefore, multiple genes, proteins and macrophages are involved to stimulate the tumor cells to cross the endothelial barrier. [7]

Colonization
Once the tumor cell enters the secondary tissue it has to survive the secretion of anti-metastatic signals by stromal cells, the attack by immune cells and the completely new microenvironment. To survive this the cancer cell needs the right set of genes for the specific secondary tumor site. The set of genes should modify the environment and improve signalling to make proliferation possible. These genetic changes are called mesenchymal to epithelial transitions (MET) and are the reverse process of EMT. All together this makes proliferation and attachment, and thus formation of a secondary tumor, possible. [5, 7]

2.1.3 Circulating tumor cells

As described above, circulating tumor cells are cancer cells in the blood or lymph circulation. CTCs can be found alone or in clusters, bound by cadherins or other cell-cell adhesions. Because of the fact that CTCs (20-30 μm) are bigger than the smallest capillaries (8 μm), they are often trapped within minutes in circulation. [13] Yet, some cells continue circulating for far longer times with measured half-lives between 30 minutes and 2 hours. Which is often explained by the fact that only smaller or plastic CTCs can survive longer. [14] During circulation, the CTCs are exposed to shear stresses caused by the blood flow. This shear stress is defined as the internal frictional force between moving layers in laminar flow. In the human vasculature it ranges from 0.5 to 30 dyne/cm^2 [15]. Little is known about the viability of CTCs affected by this shear stress. Due to the shear stress and other challenges CTCs undergo in blood, some of them undergo apoptosis. It has been shown that the presence of CTCs correlates to the disease progression and metastasis. [12] To investigate this, technologies have been designed that can measure CTCs. For example, a study from Christofanilli et al. uses a cell search system that detects the CTCs based on their expression of certain proteins. It was shown that only 5 or more CTCs per 7,5mL blood already results in a lower survival chance of the patient. [16]

Studies have shown that CTCs react to the shear stress by a change in phenotype. These changes in phentoype can be characterised by EMT. Carcinomas are tumors that arrived from epithelial tissues. These epithelial tissues are characterized by their tight bound to neighboring cells and to underlying membranes by adherens junctions, tight junctions and desmosomes. These tight physical constraints thus keep the epithelial cells together. However, in malignant tumors the cells are able to let go from these physical constraints, making it possible for them to disseminate from the primary tumor. [1] These malignant tumor cells shows more mesenchymal characteristics. In this study E-cadherin, N-cadherin and Vimentin are investigated. E-cadherin is a transmembrane protein and plays an important role in maintaining the epithelial phenotype and regulating homeostasis. It is therefore a tumor suppressor. It has been found that the loss of E-cadherin results in metastatic dissemination and activation of EMT. On the other hand, N-cadherin, also a transmembrane protein, is known to be present in non-epithelial tissues. It therefore indicates the presence of EMT and the development of carcinomas. [17] Lastly, an increase of Vimentin, a protein of the intermidate filaments, is also characteristic for EMT. This protein ensures more stability for the cell, which makes it more resistant to shear stress. [17, 18]

2.2 Breast cancer metastasis models

2.2.1 General

Over the years multiple types of models have been used to study breast cancer metastasis. 2D culture is the most simplified model, but lacks the cell-cell interactions and dimensional organization. Animal models are a better representation of the physiological situation, but this method raises ethical concerns. [9] The most recent advances are 3D, in vitro models. These are called lab-on-a-chips and are microfluidic devices that have been designed to be able to move low amounts of fluid through microchannels. These models have been widely used in breast cancer studies, including models to mimic different steps of the metastatic cascade. [9]

2.2.2 Organ-on-a-chip

This report focuses on the lab-on-a-chips designed to study the effects of shear stress on CTCs. Multiple systems have been designed to trap single cells on a chip and create a flow over them to induce shear stress. For example in a study from Landwehr et al. Here, single breast cancer cells MCF-7 and MDA-MB-231 were trapped in the microfluidic chip, as shown in figure 2.2. A flow of medium was added, which leads to a controlled shear stress (of 5, 10 and 15 $dyne/cm^2$ for 5,10 and 15 minutes) to the cells. The deformation of the cells was investigated on the chips, based on the area and circularity of the cells. The result was that a greater shear stress and a greater duration resulted in a greater deformation. MCF-7 cells deformed quicker, but MDA-MB-231 cells deformed to a greater extent. These morphological changes can be correlated to EMT, where decreased stiffness is known to increase invasiveness. [19] This study gives an insight into the effect of shear stress on the CTCs, but the cells are trapped, which is not the case in the *in vivo* situation.

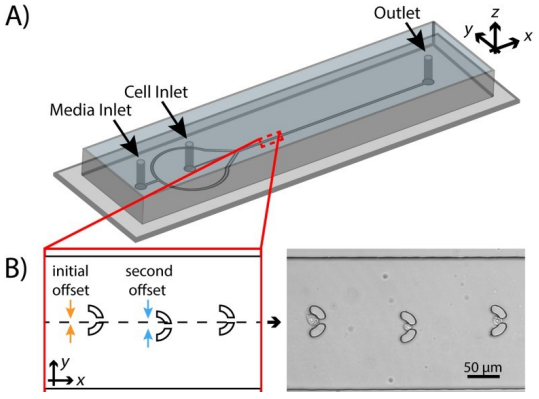

Figure 2.2: Microfluidic chip with cell traps to mimic shear stress on CTCs. Traps location with their offset and trapped cells. [19]

In another study from Quesada et al. cells were flown through a chip and afterwards part of the cells were trapped in a microwell to perform immunostaining. The other part was collected and western blot was performed. The shearing and trapping devices are shown in figure 2.3. Exposing MCF-7 breast cancer cells to shear stress (10 $dyne/cm^2$) resulted in an increased expression of biological marker Antigen Kiel 67 (Ki67), which indicates an increase of proliferation and aggresiveness. Moreover, phosphorylation of Protein kinase B (AKT) and Signal transducer and activator of transcription 3 (STAT3) was observed after applying shear stress. Showing that shear stress enhances proliferative biomarkers and results in phenotypic changes in growth factor signalling pathways. This indicates that shear stress can increase the phosphorylation of proteins associated with a more aggresive phenotype in CTCs. Concluding that CTCs survive metastasis due to the shear stress, thus increasing viability of CTCs and increasing their metastatic potential. [20]

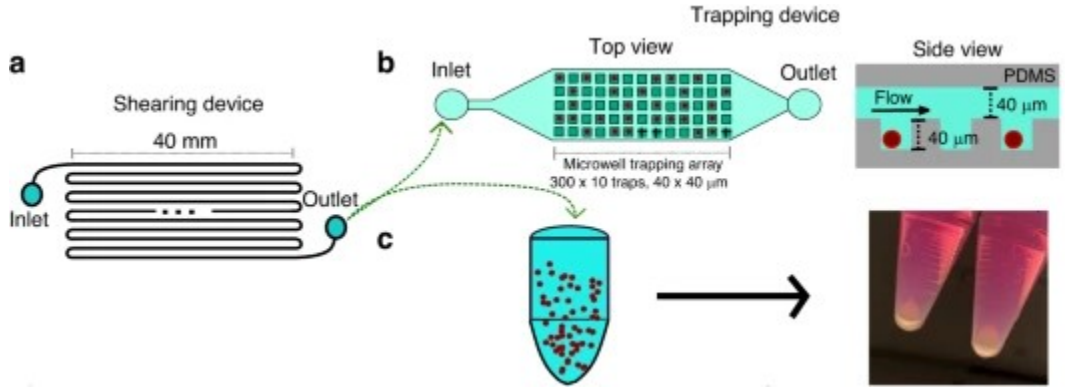

Figure 2.3: Microfluidic chip to mimic shear stress on CTCs. Part of the cells will enter the microwell trapping array while the other part will stay in suspension [20]

In this research, microfluidic methods are used to circulate a cell suspension through a chip. This method was chosen, because these models are known to be able to create controlled, approximately uniform shear stresses. Other advantages are low consumption of reagents, automatization and high reproducibility of the experiments. [20]

Chapter 3

Design of the setup

3.1 Requirements

To be able to mimic the blood circulation, a setup is needed that can create a stable, controlled and continuous flow unidirectionally through a chip. Thereby inducing physiologically relevant shear stresses. The system should also be able to create this flow for longer periods of time, e.g. days. These requirements are important to be able to model breast cancer metastasis on a chip in the future. The system should then be able to be connected to this breast cancer on a chip model, where the setup induces the flow and shear stresses through the chip.

To induce physiologically relevant shear stresses, a microfluidic chip with a long meandering channel is used, as illustrated in figure 3.1. The chip was designed to specifically mimic the blood flow in small arteries and capillaries of the human body. The channel has a height and width of 200 μm and a total path length of 1277 mm. The connection of the chip to the tubing of the setup has a diameter of 1.45 cm.

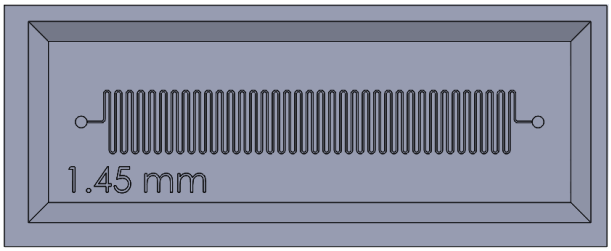

Figure 3.1: Microfluidic chip with meandering channel designed to mimic shear stress in the human vasculature. The height and width of the channel are 200 μm and the total path length 1277 mm. The connection of the chip to the tubing has a diameter of 1.45 cm.

3.1.1 Shear stress and flow control

The shear stress is directly related to the flow rate. In case of rectangular channels, like the one in this research, the shear stress calculation is very complex. Therefore, the parallel plate chamber is often used as an analytical approach to model shear stresses. In this approach the channels are represented by two infinite parallel plates. [21]. This results in a formula derived from the Navier-Stokes equation as shown in equation 3.1.

$$\tau = \frac{6\mu Q}{h^2 w} \tag{3.1}$$

τ = shear stress $(dyne/cm^2)$ = 10 $dyne/cm^2$ or 20 $dyne/cm^2$
μ = dynamic viscosity $(dyne * s/cm^2)$ = 0.01 $dyne * s/cm^2$ for cell culture medium at 37 °C [22]
Q = flow rate (mL/s)

h = height of the channel (cm) = 0.02 cm
w = width of the channel (cm) = 0.02 cm

In this research formula 3.1 is used to calculate the flow rate at the desired shear stress. When calculating this flow rate Q the formula can be rearranged to: $Q = \frac{\tau h^2 w}{6\mu}$. By filling in the the values stated above this will lead to a flow rate Q of $1.33 * 10^{-3}$ cm^3/s for a shear stress of 10 $dyne/cm^2$, which is equal to 80 μL/min. The same can be done for a shear stress of 20 $dyne/cm^2$, which results in a flow rate Q of 160 μL/min. For a shear stress of 1.25 $dyne/cm^2$ the flow rate Q is 10 μL/min.

