Changing Cancer Therapy with Personalised Testing

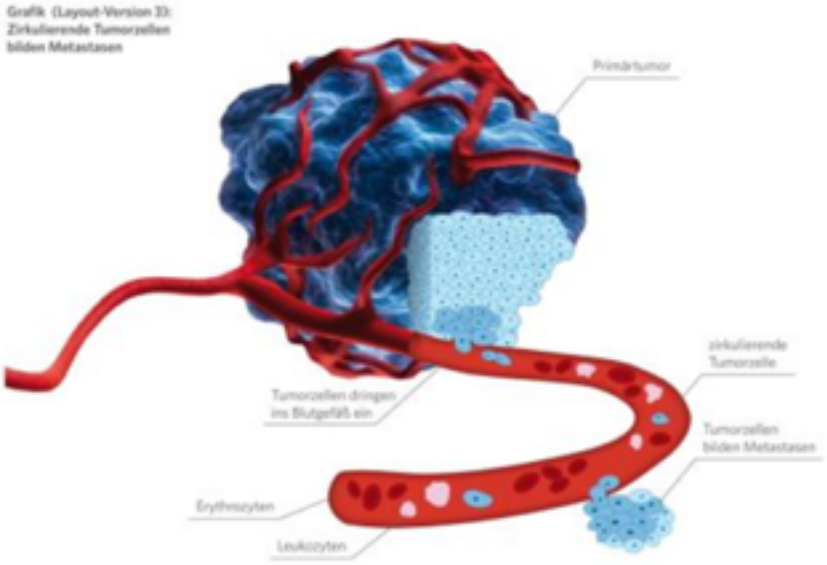
Professor Katharina Pachmann,

SIMFO GmbH and Transfusion Medical Laboratory Bayreuth, Germany

What is the "Maintrac" test?

Maintrac® is a method of tracking potential cancer cells circulating in the blood that has been used for many years in Germany and around the world.
The three main types of test available are:
1) Cell count – a figure for the number of circulating epithelial tumour cells (CETCs) in 1 ml of the patient's blood.
2) Chemosensitivity – the chemotherapy planned for use can be tested on the CETCs.
3) Cytotoxicity of natural agents – You can test natural agents to see how strong their cell-killing effect is on the patient's own CETCs.

Circulating tumour cells from solid tumours



- Carcinomas are of **epithelial origin**
- Carcinomas **disseminate** epithelial cells

⇒ **CETCs** (circulating epithelial tumour cells)

Y. Shiozawa, A M Havens, K J Pienta and R S Taichman, Leukemia (2008) 22, 941–950

TZB

The advantages of a "liquid biopsy"

The detection of circulating tumour cells is often called a liquid biopsy: this has several advantages over traditional tissue biopsies. Blood tests are easy and safe to perform. The test is non-invasive and can be used repeatedly. Much smaller tumours than can be detected in imaging can sometimes be picked up by a rising trajectory. Other types of analysis require invasive procedures that may limit patient compliance. CETC analysis allows you to modify therapy, potentially improving their prognosis and quality of life. It's important to mention that this technique does not replace the need for imaging/surgery of course, but helps to indicate where it is necessary, and where perhaps not.

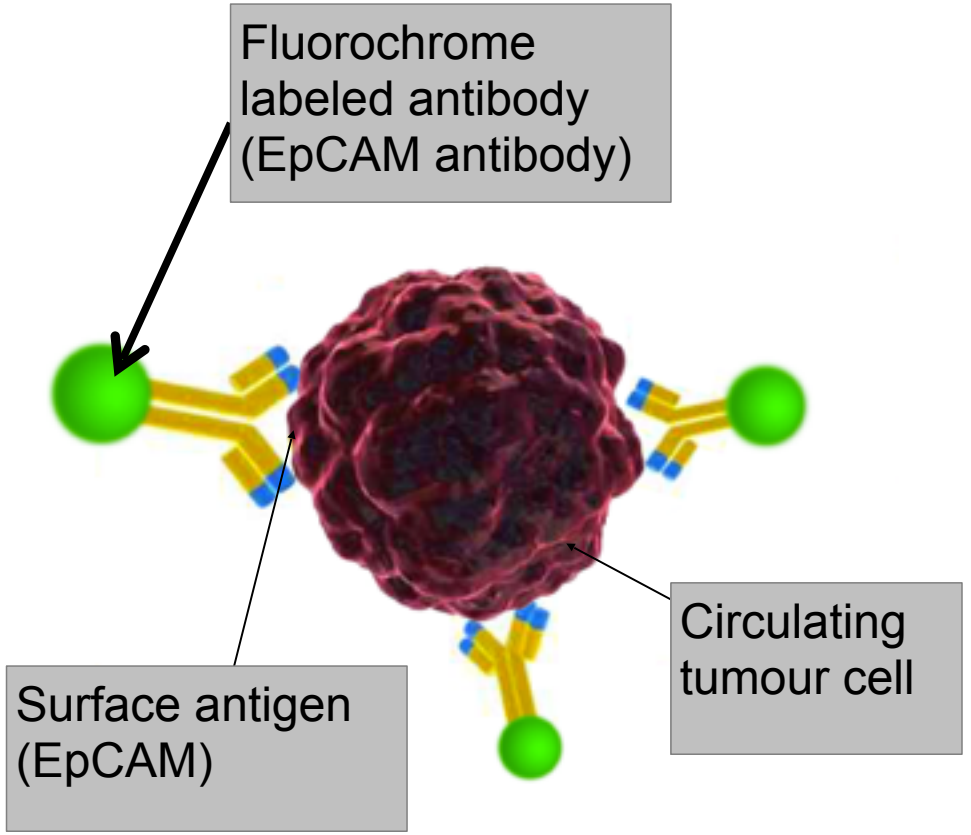
Liquid biopsy technique

Maintrac **liquid biopsy** cell staining allows **quantitative** detection of live circulating tumour cells

NO fixation.

NO isolation.

NO enrichment.

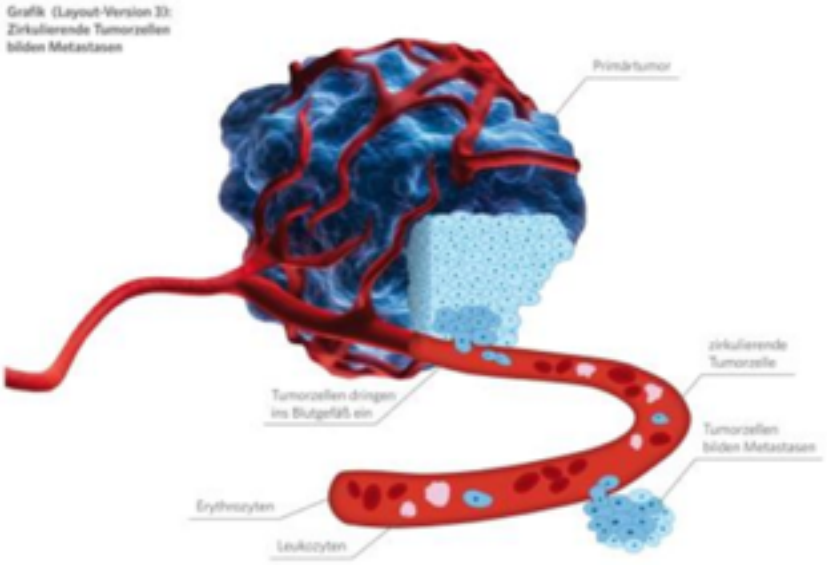


Stemtrac and tests of therapy-relevant CETC properties

Many additional tests are also possible, such as seeing whether the circulating cancer cells captured from a patient's blood develop into cancer stem cells – the most pernicious type of cell for metastatic activity (this test is called Stemtrac).

The properties of the CETCs can also be tested to detect further details. One of the multiple further tests available is to see whether a triple negative cancer has remained negative along all three dimensions. The nature of tumour cells can change over time: if for instance oestrogen-negative cells have become oestrogen-positive, this opens up new therapy options.

CETCs are shed from solid tumours …



- Vascularisation begins when the tumour has reached a size of about 1mm (1 million cells)
- Together with the uptake of nutrition by the tumour, debris and cells are shed into the circulation
- Seeding starts from the time of vascularisation

… not from blood cancers

The following are the cancers that cannot be tested for using the Maintrac method - **all the others can be**:
Acute myeloid **leukaemias**,
Acute lymphoid leukaemias
Chronic myeloid leukaemias
Chronic lymphoid leukaemias
Lymphomas
Most Hodgkin's lymphomas (some may express the EpCAM antigen)

How the results can be used

An increase in CETC numbers has been shown in numerous studies to be correlated with increased tumour activity. Maintrac does not use the single cell count as a prognostic marker, it uses the dynamics of the cell count. A rising cell count is an important indicator that tumour activity is ongoing, while decreasing cell counts tend to signal successful therapy. This means test trajectories can be used to monitor the success of the therapies being used, whether chemotherapy, hormone therapy, or natural approaches

Sources: Pachmann, Katharina (5 April 2015). "Current and potential use of MAINTRAC method for cancer diagnosis and prediction of metastasis". *Expert Review of Molecular Diagnostics*. **15** (5): 597–605; Lobodasch, Kurt; Fröhlich, Frank; Rengsberger, Matthias; Schubert, Rene; Dengler, Robert; Pachmann, Ulrich; Pachmann, Katharina (April 2007). "Quantification of circulating tumour cells for the monitoring of adjuvant therapy in breast cancer: An increase in cell number at completion of therapy is a predictor of early relapse". *The Breast*. **16** (2): 211–218

TZB

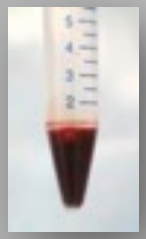
Maintrac methodology (1/2)

The Maintrac platform is based on microscopic identification of circulating tumour cells using EpCAM-specific antibodies. EpCAM stands for epithelial cell adhesion molecules. These are surface markers expressed on epithelial cells but not on blood cells. These cells should not normally be in the blood. As mentioned, carcinomas are of epithelial origin and disseminate these epithelial cells, so - if found - they are most likely from a tumour.
The EpCAM antibodies are used as a fluorescent marker to identify those cells. A staining method is used to distinguish between dead and living cells. To obtain live cells and reduce stress on these cells, blood cells are prepared by erythrocyte lysis and then only one centrifugation step. The suspension is analysed by fluorescence microscopy, which automatically counts the events. Simultaneous event galleries are recorded to verify whether the software found a true living cell and to differentiate between skin epithelial cells, for example.

Maintrac methodology (2/2)

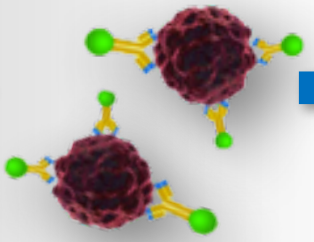
1. Blood sample quality control

2. Erythrocyte lysis



3. Centrifugation

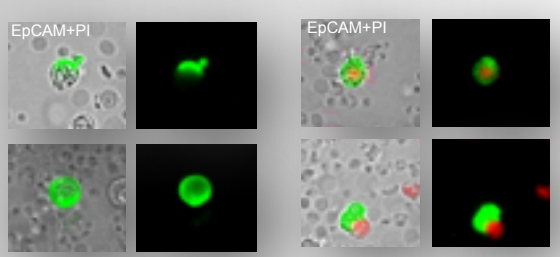
4. CETC labeling with green fluorescent EpCAM antibody



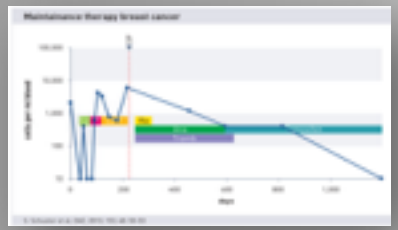
5. Transfer of suspension in 96 - well plate

