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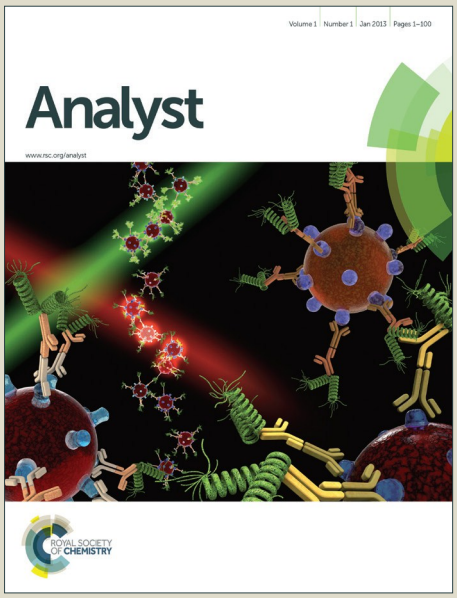

Clinical evaluation of a novel microfluidic device for epitope-independent enrichment of circulating tumour cells in patients with small cell lung cancer

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Abstract

Circulating tumour cells (CTCs) have potential utility as minimally-invasive biomarkers to aid cancer treatment decision making. However, many current CTC technologies enrich CTCs using specific surface epitopes that do not necessarily reflect CTC heterogeneity. Here we evaluated the epitope-independent Parsortix system which enriches CTCs based on size and rigidity using both healthy normal volunteer blood samples spiked with tumour cells and blood samples from patients with small cell lung cancer (SCLC). Blood samples were maintained unfractionated at room temperature for up to 4 days followed by plasma removal for circulating free DNA (cfDNA) isolation and direct application of the remaining cell component to the Parsortix system. For tumour cells expressing the EpCAM cell surface marker the numbers of spiked cells retained using the Parsortix system and by EpCAM-positive selection using CellSearch® were not significantly different, whereas only the Parsortix system showed strong enrichment of cells with undetectable EpCAM expression. In a pilot clinical study we banked both enriched CTCs as well as plasma from SCLC patient blood samples. Upon retrieval of the banked Parsortix cellular samples we could detect cytokeratin positive CTCs in all 12 SCLC patients tested. Interestingly, processing parallel samples from the same patients by EpCAM enrichment using CellSearch® revealed only 83% (10/12) with cytokeratin positive CTCs indicating the Parsortix system is enriching for EpCAM negative SCLC CTCs. Our combined results indicate the Parsortix system is a valuable tool for combined cfDNA isolation and CTC enrichment that enables CTC analysis to be extended beyond dependence on surface epitopes.

Introduction

Metastatic spread of disease to distant sites is the cause of over 90% of cancer related deaths and is thought to be primarily driven by circulating tumour cells (CTCs) present in the bloodstream, which allows them to disseminate from the primary tumour and form distal metastases.[1,2] The current "gold standard" for CTC detection and enumeration is the CellSearch® system, which utilises ferromagnetic beads coated in antibodies targeting the epithelial cell adhesion molecule (EpCAM) to extract CTCs from a 7.5 mL blood sample. The extracted CTCs are then enumerated based on their morphology and expression of cytokeratins, as well as a lack of the CD45 leukocyte marker.[3] The CellSearch® technology is the only CTC technology to be approved by the FDA for assessing overall patient survival in metastatic breast, colorectal and prostate cancer.[4] CTCs have also been studied as novel biomarkers in a variety of disease settings and have been shown to be a significant prognostic factor in small cell and non-small cell lung cancer (NSCLC), as well as in metastatic cutaneous melanoma and castration-resistant prostate cancer.[5-9]

CTCs are a very attractive "liquid biopsy" since, as well as aiding in prognosis, they can provide a current snapshot of the genetic status of the patient's tumour. In addition, since CTCs are a blood based biomarker they provide ease of access and the possibility of repeat sampling, thereby enabling disease monitoring in real time.[10] Furthermore, liquid biopsies are especially useful in cases where acquiring tumour biopsies is particularly challenging, such as in lung cancer, where CTCs from patient blood are being used both as a biomarker as well as to generate tumour xenografts in immunocompromised mice.[11-13] The ultimate utility of CTCs in a clinical setting will be determined by the number of CTCs in a blood sample and the expression of CTC specific markers which are in turn largely driven by the type of cancer and stage of disease. For example large numbers of EpCAM expressing CTCs are seen in advanced SCLC, whereas in NSCLC fewer EpCAM expressing CTCs are detected and CTCs have been shown to display epithelial, mesenchymal or hybrid epithelial/mesenchymal phenotypes.[6,14] This heterogeneity in CTCs suggests that differing CTC enrichment strategies need to be tailored for each cancer type.[15,16] This requirement has given rise to the concept of "epitope-independent", relying on the physical characteristics of cells (e.g. size, rigidity, density).[12,15] A number of different technologies have been developed in the attempt to provide alternative sources of CTCs and have been shown to work well in disease settings in which there are very few CTCs detectable by antibody-dependent methods, such as NSCLC and colorectal cancer.[6,17-22] In addition, the rapid rise in molecular analysis of both isolated CTCs as well as circulating cell free DNA (cfDNA) from plasma raises a need for enrichment methodologies that are compatible with both retrieval of CTCs, as well as cfDNA.[23-25] In considering the eventual widespread application of any novel CTC enrichment method it is also important that blood collection is made

"clinic friendly" by using standard blood collection tubes (BCT) and including blood stabilisation agents that enable the collected whole blood to be stored or shipped several days prior to CTC enrichment.

In designing this current study we looked for a CTC enrichment system that: (1) is epitope independent to encompass CTC heterogeneity; (2) is compatible with stabilised blood collection allowing maintaining whole blood for up to 4 days at room temperature; (3) provides both plasma for cfDNA and cells for CTC enrichment from the same tube; (4) requires minimum pre-enrichment blood processing; (5) utilises a sealed enrichment process to avoid potential contamination; (6) readily delivers enriched cells ready for either storage or analysis. To encompass all of the above features we have evaluated the Parsortix system, a microfluidic, marker-independent enrichment device with which we have established a robust and reproducible protocol that can be used to combine CTC enrichment, CTC isolation and cfDNA isolation from a single blood sample. Our evaluation also includes the first study of the use of the Parsortix for the enrichment of clinical samples from patients with SCLC.