Now that the values of flow rate are known, the question remains how to control this flow. There are multiple mechanisms for flow control in microfluidics. The ones used most often are the syringe pump, peristaltic pump and pressure driven pump. [2] The syringe pump works by a mechanical stimulus that pushes a syringe. It works well for small volumes, but is not precise and can result in flow pulsations. The peristaltic pump compresses and relaxes tubing and thereby pushes the fluid. It is better for large volumes, but also results in a flow with pulses. The last flow controller, which is used in this research, is the pressure driven pump. In this pumping system a controlled gas pressure pushes fluid from an airtight reservoir into the tubing. The advantage of this method is that the created flow rate is very stable and can be precisely controlled. Furthermore, it is possible to connect the pressure driven pump to a flow meter. The pressure will then automatically adjusted based on the measured flow rate. [2] Because of this stability and precise control the decision for pressure based control was made. The pressure controllers used in this research are from Fluigent. These controllers are easy-to-use and widely applicable. Therefore, it is possible to create a setup that meets the specific requirements of a research project.

3.1.2 Circulation time

A total volume inside the system of 3 mL was selected. To determine this value it was decided that each reservoir should contain at least 0.5 mL to ensure that the tubing remains in contact with the liquid and does not come in contact with the magnet. It is important to note that the total volume should not be too large, to ensure that each cells goes multiple times through the chip. Consequently, a large volume results in a small average time that each cell is inside the microfluidic chip. On the other hand, the total volume should not be too small, since this results in a high frequency of switching between reservoirs and switch positions, which affects the flow by causing interruptions of a 100 μs every time. Therefore, it was determined that the total volume would be 3 mL.

The volume inside the tubing and chip together is approximately 0.7 mL. Given a total volume within the system of 3 mL and the requirement that 0.5 mL fluid has to be present in each reservoir it was then calculated that the initial volume in reservoir one should be 1.8 mL and 0.5 mL in reservoir two. Hence, a volume of 1.3 mL cell suspension was flown through the system in each iteration of the protocol.

With all variables known, the average time that each cell is exposed to shear stress can be determined:

Total volume inside system = 3 mL
Volume inside chip = 0.051 mL
Flow rate Q = $1.33 * 10^{-3}$ mL/s for shear stress of 10 $dyne/cm^2$.
Flow rate Q = $1.67 * 10^{-3}$ mL/s for shear stress of 20 $dyne/cm^2$.
Flow rate Q = $1.67 * 10^{-4}$ mL/s for shear stress of 1.25 $dyne/cm^2$.
Total time of circulation = 2 hours = 120 min

Time it takes to circulate total volume one time = total volume inside system / flow rate = 3 mL/$1.33 * 10^{-3}$ mL/s = 37.6 min
Time of each cell in chip = volume inside chip / flow rate = 0.051 mL/$1.33 * 10^{-3}$ = 38 s
Number of times a cell goes through the chip = total time of circulation / time it takes to circulate total

volume = 120 min/37.6 min = 3.2x
Total time a cell is in the chip = Amount of times a cell goes through chip * time of each cell in chip = 3.2x * 38 s $\approx$ 2 min

Thus the average time that each cell is exposed to shear stress is 2 minutes for a shear stress of 10 $dyne/cm^2$. The same can be done for a shear stress of 20 $dyne/cm^2$, which will also result in a total time that each cell is in the chip of 2 min. For a shear stress of 1.25 $dyne/cm^2$ the cells went only once through the chip. The average time each cell is inside the chip is then: volume inside chip / flow rate = 0.051 / $10 * 10^{-3} \mu L/min$ = 5.1 min.

3.2 Recirculation system

Based on the requirements stated above, the microfluidic recirculation system from Fluigent was chosen. This system makes it possible to create the desired flow, which is stable, controlled and continuous. Moreover, it can resume for a long period of time. Thereby, it creates a continuous shear stress on the cells. The complete setup is schematically shown in figure 3.2. [23] The setup consits of the following components:

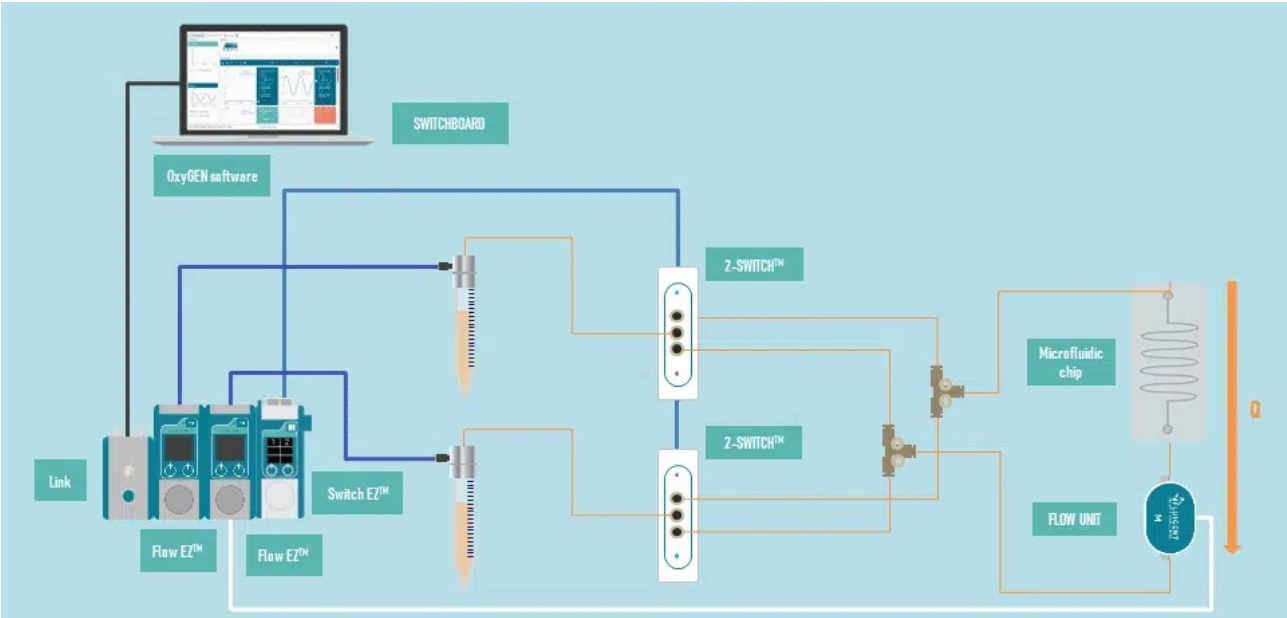

Figure 3.2: Microfluidic recirculation system from Fluigent. The system consits of two reservoirs, two Flow EZs, a switch EZ, two 2-switches, a Flow unit and a Link connected to the oxyGEN software [23]

Pressure controllers
The setup contains two pressure controllers (Flow EZs) to generate a pulseless flow. A pressure is added to one of the closed reservoir filled with cell suspension. Because of the increasing pressure in the reservoir the cell suspension will flow into the tubing. [23]

2-switches and switch controller
The two 2-switches are 3 port/2 way solenoid valves. A solenoid valve is an electromechanically operated valve. In this case it has 3 ports and can operate in 2 ways. These 2-switches determine in which direction the cell suspension will flow by opening and closing the entrance of the tubing. The switches can be operated with the switch controller (Switch EZ). [23]

Flow controller
The flow controller (Flow unit) is a bidirectional flow sensor that measures the flow rate of the liquid. A small amount of heat is added to the fluid inside the sensor. Two temperature sensors, located on both sides of the heat source measure the change in temperature. The flow rate is then calculated based on the thermal desorption. The flow unit is connected to the pressure controllers. When a desired flow is set, the flow unit will give the measured flow as feedback to the operating pressure controller. The pressure controller will then automatically adjust the pressure based on the measurements. [23]

Link module and oxyGEN software
The Link module is connected to a computer and the flow and switch controllers. With the oxyGEN software all instruments can be controlled live. Another option is to create a protocol to automate the experiment. In this case, a protocol for recirculation is used, where the pressure, flow rate, switch positions and duration of the experiments are defined. [23]. The protocol is depicted in appendix A. The initial step is to set all pressure values to zero and to set the regulation: the flow unit determines the pressure of pressure controller one (and pressure controller two remains zero). Subsequently, the positions of the switches are set, a period of 100 ms passes by and the flow rate is set. The system runs until the set volume has passed the flow detector. At that moment, the pressure of pressure controller one is set to zero and all positions and regulators are changed to the second switch position. This entire process is repeated as often as required. At the beginning and the end of the entire protocol all pressures are set to zero.

The combination of these components can create a recirculated flow. This recirculation enables a flow that always goes the same direction through the microfluidic chip. In figure 3.3 a schematic picture shows the working of the recirculation system. The system starts in Switch position 1, shown at the top of figure 3.3. A pressure is applied to pressure controller 1 and the pressure of pressure controller 2 is 0. This creates a pressure in reservoir 1 and because of that the fluid from reservoir 1 flows into the tubing. The orange tubing shows the path that the fluid is taking. The fluid comes into a switch and takes the open valve (in this case the top one). Then it flows through the chip, flow meter and the second switch and ends up in reservoir two. When reservoir 1 is almost empty the entire system changes to switch position 2, shown at the bottom of the figure. Here, a pressure is applied to pressure controller 2 and the pressure of pressure controller 1 is 0. Fluid now flows from reservoir two into the tubing and the switches have changed position resulting in a flow through the chip in the same direction as before. The fluid ends up in reservoir 1. Switching between these two switch positions can be done multiple times, resulting in unidirectional, continuous flow through the chip.

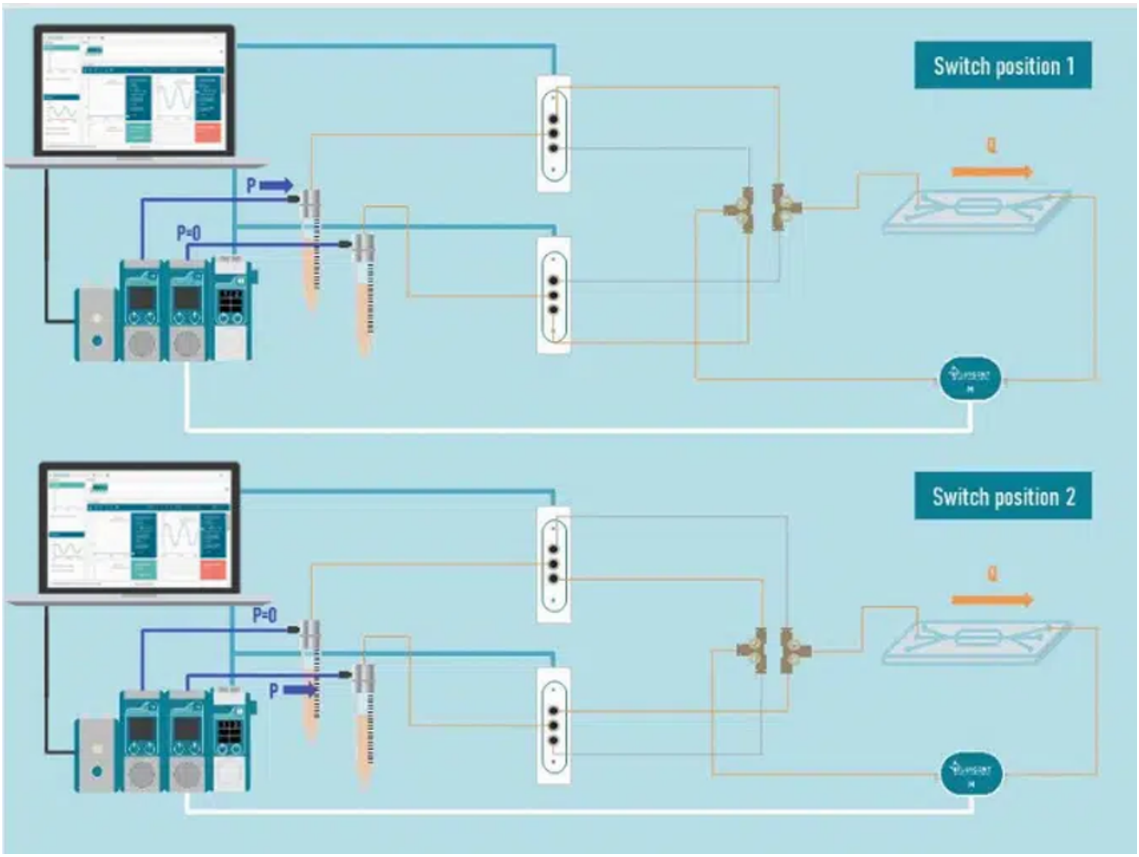

Figure 3.3: Schematic overwiew of the working of the microfluidic recirculation system. the dark blue tubing shows the applied pressures. The orange tubing shows the path the cell suspension is taking. [23]

3.3 Validation of the system

The system will be validated with cells that are normally present in the blood flow to check if the system correctly replicates the circulation and doesn't damage the cells. The morphology and viability of the cells will be investigated to see whether the cells survive the circulation. Lastly, the microfluidic chip will be visualized under a microscope to confirm that a flow of cells is present. In this study the THP-1 cell line will be used to validate the system. This is a monocyte cell line derived from a 1-year old patient with acute leukemia. [24] Since these cells are adapted to the blood stream and therefore resistant to shear stresses it is expected that these cells will not be affected by the recirculation system.