6. Microscopic identification of live cells

7. Quantification of live CETCs

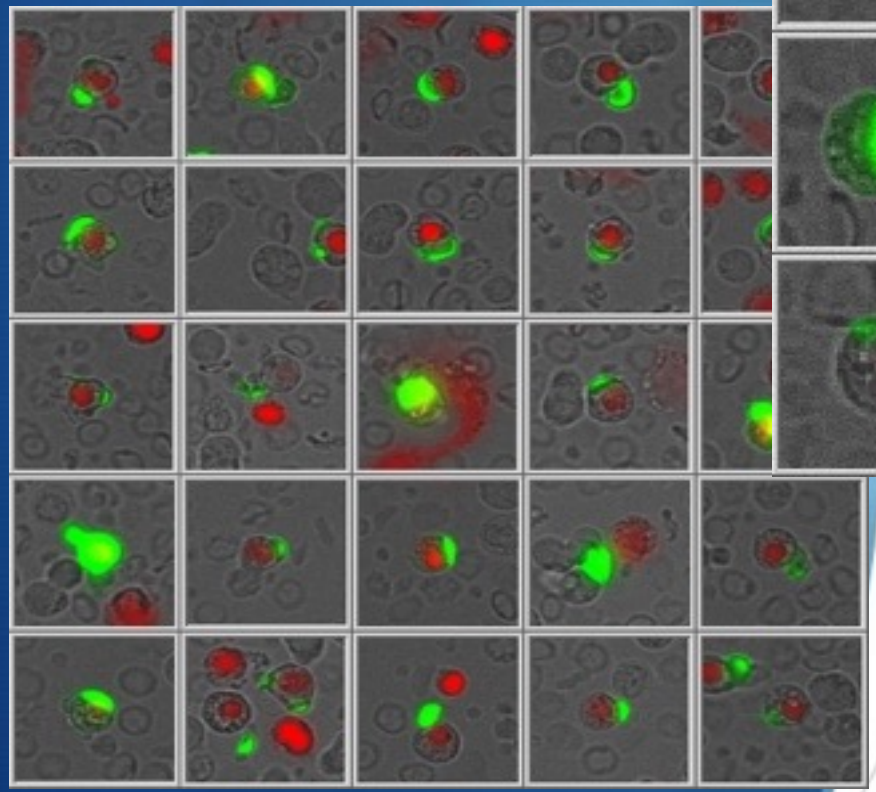


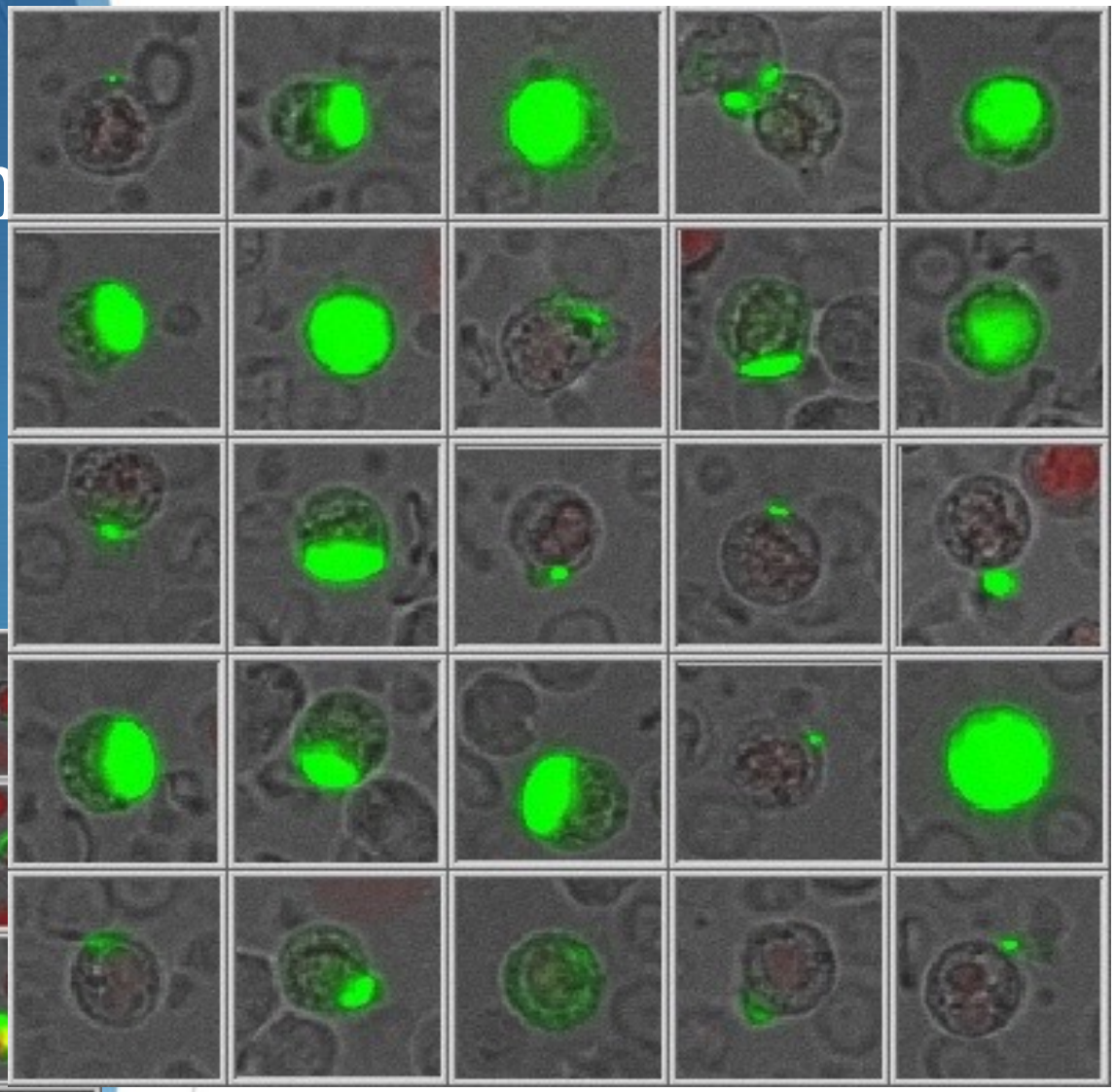
8. Determination of the CETC dynamics



TZB

Heterogeneity in cells from one patient





Red-stained nucleus
= dead cell

Duplicate analyses from one blood sample in 80 patients

How the circulating epithelial cancer cell count is presented

Diagnosis:
Adenocarcinoma of the caecum (initial diagnosis: 12/2015)

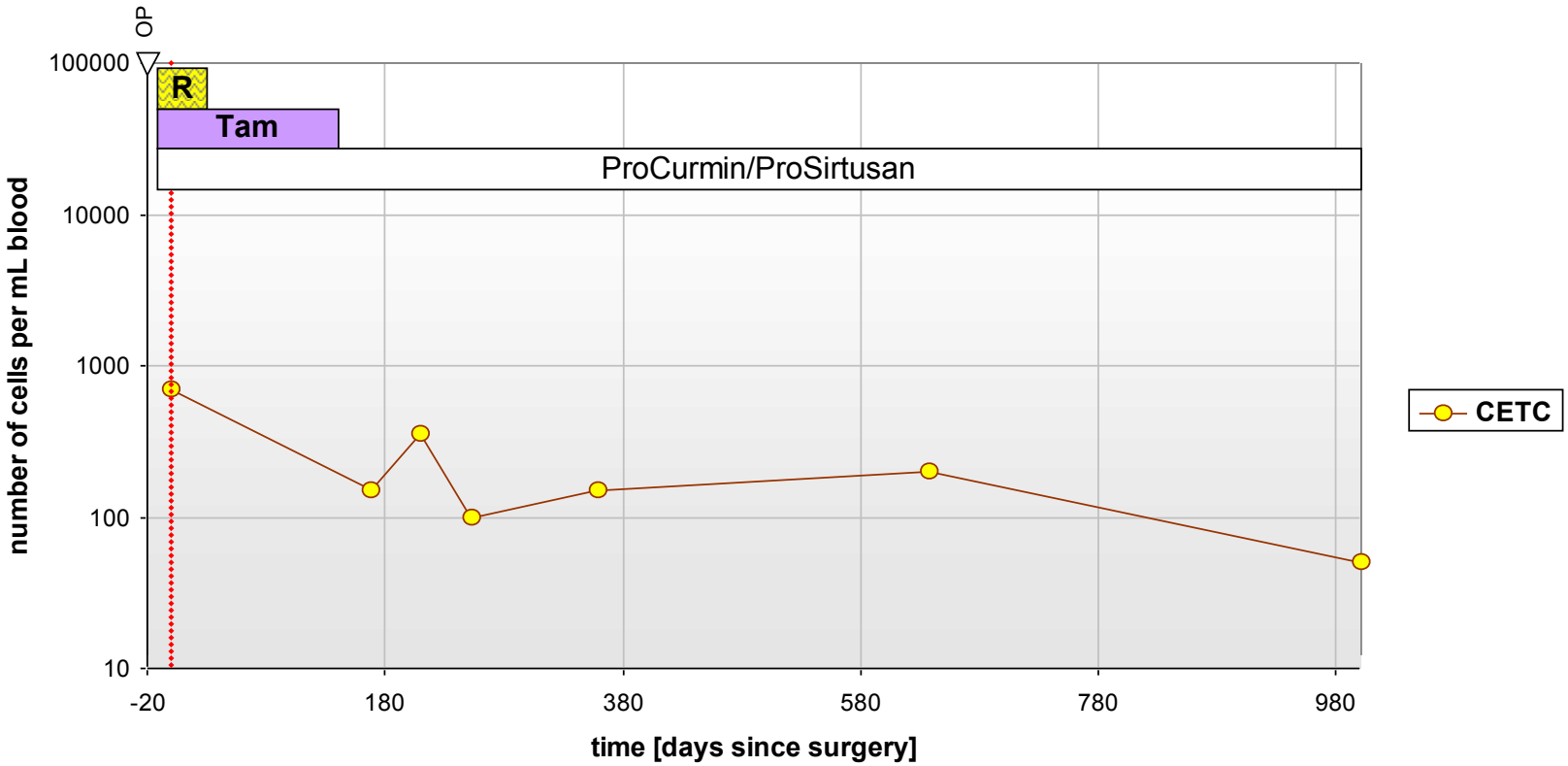
TNM:	T3 N2 M1, KRAS Exon 2 Codon 12 Mutation (Gly12Val)
-	Liver, lung and omental metastases
-12/15:	right hemicolectomy
- 03-08/16:	Chemotherapy with Capecitabine and Avastin
- 13.09.16:	left lung ablation
- 28.11.16:	left and right hemihepatectomy and non anatomical liver resection in S6 right hemihepatectomy

The automated microfluorimetric image analysis of the **epithelial cell adhesion molecule (EpCAM)**-positive cells with visual control (MAINTRAC) from **1 ml EDTA blood** resulted in following findings (detection limit is at 10 cells/ml):

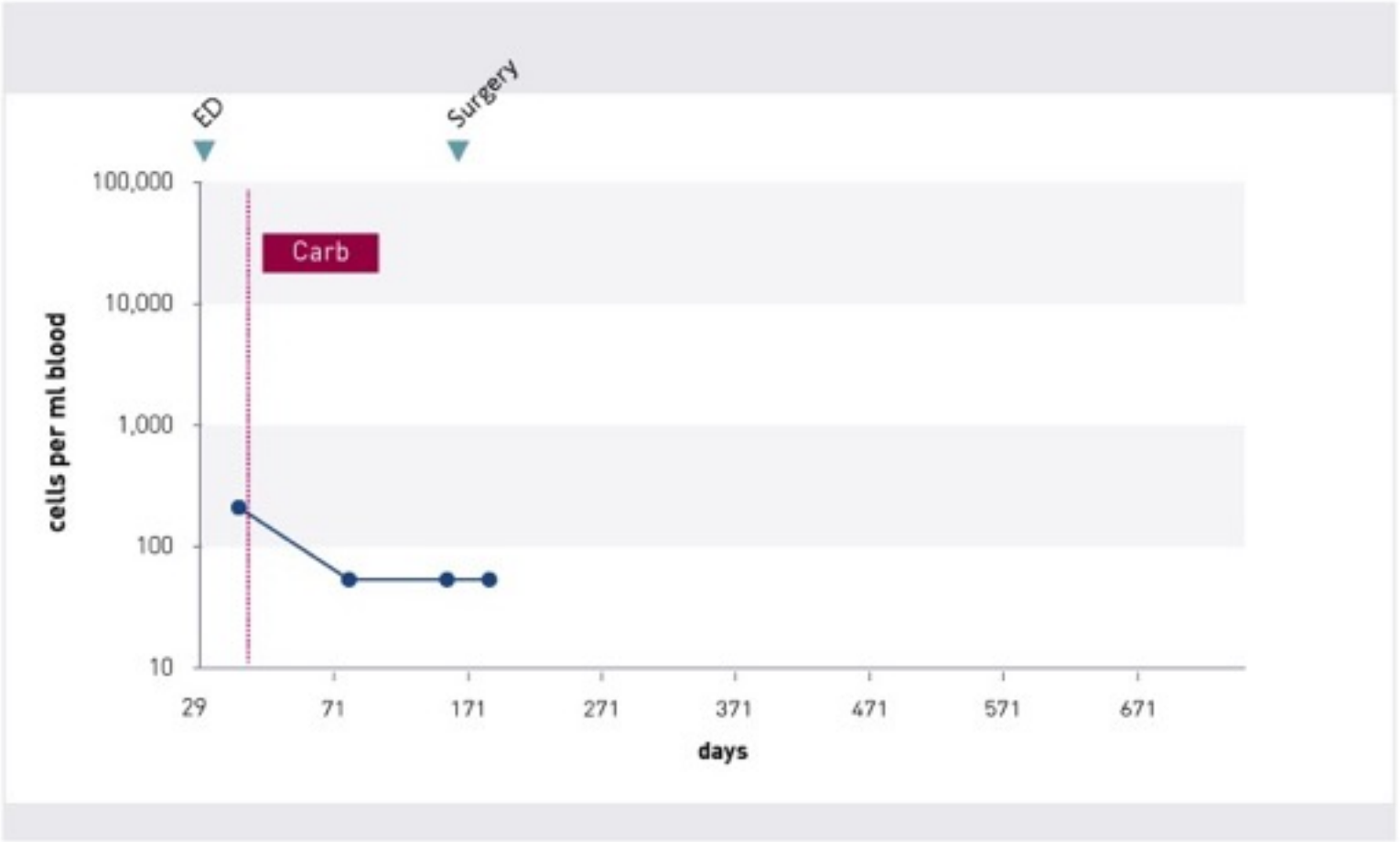
	Number of potential tumor cells			
Examination parameter	In the sample (1ml)	In circulation (5l) (in millions)	In addit. examination: % of EpCAM-pos. cells	Cell fragments
EpCAM	**250**	**1,25**		some

The material for examination could be thoroughly evaluated.
Under current therapy we found only **a slightly increased number of live tumor suspected cells circulating in the blood.**
In addition, there were some specific cell fragments detected. Specific cell fragments occur, for example, after chemotherapy or radiation, or as part of an immune response and indicate damaged

Trajectories



Changes in cell numbers during the course of disease (good reduction in cell numbers during neoadjuvant chemotherapy)



Changes in cell numbers during the course of disease (steep increase in cell numbers during radiotherapy)

Changes in cell numbers during the course of disease

(PET-CT due to high cell numbers revealed an asymptomatic brain metastasis which could be removed by surgery)

Testing the sensitivity of apoptotic agents

Chemo-sensitivity

J Cancer Therapy 2013, 4:597-605

Chemosensitivity Testing of Circulating Epithelial tumour Cells (CETC) in Vitro: Correlation to in Vivo Sensitivity and Clinical Outcome.

