

Gravity-based microfiltration reveals unexpected prevalence of circulating tumor cell clusters in ovarian and colorectal cancer

Corresponding Author: Professor David Juncker

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the submitted manuscript, the authors present a novel gravity-based microfiltration (G μ F) technique for the isolation of circulating tumor cells, which is said to be particularly suitable for the isolation of intact cell clusters. These clusters are suspected of forming metastases particularly frequently, and their diagnosis is thus of special interest. However, the fragility of these clusters is high, and previously published filtration techniques are supposed to disjoint these clusters (cCTCs) by shear forces.

To identify the optimal filtration conditions, the authors assessed several characteristics, like pore size, column height, flow rate and pressure. The final conditions were used to evaluate the filtration efficiency in blood samples from patient with ovarian and colorectal cancer, and in both cancer types single CTCs and clusters were found in all patients. Besides the interesting observation that G μ F might be more potent to capture single and clustered CTCs than other filtration based methods, also other interesting observations were made. E.g that the size of the largest cell in a cluster determines the retention of the cluster, and also that the size of the single CTCs and clusters may be influenced by chemotherapy. Overall, the manuscript is well written and the results are clearly presented. The study has appropriate scientific quality and high technical soundness.

However there are some comments, which may be addressed by the authors (minor issues):

- 1) In the chapter describing the protein expression of xenograft OV-90 and OVCAR-3 cells in blood and ascites samples of mice, it could be interesting to know the protein expression of the respective markers (E-cadh, ZO-1,...) of the original cell lines.
- 2) it is a pity that immunofluorescent staining was not done with the patient samples (ovarian, colorectal). It would be interesting to know if the protein profile would be altered by chemotherapy as well.

Reviewer #2

(Remarks to the Author)

The authors designed a gravity-based microfiltration chip for facile isolation of CTC clusters from ovarian cancer mouse model. Some comments:

1. CTC microclusters have been detected with other microfluidic devices, such as those based on inertial focusing. Can you elaborate on the novelty and advantages of this approach compared to others?
2. Please discuss the size of the setup and its implications for clinical utility
3. please discuss how did the researchers confirm that clusters were not formed during the sorting process
4. Figure 2 states that the capture efficiency varies extensively. Please elaborate
5. Did the authors observe clogging/biofouling in the device
6. what is the extent of heterogeneity observed across the single CTCs, and how does it vary regarding cCTCs?
7. Were clinical samples also stained with CD45? what was the proportion of WBCs in clusters

Reviewer #3

(Remarks to the Author)

The authors report the result of a new method for detection of circulating tumor cells (CTC) with gravity-based microfiltration (G μ F) which facilitates isolation of cluster of CTCs. Their method seems minimizing unwanted cluster disaggregation, with a high capture efficiency. Detection of CTC clusters is of clear interest in order to decipher the metastatic process and in the clinic as a pejorative prognostic marker in various types of cancer.

The method reported is original and could be a new contribution in the field of CTC study.

However, there are several points in the report that could weaken the value of the results and:

1/ the experimental results are obtained on only one model which is an orthotopic ovarian cancer mouse model.

Reproducibility of the experiments on another type of cells would have strengthened the value of the observations.

2/ the main issue is the very high number of CTC detected in all cases of cancer patients (ovarian and colo-rectal cancer).

This contrast with reports of low detection rate of CTC in ovarian cancer and colon with other methods as positive immunoselection based. Comparison with another method of detection on cells and/or patients samples would have confirmed the advantage in terms of sensitivity of the new reported method.

3/ reporting clinical results, rate of detection in patients with a new method should provide negative control results. Could the authors provide negative control, i.e. analysis of blood from healthy donors in order to prove the absence of detection of CTC and CTC clusters in these cases? This would discard the hypothesis of false positivity.

4/ in Table 1: why not providing the number of scCTC and cCTC in the same table for each patient? This could help to visualize correlation with CA 125 or clinical stage.

5/ I would suggest to quote in the discussion the recent paper of Mert Boya, in Nature Commun. 2022; 13: 3385. The authors report High throughput, label-free isolation of circulating tumor cell clusters in meshed microwells. They isolated CTC clusters ranging from 2 to 100+ cells from prostate and ovarian cancer patients

Minor comment

Line 64: explain acronym before the following line

line 77: progression-free

line 407 :being

line 699: correct the sentence

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors provide a revised version of the originally submitted manuscript, and all questions raised have been adequately answered.

Reviewer #2

(Remarks to the Author)

The authors have addressed most comments adequately.

A minor comment is to characterize the isolated clusters more deeply, using more specific markers pertaining to this cancer, instead of EpCAM and CD45.

Reviewer #3

(Remarks to the Author)

The authors should be thanked for their answers to the various comments. However, the answer to the question about the results of their technique applied to blood samples from healthy donor is still missing. Their previous work (ref 47) provided results of percent of recovery of a number of cells from tumor cell line spiked in blood of healthy donor. What is the percent of scCTC and sCTC detected in samples without spiked tumor cells ?

The authors report now a very high percentage of CD45+ cells (nearly 50% in some cases) associated with the cells counted as sCTC and SCCTC. Why was this double staining not performed on all samples in order to rule out any false positive for cancer cells? Did they observe double positive cells? (i.e., CD45+ and CK+)?

How do the authors explain that they detect CTC in a case of a border line tumor of the ovary which is supposed to be noninvasive tumor? How do they explain the complete absence of correlation of the number of scCTC and cCTC with the clinical stage of the disease for ovarian cancer (same range of detected CTC in stage I and stage IV)? All their cases in ovarian cancer and colon cancer are positive for CTC detection. Controls for negativity with non-malignant tumor or disease in a significant number of cases to rule out false positive cases seems to be a major point and is missing in this work.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Regarding the concerns of reviewer 3 I may add some comments:

I agree that the number of scCTCs is unusually high in the ovarian cancer samples. However, the authors describe a new enrichment technology, and it may well be that this technology is more potent to isolate CTCs than previous ones. However, in my opinion the focus of the manuscript is on clustered CTCs.

I totally agree with the concerns regarding healthy controls. First, just 2 controls is pretty few. And here the number of captured cells is relatively high (up to 4). However, in patients the smallest number of scCTCs is 22. I would suggest to add the number of healthy donors in the m&m section (total 7, but indeed 2).

In my opinion the most relevant concern is regarding the coagulation issue. It is very uncommon that coagulation occurs during enrichment in so many samples (5/7). In my experience this only occurs when other than EDTA blood collection tubes were used (e.g. citrate, heparin,...). How would the authors take for granted that in cancer patients, when abnormal coagulation is a quite common phenomenon, this could be the reason for clustered cells? The authors mention that for spiking experiments a citrate based anticoagulant was used. In our hand these blood samples coagulate during the microfluidic separation, and we found that citrate samples were not at all appropriate for that type of enrichment. It would be interesting to know if the authors are aware of that, and what was the rationale for using this type of vacutainers. It is also necessary to add the information on the vacutainers used for the healthy and patient samples in the material and methods section.

Reviewer #3

(Remarks to the Author)

The authors should be thanked for their answers to the comments.

However, as they observed, their results provide unexpected levels of detection of CTC. They report very high numbers of CTC, isolated and in clusters in two types of cancers (colorectal and ovarian). The level of CTC detection in ovarian cancer in the literature is usually low and the hematogenous dissemination of this cancer is usually considered as occurring late in disease with high clinical stage.

The high level of CTC independent of the clinical, including noninvasive tumor remains puzzling.

Moreover, as requested the authors have proceeded to detection of CTC in healthy controls. They can provide results only in two cases, which is very few for a control. These controls are positive for isolated CTC, slightly above the threshold previously reported in their previous publication. What is the explanation for clotting and failure in the five other cases? Does it question the reliability of the technique with a rate of failure of 5/7?

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

The authors did not provide a sufficient number of negative healthy controls

The rate of false positivity is 33% (3 cases with detected CK+ cells out of 9). Moreover, the references given in their rebuttal letter to discuss false positivity are based on detection techniques, which were not considered as reference.

Their new controls were obtained without clotting and using EDTA tubes. It is not clear if the same technique was applied for their previous clinical results.

Main concerns about clinical results provided remain unchanged:

How do the authors explain that they detect CTC in a case of a borderline tumor of the ovary which is supposed to be noninvasive tumor? How do they explain the complete absence of correlation of the number of scCTC and cCTC with the clinical stage of the disease for ovarian cancer (same range of detected CTC in stage I and stage IV)? All their cases in ovarian cancer and colon cancer are positive for CTC detection.