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Materials and Methods

Cell lines and culture

The tumour cell lines HT29, H2009 and DMS114 were cultured in RPMI-1640 (Sigma-Aldrich) with additional 10% foetal bovine serum (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). The cell lines were cultured at 37°C under humidified 5% CO2/95% air.

Collection and spiking of normal healthy volunteer blood

Blood was collected from normal healthy volunteers in accordance with on-site ethics (UoM Research Ethics Committee 1, ref. 12324). Samples of 10 mL were collected in CellSave tubes (Janssen Diagnostics) or Streck Cell-Free DNA blood collection tubes (Streck).[26] The collected blood samples were spiked with the appropriate number and type of cells within 1 hour of collection.

Cancer cell line cells for spike in were harvested by incubating with trypsin/EDTA (Sigma-Aldrich) for 10 minutes at 37°C, after which they were washed using culture media. The resulting cell suspension was then centrifuged for 5 minutes at 800 rcf, following which the supernatant was removed and the cell pellet was resuspended in culture media. The concentration of cells was then established using a Neubauer C-Chip haemocytometer, following which the appropriate volume of cells was added to the blood.

All spiked samples were enriched within 96 hours of collection.

Plasma Isolation and cfDNA preparation

Prior to CTC enrichment plasma was isolated from all Streck cfDNA BCT blood samples within 96 hours of blood collection by centrifuging the blood samples at 2000 rcf for 10 minutes with the centrifuge break switched off. Following this, the plasma layer was removed, centrifuged again at 2000 rcf for 10 minutes, after which the plasma supernatant was stored at –80°C prior to cfDNA analysis. cfDNA was isolated using the QIAmp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions. cfDNA yields were quantified using the TaqMan RNase P Detection Reagents Kit (Life Technologies).

CTC enrichment using the Parsortix system

The Parsortix system (Parsortix) (Figure 1A) used in this study is a benchtop microfluidic device for the enrichment of rare cells from blood samples. The single use disposable cassettes (Figure 1B, 1C)

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contain a stepped structure the width of which gradually decreases until it reaches its narrowest point (*critical gap*) at the width of 10 µm. As CTCs are often larger in size and more rigid when compared to cells normally found in the circulation, it was hypothesised that a critical gap of such width should be sufficient for the selective enrichment of CTCs within the background of normal blood cells.[27-29] Through the process of enrichment circulating tumour cells will be trapped at the critical gap, with the remaining blood cells passing through and going in the waste chamber (Figure 1D).

Following plasma removal from the blood samples (above), the volume of plasma removed was replaced with HEPES buffered saline (HBS, 150 mM NaCl (Sigma-Aldrich), 20 mM HEPES (Sigma-Aldrich)) and mixed by pipetting five times. Samples were enriched on the Parsortix system using a software protocol provided by the manufacturer, which involved passing the blood through the disposable cassette followed by two wash steps. Following enrichment the samples were harvested in a total volume of 1 mL of HBS deposited into 1.5 mL Eppendorf tubes by applying a reverse flow to the cassette, using a software protocol provided by the manufacturer.

Sample enrichment could optionally be followed by a second round of enrichment in order to minimise residual leukocyte contamination. For the second enrichment step HBS containing added 2% bovine serum albumin (BSA, Sigma-Aldrich) was used to prime the cassette in place of HBS, in order to maximise sample harvest. The enrichment was also carried out using a shortened enrichment software protocol (containing only a single wash step) supplied by the manufacturer.

For long term storage of the entire population of enriched cells, the harvested samples were centrifuged at 800 rcf for 5 minutes, after which the supernatant was removed to yield a final sample volume of 100 µL. 100 µL of glycerol (Sigma-Aldrich) was then added to each sample and mixed by pipetting, following which samples were stored at –20°C.

All steps prior to long term storage were carried out at room temperature (RT).

Cell labelling using CellTracker™ Green

For ease of identification, cell lines were labelled prior to spiking using CellTracker™ Green (Life Technologies), following the manufacturer's instructions. A working solution of CellTracker Green compound in pre-warmed, serum-free RPMI was prepared at a concentration of 1 µM. Culture media was then removed from the cultured cells, after which they were washed with HBS after which 10 mL of the working solution was added to the cells. The cells were then incubated at 37°C under humidified 5% CO_2/95% air for 45 minutes, after which the working solution was removed. The cells were then washed with HBS and 10 mL of culture media was added, following which the

cells were incubated under the same conditions for a further 30 minutes. Success of the labelling was determined using a microscope containing the appropriate excitation and detection filters.

CTC analysis using CellSearch®

Blood samples preserved in CellSave were analysed using the CellSearch® system as previously described.[3,30] CTCs were defined as cells expressing both EpCAM and cytokeratins[8,18,19] and not expressing CD45, as well as having a nucleus stained with DAPI (4',6-diamidino-2-phenylindole). CTC analysis was carried out by trained CellSearch® analysts.[5]

Immunofluorescent labelling and enumeration of stored enriched Parsortix SCLC CTCs

