

# How Circulating Cancer Cells Disguise: the Role of Platelets

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## Short Report

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# Abstract

Solid tumors are notorious for their ability to form lethal metastases, sometimes several decades following initial cancer diagnosis. Development of distant metastases is a result of the primary tumor shedding cells that travel via lymphatics and the blood to distant sites where they can form metastases. Platelets are known to specifically enhance tumor cells' survival in the bloodstream by as yet poorly understood mechanisms. To study the interplay of platelets with circulating tumor cells, we implemented our published approach to label both circulating epithelial tumor cells and platelets. Blood samples were collected avoiding fixation from patients with non-metastatic cancer diagnoses and processed at 4 time points following blood collection. Circulating epithelial tumor cells were undetectable directly after blood collection but became visible after overnight storage at room temperature presumably due to release of platelets from the tumor cells. Our results suggest that platelets play a key role in masking circulating tumor cells. Masking may explain the difficulties in detection of these cells and prevention of their elimination by the immune system. Our unmasking approach detects sufficient numbers of circulating tumor cells to monitor the effect on blood tumor cells of different therapeutic measures, thus contributing to improved systemic therapies for cancer.

## Introduction

The most devastating aspect of malignant tumors is their ability to form distant metastases in vital organs, eventually leading to a fatal outcome. The "embolic theory" was proposed 140 years back to explain why metastases could occur in spite of a "wide interval of healthy structure" in the intervening lymph vessels[1]. In contrast, the "permeation theory" purported that tumors metastasized by centrifugal, contiguous spread and maximally extensive surgery was necessary to cure breast cancer[2]. Yet, even 30% of lymph node-negative patients receiving extensive and often mutilating mastectomy still died from distant relapse.

It took another 50 years to confirm that disseminated tumor cells were the cause of distant relapses, making, for example, breast cancer a systemic disease from the outset. This was confirmed by the success of systemic chemotherapies designed to eliminate these disseminated cells[3]. Although this postulated that tumor cells should be present in the blood, most groups were unable to detect such cells directly in freshly drawn samples or samples drawn into preservatives to retain their current status quo[3]. Subsequent therapies were performed in a "blinded way" causing a statistically significant but "modest" improvement in outcome[3]. But even with adjuvant chemotherapy, metastases can still occur up to 30 years after primary tumor diagnosis, indicating that long-term tumor cells must be able to survive in patients' blood [4]. But why are these cells so elusive?

Already in our earlier standardization studies[5] it turned out that the numbers of epithelial antigen positive cells, presumably tumor cells from a sample drawn into EDTA anticoagulant tubes remained stable from day 1 after blood drawing up to 4 days in room temperature.

Such cells are detectable in all cancer patients at almost all times during the course of disease, that the numbers of circulating epithelial tumor cells (CETCs) retrieved are dependent on surgery[6, 7] and chemotherapy[8, 9], and the trajectory of numbers (increase or decrease) correlates with poor or favorable clinical outcome<sup>9</sup>. Thus, in contrast to other groups, we apparently were successful in detecting cells in blood the presence of which was related to tumor outcome: most possibly circulating tumor cells before metastases were apparent.

Only during the further development of the test it became evident, that indeed, like for other research groups, CETCs were rarely identifiable at the day of blood draw in freshly drawn blood. The question arose, why this was possible after one day of the sample resting at room temperature.

Platelets play an important role in cancer metastasis (promoting tumor cell migration, invasion and control of tumorangiogenesis)[10–12], and they promote the survival of circulating tumor cells<sup>13</sup>, but the exact mechanism by which this latter function is achieved is not fully understood. In this study, we investigated the interaction of platelets with CETCs. Blood samples were processed at 4 timepoints and visualized following specific antibody staining for CETCs and platelets. Our results suggest that future approaches for circulating tumor cell detection should address platelets' masking effects on CETCs and their key role in the metastatic potential of CETCs.