3.4 Breast cancer cell lines

In this research, the luminal, ER+ MCF-7 cell line and the triple negative human breast cancer cell line MDA-MB-231 will be investigated to test the effect of shear stress. The MCF-7 cell line is an adherent cell line derived from a female patient with breast adenocarcinoma and expresses estrogen and progesteron receptors. The cell line contains characteristics from mammary epithelium. [25] The MDA-MB-231 cell line is an adherent cell line derived from a female patient with breast adenocarcinoma and often used to model breast cancer in a later stage. The cells have a more mesenchymal phenotype than the MCF-7 cell line and are therefore also known to have a higher metastatic potential. [26] These cell lines were selected, because they represent two different types of breast cancer. Therefore, the difference in the impact due to shear stress can be investigated.

Chapter 4

Materials and methods

4.1 Cell culture and reagents

THP-1 cells (passage number 14) were maintained with Roswell Park Memorial Institute (RPMI) 1640 (Gibco) medium supplemented with 10% Fetal bovine serum (FBS) (Gibco) and penicillin G and streptomycin (pen/strep) (Gibco). Cells were maintained sideways in T75 flask (Greiner Bio-One) in a humidified incubator at 37 °C and 5% CO_2. Cells were subcultured once a week. During experimentation cells were in a suspension of $2 * 10^5$ cells/mL medium to avoid cell-cell interactions and blocking of the channels.

MCF-7 (passage number 22-28) and MDA-MB-231 (passage number 14-20) cells were maintained with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS, pen/strep and Glutamax (Gibco). Cells were maintained in T75 flask in a humidified incubator at 37 °C and 5% CO_2. Cells were subcultered once a week when confluency reached 80-90%. During experimentation cells were in a suspension of $2 * 10^5$ cells/mL medium to avoid cell-cell interactions and blocking of the channels.

4.2 Chip fabrication

Microfluidic chips were made by mixing Polydimethylsiloxane (PDMS) (Sylgard) and curing agent (Sylgard 184) in a ratio 10:1 and pouring it into the V4 clear resin (Formlabs) mold. The mixture was degassed in a vacuum chamber with pressure -0.08 bar to remove bubbles. Afterwards, chips were cured in the oven at 65 °C overnight. Once cured, the PDMS chips were removed from the mold. The chips were bonded to microscopic glass slides of 25x75x1 mm (Epredia) by exposing them 50 seconds to oxygen plasma treatment in a plasma oven (Femto Science Cute). Lastly, the bonded chips were put in the oven at 65 °C for 4 hours or overnight to secure the bonding between the PDMS and glass.

4.3 Design and fabrication of the microfluidic setup

The microfluidic setup consists of a Recirculation Package (Fluigent), shown in figure 3.2, which includes two pressure controllers (Flow EZ 2000 mbar), two 2-switches, one switch controller (Switch EZ), one Link module, two Fluidic T-junctions, two 15 mL Pcaps with 15 mL Falcon tubes, one Flow meter (Flow unit M) and fluorinated ethylene propylene (FEB) tubing with an inner diameter of 500 μm. The system is assembled following instructions of the manufacturer. The first pressure controller was connected to an air inlet. The air was first dehumidified by a pressure filter (Norgren) and then connected to a pressure control valve (Festo) to be able to adjust the incoming air in the pressure controller. A microfluidic chip (figure 3.1) with channel length of 1277 mm and height and width of 200 μm was connected to the recirculation system by inserting the tubing in the inlet ports of the chip with a size of 1.45 mm. The chip was placed inside a K-frame (Ibidi), which was connected to a gas mixer (Ibidi) and temperature controller (Ibidi) to regulate the temperature, humidity and O_2 and CO_2 concentrations. The Ibidi was assembled and settings were adjusted following instructions of the manufacturer. The Falcon tubes were placed on a magnetic stirrer (Fisher Scientific Isotemp) with magnets of size 6x3mm (Thermo Scientific). Lastly, the LINK module was

connected to a laptop with oxyGEN software (Fluigent, version 2.3.2.0). A build in protocol for recirculation was used and adapted to the experiments. The protocol is shown in appendix A. In the first experiments cells were introduced in the system with a septum (B. Braun) in the tubing close to the first reservoir. Before each experiment cells were injected with a syringe with needle (Sol-Vet 0.45x10mm) through this septum. Following, an equal amount of cell culture medium was injected via the same way to make sure all cells enter the tubing. Later, the septum was not used anymore and cells were introduced in the system by connecting the Falcon tube with cell suspension to the P-cap.

4.4 Circulation studies

Before each experiment the tubing was filled with medium corresponding to the used cell line. The cells were introduced to the system as described above and the oxyGEN protocol was switched on. MCF-7 and MDA-MB-231 cells with a concentration of $0.2 * 10^5$ cells/mL were circulated with a flow rate of 80 μL/min. Following circulation, the cells were analysed with a presto blue assay, live/dead assay, immunostaining and qPCR. After each experiment, the setup was emptied and cleaned by flowing, under a pressure of 400mbar, 4mL RBS 25 solution (Sigma Aldrich), demi water (MilliQ) and air, respectively.

4.5 Validation

4.5.1 Visualizing cells in circulation using fluorescent cell tracker

THP-1 cells stained with green cell tracker (Thermo Fischer Scientific) were used to test if a flow of cells was present during circulation by the system. The green cell tracker solution with 1:1000 dilution was used. After adding this the cells were incubated at 37 °C and 5% CO_2 for 30 minutes. Subsequently, a solution with appropriate medium was made and cells were injected into the circulation system as described above. During circulation the cells inside the chip were visualized using microscopy (Nikon Eclipse TS100) and afterwards using fluorescence microscopy (EVOS) with the GFP filter cube (Ex: 470/22 Em: 525/50). The experiment was repeated with MCF-7 cells to verify that these cells are as well flowing during circulation.

4.5.2 Validation of the microfluidic setup with monocytes

The system was validated by circulating THP-1 cells for 4 hours with a shear stress of 20 $dyne/cm^2$/ The system was prepared and the cells injected as described above. The oxyGEN software was turned on. Subsequently, presto blue and live/dead assays were performed to investigate the viability of the cells.

4.6 Control experiments

4.6.1 Magnetic stirring

To investigate the effect of magnetic stirring on the viability of the cells, a 1.8 mL THP-1 cell suspension with a concentration of $0.5 * 10^5$ cells/mL was stirred for 1 hour at 400 rpm at room temperature. As control a THP-1 cell suspension with the same volume and concentration were exposed to the same conditions without stirring. Afterwards, presto blue and live/dead assays were performed.

4.6.2 Circulation 1-way

To test the effect of recirculation and flow rate MDA-MB-231 cells were flown through the chip with a flow rate of 10 μL/min. The setup was prepared and the cells injected as described above. The cells went one time through the chip, meaning that there was no recirculation. Subsequently, the amount of cells was counted with a cell counter (EVE, NanoTek).

4.6.3 Testing injection

To test the effect of injection via a needle on the amount of cells $0.6 * 10^5$ MCF-7 cells in 150 μL medium were injected with a needle to 2.85 mL medium. As control a cell suspension of $0.6 * 10^5$ cells in 3 mL medium was used. The amount of cells for both suspensions were counted with a cell counter (EVE, NanoTek) and compared.

4.7 Cell viability

4.7.1 Presto blue assay

To determine the cell viability based on metabolism a presto blue assay was performed. Following circulation the cells were seeded on a 96 wells plate (Corning) and incubated overnight at 37 °C and 5% CO_2. Presto medium, containing presto blue (Invitrogen) and medium corresponding to the cell line in a ratio of 1:10, was added and the cells were again incubated at 37 °C and 5% CO_2 for 1 hour. The medium was transferred to a black plate and the absorbance was read using a plate reader (Perkin Elmer Victor 3, Ex: 560 nm Em: 590 nm).

4.7.2 Live/dead assay

The number of live and dead cells were quantified using a live/dead assay. The suspended tumor cells were seeded on a 96 wells plate after circulation and incubated overnight at 37 °C and 5% CO_2. Afterwards, the cells were incubated with a PBS solution containing Calcein AM (Sigma Aldrich) and Ethidium homodimer (Sigma Aldrich) for 30 minutes at 37 °C and 5% CO_2. The cells were washed with PBS and imaged using fluorescent microscopy (EVOS) with filter cubes GFP and RFP (Ex: 531/40 nm Em: 593/40 nm). To determine the percentage of the dead cells, the pictures were analyzed using ImageJ software (Fiji).

4.8 Epithelial to mesenchymal transition

4.8.1 Immunostaining

To detect a change in the presence of proteins in the cells an immunostaining was performed. After circulation the cells were seeded on a 96 wells plate and incubated overnight at 37 °C and 5% CO_2. Then, the cells were fixated by incubating them with 4% paraformaldehyde (Sigma Aldrich) for 30 minutes at room temperature. After that the cells were washed with PBS and incubated with 0.1% Triton-X100 (Sigma Aldrich) in PBS (Gibco) for 20 minutes at room temperature. The cells were washed with PBS again and blocking buffer containing 1% Bovine Serum Albumin (BSA) (Sigma Aldrich) in PBS was added and the cells were incubated for 1 hour at room temperature. 50 μL of primary antibody solution, containing N-cadherin, E-cadherin and Vimentin (RD systems) in a 1:200 ratio with 1% BSA buffer, was added to each well and these were incubated for 1,5 hour at 37 °C. Afterwards, cells were washed with PBS and 50 μL secondary antibody solution, containing goat, mouse and rat secondary antibodies (Life Technologies) corresponding to the primary antibodies, were added in a 1:200 ratio with 1% BSA buffer. The cells were again incubated for 1,5 hour in 37 °C and afterwards washed with PBS. The cells were visualized using fluorescence microscopy (EVOS), with filter cubes GFP, Texas Red (Ex: 585/29 nm Em: 628/32 nm) and Cy5 (Ex: 628/40 nm Em: 685/40 nm).