Clinical outcome of the patients and correlation of CTC and scCTC with prognosis are lacking

Version 4:

Reviewer comments:

Reviewer #4

(Remarks to the Author)

Control samples are of utmost importance in evaluating a technology for detection of CTC!! The authors clearly have not recognized this as exemplified by their remark in the rebuttal "nor our standard practice ", as part of the revision they included the results of 9 healthy donors for which blood was collected in EDTA (blood collection tube of patients not reported but I assume it's the same). No cCTC were detected in 3 ml blood of all 9 samples and 0,0,0,0,1, 3 and 4 scCTC cells in the 9 samples. The authors should use these findings as the basis of setting a threshold and surely not refer to thresholds in papers (ref 60 =ref 20! in this ref 145 healthy donors serve as controls for the technology) in which thresholds are based on a different blood volume, technology and CTC definition. Using this as a basis they simply can reports the background level detected in healthy donors (mean, SD), and for example use the mean + 2SD to be able to discriminate positive versus negative. Than report the mean and SD of the scCTC and cCTC in the 31 patients which simply can be added to table 1 and 2. One can divide the patients as is attempted in the manuscript but at this stage I would not put too much emphasis on this as the numbers are too small to suggest a relation and as such section "Capture of cCTCs from cancer patients" and "Differential reduction of scCTC and cCTC during treatment of metastatic EOC high grade serous carcinoma patient" can be reduced and simplified using the numbers reported in table 1 and 2.

Version 5:

Reviewer comments:

Reviewer #4

(Remarks to the Author)

One cannot report background of CTC detected with different systems and different definitions as part of the introduced CTC technology so : "While the positive detection threshold for cancer is commonly set at ≥ 2 CK+/CD45- scCTCs per 7.5 mL of blood 20" and "epithelial non-CTCs are commonly detected from the blood of healthy individuals at cell counts from 1.5 to 2 cells per mL60,61 , and reaching as high as 21 cells per mL 62, and has been ascribed to shedding of epithelial cells into the bloodstream and/or blood collection device during venipuncture 63" will have to be removed from the result section. If desired one can mention something in the discussion but keep in mind that the actual CTC definitions are quite different so one should be careful making any comparisons. So better to caution the reader and mention that the controls are limited in this study.

The results of the nine controls should be added to either table 1 or table 2 and next to the per ml the actual number of ssCTC / cCTC should be provided

Version 6:

Reviewer comments:

Reviewer #4

(Remarks to the Author)

The changes clearly improved the manuscript

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We thank all 3 reviewers for their careful review and insightful questions. Our response to the comments is detailed below. We revised the manuscript with considerable additions and edits to address the comments. Added text and revisions are included in the response, and highlighted in yellow in the revised manuscript to facilitate the review process.

Reviewer #1 (Remarks to the Author):

In the submitted manuscript, the authors present a novel gravity-based microfiltration (G μ F) technique for the isolation of circulating tumor cells, which is said to be particularly suitable for the isolation of intact cell clusters. These clusters are suspected of forming metastases particularly frequently, and their diagnosis is thus of special interest. However, the fragility of these clusters is high, and previously published filtration techniques are supposed to disjoint these clusters (cCTCs) by shear forces.

To identify the optimal filtration conditions, the authors assessed several characteristics, like pore size, column height, flow rate and pressure. The final conditions were used to evaluate the filtration efficiency in blood samples from patient with ovarian and colorectal cancer, and in both cancer types single CTCs and clusters were found in all patients. Besides the interesting observation that G μ F might be more potent to capture single and clustered CTCs than other filtration based methods, also other interesting observations were made. E.g that the size of the largest cell in a cluster determines the retention of the cluster, and also that the size of the single CTCs and clusters may be influenced by chemotherapy.

Overall, the manuscript is well written and the results are clearly presented. The study has appropriate scientific quality and high technical soundness.

However there are some comments, which may be addressed by the authors (minor issues):

We thank Reviewer #1 for appreciating the performance of our method for CTC isolation, and interest in our findings on patient samples. Please see below our response to the reviewer's suggestions.

1) In the chapter describing the protein expression of xenograft OV-90 and OVCAR-3 cells in blood and ascites samples of mice, it could be interesting to know the protein expression of the respective markers (E-cadh, ZO-1,...) of the original cell lines.

We thank the reviewer for this suggestion. We compared our result obtained from mouse models with recent studies using cell cultures and human samples of ovarian cancer at various stages. We completely rewrote the subsection "cCTCs can help understand dissemination in ovarian cancer" with a summary of our observations and how they compare with studies by others, see [lines 295 – 322 in the manuscript](#).

2) it is a pity that immunofluorescent staining was not done with the patient samples (ovarian, colorectal). It would be interesting to know if the protein profile would be altered by chemotherapy as well.

We thank the reviewer for this suggestion, and we agree that it would be interesting to analyze the protein profile of the collected CTCs from patient samples. Our ongoing work is in fact focusing on collecting serial cancer patient samples at diagnosis, pre- and post-surgery treatments using high multiplex fluorescence imaging.

Reviewer #2 (Remarks to the Author):

The authors designed a gravity-based microfiltration chip for facile isolation of CTC clusters from ovarian cancer mouse model. Some comments:

1. CTC microclusters have been detected with other microfluidic devices, such as those based on inertial focusing. Can you elaborate on the novelty and advantages of this approach compared to others?

We thank the reviewer for this suggestion. We added additional text in the revised manuscript.

“A more recent microfluidic chip-based device developed by the same group combined inertial focusing with repeated flow-shifting for cCTC isolation, enabling large volume blood samples processing capacity at a flow rate higher than 30 mL h⁻¹.” (Line 86 – 88)

“Inertial focusing produces forces that are expected to cause cluster breakage, along with the disadvantage of losing small cells resulting in additional loss of information.” (Line 101 – 103)

We highlighted G μ F’s capacity of isolating both scCTC and cCTC with the following text.

“G μ F... is an effective method for capturing and selectively enriching the compendium of single cell CTCs and fragile clusters.” (Line 417– 419)

“Cluster-Wells, a recent method developed for cCTC isolation, employs a 47 mm-diameter membrane etched with >100,000 microwells that was developed specifically for cCTC isolation. The geometry of the microwells retains clusters of 3 cells and more at nearly 100% efficiency, but at the expense of losing scCTCs. G μ F efficiently captures both scCTCs and cCTCs, for which we ascribe the high capture yield to higher porosity filters (up to 40% porosity), gravity (pressure) driven flow, larger pores, and painstaking optimization.” (Line 428 – 433)

2. Please discuss the size of the setup and its implications for clinical utility

The fabrication method of the components was described in detail in our previous publication (Meunier et al. 2016). Specifically, the setup consists of a typical laboratory retort stand with a 12 × 22 cm² footprint, on which the syringe, tubing, filtration cartridge and collection tube are assembled.

In this study, the microfiltration setup was fabricated in the laboratory, transported, assembled and used at the clinic for CTC extraction from fresh blood samples. The size and operation of the setup have not been a hindrance in clinical laboratory, we thus conclude that the method would be suitable for clinical use.

We updated the text with the following statement following the description of filter cartridge and total column heights:

“The entire setup was assembled on a typical laboratory retort stand, with a footprint of $12 \times 22 \text{ cm}^2$, thus occupying little bench space with minimal hindrance to activities in the clinical laboratory.” (Line 138 – 140)

We updated Fig. 1 to show the dimension of the G μ F setup.

3. please discuss how did the researchers confirm that clusters were not formed during the sorting process

In G μ F, the 8-mm diameter microfilter has a surface area of 201 mm^2 , whereas the diameter of a typical tumor cell is $\sim 8 \text{ }\mu\text{m}$, or an area of 0.000201 mm^2 ($201 \text{ }\mu\text{m}^2$), a difference of six orders of magnitude. Given the rarity of CTC (< 600 scCTCs observed in patient sample in this work), and the very small area fraction of the filter covered by CTCs (typically $\ll 0.1\%$), we confidently exclude artefactual scCTC aggregation on the filter as a source of cCTCs.

In our previous work (Meunier et al., 2016), single cells spiked in normal human blood did not produce clusters after G μ F, thus proving that G μ F does not cause cluster formation.