## Results

In 86 patients that had presented at our center (Transfusion Center Bayreuth, TZB) for blood draw CETCs were detected in none of the patients at day zero but in 94% of the patients more than 50 cells/ml were detected on day 1. No CETCs were observed in 2 individuals with no cancer diagnosis, neither on day zero, nor on day 1.

Platelets are difficult to detect in transmitted light, due to their small size and lack of a nucleus. However, platelets (in their resting and activated stages) abundantly express CD36, a protease-resistant surface glycoprotein IV scavenger receptor that contributes to platelet adhesion and collagen dependent thrombus stabilization[14]. CETCs express epithelial cell adhesion molecules (EpCAM). Thus, we were able to label platelets with anti-CD36-phycoerythrin (PE) antibodies (red staining) and CETCs with anti-EpCAM-fluorescein-isothiocyanate (FITC) antibodies (green staining).

Typically, at 0 hours (h) post-blood draw and processing from patients with non-metastatic cancers, we observed platelet aggregates strictly attached to single cells that did not stain with anti-EpCAM antibody (Fig. 1). These clogged unstained cells were, by far, outnumbered by surrounding white blood cells with no platelets attached.

After keeping blood samples at room temperature for 24 h, then processing an aliquot of the sample in an identical way to time 0h, a completely different picture became apparent: there were still cells detectable with platelets attached to them but the underlying cell now became accessible to the anti-EpCAM

antibody leading to green fluorescence staining (Fig. 2). The increase in single platelets in the background could be a sign that after 24 h, platelets detach from CETCs.

We also observed that many platelets had changed their shape to become spread out into multilobular particles with pseudopodia (Figure. 2), like those seen during activation.

After another 24h platelets had moved into caps at a pole of the tumor cell and the EpCAM staining became brighter (Fig. 3).

At 72 h following initial blood drawing, platelets had almost completely disappeared from the cell surface (Fig. 4a). Numbers of EpCAM-positive cells remained stable between days 1 and 3, as reported previously [5].

These changes over time were seen in all 10 unfixed samples. Note that when the blood had been drawn into Streck-Tubes containing a fixative, platelet-clogged cells were observed in most patients at all time points and no EpCAM staining occurred at any time (Fig. 4b).

## Discussion

In most solid malignant tumors, no metastases are present at diagnosis but still, up to 50% of patients suffer relapse, which is assumed to be due to cells shed either directly into the circulation or via the lymphatic system. Lymph nodes are no barrier to dissemination[15]. The key questions are when and how dissemination occurs, how disseminated cells survive, and what induces them to form metastases. In blood, tumor cells are not readily detectable. In fact, we have noted that in freshly drawn unfixed blood samples, circulating tumor cells become detectable only after the sample rests at room temperature overnight or is treated with a surfactant[16], indicating that their surface molecules initially are somehow masked.

A critical step for distant metastasis formation is survival of cancer cells in the bloodstream. The main threats to circulating tumor cells are shear stress and natural killer (NK) cytotoxic cells. It has been reported that platelets protect circulating tumor cells[17], which correlates with the tumor cells' abilities to aggregate platelets[18]. Indeed, platelets are known to bind benign exfoliated epithelial cells that enter the circulation (e.g. upon wounding). Whereas normal epithelial cells may not be capable to proliferate, and seem to become eliminated after days or weeks[7] circulating tumor cells persist[13] when coated with platelets[19]. Surface coating of the tumor cells with platelets may serve as a shield against immune assault because the effect of anti-tumor attacks mediated by NK cells is primarily based on the direct interaction with circulating tumor cells[20].

Platelets that coat CETCs also prevent antibodies from accessing the tumor cell surface and it is likely the reason for the inability to detect these cells in freshly drawn blood. This evasion of circulating tumor cells from immune recognition may also be the reason why anti-EpCAM- antibodies have, so far, not been

successful in treating epithelial tumors[21]. Drugs that theoretically would be effective may not be able to access the clogged cells, an important consideration in adjuvant or neoadjuvant systemic chemotherapy.