Samples stored in glycerol were first diluted with a 10-fold volume of PBS, after which they were centrifuged at 800 rcf for 5 minutes. The supernatant was then removed to yield a final volume of 100 µL. Following this, 100 µL of 0.25% paraformaldehyde (PFA, Affymetrix) was added to the sample, which was then incubated on ice for 1 hour. The sample was then diluted with 1 mL of PBS, centrifuged at 800 rcf for 5 minutes, the supernatant was removed and the cell pellet was then resuspended in 100 µL of PBS and 5 µL of CD45 antibody conjugated to allophycocyanin (APC) (clone H130, Biolegend) was added. The sample was incubated at 4°C for 20 minutes, after which 1 mL of PBS was added followed by centrifugation at 800 rcf for 5 minutes and the supernatant was removed. The sample was then resuspended in 100 µL of 0.1% Triton100 (Sigma-Aldrich) and 5 µL of pan-cytokeratin antibody conjugated to phycoerythrin (PE) (clone C-11, Sigma-Aldrich) was added. The sample was incubated at 4°C for 20 minutes, after which 1 mL of SB115 (Silicon Biosystems) was added, the sample was centrifuged at 800 rcf for 5 minutes and the supernatant was removed. The sample was resuspended in 1 mL of SB115 and two drops of NucBlue Fixed Cell ReadyProbes Reagent (Life Technologies) was added. The sample was incubated at room temperature for 5 minutes, after which it was centrifuged at 800 rcf for 5 minutes and the sample volume was reduced to 13.5 µL. Labelled cells were imaged and counted using the DEPArray system (Silicon Biosystems) as per manufacturer's instructions.[31] Parsortix system enriched CTCs were defined as those cells showing positive staining for cytokeratin, undetectable CD45 labelling and clear nuclear staining (NucBlue). CTC counts obtained from the DEPArray system for Parsortix system enriched samples were adjusted to extrapolated CTC counts per 7.5 ml of blood (rounded to the nearest whole cell) in order to compare to the standard CellSearch® readout of a 7.5 ml blood sample. Cell images of both Parsortix enriched and stained CTCs as well as selected CellSearch® labelled CTCs were obtained from the DEPArray system.

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Patient sample collection

Patient samples were collected as part of an ethically approved prospective, single-centre study carried out at the Christie Hospital, Manchester, United Kingdom. Patients had histologically or cytopathologically confirmed SCLC, staged and treated using standard protocols in accordance with international guidelines.[5,32] Duplicate blood samples were collected from each patient, one preserved in CellSave (for CellSearch® analysis) and the other preserved in Streck preservative (to be enriched using the Parsortix system). Blood samples were collected up to 7 days prior to commencing treatment (baseline), following end of chemotherapy (EndTx) and, if possible, upon the development of relapsed disease (relapse).[5]

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Results and Discussion

Evaluation of enrichment potential using spiked blood samples

Initial evaluation of the enrichment potential of the Parsortix system was established using preserved blood samples from healthy volunteers spiked with HT29 cells, a colorectal cancer cell line that was pre-labelled with CellTracker™ Green to facilitate detection and enumeration. Samples from two donors (Donor A and Donor B) were spiked at a final concentration of 50 HT29 cells per mL of blood. Following storage of the blood for four days at RT, duplicate runs of both 0.5 mL and 4 mL aliquots of blood from both donors were enriched to investigate the relationship between sample volume and cell recovery.

For the 0.5 mL blood samples the average number of spiked cells recovered across both donors was 17.5 (range 14-21) (Figure 2A), which corresponds to a recovery efficiency of 70% ±10 (range 56-84%) (Figure 2B). For the 4 mL blood samples the average number of cell recovered across both donors was 138 (range 114-165) (Figure 2A), which corresponds to a recovery efficiency of 69% ±10 (range 57-83%) (Figure 2B). These results showed that the efficiency of recovery of spiked cells was consistent across sample volume processed.

By counting DAPI stained non-CellTracker™ Green positive cells the number of contaminating leukocytes could also be enumerated for each run. In contrast to the spiked cancer cells the number of contaminating leukocytes did not significantly increase with volume of sample processed, with an average of 2439 leukocytes (range 2403-2475) recovered from the 0.5 mL samples of Donor A and 2840 (range 2610-3069) from 4 mL of the same donor (Figure 2A). This discontinuity in leukocyte recovery versus volume of blood processed was also seen for donor B, with an average of 509 for 0.5 mL blood (range 504-513) and 765 for 4 mL (range 756-774). Similar trends in leukocyte recovery were also seen with additional healthy donors (not shown) indicating that with the current enrichment protocol the absolute numbers of leukocytes recovered is donor dependent. From this we conclude that increasing the blood sample volume will result in a larger number of CTCs being recovered, without having to deal with significant increases in leukocyte contamination, which we have found is a variable feature of the input blood.

Evaluation of a high-purity enrichment protocol

Though the initial experiments with the Parsortix system showed good recovery of spiked cells from HNV blood, a significant number of contaminating leukocytes was seen in all recoveries (Figure 2A). This carryover of leukocytes is seen with all CTC enrichment platforms and is not a major problem

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for the simple enumeration of CTCs. However, one of the strengths of the Parsortix platform is that the enriched cells can be readily recovered from the cassette using a simple back flush cycle (Materials and Methods). This provides the ability for molecular analysis of the captured cells, which has immense clinical potential. For bulk analysis of the entire enriched population of cells the carryover of contaminating leukocytes will result in unwanted dilution of any CTC derived readouts. In an attempt to reduce the leukocyte carryover, a double-enrichment approach was assessed using blood samples spiked with HT29 and H2009 (NSCLC cell line) cell lines, both pre-labelled with CellTracker™ Green. Blood samples from three donors were spiked with HT29 cells and three donors were spiked with H2009 to a final concentration of 100 spiked cells per mL of blood. 1 mL of each sample (100 spiked cells total) was passed through a first Parsortix system cassette, the captured cells were then recovered using a back-flush cycle and these enriched cells run through a second Parsortix system cassette. The resulting double-enrichment captured cells were then enumerated.

In the case of the HT29 spiked samples, the average number of recovered spiked cells was 42 (range of cells recovered 37-46) (Figure 3A). This gave an efficiency of recovery of 41% ±3, which was lower than the single enrichment (69% across all samples, Figure 2A) showing loss of target cells following the second enrichment. However, the range of contaminating leukocytes was greatly reduced (Figure 3B) with an average cell number of 115 (range 69–178). For the H2009 spiked samples, the average number of target cells recovered was 51 (range of cells recovered 31-70) (Figure 3A). This gave a slightly better efficiency of recovery than the HT29 samples of 51% ±13, though this was more variable. Again, the level of contaminating leukocytes was greatly reduced compared to a single cassette with an average cell number of 108 (range 72–141) (Figure 3B).

These experiments show that a double enrichment approach significantly reduces the leukocyte contamination of recovered cells compared to a single enrichment. The second step however, also reduces the efficiency of recovery of the target cell population. Despite spiked cell losses the maximum number of leukocytes present following double enrichment was 178 indicating that if similar reduced leukocyte numbers are also seen for clinical samples, the presence of a single CTC would account for at least 0.5% of the entire enriched sample. Given the above characteristics of double enrichment, bulk analysis may be applicable for specific projects where CTC specific detection sensitivity is +> 0.5%.