Journal of Cancer Therapy, 2013, 4, 597-605
doi:10.4236/jct.2013.42077 Published Online April 2013 (http://www.scirp.org/journal/jct)

Scientific Research

Chemosensitivity Testing of Circulating Epithelial Tumor Cells (CETC) *in Vitro*: Correlation to *in Vivo* Sensitivity and Clinical Outcome

Nadine Rüdiger[1], Ernst-Ludwig Stein[2], Erika Schill[2], Gabriele Spitz[2], Carola Rabenstein[2], Martina Stauch[3], Matthias Rengsberger[4], Ingo B. Runnebaum[4], Ulrich Pachmann[2], Katharina Pachmann[1,2*]

[1]Clinic for Internal Medicine II, University Hospital, Friedrich Schiller University, Jena, Germany; [2]Transfusionsmedizinisches Zentrum, Bayreuth, Germany; [3]Onkologische Schwerpunktpraxis, Kronach, Germany; [4]Women's Hospital, University Hospital, Friedrich Schiller University, Jena, Germany
Email: *kpachmann@laborpachmann.de

Received February 15th, 2013; revised March 20th, 2013; accepted April 2nd, 2013

ABSTRACT

Background: Chemotherapy is a mainstay of tumor therapy, however, it is predominantly applied according to empirically developed recommendations derived from statistical relapse rates occurring years after the treatment in the adjuvant situation and from progression-free interval data in the metastatic situation, without any possibility of individually determining the efficacy in the adjuvant situation and with loss of time and quality of life in the metastatic situation if the drugs chosen are not effective. Here, we present a method to determine the efficiency of chemotherapeutic drugs using tumor cells circulating in blood as the part of the tumor actually available in the patient's body for chemosensitivity testing. Methodology/Principal Findings: After only red blood cell lysis, omitting any enrichment (analogous to other blood cell enumeration methods, including rare CD34 cells), the white cells comprising the circulating epithelial tumor cells (CETC) are exposed to the drugs in question in different concentrations and for different periods of time. Staining with a fluorescence-labeled anti-epithelial antibody detects both vital and dying tumor cells, distinguishing vital from dying cells through membrane permeability and nuclear staining with propidium iodide. Increasing percentages of dying tumor cells are observed dependent on time and concentration. The sensitivity can vary during therapy and was correlated with decrease or increase in CETC and clinical outcome. Conclusions/Significance: Thus, we are able to show that chemosensitivity testing of circulating tumor cells provides real-time information about the sensitivity of the tumor present in the patient, even at different times during therapy, and correlates with treatment success.

Keywords: Circulating Epithelial Tumor Cells; Chemosensitivity Testing; Breast Cancer; Ovarian Cancer

1. Introduction

For patients diagnosed with a malignant tumor, cure is presumably only possible if the tumor is completely eradicated. Initially, the main aim is to eliminate the primary tumor, the major tumor burden, preferentially by surgery. However, most cancer patients do not die from their primary tumor but from distant metastases, developing some years after the removal of the primary tumor. During tumor growth, cells from the tumor are disseminated continuously via lymph vessels or directly into blood [1]. These cells are assumed to be the source of metastasis formation. Patients with affected lymph nodes have a less favorable chance of disease-free survival than patients without lymph node-positive disease, indicating that cells detached from the tumor were able to settle and grow as foreign tissue. Therefore, as the second pillar of tumor therapy, chemotherapy has evolved and is applied after surgery as adjuvant chemotherapy, e.g. in breast and ovarian cancer, to eliminate such early disseminated cells, when no detectable tumor is present. Such therapies have been shown to avert metastasis formation and ultimately save lives in breast cancer patients [2]. In the adjuvant situation, these therapies have been developed in clinical trials using the statistical improvement of relapse-free survival as a measure. This cannot, however, predict for the individual patient whether the

*Corresponding author.

Comparison of different agents

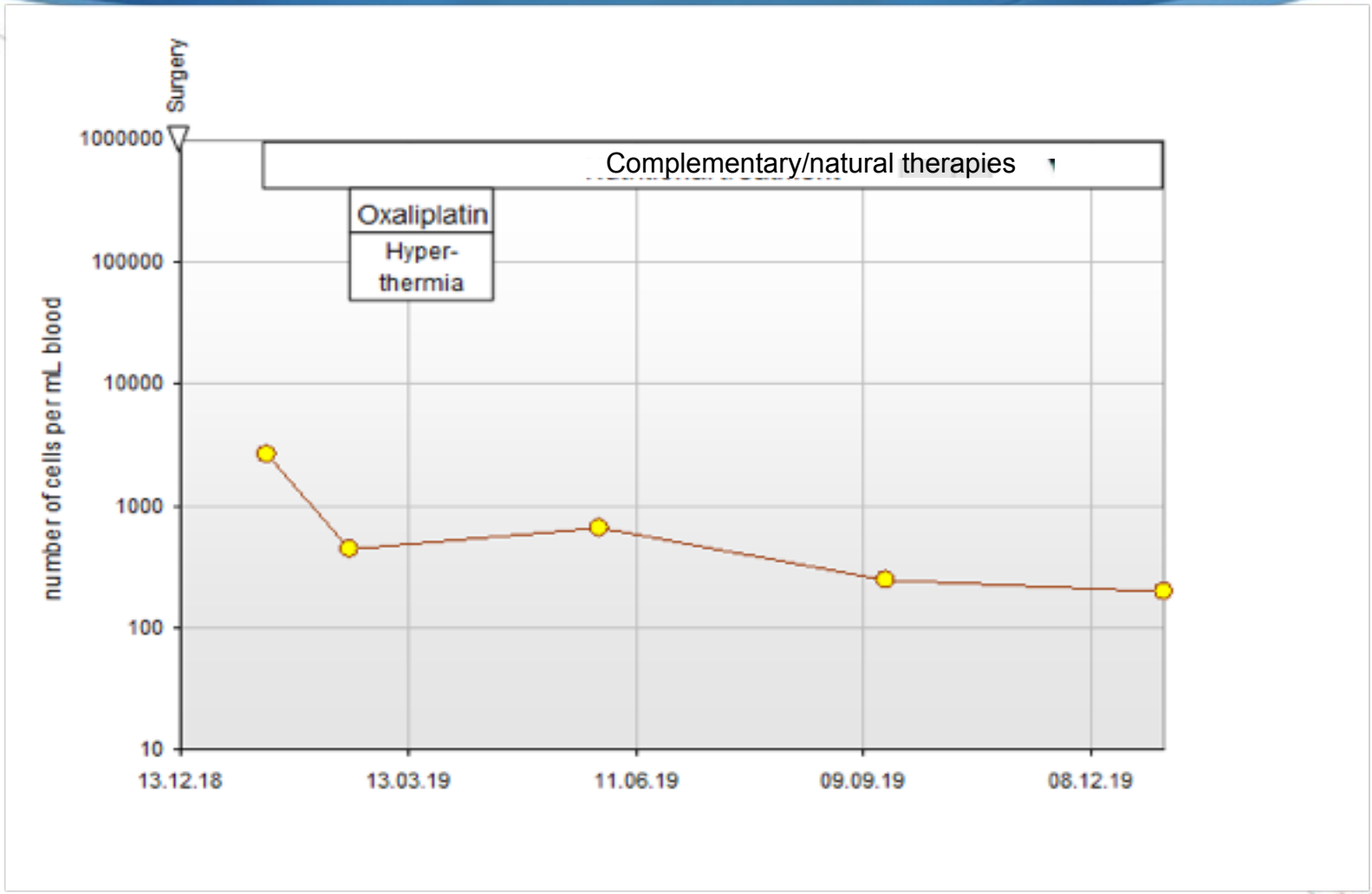
Examination parameter	Number of potential tumor cells: In the sample (1ml)	In circulation (5l) (in millions)	In addit. examination: % of EpCAM-pos. cells	Cell fragments
EpCAM	**2,650**	**13.25**		numerous

in-vitro-vitality reduction in relation to concentration and time (in%)
with eutherapeutic concentrations of
The ideal is a reduction by 100% in short-term cell culture

5-Fluoruracil 0.1-fold	**<10**	5-Fluoruracil 1-fold	**30**	5-Fluoruracil 10-fold	**45**
Oxaliplatin 0.1-fold	**65**	Oxaliplatin 1-fold	**75**	Oxaliplatin 10-fold	**85**
Artesunate 250mg 0.1-fold	**<10**	Artesunate 250mg 1-fold	**80**	Artesunate 250mg 10-fold	**n.a.**
Curcumin 450mg 0.1-fold	**80**	Curcumin 450mg 1-fold	**90**	Curcumin 450mg 10-fold	**99**

TZB

Monitoring the efficacy of therapy



Testing the sensitivity of chemotherapy vs. natural agents

Diagnosis:
Lung Cancer, initial diagnosis: 26.06.2017
TNM: T4 N3 M1b, Stage IV
- no Surgery
- no Radiation therapy
- post Complementary therapy
- no current therapy
- Medication: Herbal supplements

The automated microfluorimetric image analysis of the **epithelial cell adhesion molecule (EpCAM)**-positive cells with visual control (MAINTRAC) from **1 ml EDTA blood** resulted in following findings (detection limit is at 10 cells/ml):

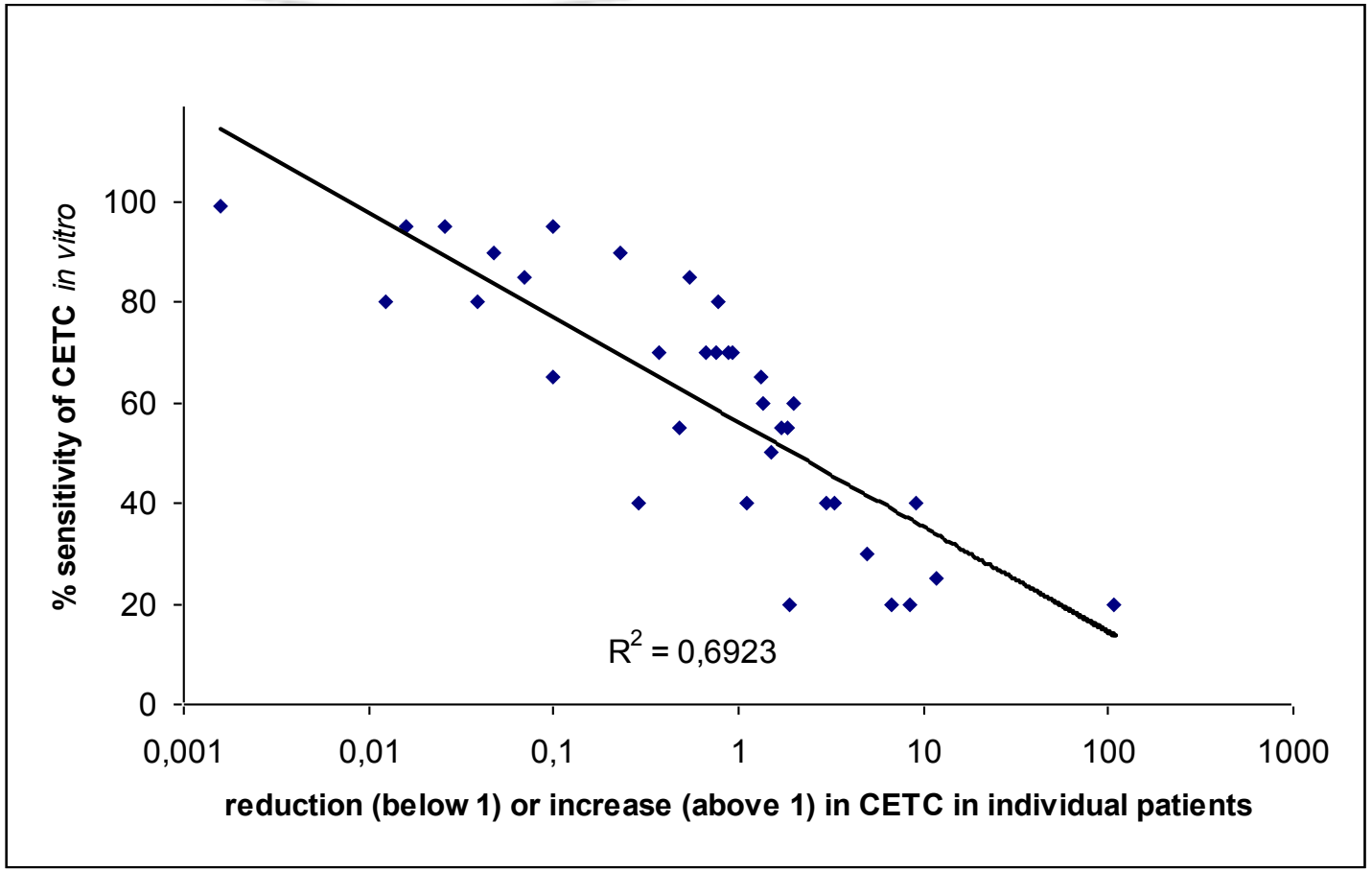
	Number of potential tumor cells			
Examination parameter	In the sample (1ml)	In circulation (5l) (in millions)	In addit. examination: % of EpCAM-pos. cells	Cell fragments
EpCAM	**150**	**0,75**		numerous

in-vitro-vitality reduction in relation to concentration and time (in%) with eutherapeutic concentrations of				
Avastin	**20**	Alimta	**60**	The ideal is a reduction by 100% in short-term cell culture
Cisplatin	**65**	Vitamin C	**40**	
Curcumin	**90**	Artemisia	**80**	