In our study, platelet inactivation on day 1 was likely due to "platelet storage lesion", a phenomenon resulting in the platelet's disc-to-sphere transformation and known to be associated with reduced responsiveness to adenosine diphosphate for platelet aggregation[22]. We believe our observation was due to hypoxia, an effect of the sample resting on the bench. Platelets were released from the epithelial cells during overnight storage, making the CETCs accessible to the specific antibody. The subsequent cap formation may be a particular characteristic of epithelial cells[23] and the joint movement of EpCAM on the tumor cells with the platelets is suggestive of a preferential binding of the platelets to EpCAM. Activation of platelets did not lead to thrombus formation due to the coagulation-inhibitory environment in this artificial system.

Our *in vitro* observations may differ from what occurs *in vivo*, where an intact coagulation system is present. The association between abnormalities of haemostasis and cancer is described as recurrent migratory thrombophlebitis[24] with platelet-rich microthrombi in the vasculature. The link between cancer and risk of venous thromboembolism (VTE) has been well-documented[25]. Cancer patients on chemotherapy are 47 times more likely to experience VTE[26] and this correlates with the metastatic potential of cancer cells.

Circulating platelet/tumor cell complexes (like the ones visualized in this study) may arrest in thrombi or sites where the blood flow slows down, like the liver, lung or the bone marrow, contributing to metastasis formation in these organs. Under physiological-normal conditions, the endothelium prevents thrombosis by providing a surface that discourages the attachment of cells and clotting proteins. Upon vascular injury, initiation of clot formation occurs, with resultant activation of the endothelium[27]. Activated endothelial cells express a variety of molecules and receptors that increase platelet adhesion to the site of injury. If, accidentally, a platelet covered tumor cell is trapped in the clot, platelets detach from the tumor cells. At the same time they release growth factors such as platelet-derived growth factor, vasoactive agents and chemokines, and pro-angiogenic growth factors (VEGF). A small fraction of tumor cells subsequently clonally expand to form tumor metastases.

## Conclusion

In this work, we could show that circulating tumor cells are covered by platelets in cancer patients. We show proof-of-principle that coated circulating tumor cells exist in significant numbers directly after blood draw thereby impeding their visualization with antibody fluorescence staining. Storage of blood samples overnight at room temperature activates platelets which led to the detachment of platelets from circulating tumor cells. Further studies are needed to determine the clinical significance of platelet-coated circulating tumor cells.

## Materials And Methods

During the last 3 years an analysis was done at the same day of blood draw and 24 h later after the samples had been left at room temperature in 86 patients that had presented at our center (Transfusion Center Bayreuth, TZB) for blood draw for the determination of circulating tumor cells (54 breast, 9 prostate, 5 colon, 4 lung cancer, 12 other cancers and 2 with no cancer diagnosis). We used our previously published maintrac® approach [5] to isolate and detect CETCs from the blood samples. Briefly, all blood draws were collected into blood count tubes containing the anticoagulant EDTA but without any fixative[5]. We more closely analyzed 10 blood samples from patients with breast (n = 5), prostate (n = 3) and colon (n = 2) as their primary sites of cancer which had been collected with informed consent into either blood count tubes containing the anticoagulant EDTA but without any fixative[5] and Streck tubes (containing fixative). Samples were maintained at room temperature and 1 ml of blood was processed at each of four time points: 0 h, 24 h, 48 h, and 72 h after first blood collection. For each time point, we used an immunocytochemistry approach to identify CETCs. After red blood cell lysis, cell pellets were stained with a fluorescein-isothiocyanate (FITC)-conjugated anti-human epithelial cell adhesion molecule antibody (EpCAM) (clone HEA-125, Miltenyi Biotec GmbH, Germany) at a final concentration of up to  $10^7$  cells/100  $\mu$ l. The corresponding isotypic control for EpCAM (mouse IgG1K FITC, miltenyi Biotec GmbH, Germany) was used at the same concentration. To visualize platelets, additional staining of cell pellets was done using anti-human CD36 antibody (clone 5-271, BioLegend) conjugated to phycoerythrin (PE) at a final concentration of 0.08 $\mu$ g/ml. Analysis of green (FITC) and red (PE) fluorescence of the cells was performed using a fluorescence scanning microscope, ScanR (Olympus, Hamburg, Germany), enabling visual examination for detection and localization of EpCAM- and CD36-positive cells.

