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Screening for Circulating Tumour Cells Allows Early Detection of Cancer and Monitoring of Treatment Effectiveness: An Observational Study

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Abstract

Background: Circulating-Tumour-Cells (CTC) provide a blood biomarker for early carcinogenesis, cancer progression and treatment effectiveness. An increase in CTCs is associated with cancer progression, a CTC decrease with cancer containment or remission. Several technologies have been developed to identify CTC, including the validated Isolation-by-Size-of-Epithelial-Tumour (ISET, Rarecells) technology, combining blood filtration and microscopy using standard histo-pathological criteria.

Methods: This study compared CTC count to cancer status and cancer risk, by monitoring treatment effectiveness in cancer patients and by screening for CTC in asymptomatic patients with risk factors, including family history of cancer.

Results: Between Sept-2014 and Dec-2016 we undertook 600 CTC tests (542 patients), including 50% screening requests of patients without cancer diagnosis but with risk factors. CTC were detected in all cancer patients (n=277, 100%), and in half of the asymptomatic patients screened (50%, 132 out of 265 patients). Follow-up tests including scans were scheduled within 1-6 months of CTC tests. In up to 50% of male patients with normal PSA (prostate-specific-antigen) levels but detected CTC, PET scans using PSMA (Ga-68-prostate-specific-membrane-antigens) revealed increased uptake in the prostate, indicative of early prostate cancer. Other types of cancer, including early breast, ovarian, lung, or renal cancer were detected in a small number of asymptomatic men or women with a positive CTC count.

A subgroup of patients with detected CTC underwent interventions, including nutritional therapy with immune-stimulating and anti-carcinogenic nutrients. CTC repeat tests were available in 10% of patients with detected CTC (40 out of 409 patients, n=98 CTC tests) to assess treatment effectiveness.

Conclusion: CTC screening provided a highly sensitive biomarker for the early detection of cancer, with higher CTC counts being associated with higher risk of malignancy. CTC monitoring over time indicated treatment effectiveness. Nutrients with anti-carcinogenic properties could reduce CTC count, and included curcumin, garlic, green tea, grape seed, modified-citrus-pectin, and medicinal mushroom-extract.

Keywords: Circulating tumour cells (CTC); Cancer screening; Early detection; Treatment effectiveness; Prostate cancer; Breast cancer; Integrative nutritional therapy

List of Abbreviations: CTC: Circulating Tumour Cells; EDTA: Ethylene-Diamine-Tetra-Acetic-acid, EpCAM: Epithelial Cell Adhesion Molecule; ISET: Isolation by Size of Epithelial Tumours; PET: Positron Emission Tomography; PSA: Prostate Specific Antigen; PSMA: Prostate Specific Membrane Antigen

Introduction

Circulating Tumour Cells (CTC) provide a biomarker for cancer prognosis and treatment effectiveness, whereby an increase in CTC count is associated with cancer progression, shorter progression free survival, and shorter overall survival compared to a decrease in CTC count [1,2]. In a group of 177 women with metastatic breast cancer, CTC count was directly related to disease progression and survival, whereby a CTC count of less than 0.7 CTC/ml (5 CTC in 7.5 ml of whole blood) had a longer progression free survival and overall survival compared to a CTC count of more than 0.7 CTC/ml (median progression-free survival 2.7 months versus 7.0 months, $p<0.001$), and median overall survival (10.1 months versus >18 months, $p<0.001$) [1]. Furthermore, the type of CTC cells, either single cells or CTC clusters, are a prognostic predictor of metastasizing potential and overall survival, with a hazard ratio of 14.5 ($p<0.001$) for ≥ 3-cell CTC clusters compared to no CTC [3].

Presence of CTC has also been associated with early carcinogenesis and risk of cancer [4]. In a study of cancer-free patients with chronic obstructive pulmonary disease (COPD), CTC were detected in 3% of the patients, who developed lung cancer within 1-4 years after CTC screening.

Several technologies have been developed to identify CTC, including the Isolation-by-Size-of-Epithelial-Tumour (ISET) technique (Rarecells, France) [5], which involves blood filtration, and analysis by microscopy using standard histo-pathological/ cyto-morphological criteria [6,7]. Blood is treated to lyse red blood cells, and remaining rare cells, including CTC and inflammatory (white blood) cells, are then enriched on a filter, stained and analysed by standard cytological microscopy. The ISET technology allows direct identification of CTC, independent of the presence of tumour markers [7].

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For example, the Cellsearch or Maintrac technologies use Epithelial-Cell-Adhesion Molecule (EpCAM) markers to detect CTC [8,9]. The ISET technology enables CTC to be detected in all types of cancer, including small-cell type cancers and blood type tumour cells. All CTC are larger than the filter holes of 8 microns, including solid tumour cells of 11.7-23.8 microns, small-cell type cancers (e.g. small cell lung carcinoma of 7.2-10 microns) and blood type cancers (e.g. leukemia cells of 8.9-15.3 microns) [10,11]. Furthermore, blood type cancer cells don't express the EpCAM markers, and in cancer cells undergoing normal morphogenetic processes, also known as epithelial mesenchymal transition (EMT), which can lead to loss or gain of tumour markers including EpCAM markers [12].

In addition, the ISET technology allows observation of morphological changes of atypical cells, and therefore allows distinction between CTC with malignant features (3-4 criteria out of 4 for malignancy), 'CTC' with uncertain malignant features (2-3 criteria), and benign circulating epithelial cells and cell clusters (CEC), as well as reactive inflammatory cells [13]. Changes of the normal morphology of cells into atypical cells are meaningful, and can be regarded as precursors in cancer development [14,15].

Because of the morphological changes of cells during carcinogenesis, the identification of (atypical cells or) CTC by ISET technology may be superior to other indirect tumour marker dependent methodologies. For example, the CTC count was more accurate on average with the ISET methodology compared to the Cell search methodology in metastatic prostate and lung cancer patients [16].