4.8.2 qPCR

Cells were analyzed with quantitative polymerase chain reaction (qPCR) to detect genetic changes due to the circulation. The suspended cells were seeded on a 96 wells plate and incubated overnight at 37 °C and 5% CO_2. Afterwards, the cells were lysed by adding lysis buffer (Qiagen buffer RLT plus) and freezed until further evaluation.

RNA isolation
RNA isolation was performed using the Qiagen RNAEasy Microkit. After lysing the samples were transferred to a MinElute spin column, centrifuged for 15s at 10000g and washed and centrifuged (Eppendorf 5424 R) with Buffer RW1. Following, DNAse 1 incubation mix, containing DNAse stock solution and Buffer RDD was added onto the MinElute spin column membrane and incubated for 15 minutes at room temperature. Afterwards the spin column membrane was washed and centrifuged with Buffer RW1, Buffer RPE and 80% ethanol. The spin column was dried by centrifuging for 5 min with open lid. Lastly, RNAse free water was added onto the spin column membrane and centrifuged 1 minute to elute RNA and the samples were analysed using the Nanodrop (Nanodrop ND-100 spectrophotometer, Thermo Scientific). Absorbance measurements were taken at 260nm to determine RNA concentration and the 260/280 ratio was used to determine RNA purity.

cDNA synthesis
cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad). From the nanodrop measurements the amount of RNA sample was calculated and diluted in RNAse free water. A mixture of 5x iScript reaction mix, iScript reverse transcriptasse the RNA dilution was made for each condition and a no template control (NTC) with nuclease free water. The samples were run in the thermocycler (Bio-Rad T100) with iScript program (5min at 25°C, 30min at 42°C, 5min at 85°C and hold at 4°C). The cDNA was stored at -20°C.

PCR
cDNA samples were diluted in RNAse free water 1:10. For each gene a master mix was made containing SensiMix SYBR Fluorescein Kit (Bioline), forward primer for N-cadherin, E-cadherin, Vimentin, PCBP1, CCSER2 and SYMPK and reverse primer for the same genes (Sigma Aldrich), all shown in table 4.1. The samples were added to a 384 wells plate (Corning) with three technical replicates for each sample. Lastly, the plate was loaded into the Bio-Rad CFX96 device and qPCR program was ran (10min at 95 °C, 40 cycles of 15s at 95°C, 15s at 60 °C, 25s at 72 °C followed by a melting curve from 65 °C to 95°C). The data was analysed using the $\Delta\Delta C_t$ method.

Table 4.1: Used (housekeeping) genes with their forward and reverse primers

	Gene	Forward/reverse	Primers
1	N-cadherin (mesenchymal)	Forward	CAGACCGACCCAAACAGAAC
2	N-cadherin	Reverse	GCAGCAACAGTAAGGACAAACATC
3	E-cadherin (epithelial)	Forward	CCCGGTATCTTCCCCGC
4	E-cadherin	Reverse	CAGCCGCTTTCAGATTTTCAT
5	Vimentin (mesenchymal)	Forward	CCAAACTTTTCCTCCCTGAACC
6	Vimentin	Reverse	CGTGATGCTGAGAAGTTTCGTTGA
7	PCBP1 (housekeeping gene)	Forward	ACTCTCACCATTCGGCTTCTT
8	PCBP1	Reverse	TCTTACACCCGCCTTTCCCA
9	CCSER2 (housekeeping gene)	Forward	AGCCACCCTGACCACTATCA
10	CCSER2	Reverse	GAGCCCTGGAAAAAGGGCATC
11	SYMPK (housekeeping gene)	Forward	ACAGYGCTCAAACAGGTCCA
12	SYMPK	Reverse	GCCGTCATTGTCAGAGTCCA

4.9 Statistical analysis

Each experiment was performed one time. Following each experiment, three technical triplicates were taken for each separate analysis (presto blue assay, live/dead assay, immunostaining and qPCR). The results were analysed based on their mean and standard deviation.

Chapter 5

Results

5.1 Setup design and validation

5.1.1 Design and settings

Combining the Fluigent recirculation package, magnetic stirrer and mini incubator, the final design of system was assembled. The complete setup is shown in figure 5.1.

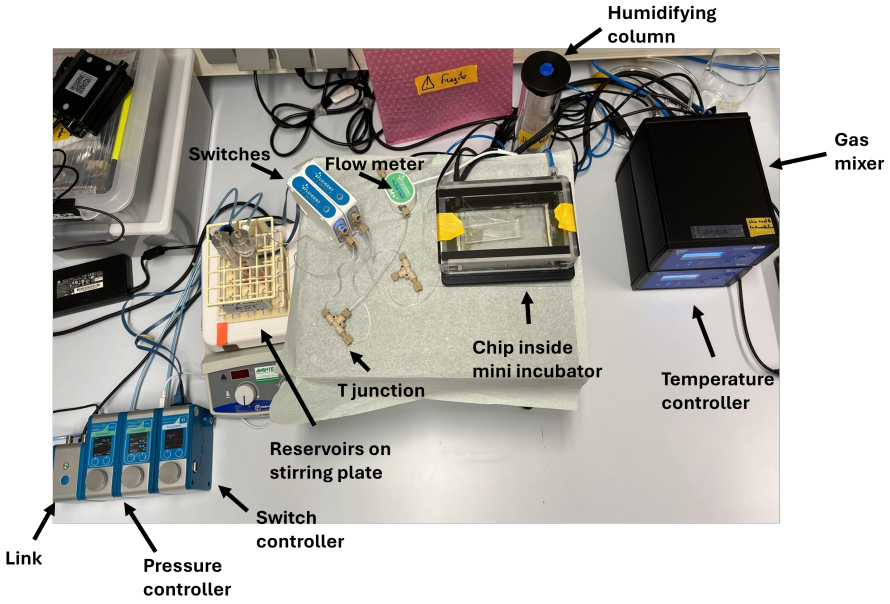

Figure 5.1: Actual microfluidic setup consisting Fluigent recirculation package, magnetic stirrer and mini incubator.

With this setup shear stress values of 1.25, 10 and 20 dyne/cm^2 were created. Implementing these values in the Navier-Stokes equation resulted in a flow rate of 10, 80 and 160 µL/min respectively. All conditions and their variable are shown in table 5.1. The complete calculations are shown in section 3.1

Table 5.1: Overview of all conditions and corresponding variables tested

Cell line	Shear stress ($dyne/cm^2$)	Flow rate (μL/min)	Duration (hours)	Time inside chip (minutes)
THP-1	20	160	4	4
MCF-7	10	80	2	2
MDA-MB-231	10	80	2	2
MDA-MB-231	1.25	10	2	5.1

5.1.2 Magnetic stirring does not affect cell viability

Prior to circulation of the breast cancer cells, the impact of magnetic stirring was tested to ensure that the viability of the cells is not significantly reduced as a consequence of the stirring. To test this, a THP-1 cell suspension with volume of 1.8 mL and a concentration of 0,5 million cells/ml was subjected to magnetic stirring for one hour at 400 rpm at room temperature. As a control, the same cell suspension with the same conditions was used without stirring. Afterwards, presto blue and live/dead assays were performed. The results were normalized to the not stirred control and are presented in bar charts in figures 5.2 and 5.3. The presto blue assay yielded an absorbance of 142 +/- 26 % for the stirred cells and an absorbance of 100 +/- 10 % for the non-stirred cells, when comparing to the not stirred control. The live/dead assay was performed to determine the percentage of dead cells. Both the stirred cell suspension and the non-stirred cell suspension showed a greater percentage of live than dead cells. The percentage of dead cells was 11,9 +/- 5,7% for the stirred cell suspension and 11,3 +/- 7,0% for the non-stirred cell suspension.

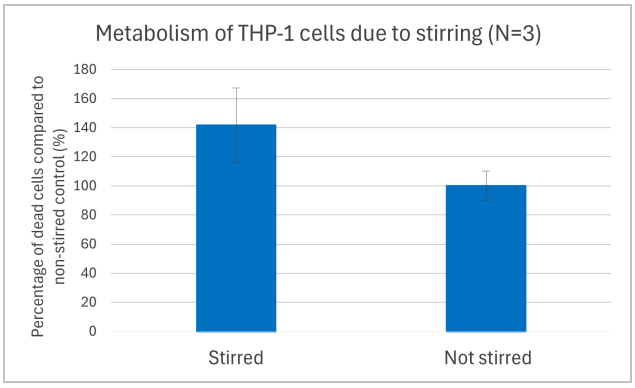

Figure 5.2: Presto blue assay showing an slight increase in metabolism of stirred (400 rpm, 1 hour) THP-1 cells compared to the not stirred control.

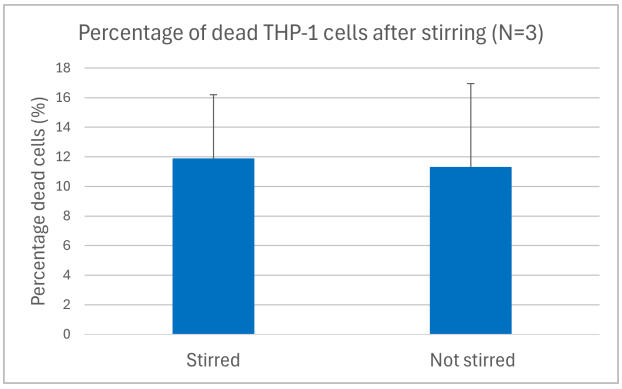

Figure 5.3: Live/dead assay showing a consistent percentage of dead cells after stirring (400 rpm, 1 hour) and for the not stirred control.

5.1.3 A flow of THP-1 and MCF-7 cells is present

The next step was to examine whether a flow of cells was present. THP-1 and MCF-7 cell suspensions ($0.2 * 10^5$ cells/mL) with green cell tracker were circulated for two hours with a shear stress of 20 $dyne/cm^2$. During circulation the microfluidic chip was visualized under a microscope and a flow of cells was observed. The cells seemed to be distributed throughout the entire chip with no cells adhering to the walls of the channel. Following circulation, the microfluidic chip and cell suspension were visualized to determine whether the cells had survived the circulation. In figure 5.4 THP-1 cells inside the chip after circulation are shown. The cells appear to be healthy and have a round morphology. In figure 5.5 a MCF-7 cell suspension after circulation is shown. During this experiment the tubing detached from the microfluidic chip after 1,5 hours, resulting in the introduction of air into the system. Healthy cells with a round morphology are present, but cell debris of dead cells is also observed. Moreover, an attempt was made to visualize the cells inside the tubing. However, this was not possible due to too much autofluorescence of the tubing.