Comparison of Parsortix and CellSearch® systems using spiked blood samples

As the Veridex CellSearch® is currently the only CTC enrichment platform with FDA approval we next compared the enrichment potential of the Parsortix system to the CellSearch® system.[3] To this end

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healthy volunteer bloods were spiked with the cancer cell line HT29, that we had shown to be highly EpCAM positive (Figure 4A). The HT29 cells were pre-labelled with CellTracker™ Green and spiked into paired blood samples at 133 cells per mL (1000 cells in 7.5 mL of blood). The paired samples were collected in CellSave tube for analysis on CellSearch® and Streck cfDNA blood collection tubes for analysis on the Parsortix system.

Four pairs of samples were evaluated using the EpCAM positive HT29 cell line with similar capture efficiencies seen on both platforms (Figure 4B). The average percentage of spiked cells captured by the Parsortix system was 78% ±11 (range 59-86%). For the CellSearch® system the average recovery was 83% ±6 (range 73-88%). A paired sample two tailed t-test showed that the capture efficiencies of the Parsortix system and the CellSearch® were not significantly different (p=0.23).

To demonstrate the epitope independence of the Parsortix system we next compared the two systems using DMS114 cells, that we had shown to have low EpCAM expression (Figure 4C). Two pairs of samples were evaluated using the same approach as described above, with recovered cells enumerated by detection of CellTracker™ Green cells. The average percentage of DMS114 cells captured by the Parsortix system was 41% ±1 (range 40-42%). In contrast the CellSearch® platform failed to recover appreciable numbers of DMS114 cells with an average recovery 0.35% ±0.05 (range 0.3-0.4%). A paired sample two tailed t-test showed that the capture efficiencies of DMS114 EpCAM low cells by the Parsortix system and the CellSearch® were significantly different (p=0.02) (Figure 4C).

These data demonstrate that the Parsortix system has comparable recovery efficiencies as the CellSearch® system for the enrichment of EpCAM positive cells, and a greatly improved recovery of EpCAM negative or low expressing cells. This confirms that use of enrichment based on physical characteristics (size and rigidity), rather than on the expression of specific surface epitopes, enables access to an additional subset of CTCs which may be otherwise missed when using epitope dependent systems, a significant advantage of an epitope independent CTC enrichment platform.

Enrichment of SCLC clinical samples using the Parsortix system

Following the success of initial spiked cell line experiments, we went on to evaluate the Parsortix system enrichment of cytokeratin positive CTCs in a clinical head-to-head comparison with CellSearch®, the only technology currently approved by the FDA as a prognostic indicator for metastatic breast, prostate and colorectal cancer, and thus the current "gold standard" in CTC detection. We chose SCLC because: 1) it is a major biological interest of the group 2) large numbers of EpCAM +ve CellSearch® CTCs have been previously reported; and 3) we are particularly interested in enrichment technologies that could help dissect the heterogeneity of SCLC CTCs.[13] Paired blood

samples were taken from 12 patients with SCLC (Figure 5A) for enrichment and enumerated by both CellSearch® and the Parsortix system. To maximise the amount of clinical data elucidated from a single blood sample we developed a Parsortix-based protocol that enabled the isolation of the plasma from patient blood samples prior to CTC enrichment (Materials and Methods). We utilised this approach on 11 of the SCLC samples and performed cfDNA isolation from the plasma. Due to an extremely low sample volume for patient 1 (2.5 mL of blood), plasma was not collected for this patient. An RNAseP qPCR assay was used to quantify the yield of cfDNA from each sample (Figure 5B) and found that significant levels of cfDNA could be purified for all 11 patients, providing a valuable clinical resource that can be utilised to monitor and evaluate the molecular status of the patients' disease.

Following separation of the plasma, the cellular component of each patient sample was enriched on the Parsortix system followed by staining and enumeration of CTCs as described (materials and methods). A parallel sample was enumerated on the CellSearch® system, on both systems CTCs were identified as CK+, CD45–, DAPI+ and white blood cells (WBC) identified as CK–, CD45+, DAPI+ (Figure 5C). For the CellSearch® system, CTCs were detected in 10 out of 12 samples with a range of 1-3780 CTCs per 7.5 mL of blood (Figure 5D). In the paired samples enriched by the Parsortix system CTCs were detected in 12 out of 12 samples, with a range of 20–1474 (following extrapolation to match the volume of blood analysed on the CellSearch®) (Figure 5D, SI1). For the 10 patients with CTCs detected by both CellSearch® and the Parsortix system, CTC counts were within 2 fold for both systems for 4 patients, 5 patients showed higher Parsortix system numbers than CellSearch® and 1 patient showed higher CellSearch® numbers than Parsortix system (Figure 5D). The combined results indicate patient to patient CTC heterogeneity, including some patients with EpCAM negative or low expressing CTCs that fail to be captured by CellSearch®, and others with EpCAM positive CTCs not retained by the Parsortix system. This emphasises the need for multiple approaches to CTC enrichment and raises the important clinical question of what the biological consequence of these different CTC subsets is.

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Conclusion

In evaluating the Parsortix system for its utility as a CTC enrichment device we devised a sample workflow that would fit readily into standard clinical practice as well providing flexible stop off points where processed samples could be stored prior to further, more intensive analysis (Figure SI2). We specifically set out to use blood collection tubes that are clinic ready and provide both cells as well cfDNA following maintaining the samples as whole unfractionated blood for at least 96 hours at room temperature. This would make it possible to process samples from multiple sites and, more importantly, avoid the processing variation due to handling living blood samples. For routine use we found the Streck cfDNA tubes provided both cell stability suitable for cell enrichment (Figures 2-4) and plasma suitable for cfDNA preparation (Figure 5). We also found similar results with the Parsortix system using spiked cells with CellSave, Streck Cell-Free RNA and Streck CytoChex preservative (Figure SI3) indicating that Parsortix system is compatible with a wide variety of blood preservative tubes.