The ISET technology has been validated in several published studies, providing high specificity (1 CTC/ml), and high sensitivity (0 CTC/ml in 600 healthy donors) in cancer patients with various types of cancer including liver, lung, pancreatic cancer, soft-tissue sarcoma, and melanoma [4,14,17-24].

In this study we used the ISET technology for the detection of CTC in cancer patients and as screening tool in patients with higher risk of malignancy, e.g. family history, smoking, age (>50 years). Here we provide evidence that screening for CTC allows for early detection of cancer. We further summarise follow-up results by CTC repeat test of patients with detected CTC who undertook immune-stimulating nutritional therapy.

Materials and Methods

Aims

The study aimed to compare CTC count to cancer status and cancer risk, by monitoring treatment effectiveness in cancer patients and to screen for CTC in patients with a family history of cancer or clinical indication but no tumour mass.

Study design and patients

For this cohort study, patients were recruited from two medical clinics in Melbourne, Australia, the National Institute of Integrative Medicine 'NIIM' Clinic, and the Eng Medical Centre, between Sept 2014 and Dec 2016. CTC tests were performed to monitor treatment effectiveness in cancer patients, and for early detection screening in patients with an increased risk of cancer, including patients with a family history of cancer, smoking habits, long term oral contraceptive use or hormone replacement therapy in women, advanced age (>50 years) in men, or other medical indication.

The study was approved by the NHMRC-endorsed NIIM Human Research Ethics Committee. Participating patients provided written informed consent. No individual patient data is divulged in this article.

The authors confirm that all ongoing and related trials for this study are registered.

Circulating tumour cell (CTC) detection

In this study we used the Isolation-by-Size-of-Epithelial-Tumour (ISET) methodology (Rarecells, France), combining blood filtration and analysis by microscopy using standard histo-pathological criteria [13,17]. We followed standardised validated protocols described previously [6].

Briefly, the ISET method is a blood filtration-based approach, which enriches rare cells on a polycarbonate membrane with 8 micron holes. 10 mL of peripheral blood was collected in buffered EDTA, maintained at room temperature and processed within 2 hours of collection. Blood was then diluted 1:10 with buffer containing 0.175% saponin, 0.2% paraformaldehyde, 0.0372% EDTA, and 0.1% bovine serum albumin, shaken for 10 minutes at room temperature, and filtered with the ISET filtration blocks and device (Rarecells, France) [6].

The dried filter membrane was stained with May-Gruenwald-Giemsa for cytological analysis.

A trained and experienced cancer cytologist conducted the analysis using a Leica DMLB microscope with 63 × 10 magnification and standard histo-pathological criteria to identify the degree of malignancy.

Circulating malignant cells were defined by the presence of 4 of the following criteria: a) anisonucleosis (ratio >0.5), b) nuclei larger than 2-3 calibrated pore sizes (8 microns) of the membrane (i.e. >24 microns), c) irregular nuclear borders, d) high nuclei-cytoplasmic ratio, and/or e) presence of three-dimensional sheets. Cells displaying 1-3 criteria were defined as atypical cells with uncertain malignant potential. Circulating benign cells were characterized by the absence of these criteria [17].

Images of CTC and atypical cells were taken with a digital Leica EC3 camera, and all images were reviewed independently by a second cytologist and any discrepancies discussed. All images were added to a library of digital images for future cross-reference.

Patient follow-up

Patients with detected CTC were advised on follow-up tests including scans by the consulting doctor. Asymptomatic men with detected CTC, and Ki-67, PSA or androgen receptor (AR) expression [25], and PSA (prostate specific antigen) levels in the normal range, had a pelvic PET scan using Ga-68 PSMA (Gallium-68 Prostate-Specific-Membrane-Antigens) [26]. The Ga-PSMA-PET/CT scan is a highly sensitive test detecting lesions of ≥ 2.4 mm short axis diameter [27-29]. Asymptomatic women with detected CTC, and endocrine receptor (HER2) positive expression, had an MRI scan of the breast. Symptomatic patients had a scan relevant to the area of their symptoms. CTC testing was repeated within 3-6 months in patients with detected CTC.

All patients with or without cancer diagnosis but with detected CTC were advised about immune-stimulating therapy. Protocols included nutrients with evidence of anti-carcinogenic properties.

Analysis

Descriptive analysis was used to compare CTC count and cancer status or risk at baseline, the primary outcome and observational

component of the study. Simple comparative analyses were conducted for the subgroups of patients who undertook a repeat CTC test after a variety of treatments as intervention.

Results

Between Sept-2014 and Dec-2016 we undertook 600 CTC tests in 542 patients, including 50% screening requests (n=286 tests) of patients without cancer diagnosis but with risk factors. CTC were detected in all cancer patients (n=277, 100%), and in half of the asymptomatic patients screened (50%, n=132 out of 265 patients). A subgroup of patients with detected CTC underwent interventions (n=161). CTC repeat tests were done for 10% of patients with detected CTC (40 out of 409 patients, n=98 CTC tests). Figure 1 summarises the trial flow.