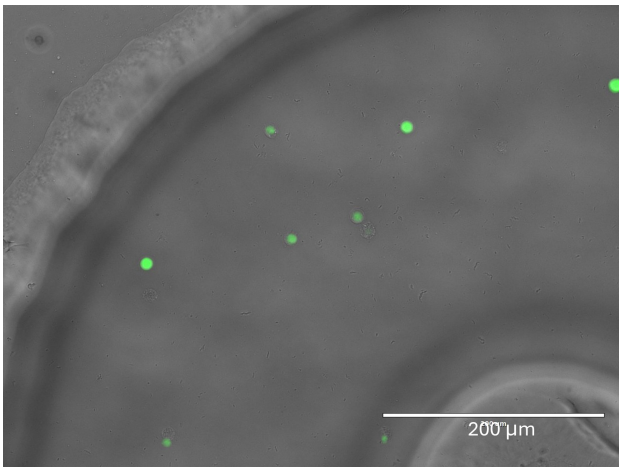

Figure 5.4: THP-1 cells with green cell tracker inside chip after circulation (two hours, 20 $dyne/cm^2$)

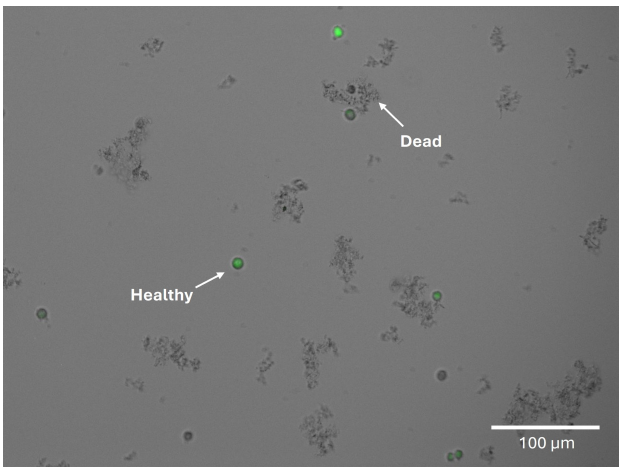

Figure 5.5: MCF-7 cells with green cell tracker after circulation (1,5 hours, 20 $dyne/cm^2$).

5.1.4 Shear flow does not affect THP-1 viability

To validate the system THP-1 monocytes were circulated and their viability was determined. A suspension of THP-1 cells with a concentration of $0.2 * 10^5$ cells/mL was used and circulated for four hours at a shear stress of 20 $dyne/cm^2$. As controls a cell suspension that was only stirred by the magnetic stirrer and a cell suspension that was in the same conditions, but neither circulated nor stirred were used. Following circulation, a presto blue and a live dead assay were performed to investigate the viability of the cells. Figure 5.6 illustrates the results of the presto blue assay. The absorbance of each group was normalized to the not stirred control. This resulted in metabolic activity of 98.4 +/- 16.6 % for the circulated cells, 96.9 +/- 63.6 % for the stirred cells and 100.0 +/- 13.4 % for the not stirred cells. The results of the live/dead assay are presented in figure 5.7. Here it can be observed that the number of live cells present is greater than that of dead cells.

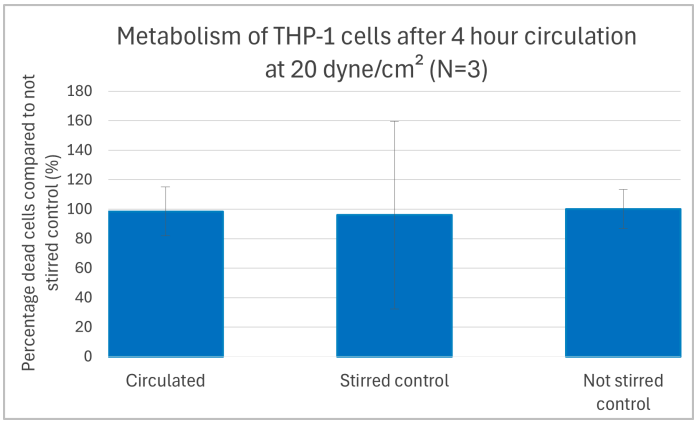

Figure 5.6: Presto blue assay showing that the metabolism of THP-1 cells remained constant. Circulated cells were in circulation for 4 hours with a shear stress of 20 $dyne/cm^2$ The stirred control was on a stirring plate with 400 rpm.

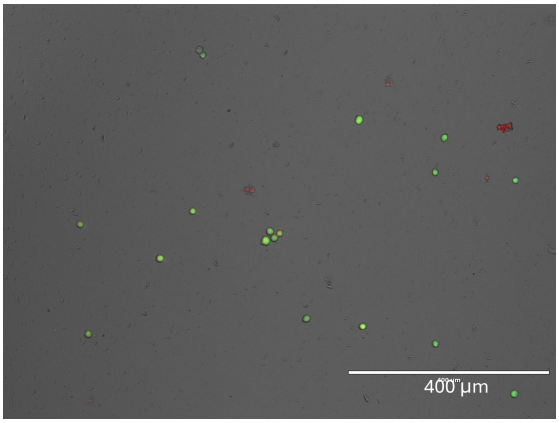

Figure 5.7: Figure showing THP-1 cells after 4 hours of circulation at 20 dyne $dyne/cm^2$ with live/dead staining. Live cells are shown in green and dead cells in red.

5.2 Viability of breast cancer cells decreases due to shear stress

Following the validation of the system, the impact of shear stress on the breast cancer cell lines MCF-7 and MDA-MB-231 was investigated. The breast cancer cell lines were subjected to a shear stress of 10 $dyne/cm^2$ for a period of two hours. As controls two cell suspensions were used: one that was on the magnetic stirrer with 400 rpm and and a second that was neither on the magnetic stirrer nor in circulation. All other variables remained the same. After circulation the cells were examined based on their cell viability with a presto blue and a live dead assay.

5.2.1 Metabolism of breast cancer cells decreases due to shear stress

The results of the presto blue assay are shown in figure 5.8. The metabolic activity, normalized to the not stirred control, of the MCF-7 cells for the circulated, stirred and not stirred cells were 6.5 +/- 1.4 %, 69.1 +/- 7.0 % and 100.0 +/- 51.9 % respectively. The metabolic activity, normalized to the not stirred control, of the MDA-MB-231 cells for the circulated, stirred and not stirred cells were 33.3 +/- 21.3 %, 129.6 +/- 34.5 % and 100.0 +/- 8.2 %. Upon analysis of the circulated condition, both cell lines showed lower values than the controls. Moreover, it can be observed that stirring causes the metabolic activity of the MDA-MB-231 cells to increase, while it has the opposite effect on MCF-7 cells. Lastly, the metabolic activity of the MCF-7 cells decreases to a greater extent than that of the MDA-MB-231 cells due to circulation compared to their not stirred control.

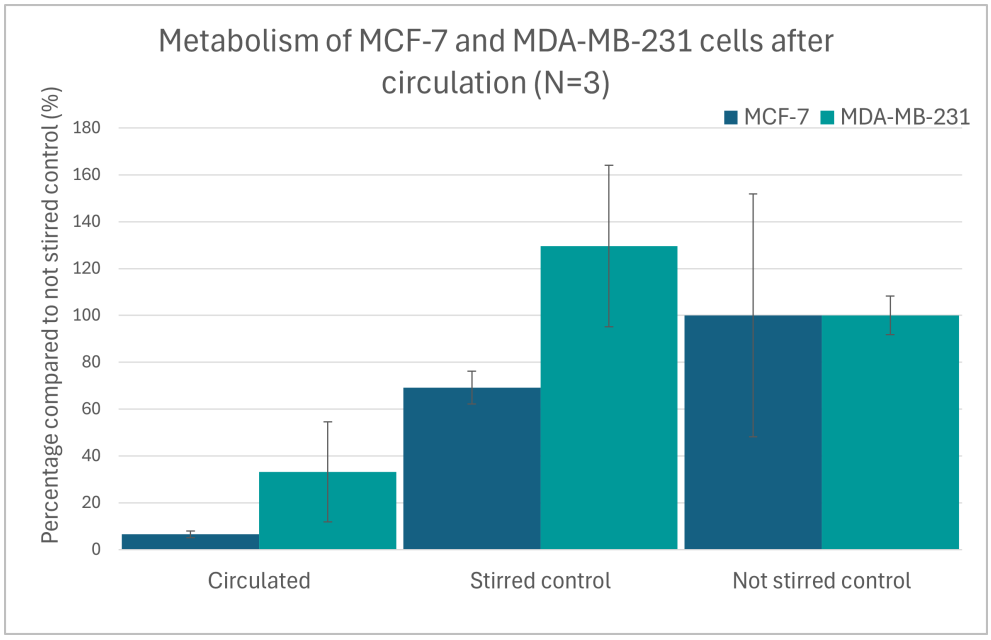

Figure 5.8: Presto blue assay illustrating the decrease in metabolic activity of MCF-7 and MDA-MB-231 cells due to shear stress. Circulated cells were circulated for two hours at 10 $dyne/cm^2$. The stirred control was stirred with 400 rpm.

5.2.2 Percentage of dead cells increases due to shear stress

Figure 5.9 depicts the results of the live dead assay from circulating MCF-7 and MDA-MB-231 cells. The percentage of dead cells for the MCF-7 cell line was found to be 11.7 +/- 3.6 % for the circulated cells, 14.1 +/- 3.5 % for the stirred cells and 5.2 +/- 1.7 % for the not stirred cells. For the MDA-MB-231 cell line the percentage of dead cells was 16.3 +/- 5.7 % for the circulated cells, 4.3 +/- 2.8 % for the stirred cells and 1.7 +/- 1/4 % for the not stirred cells. It can be observed that, for both cell lines, the percentage of dead cells is higher after circulation than in the controls, with the exception of the stirred control of the MCF-7 cell line.

Furthermore, the percentage of dead cells increased more for the MDA-MB-231 cells than for the MCF-7 cells when comparing the not stirred control and the circulated cells.

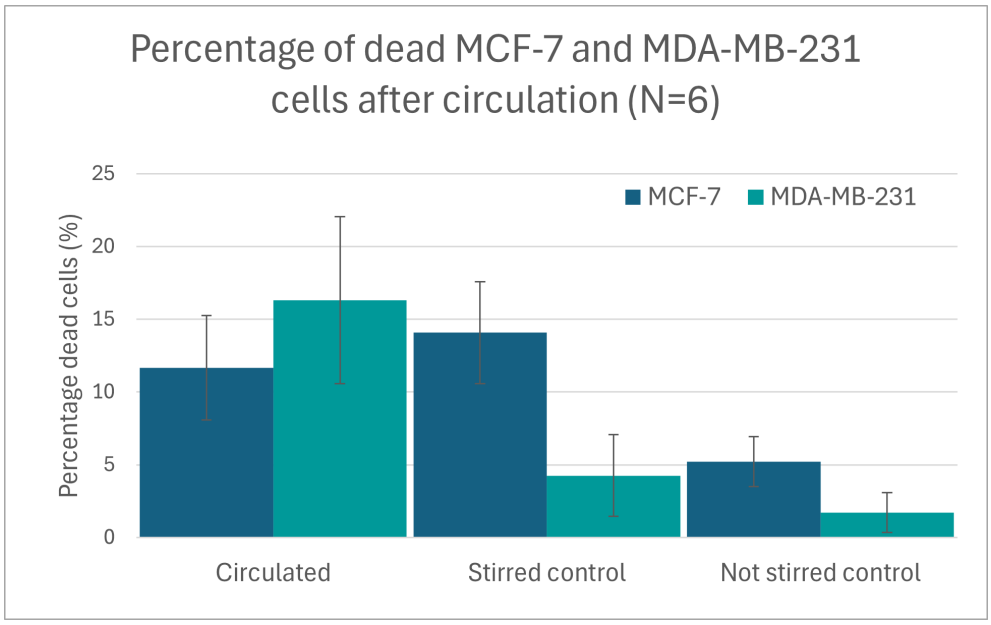

Figure 5.9: Percentage of dead cells indicating viability of MCF-7 and MDA-MB-231 cells. Showing increase in percentage of dead cells due to shear stress. Circulated cells were circulated for two hours with a shear stress of 10 $dyne/cm^2$. Stirred cells were stirred with 400 rpm.

