Kijken Denken Doen (Look Think Do)

Lecture presented at the occasion of the appointment as professor for Medical Cell Biophysics at the Faculty of Science and Technology of the University of Twente on the 27th of September 2007 by Prof. Leon WMM Terstappen, MD, PhD

Cover Illustration Art or Science, DNA leaking from a white blood cell. Experiment by PhD student Sjoerd Ligthart.
Prelude

Mijnheer de Rector Magnificus,
Ladies and Gentlemen

Kijken Denken Doen (KDD) / Look Think Do, a phrase Guus Terstappen my dad, a surgeon, introduced to highlight a sequence that when followed diligently provides the key ingredients to become successful in practicing medicine, Figure 1. Before I ever opened a book on medicine it also appeared to be a fruitful strategy to tackle normal life problems like “water coming from the ceiling”, “no lights in the kitchen” “smoke coming out of the engine”. Within my extended family such problems are now tackled with the KDD principle and even those that I considered to be in possession of two left hands are now able to solve simple problems.

Figure 1 Watercolor by Guus Terstappen showing an artistic impression of the tools necessary to Look, Think & Do (Kijken, Denken Doen). The Eye to Look, the Brain to Think and the Hand to Do.
While finishing up my medical degree I was witnessing a brutal procedure in which a radiologist was poking a needle in a women’s foot for more than an hour in an attempt to find a lymph vessel and inject contrast media to visualize the lymph vessels draining the legs, Figure 2 Panel A. Within the differential diagnosis was hereditary lymphoedema in which case there would be nothing to poke in. I reasoned that when I would inject a substance subcutaneously that was too large to be taken up by the capillaries it would end up in the lymph vessels. Sugars seemed harmless to me so I choose a dextran with sufficient molecular weight coupled it to the radioactive metal Technetium-99m, injected it into my feet and made a “lymphscintigraph”. Figure 2 Panel B shows my lymph vessels. The patient was shown to have a hereditary form of lymphedema and I was destined to go into science. The KDD principles I have used ever since and the most likely reason I am standing here today. My scientific interests have lead me in many directions with one common trust, identification of tools used in fundamental- and applied science that can be put to use to solve issues faced in the diagnosis and treatment of medical conditions. Today’s medicine is flooded with technology and knowledge of underlying technological principles is becoming increasingly more important to adequately practice medicine. One of my aims is to make a contribution in the fight against cancer and believe that collaboration between the different scientific disciplines can significantly increase our chances to contain this disease.

Figure 2, Panel A, Feet from a patient after lymphangiography,
Panel B, lymphscintigraphy showing the lymphvessels of Leon Terstappen after he injected his feet with TC-99m labeled dextran.
Introduction

Cancer is the second largest cause of death and it is becoming an increasingly larger problem with the aging of the population. Review of the age adjusted death rates in the USA between 1960 and 2004 shows striking decreases in death by heart disease, cerebrovascular disease and accidents but only a slight improvement in death caused by cancer, Figure 3.

This leads one to believe that more emphasis has been placed on tackling cardiovascular disease or cancer is simply a much more difficult problem to deal with. One of the impedances in tackling cancer is our lack of understanding of the disease and the slow pace at which scientific findings are being translated into new diagnostic tools and therapies.

Many questions arise when reviewing case histories of cancer patients. Figure 4 shows a case history of a 38 year old woman diagnosed with breast cancer. A routine mammogram showed a suspicious mass, and a needle
biopsy showed the presence of cells the pathologist deemed malignant. The surgically removed tumor was less than one cm in diameter. The margins of the tumor were clear of tumor and no tumor cells were detected in the lymph nodes draining the region. The tumor grade was low and expressed the hormone receptors estrogen and progesterone. There was no evidence of other disease locations. No further treatment was given and the patient had no problems until four years after surgery when she developed local pain on her upper body. X-rays showed a suspicious mass in a rib which was pathologically confirmed as a breast cancer metastasis. Further investigation showed some hot spots on the bone scans, but no visceral disease. The patient received hormonal therapy and symptoms relieved, 5 months later symptoms reappeared and a second line of hormonal therapy was started. Disease evaluation after 3 month of therapy revealed progression of disease with new lesions in liver and lung. The patient received 3 lines of chemotherapy over a period of 9 months, was transferred to a hospice and died one month later.

Observations:
1. The radiological techniques evaluate the presence or absence of a mass: shouldn’t future technology detect the presence or absence of malignant cells.
2. The pathological evaluation is restricted to morphological assessment of portions of the tumor and lymph nodes: shouldn’t future technology assess the make-up of each individual cell in the tumor and reveal its molecular make-up.
3. At diagnosis no disease was detected beyond the primary tumor, still tumor cells must have been present for 4 years: shouldn’t future technology detect these dormant cells and understand what keeps them asleep and what awakes them.
4. The choice not to administer adjuvant therapy was based on the low anticipated risk for disease recurrence of patients with this disease profile: shouldn’t future technology assess the dissemination of disease and specify what systemic therapies will have a high likelihood of success for the individual patient.

Although the need for more advanced technology in the fight against cancer is desperately needed, we should not forget that the psychological well being of the patient may well be of greater importance than the physical status. Earlier this year the neurologist Prof Jan van Gijn stated during his farewell speech “the psyche for now is an elusive brain activity that ultimately
resides within the function of cells, their molecules and their connections". I believe the day will come that we uncover the secrets contained within the cells and can explain the psychological status just like we measure the status of other vital organs. Until that time physicians will have to take the time to carefully listen to their patients before starting to Look, Think and Do. The emphasis put forward by the University of Twente to support the creation of a Chair in Medical Cell Biophysics opens the path towards this challenging aim.

In the remaining of this lecture I will lay out the KDD strategy I have taken during my career to address some of the questions and provide you with a flavor of the steps needed to build the blocks required to make cancer a chronic disease.

Before I started my PhD work, a team consisting of the biophysicists Bart de Grooth and Jan Greve and the medical doctors Chris ten Napel, Wim van Berkel and myself gathered to discuss malignant blood diseases and came to the conclusion that only after we improved the understanding of normal blood cell formation or hematopoiesis we could start to unlock the secrets behind malignant blood cell formation. The results of these discussions were
written into a grant proposal and submitted to the Dutch Cancer Foundation. The reviewers of the grant did not agree with what we believed was a rational approach to the issue at hand and graded it as a whole lot of baloney. Jan Greve, however, believed that we had had a good Look at the problem, had Thought carefully and logically about the problem and decided to use funds from the University of Twente to let us Do the project. So of we go.