In Figure 3C, normal blood spiked with 150 OC-90 single cells and 100 OV-90 clusters was filtered by serial G μ F through filters with decreasing pore size (28, 20, 15, 12, 10 and $8 \text{ }\mu\text{m}$). We did not detect large clusters (which were captured by the upstream filters with larger pores) on the smaller-pore filters downstream. This observation also indicates G μ F did not cause cluster formation.

We updated the manuscript for clarification:

“Small pore filters ($8, 10, \text{ and } 12 \text{ }\mu\text{m}$) mostly captured 2- and 3-cell clusters with areas $\sim 150\text{-}300 \text{ }\mu\text{m}^2$ ($\text{O}_{\text{eq}} \sim 14\text{-}19 \text{ }\mu\text{m}$) that could pass through the preceding larger pore filters. Small clusters were also found on larger pore size filters but to a lesser extent. Based on the absence of large clusters on downstream small pore filters, together with the very small area fraction of the filter covered by CTCs (typically $\ll 0.1\%$) and our previous work with single cells of other cell lines, we confidently exclude artefactual scCTC aggregation on the filter as a source of cCTCs during G μ F.” (Line 204 – 209)

4. Figure 2 states that the capture efficiency varies extensively. Please elaborate

Figure 2 details our experiments exploring how the differences between G μ F and syringe pump-driven flow affect scCTC and cCTC capture efficiency. We observed that G μ F consistently yielded higher capture efficiency than pump-driven flow. Increasing the steady state flow rate observed in Domain II (0.1 mL min^{-1}) to a higher flow rate observed in Domain I (0.5 mL min^{-1}) (see Fig. 1) led to decrease in cCTC capture efficiency in both methods; whereas scCTC capture increased more than 3-fold for G μ F, and almost 5-fold for pump-driven flow. We reasoned that cell cluster break up due to increased flow rate is a possible explanation for the variation in capture efficiency for both scCTC and cCTC, and that pump-driven flow already disrupted the clusters even at low flow rate.

To highlight the difference in capture efficiency between using gravity- and pump-driven flow, we added the following text in the discussion.

“We attributed the lower cluster recovery rate to higher cluster disruption under pump-driven flow even at low flow rate, which is consistent with the observation that clusters more than 4 cells were essentially absent using pump-driven filtration.” (Line 439 – 441)

We also updated the text to emphasize that the same flow rate at 0.1 mL min^{-1} was used in subsequent CTC isolation experiments, and capture efficiency is reproducible. We provided standard deviation of the capture efficiencies.

We clarified the flow rate used in the experiments.

“Next, blood from a single OVCAR-3-GFP (green fluorescent protein) mouse was diluted and divided into four identical aliquots. One quarter was saved for further growth analysis, and the three others filtered through $15 \mu\text{m}$ filters at 0.1 mL min^{-1} .” (Line 263 – 265)

“The blood samples were processed by filtering first with $15 \mu\text{m}$ followed by $8 \mu\text{m}$ filters to capture CTCs at 0.1 mL min^{-1} .” (Line 332 – 333)

“A flow rate of 0.1 mL min^{-1} was thus deemed optimal for both capture and release of clusters with our setup.” (Line 451– 452)

5. Did the authors observe clogging/biofouling in the device

Our previous characterization of the microfiltration established $8 \mu\text{m}$ -diameter pores as the lower limit of pore size before clogging by WBCs becomes significant (Meunier et al., 2016). With optimization, the number of WBCs remaining on the filter after filtration and rinsing was only ~ 1000 WBCs, filling $\sim 1\%$ of the total number of pores without causing clogging issues.

We added in the manuscript the following clarification.

“After filtration, filters were rinsed with PBS... We did not observe significant clogging or biofouling of the device with flow remaining steady throughout the experiment.” (Line 157 – 161)

“Our previous work established $8 \mu\text{m}$ pores as the lower limit of pore size before clogging by WBCs becomes significant, and optimization showed that the number of clogged pores after filtration and rinsing was negligible ($\sim 1\%$).” (Line 175– 176)

6. what is the extent of heterogeneity observed across the single CTCs, and how does it vary regarding cCTCs?

In the manuscript, we described how scCTC and cCTC counts, scCTC:cCTC ratio, and cluster size of the cCTCs vary in different samples, cancers and cancer subtypes and stages. Notably, scCTC counts ranged from a few cells to ~ 600 single cells, and cCTC counts from 1 to ~ 60 events. The number of cells in each of the clusters vary, and depend on the cancer type for

ovarian cancer: up to 60 cells in high grade ovarian cancer cCTCs; whereas cCTCs from patients with lower stage disease could comprise more than 100 cells. On the other hand, CRCLM cCTCs were mostly small clusters with less than 10 cells.

To better discuss the phenotypic heterogeneity of the isolated CTCs, we added in this revision the percentage of cCTCs containing WBCs.

We performed CD45 staining for all samples. 10 EOC patients and all CRCLM patients were analyzed for cCTC associated CD45⁺ cells that are assumed to be WBCs.

“CD45⁺ cells counts were performed on 10 patient samples (OC1-5, and OC11-15). WBCs (i.e. CD45⁺ cells) were detected in the isolated cCTCs of 5/10 EOC patients, and in 3.2% (OC13) to 50% (OC4) of the isolated clusters. WBCs accounted for between 20% (OC13) to 60% (OC12) of total clustered cells in each sample.” (Line 374 – 377)

“WBCs were detected in the isolated cCTCs of 4/13 patients, and in 17% (CR1) to 50% (CR9) of the isolated clusters. The proportion of WBC ranged from 9% (CR1) to 39% (CR9) of all clustered cells.” (Line 383 – 385)

The observation of large cCTCs is in agreement with a recent study using Cluster-Wells.

“...our result is in agreement with the detection of ovarian cancer cCTCs of >150 cells by Cluster-Wells. We also found that, using G μ F, more cCTCs that are associated with WBCs were detected (up to 50%) than in the work by Boya et al. using Cluster-Wells (up to 26.4% in EOC cCTCs).” (Line 484 – 487)

7. *Were clinical samples also stained with CD45? what was the proportion of WBCs in clusters*

Yes, CD45 staining was used to distinguish WBCs for clinical samples of EOC and CRCLM patients. We clarified the procedure in the Methods section.

“Then, for identification, cells were stained with anti-CK (cytokeratin) 18-Alexa Fluor 488 (2.0 μ g mL⁻¹) and anti-human CD45-PE (cluster of differentiation 45, labeled with phycoerythrin, 1.0 μ g mL⁻¹) to further sort cancer cells from blood cells in spiked and clinical samples.” (Line 670 – 673)

We added the detection of WBC in cCTCs in the result section (see answer to 6).

“CD45⁺ cells counts were performed on 10 patient samples (OC1-5, and OC11-15). WBCs (i.e. CD45⁺ cells) were detected in the isolated cCTCs of 5/10 EOC patients, and in 3.2% (OC13) to 50% (OC4) of the isolated clusters. WBCs accounted for between 20% (OC13) to 60% (OC12) of total clustered cells in each sample.” (Line 374 – 377)

“WBCs were detected in the isolated cCTCs of 4/13 patients, and in 17% (CR1) to 50% (CR9) of the isolated clusters. The proportion of WBC ranged from 9% (CR1) to 39% (CR9) of all clustered cells.” (Line 383 – 385)

We also noted the proportion of cCTC that are associated with WBCs is higher than the result of Boya et al using Cluster-Wells.

“We also found that, using G μ F, more cCTCs that are associated with WBCs were detected (up to 50% of all cCTC events) than in the work by Boya et al. using Cluster-Wells (up to 26.4% in EOC cCTCs).” (Line 485 – 487)

Reviewer #3 (Remarks to the Author):

The authors report the result of a new method for detection of circulating tumor cells (CTC) with gravity-based microfiltration (G μ F) which facilitates isolation of cluster of CTCs. Their method seems minimizing unwanted cluster disaggregation, with a high capture efficiency. Detection of CTC clusters is of clear interest in order to decipher the metastatic process and in the clinic as a pejorative prognostic marker in various types of cancer.

The method reported is original and could be a new contribution in the field of CTC study. However, there are several points in the report that could weaken the value of the results and:

1/ the experimental results are obtained on only one model which is an orthotopic ovarian cancer mouse model. Reproducibility o of the experiments on another type of cells would have strengthen the value of the observations.

