Detection of circulating and disseminated neuroblastoma cells using the Imagestream Imaging Flow Cytometer for use as predictive and pharmacodynamic biomarkers

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Conflict of Interest

The authors declare no potential conflicts of interest

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Statement of translational relevance

This the first study to show that circulating tumour cell (CTCs) and disseminated tumour cells (DTCs) are detectable in neuroblastoma (NB) patient samples at diagnosis and relapse using the Imagestream imaging flow cytometer. Increased p53 and p21 expression in CTCs/DTCs following MDM2 antagonist treatment may be a useful pharmacodynamic proof-of-mechanism biomarker for early phase clinical trials. Enumeration of CTCs at diagnosis in high-risk NB patients with bone marrow infiltration should be further investigated as a predictive biomarker of bone marrow response to first line induction chemotherapy.
Abstract

Purpose: Circulating tumour cells (CTCs) serve as non-invasive tumour biomarkers in many types of cancer. Our aim was to detect CTCs from patients with neuroblastoma for use as predictive and pharmacodynamic biomarkers. Experimental Design: We collected matched blood and bone marrow samples from 40 neuroblastoma patients to detect GD2+ve/CD45-ve neuroblastoma CTCs from blood and disseminated tumour cells (DTCs) from bone marrow (BM) using the Imagestream Imaging flow cytometer (ISx). In 6 cases circulating free DNA (cfDNA) extracted from plasma isolated from the CTC sample was analysed by high-density single nucleotide polymorphism (SNP) arrays. Results: CTCs were detected in 26/42 blood samples (1-264/ml) and DTCs in 25/35 BM samples (57-291544/ml). Higher numbers of CTCs in newly diagnosed, high-risk neuroblastoma patients correlated with failure to obtain a complete BM metastatic response after induction chemotherapy (p<0.01). Ex-vivo Nutlin-3 (MDM2 inhibitor) treatment of blood and BM increased p53 and p21 expression in CTCs and DTCs compared with DMSO controls. In 5/6 cases cfDNA analysed by SNP arrays revealed copy number abnormalities associated with neuroblastoma. Conclusion: This is the first study to show that CTCs and DTCs are detectable in NB using the ISx, with concurrently extracted cfDNA used for copy number profiling, and may be useful as pharmacodynamic biomarkers in early phase clinical trials. Further investigation is required to determine if CTC numbers are predictive biomarkers of BM response to first line induction chemotherapy.
**Introduction**

Neuroblastoma (NB) is a heterogeneous tumour occurring in 10.2 per million children. It has one of the lowest survival rates of all childhood cancers, with only 67 percent of patients surviving to five years (1). Treatment of NB depends on the International Neuroblastoma Risk Group (INRG) classification which is determined by patient age, stage and genetics including \textit{MYCN} gene amplification, classifying patients as low, intermediate or high-risk (2). For high-risk patients defined in Europe as metastatic disease over 1 year of age or \textit{MYCN} amplified, intensive multimodality approaches are used including induction chemotherapy, surgical resection of the primary tumour, consolidation with myeloablative therapy and autologous stem cell rescue and local radiotherapy followed by immunotherapy and differentiation therapy. Despite an improved initial response to treatment survival remains poor (<50% at 5 years) due to eventual drug resistant relapse (3).

To better understand tumour evolution and drug resistance, it is important to consider intra-tumoural heterogeneity (4), but performing multiple biopsies from different tumour areas is not feasible in most patients. Studying circulating tumour cells (CTCs) from blood may inform intra-tumour heterogeneity and tumour evolution (5,6), and such studies are gaining importance in the clinic as their detection is a non-invasive procedure involving only the collection of peripheral blood (7). However, as CTCs are very rare, present in around one in 10\textsuperscript{6} leucocytes in prostate cancer patients (8), it is important to increase sensitivity in detection assays by including an enrichment step.

Technologies recently developed for CTC analysis include microfluidic devices with antibody-coated microspots (CTC chip), high-throughput microfluidic mixing devices (Herringbone-Chip) (9), or ultrasound-based isolation in microfluidic devices (10). A dielectrophoretic array method has been used to isolate disseminated tumour cells (DTCs) from bone marrow (BM)
from NB patients for tumour genetic analysis (11), and magnetic bead-based enrichment has been developed to isolate DTCs from BM samples using anti- GD2 and NCAM antibodies (12).

The Amnis Image Stream Imaging flow Cytometer (ISx) combines the features of classical flow cytometry, including an impartial analysis of a large number of cells in a short period of time with essential features of fluorescence microscopy allowing multiparameter cell analysis (13).