**Normal Hematopoietic Cell Differentiation**

By traditional light microscopic examination of normal blood smears erythrocytes, platelets, neutrophilic granulocytes, lymphocytes, monocytes, eosinophilic granulocytes and basophilic granulocytes can be identified. The large differences in frequency of these cell types make accurate and reliable quantification difficult, Figure 5. Many diseases are accompanied by changes in the composition of these cell types and some diseases can be recognized based on a specific change of the peripheral blood cell composition. Flowcytometry a technology that permits characterization of individual cells by passing them individually at high speed through a laser beam has accelerated the accuracy and reliability of counting different cell types and provided us with the opportunity to uncover new information.

![Figure 5](image)

**Figure 5** Frequency of different cell types in peripheral blood

Our contribution to this field was to measure differences in the light scattered by the cells while passing through a laser beam. By simultaneous
measurement of light scattered at three different angles and differences in the polarization of the light we could identify the different peripheral blood cell types without the need of cell staining. Figure 6 illustrates the position of the different cell clusters in the four parameter light scatter measurement 1,2. To prove that the cell clusters indeed were composed of the different cell types a cell sorter was built that permitted conformation of the identity of the cells in the clusters. This technology has been successfully commercialized and I believe in today's Dutch language the trendy name for this process is “valorized” 3.

Figure 6  Four-parameter light scatter measurement of peripheral blood leukocytes. Panel A scatter plot of light scattered at an angle between 0°-2° versus light scattered at an angle between 3°-11°. Gate 1 contains lymphocytes, Gate 2 is enriched for basophils, Gate 3 contains monocytes and gate 4 the neutrophilic and eosinophilic granulocytes. Panel B scatter plot of orthogonal light scatter (65°-115°) and depolarized orthogonal light scatter (65°-115° & polaroid filter absorbing the scattered light in the direction of the sample stream). Gate 5 contains the neutrophils and Gate 6 the eosinophils.

In many diseases the frequency and distribution of the major blood cells is altered, and often immature blood cells mobilized from the bone marrow appear. The bone marrow is the source of all blood cells and it maintains the peripheral blood cell levels at equilibrium. An amazing engine considering the lifespan of blood cells (red blood cell ~120 days, granulocyte ~24 hours) and the speed at which it responds to blood losses and infections. The continuous production of blood cells is made possible by a source of cells referred to as hematopoietic stem cells. These stem cells have the capability to self renew as well as to differentiate into all the different types of blood cells. By examination of morphological appearances of the different cells
found in bone marrow, hypotheses were generated that defined differentiation and maturation pathways that cells had to undergo, before entering the peripheral blood. An example of a cell differentiation schema is shown in Figure 7.

![Figure 7 Hematopoietic cell differentiation, proliferation and maturation. Mature cells are present in blood, tissue and lymphoid tissue (node). T-lymphocyte and Natural Killer (NK) cell development reside in the thymus and development of other hematopoietic cells occurs in the bone marrow. The early hematopoietic progenitors do circulate in blood at low frequency and can also be found in the liver.

With the introduction of monoclonal antibody technology and fluorescence activated flowcytometry it became possible to prove or disprove these schemas. The monoclonal antibody HPCA-1 (CD34) made by the group of Curt Civin was found to be expressed on approximately 1% of bone marrow cells. Colony formation was restricted to cells expressing the CD34 antigen suggesting that the CD34+ cells contained all hematopoietic progenitor cells. Evaluation of expression of different antibodies on cells expressing the CD34 antigens yielded a typical co-expression pattern with the CD38
antibody. The flowcytometric data display of the expression of the CD34 and CD38 was suggestive for a differentiation pathway as illustrated in Figure 8. Cells expressing the highest level of CD34 lacked the CD38 antigen and represented only a small portion of the CD34+ cells. The location of these cells in the forward and orthogonal light scatter plot suggested a homogenous morphological appearance, generating the hypothesis that this cell population contained hematopoietic stem cells. Cell differentiation was thus accompanied by a gradual loss of the CD34 antigen and the acquisition of the CD38 antigen.

Morphological examination of cells sorted along this differentiation pathway supported the hypothesis that the cells that lacked the CD38 antigen were a homogenous population of cells with primitive features. While cells with increasing CD38 antigen expression were heterogeneous, and showed features believed to be associated with cell differentiation, Figure 9. To prove that these CD34+, CD38- cells were indeed hematopoietic stem cells capable of forming all the different types of blood cells, cells present along the differentiation pathways were sorted individually in wells which contained media that would support their growth. Only cells within the CD34+, CD38- cell population were capable to differentiate into all blood cell types. Thus proving the original hypothesis, Figure 9.
Cells in the PI => PIV gates shown in Figure 8 were sorted. Cytospins were made of the 4 populations and stained with Wright Giemsa. The slides were examined on a microscope with an 100X objective and typical examples of the cell morphology of cells in PI, PII, PIII and PIV are shown in Panels A, B, C and D respectively. While the morphology of cells in PI and II was homogenous with features consistent with primitive blast, cells from PIII and PIV showed a more heterogeneous morphology with clear differentiation features. Individual cells of the PI => PIV populations were sorted into wells to which appropriate growth media was added. Only cells in PI had the ability to generate cells from all hematopoietic lineages. Panel E shows a well after 10 days of culture showing a multiplication of the cells. Replating of these cells ultimately lead to differentiation of the cells into the various hematopoietic cell lineages. Wright Giemsa staining of the cultured cells is shown in Panel ---in which various cell types can be identified such as a macrophage (ma) a megakaryocyte (me), cells of the erythroid lineage (e), cells of the neutrophilic granulocyte lineages (n) and undifferentiated blast (b).
This work formed the basis for the mapping of the normal differentiation pathways of each of the hematopoietic cell lineages Figure 7 6-27. With the extensive knowledge of the normal differentiation pathways we could start comparing normal hematopoietic cell differentiation with that found in hematopoietic cancers.

**Cancerous versus Normal Hematopoietic Cell Differentiation**

Hematopoietic malignancies are traditionally identified by a predominance of cells with similar morphological appearance in bone marrow or lymphnodes, and subdivided into cells resembling cells of myeloid or lymphoid origin. Questions arise at which stage of normal cell differentiation cells derail and turn malignant. To investigate whether cell differentiation patterns can be identified in patients with hematopoietic malignancies the expression of CD34 and CD38 was determined in bone marrows that were predominantly occupied by the leukemia. Figure 10 shows the relation between CD34 and CD38 expression in a bone marrow from a normal donor and 5 patients with acute myeloid leukemia 28. The remarkable resemblance in differentiation pathways detected in normal bone marrow and these leukemias suggests the presence of leukemic stem cells with similar phenotypic features as those from normal hematopoietic stem cells. This also indicates that although the leukemia may have features from the more mature lymphoid or myeloid lineages the actual event that made them turn malignant is occuring at the stem cell level.