In our 2016 publication (Meunier et al., 2016), the G μ F setup was validated with two breast cancer cell lines (MDA-MB-231 and MCF-7) and two kidney cancer cell lines (786-O and A-498) spiked in normal human blood. In this work, we further optimized and validated the method using a fifth cell line (OV-90), along with mouse models injected with two different ovarian cancer cells (OV-90 and OVCAR-3) mimicking metastasis. The extensive optimization data and reproducibility experiments published previously and in the present work provided a solid validation of the performance of our method, and motivated its use for analyzing patient samples.

We added the following text:

“We previously validated G μ F for CTC isolation using two breast cancer cell lines (MDA-MB231 and MCF-7) and two kidney cancer cell lines (786-O and A-498) spiked in normal human blood.” (Line 150 – 151)

2/ the main issue is the very high number of CTC detected in all cases of cancer patients (ovarian and colo-rectal cancer). This contrast with reports of low detection rate of CTC in ovarian cancer and colon with other methods as positive immunoselection based. Comparison with another method of detection on cells and/or patients samples would have confirmed the advantage in terms of sensitivity of the new reported method.

We previously validated the microfiltration setup using cancer cells spiked in blood samples of healthy individuals (see response above). The number of cells that were spiked were precisely determined by manual counting of the diluted cell suspensions, and the number of cells isolated from the spiked samples determined. The number of isolated cells were never found to be higher than the number of spiked cells, and matched the expected number for the amount of spiked cells. The capture efficiency was not dependent on the number of spiked cells, thus validating our method and providing support that our microfiltration method does

not introduce false positive cell counts arising from non-cancer cells in the normal blood samples.

We thank the reviewer for suggesting a performance comparison between GuF and another method. To perform the comparison, CTC isolation would need to be performed on the same patient blood samples using both methods. As CTC isolation requires fresh blood, comparison is not possible without analyzing new patient cohorts of comparable size. Patient recruitment and sample collection is not trivial and had been a challenge (a significant delay was caused by COVID-19). Performing the comparison essentially represents a complete replicate of the study which had taken multiple years of experiment and analysis, and which we consider not practical.

Notably, our results on scCTC and cCTC counts, prevalence of cCTC, cluster size, and occurrence of WBC in cCTCs are in fact comparable and in agreement with a recent study using Cluster-Wells by Boya et al, a work also mentioned by the reviewer. In comparison, our patient cohort contains 10 more patients, including a time-course monitoring of one ovarian patient, thus providing considerably more data points. As discussed in the manuscript, thanks to the advance of CTC isolation technology, especially for cCTCs, the commonly held notions about the extreme rarity of scCTC and cCTC are challenged and need to be re-examined.

3/ reporting clinical results, rate of detection in patients with a new method should provide negative control results. Could the authors provide negative control, i.e. analysis of blood from healthy donors in order to prove the absence of detection of CTC and CTC clusters in these cases? This would discard the hypothesis of false positivity.

Please see the first part of our answer to comment #2 on the use of cultured cells spiked in healthy blood samples in our optimization and validation experiments, which provided proof for the absence of CTC detection in normal human blood.

4/ in Table 1: why not providing the number of scCTC and cCTC in the same table for each patient? This could help to visualize correlation with CA 125 or clinical stage.

We thank the reviewer for this suggestion. To facilitate data interpretation, we updated Table 1 and 2 which now contain scCTC and cCTC events per mL.

5/ I would suggest to quote in the discussion the recent paper of Mert Boya, in Nature Commun. 2022; 13: 3385. The authors report High throughput, label-free isolation of circulating tumor cell clusters in meshed microwells. They isolated CTC clusters ranging from 2 to 100+ cells from prostate and ovarian cancer patients

We added new text in the discussion about the cell cluster isolation principle of Cluster-Wells, its performance of large cluster isolation, and the discovery of cCTCs in prostate and ovarian cancer patients by Boya et al.

“Cluster-Wells, a recent method developed for cCTC isolation, employs a 47 mm-diameter membrane etched with >100,000 microwells that was developed specifically for cCTC isolation. The geometry of the microwells retains clusters of 3 cells and more at nearly 100% efficiency, but at the expense of losing scCTCs.” (Line 428 – 431)

“...whereas Boya et al. isolated cCTCs in 75% and 89% prostate and ovarian cancer patients using Cluster-Wells.” (Line 467 – 468)

“..., our result is in agreement with the detection of ovarian cancer cCTCs of >150 cells by Cluster-Wells. We also found that, using G μ F, more cCTCs that are associated with WBCs were detected (up to 50%) than in the work by Boya et al. using Cluster-Wells (up to 26.4% in EOC cCTCs).” (Line 484-487)

Minor comments

Line 64: explain acronym before the following line. We corrected the error.

line 77: progression-free. We corrected the error.

line 407 :being. Here, we meant “cell number bin”. We kept the text as-is.

line 699: correct the sentence. We corrected the error.

Response to Reviewers' comments to Revision 1

Reviewer #1 (Remarks to the Author):

The authors provide a revised version of the originally submitted manuscript, and all questions raised have been adequately answered.

We thank Reviewer 1 again for the constructive comments which helped us to improve the manuscript.

Reviewer #2 (Remarks to the Author):

The authors have addressed most comments adequately.

A minor comment is to characterize the isolated clusters more deeply, using more specific markers pertaining to this cancer, instead of EpCAM and CD45.

We thank Reviewer 2 for the suggestion. While we find a deeper protein profiling of the isolated CTC would be beyond the scope of this manuscript, our ongoing research indeed aims to characterize the capture scCTCs and cCTCs more deeply through multiplex measurement of cancer-related proteins.

Reviewer #3 (Remarks to the Author):

The authors should be thanked for their answers to the various comments. However, the answer to the question about the results of their technique applied to blood samples from healthy donor is still missing. Their previous work (ref 47) provided results of percent of recovery of a number of cells from tumor cell line spiked in blood of healthy donor. What is the percent of scCTC and sCTC detected in samples without spiked tumor cells ?

We agree that analysis of healthy samples is an important control experiments in CTC research, and although we had tested it with spike-ins, we also agree that it did not address the question of whether scCTCs and cCTCs could be found in healthy individuals. In this revision, we thus performed GuFs on blood collected from healthy individuals and included the data in the revised manuscript (please see answer below).

The authors report now a very high percentage of CD45+ cells (nearly 50% in some cases) associated with the cells counted as sCTC and SCCTC. Why was this double staining not performed on all samples in order to rule out any false positive for cancer cells? Did they observe double positive cells? (i.e., CD45+ and CK+)?

We thank the reviewer for this question. CK/CD45 double staining was indeed performed.

We initially reported the prevalence of WBC as the percentage of WBCs in each cCTCs detected in each patient sample. We realized this way of presentation might not accurately reflect the overall prevalence of WBC, especially when the number of cCTC detected in the

sample is low (e.g., 2 out of 4 detected cCTCs in OC4 were WBC⁺, or 50%), and that they are small clusters comprising < 5 cells. Boya *et al* reported WBC prevalence as the % of WBC⁺ cCTCs in all isolated cCTCs. Using the same measure, we found among the 551 cCTCs isolated in 10 EOC patient samples, 18 cCTC were WBC⁺, corresponding to 2.9% of all detected cCTCs. Of the 18 WBC⁺ cCTCs, one contained 3 WBCs out of 5 clustered cells, two contained 2 WBCs out of 4 clustered cells, and the remaining 15 cCTCs contained only 1 WBC out of 3-5 clustered cells.

For CRCLM, 7/13 patients had WBC⁺ cCTCs, 75/336 cCTC were WBC⁺ (22.6% of all isolated cCTCs). On a per patient basis, WBC⁺ cCTCs ranged from 12% to 40%, but note that the total number of cCTCs isolated varied considerably among the CRCLM patients. For example, in CR12, 9/74 cCTCs (12%) were WBC⁺; while in CR9, WBC were found in 4/10 cCTCs (40%). We isolated the highest number of WBC⁺ cCTCs from CR11(27/77 cCTCs, or 35%).

We clarified the prevalence of WBC by reporting: 1. the number of patients who had cCTCs associated with WBCs; 2. the percentage of WBC⁺ cCTCs in each patient sample; 3. the number of WBCs in each WBC⁺ cCTC. We summarized the data above in the main text (see highlighted text), and added two tables (Table S4, S5) in the supplementary information listing the WBC counts in the WBC⁺ cCTCs.