Detection of circulating free DNA and circulating tumour DNA (ctDNA) in plasma or serum of cancer patient blood is another type of liquid biopsy with higher levels detected in patients with metastatic disease (14). Recent studies have shown the feasibility of detecting ctDNA in cell free DNA (cfDNA) extracted from plasma to determine genetic aberrations including in NB (15-19).

In the present study, we detected CTCs and DTCs from NB patient blood and BM samples identified by GD2 expression and the absence of CD45 expression using the ISx. We show that higher numbers of CTCs in newly diagnosed patients with high-risk NB are associated with a failure to achieve a complete BM metastatic response after first line induction therapy and that CTCs and DTCs can be used as pharmacodynamic biomarkers of novel targeted treatments e.g. MDM2 inhibitors in early phase clinical trials. To our knowledge this is the first study to report use of the ISx to detect NB CTCs and DTCs in NB clinical samples.
Materials and Methods

Cell Culture

A panel of human NB cell lines, N-type (SHSY5Y (20-22), NGP (23), and NBLW-N (24,25)) and S-type (SHEP, SKNAS & NBLW-S (20,21)) were used to develop a protocol for detection of NB cells using the ISx. Cell lines were routinely maintained in RPMI medium supplemented with 10% v/v Fetal Bovine Serum (FBS, Gibco) and 1% v/v penicillin-streptomycin (Sigma) in 5% CO₂ in air in a humidified incubator at 37oC, regularly tested and found to be free from Mycoplasma using MycoAlert™ (Lonza, Basel, Switzerland). Authentication of the NBLW cell line was performed as described previously (26). NGP and SHSY5Y cell lines were authenticated using STR genotyping (New Gene Limited, International Centre for Life, Newcastle, U.K). SKNAS, SHEP and SKNBE(2c) (Be2C) cells were authenticated using cytogenetic analysis (27). All cell lines were used within 6 passages or within a period of 6 months.

Detection of NB cells using the ISx

A GD₂ antibody conjugated with PerCP Cy5.5 (Peridinin chlorophyll protein, BD Pharmingen, UK, 563438, 14.G2a) and anti-neural cell adhesion molecule (NCAM) conjugated with PE CF594 antibodies (BD Pharmingen, UK, 562328, B159) were used as NB markers in different cell lines alongside DAPI (BD Pharmingen, UK, 564907) nuclear staining. Imagestream data was analysed using IDEAS image stream analysis software using methods described previously (28).

Patient samples and Clinical Study design

This study was undertaken in accordance with the ethical principles of the Declaration of Helsinki. Following institutional review board approval (ethics reference number 14/NW/0154), local institutional approval and written informed consent from the patient or
carer, clinical samples (4-8ml blood and 1-3ml BM) were obtained from patients with newly
diagnosed or relapsed NB from 5 UK Paediatric Oncology Principal Treatment Centres
(Supplementary Fig. S1b-c). Samples were collected in Cell Save® (Veridex®, Menarine
diagnostics, UK) tubes, sent by post at room temperature within 72 h of collection, and
processed within 96 h of collection. In the clinical study 40 NB patients were recruited (32
high, 6 low and 2 intermediate risk), 24 were studied solely at diagnosis, 14 solely at relapse
and 2 at both diagnosis and relapse (Supplementary Fig. S1a & d). In total 42 blood samples
were studied for CTCs (1 fail) and 35 paired bone marrow samples. In 7 cases only a blood
sample was obtained (Supplementary Fig. S1c). International Neuroblastoma Risk Group
(INRG) and other clinical characteristics of patients are shown in Table 1 and Supplementary
Table T2. For ex-vivo Nutlin-3 treatment 4 blood and 2 bone marrow samples were collected
in EDTA tubes from 4 patients. Clinical information is correct up to 31/5/2018. All newly
diagnosed high-risk patients studied were treated on the European High Risk Neuroblastoma
trial (HR-NBL1) (29).

Analysis of patient samples using the ISx

Samples (blood and BM) were blocked to prevent non-specific antigen binding, red cell lysed
and enriched for non-haematopoietic cells using previously reported methods (30,31). Cells
were then permeabilised in perm-wash buffer (BD Pharmingen) and stained with
immunofluorescent antibodies including GD2-PerCp, NCAM-PE, and CD45- PE-Cy 7 (Bio
Legend, UK, 560915, H130) and nuclei stained with DAPI. Following incubation for 1h,
stained cells were washed, resuspended in PBS and processed on the ISx according to the
manufacturer’s protocol, and the presence of CTCs and DTCs detected using IDEAS software
(See Supplementary Methods- & Supplementary Fig. S2).