The distribution of the leukemic cells along the differentiation pathways has implications for the prognosis. When patients were divided into those with a predominance of cells in the early differentiation stages, in the intermediate stages and in later stages of differentiation, a clear difference in progression free survival was observed, Figure 11 29. Patients with a predominance of cells in the early stages of differentiation having the worst prognosis.
Figure 10  Expression of CD34 and CD38 on bone marrow cells from a healthy adult, Panel A, and 5 patients diagnosed with acute myeloid leukemia (AML) Panels B=>F. In Panel A the arrow starting blue and ending in red for the lymphoid and green for the myeloid cells indicate the pathways along which the hematopoietic cells differentiate. A remarkable resemblance can be seen between the normal and malignant differentiation pathways. The relative frequencies of the cells along the differentiation pathways are different from normal cell differentiation and quite different between AML patients.
Figure 11 Kaplan Meier analysis for progression free survival of 51 AML patients. The 22 patients with primitive cells had progression free survival of 6 months. The 26 patients with intermediate differentiated cells had progression free survival of 8 months and the 19 patients with a predominance of mature cells had progression free survival of 18 months.

Further characterization of these “leukemic” stem cells and their distinction from “normal” hematopoietic stem cells can provide valuable insights that can lead to the development of targeted therapies. Aberrant expression of antigens is well documented in hematopoietic malignancies \(^{30-33}\) opening the door to the development of targeted therapies. First successes of targeted therapies have already been made. For example, the aberrant expression of the bcr-abl protein produced by the Ph chromosome in chronic myeloid leukemia has for example lead to the development of imatinib mesylate. This drug blocks the signal of the bcr-abl protein and drastically changed the course of this disease. The aberrant expression of antigens can also be used to detect residual disease. The ability to detect residual disease after treatment allows for the determination of its effectiveness and provides a tool to monitor the detection of early recurrence of the disease. Simultaneous assessment of multiple parameters on both bone marrow and peripheral blood cells have been used to demonstrate the presence of residual leukemic cells after treatment and their presence was associated with poor outcome \(^{34-35}\). The question arises whether an earlier start of
therapy, can prolong survival. This may not be the case for diseases for which no or few effective therapies are available. New targeted therapies are however emerging and may be effective upon detection of residual disease. This will have a greater impact when specific disease mutations can be detected in the leukemic cells and targeted therapies for these mutations become available.

**Non-Hematopoietic malignancies**

The difficulty in the detection of hematopoietic malignancies in blood and bone marrow is the discrimination from normal hematopoietic cells, which is increasingly more difficult when the frequencies are low and the aberrancies from normal hematopoietic cells are relatively minor. The advantage of hematopoietic diseases is that the source is easily accessible i.e. a blood or bone marrow aspirate can be obtained easier and more frequently than a biopsy from a suspected lesion in a solid organ. An important difference between hematopoietic and non-hematopoietic malignancies is that hematopoietic malignancies remain in the hematopoietic organs while non-hematopoietic malignancies are mostly characterized by invasion into other organs. As a consequence cause of death in non-hematopoietic malignancies is in most instances not caused by the loss of function from the site the tumor arises, but through dissemination of the disease to other organs causing their destruction. For example, in colon cancer the organ mostly affected is the liver, in prostate cancer the bones and in breast cancer, bone, liver and lung. A cancer cell or an expanded group of cancer cells are relatively harmless, but become dangerous when the cells break away from the primary site and find homes elsewhere in the body. The cascade of events involved in the metastatic process is shown in Figure 12. The figure depicts the penetration of tumor cells through the surrounding tissue and blood vessel wall. The majority of tumor cells will be trapped in the capillary beds succumb and are removed by phagocytes. Occasionally they will adapt to the environment and proliferate. Few tumor cells will however extravasate. Some will adapt to the environment and either stay dormant for an undetermined period of time or proliferate and form a metasasis. Another route tumor cells take is penetration through the lymph vessel walls or they are simply carried away by the draining lymph vessels. These tumor cells pass by the lymph nodes in which they will be destroyed, multiply or simply be travelling through and end up in the blood.
Figure 12  Formation of metastasis. Cells in the primary tumor gain properties to invade the surrounding stroma and penetrate the local blood vessels. Most of the tumor cells penetrating the blood vessels will be trapped in the capillary beds and are destroyed. Some will become trapped in the capillaries and adapt to the environment, others adhere specifically to molecules on the surface of the endothelial cells lining the blood vessels. Some of these cells will extravasate, adapt to the environment and form micro or macro metastasis, some will persist in a dormant state and may be revived at a later time to give rise to a disease recurrence, others will simply die.

Metastasis interfere with the function of organs and use up the body’s resources and ultimately lead to death. Rarely patients die from the primary tumor. Preventing metastasis would therefore greatly advance the fight against cancer. Although it will not cure the disease it would make it a chronic disease that could be managed. A more thorough understanding of the processes involved in the escape of tumor cells from the primary tumor is however needed. One approach is to prevent the outgrowth of blood vessels that are needed to support the growth of the metastases as it would
limit the supply of nutrients and oxygen to the tumors. The first therapeutic agents taking this approach have been introduced. The therapeutic antibody bevacizumab targeting the vascular endothelial growth factor for example prolongs survival of patients with metastatic colorectal cancer. Only a few factors that play a role in the metastatic process are known and a more thorough understanding will be necessary to get a real grip on the cancer problem.

Not only is the prevention of metastasis vital, but also the knowledge of whether or not the disease has been disseminated is of great importance for the management of the disease. The question that arises is what technology can be used to detect the presence of tumor cells that have settled at distant sites. The term “metastasis” was introduced by Recamier in 1829 and he speculated that tumor cells should be present in blood. This was confirmed by Ashford in 1859 and Engell in 1955 by showing the presence of cancer cells in the peripheral blood. This spawned numerous studies over the next twenty years and carcinocythemia and carcinoma cell leukemia were introduced in the seventies to describe the presence of circulating tumor cells in patients with carcinomas. The presence of tumor cells in these case reports were associated with short survival, but no clinical studies were performed mainly due to extremely low frequencies of these cells and lack of standardized technology with the necessary sensitivity and specificity to perform such studies. Observations of tumor cells in stem cell collections used for autologous transplantation and more advanced detection methodologies renewed attempts to identify occult tumor cells.