5.3 Shear stress affects gene expression to a more epithelial phenotype, but does not affect protein expression

The circulated breast cancer cells were not only investigated based on their changes in viability, but also on their epithelial to mesenchymal transition. In this study, immunostaining and qPCR were performed to invastigate the impact of shear stress on EMT. E-cadherin, N-cadherin and Vimentin RNA and proteins were used. The same cell suspensions were used with breast cancer cells that were subjected to a shear stress of 10 $dyne/cm^2$ for a period of two hours. As well as the same controls: one cell suspension that was on the magnetic stirrer with a 400 rpm and and a second that was neither on the magnetic stirrer nor in circulation. All other variables remained the same.

5.3.1 Shear stress has no effect on protein expression

Immunostaining was performed to visualize the proteins related to EMT. The proteins vimentin, E-cadherin and N-cadherin were used, given their association to changes in cell adhesion and structure during EMT. Vimentin is present throughout the whole cell while E-cadherin and N-cadherin are predominantly located at the cell membrane, as can be seen in figures 5.10 and 5.11. Furthermore, all three proteins are present in all conditions.

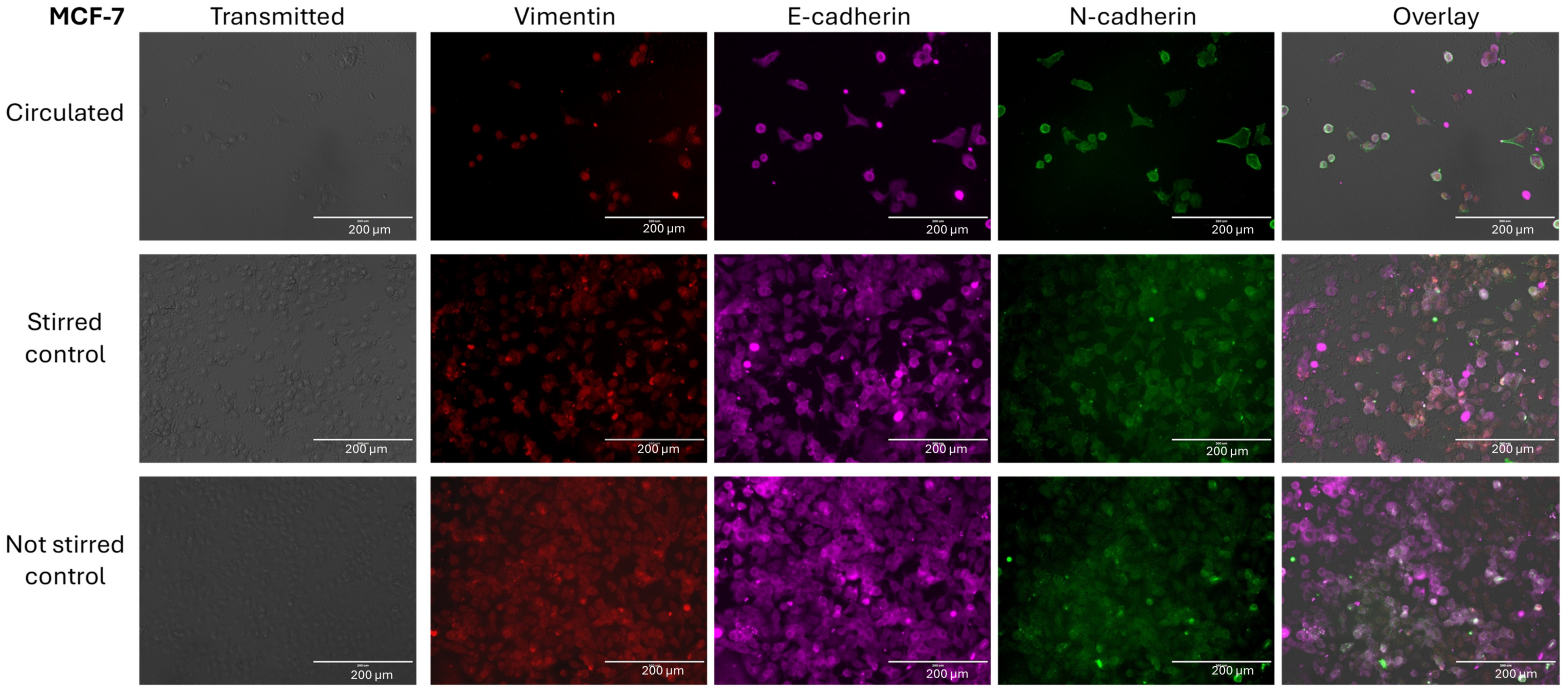

Figure 5.10: Results of immunostaining of MCF-7 cells. Shown are the proteins: Vimentin: E-cadherin and N-cadherin. The circulated cells were circulated for two hours with a shear stress of 10 $dyne/cm^2$. The stirred control was stirred at 400 rpm.

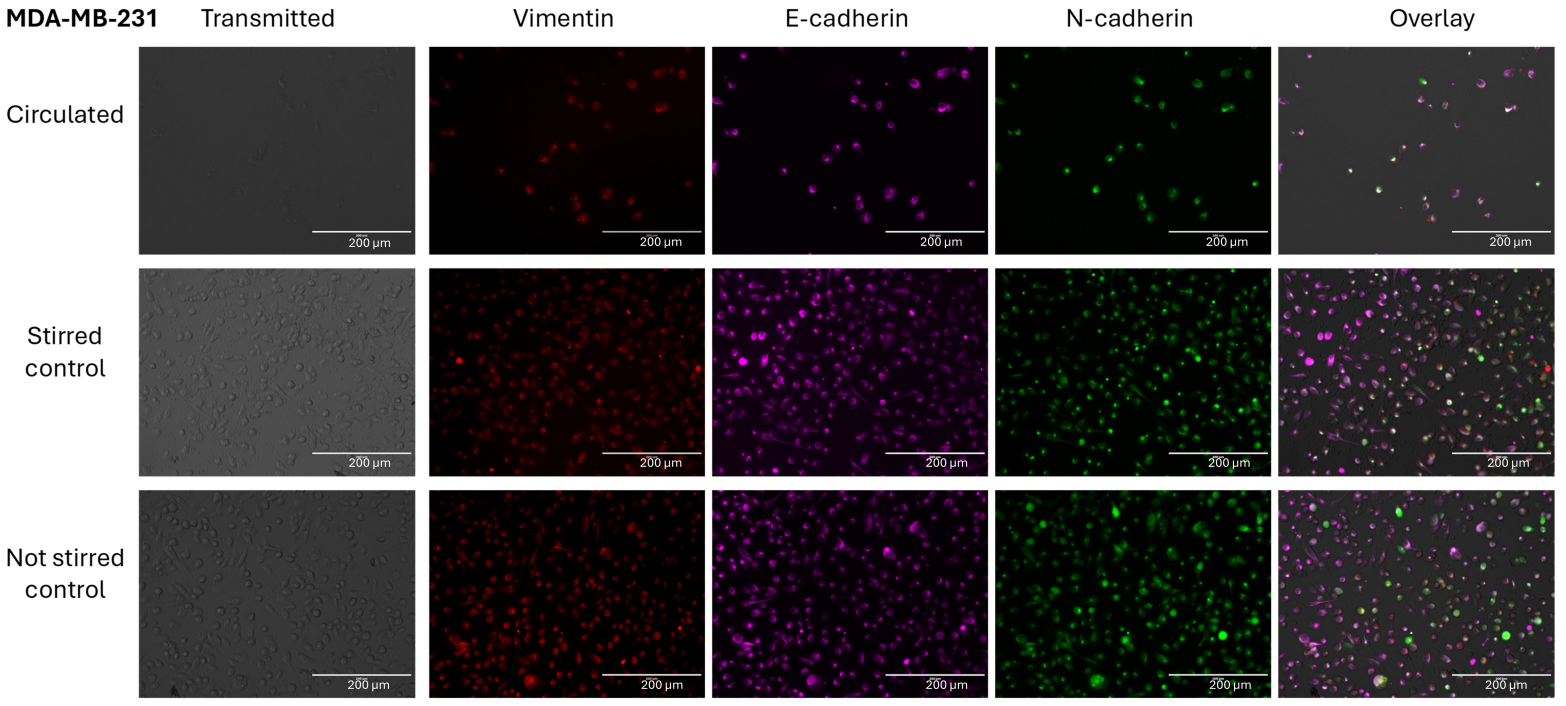

Figure 5.11: Results of immunostaining of MDA-MB-231 cells. Shown are the proteins: vimentin: E-cadherin and N-cadherin. The circulated cells were circulated for two hours with a shear stress of 10 $dyne/cm^2$. The stirred control was stirred at 400 rpm.

5.3.2 Shear stress changes expression of genes to epithelial phenotype

To study the expression of the genes connected to EMT, a qPCR was performed with Vimentin, E-cadherin and N-cadherin. First, the concentration RNA was determined with Nanodrop and the results were used to determine the used volume. The 260/280 ratio was used to determine the purity of the RNA. The values are shown in table 5.2.

Table 5.2: Results of the absorbance measurements with Nanodrop

Cell line	Condition	Concentration RNA (μL/mg)	260/280 ratio
MCF-7	Circulated	11.5	1.38
MCF-7	Stirred control	16.1	1.75
MCF-7	Not stirred control	20.6	2.64
MDA-MB-231	Circulated	15.4	1.53
MDA-MB-231	Stirred control	26.8	2.39
MDA-MB-231	Not stirred control	21.9	2.38

Afterwards, qPCR was continued and the expression of N-cadherin, E-cadherin and Vimentin was determined. N-cadherin was not present in all conditions for both the MCF-7 and the MDA-MB-231 cell lines. E-cadherin was only present in the MCF-7 cells, which is shown in figure 5.12. Although the standard deviation of the stirred control is large, a trend can be found where circulation increases the presence of E-cadherin.

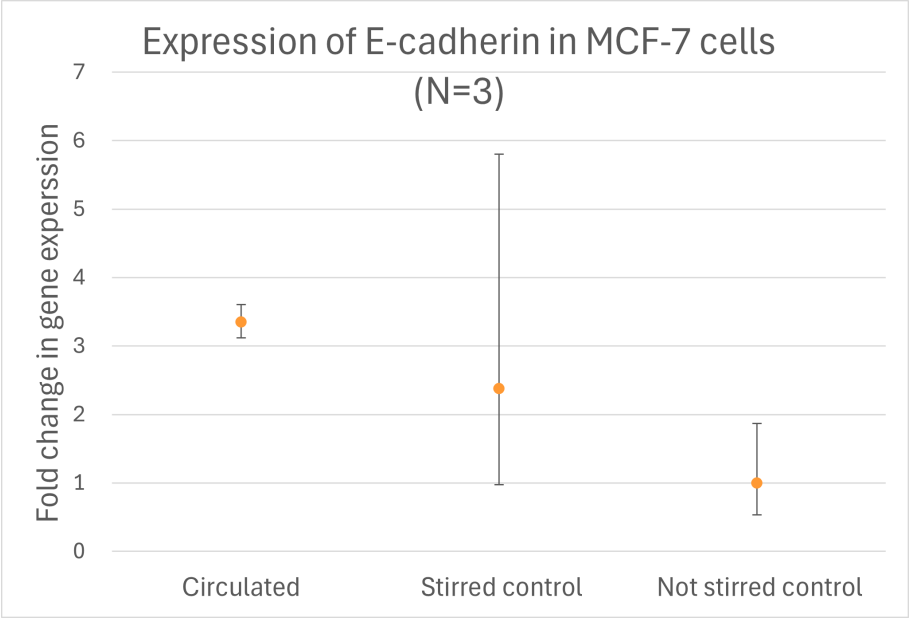

Figure 5.12: Expression of E-cadherin in MCF-7 cells increases in the circulated condition (two hours, 10 $dyne/cm^2$) compared to the stirred (400 rpm) and not stirred controls.