CTCs and DTCs were detected based on brightfield morphology, GD2 expression, a nuclear
signal and the absence of CD45 expression. Potential CTCs were gated in GD2 and CD45
scatter plots and visually confirmed from immunofluorescence images, tagged and counted manually. The numbers of DTCs were counted using IDEAS software as there were too many to count manually. The diameter of CTCs and DTCs in NB patient blood and BM samples was calculated as previously described (28). The mean diameter of CTCs/DTCs was compared with the mean diameter of white blood cells (WBCs; n=5000). The number of CTCs used to calculate diameter ranged from 4-100 and for DTCs from 4-2000. DNA ploidy was determined in all blood and BM samples with ≥ 4 cells based on WBC DNA and CTC/DTC DNA ratio from a DNA histogram using IDEAS software using the formula CTC DNA Ploidy = Mean CTC DNA/ Mean WBC DNA. If the ratio of CTC DNA was > 1.25 x WBC DNA ploidy then it was considered hyperdiploid, if not diploid (32).

**Collection of plasma from NB patient blood and BM samples**

Plasma was separated from blood (n=3 cases) and BM (n=3 cases) samples in Cell Save tubes by two sequential centrifugations (2,000 g, 10 min) and stored at -80°C in 1 ml aliquots. cfDNA was isolated from aliquots of double spun plasma using the QIAamp DNA Blood Maxi Kit (Qiagen). Following isolation the cfDNA yield was quantified using the Qubit® Fluorometer (Thermo Fisher Scientific) as per the manufacturer’s instructions. cfDNA fragment size was determined using 1% agarose gel electrophoresis.

**Nutlin-3 treatment of NB cell lines and patient samples**

Two NB cell lines, p53 wt (SHSY5Y) and p53 mutant (Be2C) (27), and blood and BM samples were exposed to Nutlin-3 an MDM2 inhibitor, and upregulation of p53 and p21 detected by immunofluorescence using the ISx and compared with DMSO (dimethyl sulfoxide) controls (Sigma-Aldrich, UK, D2650). Nutlin-3 (Selleckchem, UK) was dissolved in DMSO to a stock concentration of 100µM. Cells were treated with 10µM Nutlin-3 or an equal volume of DMSO for 24 h prior to fixation, followed by incubation with GD2 PerCp (BD Pharmingen, UK), p53 Alexa Fluor-647 (Cell Signaling technologies, UK, 2533S, 1C12) at 1:50, p21 Alexa Fluor-
488 (Cell Signaling technologies, UK, 5487, 12D1) at 1:50) and DAPI (BD Pharmingen, UK) and run on the ISx as described above. Cell cycle analysis was performed using IDEAS software with round single in focussed cells gated on scatter plots. Using the intensity of the DAPI histogram, mitotic cells and cells in G1, S & G2/M were gated to observe the effect of Nutlin-3 treatment and DMSO in NB cell lines, healthy volunteer blood samples and NB patient blood and BM samples. (See supplementary methods for more details).

**Cytogenetic analysis of NB tumours and cfDNA**

Using single nucleotide polymorphism arrays (SNP) (n=11 cases), array comparative genomic hybridisation (CGH) (n=21 cases) and multiplex ligation PCR dependent amplification (MLPA) (n=6 cases), cytogenetic analysis was undertaken on primary tumours or bone marrow metastases from all except 4 cases (Supplementary Table T2). For SNP arrays DNA samples were hybridised to Infinium CytoSNP-850K v1.1 BeadChip (Illumina, Inc) according to the manufacturer's instructions. Illumina IDAT files were analysed using BlueFuse Multi software. Using targeted next generation sequencing (NGS) (26) p53 gene mutational status was determined for the cases treated with Nutlin 3 (Supplementary Table T3). Affymetrix Oncoscan arrays (OncoScan FFPE CNV) performed by Eurofins Genomics (Ebersberg, Germany) were used for detecting copy number abnormalities from cfDNA from plasma with CEL files analysed using Nexus (Biodiscovery) software.