After we took a good look at the problem we realized that the anticipated frequency of tumor cells circulating in blood was too low to detect reliably with current available technology. We believed that the frequency of circulating tumor cells (CTC) was less than 1 cell per mL of blood which thus needed to be found in a haystack containing at least 5 x 10^6 leukocytes and 4 x 10^9 erythrocytes, Figure 5. We reasoned that under normal circumstances blood should only contain cells of hematopoietic origin with maybe an occasional endothelial cell that escaped the blood vessel wall.

The embryonic origin of blood is the mesoderm, which is separate from the ectoderm and endoderm, all three of which are formed at the earliest phase of the developing embryo. It can thus be expected that cell surface antigens exist that are uniquely expressed by these three lineages and consequently can be used to differentiate among them. Examples of antigens with an
expression restricted to cells of epithelial origin are Epithelial Cell Adhesion Molecule (EpCAM) a glycoprotein involved in homotypic intercellular adhesion of epithelial cells and cytokeratins that form the cytoskeleton and maintain the structural integrity of the cells. Likewise, the expression of the CD45 antigen is restricted to cells of hematopoietic origin. We have used these antigens to discriminate carcinoma cells which are of epithelial cell origin from hematopoietic cells. Ferrofluids, with an approximate size of ~170nm, a core of Fe₃O₄ and coated with albumin were conjugated with monoclonal antibodies directed against EpCAM to create immunomagnetic ferrofluids specific for epithelial cells. After incubation of these ferrofluids with 20 mL of blood and magnetic separation, the blood volume was reduced while maintaining the epithelial cells and reducing the hematopoietic cells with more than 4 logs. The cells were fluorescently labeled with a nucleic acid dye, monoclonal antibodies directed against cytokeratins and CD45 were analyzed by flowcytometry. To assess whether cells of epithelial cell origin could be detected, 20mL blood samples from healthy donors and patients with primary and metastatic carcinomas were processed and analyzed by flowcytometry. Figure 13 shows the analysis of a blood sample from a normal donor and three carcinoma patients. The large black dots in the figure represent epithelial cells. Two were present in blood of a normal donor and a larger number were detected in the analysis of the patient blood samples. The highest number were detected in blood from a patient with metastatic breast carcinoma. The results of this study warranted further development of this technology. However, upfront some key observations were made that needed to be addressed before one truly could investigate the clinical implications of the presence of CTC. The identity of the epithelial cells needed to be confirmed as indeed being cells and having features consistent with those of cancer cells and not normal epithelial cells. Furthermore the sample processing needed to be automated to avoid variable as well as erroneous results.
Figure 13 Flowcytometric analysis of immunomagnetically enriched and fluorescently labeled epithelial cells from 20 mL of blood. A threshold on a nucleic acid dye was used to allow only events with the nucleic acid content of nucleated cells and gates on forward and orthogonal light scatter to exclude events below a size of 4µm. Panel A shows the CD45 versus cytokeratin staining of a normal blood sample, panel B from a patient with organ confined breast cancer, Panel C from a patient with organ confined prostate cancer and Panel D from a patient with metastatic breast cancer. The leukocytes (CD45+, cytokeratin-) are depicted as small black dots, debris as small grey dots and the epithelial cells as large black dots.

An instrument was designed to automate the preparation of blood samples for CTC analysis. The configuration of the instrument is illustrated in Figure 14. A 7.5 mL blood sample placed on the system passes through 9 stations before the sample is placed in an analysis chamber. During the sample processing the volume of the sample is reduced from 7.5mL to 300µl. Erythrocytes and platelets are virtually eliminated and the leukocytes are reduced to an average of ~5,000. The majority of the epithelial cells are retained and the cells are stained with the DNA specific fluorescent dye DAPI, Phycoerythrin labeled monoclonal antibodies recognizing cytokeratins 4, 5, 6, 8, 10, 13, 18 and 19 and Allophycocyan labeled monoclonal antibodies recognizing CD45.
Figure 14  Automation of isolation and labeling of epithelial cells from 7.5 mL of blood. Panel A shows the mechanical design of the instrument with a circle of 9 processing stations around a reagent reservoir. Panel B shows a disposable reagent tray from which the reagents are taken. Panel C shows the automated sample preparation instrument capable of processing blood samples from 8 patients in approximately 3 hours. Panel D shows the functions for each individual station. Original engineering under supervision of Michael Kagen and original reagent development under supervision of Chandra Rao and Mark Connelly.

Now the challenge was how to analyze all the cells present in the sample. Introduction of the sample in a flowcytometry does not permit the analysis of the complete volume and cytospins traditionally used to prepare samples for microscopic analysis, which are hampered with significant and variable cell losses. We took a good look at the problem and reasoned that we should be able to use the magnetic labels of the cells to direct them to an analysis surface. A computer program was written to simulate the trajectories of magnetically labeled cells in between two magnets. Figure 15 shows two angled magnets with a small opening in between. The magnetic gradients created by the magnets permitted the travel of all cells present in an analysis chamber to its upper surface for microscopic analysis.
Figure 15  Computer simulation of trajectory of magnetically labeled cells present in between two magnets. The magnets with a South and North Pole are angled their upper surface is spaced 3 mm apart. Each of the dotted lines represents the trajectory of a magnetically labeled cell. All cells move vertically in the middle portion below the surface of the two magnets. Magnetically labeled cells in a chamber placed at this position (red rectangular box) will thus move vertically and distribute over the surface forming a homogenous layer of cells. Simulations by Jerry Dolan.