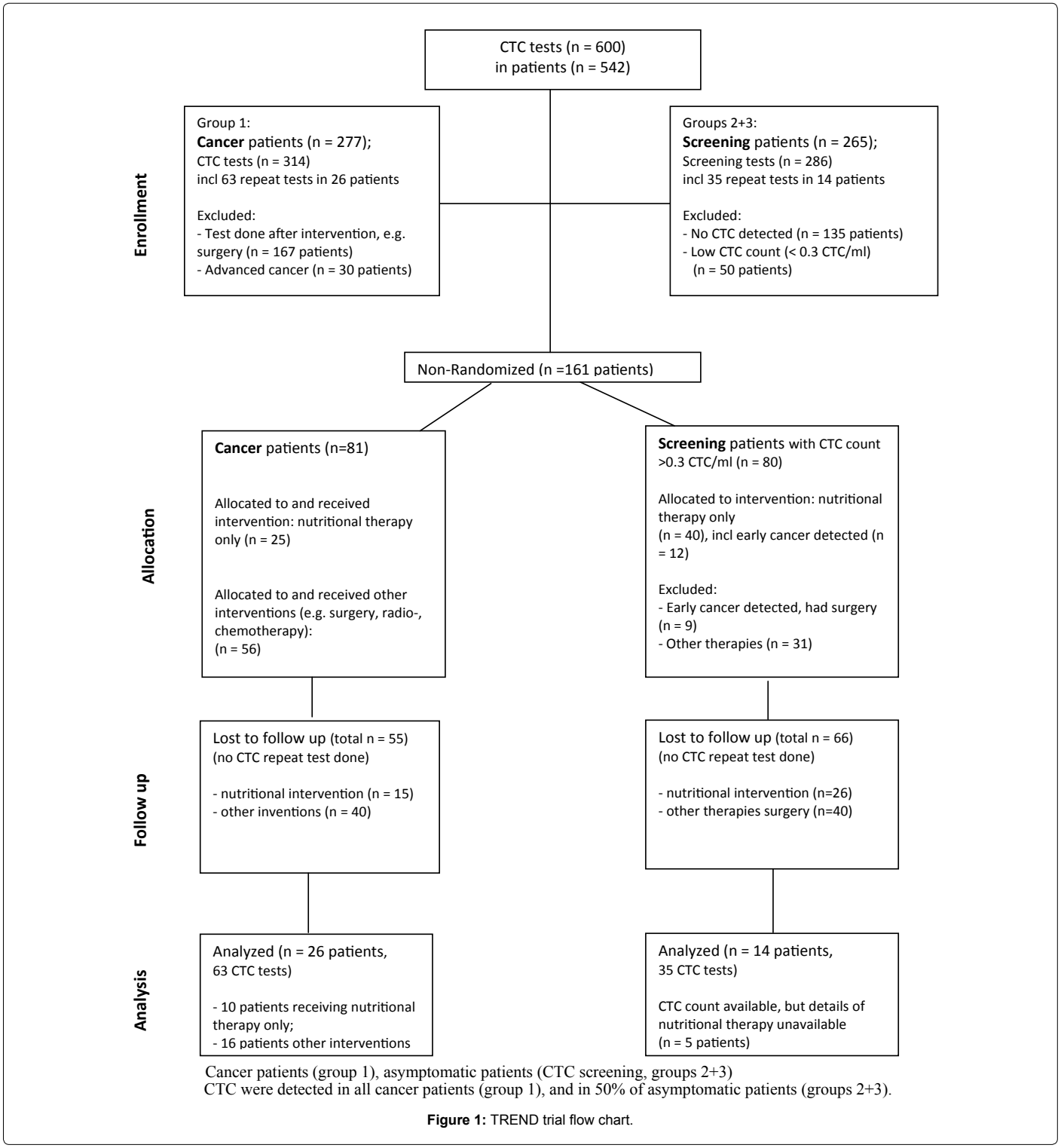


Figure 1: TREND trial flow chart.

Cancer Patients (group 1)

All patients with diagnosed cancer (group 1, Table 1) had a positive CTC count, detected with the ISET technology in patients with solid tumours and blood type tumours (e.g. non-Hodgkin's lymphoma, multiple myeloma). The CTC count ranged from 0.2 CTC/ml to 65.4 CTC/ml including single CTC and CTC clusters. CTC baseline count usually correlated to patient's cancer status and symptoms, with higher CTC counts presented in more advanced cases. Our data suggests a count of less than 0.3 CTC/ml to be usually associated with mild risk of malignancy, a count of 0.3-20 CTC/ml with moderate risk, and >20 CTC/ml with high risk of malignancy including metastasis, recurrence, and cancer progression. CTC count profile was similar in patients with other types of cancer. Follow-up is ongoing.

Figure 2 illustrates examples of CTC detected with the ISET method using cyto-morphological criteria.