Vimentin was present in both cell lines, as shown in figure 5.13. Here, it was observed that the presence of vimentin decreases as a result of circulation in both cell lines. This decrease is steeper for the MDA-MB-231 cells than for the MCF-7 cells.

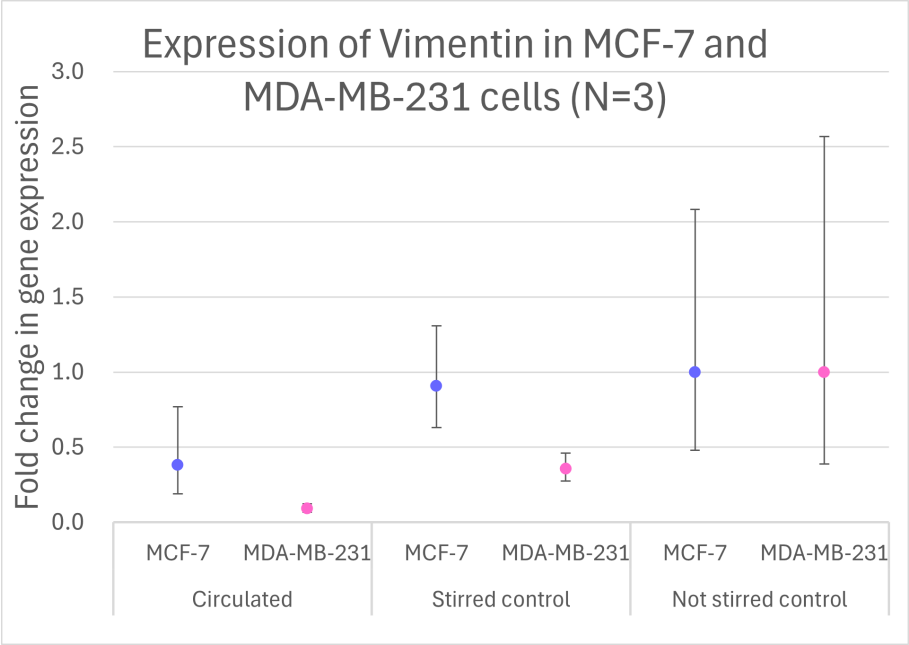

Figure 5.13: Expression of Vimentin in MCF-7 and MDA-MB-231 cells decreases in circulated condition (two hours, 10 $dyne/cm^2$) compared to the stirred (400 rpm) and not stirred controls.

5.4 The amount of cells decreases due to circulation

After the circulation of MCF-7 cells it was observed that there were significantly less cells left after circulation compared to the controls. Because of this cell loss it was decided that the MDA-MB-231 cells would be injected directly inside the tubing and not via the septum. This is done to rule out that cells were sticking inside the septum and therefore never enter the circulation. However, as can be seen in figure 5.14, the amount of cells after circulation is in this case still significantly lower than in the controls.

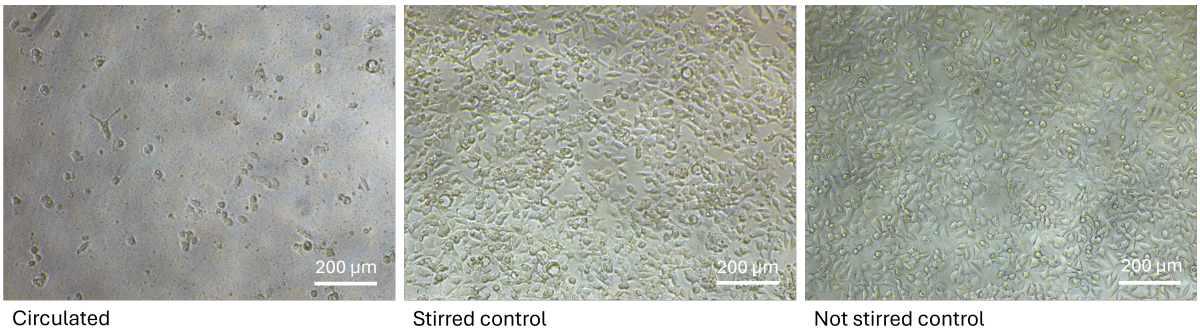

Figure 5.14: The amount of MDA-MB-231 cells seeded after circulation (10 $dyne/cm^2$, 2 hours) is significantly lower than in the stirred (400rpm) and not stirred controls.

5.4.1 Flow rate has no effect on the amount of cells

It was hypothesized that the flow rate was too high and therefore the cells could be destroyed. Another option was that the cell loss was caused by the recirculation. To test this, an experiment was performed at a lower flow rate of 10 μL/min where the cells went only one time through the microfluidic chip. MDA-MB-231 cells were used in concentration of $2 * 10^5$ cells/mL and flown through the chip one time with a shear stress of 1.25 $dyne/cm^2$ (10 μL/min). As controls the same cell suspension that was only stirred and the same cell suspension that was not stirred were used. Afterwards, the cells were counted with a cell counter and visualized with microscopy. The amount of cells counted after circulation were still significantly less: $0.45 * 10^5$ +/- $0.07 * 10^5$ cells/mL. The amount of cells for the stirred control and not stirred control

were respectively: $2.6 * 10^5$ +/- $0.28 * 10^5$ cells/mL and $2.6 * 10^5$ +/- $0.15 * 10^5$ cells/mL. Pictures from the miscroscope confirm that there are less cells present after circulation that in the controls, as shown in figure 5.15.

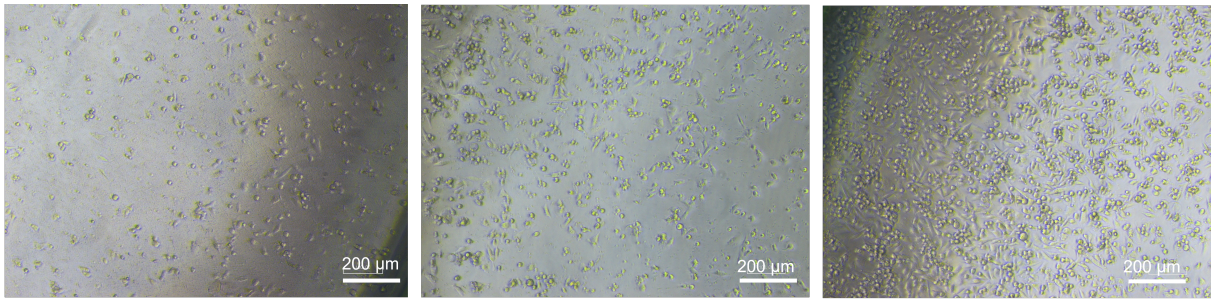

Figure 5.15: The amount of MDA-MB-231 cells seeded after circulation (1.25 $dyne/cm^2$, 2 hours) is significantly lower than the stirred (400 rpm) and not stirred controls.

After circulation the chip was emptied from the fluid and visualized. This showed the presence of attached cells to the channels of the chip, as shown in figure 5.16. Indicating that due to the reduces flow rate, the cells had more time to interact with the channels and therefore could attach.

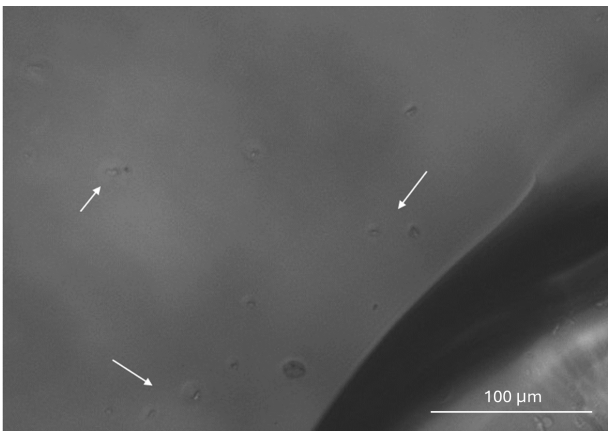

Figure 5.16: Microfluidic chip after circulation of MDA-MB-231 cells. Attatched cells to the channel shown with white arrow.

5.4.2 Cell injection via needle has no effect on the amount of cells

In the first design of the setup a septum was included to inject the cells with a needle inside the system. This was done to prevent the formation of air bubbles when introducing the cells. As described earlier, the septum was not used anymore for the experiment with MDA-MB-231 cells to test if this caused the observed loss of cells. Since that was not the case it was hypothesized that the cells might stay behind in the needle itself. Therefore, a test was done to determine if this was the case. $6 * 10^5$ MDA-MB-231 cells in 150 μL were injected with a neelde to 2.85 mL cell culture medium and counted with a cell counter and visualized under the microscope. As control a 3 mL cell suspension of $2 * 10^5$ cells/mL was used. This resulted in $2.8 * 10^5 +/- 0.5 * 10^5$ cells/mL after circulation and $2.3 * 10^5 +/- 0.1 * 10^5$ cell/mL in the control. Showing that injection via needle does not result in a loss of cells.

Chapter 6

Discussion

6.1 Setup

A crucial aspect in understanding the metastatic process is the change in characteristics of the cancer cells. However, modelling cancer metastasis remains a widely known challenge. Most studies use peristaltic or syringe pumps to flow cell culture medium over cancer cells that are adhered to a surface. [2] Given that in the human body CTCs flow in the bloodstream, this study uses a cell suspension which is flown through a microfluidic chip by pressure pumps. Although this approach represents the physiological conditions in the human body more accurately, it is not yet a complete replication. For instance, the flow of cell suspension is continuous, but each individual cell experiences shear stress multiple times for a short period. The majority of the time each cell resides in the reservoirs. In addition, the system does not account for the viscoelastic properties of blood, turbulent flows in the heart and major arteries and adhesive reactions with vessel walls and other cells. [27] Moreover, the dimensions of the chip used in this study are far larger than micro channels due to the limitations of the 3D printer. Therefore this is not an accurate representation of the capillaries and small arteries. This also leads to much higher flow rates to have sufficient shear stress.

Furthermore, breast cancer cells are affected by only two minutes of shear stress in this research, which may not be sufficient to observe the effects. The half-life of CTCs is not yet know, but studies estimate that it lies between 30 minutes and 2 hours. Nevertheless, the majority of cells get trapped in a vascular bed within minutes. [14, 27] To be able to circulate the cells longer, multiple microfluidic chips could be placed in series to increase the time that each cell experiences shear stress. Moreover, the setup could be placed in an incubator at 37 °C and 5% CO_2 to prevent cells from dying. Another option is to cool the reservoirs in the fridge and heat up the cell suspension in the tubing before it reaches the microfluidic chip. The last two options increase the viability of the cells, therefore making it possible to do experiments with longer circulation times.