**Statistical analysis**

Statistical tests were performed using Graph Pad Prism (version 6.04) and IDEAS® software (Amnis-Imagestream Imaging Flow cytometer). The number of cases with BM involvement and the presence or absence of CTCs and DTCs was assessed using a Fisher’s exact test. A Mann Whitney U-test was used to calculate the relationship between numbers of CTCs and DTCs with BM involvement and BM response to induction chemotherapy. To determine the correlation between numbers of CTCs/DTCs detected by qRT-PCR for the NB mRNAs
tyrosine hydroxylase (TH) and PHOX2B) (33) a Spearman rank correlation test was used (SPSS). All p values reported were two-tailed and considered significant if $p \leq 0.05$. Data is presented as mean ± SEM.
Results

Detection of GD2 & NCAM expression in cell lines using the ISx

A panel of NB cell lines (n=6) comprising neuronal (N) and substrate-adherent (S) types were used to determine expression of the NB cell surface markers (GD2 and NCAM) using the ISx. N-type cell lines (NGP, SHSY5Y and NBLW-N) were found to express a higher percentage of GD2 and NCAM +ve cells than S type cell lines (SHEP, NBLW-S, and SKNAS). NGP cells were the most strongly positive N type cell line for GD2 and NCAM (Supplementary Table T1).

Detection of CTCs and DTCs from NB samples using the ISx

CTCs and DTCs were detected from a scatter plot of cells that were GD2+ve/CD45-ve with an intact nucleus (Fig. 1a & e). Potential CTCs/DTCs were gated based on intensity scatter plots of GD2 versus CD45 for one patient sample and the template saved as a standard and used for all remaining clinical samples using IDEAS software. Dots in scatter plots were linked to corresponding cell imagery and visualised to help define gating boundaries (Fig. 1a-f). Once a gate was defined, cell imagery of that population could be inspected and CTCs/DTCs were visually confirmed based on a round single cell from the bright field image (BF), with positive GD2 expression, negative CD45 expression and an intact DAPI stained nucleus (Fig. 1a, b & c). Confirmed CTCs/DTCs were then tagged and saved for further analysis of cell diameter and DNA ploidy.

CTCs were detected in 26/42 patient blood samples (mean=40/ml, range, 1-264/ml at diagnosis; mean=6/ml, range, 1-39/ml at relapse, Fig. 1g). DTCs were detected in 25/35 BM samples (mean=30,342/ml, range, 57-291,635/ml at diagnosis; mean=2,124/ml, range, 112-15,688/ml at relapse, Fig. 1h). Table 1 shows a summary of cases studied in relation to clinical risk group
and MYCN status with 32/40 (80%) cases high-risk and 26/40 (65%) studied at diagnosis (Supplementary Fig S1a, d & e).

BM involvement with NB on either 1 or 2 aspirates and/or trephines as reported by a consultant haematologist based on morphology was present in 24/41 cases (including 1 case at diagnosis and relapse). In patients with reported BM involvement, CTCs were detected in 19/24 cases and DTCs in 20/21 cases (in 3 cases DTCs not studied)(Supplementary Fig S1e). In patients without reported BM involvement CTCs were detected in 6/17 and DTCs in 5/17 cases (Table 1). The presence of CTCs or DTCs was associated with BM involvement (p<0.01 for CTCs and p<0.0001 for DTCs, Fisher’s exact test). Similarly there was an association between the number of CTCs and DTCs and BM involvement (p<0.0001 Mann Whitney test, Fig. 1i & j).

CTCs were detected in 2/42 cases in the absence of DTCs and BM involvement (one low-risk and one high-risk), and in one case in the absence of DTCs and the presence of BM involvement (Supplementary Fig 1e). In 3/35 cases DTCs were detected in the absence of CTCs and presence of BM involvement, and in two cases in the absence of CTCs and absence of BM involvement. There was no association between the numbers of CTCs or DTCs and the presence of MYCN amplification in the primary tumour or other metastatic site biopsied. CTC and DTC numbers from 16 and 21 patients respectively with untreated high-risk NB treated on the SIOPEN HR-NBL-1 trial were compared with NB- specific mRNA detected by qRT-PCR for TH and PHOX2B in blood and BM. There was a weak correlation between CTC numbers and level of PHOX2B mRNA expression in BM only (r =0.45, p< 0.05) (data not shown).

NCAM was expressed in only 3/11 GD 2+ve/CD45-ve blood samples initially examined, whereas in GD2+ve/CD45-ve cells from BM aspirates weak NCAM+ve DTCs were observed in 9/11 samples. From these early observations, it appears that there are differences in NCAM expression in NB cell lines compared with NB CTCs and DTCs. It has been reported that...
polysialylated NCAM and non-polysialylated NCAM expression differ between *in vitro* and *in vivo* conditions (34). Hence NCAM expression was not considered further to detect and confirm CTCs/DTCs in this study.