In a pilot clinical study we successfully processed all 12 SCLC patient blood samples using the Parsortix system and banked both plasma and enriched cells for future analysis. For all banked 12 cellular samples we were able to retrieve cells, carry out immunofluorescent labelling followed by successful CTC enumeration based on CK+, CD45–, DAPI+ staining (Figure 5). For all 11 plasma samples processed we obtained cfDNA that could be analysed by qPCR and ranged in concentration from 1.6 to 633.6 ng/mL of plasma (Figure 5A). In a matched comparison of CK+, CD45–, DAPI+ CTCs enriched either by the EpCAM dependent CellSearch® system or by Parsortix we identified differences between the 2 enrichment protocols which indicates clear CTC heterogeneity (Figure 5). Importantly, we identified ≥20 CTCs in all 12 samples enriched by Parsortix whereas 2 samples enriched by CellSearch® showed no CTCs and an additional 3 samples contained fewer than 5 CTCs. Based on these encouraging results we have now incorporated the Parsortix chip workflow into multiple clinical trials and have accumulating many 100s of stored enriched samples that will be of immense value in our future CTC studies.

In summary, the approach we have described offers a unique combination of features making it suitable for routine clinical analysis of patient blood samples. Firstly, the epitope independent CTC enrichment approach we have devised is compatible with whole blood stabilisation reagents suitable for at least four days at room temperature. Secondly, CTC enrichment takes place in a simple plug and play device that remains sealed throughout the enrichment step and the enriched cells are eluted directly into a collection tube for further analysis. Thirdly, the approach delivers both plasma for cfDNA analysis and cells for CTC enrichment, enabling direct comparisons of molecular readouts

from both cfDNA and CTC. Fourthly, enriched CTC samples can be banked for later analysis providing the much needed flexibility often required to select relevant subsets of samples as well as analytical approaches appropriate to specific clinical trials and biological questions. Finally, our initial clinical results with SCLC patient blood samples clearly demonstrates that the CTC enrichment process we have developed identifies subsets of CTCs not readily detected by epitope dependent technologies thereby facilitating more extensive CTC analysis which may help determine the underlying patient cancer status providing the potential for improving patient outcomes.

In the course of submitting this manuscript, Xu et al. published an evaluation of the Parsortix which included a pilot study with EDTA blood samples from patients with prostate cancer, demonstrating that Parsortix is capable of enriching for prostate CTCs, indicating the Parsortix system is applicable to both fresh and preserved blood samples.[33]

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Figure 1

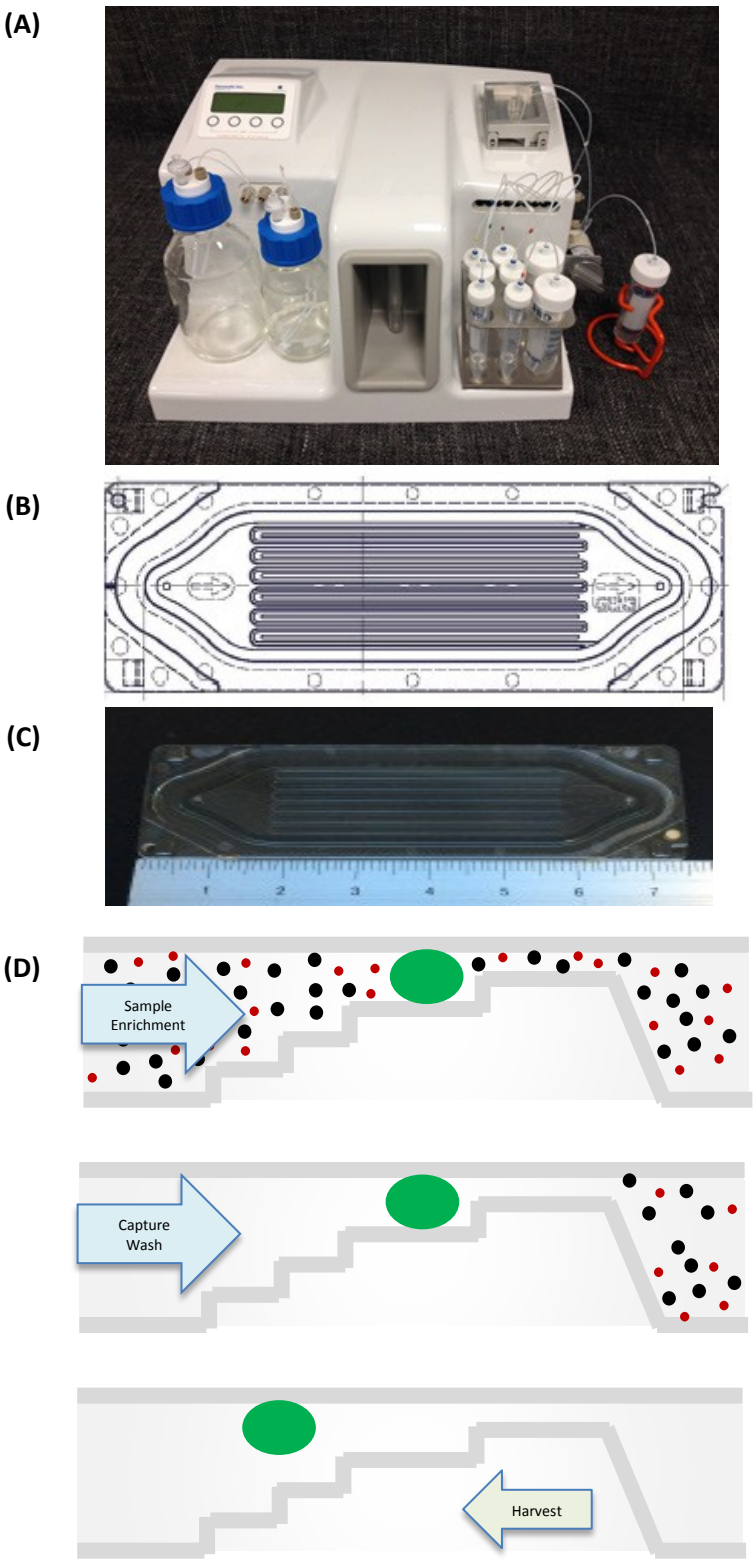

Fig. 1 Parsortix system design and principle of operation. (A) The system consists of a microfluidic pump that pushes the blood sample through a disposable cassette at a set pressure of 23 mBar, resulting in a flow rate of approx. 2 mL of blood per hour. (B) Schematic of the disposable cassette,

courtesy of Angle plc. (C) Image of the disposable cassette. (D) Graphical representation of the cross-section of the cassette. The cassette contains a stepped structure which gets progressively narrower until it reaches the width of 10 µm (termed the critical gap). CTCs are retained at this gap, while contaminating red cells and white cells pass through into the waste receptacle. Captured cells can then be recovered (harvested) for further analysis by applying a reverse flow. Image adapted from Xu, L., *et al.*[33]