Moreover, a loss of cells due to circulation was observed in this research. Multiple possibilities were investigated, e.g. cells sticking inside the chip, septum and needle. None of these components caused the loss of cells. Therefore, it was believed that some of the cells get destroyed due to the shear stress and are thus not visible anymore. However, it is not known what happens to the cells inside the tubing, since it is not possible to look inside. It is very unlikely that the cells get stuck here, since the flow rate is high. However, it would be of added value to confirm this and observe the behaviour of the cells here, e.g. whether clump formation occurs. On the other hand, the loss of cells is not necessarily bad. In patients with metastatic cancer, only 5 CTCs per 7.5mL blood are found. [12] Meaning that in the normal physiological conditions a lot of CTCs do not survive circulation either. The key point of the setup is that it accurately mimics the *in vivo* situation and that there should be sufficient cells remaining for characterisation.

6.2 Presto blue and live/dead assay: cell viability

While previous studies have investigated the effect of shear stress on CTCs, little is known about the cell viability of CTCs affected by shear stress [12]. In this study, a loss of cells was observed indicating that the cells are destroyed due to circulation. Therefore the viability decreased. In the presto blue assay (figure 5.8) a low metabolism was observed for both the MCF-7 and MDA-MB-231 cells. To be able to directly compare the circulated cells to the controls, one would need to correct for the number of cells. This figure does however give an indication of the loss of viable cells. Moreover, the more invasive MDA-MB-231 cell line shows a higher metabolism than the less invasive MCF-7 cell line compared to their not stirred control. Thus, the cell viability of MDA-MB-231 cells is higher than the MCF-7 cells. Both cell lines experience a decrease in cell viability as result of the shear stresses. On the other hand, the live dead assay (figure 5.9) shows a steeper increase in the percentage of dead cells for the MDA-MB-231 cells than the MCF-7 cells. This indicates a lower cell viability for the invasive MDA-MB-231 cells than the non-invasive MCF-7 cells. However, since the metabolism of MDA-MB-231 cells is higher than MCF-7 cells it is possible that there are more MDA-MB-231 cells left and of these cells the percentage of dead cells is higher. Thus, meaning that there are more live MDA-MB-231 cells left.

Previous studies using peristaltic pumps and syringe pumps have shown diverse viabilities. Often in these studies cells were adhered to a surface while a flow was exerted on them. In a research from Connolly et al. cell suspensions of MCF-7 and MDA-MB-231 cells were circulated by a syringe pump and analyzed based on their cell viability. This showed that the shear stress did not have a significant effect on the MDA-MB-231 cells and only MCF-7 to a very slight degree. [28] In another study from Remgi et al. MCF-7 and MDA-MB-231 cells were circulated with a peristaltic pump at different shear stresses. This resulted in a viability of 86% after 4 hours circulation at 15 $dyne/cm^2$ for MDA-MB-231 cells and a viability of 25% for the less invasive UACC-983 breast cancer cell line. Showing that a shear stress of 15 $dyne/cm^2$ can induce apoptosis in nonmetastatic breast cancer, but not in metastatic breast cancer. [29] Both studies confirm that the invasiveness of tumor cells increases their viability in circulatory conditions. In further research with the setup presto blue and live/dead assays could be done on the cell suspension directly after circulation, instead of first adhering them to a wells plate overnight. In this way cells that might not be able to attach anymore are also considered in the measurements. Furthermore, the cells don't have time to recover from the circulation, thereby increasing their metabolism. In this way, all circulated cells can be analysed directly after circulation, giving a more representative result.

6.3 qPCR and immunostaining: EMT

Because epithelial to mesenchymal transitions are an import step in the first stages of metastasis it was hypothesized that shear stress would induce EMT in breast cancer cell lines. Expected would be an increase in mesenchymal gene expression and a decrease in epithelial gene expression in the MCF-7 and MDA-MB-231 cells. [5, 30] However, in this research, the opposite was found from qPCR (figures 5.12 and 5.13). An explanation for this could be that the circulated cells were seeded and incubated before qPCR was performed. Therefore only the adhered cells were analyzed. If there were breast cancer cells that were too mesenchymal after circulating they would not adhere to cell culture plate and thus their RNA would not be detected. Furthermore, it should be noted that EMT is a reversible process. The reverse process is called mesenchymal to epithelial transitions (MET) and is characterized by an increase in the epithelial phenotype. In breast cancer metastasis, MET is used by the cancer cells for colonization in the final stage of the metastatic cascade. [31] This raises the possibility that the cells in this research underwent MET when they were attached and incubated after circulation. This could therefore explain the increased epithelial and decreased mesenchymal phenotype observed in this research. To prevent this from occuring it would be recommended to perform immunostaining and qPCR on the cells while they are still in suspension. Thereby, all RNA is included and the cells have no time to undergo MET. Thus, only the effect of the shear stress in circulation is considered.

Something else that should be considered is that in the immunostaining (figures 5.10 and 5.11) the proteins N-cadherin, E-cadherin and Vimentin are expressed in both cell lines in all conditions. This does not correlate to the data from qPCR, where RNA of N-cadherin is not present at all and RNA of E-cadherin only in the MCF-7 cells. It is most likely that qPCR the primers for N-cadherin were invalid.

Previous studies have found that complete EMT is not necessary to form a secondary tumor. For example, downregulation of E-cadherin and upregulation of N-cadherin and Vimentin is often correlated with invasive carcinomas. Nevertheless, multiple studies show cases where the presence of E-cadherin does not relate directly to invasiveness and metastasis. [30] For example in a study from Lipponen et al. 208 biopsies from breast cancer carcinomas were taken. From these biopsies 72% showed E-cadherin positivity in more than 50% of the cells. This resulted in a conclusion that presence of E-cadherin does not directly correlate to the metastatic potential. [32]

Taking this all together, it is recommended to adjust the setup in a way that it is suitable to put inside an incubator or the reservoirs inside a refrigerator and heat up the cells before they flow into the chip. Both options make it possible to increase the circulation times. Another option is to put microfluidic chips in series, thereby increasing the time that each cell is affected by shear stress. The main adjustment in analysis would be to perform each assay directly on the cell suspensions following circulation, rather then first adhering the cells to a cell culture plate. Combining these adjustments would result in a more comprehensive and representative overview of the effects of shear stress on CTCs.

Chapter 7

Conclusion

This study demonstrated the development of a microfluidic recirculation setup to mimic shear stresses in the blood flow. The setup enabled a stable, controlled, continuous and unidirectional flow through a microfluidic chip, which was used to analyse the effect of shear stress on CTCs in breast cancer metastasis. Following validation with THP-1 monocytes, it was concluded that shear flow did not affect THP-1 viability. The non-invasive MCF-7 and invasive MDA-MB-231 breast cancer cell lines were circulated for a period of two hours, thereby subjecting them to a shear stress of 10 $dyne/cm^2$ for two minutes. The presto blue and live/dead assays indicated a decrease in viability due to shear stress. Immunostaining showed that expression of proteins related to EMT remained constant, whereas qPCR indicated that expression of genes related to EMT changed to a more epithelial phenotype. These results demonstrate the possibility to use the developed microfluidic setup in CTC analyzation. Overall, the microfluidic recirculation setup offers a valuable tool for investigating the responses of CTCs to shear stress, contributing to a better understanding of breast cancer metastasis. The setup provides a suitable basis for further research into the development of a breast cancer metastasis on chip model.

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Appendix A

oxyGEN protocol

oxyGEN software version 2.3.2.0

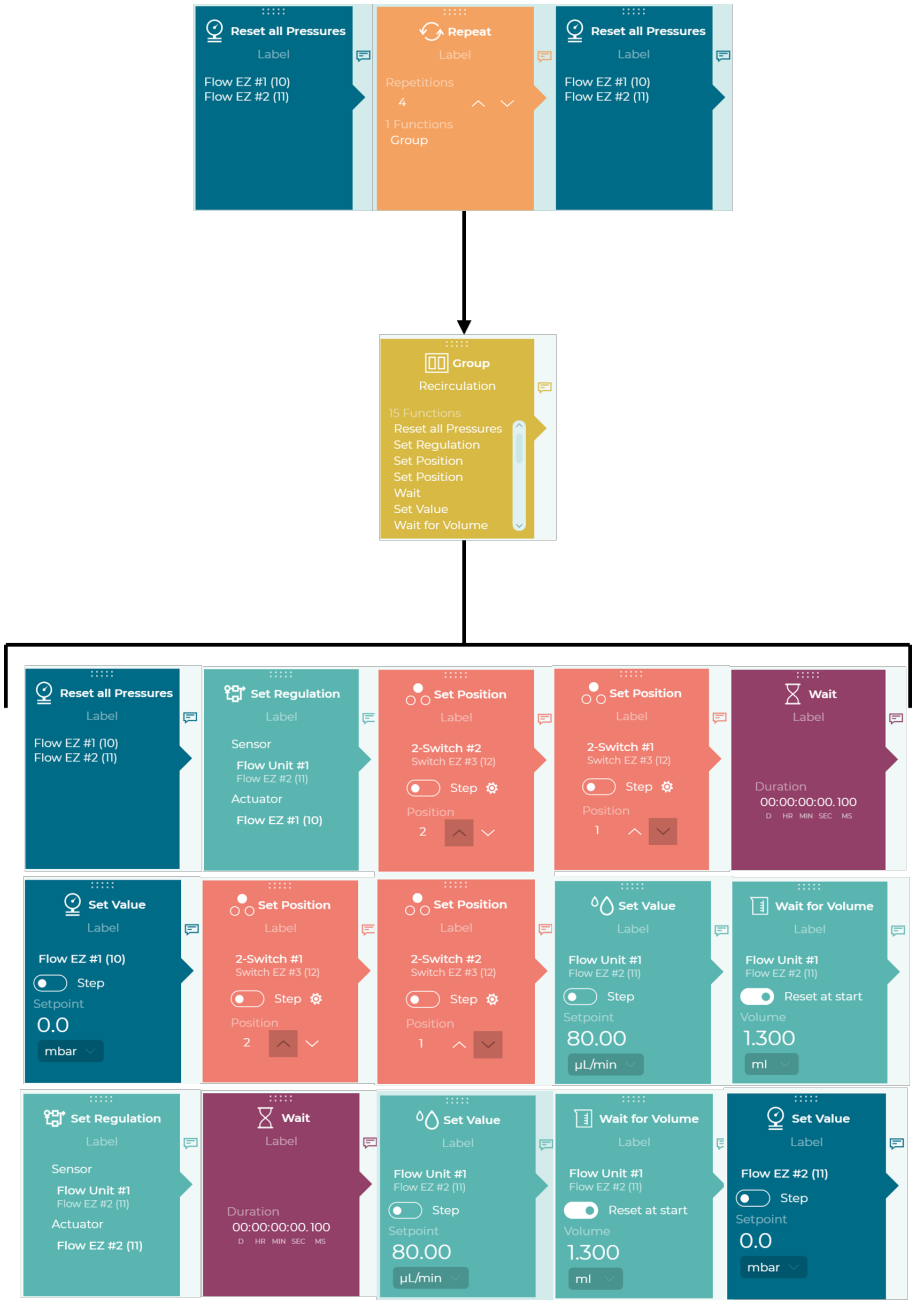

Figure A.1: Protocol for recirculation in oxyGEN software