**Higher CTC numbers are associated with incomplete bone marrow response to induction chemotherapy**

To determine whether the number of CTCs or DTCs at diagnosis were associated with BM response to chemotherapy in high-risk NB patients with BM involvement at diagnosis (n=21), numbers of CTCs/DTCs were plotted against BM response after first line induction therapy (COJEC or N7) (Fig. 1k & l). BM response after induction chemotherapy was determined by the presence of neuroblastoma cell infiltration in either BM aspirate or trephine biopsy according to the International Neuroblastoma Response Criteria Bone Marrow Working Group classification (35). Absence of morphological evidence of NB on 2 aspirates and 2 trephines was considered a complete response (CR) i.e. BM in CR and a +ve BM aspirate or trephine at 1 or more sites was considered an incomplete response i.e. BM not in CR (35,36). Patients with higher numbers of CTCs at diagnosis were found to have an incomplete BM response after first line induction therapy (Fig. 1k, p<0.01, Mann Whitney U test). In contrast, there was no association between numbers of DTCs at diagnosis and BM response to first line induction chemotherapy (Fig. 1l, p=0.38).

**Detection of ploidy using the ISx**

The DNA content of WBCs and CTCs/DTCs in the CD45 depleted blood cell population was determined from a DNA histogram plotted using IDEAS software. On the DNA histogram of CD45 depleted cells, visually confirmed CTCs/DTCs were overlaid to determine DNA content of CTCs/DTCs, from which ploidy could be extrapolated (Fig. 2a & c). The ploidy status determined in this way from 24/42 CTC samples and 25/35 DTC samples is shown in
Supplementary Table T2. For CTCs 19/24 samples were diploid and 5/24 hyperdiploid and for DTCs 17/25 diploid and 8/25 hyperdiploid.

The DNA ploidy of CTCs in five cases determined using the ISx was compared with ploidy from a corresponding primary tumour or BM aspirate determined using high density SNP arrays. Case-No-15 CTCs were found to be diploid using the ISx (Fig. 2a) and the corresponding primary tumour SNP array showed diploidy with multiple segmental chromosomal abnormalities (SCA) without MYCN amplification (Fig. 2b). Case-No-33 CTCs detected at 1st relapse were hyperdiploid using the ISx (Fig. 2c) and a SNP array performed 15 months later at further relapse on a BM aspirate showed near-triploidy (Fig. 2d). MYCN and ALK amplification were detected in the SNP array profile from this case as well as 1p and 6q loss as shown in Fig. 2d. MYCN and ALK amplification were the only SCAs detected in the SNP array from a lymph node metastasis from the same patient at diagnosis when diploidy was present. The concordance of ploidy results from 5/5 cases with primary tumour or BM aspirate SNP arrays with CTC ploidy results from the same patients suggests that measurement of ploidy status using the ISx is accurate and reliable (Supplementary Table T2). In addition the ploidy from CTCs/DTCs of 3 cases determined using the ISx was compared with the ploidy from SNP arrays performed on cfDNA collected at the same time and found to be concordant (Supplementary Table T2). However, the low frequency of hyperploidy indicates that ploidy could only be used to distinguish a diploid WBC from a hyperploid CTC/DTC in a minority of cases.

Measurement of CTC/DTC diameter

Cell diameter was also investigated as an additional feature to differentiate CTCs/DTCs from WBCs. The diameter of all CTCs and DTCs in samples with \( \geq 4 \) CTCs/DTCs was measured using IDEAS software in 21 and 25 samples respectively (Supplementary Fig S3a & b). A
significant difference was observed between the diameter of WBCs in blood or BM samples versus CTCs or DTCs respectively (Wilcoxon signed rank test, p<0.0001, Supplementary Fig S3g & h). However, after plotting the values for each patient the intra-patient variability between CTC/DTC diameter and WBC diameter suggested cell size would not be a useful parameter to isolate NB CTCs/DTCs from residual WBCs.

cfDNA copy number analysis using SNP arrays
cfDNA collected in Cell Save tubes (n=6 samples) was analysed by Oncoscan FFPE arrays and copy number variations (CNV) successfully detected in 5/6 samples. In one case blood and BM plasma were collected at the same time and the CNV found to be identical in both samples (Fig. 3a & b). In 3 cases MYCN amplification was detected by SNP arrays in cfDNA (Fig 3c & d). In 2 cases the numbers of SCAs detected in the blood plasma at relapse increased from diagnosis illustrating temporal heterogeneity (Fig. 3 c & d).