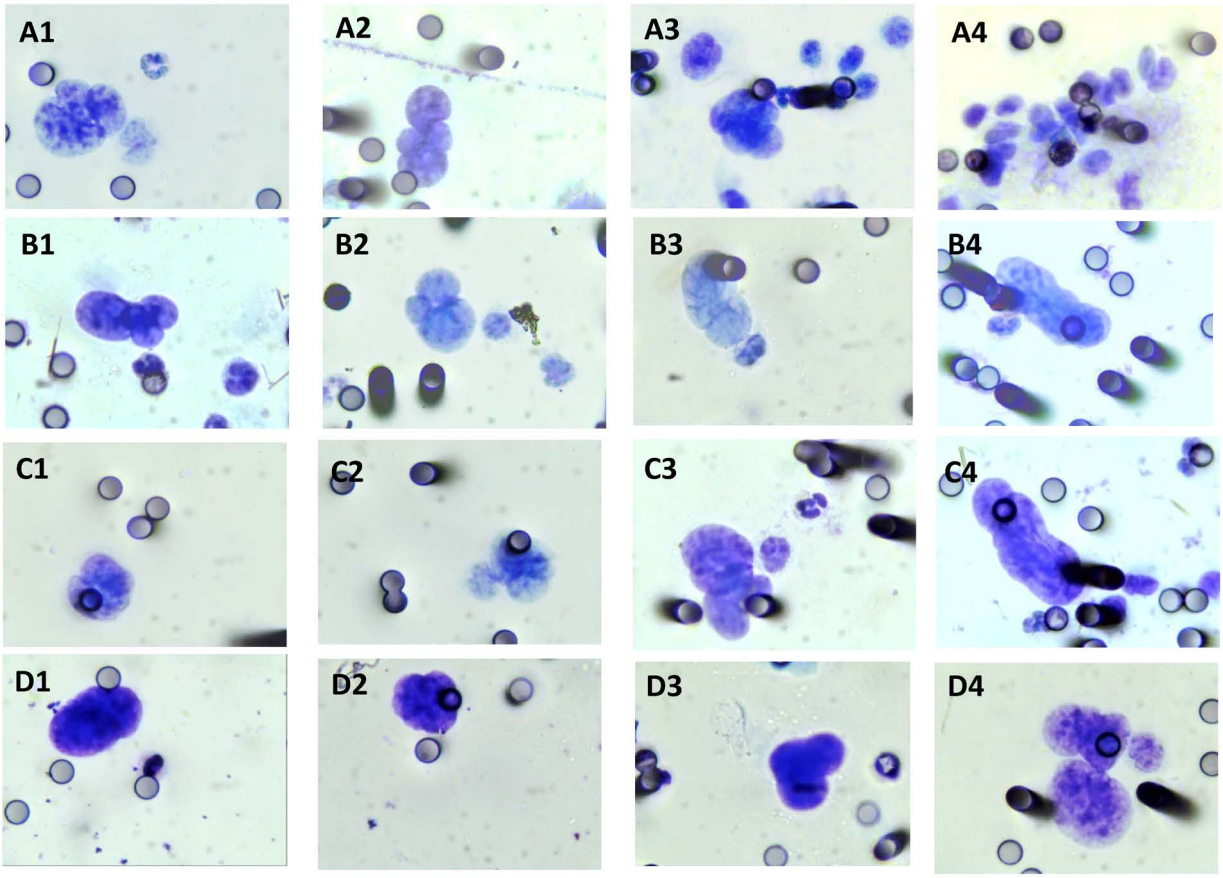
To monitor treatment effectiveness, CTC testing was repeated 3-4 weeks after conclusion of a treatment cycle around 3 months in 10% of cancer patients (n=26). Treatment could include surgery, chemotherapy, radiotherapy, hyperthermia, and nutritional therapies. CTC count correlated to patient's cancer status (Table 1), with an increase in CTC count over time indicating cancer progression or metastases, and a decrease in CTC count over time indicating cancer remission (Table 2).

Table 2 summarises the CTC count over time in cancer patients who underwent treatment other than nutritional therapies. In this group of patients, surgery treatment generally resulted in a decrease of CTC, standard chemo- and radiotherapy treatment did not.

Early Detection Screening (groups 2 + 3)

CTC screening tests were undertaken in mostly asymptomatic patients without diagnosed cancer but with increased cancer risk, including family history of cancer or advanced age (>50 years). In CTC screening patients, baseline CTC count ranged from 0.2-50 CTC/ml (mean=16 CTC/ml). For those patients with detected CTC (group 2), follow-up tests including scans and repeat CTC tests were scheduled within 0.5-10 months (mean=3.5 months). Follow-up scans taken within 1-6 months revealed early cancerous lesions in about 20% of patients with detected CTC (Table 3).

In up to 50% of male patients with normal PSA (prostate specific antigen) levels but with detected CTC, PET scans using PSMA (Ga-68 prostate-specific-membrane-antigens) revealed increased uptake in the



CTC are stained blue, filter holes of 8 microns appear black.

Panel A: breast cancer, B: prostate cancer, C: colorectal cancer, D: renal/ bladder cancer

Figure 2: Histo-pathological/ cyto-morphological detection of CTC using the ISET method.

		CTC count [1]		
Type of cancer	**Number of patients**	**Stage 1**	**Stage 2-3**	**Stage 4**
		<3 CTC/ml	**3-20 CTC/ml**	**>20 CTC/ml**
		N (% of type)	**N (% of type)**	**N (% of type)**
All	277			
Breast	81	52 (64)	20 (25)	9 (11)
Prostate	69	54 (78)	11(14)	4 (5)
Colorectal, gastric	37	26 (70)	7 (19)	4 (11)
Kidney, bladder	19	11	6	2
Blood type cancers: Lymphoma, NHL, HL, MCL, MM	17	10	2	5
Ovarian, endometrial, uterine, cervical	15	10	4	1
Lung	6	2	1	3
Melanoma	9	9	-	
Pancreatic	3	2	-	1
Thyroid	5	5	-	-
Other, e.g. tongue, brain, SCC	16	9	6	1

[1] CTC baseline count, CTC repeat tests of same patient not included in this table.
Abbreviations: HL, Hodgkin's lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; NHL, Non-Hodgkin's lymphoma; SCC, squamous cell carcinoma

Table 1: CTC count by type of cancer (Group 1: Cancer patients).