A fluorescent microscope equipped with a 10X objective (NA0.45), a mercury arc lamp, a CCD camera, a computer controlled filter wheel and X, Y, Z stage is used to take images of the DAPI, APC and PE fluorescence covering the complete surface of the analysis chamber. Panel A of Figure 16 shows the 140 PE images needed to cover the 2.7 mm by 33mm surface of the analysis chamber. Panel B shows a DAPI, PE and APC image. The PE image shows a few fluorescent events, in contrast with the DAPI and APC images, that show many fluorescent events. Most of these events are leukocytes characterized by a nucleus (DAPI) and CD45 staining. A computer program is used to identify locations that fluoresce with both DAPI and PE. The two rectangular
boxes shown in the images of Panel B represent two such events. All images of classified events are presented to an operator that makes the final classification. Panel C shows four events derived from a blood sample of a metastatic carcinoma patient. The event in the top row classifies as a CTC with a nucleus, cytoplasmic staining with cytokeratin and no staining with CD45. The event in the second row has a nucleus, cytokeratin staining not associated with the nucleus and staining with CD45 and classifies as a leukocyte with some free cytokeratin in close association. The event in the third row has a nucleus, a cytoplasm staining with cytokeratin and no CD45 staining. The event classifies as a CTC but does not appear as “healthy” as the CTC in the top row. Two events are in the bottom row. One is a leukocyte with a nucleus, no cytokeratin staining, but staining with CD45. The other is a micro particle staining with only cytokeratin.

**Figure 16** Analysis of the cells in the analysis chamber by fluorescence microscopy. Panel A shows the images needed to cover the entire surface. Panel B shows the DAPI, PE and APC images at one position in the chamber and the two rectangular boxes in each of the images indicates the two positions with DAPI as well as PE signals. Panel C shows the DAPI, PE, APC and DAPI/PE overlap images of four events identified by the computer program as candidate CTC. Original software design by John Sylvia.
The morphology of CTC within and between patients is extremely heterogeneous as illustrated in Figure 17. The reproducibility of the system was established by spiking blood of healthy donors with cells of a variety of tumor cells expressing different levels of EpCAM and cytokeratin. The same study showed the presence of CTC in patients with a variety of carcinomas and an absence in blood from healthy donors 53.

Figure 17 Gallery of CTCs detected in a variety of patients. Green represents the cytoplasm identified by cytokeratin and purple or white the nucleus identified by DAPI.

To evaluate the clinical significance of CTC prospective international multicenter clinical trials were conducted, for which patients with metastatic breast-, colorectal- and hormone refractory prostate cancer were enrolled54-59. Blood samples were analyzed for CTC before and at monthly intervals after initiation of therapy. The relation between the presence of CTC before initiation of therapy and survival of the patients is illustrated in Figure 18. The green bars in Panels A, B and C represent the patients with zero CTC in 7.5mL of blood and the red bars patients with increasing numbers of CTC. The percentage of represent patients in each group is indicated on top of
each bar. Error bars indicate the 95% confidence around the median survival. The median survival of patients with no CTC is significantly longer as compared to those patients with one or more CTC. The survival prospects for patients with and without CTC are remarkably similar between the three cancers, while the proportions of patients with tumor cells detected in the blood is quite different. The prognosis does not alter much with an increasing number of tumor cells in the blood and only at a higher CTC number is a decrease in survival noticed.

Figure 18  Relation between the presence of Circulating Tumor Cells and survival of patients with metastatic breast cancer Panel A, colorectal cancer Panel B and hormone refractory prostate cancer Panel C before starting a new line of therapy. The error bars indicate the 95% confidence interval of the median survival of the patients with the specified CTC. Original clinical trial designs by Gerald Doyle. Sample analysis under supervision of Madeline Repollet (USA) and Arjan Tibbe (Europe). Data analysis by Craig Miller.

For breast and prostate cancer a threshold of 5 CTC and for colorectal cancer a threshold of 3 CTC was chosen to divide patients into those with Favorable and those with Unfavorable CTC. The Kaplan Meier graphs in Figure 19 shows the probability of survival for metastatic breast, colorectal and prostate cancer patients with Favorable and Unfavorable CTC at monthly intervals after initiation of therapy. At all time points tested the difference in survival between the Favorable and Unfavorable groups is highly significant. Implications of these findings are that the presence of CTC are
predictive of outcome at any time during the treatment and suggests that the patients that have Unfavorable CTC after initiation of therapy are on a futile therapy.

**Figure 19** Kaplan Meier Analysis of overall survival. Median overall survival of breast cancer patients with Favorable CTC after 3-5 Weeks (n=92), 6-8 Weeks (n=77), 9-14 Weeks (n=105) and 15-20 Weeks (n=70) of treatment median was 21.7, 19.1, 20.8 and 20.1 months. Median overall survival of breast cancer patients with Unfavorable CTC after 3-5 Weeks (n=40), 6-8 Weeks (n=22) 9-14 Weeks (n= 24) and 15-20 Weeks (n=15) of treatment was 6.2, 6.3, 6.4 and 11.3 months. Median overall survival of colorectal cancer patients with Favorable CTC after 1-2 Weeks (n=316), 3-5 Weeks (n=292), 6-12 Weeks (n=285), and 13-20 Weeks (n=172) of treatment was 15.7, 16.4, 15.8 and 14.6 months. Median overall survival of colorectal cancer patients with Unfavorable CTC after 1-2 Weeks (n=41) 3-5 Weeks (n=41), 6-12 Weeks (n=25), 13-20 Weeks (n=21) of treatment median was 6.1, 4.4, 3.3 and 3.3 months. Median overall survival of prostate patients with Favorable CTC after 2-5 Weeks (n=123), 6-8 Weeks (n=110), 9-12 Weeks (n=100) and 13-20 Weeks (n=99) of treatment median was 20.7, 19.9, 19.6 and 19.8 months. Median overall survival of prostate cancer patients with Unfavorable CTC after 2-5 Weeks (n=80), 6-8 Weeks (n=53), 9-12 Weeks (n=49), 13-20 Weeks (n=44) of treatment was 9.5, 8.5, 7.6 and 6.7 months.

Further Kaplan Meier analysis was performed to determine whether a change in CTC after treatment would alter survival prospects. Patients were divided into those that remained Favorable and Unfavorable and those that changed to Unfavorable or to Favorable during the course of therapy, **Figure 20**. Regardless of the type of cancer, patients in which CTC were not
eliminated have an extremely poor outcome. Likewise patients that develop 
CTC during the course of therapy convert from a Favorable to Unfavorable 
prognosis. The data suggest that after the first cycle of therapy one already 
can determine that patients with Unfavorable CTC are on a futile therapy 
and other alternatives should be considered. This however represents a large 
shift in the management of the disease as currently disease assessment is 
performed after months of treatment and not weeks. Clinical studies are 
underway to demonstrate that an early change of therapy based on 
Unfavorable CTC can improve survival. Effective therapies result in an 
elimination of CTC and do prolong survival as is shown by the improvement 
of survival in patients of all three cancers when CTC convert from 
Unfavorable to Favorable. These conversions were found after first as well as 
later lines of therapies, indicating that therapies can still be effective after 
previous ones have failed. Significant progress can however only be made 
when we have the technology available that can tell what treatment targets 
are present on the tumor cells in the individual patient at the time therapy 
is to be administered and the availability of effective drugs against these 
targets.