We observed CK⁺/CD45⁺ cells. These cells were not included in any counts in this work. CK⁺/CD45⁺ cells have been reported but not widely investigated. These dual-positive cells are not artefacts (Lustberg et al, *Breast Cancer Res*, 2014; 16: R23 and Reduzzi et al, *Cancer Research*, 2023; 83: P2-26-06-P22-26-06), and have been suggested to be hybrids deriving from the fusion of tumor cells and macrophages (Clawson et al, *PLoS One*, 2017; 12: e0184451), and could therefore contribute to CTC heterogeneity. However further analysis is required to characterize and classify these atypical cells. We clarified that these cells are not included in the CTC counts in this work.

How do the authors explain that they detect CTC in a case of a border line tumor of the ovary which is supposed to be noninvasive tumor? How do they explain the complete absence of correlation of the number of scCTC and cCTC with the clinical stage of the disease for ovarian cancer (same range of detected CTC in stage I and stage IV)?

Borderline ovarian tumors are a heterogeneous group of supposedly non-invasive tumors of uncertain malignant potential (Fischerova et al., *Oncologist*, 2012; 17: 1515-1533). While the blood sample of the single borderline disease in our cohort was analyzed pre-surgery and pre-chemotherapy, the time between disease classification and blood analysis remained unknown to us. It is also unknown whether the borderline disease had progressed to invasive tumor, or whether the classification was performed with sufficient sampling of the tumor and areas of microinvasion.

Due to the small size of the EOC patient cohort, we remain cautious and chose to refrain from making additional conclusion on the correlation of our observed CTC counts with disease stage. In the revised discussion section, we acknowledged the size of our patient cohorts, and indicated that further studies with larger patient cohort will be needed to further support our findings.

All their cases in ovarian cancer and colon cancer are positive for CTC detection. Controls for negativity with non-malignant tumor or disease in a significant number of cases to rule out false positive cases seems to be a major point and is missing in this work.

We agree with the importance to rule out false positive CTC detection. We performed G μ F on seven 3-mL healthy blood samples, but blood coagulation occurred in 5 of the 7 samples during G μ F thus forcing us to abort the analysis, leaving us with 2 measurements. We found 3 and 4 CK⁺/CD45⁻ cells in the 2 samples, but however did not detect cell clusters in healthy controls, thus confirming our findings.

The presence of CK⁺/CD45⁻ single cells has been explained by shedding of epithelial cells into the bloodstream or blood collection device during venipuncture (Castle et al, *PLoS One* 12: e0175647). While we found ~1 cell/mL in our healthy controls, epithelial non-tumor cells have been found in healthy blood from 1.5-2 cells/mL (Sun et al, *EBioMedicine* 46, 133-149; Green et al, *Anal Chem* 91, 9348-9355) to as high as 21 cells/mL (Tsai et al, *Biomed J* 44, S190-S200).

We added the result of healthy blood analysis in the main text, and indicated the number of healthy controls in this work, and that further studies with larger patient and healthy cohorts are needed to provide further support for the unexpected prevalence of cCTC found in the two cancers studied here.

Response to Reviewers' comments

We thank the reviewers for their time and effort in reviewing our manuscript, and providing insightful comments and recommendations. As the reviewers' main concerns are on the analysis of the healthy control samples, we consolidate our explanations, clarifications and new experimental data to address their comments. Revisions of the manuscript are **highlighted in yellow** in the **main text file** and in the **response to comments** here.

The reviewers' concerns are the following:

1. The observed clotting of the control blood samples
2. The number of control samples
3. The number of CK⁺/CD45⁻ cells detected in the microfiltration analyses of the control samples in our previous revision

1. Clotting was no longer observed in the healthy control samples

We agree that the clotting observed in our previous analysis is unusual. Due to the limited access to fresh blood from healthy individuals, in our previous revision we were not able to perform additional experiments to determine the cause of the unexpected clotting, and therefore chose to report our observation.

In this revision, we ensured, to the best of our ability, every step in the workflow, including storage condition of the collected blood, and time between collection and microfiltration, were identical to the patient blood analyses performed in the hospital. All blood tubes, buffers and diluents were new and freshly prepared.

We clarify that EDTA vacutainers were used for patient and healthy controls. Blood from healthy individuals that were previously collected in CTAD tubes were used only for the single cells and cluster spike-in experiments at the method development and characterization stage.

We fabricated additional microfiltration devices, and performed 4 microfiltrations in parallel, thereby completing the analysis of the control samples within a timeframe comparable to the patient sample analyzes.

We are pleased to report that no clotting was observed throughout the microfiltration procedure. The nature of the samples and filtrates, observed flow rate and volume were as expected.

2. Number of control samples increased to 9

We agree that a larger number of healthy controls would further validate our microfiltration method and strengthen the manuscript. **In this revision, we include CTC analysis of 9 healthy controls.**

3. Number of CK⁺/CD45⁻ cells and clusters observed in healthy controls

The control experiments validate our microfiltration method, and lend support to our findings. Indeed, no cell clusters (cCTCs) were detected in healthy control samples, and no CK⁺/CD45⁻ single cells were detected in 6 out of 9. In three controls, 1, 3 and 4 CK⁺/CD45⁻ cells were detected in 3 mL of sample (i.e. ~1 cell per mL).

While the positive detection threshold for cancer is commonly set at ≥ 2 CK⁺/CD45⁻ scCTCs per 7.5 mL of blood, epithelial non-CTCs are commonly detected from the blood of healthy individuals at cells counts from 1.5 to 2 cells per mL (Sun et al, *EBioMedicine* **46**, 133-149; Green et al, *Anal Chem* **91**, 9348-9355), and reaching as high as 21 cells per mL (Tsai et al, *Biomed J* **44**, S190-S200), and has

been ascribed to shedding of epithelial cells into the bloodstream and/or blood collection device during venipuncture (Tsai et al, *Biomed J* **44**, S190-S200).

We added the details and result of the healthy control analyses in the Methods section and main text. We clarify in the Methods section the number of healthy control samples, and the type of vacutainer used for patient and healthy blood collection.

Our point-by-point response

Reviewer #1 (Remarks to the Author):

Regarding the concerns of reviewer 3 I may add some comments:

I agree that the number of scCTCs is unusually high in the ovarian cancer samples. However, the authors describe a new enrichment technology, and it may well be that this technology is more potent to isolate CTCs than previous ones. However, in my opinion the focus of the manuscript is on clustered CTCs.

I totally agree with the concerns regarding healthy controls. First, just 2 controls is pretty few. And here the number of captured cells is relatively high (up to 4). However, in patients the smallest number of scCTCs is 22. I would suggest to add the number of healthy donors in the m&m section (total 7, but indeed 2).

Thank you for this comment. We increased the number of controls to 9. We did not detect cell cluster and CK⁺/CD45⁻ cells in 6 of the 9 controls, and in 3 control samples, 1, 3 and 4 CK⁺/CD45⁻ cells were detected in 3 mL of sample (i.e. ~1 cell/mL). Non-CTCs have been detected in healthy blood at 1.5-2 cells per mL, and as much as 21 non-CTCs per mL. Please see our full answer in **Response 3** above. **We added the number of healthy control samples in the Methods section.**

In my opinion the most relevant concern is regarding the coagulation issue. It is very uncommon that coagulation occurs during enrichment in so many samples (5/7). In my experience this only occurs when other than EDTA blood collection tubes were used (e.g. citrate, heparin,...). How would the authors take for granted that in cancer patients, when abnormal coagulation is a quite common phenomenon, this could be the reason for clustered cells? The authors mention that for spiking experiments a citrate based anticoagulant was used. In our hand these blood samples coagulate during the microfluidic separation, and we found that citrate samples were not at all appropriate for that type of enrichment. It would be interesting to know if the authors are aware of that, and what was the rationale for using this type of vacutainers. It is also necessary to add the information on the vacutainers used for the healthy and patient samples in the material and methods section.

We resolved the issue of coagulation in the blood samples by ensuring the complete workflow follows as close as possible to that for patient sample analyzes as possible. We used fresh blood tubes, buffers and diluents. We also made additional devices to enable simultaneous processing of up to 4 samples to minimize sample storage time. Please see **Response 1** above.