CTCs/DTCs can be used as PD biomarkers of MDM2 inhibitor activity
MDM2 inhibitors prevent binding of MDM2 to p53 so stabilising and activating p53 leading to increased transcription of target genes including p21, which mediates a G1 cell cycle arrest. p53 mutant Be2C cells and p53 wt SHSY5Y cells were treated with the MDM2 inhibitor Nutlin-3 for 24h or DMSO control, immunostained with GD2, p53, p21, and DAPI and run on the ISx. p53 and p21 expression was determined by the percentage of p53 and p21 positive cells from intensity histograms of p53 and p21 (Fig. 4a and Supplementary Fig. S4a & b). Mutant Be2C cells expressed a higher percentage of p53 positive cells at baseline compared with SHSY5Y cells with no increase following Nutlin-3 treatment, whereas in SHSY5Y cells there was a statistically significant increase in p53 expression following Nutlin-3 treatment (Fig. 4a and Supplementary Fig. S4a & b and Fig. 5g). In p53 mutant Be2C cells there was low baseline expression of p21 and no increase following Nutlin-3 treatment (Supplementary Fig.
S4b and Fig. 5h), resulting in a virtually unchanged cell cycle profile (Fig. 5f), whereas in p53 wt SHSY5Y cells there was an almost 10 fold increase in p21 expression (Fig. 4a and Supplementary Fig. S4a) resulting in a strong G1/S cell cycle arrest and increase in G1/S ratio from 6.4 to 33 (Supplementary Fig. S4c and Fig. 5f).

In order to validate the panel of antibodies with GD2-PerCp, PE Cy7 CD45, p53-Alexa Fluor-647, p21 Alexa Fluor-488 and DAPI and to compensate data with all fluorochromes, healthy volunteer blood samples (n=3) were spiked with SHSY5Y cells or Be2c cells, treated with Nutlin-3 for 24h or DMSO control and p53 and p21 expression measured. After 24h there was increased expression of p53 and p21 in GD2+ve/CD45-ve SHSY5Y NB cells and residual GD2-ve, CD45+ve WBC remaining after WBC depletion (Fig. 4b & Fig. 5g & h). The fold increase in p21 in CD45+ve cells, although statistically significant, was not as great as for SHSY5Y cells (Fig. 5h), resulting in an increased G1 population but unchanged G1/S ratio after Nutlin-3 treatment (Fig. 5f). In contrast there was no change in p53, p21 or cell cycle profile after Nutlin 3 treatment of spiked p53 mutant Be2c cells compared with DMSO control cells (Fig 5f-h).

We next treated NB patient blood (n=4) and BM samples (n=2) with Nutlin-3 ex- vivo for 24 hr or DMSO control and measured p53 and p21 expression (Supplementary Table T3). In 4/6 samples GD2+ve cells were present but a BM and blood sample from a patient with low-risk NB at diagnosis had no detectable GD2+ve /CD45-ve CTCs or DTCs. In the 4 Nutlin-3 treated samples (3 blood and 1 BM) with CTCs/DTCs present, increased p53 and p21 expression was seen in GD2+ve/CD45-ve CTCs and DTCs compared with DMSO control (Fig. 4e & f, Fig. 5a, b, g & h). In Case-No-12 a high number of CTCs (n=264/ml) and DTCs (n=6383/ml) were detected (Supp Table T3), and a histogram was generated for cell cycle changes in DTCs and p53 and p21 expression in CTCs and DTCs, before and after 10µM Nutlin-3 treatment (Fig. 4e
& f and Fig. 5a-b & e-h). It was not possible to generate histograms from CTCs from the two blood samples taken at relapse due to low numbers of CTCs (Supp Table T3). In CTCs and DTCs baseline nuclear and cytoplasmic p53 was detectable with nuclear accumulation of p53 following Nutlin-3 treatment (Fig. 4e & f and Fig. 5a & g). Consistent with p53 activation there was increased expression of p21 in CTCs and DTCs following Nutlin-3 treatment (Fig. 4e & f, Fig. 5b and h), with a 12 fold increase in p21 in DTCs (Fig. 5b). This led to an increase in the G1 population of Case-No-12 DTCs (56% for DMSO treated and 70%-Nutlin-3 treated), but no reduction in S phase in DTCs (Fig. 5e & f), so an unchanged G1/S ratio (9.8-DMSO and 9.2-Nutlin-3).