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Figure 2

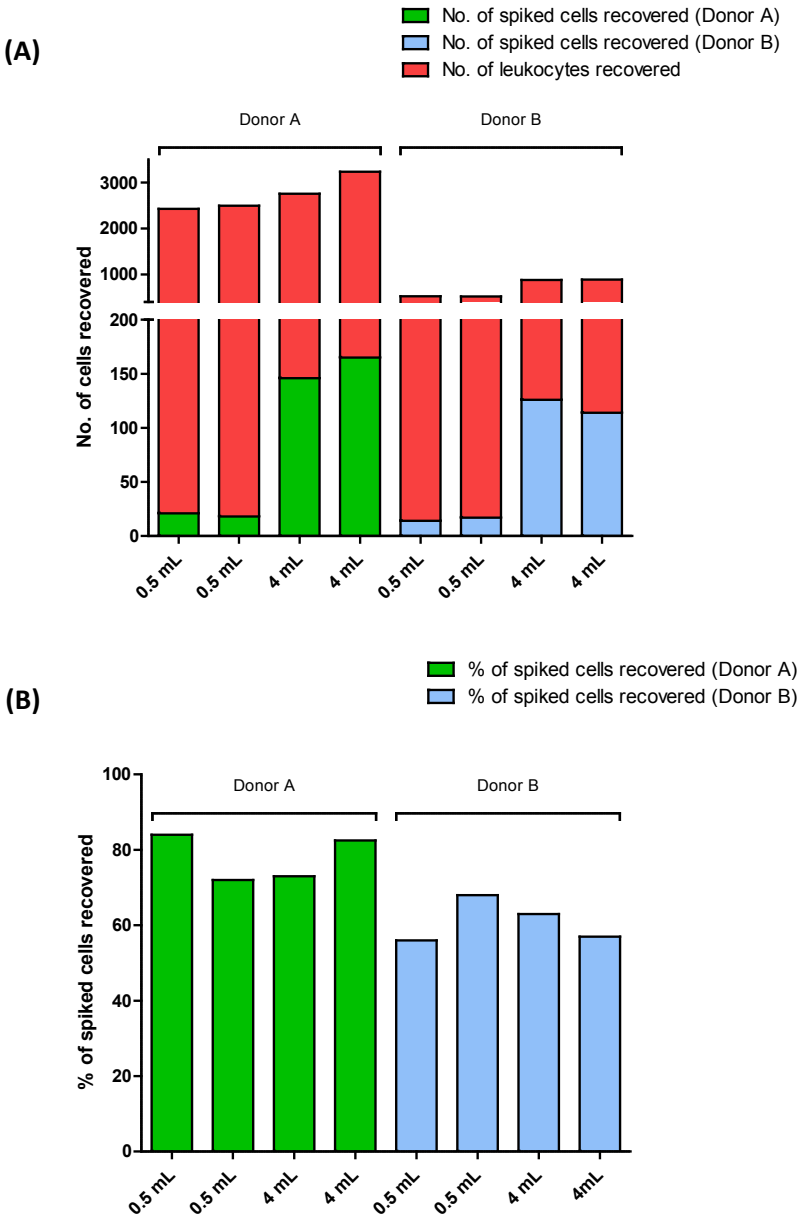

Fig. 2 Evaluation of the enrichment potential of the Parsortix single enrichment protocol using healthy blood spiked with HT29 cancer cell line. (A) Duplicate samples of 0.5 mL and 4 mL of blood from two separate donors, spiked at a concentration of 50 cells / mL of blood, were enriched using the Parsortix system, after which they were recovered from the cassette. Both the number of spiked cells and contaminating leukocytes was assessed. Increasing the volume of enriched blood 8-fold resulted in an 8-fold increase in the number of captured spiked cells, but only a slight increase in the number of contaminating leukocytes. (B) Increasing the volume of enriched blood did not impact the capture percentage of the device.