We used EDTA vacutainers for patient and control samples. The CTAD tubes were previously used for collecting healthy blood samples for the scCTC and cCTC spike-in experiments in the method

development and characterization stage. We did not observe any coagulation or clogging issue with the spiked samples. **We clarified the type of vacutainers used in Methods section.**

Reviewer #3 (Remarks to the Author):

The authors should be thanked for their answers to the comments.

We thank Reviewer #3 for the insightful comments.

However, as they observed, their results provide unexpected levels of detection of CTC. They report very high numbers of CTC, isolated and in clusters in two types of cancers (colorectal and ovarian). The level of CTC detection in ovarian cancer in the literature is usually low and the hematogenous dissemination of this cancer is usually considered as occurring late in disease with high clinical stage. The high level of CTC independent of the clinical, including noninvasive tumor remains puzzling.

The high prevalence of scCTC, and in particular cCTCs is indeed unexpected. Nevertheless, recent studies using other low shear cCTC capture technologies also found cCTC to be prevalent in early breast cancer (Reduzzi et al, *Cancers (Basel)* **13**, 2356-2375; Krol et al, *Br J Cancer* **125**, 23-27), thus challenging the commonly held notion that cCTCs are found rarely.

Moreover, as requested the authors have proceeded to detection of CTC in healthy controls. They can provide results only in two cases, which is very few for a control. These controls are positive for isolated CTC, slightly above the threshold previously reported in their previous publication.

We increased the number of healthy controls to 9 in this revision. We did not detect cell cluster and CK⁺/CD45⁻ cells in 6 of the 9 controls, and in 3 control samples, 1, 3 and 4 CK⁺/CD45⁻ cells were detected in 3 mL of sample (i.e. ~1 cell/mL). The presence of epithelial non-CTCs are commonly detected from the blood of healthy individuals at cells counts from 1.5 to 2 cells per mL, and reaching as high as 21 cells per mL. Please see our full answer in **Response 3** above.

What is the explanation for clotting and failure in the five other cases? Does it question the reliability of the technique with a rate of failure of 5/7?

We suspect the cause of clotting was due to the difference in storage time of the collected healthy blood samples, as well as the freshness of the buffers and diluents. We are pleased to report that the clotting issues are resolved after replacing the buffers and diluents. In addition, we fabricated additional microfiltration devices, enabling us to perform 4 CTC isolations in parallel, thereby minimizing the blood storage time. Please see our full answer in **Response 1** above.

Communications Medicine Appeal Form

This form is intended to help authors efficiently present their arguments for reconsideration of a decision. Please fill out only those sections that apply to your paper. Brevity is greatly encouraged.

Manuscript information

Manuscript number: COMMSMED-22-0255C

Title: Gravity-based microfiltration reveals unexpected prevalence of circulating tumor cell clusters in ovarian and colorectal cancer

Corresponding author: David Juncker

Date of contested decision: 12 November 2023

Date of form submission: 8 December 2023

Manuscripts rejected after peer review - Reason(s) for appeal

We addressed the reviewers' comments and questions in the previous revisions by extensively revising the manuscript to provide more clarity, and collecting new healthy control samples in response to the reviewers' concerns.

Reviewer 3's comment on our experiments with healthy controls implies we did not resolve the clotting problems, which is baseless. We clarified the experimental procedure in the manuscript, and provided a list of measures addressing the clotting issue, including performing 4 microfiltrations in parallel to minimize blood storage time. But generally speaking, EDTA is a commonly used anticoagulant and has been used in other CTC studies, and there is nothing 'special' in what we do in that respect.

The remaining concerns by Reviewer 3's are on matters of clinical diagnosis, staging, and prognosis. We do not recognize our manuscript in these concerns, because our manuscript is on the unexpected prevalence of circulating tumor cell (CTC) in previously diagnosed cancer patients undergoing surgery, while our manuscript makes no claims regarding diagnosis, staging or prognosis – we only include the time course of response to therapy for one patient as potential future direction. Our claim is simply that in 17 and 13 patients with Ovarian Cancer and Colorectal Cancer Liver Metastasis, respectively, there is a higher prevalence than expected of CTC clusters.

New data

We provide a summary of new experiments/data performed previously in response to reviewers' comments in the 2nd and 3rd revisions.

2nd revision

Responding to the reviewers' comments, we included CTC microfiltration experiments obtained from two healthy control samples as a validation of the microfiltration process. We disclosed, with full honesty and transparency, a clotting issue with other control samples that have not been encountered before. However, we did not investigate further the causes of the clotting due to the limited availability of control samples.

3rd revision

1. Responding to the reviewers' comment for the 2nd revision, significant efforts had been spent to collect blood samples from additional healthy controls. CTC microfiltrations were performed on a total of 9 healthy control samples.
2. We provided in our response a summary of the steps to minimize the clotting issues encountered with the healthy blood samples, including fresh buffers, fresh sample diluents, minimization of the

blood storage time between collection and microfiltration, as well as fabrication of additional microfiltration devices to conduct 4 microfiltrations in parallel. We clarified the clotting was no longer observed with the measures taken, and that the clotting issue was therefore resolved.

New analysis

Please provide a numbered list of all new analyses of existing data and explain how the new information affects your conclusions or addresses the concerns of the referees.

1st revision

1. The manuscript was revised extensively to address the reviewers' comments.
2. The introduction and discussion sections were revised to highlight novelty and advantages of our approach.
3. We clarified in the text how we confirm that (i) our microfiltration method does not introduce false positive cell counts from non-cancer cells, (ii) clusters were not formed during the filtration process, and (iii) how CTCs were distinguished from white blood cells in clinical samples.
4. We added a discussion on the number and phenotypic heterogeneity of the isolated CTCs and compared with recent studies suggested by the reviewers.
5. The subsection "cCTCs can help understand dissemination in ovarian cancer" was completely rewritten with a summary of our observations and how they compare with studies by others.

2nd revision

1. We clarified the prevalence of WBC and summarized the data in two tables in the supplementary information.
2. We acknowledged in the revised discussion section that our patient cohorts are small. We remain cautious and chose to refrain from making additional conclusions, especially on the correlation of our observed CTC counts with disease stage. While we reported detection of CTC in borderline ovarian tumor (one case in our cohort), a tumor supposedly of uncertain malignant potential, we did not claim any correlation between this type of disease and CTC detection. On the other hand, we provided recent studies using other low shear CTC capture technologies that also found CTC (especially CTC clusters) to be prevalent in early cancer (e.g. breast cancer).
3. We analyzed the microfiltration data obtained from 2 healthy control samples as validation of the microfiltration process. We reported ~ 1 CK⁺/CD45⁻ single cell/mL in the control samples, but did not detect cell clusters in healthy controls, thus confirming our findings. We provided an explanation for the detection of non-tumor epithelial cells and support from 3 references.

3rd revision

1. We analyzed a total of 9 healthy control samples, and reported the detection of ~ 1 CK⁺/CD45⁻ single cell/mL in 3 control samples. No CTC clusters were found, again confirming our findings.
2. We reported that clotting is no longer observed after using new reagents, and minimizing blood storage time by conducting 4 microfiltrations in parallel.

Factual errors by a referee

Reviewer 3's comments are not directly factually wrong, but they are factually wrong by implication, because the questions imply that we should address the questions of clinical relevance such as screening (i.e. early diagnosis), prognosis, or staging. However, our study was not designed to address these questions, nor did we claim to address these questions, hence we consider that it is factually incorrect to keep repeating those questions that are not directly relevant to our findings.

Where we agree with the reviewer is that we should provide technical validation of our method. We did respond to the reviewer concerns, and conducted additional experiments with healthy controls as technical validation of microfiltration, but not as a clinical validation for the use of our method for screening, prognosis or staging (see point above).

The reviewer implies that we should see a difference between different stages, but it is well known that there is a high heterogeneity between CTC counts and stage, and hence it is clear that our study is statistically underpowered to address such questions (again, at the risk of being tedious, we do not perform screening, prognosis, or staging), and hence the implication that we should observe a statistically significant difference in our data is factually incorrect; given the small cohort size, results that are both consistent and inconsistent with higher CTC counts for late stage are plausible.

Please see our point-by-point respond to the comments. Comments are in *black* and our response in *blue*.