To determine the effect of Nutlin-3 on WBCs, 4 patient samples and 3 spiked healthy volunteer blood samples were treated with Nutlin-3 or DMSO control and p53, p21 and cell cycle arrest measured. Compared with NB cell lines, CTCs and DTCs there was very low baseline expression of p53 in WBCs, but in all cases there was nuclear p53 accumulation following Nutlin-3 treatment (Fig. 4c & d, Fig. 5c & g, Supplementary Fig. S4e), and increased p21 expression compared with DMSO controls (Fig. 4c & d, Fig. 5d & h, Supplementary Fig. S4f). This led to an increase in G1 population after Nutlin-3 treatment but no change in G1/S phase ratio i.e., 9.1 after Nutlin-3 treatment compared to DMSO control (8.7) in WBCs from Case-No-12 BM (Fig. 5f & Supplementary Fig. S4d).
Discussion

The aim of the present study was to detect NB CTCs from blood and DTCs from BM using the high-resolution ISx to use as biomarkers in NB. Recently we reported CTC detection and characterisation from patients with oesophageal, hepatocellular, thyroid and ovarian cancers using the ISx (28,37). Various techniques have been used to detect CTCs such as DEPArray and CTC-iChip. DEPArray has been previously used for NB cell lines and patient BM samples (11) but the current study is, to our knowledge, the first to detect NB CTCs and DTCs using the ISx. Previously we reported between 0-118 CTCs /ml in thyroid cancer and 0-20 CTCs/ml in hepatocellular carcinoma (28) using similar methods on the ISx. This compares with 0-264 NB CTCs/ml and a mean of 12 CTCs/ml in the current study.

Seeger et al reported >10⁴ NB cells per 10⁵ nucleated cells in BM samples from 103/267 patients and >10⁴ NB cells per 10⁵ nucleated cells in 2/174 patient blood samples from high-risk metastatic NB patients at diagnosis using anti-GD2 immunocytoLOGY. They concluded that quantifying NB cells in BM and peripheral blood at diagnosis and during induction therapy provides an important poor prognostic marker for patients with stage IV NB (38). In our study higher number of CTCs were detected at diagnosis (1-264/ml) compared to relapse (1-39/ml) due to clearance of CTCs from the blood by chemotherapy and likely earlier detection of relapsed disease. It is not possible to compare the sensitivity of our method for detection of CTCs with other published studies as CD45+ve cells were depleted prior to analysis. In 5/17 cases where DTCs were detected without bone marrow involvement this may have been due to sampling variation, the assessment of bone marrow on the basis of morphology alone or NB cells passing through the bone marrow rather than homing there. Although GD2 is expressed on >90% of neuroblastoma bone marrow metastases at diagnosis and relapse (39), it is possible that using this technique we are missing a small proportion of tumour cells which do not express GD2. A future study should compare the sensitivity of the ISx to detect DTCs with bone
marrow examination using immunocytology and also compare with NB specific mRNA expression by qRT-PCR. In untreated high-risk NB patients there was a weak correlation between numbers of CTCs with the level of the NB-specific mRNA PHOX2B detected by qRT-PCR in BM but only small numbers of patients were studied (n = 22).

Higher numbers of CTCs were detected in untreated patients with high-risk NB who did not achieve a BM CR after first line induction therapy versus those who did, suggesting that CTC enumeration may prove useful to guide the length of induction chemotherapy in patients with high-risk NB in the future. However, this was not the case for DTCs, which could be due to much higher numbers of DTCs which were not visually confirmed. These observations now need to be extended to a larger, prospective study of high-risk NB. Due to our sample size of 40 patients including 16 relapse cases, it was not possible to evaluate the prognostic significance of CTC/DTC numbers. DNA ploidy of tumour cells was not useful for distinguishing CTCs from WBCs due to the presence of diploidy in the majority of NB CTCs studied, but ploidy of CTCs/DTCs did reflect the ploidy status of the primary tumour.

We also evaluated the use of cfDNA for detection of circulating nucleic acids from blood and BM plasma collected for CTC/DTC studies in Cell Save tubes. In NB the genomic profile of the tumour is necessary for treatment stratification. Various methodologies have been reported for detecting NB genomic profiles with SNP arrays now frequently used in national reference laboratories (40). Chicard et al reported ctDNA copy number analysis using Oncoscan arrays in 66/70 patients with copy number profiles obtained in 74% of patients (19).

In 5/6 blood or bone marrow plasma samples collected at the same time as CTCs/DTCs, CNA were detected in ctDNA using Oncoscan FFPE arrays. In one case the paired blood and bone marrow plasma showed the same CNA confirming the usefulness of BM plasma for detecting CNA in NB as previously reported (41), and in another case the CNA from plasma cfDNA.
were identical to those in a concurrently biopsied metastatic disease site. cfDNA extracted from
blood and BM plasma isolated from samples collected in Cell Save tubes for CTCs/DTCs could
be used to detect metastatic NB tumour specific genomic alterations and should be evaluated
prospectively in future clinical trials.