Patient ID, age	Cancer type	Test ID	CTC test time points (A-D)	CTC count/ ml	N months between CTC tests	Treatment details & comments
F1, 62 yrs	Colorectal	292GL 383GL 437GL 595GL	A: Sep-15 B: Jan-16 C: Mar-16 D: Jul-16	0.4 1.2 3.5 1.9	 4 3 4	A: After surgery, radio, chemo C: Liver metastases detected
F2, 60 yrs	Colorectal	338VD 405VD 592VD	A: Nov-15 B: Feb-16 C: Jul-16	0 0 21.1	 3 5	C: Lung metastases detected
F3, 41 yrs	Colorectal	343NZ 609NZ 782NZ	A: Nov-15 B: Jul-16 C: Dec-16	2.0 6.1 13.3	 8 5	B: Ongoing herbal therapy, details unknown
F4, 33 yrs	Colorectal, sigmoid	691GK 725GK	A: Oct-16 B: Nov-16	13.2 1.0	 1	B: After hyperthermia, IVC, IV-Curcumin
F5, 71 yrs	Breast	171WS 291WS 458WS	A: May-15 B: Sept-15 C: Apr-16	0.6 19.2 0.1	 4 7	A: After surgery, radio B: Ongoing hormonal therapy, low Vit D level C: After vitamin D, curcumin, relaxation
F6, 66 yrs	Breast	296JWK 483JWK	A: Sep-15 B: Apr-16	0.5 2.5	 7	A: After surgery B: On chemo
F7, 65 yrs	Breast, bone, liver	417SM 496SM	A: Feb-16 B:May-16	1.2 2.6	 3	A: Surgery 5 yrs ago B: Ongoing chemo
F8, 46 yrs	Breast	255JB 497JB	A: Jul-15 B: May-16	0.1 6.6	 10	A: After surgery, chemo, radio a year earlier
F9, 44 yrs	Breast	153AB 390AB	A: May-15 B: Jan-16	2.6 0.7	 8	B: After surgery
F10, 42 yrs	Breast	579DM 731DM	A: Jul-16 B: Nov-16	2.4 13.0	 4	B: After surgery, radio, chemo
F11, 63 yrs	Breast	656DM 763DM	A: Aug-16 B: Nov-16	0.3 3.2	 3	B: After radio, chemo, supplements
M12, 35 yrs	Gastric	460MM 690MM	A: Apr-16 B: Oct-16	4.7 0.1	 6	A: Ongoing chemo B: Chemo + immunotherapy drug
F13, 57 yrs	Melanoma	27EN 449EN	A: Nov-14 B: Mar-16	7.2 1.1	 16	A: Melanoma detected B: After surgery
M14, 51 yrs	Lung	64SW 427SW	A: Nov-15 B: Feb-16	1.2 0.9	 3	A: After CTC screening 4 mm tumour detected B: After surgery
F15, 48 yrs	Ovarian	602GN 775GN	A: Jul-16 B: Dec-16	1.1 1.1	 5	B: Ongoing chemo
M16, 65 yrs	Prostate	534NM 757NM	A: May-16 B: Nov-16	6.2 0.8	 6	B: Sonotherapy, supplements

F, female; M, male

Figure3a illustrates CTC count over time available for 9 cancer patients with mild disease, who had not undergone surgery, chemo- or radiotherapy for a variety of reasons, but undertook evidence-based nutritional immune-stimulating and anti-carcinogenic therapy. In this group (group 1, n=9), CTC count ranged between 25 and 1.4 CTC/ml at baseline and decreased over time for all patients, down to no detected CTC for a third, after 3-12 months with adjuvant nutritional therapy.

Table 2: CTC repeat test results of cancer patients undergoing treatment incl. surgery, radio-, FDA-approved chemotherapy (group 1).

Patient ID, age	CTC test method	Date CTC test	CTC number/ ml	Receptor expression (%)	PSA ug/L	Date scan	N months between CTC and scan	Scan results/ Tumour detected	Results comments
F1, 37 yrs	Maintrac	Mar-15	2	n/a	n/a	Apr-15	1	Breast	MRI: 0.5 × 0.8 × 0.4 cm lesion right breast confirmed with FNA
F2, 37 yrs	ISET	May-15	0.8	n/a	n/a	Jul-15	3	Breast	CT scan: 0.7 × 0.6 × 0.7 cm tumour left breast, biopsy confirms neoplasm
F3, 44 yrs	ISET	May-16	101	n/a	n/a	May-16	0.2	Ovarian	Ultrasound: had ovarian cystectomy, ISET-CTC test after surgery: 0 CTC/ml
F4, 57 yrs	ISET	Nov-14	7.2	n/a	n/a	Dec-14	1	Melanoma	Biopsy, surgery
M5, 50 yrs	ISET	Dec-14	1.2	n/a	n/a	Dec-14	0.5	Lung	PET scan: 4mm right upper pulmonary tumour with radiotracer (FDG) uptake
M6, 54 yrs	ISET	Jun-16	7.2	n/a	n/a	Jul-16	1	Kidney	Nephrectomy in 12/16; CTC repeat after surgery 1/2017 1 CTC/ml
F7, 42 yrs	ISET	Jun-16	8.1	n/a	n/a	Jun-16	0.5	Lung, Mesothelioma	Symptoms at time of CTC test: Abdominal pain, pelvic fluid, bloating; Mesothelioma
M8a, 59 yrs	ISET;	Dec-14;	2.6;		1.44				
M8b	Maintrac	Mar-15	33.5	Ki67=19.3		Jun-15	6	Prostate	PSMA-PET: very mildly increased activity in the right side of the prostate
M9, 55 yrs	Maintrac	Oct-15	0.5	Ki67=78.9 AR=95.2; PSA=68.4	0.87	Nov-15	1	Prostate	PSMA-PET: low volume, low grade carcinoma
M10, 73 yrs	Maintrac	Sep-15	11	Ki67=77.1; PSA=31.8	1.5	Oct-15	1	Prostate	PSMA-PET: Moderate uptake right lobe, low grade left lobe
M11, 58 yrs	Maintrac	Sep-15	10	Ki67=85.7; PSA=50	4.4	Oct-15	1	Prostate	PSMA-PET: low volume low Gleason score prostatic malignancy; minimally increased uptake base of prostate right posterior, bilaterally mid-prostate anterior right, mid left, apex right
M12a, 71 yrs	ISET;	Feb-15;	3.1;		1.97				
M12b	Maintrac	Oct-15	4.5	Ki67=74.1 PSA=63.5 AR=51.8		Oct-15	8	Prostate	PSMA-PET: moderate grade prostate carcinoma, central aspect of the left lobe; linear low grade uptake in oesophagus most likely physiologic/salivary
M13a, 66 yrs	ISET;	Sep-15;	1.1;		0.33				
M13b	Maintrac	Oct-15	3.5	Ki67=83.3; PSA=59; AR=71		Oct-15	1	Prostate	PSMA-PET: low grade prostate cancer
M14a, 76 yrs	ISET;	Jan-15;	4.9;						
M14b	Maintrac	Sep-15	9	Ki67=61.8; PSA=69 ; AR=65.2	2.19	Oct-15	10	Prostate	PSMA-PET: mild uptake in both lobes; likely to be true positive
M15, 65 yrs	Maintrac	Oct-15	5	PSA=40 AR=40	2.74	Nov-15	1	Prostate	PSMA-PET: very low volume low grade prostate cancer
M16a, 53 yrs	ISET;	Feb-15;							
M16b	Maintrac	Jun-15	4.9	Ki67=67.4	1.95	Nov-15	10	Prostate	MRI normal, but PSMA-PET abnormal
M17a, 69 yrs	ISET;	Sep-15;	0.5 + inflammation;		3.7				PSMA-PET: no significant accumulation, no evidence of nodal or distant metastases; marked prostatomegaly, but no tumour; ISET-CTC: inflammation, atypical cells due to infection;
M17b	Maintrac	Oct-15	3	PSA=100; AR=46.2; Ki67=60		Nov-15	2.5	Prostate – no uptake	Maintrac-CTC does not distinguish between CTC and atypical inflammatory cells;
M18, 65 yrs	Maintrac	Oct-15	12	Ki67=53.8; PSA=66.7; AR=53.8	14.2	Sep-15	-1 (MRI before CTC)	Prostate	MRI prostate: multiple lesions (1.7 cm; 0.7 cm); had surgery, CTC count dropped to M: 4.7 CTC/ml
M19, 71 yrs	Maintrac	Feb-16	2.5		1.63	Apr-16	2.5	Prostate	PSMA-PET: low grade uptake right prostatic base
M20a, 68 yrs	Maintrac	Dec-15; Feb-16	6.5; 7.5	Ki67=72.6	<0.01	Jan-16	1	Prostate	Had bladder cancer in 2014; prostectomy Jan 16; minimal uptake non-specific; NIIM CTC + lipoblast masses
M20b	ISET	May-16	2.8						
M21a, 67 yrs	ISET;	Aug-15;	0.6;						
M21b	Maintrac	Dec-15	3	Ki67=50; AR=45.9	1.21	Mar-16	7	Prostate	PSMA-PET: possible low-grade prostate cancer in left posterior peripheral zone, more concerning uptake in right hepatic lobe
	ISET	Apr-16	5.4						