Comment to 3rd revision by Reviewer 3

The authors did not provide a sufficient number of negative healthy controls

Reviewer 3 comments and focuses on negative controls primarily linked to single CTCs (not clusters), and to the clinical implications, which as they are not part of our claims, were peripheral questions to us. Regarding controls, we do not claim that we can distinguish between healthy and diseased, and hence healthy samples are technical controls, and 9 controls is significant in that context. As a side note, our clinical collaborators do not have access to healthy individuals, and our lab had to organize a separate effort to recruit healthy controls and obtain blood which was a challenging undertaking as it was not part of the planned study, nor our standard practice, and increased the total number of samples analyzed by 30%.

Given our focus of clusters, and based on Reviewer 1's comments who recognized that we are not looking at single CTCs ("*...in my opinion the focus of the manuscript is on clustered CTCs.*" , Reviewer #1 comment in the 2nd review), we believe that our previous response adequately addressed the overall reviewers' comments, including Reviewer 3's comment on CTCs in early disease. Indeed, we provided reference to a breast cancer study that also finds CTCs in early disease. But Reviewer 3 thought differently, yet does not question the core findings that we see clusters, nor explain why this work should be held to different, higher standards than other studies (see next question).

The rate of false positivity is 33% (3 cases with detected CK+ cells out of 9). Moreover, the references given in their rebuttal letter to discuss false positivity are based of detection techniques, which were not considered as reference.

The "false positive" single cell count observed in 3 of 9 controls is a misinterpretation of the implication of the result. As we wrote, the control test is a technical assessment as we are not presenting our work as a screening, staging or prognostic method, but one that uncovers clusters in cancer patients (where others could not). We had no false positive clusters (i.e. 0 false positive clusters), and we consider that the new experimental results strengthen our claims. While we observed staining of single cells, it has been shown previously, and is consistent with other publications (Sun et al., *EBioMedicine* **46**, 133-149; Green et al., *Anal Chem* **91**, 9348-9355; Tsai et al., *Biomed J* **44**, S190-S200). One explanation is that the insertion of the needle disrupts the skin and can lead to release of epithelial cells that will be stained (Castle et al, *PLoS One* **12**: e0175647).

Also, it is generally expected that higher sensitivity, in our case owing to low shear stress allowing us to capture clusters, it would increase the rate of false positive single cells. But again, at the risk of sounding tedious, our focus is clusters. Hence we are puzzled that Reviewer #3 continues to point out the lack of correlation between our CTC counts and disease stage based on only a few data points, and requires us to provide explanation for the lack of correlation, which is not a claim we make, and for which the number of samples are underpowered in any case.

This work's contribution is to identify clustered CTC in patient samples at a much higher rate and frequency than previously observed, which we attribute to the higher sensitivity of the gravity microfiltration (GµF) method; we also capture isolated CTCs. To reiterate, as analytical sensitivity increases, one would indeed expect more positive detections to occur, including false positives. One example is the detection of Troponin T as myocardial infraction biomarker. As assay sensitivity (i.e

analytical, and not clinical sensitivity) have become more sensitive, more so-called “false positive” occurred, and new way of interpreting troponin test results have therefore been recommended (<https://www.acc.org/latest-in-cardiology/articles/2017/08/07/07/46/a-brief-review-of-troponin-testing-for-clinicians>). Likewise, revised and refined clinical interpretation of CTC data would likely be necessary as the sensitivity of CTC isolation technologies continue to improve; and to reiterate, single CTCs are peripheral to our focus which is on clustered CTCs.

Mains concerns about clinical results provided remain unchanged:

How do the authors explain that they detect CTC in a case of a borderline tumor of the ovary which is supposed to be noninvasive tumor? How do they explain the complete absence of correlation of the number of scCTC and cCTC with the clinical stage of the disease for ovarian cancer (same range of detected CTC in stage I and stage IV)? All their cases in ovarian cancer and colon cancer are positive for CTC detection.

Clinical outcome of the patients and correlation of CTC and scCTC with prognosis are lacking

Reviewer #3 did not fully consider our response regarding the single sample that was graded as “borderline” tumor. In the 2nd rebuttal, we clarified that “borderline” tumor is supposedly a non-invasive tumor with uncertain malignant potential (Fischerova et al., *Oncologist*, **17**, 1515-1533, 2012). We argue here that, while not classified as metastatic disease, CTCs could disseminate from the tumor and detected by high sensitivity methods such as G μ F. CTCs have been detected in patients at early cancer stage (Lawrence et al, *Nat Rev Clin Oncol* **20**, 487–500, 2023 and references herein), with ovarian benign tumor (Cheng et al, *Chin J Cancer Res* **33**, 256–270, 2021) and even preceding a clinical cancer diagnosis (Illie et al., *PLoS ONE*, **9**, e111597, 2014). For ovarian cancer, CTCs have in fact been detected in 8 mL of venous blood collected using 1.6 mg EDTA/mL as anticoagulant, from patients across FIGO stages I, II, III and IV, as well as disease classified as serous/borderline (Kolostova et al., *Am J Cancer Res*. 2015; 5(11): 3363–3375).

In the previous round, Reviewer #3 indicated that “hematogenous dissemination of this cancer is usually considered as occurring late in disease with high clinical stage”. We think the reviewer conflates clinical presentation of metastatic disease with the presence of CTCs. We do not make claims about metastasis of ovarian cancer, but simply about the presence of CTCs (and more specifically clusters) in the blood. The presence of CTCs in the blood does not imply metastasis, and only a minuscule fraction of CTCs will eventually seed a metastasis (Chemi et al., *Front. Oncol* **11**, 672195, 2021). Hence there is no contradiction between detecting CTCs at an early stage already, and metastasis occurring at a later stage, which might occur as a result of the accumulation of CTCs circulating and colonizing distant organs. Our results are consistent with the observed initial metastasis in the peritoneal cavity (Yousefi et al., *Cell Oncol* **43**, 515-538, 2020) by disseminated tumor cells that seed the. Indeed, despite seeing CTCs in the blood early on, one would still expect that dissemination and metastasis would initially progress within peritoneal cavity. In summary, the reviewer is making a wrong inference, and drawing the wrong conclusion.

Their new controls were obtained without clotting and using EDTA tubes. It is not clear if the same technique was applied for their previous clinical results.

We already clarified in the methods section in our 3rd revision the type of blood collection tube used. As pointed out in our 3rd response to reviewers, microfiltrations on the additional control samples followed as close as possible to that for patient sample analyzes in the hospital as possible (which is typically performed one at a time). The clotting that the reviewer highlights was never a problem with patient samples, and occurred only in the control experiments done at the request of the reviewer after the 1st revision. EDTA is commonly used as anticoagulant in CTC analyzes (*Am J Cancer Res*. 2015; 5(11): 3363–3375; Hendricks et al., *Cancers (Basel)* **12**, 2643, 2020; Boya et al., *Nat Commun* **13**, 3385, 2022))

In our previous response we also pointed out that fresh buffers and diluents were used (which we found out later PBS supplemented with calcium might have been mistakenly used for the control performed in

the 2nd revision). We also performed 4 microfiltrations in parallel in order to minimize storage time of the collected blood, which might result in undesired clotting.

The basic principle of CTC filtration is well established and has been used by thousands of studies, and we do not apply any special treatment to the blood, or to our microfilter, and clotting is not an issue generally speaking in the field and simply a question of providing sufficient anti-clotting reagents. Moreover, we apply less shear than others, so there is less reason for clotting. The reviewer statements imply we have a particular issue with clotting which is not the case.

Disagreement with technical or statistical concerns raised by a referee

N/A

Concern that a referee is biased

Please describe your evidence. We ask that you be as specific as possible, bearing in mind that robust criticism does not necessarily reflect referee bias.

As presented point-by-point above on the reviewer's factual errors, we believe Reviewer 3 does not question the core findings that we see clusters, but only peripheral concerns, while adopting a clinical perspective that leads to strawman arguments, but which are not part of our study aims nor claims.

We also remain puzzled that Reviewer #3 continues to point out the lack of correlation between our CTC counts and disease stage, and did not acknowledge that that is not our aim, and that any correlation, or lack thereof, would be unlikely to be achievable given the limited number of patient data points shown here, unless the effect was very pronounced; but again, this was not a focus of our study, nor do we claim it. In studies that found correlation between CTC count and disease stage, there are large individual variations in CTC counts (Zhang et al., *Cell Physiol Biochem* **48**, 1983–1994, 2018), highlighting the need for large number of samples for correlation to become apparent and statistically significant. Hence the request by Reviewer #3 to provide an explanation for the lack of correlation, clinical significance and outcomes is unjustified as lack of correlation in such small cohort is the expectation, which is also why we did not make such a claim of correlation nor particularly comment on it.