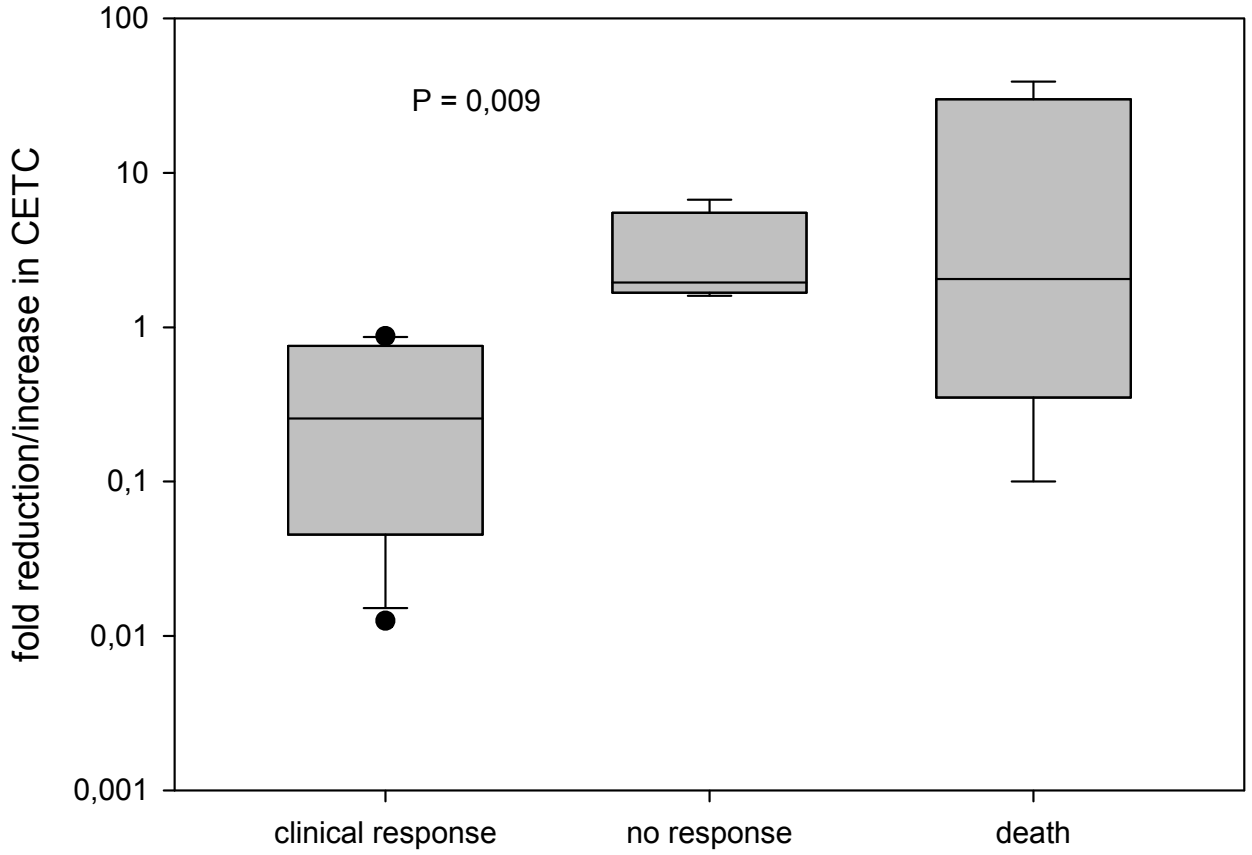
The material for examination could be thoroughly evaluated.
Under Therapy with herbal supplements we found only **a slightly increased number of live, potentially malignant tumor cells circulating in the blood.**
In addition, there were numerous specific cell fragments detected.
Specific cell fragments occur, for example, after chemotherapy or radiation, or as part of an immune response and indicate damaged cells.