Figure 20  CTC changes after treatment of 
patients with metastatic breast, 
colorectal and prostate cancer. 
After initiation of therapy CTC in 
83 (47%) breast cancer patients 
remained Favorable with median 
overall survival (OS) of 22.6 months, 
CTC in 39 (22%) patients remained 
Unfavorable median OS 4.1 months, 
CTC in 38 (21%) patients converted 
to Favorable CTC median OS 19.8 
month and CTC converted to 
Unfavorable in 17 (10%) patients 
median OS 10.6 month. CTC in 303 
(70%) colorectal cancer patients 
remained Favorable median OS 
18.6 months, CTC in 24 (6%) 
patients remained Unfavorable 
median OS 3.9 months. CTC in 74 
(17%) patients converted to
Favorable CTC median OS 11.7 month and CTC converted to Unfavorable in 29 (7\%) patients median OS 7.1 month. CTC in 88 (38\%) prostate cancer patients remained Favorable median OS of more than 26 months, CTC in 71 (31\%) patients remained Unfavorable median OS 6.8 months, CTC in 45 (20\%) patients converted to Favorable CTC median OS 21.3 month and CTC converted to Unfavorable in 26 (11\%) patients median OS 9.3 month.

Disease evaluation after initiation of therapy in patients with metastatic carcinoma is performed by radiographic imaging of the disease in those patients that have measurable lesions. Changes in tumor size and appearances of new lesions can only be determined after 3 to 6 months of therapy. The question that arises is whether the disease assessment by radiographic imaging provides similar results when compared to measurement of CTC or are these two different biological phenomenons, Figure 21.

Comparison of CTC and radiographic imaging to predict survival in patients with metastatic breast cancer clearly show that they are two independent measurements of disease activity. The Kaplan Meier analysis in Figure 22 shows that the patients with progressive disease by radiographic imaging and Unfavorable CTC after initiation of therapy have the worst prognosis. Conversely patients with stable disease or a partial response of the disease
by radiographic imaging and Favorable CTC have the best prognosis. In 21% of these cases both technologies did not agree, those patients that did progress by radiographic imaging but Favorable CTC had a survival not statistical different from patients with no progressive disease by imaging. Conversely patients with no progression by radiographic imaging but Unfavorable CTC had a survival not statistically different from those that showed progressive disease by imaging. Disease assessment by CTC reflects the biological activity and the aggressiveness of the disease more accurately.

Figure 22 Measurement of the change in disease burden by radiographic imaging at the first disease evaluation versus CTC at the first follow-up after initiation of therapy and its relation to survival in patients with measurable metastatic breast cancer. S/PR = stable disease / partial response, PD = progressive disease by radiographic imaging

In those cases where no measurable lesions were present, proteins shed by the tumor were measured, these serum tumor markers are however not being produced by all tumors and one can never assess whether they are produced by a few or many cancer cells. Moreover, most of these proteins are also produced by normal cells and thus are present in serum of healthy individuals. In most cases measurable liver lesions are present in colorectal
cancer, but in breast cancer approximately 30% of the patients have non-measurable disease, and in prostate cancer only few cases have measurable disease.

**Challenges**

**Improvements in circulating tumor cell detection**

The data obtained to date suggests that the mere presence of CTC drastically changes the survival prospects of patients with metastatic carcinomas. The technology challenge is to assure that the events detected are indeed tumor cells. Evaluation of the errors in CTC identification pointed to the ability of the operator to assign an event as a CTC. Future CTC assignment therefore will need to be independent of the operator. This is of greatest importance when “tumor cells” are detected in blood of patients with no evidence of active disease. A good reason to make CTC assignment independent of the operator. Although the analysis of larger blood volumes can help to improve the accuracy of CTC detection the volume one can reasonably draw, quickly reaches its limit.

A first step to increase the specificity of the test was the demonstration that tumor cells in blood showed the same chromosomal abnormalities as those present in the primary tumor. To enable the detection of abnormal chromosome copy in, technology was developed to preserve the cells in the chamber after they have been analyzed. Samples could now be hybridized with fluorescence labeled nucleic acid probes and the copy number of each probe could be assessed after revisiting the locations of the CTC in the analysis chamber. **Figure 23** shows the enumeration of the number of copies of chromosome 1, 7, 8 and 17 in an event previously identified as a CTC.

Although these four chromosomes are the ones that are most frequently affected in the major carcinomas many other abnormalities can be present that will go undetected with this approach. The aim therefore is to drastically improve the number of probes we can assess. This challenge is subject of the PhD project of Sjoerd Ligthart. Sjoerd’s simulation in Figure 24 shows the packing of all 23 chromosome pairs identified with FISH probes providing a 650nm fluorescent signal in a 10 µm cell. I am real curious to see how far this simulation is from the one he is going to show us when he is ready to defend his thesis. Who knows he might exploit his observation on the cover of this booklet. In this picture, the cell has been permeabilized causing the DNA to leak out of the cell providing a larger surface to
investigate the composition of the DNA. Analysis of a large number of CTC by FISH showed that some of the events identified as CTC did not show any FISH signals. The review of the morphology of these events suggested that they were undergoing apoptosis. Improvement of the discrimination within the events considered as CTC is needed. A first step towards this was achieved by the PhD candidate Eric Schreuder. During his PhD project he made an image cytometer with a higher resolution and improved ability to quantify the signals from the cells. Panel A in Figure 25 illustrates a typical measurement of an immunomagnetically enriched and fluorescently labeled blood sample from a patient with metastatic cancer performed with Eric’s cytometer. The CTC candidates are represented in the scatter plot comparing the fluorescence intensity of the nucleic dye DAPI and Cytokeratin PE divided by CD45 APC. The red dots were identified as “intact” tumor cells by examination of their
morphology, the green dots as CTC undergoing apoptosis visualized by clumping of cytokeratin and the purple dots as CTC debris. The blue dots are identified as leukocytes that either stain non-specific with cytokeratin or stain because they have taken up tumor cell debris through phagocytosis, and the grey dots represent artifacts. Three representative images are illustrated on top of the panel. The analysis of blood samples from 26 metastatic cancer patients showed a clear relation between the presence of CTC and CTC debris with more debris detected than intact CTC, panel B. Patients with no intact CTC detected did not have CTC debris. This observation suggests that development of technology that reliably enumerates tumor cell derived microparticles, could be a useful tool to monitor the disease in patients treated for cancer.