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Figure 3

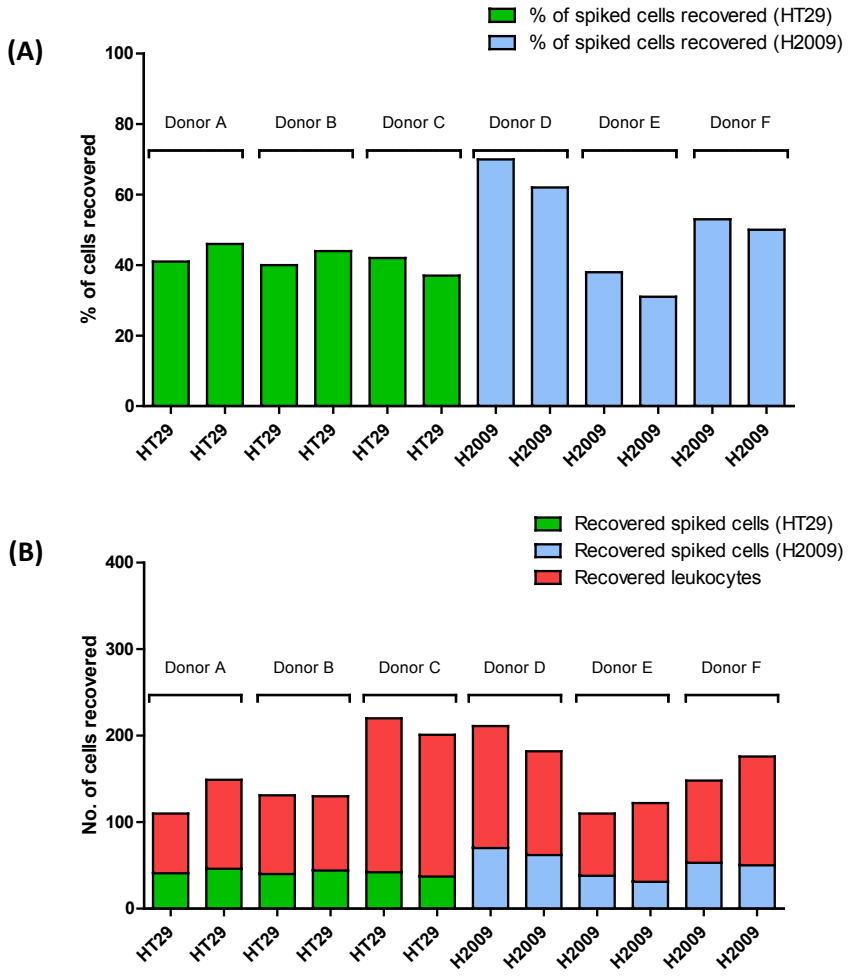

Fig. 3 Evaluation of double enrichment protocol using healthy blood spiked with HT29 and H2009 cells. (A) Duplicate samples of 1 mL of blood from 6 separate donors , spiked with either HT29 or H2009 cells at a concentration of 100 cells / mL of blood, were enriched using the Parsortix system employing the high purity double enrichment protocol, after which the captured cells were recovered from the cassette. The double enrichment protocol results in fewer spiked cells being retained compared to the single enrichment protocol. (B) The advantage of the double enrichment protocol lies with a significantly lower number of contaminating leukocytes being retained (fewer than 200 cells retained). Using different cell lines did not significantly impact the percentage of cells recovered.

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Figure 4

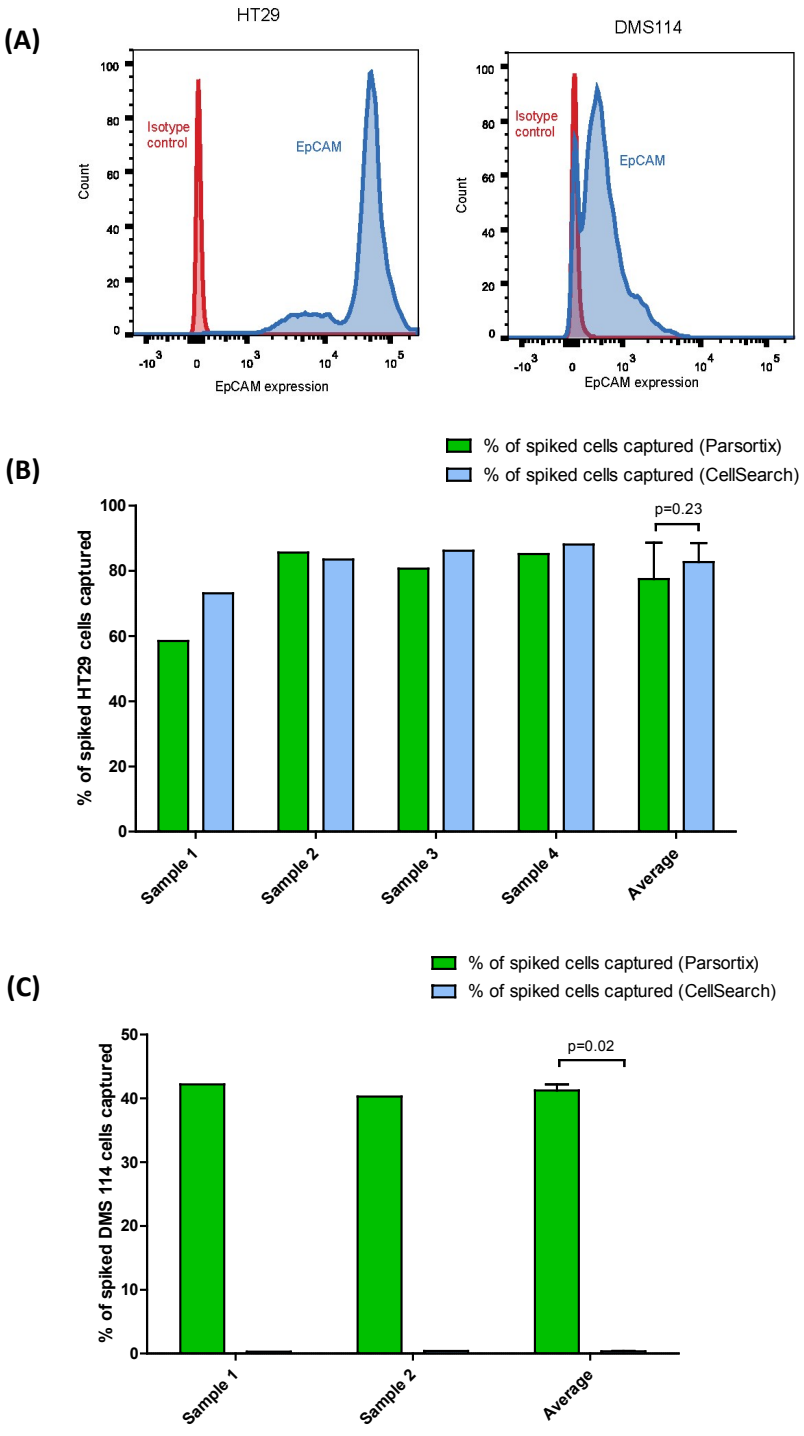

Fig. 4 Comparison of enrichment potential of the Parsortix and the CellSearch® systems. (A) Histograms of EpCAM expression in HT29 and DMS114 cells, with isotype controls in red and EpCAM expression levels in blue. (B) Comparison using healthy blood spiked with a cell line expressing high levels of EpCAM (HT29). Paired samples of 7.5 mL of blood from 4 separate donors were spiked with 1000 HT29 cells pre-labelled with CellTracker Green. One sample was enriched using the Parsortix system and the other was enriched using the CellSearch®. Numbers of captured cells for each sample