## Declarations

**Funding:** Not applicable.

**Conflicts of interest:** The authors declare no competing interests.

**Data availability:** The datasets generated and/or analyzed during the study are not publicly available due to preservation of privacy but are available from the corresponding author on reasonable request.

**Code availability:** Not applicable

**Authors' contributions:** Conceptualization, K.P., D.S., M.P.; Methodology, D.S., M.P., K.H. and K.P.; Validation, D.S., and M.P.; Investigation, D.S., M.P., E.S.; Writing-Original Draft Preparation, K.P., D.S., M.P.; Writing-Review & Editing, K.P., M.P.; Visualization, D.S. and M.P.; Supervision, K.P. and D.S.; Project Administration, D.S., K.P., M.P.

**Ethics declarations:** All subjects have provided written informed consent before participation in the study which has been approved by the Ethics Committee of the Bavarian Medical Association.

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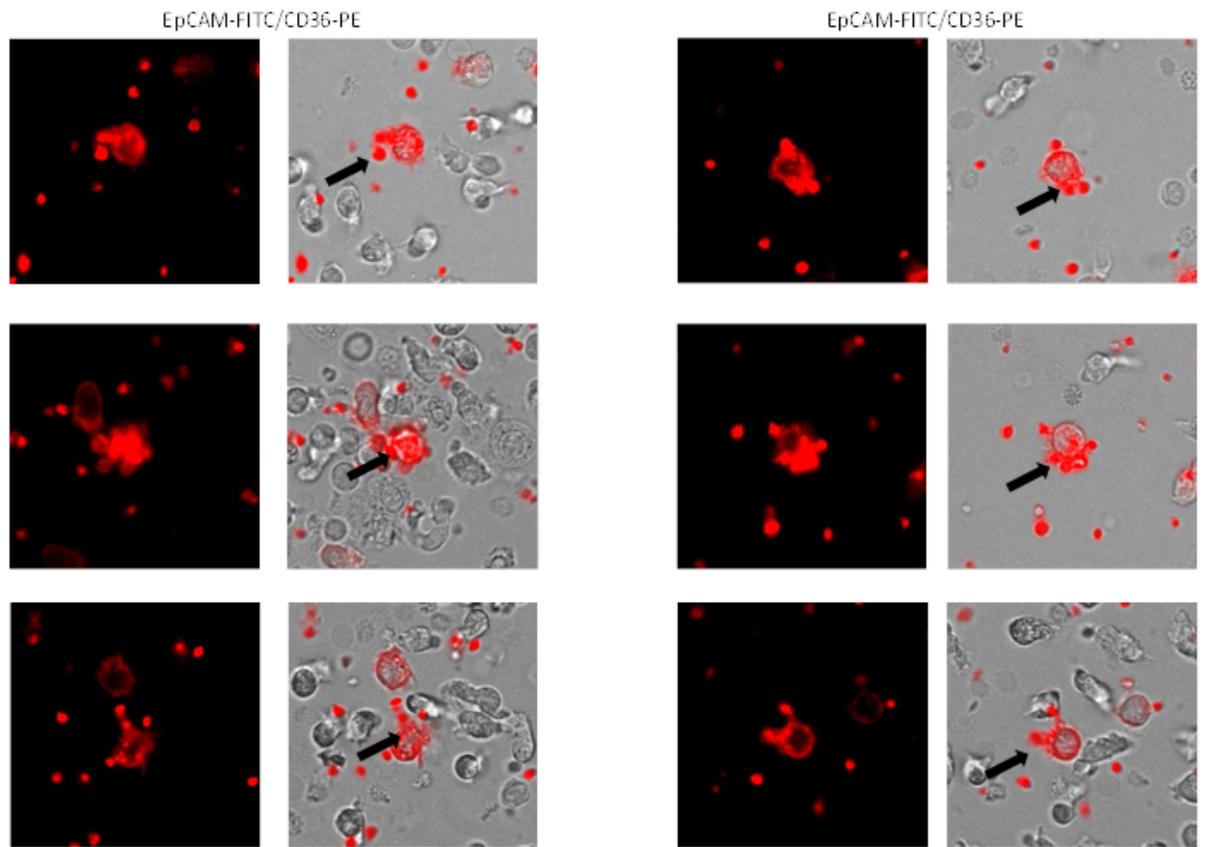
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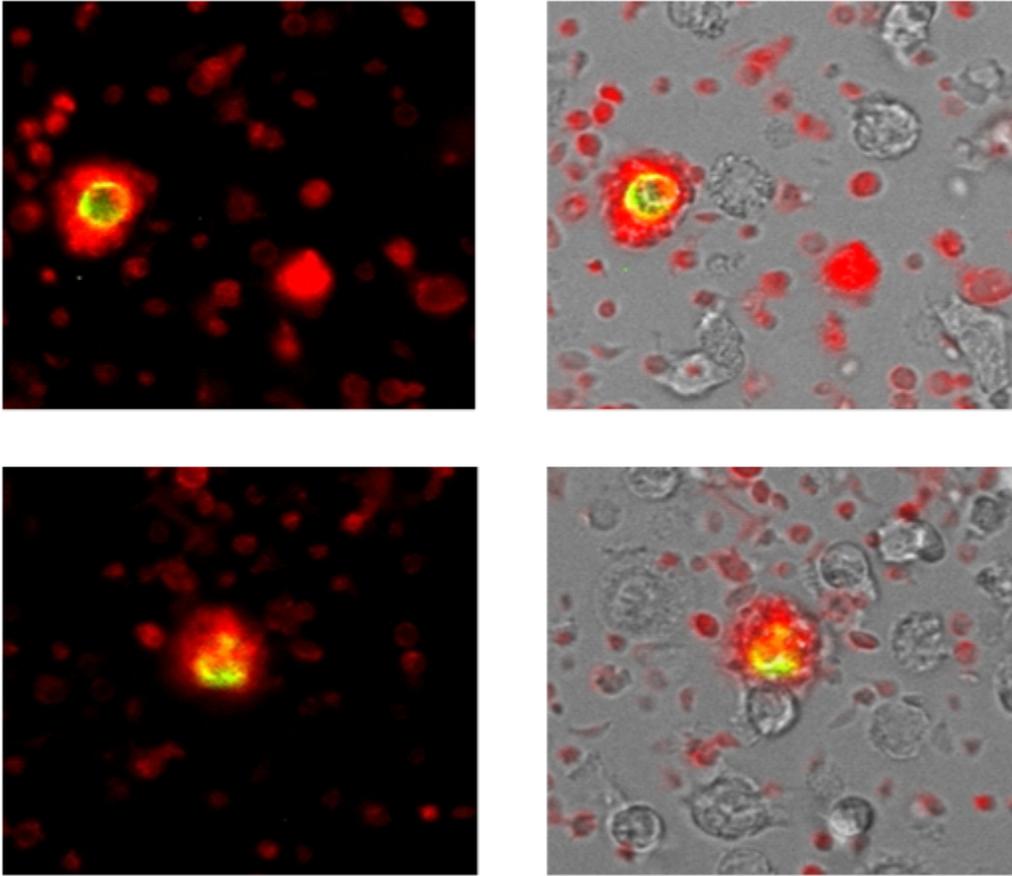
## Figures