Figure 24  Computer simulation 23 chromosome probes identified by 650nm FISH probes in a 10 µm cell. Simulation by Sjoerd Ligthart.
Figure 25 Measurement after immunomagnetic enrichment and fluorescent labeling of CTC from 7.5 mL of blood, Panel A. The arrow indicates the transition from intact CTC to tumor cell debris. Panel B shows the correlation between the number of intact CTC and CTC debris in 26 metastatic cancer patients. Experiments by Eric Schreuder.

A drawback of the immunomagnetic enrichment is that along with CTC, the ferrofluids not bound to the cells come along for the ride. The larger the blood volume, the more ferrofluids will be concentrated on the analysis surface. Ferrofluids are colloidal and the automated sample preparation device allows cells to settle under the influence of gravity after which the top layer of the sample can be aspirated without removing the cells. This reduction is not sufficient, ferrofluids still mask portions of the cells interfering with the quality of the image and decrease the fluorescence signals. Improving the ability of the detection system does not overcome this problem. The challenge for Tycho Scholten in his PhD project is to tackle this issue. Tycho has designed a new surface for the analysis chamber which is made from polydimethylsiloxane (PDMS). These PDMS structures contain channels in which cells are moved under the influence of the vertical magnetic gradients. The reduction in the scanning area provide a three fold reduction in the analysis time, but he also made his problem larger by increasing the amount of ferrofluids. Panel A of Figure 26 shows a Smooth Electronic Microscope (SEM) image of the PDMS structure. The obstruction of
the CTC analysis by the ferrofluid can be observed in Panel B, which shows
one of the channels with three leukocytes (blue) and one CTC (green). The
horizontal black lines are created by the alignment of 170nm ferrofluids
under influence of the magnetic field. Panel C shows one of the early
successes from Tycho in which he was able to reduce the ferrofluids while
maintaining the CTC. Two green colored CTC, a couple of blue colored
leukocytes, some uncolored erythrocytes and a significant reduction of the
ferrofluids can be observed. The small objects, frequently associated with the
ferrofluids represent platelets or tumor cell derived microparticles. It is

obvious that more information can be retrieved from the image shown in
Panel C as compared to the image in Panel B.

Figure 26  Panel A, SEM image of the CTC analysis surface. The two sided arrow
indicates the width of the analysis channel and the vertical arrow
indicates the adjacent surface that aids the movement of the cells in the
analysis channel. Panel B, analysis channel with ferrofluids a CTC
(green) and leukocytes (blue). Panel C, analysis channel after ferrofluid
reduction with two CTC, leukocytes, erythrocytes and platelets.
Experiments by Tycho Scholten.

This is a big step, but if we look at all the results we have obtained and think
about the approach we have taken, it can not lead to the ultimate solution.
We are only taking a 0.15% aliquot from the total blood volume and simply
can not analyze all the blood nor can we detect the presence of all the tumor
cells that may hide elsewhere in the body and can awake, proliferate and
then disseminate at any time. The challenge is to identify and develop
technology that is capable to interrogate all the blood for the presence of
tumor cells. A breakthrough would be if the technology also could eliminate
the tumor cells. Identification and elimination of dormant tumor cells will
be a greater challenge as little is known about them. What we do know is
that they do matter, can hide in the body for prolonged periods of time and
occasionally can be found in the blood [63,64,65]. Time will tell whether the
University of Twente will make an impact in this field of research. The
initiative by the University of Twente to introduce Technical Medicine as a
new interdisciplinary program linking basic science and technology with
clinical practice in medicine certainly sets the stage for this to happen. It
may well be that some of the basic science currently worked on at the
university will provide valuable tools for our challenge. Who knows it may be
in-vivo imaging technologies applied on chicken eggs in the group lead by
Prof Ton van Leeuwen or the micro-bubble technology worked on in the
group lead by Prof Detlef Lohse.

Personalized Medicine
The oncologist will have an increasing armamentarium of therapies
available to treat patients with recurrent cancer and is faced with the
challenge of finding the most effective treatment for the individual patient.
The paradigm shift towards targeted therapy will end the unrealistic attempt
to kill all cancer cells with high-dose chemotherapy, but also complicates
matters as more knowledge is required on the characteristics of the tumor in
the individual patient. In current medical practice the absence or presence of
treatment targets is assessed on the primary tumor. It is however well-
documented that the original tumor clone is genetically unstable and
continues to mutate at a rapid rate constantly giving rise to variants
resistant to the particular therapeutic regimen [66-74]. To overcome this issue
assessment of therapy targets can be performed on CTC and would
constitute a “real-time” biopsy. First attempts have been successful and
promise to be valuable tools for the development of targeted therapies [75-80].

Figure 27 shows examples of assessments of therapy targets on CTC by
investigating the presence of the target protein or the amplification of its
gene.
Figure 27  Assessment of therapy targets on CTC. Panel A, two CTC from a breast cancer patient, one CTC expresses bcl-2, but the other CTC does not. Panel B, CTC from a breast cancer patient expressing the Her2 protein and in Panel D a CTC with overamplification of the Her2 gene. Panel C, CTC in a prostate cancer patient with overamplification of the Androgen receptor and no translocation of TMPRSS/ERG.

The ability to detect the presence of a therapeutic target may not be sufficient and we may need to visualize therapeutic pathways within the cells to determine whether or not the drug indeed will initiate the cascade of events necessary to lead to the death of the tumor cells. Under the leadership of Prof Jan Greve, the University of Twente made significant contributions to the field of optical detection technology. At the time I started my PhD work in his department, Cees Otto was working on Raman spectroscopy and showed me spectra in which he pointed out signals produced by water and silver molecules in the solution he was investigating. At the time I could not envision what this could bring to the table for the advancement of medicine. Looking back, my time frame in which I expect things to happen was too short, as now 20 years later, Raman spectroscopy can be used to characterize cells and I am in active discussions with Cees to explore its potential to visualize molecules and organelles in tumor cells. To
evaluate whether there is merit to this approach a Raman spectrum was measured from white blood cells labeled with an Allophycocyan conjugated antibody recognizing CD8. Individual leukocytes were located by bright field microscopy for Raman imaging of the cells. Some cells yielded a Raman spectrum Cees never encountered, Figure 28. The unique spectrum most likely can be contributed to Allophycocyan the fluorescent label tagged onto a CD8+ lymphocyte. It definitely aroused my curiosity and I fully expect that we will merge the technologies we both developed. We hope to map the Raman signals from immunophenotypically defined cells, during their maturation and proliferation pathways, and than compare those with cancerous cells.