prostate, which is indicative of early prostate cancer. In addition, early breast cancer, melanoma, ovarian, lung or renal cancer was detected during the study period in a small number of asymptomatic women and men (n=7) who had undergone the CTC screening test (Table 3).

Nutritional Therapies

A subgroup of patients with detected CTC was advised on evidence-based immune-boosting and anti-carcinogenic nutritional therapy by the consulting doctor. Treatment was tailored towards increasing natural killer cell count, inhibition of angiogenesis and metastasis. Supplements included curcumin, green tea, garlic extract, vitamin D, grape seed, lycopene, citrus pectin, medicinal mushroom extract, black cumin seed, artemisinin, and other immune stimulants with anti-carcinogenic properties (Table 4).

M22a, 76 yrs	ISET;	Feb-16;	0.7 atypical inflammatory cells;		normal	May-16	3	Prostatitis	PSMA=PET CT: mild prostatitis; ISET-CTC identified inflammatory condition, no CTC detected; Maintrac-CTC does not distinguish between CTC and atypical inflammatory cells
M22b	Maintrac	Apr-16	2	PSA=79; AR=88.6	normal				
M23, 49 yrs	ISET;	May-16	65.4;		normal	April-16	1	Prostate	PSMA-PET: moderate uptake
	Maintrac	May-16	13	AR=62; PSA=0					
M24, 66 yrs	ISET;	May-16	10.7;		high normal	Jun-16	1	Prostate	PSMA-PET: low to moderate uptake
	Maintrac		11	PSA=79; AR=73;					

ISET, ISET technology (Rarecells, France, www.rarecells.com)
Maintrac technology (Germany, www.maintrac.com): Receptor expression and EpCAM marker based CTC testing. In our experience, the CTC count by Maintrac correlates to the ISET CTC count by a factor of 100. For comparison to ISET CTC counts Maintrac CTC counts have been divided by 100.
F, female; M, male, AR, androgen receptor; Ki67, the Ki-67 protein is a cellular marker for cell proliferation; PSA, prostate specific antigen; PSMA, prostate specific membrane antigen; PET scan, positron emission tomography scan; n/a, not applicable

Table 3: Early detection CTC screening and follow-up scans of asymptomatic patients without detected tumour at time of CTC testing (group 2).

Patient ID; (gender, age)	Group	Curcumin	Green tea	Garlic	Vit D	Grape Seed	Lycopene	Citrus Pectin	Mushroom extract	Nigella sativa	Artemisinin	Others (immune stimulants)
Group 1: Cancer patients with detected CTC, who did not undergo surgery, chemo- or radiotherapy during the intervention												
C10_TCC	1		√		√			√	√		√	Vit E, Se, NK cell activator, reveratrol, astragalus
C11_SCC (F, 68yrs)	1									√		NK Cell activator, astragalus
CJ12_prostate (M, 67 yrs)	1	√	√	√	√		√	√	√	√		Prostate formula: saw palmetto, lycopene, boswellia, pumpkin seed oil, boron, fish oil, Vit E, Se
C13_prostate (M, 71 yrs)	1	√			√				√		√	IVC, resveratrol, liver tonic, soy, Ca, Vit K2, phosphatidylserine, bromelain, salvestrol, p53, fish oil
CJ16_NHL & prostate (M, 65 yrs)	1	√	√			√		√	√			Pomegranate, fish oil, Ca, Vit K2,
CJ26_bladder (M, 53 yrs)	1	√	√	√	√	√	Vit A	√	√		√	NK cell activator, probiotic, salvestrol, astaxanthin, NAC
J52_prostate, & bladder (M, 57 yrs)	1	√			√		√	√			√	mistletoe, quercetin, bromelain, Se, soy, fucoidan (brown algae)
C73_prostate (M, 49 yrs)											√	Mg, Vit B12
C100_breast (F, 56 yrs)	1	√	√	√					√			Fish oil, pomegranate, rosemary
Group 3: Asymptomatic patients without tumour but detected CTC												
Pt1 (F 51 yrs)	3	√	√	√	√	√			√			astragalus, probiotic, Vit C, boswellia, soy, liver tonics, NAC, Vit E, Se, Ca, Vit K2
Pt 2 (M, 50 yrs)											√	Mg, Vit B12
Pt3 (F 63yrs)	3	√		√		√		√	√		√	resveratrol, Vit C, NAC, Vit E, Se, Ca, Vit K2