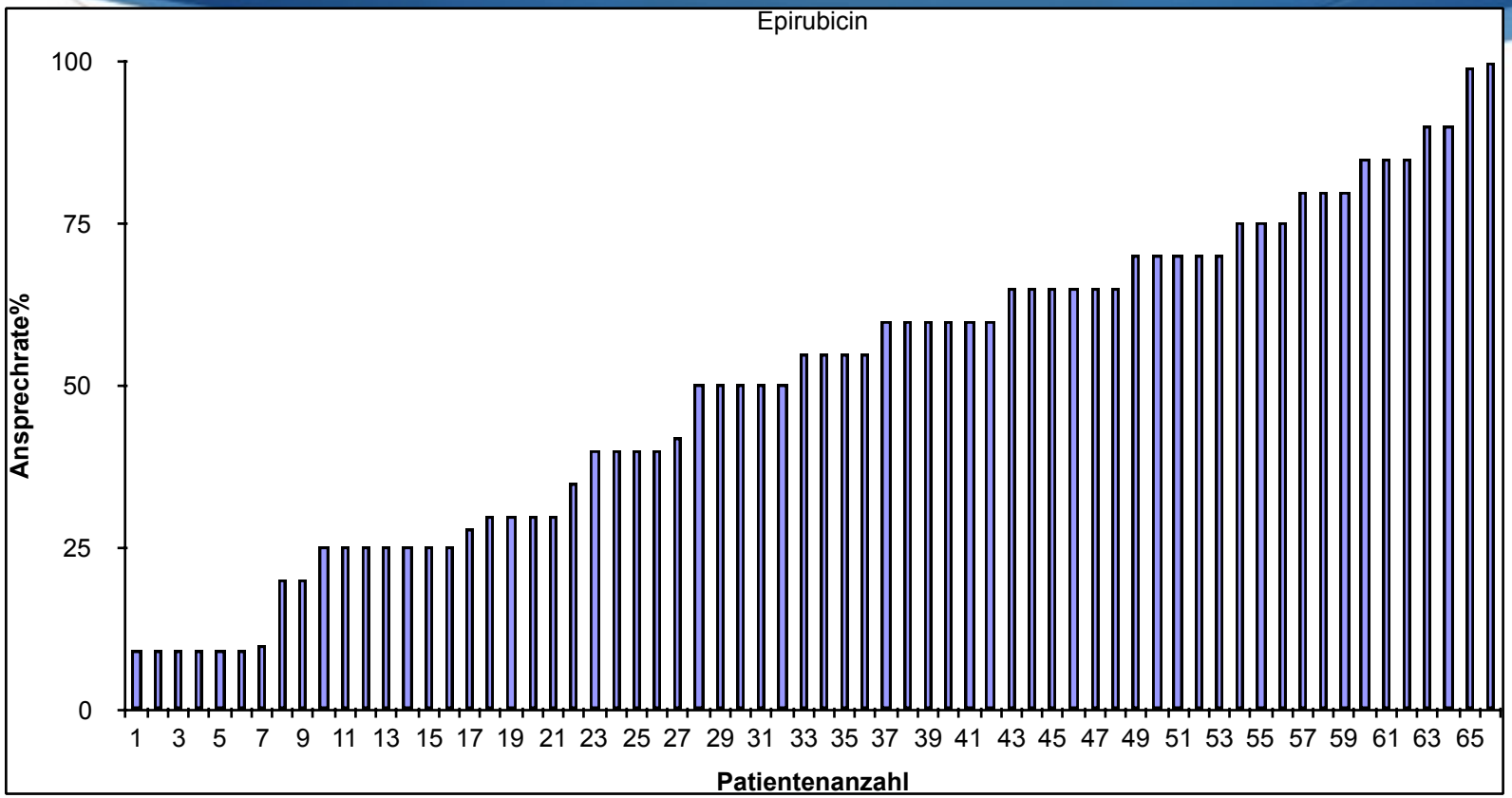
TZB

Correlation between *in vitro* chemosensitivity and *in vivo* reduction of CETC

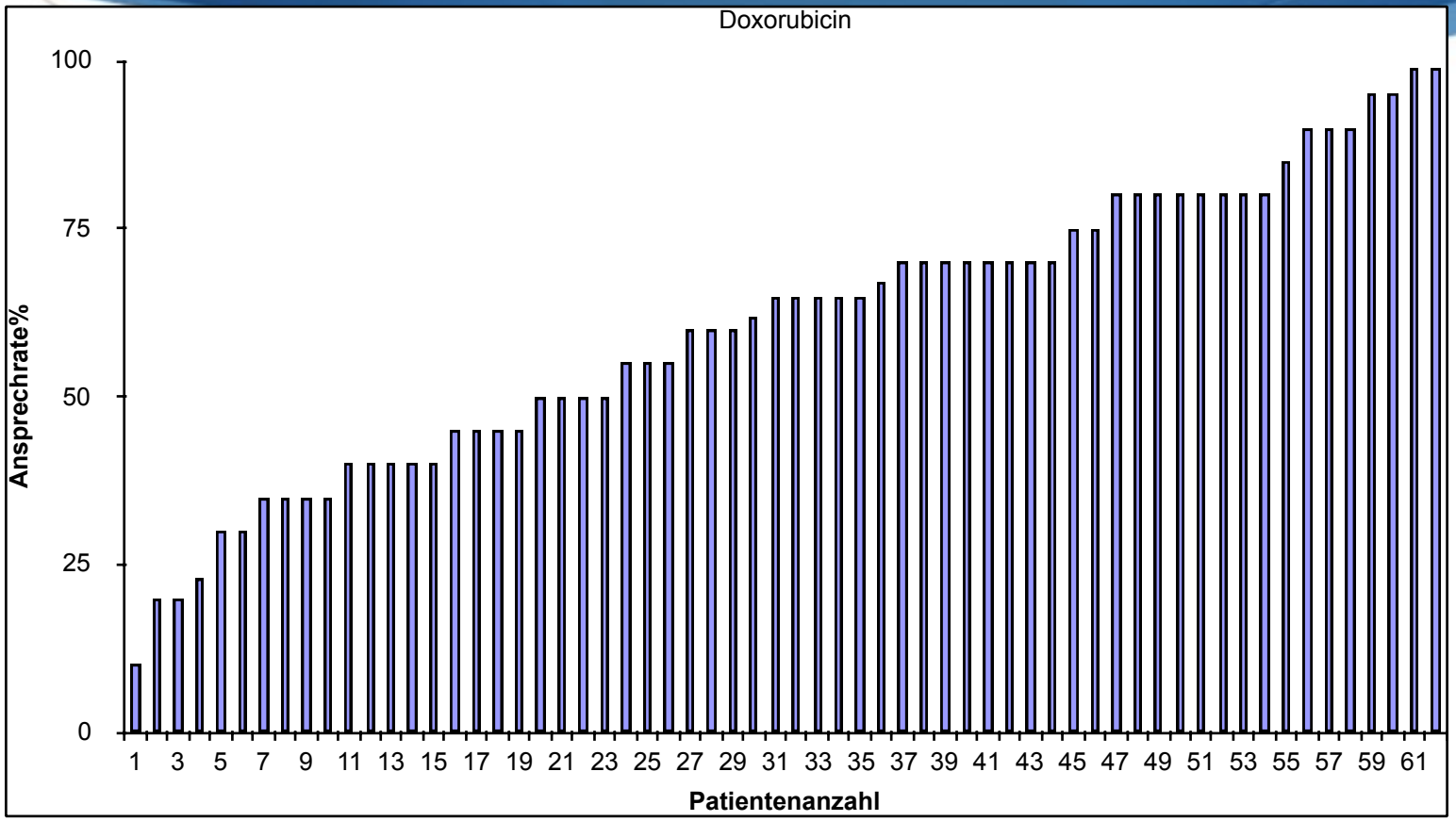


Correlation between CETC reduction/increase and clinical outcome

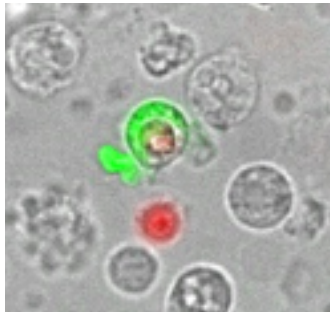
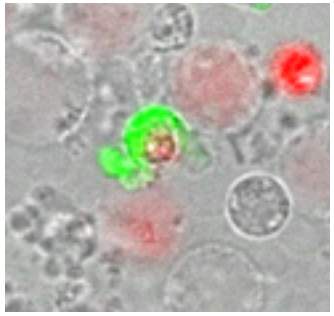
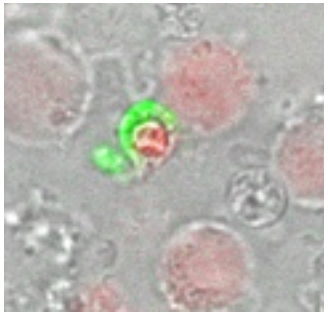
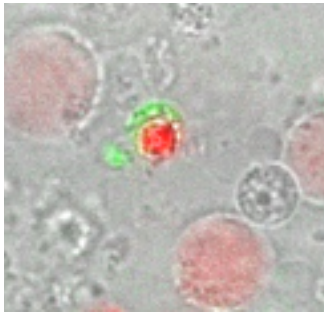
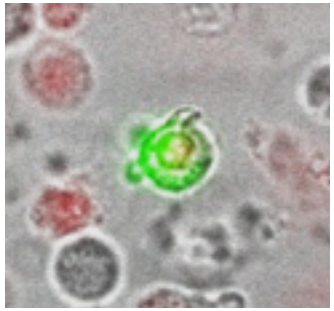
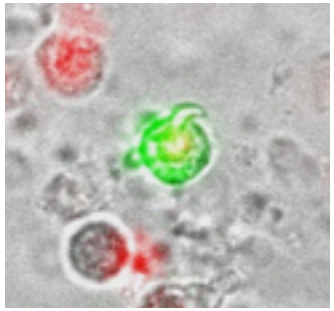
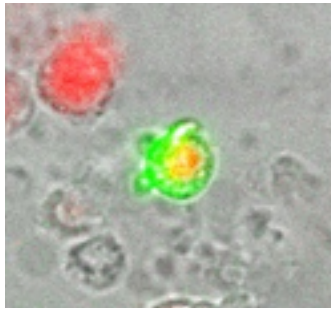
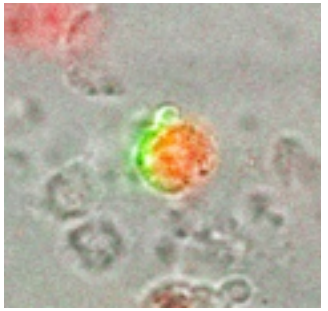
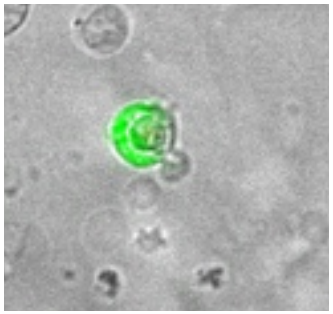
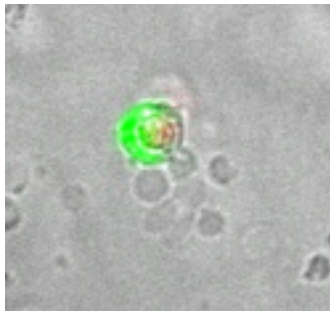
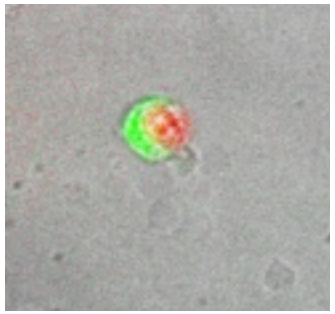
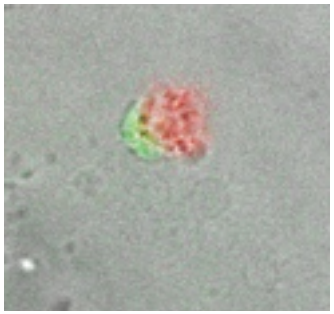




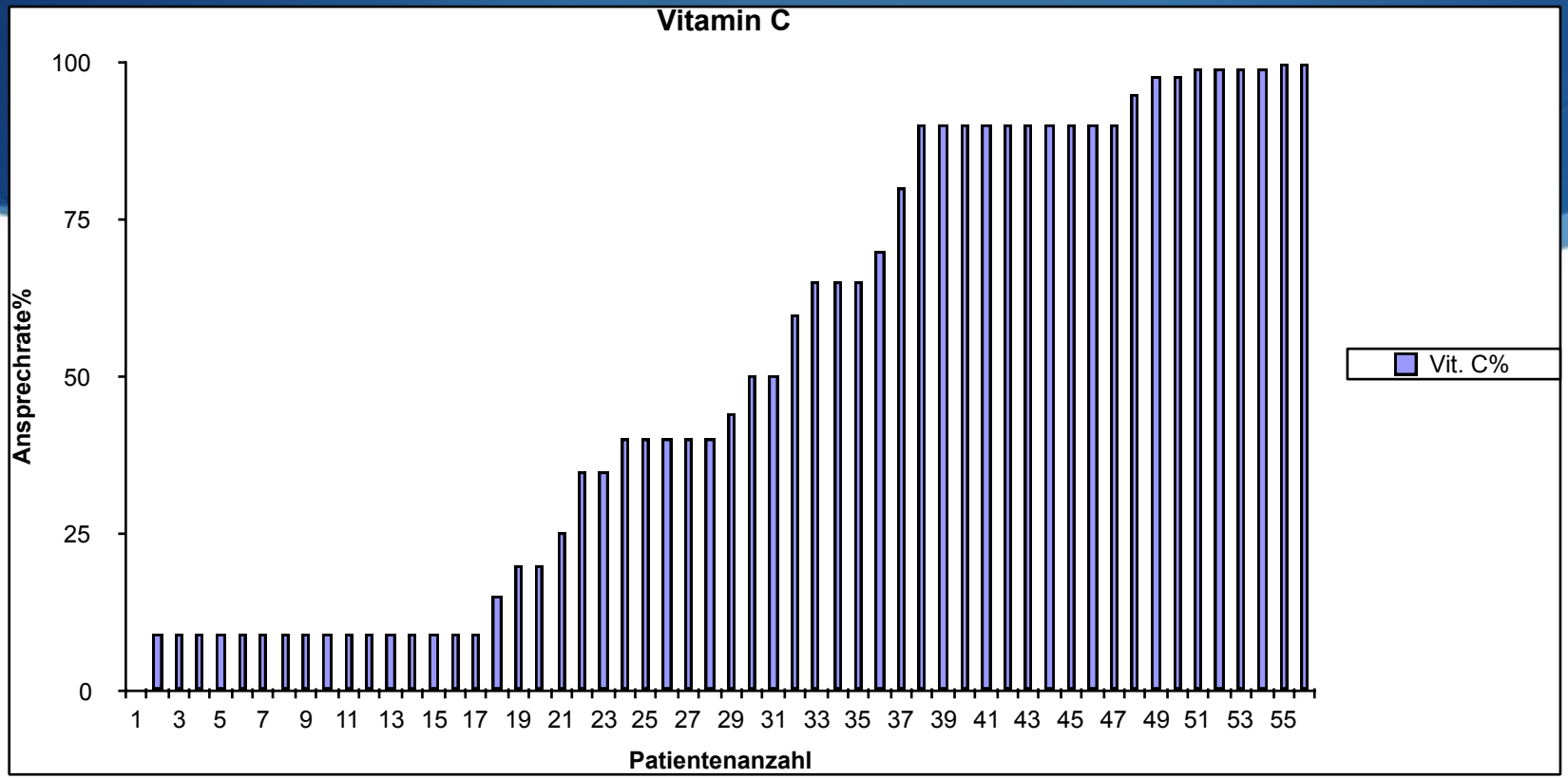
Patients total: 66		
Sensitivity > 50%	34 Patients	52%
Sensitivity < 50%	32 Patients	48%



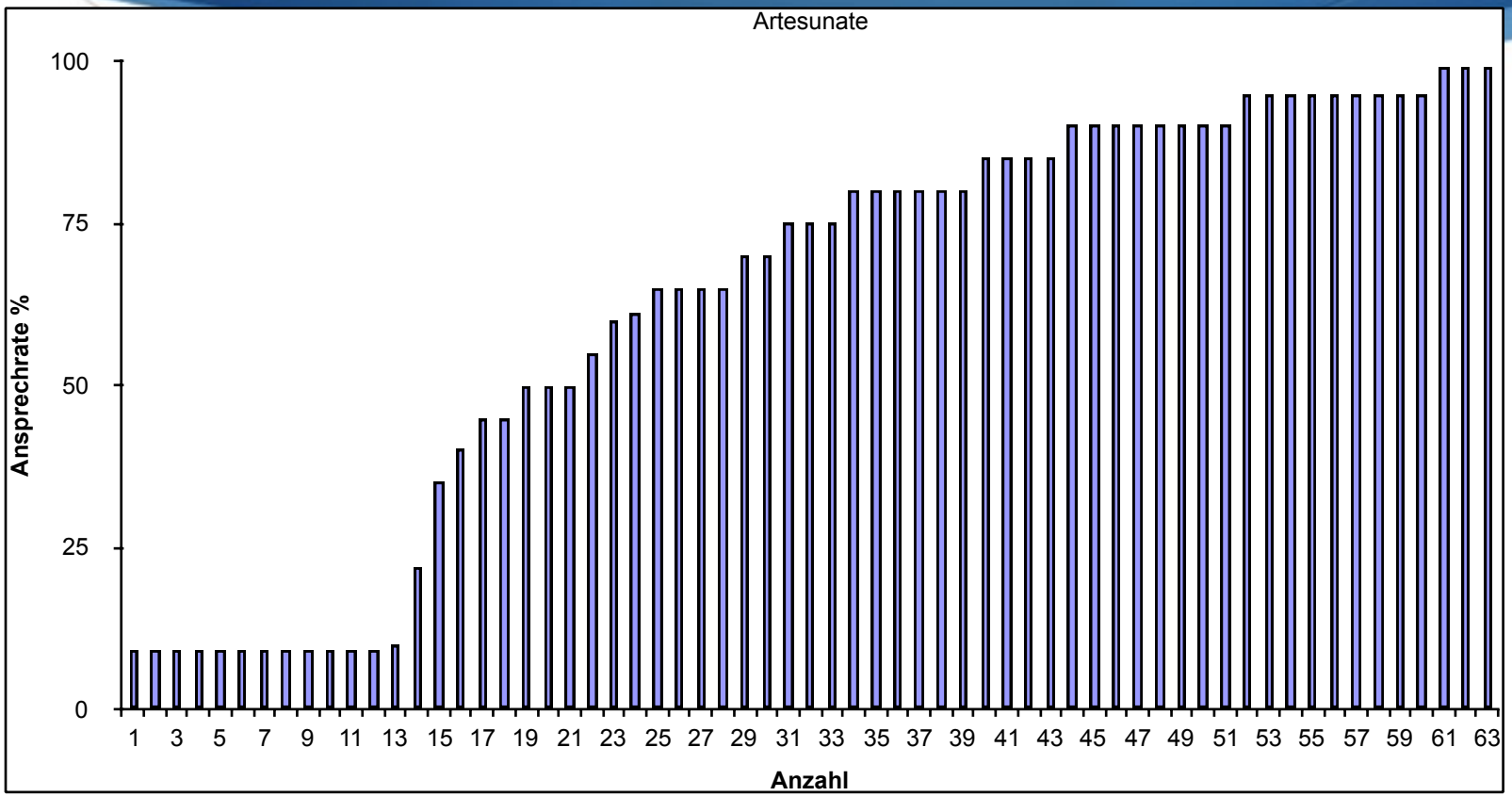
Patients total: 62		
Sensitivity > 50%	39 Patients	63%
Sensitivity < 50%	23 Patients	37%