Figure 28  Ramanspectrum of an Allophycocyan labeled CD8+ lymphocyte collected after a 10 second accumulation with 2mW power from a 647nm laser line. Experiment by Henk-Jan van Manen.

Not only do we need to develop technology which obtains more detailed information from the detected cells we will also need technology that can assess the presence and functional status of the complete arsenal of
therapeutic targets on each cell. Technology residing within the University of Twente’s institute of nanotechnology (MESA+) may well be suited to tackle this problem. Discussions with Prof Albert van der Berg lead to a concept we baptized the “Cancer Cell Chip”, Figure 29. Whether this concept is viable may be examined in future bachelors, masters and PhD assignments. First experiments conducted by Floor Wolbers and Tycho Scholten indicate, that the challenges the students will face are not trivial. This however makes science exciting and when we provide the students with a sound education they too will enjoy the great feeling of uncovering answers to a problem after they have taken a good look at a problem, arrived at an experimental plan by thoroughly thinking about the problem and than carefully execute the plan (do).

Figure 29 “Cancer Cell Chip” a bright field image of cells from the breast cancer cell line MCF7 moved into individual positions in a microfluidic device (indicated with arrows). Experiment by Floor Wolbers and Tycho Scholten. Subsequent deliveries of therapeutic target specific probes or movement of the cells to the locations of the probes will be needed. The number of probes that can be detected simultaneously is limited and technology will be needed to remove the previously used probes. For functional analysis the viability of the cells will need to be maintained which might be achieved by electroporation.
Divergence
Curiosity is a necessary evil in science as at one hand it’s the key ingredient for new discoveries, but at the other hand it unavoidably leads to deviations from the main course as well as troubles at the home front as one tends to lose track of time. It happened to me shortly after I started my PhD assignment, but had a good excuse as my supervisor the late Prof Bart de Grooth was of a similar mind. We quickly learned to harmonize each others skills and spend for example many evenings trying out techniques to measure the eating capabilities of granulocytes. I never have been able to avoid this temptation, after phagocytosis, it was primary paroxysmal nocturnal hemoglobinuria, then bone development, then development of a technology to identify specific human monoclonal antibody fragments from a synthetic phage antibody library, then gene therapy and those in the audience reading the local newspaper have already been made aware of the latest deviation from the main course, Figure 30.

Figure 30  Affordable HIV monitoring in resource poor countries.
When asked by journalists whether they could talk to the bachelor students working on the project I suggested they interview my room mate Prof Jan Greve as he was the one who initiated the project and was more familiar with the real status of the project. Indeed progress has been made on this STW funded project and PhD student Xiao Li (Lilly) has successfully evaluated the assay in Thailand this summer on the instrument designed and built by Aurel Ymeti and Christian Breukers. A more appropriate heading of the article would have been: UT-discovery might help the third world.

We have come to the end of this lecture and hope it created the excitement required to get you all thinking of how to advance the cancer field. Many of you have probably experienced the far reaching implications when someone in your environment is diagnosed with cancer and if we just try a little harder to work together we will make an impact. So in plain US terminology, let's kick butt.
Acknowledgments

Before we all leave, I would like to take the opportunity to thank the many people that have and will make contributions to this effort. I would like to start with Bart de Grooth who is no longer with us. Bart convinced me that my lack of understanding of the theory behind most of the physical principals would not hamper me in becoming a succesfull scientist. After being diagnosed with an incurable cancer, Bart tried to put together the content of his inaugural lecture, the title would have been “De Wekker” (Alarm Clock), Bart was facinated with the perfect mechanics of a clock and on the other hand he realized, that his clock was about to stop ticking. This was one more reason to work dilligently to advance the cancer field. Jan Greve, Bart and myself started the “Cell Characterization Group” at the department of Applied Physics in the early eighties (with no resources besides some gray matter). Since Jan’s official retirement he reminded me from time to time that the Cell Characterization Group would come to an end at the Univerity of Twente if no plans for its future were made. Discussions with Vinod, Cees and Ton confirmed that nobody had the time nor sufficient knowledge to keep the group alive. All eyes pointed at me, but I was living and working in the USA. Luckily, Alfred Bliek the dean of the faculty of Science and Technology at the University of Twente and Byron Hewett the Chief Operating Officer at Immunicon corporation have had quite some experience in commuting. They believed it could work, so here I stand. “Once an apprentice always an apprentice” another reminder from Jan Greve, this apprentice took some of his advice quite seriously. “You need to go to the USA to broaden your horizon”. I was happy to procure a post-doc position under the tutelage of Michael Loken in sunny Palo Alto, California. That enivironment was indeed stimulating and many people helped me do some exciting science. I lost track of time and spent many, many more years than originally planned. One day I met Paul Liberti, a Professor in Biochemistry at the Thomas Jefferson University in Philadelphia who had started a company around ferrofluids. He promised me that I could take the company in any direction I liked, and so the cancer story started. Initially I only got the dentist Jerry Doyle believing and helping, followed by software guru John Sylvia and steadily more people jumped on the bandwagon as proof of principles were demonstrated. Financing is a necessary evil, two topnotch finance dudes Jim Murphy and Ed Erickson raised the funds required to allow the science to mature, conduct the clinical studies and develop and manufacture the products. Multi-tasking is a skill I mastered.
over the years, but it would have left me empty handed when I would not have been surrounded by an excellent team of people. As we speak Craig Miller is most likely sitting behind his desk crunching data, Madeline Repollet is guiding her team processing and analyzing blood samples from cancer patients, Mark Connely and Chandra Rao are guiding a team extracting more information from cancer cells and the teams from John Verrant, Frank Coumans and Mike Kagan are trying to make the necessary adjustments to the instrumentation. The european Immunicon crew headed up by Arjan Tibbe and the current members of the “Cell Characterization Group” are in the audience and believe me they are great to work with. So if you are interested in joining the effort please feel free to contact any of us. When we will leave this room I will again have to ask Yvonne Greve the honory member of the “Cell Characterization Group” permission to let her dear husband Jan spend some time with us in advancing the field.

Last but not least I would like to dedicate this lecture to my parents Corry and Guus and to my “girlfriend” Jessy, mother of our children Wout, Jon and Stijn who has been supportive throughout this endeavor.

Ik heb gezegd
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