Moreover, implying that we did not resolve the clotting issue is baseless, and also penalizes us for being transparent and honest. We also do not understand why this work should be held to different standards than other studies, especially regarding the number of technical controls presented in this work.

In summary, we consider Reviewer 3's assessment to be unfair as the criticism is not directed at the core claims of the work, but at peripheral questions on which we make no claims, and in that sense strawman arguments. Given the errors, and that the reviewer's concerns are tangential and not aimed at our core findings, we conclude that Reviewer 3's comments are biased.

Procedural concerns, editorial consistency

N/A

Dispute over novelty

N/A

Dispute over significance or general interest

N/A

Suggested referees or advisors

N/A

Any other comments
N/A

We would like to thank all Reviewers for their comments and questions. In this revision, we addressed Reviewer #4's comments and revised the manuscript based on the suggestions. Please see our point-by-point response (in blue) below. Revised texts are highlighted in yellow in the manuscript file.

Reviewer #4 (Remarks to the Author):

Control samples are of utmost importance in evaluating a technology for detection of CTC!! The authors clearly have not recognized this as exemplified by their remark in the rebuttal "nor our standard practice", as part of the revision they included the results of 9 healthy donors for which blood was collected in EDTA (blood collection tube of patients not reported but I assume it's the same). No cCTC were detected in 3 ml blood of all 9 samples and 0,0,0,0,1, 3 and 4 scCTC cells in the 9 samples. The authors should use these findings as the basis of setting a threshold and surely not refer to thresholds in papers (ref 60 =ref 20! in this ref 145 healthy donors serve as controls for the technology) in which thresholds are based on a different blood volume, technology and CTC definition. Using this as a basis they simply can reports the background level detected in healthy donors (mean, SD), and for example use the mean + 2SD to be able to discriminate positive versus negative. Than report the mean and SD of the scCTC and cCTC in the 31 patients which simply can be added to table 1 and 2. One can divide the patients as is attempted in the manuscript but at this stage I would not put too much emphasis on this as the numbers are too small to suggest a relation and as such section "Capture of cCTCs from cancer patients" and "Differential reduction of scCTC and cCTC during treatment of metastatic EOC high grade serous carcinoma patient" can be reduced and simplified using the numbers reported in table 1 and 2.

"blood collection tube of patients not reported but I assume it's the same"

Blood collection tube used for:

- Healthy volunteer blood that are subsequently spiked with OV-90 cells: CTAD tubes (previous version line 651-652; line 663-665 in the revised version).
- EOC patient samples: EDTA tubes. We realized we omitted to indicate the type of blood tube in the previous version. We added this information on line 691-692 in the revised version.
- CRCLM patient samples: EDTA tubes (previous version line 686-687; line 698-699 in the revised version).
- 9 healthy volunteer blood samples that were analyzed by GµF: EDTA tubes (previous version line 688-690; line 701-702 in the revised version).

The authors should use these findings as the basis of setting a threshold and surely not refer to thresholds in papers...they simply can reports the background level detected in healthy donors (mean, SD), and for example use the mean + 2SD to be able to discriminate positive versus negative.

Per the reviewer's suggestion, we determined the mean CK⁺/CD45⁻ cell count from the 3-mL healthy control samples (mean = 1 cell/3 mL) and determined the standard deviation

(SD = 1.5 cells/3 mL). Using mean cell count + 2SD of the healthy samples to calculate the limit of detection, we establish a positive threshold for scCTC as > 4 cells/3 mL (or >1 cell/mL). We did not detect any cCTC in any of the healthy samples and the threshold is thus 1 cCTC.

...report the mean and SD of the scCTC and cCTC in the 31 patients which simply can be added to table 1 and 2

For EOC patients, the mean scCTC count (sample volume ranged from 3 to 5.5 mL) was 333 ± 397 cells/3 mL, while the lowest number of scCTC detected in our cohort was 66 cells/3 mL (OC4). For CRCLM patients, the mean scCTC count (sample volume ranged from 2.5 to 5 mL) was 107 ± 79 cells/3 mL while the lowest number of scCTC detected in our cohort was 12 cells/3 mL (CR2). *We added the mean and median of the CTC counts in Table 1 and 2.*

In summary, based on the small cohort of 30 patients and 9 healthy controls, we report a threshold of 4 cell/3 mL (or ~ 1 cell/mL) for scCTC, based on the mean scCTC count + 2 SD in healthy samples. *The lowest number of scCTC detected in our cohort (4 cells/mL) was 4 times above the threshold we determined here. We updated the manuscript on line 348-364 and line 553-555.*

...section “Capture of cCTCs from cancer patients” and “Differential reduction of scCTC and cCTC during treatment of metastatic EOC high grade serous carcinoma patient” can be reduced and simplified using the numbers reported in table 1 and 2.

We appreciate the reviewer’s suggestion. We decide to keep these sections separate for clarity. In particular, the section “Differential reduction of scCTC and cCTC during treatment of metastatic EOC high grade serous carcinoma patient” focuses on reporting the change in CA125 level and number of CTC events during the course of chemotherapy for one EOC patient. We did not relate the status of the tracked patient to the other patients in our small cohort, and did not make any claim regarding diagnosis.

We further clarified that our study is a pilot study with small cohorts of patients and controls. We indicated the number of patients and controls used in this study in the Introduction, Result and Discussion sections, and mentioned in the Discussion the limited cohort size and the need for a larger study to confirm the unexpected prevalence of cCTC in the two cancers studied here.

Other revisions

We corrected the citation error for Ref 20 and 60

We updated the text on line 34, 123-125, 348

We thank Reviewer #4 for the suggestions. In this revision, we updated the manuscript based on the suggestions outlined in the review. We revised the text, and added 3 tables in the SI. Text below in blue is our point-by-point response.

Reviewer #4 (Remarks to the Author):

One cannot report background of CTC detected with different systems and different definitions as part of the introduced CTC technology so : "While the positive detection threshold for cancer is commonly set at ≥ 2 CK+/CD45- scCTCs per 7.5 mL of blood 20" and "epithelial non-CTCs are commonly detected from the blood of healthy individuals at cell counts from 1.5 to 2 cells per mL60,61 , and reaching as high as 21 cells per mL 62, and has been ascribed to shedding of epithelial cells into the bloodstream and/or blood collection device during venipuncture 63" will have to be removed from the result section. If desired one can mention something in the discussion but keep in mind that the actual CTC definitions are quite different so one should be careful making any comparisons. So better to caution the reader and mention that the controls are limited in this study.

Point-by-point response

"While the positive detection threshold for cancer is commonly set at ≥ 2 CK+/CD45- scCTCs per 7.5 mL of blood 20"... will have to be removed from the result section.

Following the reviewer's suggestion, we removed the previously reported positive detection threshold numbers and references for scCTCs. Per suggestions in the previous review, we used the mean cell count of the healthy controls + $2 \times$ SD to define a threshold for scCTCs at 4 cells per 3 mL (or ~ 1 cell per mL). We acknowledged in the Result and Discussion sections that the cohort and healthy controls are limited in this pilot study.

"epithelial non-CTCs are commonly detected from the blood of healthy individuals at cell counts from 1.5 to 2 cells per mL60,61 , and reaching as high as 21 cells per mL 62, and has been ascribed to shedding of epithelial cells into the bloodstream and/or blood collection device during venipuncture 63" will have to be removed from the result section.

We removed the text and reference that provided an explanation for the detection of epithelial cells in healthy controls because it is possible the difference in CTC definitions would lead to different CTC counts.

The results of the nine controls should be added to either table 1 or table 2 and next to the per ml the actual number of ssCTC / cCTC should be provided.

To maintain clarity, we added Table S4, S5 and S6 summarizing the sample volume, actual number of scCTC and cCTC detected in EOC, CRCLM and healthy control samples, respectively, as Supplementary Information.

Reviewer #4 (Remarks to the Author):

The changes clearly improved the manuscript

Responses

We would like to thank all Reviewers for their comments and questions.

In this revision, figures are revised and all points are shown when replicate experiments were performed; data used for calculating the mean value and standard deviation are shown as individual points on the plots when applicable.