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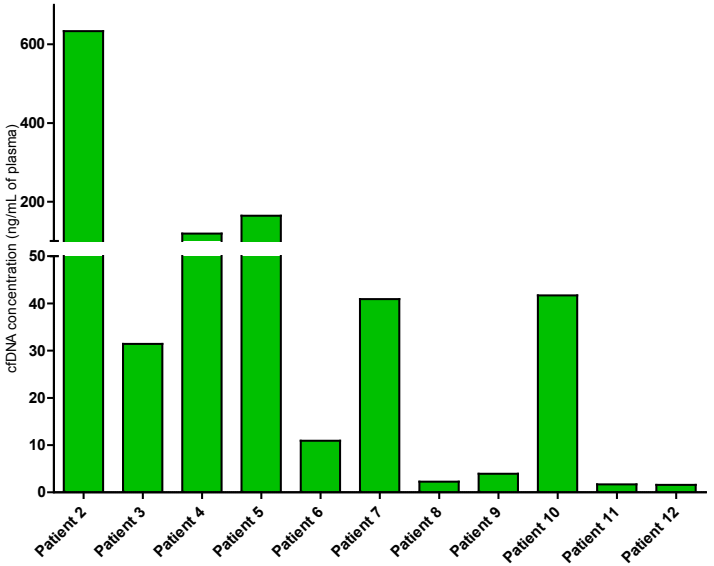
were compared, showing no significant difference in capture levels between the Parsortix system and the CellSearch® (p=0.23). (C) Comparison using healthy blood spiked with a cell line expressing low levels of EpCAM (DMS114). Paired samples of 7.5 mL of blood from 2 separate donors were spiked with 1000 DMS114 cells pre-labelled with CellTracker Green. One sample was enriched using the Parsortix and the other was enriched using the CellSearch®. Numbers of captured cells for each sample were compared, showing significantly higher capture levels for the Parsortix system compared to the CellSearch® (p=0.02).

Figure 5

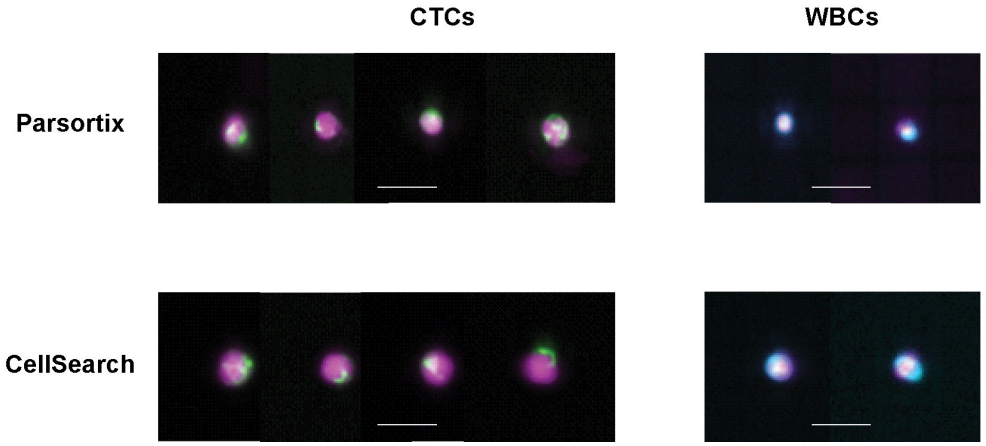
(A)

Patient ID	Gender	Age at diagnosis	SCLC Stage	Timepoint analysed
Patient 1	Female	70	Extensive	Baseline
Patient 2	Male	65	Extensive	Baseline
Patient 3	Male	65	Extensive	Baseline
Patient 4	Male	81	Extensive	Baseline
Patient 5	Male	78	Extensive	Baseline
Patient 6	Male	53	Limited	Relapse
Patient 7	Male	73	Extensive	Baseline
Patient 8	Female	50	Extensive	Relapse
Patient 9	Male	74	Extensive	EndTx
Patient 10	Female	49	Extensive	Relapse
Patient 11	Female	56	Extensive	EndTx
Patient 12	Female	62	Limited	Baseline

(B)



(C)



(D)

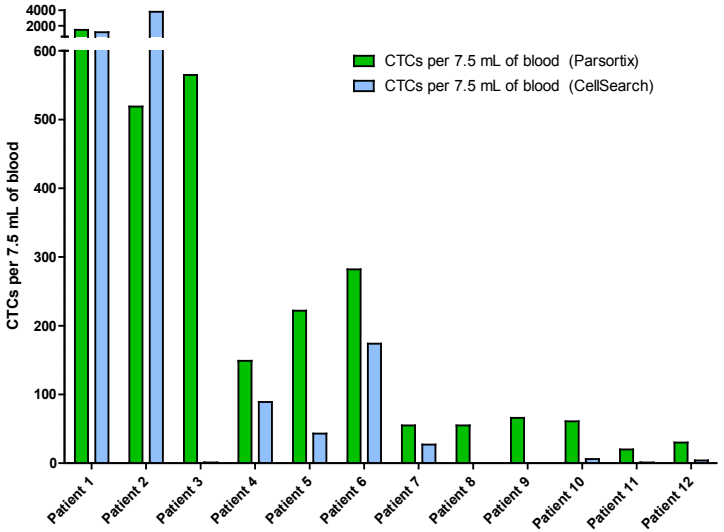


Fig. 5 Comparison of results from SCLC clinical samples enriched by the Parsortix and the CellSearch® systems. (A) Summary of the patients' clinical characteristics at time of diagnosis. 7 patients were treatment-naïve (Baseline), 2 were undergoing chemotherapy (EndTx) and 3 had relapsed following previous treatment (Relapse). (B) Yields of cfDNA extracted from plasma of 11 of the 12 patients. (C) Comparison of CTCs and white blood cells isolated by DEPArray form a paired sample enriched using both the Parsortix and the CellSearch®. Pan-cytokeratin is labelled in green, CD45 in blue and nuclear staining in pink. Scale bar represents 20 µm. (D) Summary of CTC counts from 12 SCLC samples collected from 12 patients at various stages of treatment. CTC counts for samples enriched by the Parsortix were adjusted to reflect 7.5 mL of blood – the sample volume used by the CellSearch® system.