**Figure 1**

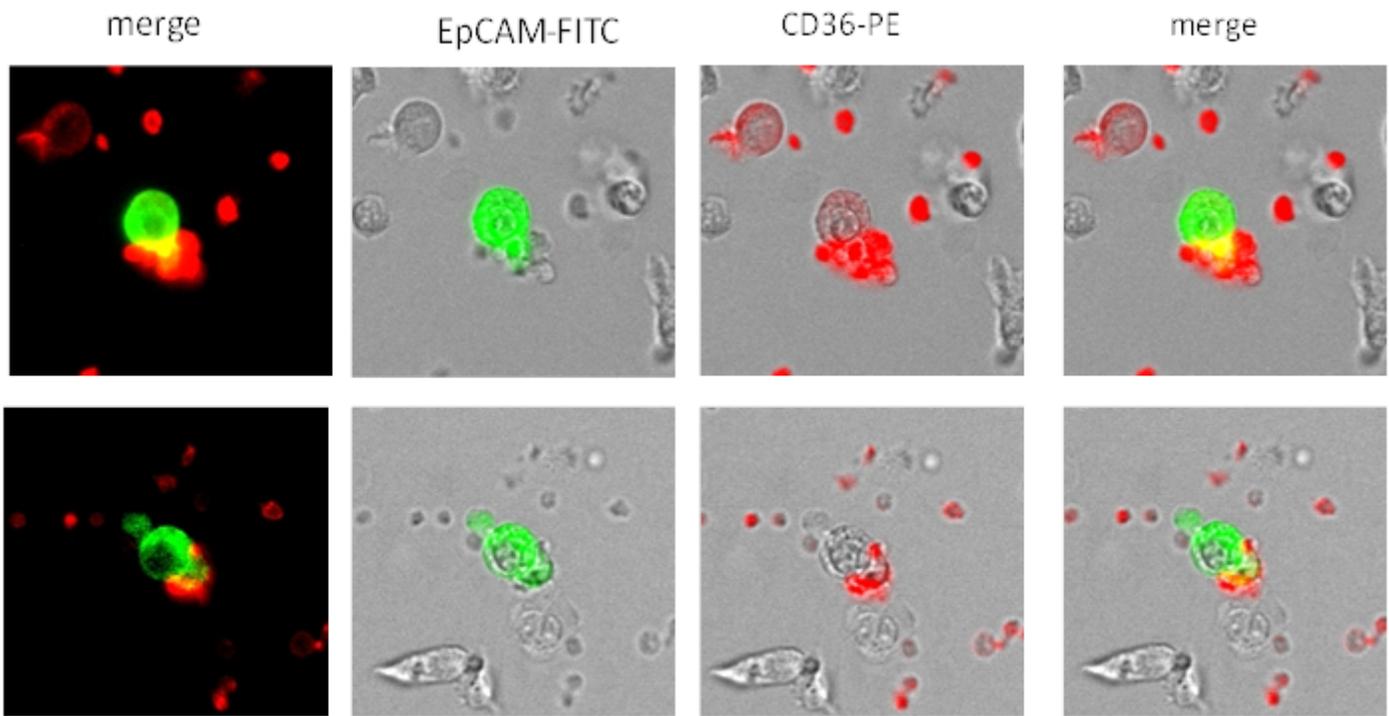
Cell pellets after erythrocyte lysis were stained with an anti-EpCAM-FITC (green) antibody and an anti-CD36-PE antibody (red). No EpCAM staining was observed 0h after blood collection in all patients. Platelets (red staining) were aggregated to suspected circulating tumor cells (arrow). Left rows: fluorescence light; right rows: additional transmitted light