	t=0 Std.	t=3,5 Std.	t=7 Std.	t=10,5 Std.
Docetaxel				
Epirubicin				
Mafosfamid				

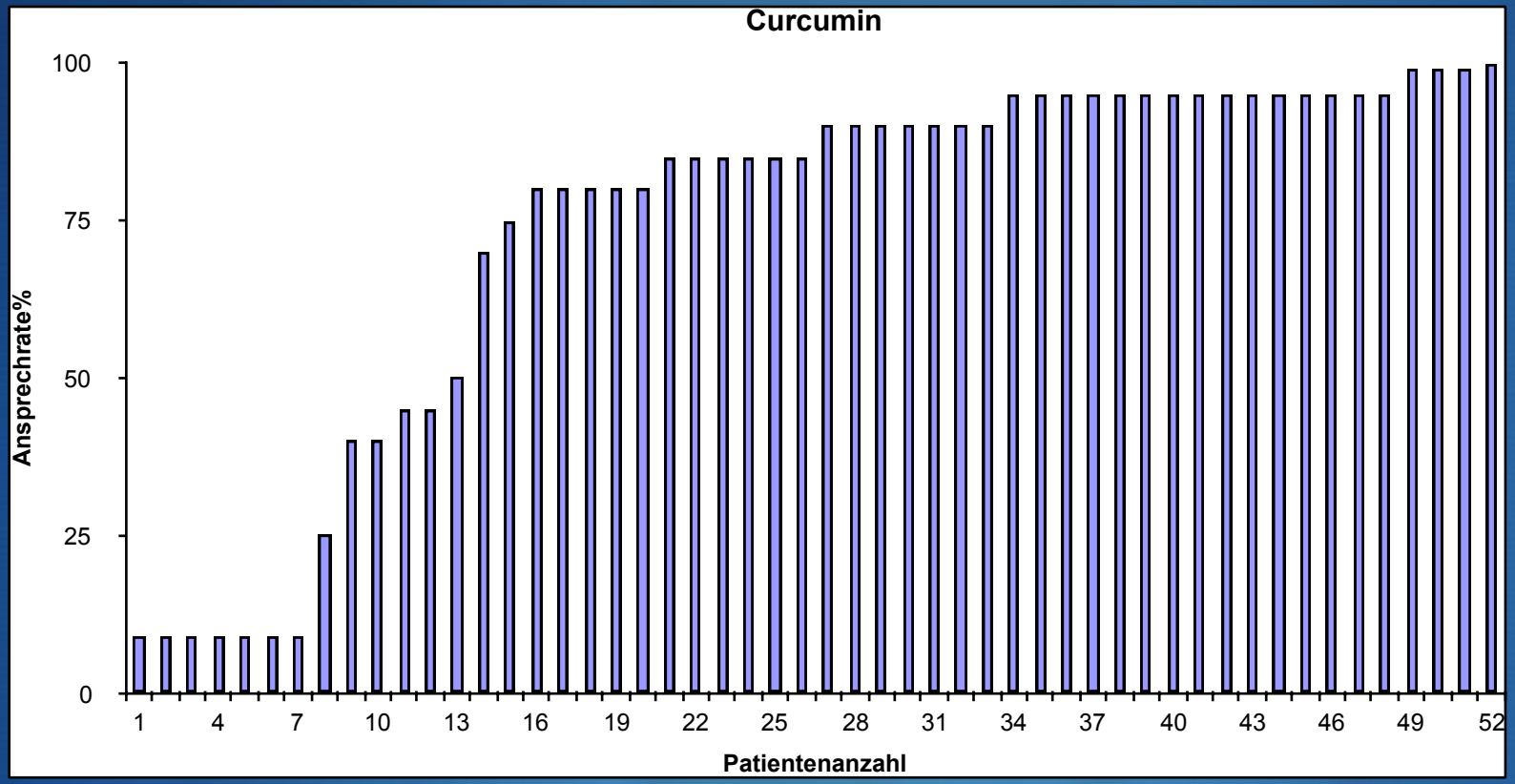
TZB



Patients total: 56		
Sensitivity > 50%	25 Patients	45%
Sensitivity < 50%	31 Patients	55%



Patients total: 63		
Sensitivity > 50%	42 Patients	67%
Sensitivity < 50%	21 Patients	33%



Patients total: 52		
Sensitivity > 50%	39 Patients	75%
Sensitivity < 50%	13 Patients	25%

Prioritisation of natural agents suggested by the results

The automated microfluorimetric image analysis of the **epithelial cell adhesion molecule (EpCAM)**-positive cells with visual control (MAINTRAC) from **1 ml EDTA blood** resulted in following findings (detection limit is at 10 cells/ml):

	Number of potential tumor cells			
Examination parameter	In the sample (1ml)	In circulation (5l) (in millions)	In addit. examination: % of EpCAM-pos. cells	Cell fragments
EpCAM	**500**	**2,5**		numerous

in-vitro-vitality reduction in relation to concentration and time (in%) with eutherapeutic concentrations of				
Vitamin C	**70**	DCA	**60**	The ideal is a reduction by 100% in short-term cell culture
Amygdalin	**70**	Curcuma*	**40**	
Artesunat	**95**	Prosanalin*	**85**	
Boswellia*	**60**			

*provided by the patient

Great flexibility

- Test natural agents for their cytotoxicity against the patient′s own cancer cells
- Send in own selection of agents (small sample required)
- And/or select from lab's list of suggestions
- Test the same agent as an infusion and an oral supplement – often very different results
- Test mixtures in one formula – you choose the combination

Maintrac sensitivity to natural agents is available in three levels of concentration

in-vitro-vitality reduction in relation to concentration and time (in%) with eutherapeutic concentrations of The ideal is a reduction by 100% in short-term cell culture					
Quercetin 0,1-fold	**85**	Quercetin 1-fold	**90**	Quercetin 10-fold	**99**
Vitamin C 30g 0,1-fold	**55**	Vitamin C 30g 1-fold	**75**	Vitamin C 30g 10-fold	**90**
Artesmisinin 250mg 0,1-fold	**25**	Artesmisinin 250mg 1-fold	**90**	Artesmisinin 250mg 10-fold	**98**
Curcumin 450mg 0,1-fold	**n.a.**	Curcumin 450mg 1-fold	**90**	Curcumin 450mg 10-fold	**n.a.**

Strength of different combinations

	Number of potential tumor cells			
Examination parameter	In the sample (1ml)	In circulation (5l) (in millions)	In addit. examination: % of EpCAM-pos. cells	Cell fragments
EpCAM	**550**	**2,75**		numerous

in-vitro-vitality reduction in relation to concentration and time (in%) with eutherapeutic concentrations of				
Quercetin +Artesunate	**85**	Vitamin C +Curcumin	**60**	The ideal is a reduction by 100% in short-term cell culture

Change in effectiveness of agents over time

2017

	Number of potential tumor cells			
Examination parameter	In the sample (1ml)	In circulation (5l) (in millions)	In addit. examination: % of EpCAM-pos. cells	Cell fragments
EpCAM	**200**	**1**		some

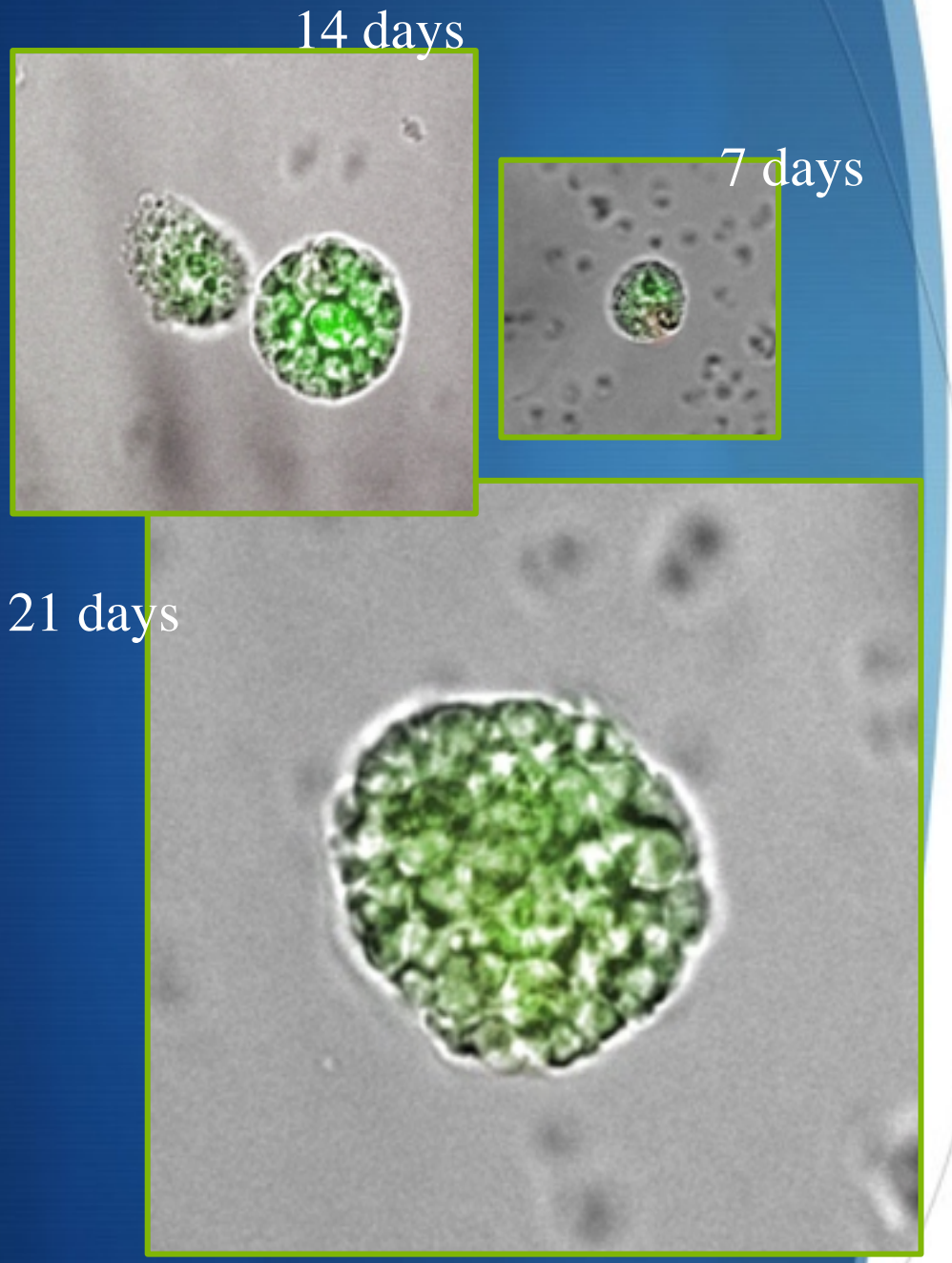
in-vitro-vitality reduction in relation to concentration and time (in%) with eutherapeutic concentrations of				
Vitamin C	**<10**	Capecitabine	**80**	The ideal is a reduction by 100% in short-term cell culture
Artesunat	**50**			

2019

in-vitro-vitality reduction in relation to concentration and time (in%) with eutherapeutic concentrations of				
5FU	**20**	5FU/ Honokiol	**25**	The ideal is a reduction by 100% in short-term cell culture
5FU/ Curcumin	**80**			

Honokiol = Magnolia

Clonal expansion of circulating tumour cells



Tumour spheres from CETC

Spheres were detected in 86 out of 109 patients **(78.9%)**;

Number of spheres varied between 50 and 1700/ml (median 200)

All spheres detected **are positive for EpCAM**.

Tumour spheres

Cancer Res 2013;73(24 Suppl): Abstract nr PD6-1

Tumour spheres growing from peripherally circulating tumour cells exhibit stem cell features

Abstract

Background: Among the cells that are disseminated from a malignant tumour only very few are capable to resettle in distant organs and grow into life-threatening metastases. Therefore, the question arises how and whether such cells which have the potential to grow into metastases can be detected. It has been shown that a subpopulation of cells from breast cancer tissue can form so-called mammospheres with stem cell features. Here we show that such tumour spheres can also be grown from peripherally circulating tumour cells from breast cancer patients in different stages of disease

Materials and Methods: Using a nondissipative approach with only one enrichment step of red blood cell lysis, the cells from the pellet, containing the white blood cells together with the putative tumour cells were cultured under conditions favoring the growth of epithelial cells. At 7, 14 and 21days the cell cultures were inspected for the appearance of spheroids staining with anti-EpCAM, anti-CD24 and anti-CD44 antibody and.expressing ALDH1.

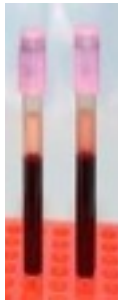
Results: Peripherally circulating cells from patients with malignant tumours in different stages of disease were analyzed for the presence of circulating epithelial tumour suspect cells and the frequencies of tumourspheres. tumourspheres could so far be grown from 79% of 36 patients in whom more than 1700/ml epithelial tumour suspect cells were detected. Numbers of tumourspheres varied from 1 to 29 /ml and correlated with the aggressiveness of the tumour. Surprsingly the numbers were highest in patients after surgery who had not yet received any systemic therapy. The size of the spheres increased from day 7 to day 21. The spheres were negative for CD24 and positive for CD44. They highly express ALDH1 and thus exhibite typical features of stem cells.

Conclusion: Here, we demonstrate that the circulating tumour cells, detected in our approach contain a subpopulation with stem cell-like properties capable of growing into tumourspheres. The frequency and growth potential of cells capable of forming spheres seems to be dependent from the properties of the primary tumour. The possibility to grow tumourspheres from peripherally circulating tumour cells may open up a new field, where the relevant cells with stem cell properties from individual patients can now be specifically analysed further for genetic endowment, transcriptional activity, heterogeneity and stem cell markers.