Pt4 (F 56 yrs)	3	√				√	√		√	√		broccoli, Vit A, CoQ10, NAC
Pt5 (F 55 yrs)	3							√	√	√		NK cell activator, astragalus, Se, Vit E, Se, Ca, Vit K2
Pt7 (M 71 yrs)	3	√	√		√		√	√	√	√	√	Vit K2, reveratrol, broccoli, NAC, milk thistle, Vit C, Vit B12
Pt9 (M, 66 yrs)	3	√		√		√	√	√	√			NK cell activator, salvestrol, glutathione, chlorophyll, broccoli, NAC, fish oil, Vit E, Se
Pt10 (F, 63 yrs)	3	√		√		√	√	√	√			Nk cell activator
Pt14 (F, 49 yrs)	3	√	√						√		√	resveratrol, salvestrol, broccoli, pomegranate, Vit B12, NAC, fish oil, Vit E, Se

Abbreviations: C, cancer; S, screening; F, female; M, male; Ca, Calcium; NAC, N-acetylcysteine; NK cell, natural killer cell; NK cell activator contains enzymatically modified rice bran; Se, Selenium; Vit, vitamin

Table 4: Adjuvant nutritional treatment of patients with detected CTC (group 1 and group 3).

Treatment effectiveness of nutritional immune-boosting therapy was assessed by repeat CTC testing. CTC counts, available for cancer patients who did not undergo other therapies, (group 1, n=10, Figure 3a), and asymptomatic patients without detected tumour but positive CTC count (n=14, group 3), decreased over time (1-15 months) with nutritional therapy in all patients (Figure 3b). No adverse effects were reported by the patients who underwent integrative nutritional therapy.

Discussion

Our study suggests testing for Circulating Tumour Cells (CTC) to be a useful prognostic tool to screen for cancer risk and to monitor treatment effectiveness in cancer patients. A positive CTC count was associated with cancer risk, whereby a low CTC count (<0.3 CTC/ml) was correlated with mild malignant potential, 0.3-20 CTC/ml with moderate malignant potential, and a higher CTC count (>20 CTC/ml) with higher risk of malignancy, recurrence and metastasis, consistent with previous reports [1,2,4]. In addition to the CTC number, the type of cells, single cells or clusters, provide valuable insights into the cancer prognosis [3,4]. In this study we employed the ISET technology (Rarecells, France) [5] for CTC detection, which provides the advantage of a direct identification of malignant cells by cyto-morphological criteria [6], permitting distinction between precursor and malignant single cells and clusters, as well as reactive inflammatory atypical cells [9,16,17].

In our study, screening for CTC in asymptomatic individuals allowed the detection of early cancer, in about 20% of patients presenting with CTC. Importantly, in up to half of the men with detected CTC (25% of all men screened), but with normal PSA levels, subsequent positive PSMA-PET scans revealed early prostate cancer. This suggests CTC screening to be a more reliable measure for the detection of early prostate cancer than standard PSA testing [30]. In addition, early breast cancer, melanoma, ovarian, lung and renal cancer was detected in a small number of asymptomatic women and men with a positive CTC count. Early detection of cancer is associated with a greater range of treatment options and better prognosis [1,2,31].

A strength of our study was to compare the CTC count to cancer status and cancer risk in a large cohort of 542 patients. While CTC repeat test results after treatment were available in only a small subgroup of patients (40 out of 409 patients, 10%, with detected CTC), early results provide a trend towards treatment effectiveness of different types of interventions. However, statistical analysis in this patient cohort was not feasible due the small sample size and variety of treatments, therefore limiting generalisability about effectiveness of interventions.

Our study provided early evidence for integrative nutritional therapy to have the potential to lower CTC count, which in turn is associated with a lower risk of malignancy. Nutritional therapy was highly tolerable, and tailored towards increasing natural killer cell count, enhancing apoptosis of cancer cells, inhibition of angiogenesis and metastasis.

Natural Killer (NK) cells are an important gatekeeper stalling the growth of atypical cells, including cancer cells. Low NK cell levels have been associated with an increased risk of death in breast cancer [32]. Additionally, reduced NK cell activity increased the risk of metastasis by 350% during a 31-month period [33].

Garlic, available in form of garlic extract or garlic powder, has shown to increase natural killer cells [34]. Other anti-carcinogenic properties of garlic include reduced infection-induced carcinogenesis, and the induction of apoptosis [35,36].

Other nutrients with anti-carcinogenic properties include curcumin, green tea, grape seed extract, black cumin seed, artemisinin, modified citrus pectin, and mushroom extract.

Curcumin enhances apoptotic death, inhibits deregulated cellular proliferation, dedifferentiation and progression towards the neoplastic phenotype by altering key signaling molecules required for cell cycle progression, in addition to inhibiting H-Ras oncogene expression [37-39] .

Green tea with its polyphenols has been shown to inhibit several pathways and enzymes engaged in carcinogenesis, including the nuclear factor-κB (NF-κB), epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF)-I, urokinase-plasminogen activator (uPA), matrix metalloproteinases (MMPs) involved in oncogene expression, and proteasome activities, and contributing to apoptosis and cell cycle arrest [40,41].

Grape seed extract inhibits advanced tumour growth and angiogenesis and upregulates insulin-like growth factor binding protein [42], and can induce apoptosis and cell cycle arrest [43].