## EpCAM-FITC/CD36-PE



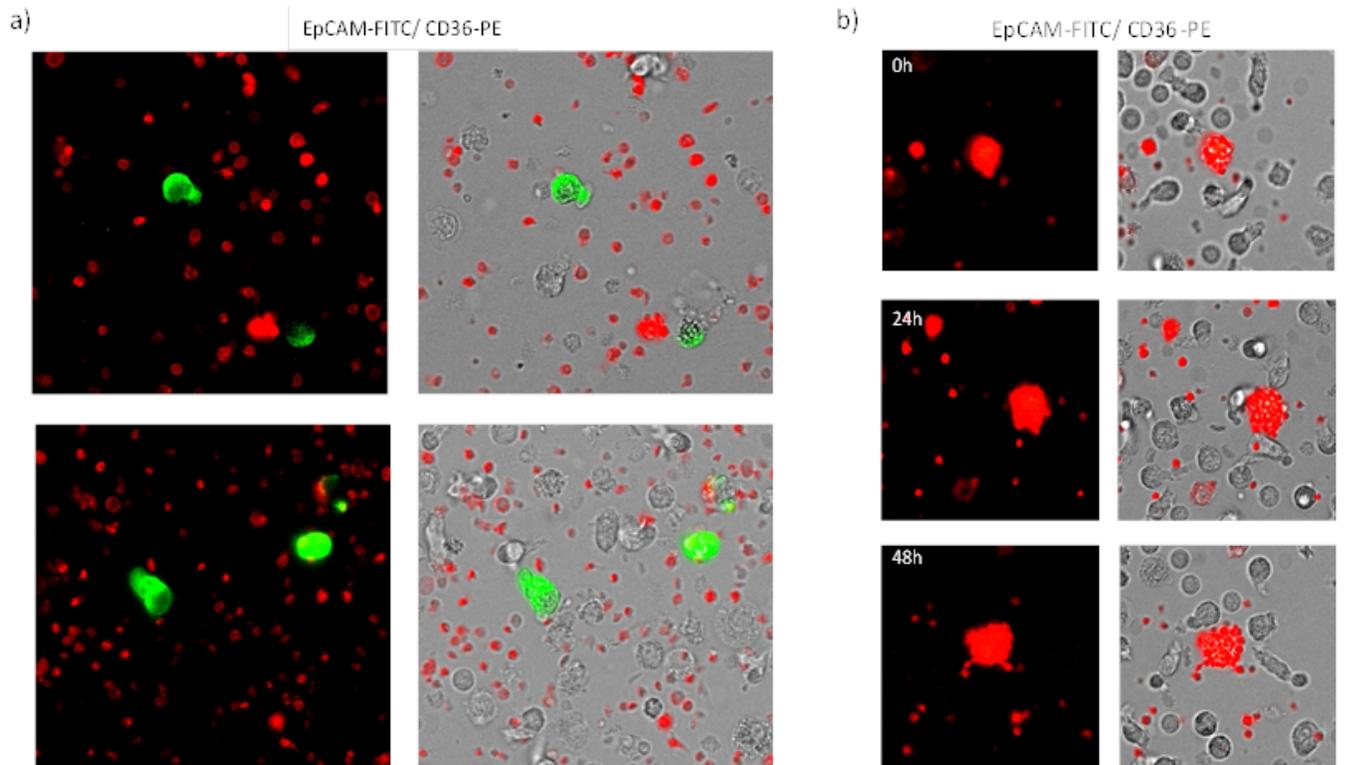
**Figure 2**

Cell pellets were stained 24h after blood collection in the same way as time 0h with an anti-EpCAM-FITC (green) and an anti-CD36-PE (red) antibody. EpCAM positive cells (green) were still aggregated with platelets (red), but were now visible. Platelets had changed their shape, enabling binding of the EpCAM antibody to cells. ). Left row: fluorescence light; right row: additional transmitted light



**Figure 3**

At 48 h post-blood collection, CETCs were still positive for EpCAM (green) and platelets (red) were located at a pole of the tumor cell. First row on the left: fluorescence light, with both red green fluorescence merged; next row: additional transmitted light, next row: only green fluorescence together with transmitted light; last row: red fluorescence together with transmitted light.



**Figure 4**

a) At 72h after blood collection CETCs were positive for EpCAM (green). Platelets (red) disappeared from the surface of CETCs and were present unbound in the cell environment. Left row: fluorescence light; right row: additional transmitted light. b) Representative circulating tumor cells clogged with platelets at time point 0h, 24h and timepoint 48h after blood drawing into Streck tubes, inhibiting binding of the EpCAM antibody. Left row: fluorescence light; right row: additional transmitted light.