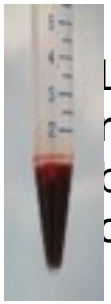
http://cancerres.aacrjournals.org/content/73/24_Supplement/PD6-1.short

TZB

Stemtrac®

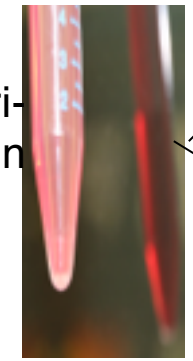


1 ml EDTA Blood

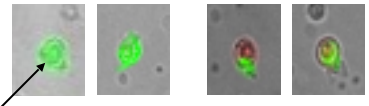


Lysis of red blood cells

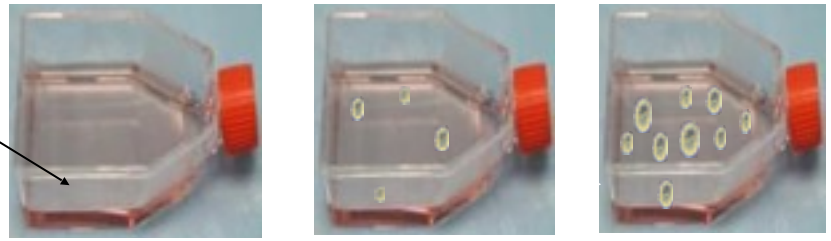
One centri-fugation step



+ Anti-EpCAM

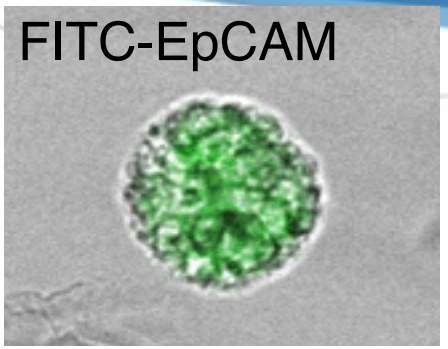


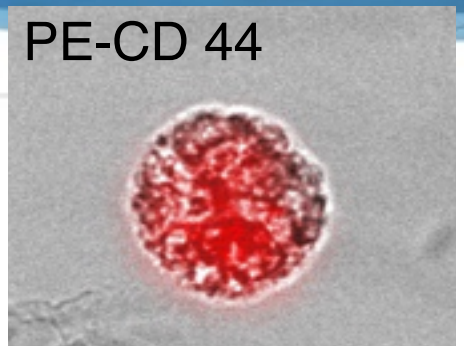
Determine live and dead EpCAM- positive cells among the white blood cells

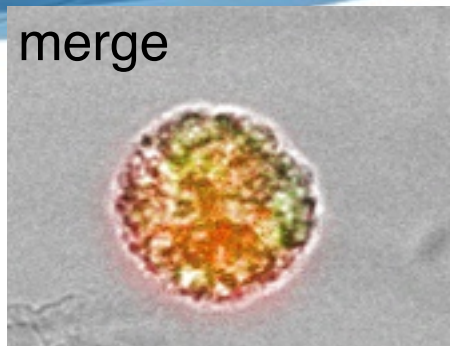


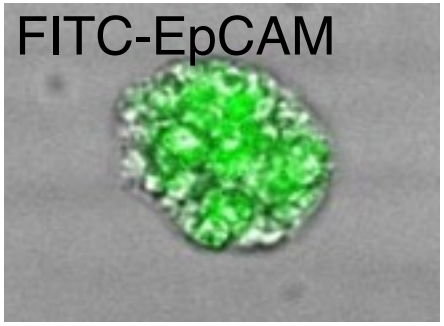
Culture of all white blood cells under conditions favouring growth of epithelial cells and determination of sphere formation at different times of culture

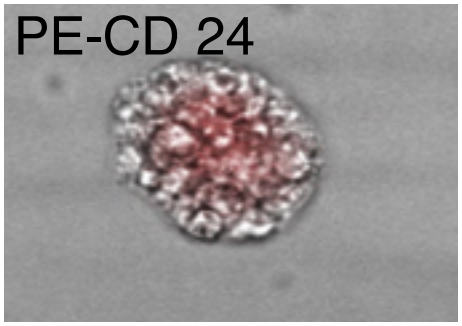
Stem cell marker expression in tumour spheres

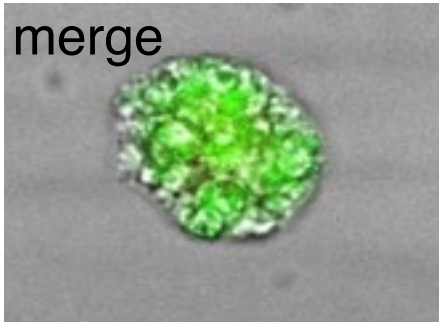


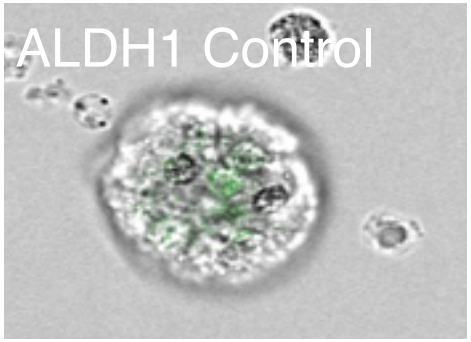


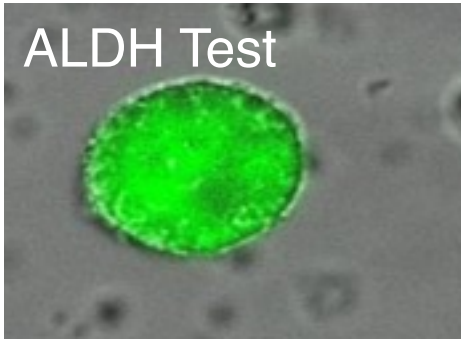






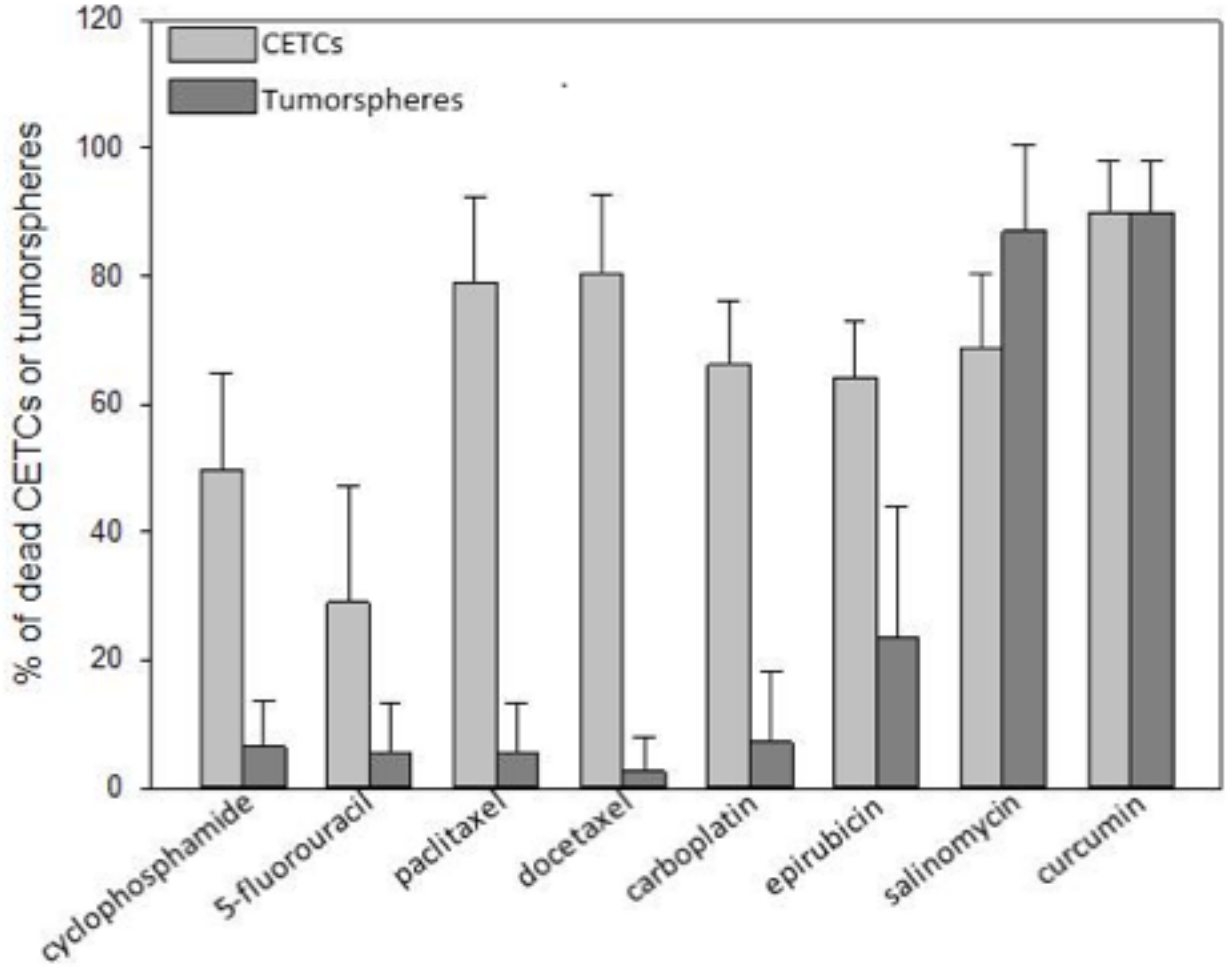






Chemo-sensitivity of tumour spheroids

Chemosensitivity of tumour spheroids vs. CETCs



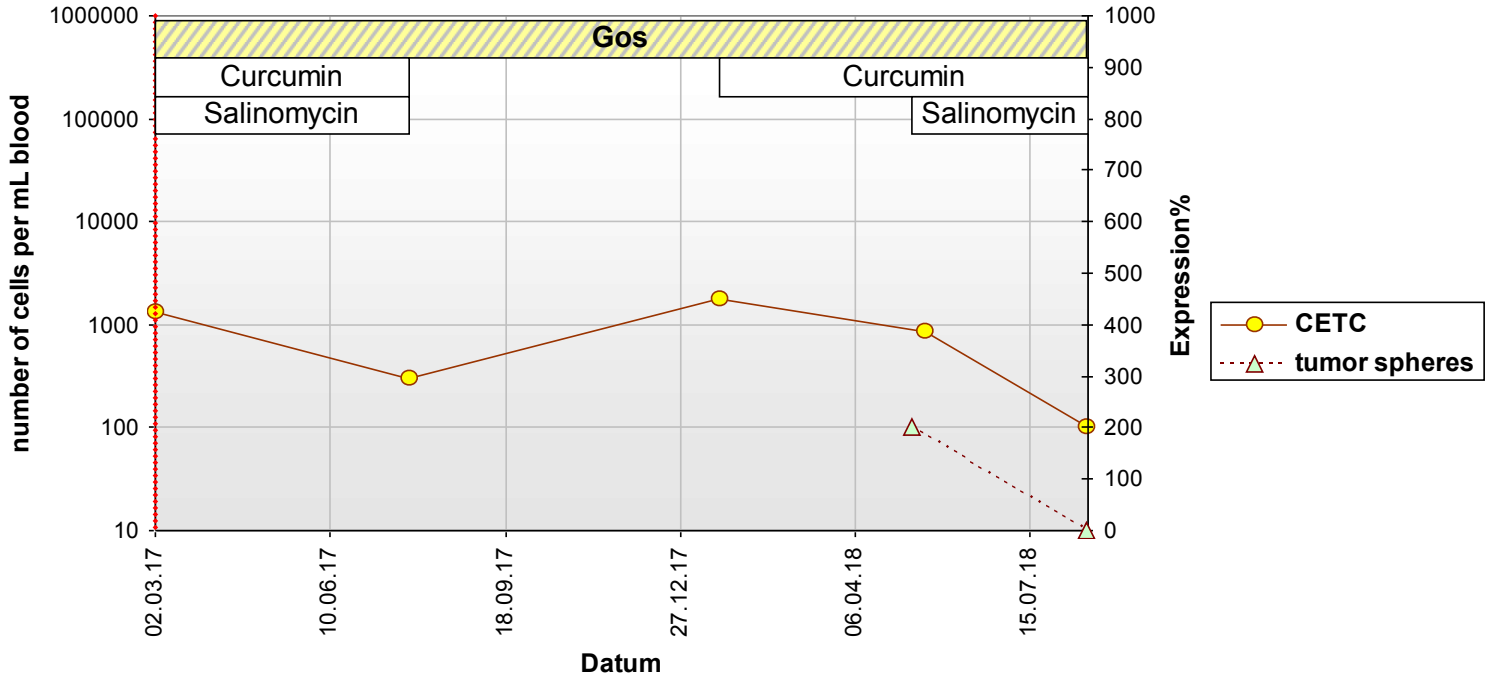
Effectiveness of agents against circulating stem cells

	Number of potential tumor cells			
Examination parameter	In the sample (1ml)	In circulation (5l) (in millions)	In addit. examination: % of EpCAM-pos. cells	Cell fragments
EpCAM	**100**	**0,5**		some
Circulating Cancer Stem Cells	**250**			