Black cumin seed (*Nigella sativa*), with its main active ingredient

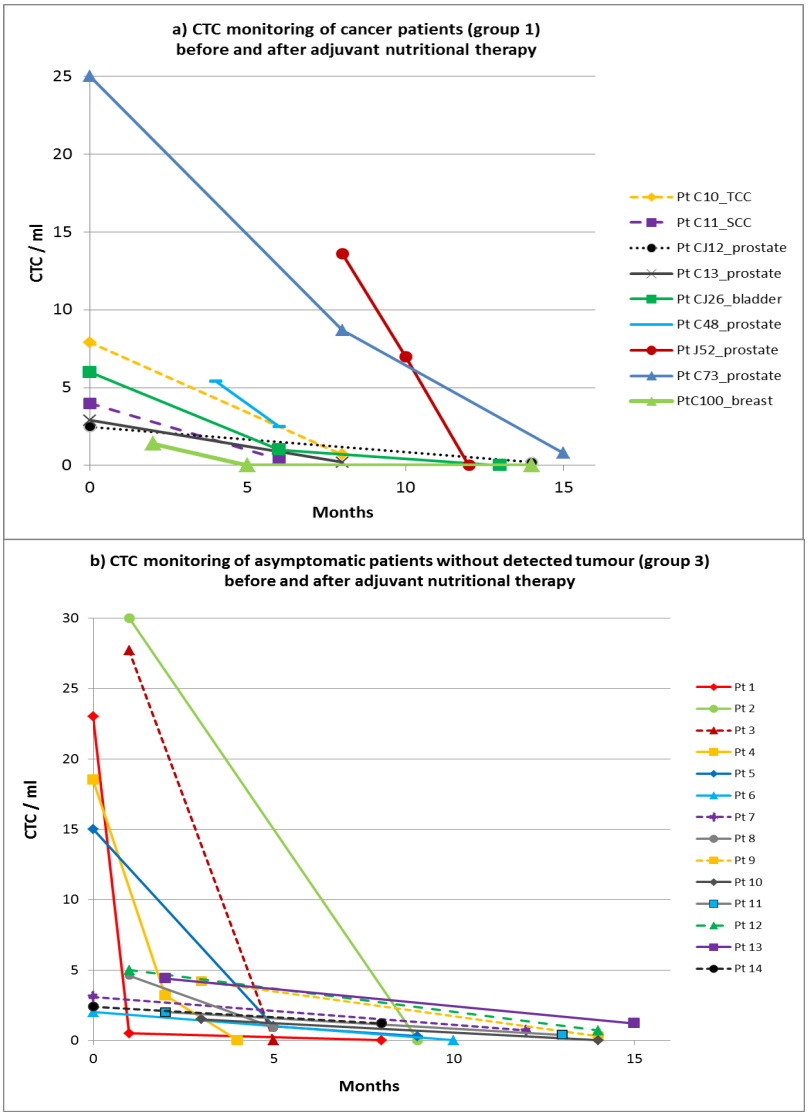


Figure 3: CTC monitoring of a) cancer patients (group1) and b) asymptomatic patients without detectable tumour (group3) before and after integrative nutritional immune-stimulating therapy.

Cancer patients (group 1) did not undergo surgery, chemo- or radiotherapy.

thymoquinone, has shown promise in inducing tumour cell death, and inhibiting proliferation, angiogenesis, invasion and metastasis [44]. Artemisinin triggers apoptosis in human cancer cells [45].

Modified citrus pectin, containing the main active ingredient galectin-3, has numerous anti-metastatic properties through anti-adhesion and apoptosis-promotion, and has shown promise in several clinical studies by halting cancer progression [46,47].

Medicinal mushroom extracts, including species of *Auricularia, Flammulina, Ganoderma, Grifola, Hericium, Lentinus (Lentinula), Pleurotus, Trametes (Coriolus), Schizophyllum, and Tremella* mushrooms, contain polysaccharides or polysaccharide–protein complexes, which enhance innate and cell-mediated immune responses, and inhibit proteins and enzymes involved in carcinogenesis, including NF-κB, protein-kinases, aromatase and sulfatase, and cyclooxygenase [48].

Additionally, a number of nutrients are essential for an active healthy immune system, including vitamin D, which has also been shown to play a role in anti-carcinogenesis.

Calcitriol derived from Vitamin D decreases the expression of aromatase, the enzyme that catalyses estrogen synthesis in breast cancer, both by a direct transcriptional repression and indirectly by reducing inflammatory prostaglandins [49].

Vitamin D, in addition to calcium, magnesium, Vitamin K, and boron, is also important for bone integrity [50], with bone always being affected in advanced breast and prostate cancer [51,52].

Lycopene, abundant particularly in tomatoes, has shown promise particularly in prostate cancer [53].

Conclusion

Here we provide evidence that screening for Circulating Tumour

Cells (CTC) allows detection of early cancer, while CTC monitoring over time allows assessment of treatment effectiveness, with higher CTC counts being associated with higher risk of malignancy. Our study suggests CTC count to be a more reliable predictor of early prostate cancer than standard testing of PSA levels, identifying early prostate cancer confirmed by PSMA-PET scan in 50% of asymptomatic men with detected CTC. Furthermore, our study provides evidence that a combination of immune-stimulating nutritional supplements can reduce CTC count, and therefore risk of malignancy. Nutrients with anti-carcinogenic properties include curcumin, garlic, green tea, grape seed, black cumin seed, artemisinin, modified citrus pectin, and medicinal mushroom extract.

Authors Contribution

All authors conceived and designed the study. NIIM Director AS introduced CTC testing to the institute, and doctors PE and AS provided patients, patient data, and treatment plans for the study. KR established and oversaw ISET-CTC testing at the NIIM lab, collated and analysed the data, and wrote the manuscript, with contributions from co-authors. All authors read and approved the final version.

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