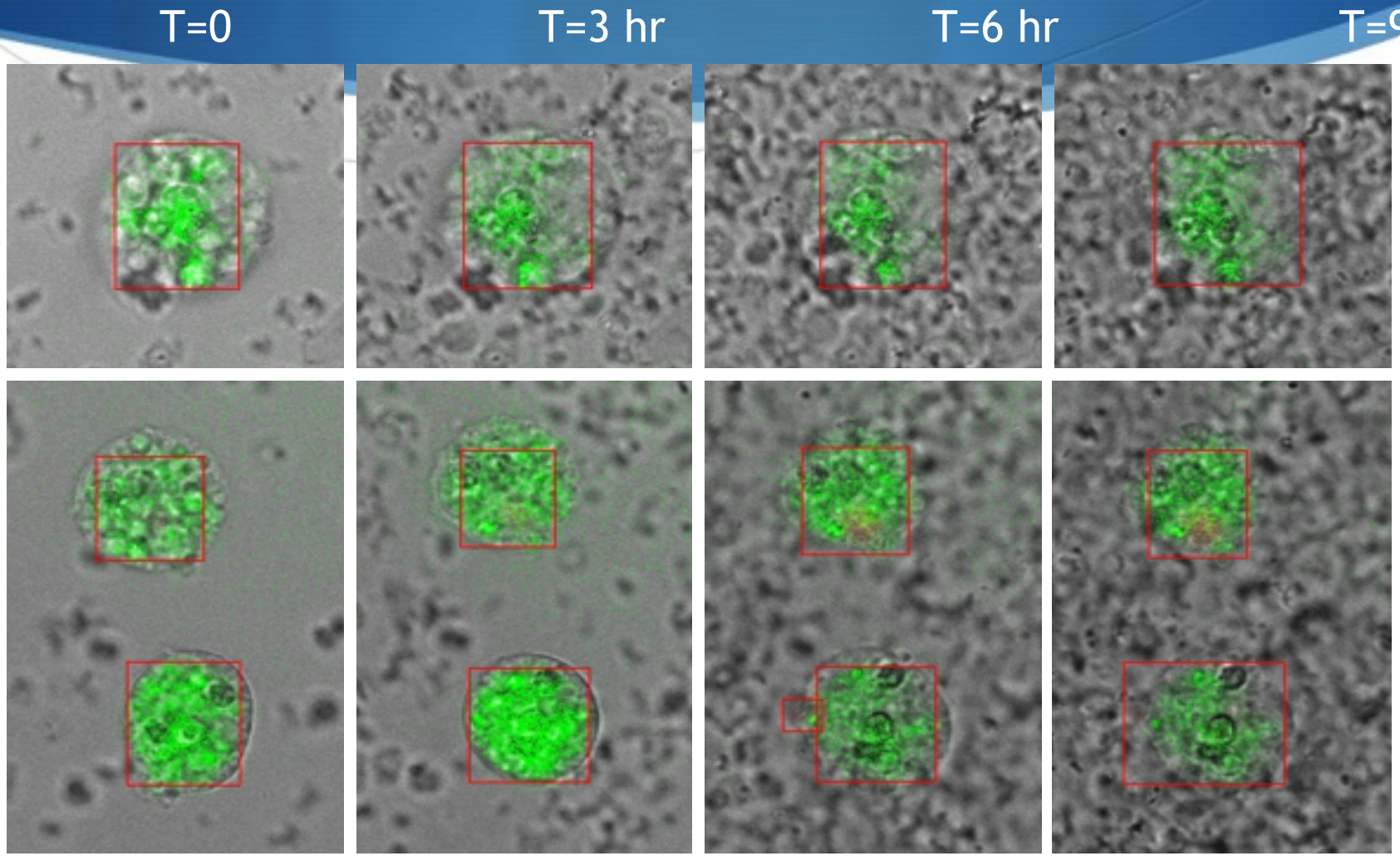
in-vitro-vitality reduction in relation to concentration and time (in%) with eutherapeutic concentrations of				
Curcumin	**90**		Salinomycin	**50**

The ideal is a reduction by 100% in short-term cell culture

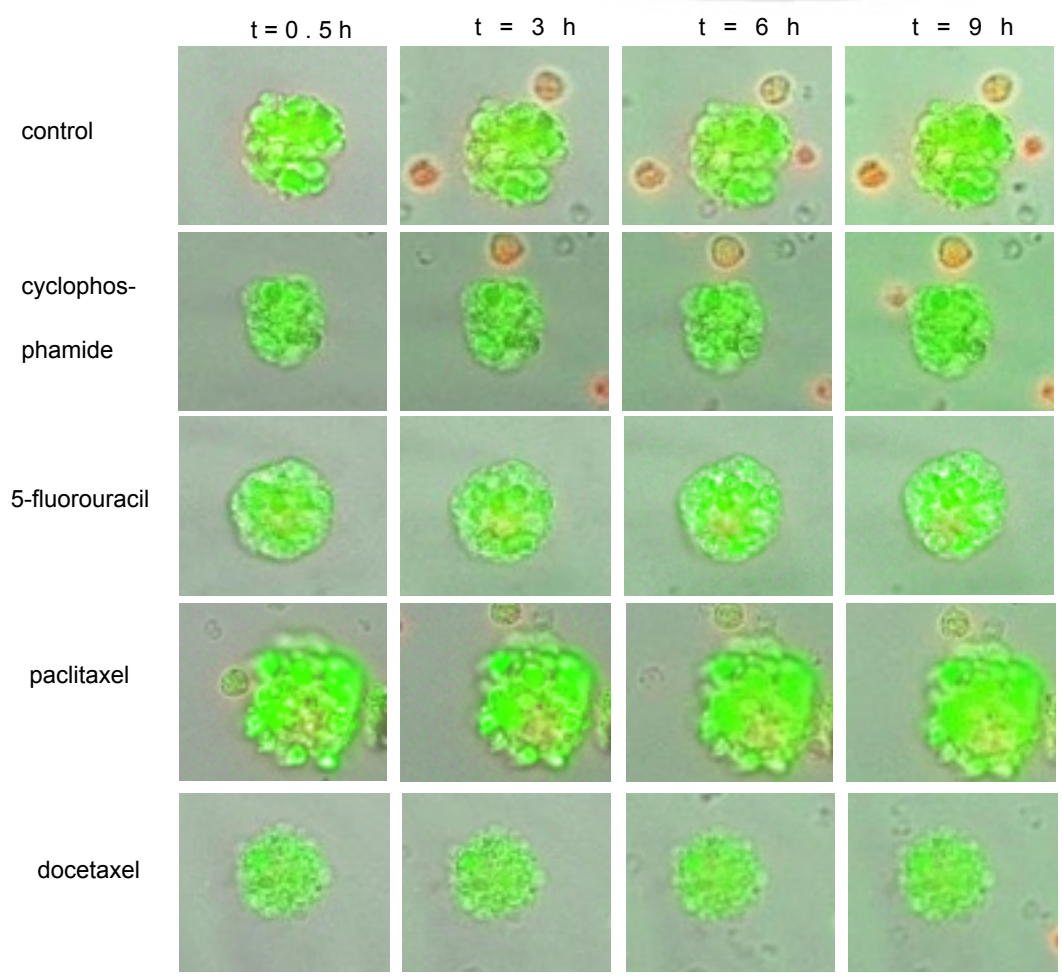
Effectiveness of agents against circulating stem cells



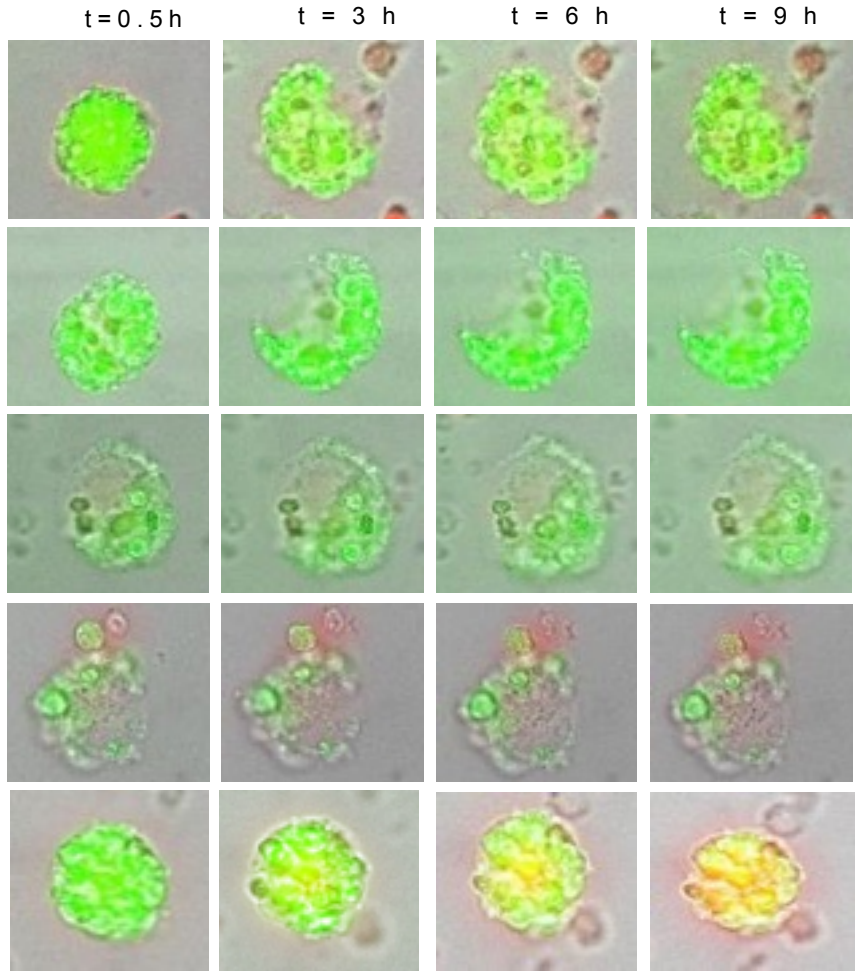
Cancer stem cells are particularly sensitive to curcumin



Fascinating to see the effectiveness of salinomycin and curcumin



Examples of tumourspheres with chemoresistance to cyclophosphamide, 5-fluorouracil, paclitaxel and docetaxel. The tumour spheres remain alive during the culture (0-9h).



tumourspheres sensitive to carobplatin, epirubicin, salinomycin and curcumin. Carboplatin and epirubicin lead to disintegration of tumourspheres with destruction of part of the cells in the spheroids. The strong cytotoxic effect of salinomycin is already observed at the first point of measurement with almost total destruction of all cells. Curcumin works by inducing cell death in all cells of the tumourspheres leading to nuclear staining with propidium iodide.

TZB

Dynamics of CETCs as a **parameter**
for personalised therapy decisions

- Is non-invasive, repeatable, reproducible
- Is **quantitative**, so can often **detect** occurrence/recurrence **before** it **could be found in imaging**, and identify points at which imaging would be prudent
- Monitors the **success of therapy**

→ **Maintrac helps to find personalised and effective therapy**

TZB

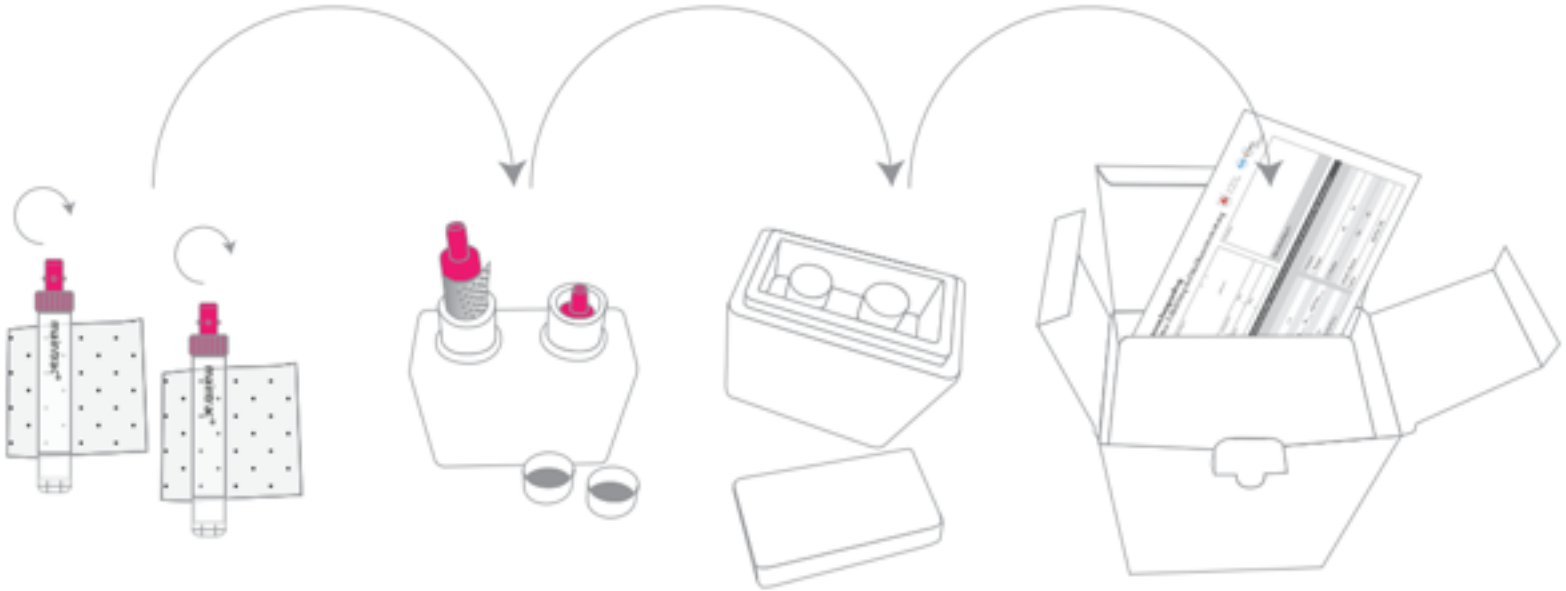
Fully accredited laboratory

Please explain the
two accreditation
how many scienti
have, and how ma
counts

TZB

Blood collection kit

Sample packaging



Shipping and results

Within 48 to max. 72 h at room temperature

to our lab in Bayreuth, Germany

Results will be sent usually 5 days after receiving the sample.

For more information about Maintrac/Stemtrac and CTC testing please contact:

info@aonm.org
03331 210 305
www.aonm.org/maintrac/

Thank you
for your attention