We are developing MDM2 inhibitors as a novel therapy for NB (42-44) and MDM2 inhibitors
are currently being evaluated in adult and paediatric early phase clinical trials therefore we
sought to establish if CTCs could be used as a circulating PD biomarker . The optimum PD
biomarker for MDM2 inhibitor activity is activation of the p53 pathway in tumour cells
detected by increased expression of a p53 induced gene. Macrophage Inhibitory cytokine
(MIC1) has been used as a surrogate marker in plasma samples (45), but elevation of MIC1
levels are not specific for tumour cells highlighting the importance of developing less invasive,
but tumour specific pharmacodynamic proof-of-mechanism biomarkers. In the current study
detection of increased p53 and p21 expression in SHSY5Y cells following Nutlin 3 treatment
but not mutant p53 Be2C cells is consistent with our previous studies of MDM2 inhibitors in
these cell lines (46,47).

In healthy volunteer blood samples exposed to MDM2 inhibitors ex-vivo, blood spiking studies
with NB cells and then patient blood and BM samples, we demonstrated increased p53 and p21
protein expression and an increase in G1 population in CTCs, DTCs and WBCs after Nutlin-3
consistent with our previous studies testing p53 function in diagnostic NB biopsies showing
induction of the p53 pathway following ex-vivo irradiation (48). The current study
demonstrates proof-of-concept to use p21 as a sensitive and specific PD biomarker of MDM2
inhibitor activity for CTCs and DTCs detected by the ISx, but its usefulness for CTCs may be
limited by small numbers of CTCs present in relapsed blood. p21 expression and the G1
population also increased in WBC following Nutlin 3 highlighting the importance of studying
tumour cells rather than surrogate WBC to study proof-of-concept PD biomarkers of MDM2 inhibitor activity.

In conclusion, this is the first study to demonstrate the clinical utility of NB CTCs detected by the ISx as non-invasive PD biomarkers of novel therapies in early phase clinical trials. A future, larger study is needed to investigate whether they are predictive biomarkers of response and also to determine if they are potential prognostic biomarkers to further refine risk stratification in high-risk NB.
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References


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Table 1: Summary of patients in study according to risk group, *MYCN* status and bone marrow involvement (n=42)a

<table>
<thead>
<tr>
<th></th>
<th>Number of patients at Diagnosis</th>
<th>Number of patients at Relapse</th>
<th>Number of patients with CTCs*</th>
<th>Number of patients with DTCs*</th>
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<td>n= 16</td>
<td>Diagnosis n= 20</td>
<td>Relapse n= 6</td>
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<td>3</td>
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<td>11</td>
<td>19</td>
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<tr>
<td>N= 42b</td>
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<tr>
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<tr>
<td>N= 42c</td>
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<tr>
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<td>17</td>
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<tr>
<td>No n= 17</td>
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<td>12</td>
<td>3</td>
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</table>

a2 patients studied at diagnosis and relapse, b1 case *MYCN* not studied, c1 case BM not examined at relapse, d42 samples studied for CTCs (1 fail), e35 samples studied for DTCs. MNA = *MYCN* amplified
**Figure 1**: CTCs and bone marrow response. 

a. Scatter plot of Intensity_MC GD2 against Intensity_MC CD45 of patient blood sample (Case-No-22) generated using IDEAS software. Potential CTCs were gated by visual inspection of images. 

b. Immunofluorescence image of a CTC (GD2+ve/CD45-ve cell) with an intact nucleus (DAPI) by analysis of a single dot (cell) from the GD2 +ve region of the scatter plot. 

c. Immunofluorescence image showing three cells in a single image: a CTC (GD2+ve/CD45-ve) and WBCs in doublets (CD45+ve/GD2-ve) to show compensation of fluorochromes. 

d. Immunofluorescence image of a CD45 +ve region showing a CD45+ve/GD2-ve WBC. 

e. Scatter plot of Intensity_MC GD2 against Intensity_MC CD45 of BM sample (Case-No-22) showing DTCs. BM cells in higher intensity GD2 gated regions were GD2+ve/CD45-ve DTCs and the scatter plot shows very few CD45+ve cells towards the X-axis, compared with the paired blood sample which had more CD45+ve cells. 

f. Immunofluorescence image showing a GD2+ve/CD45-ve DTC. 

g-h. Scatter plots showing numbers of CTCs in 41 patient blood samples (2 cases at both diagnosis and relapse, 1 fail) and numbers of DTCs in 35 patient BM samples. In 26 cases ≥1 CTC was detected and in 25 cases ≥1 DTC was detected. Horizontal line represents median with range. The mean number of CTCs and DTCs detected was 12 and 5431 per ml of blood and BM respectively. 

i. Scatter plot showing the association between numbers of CTCs in 41 blood samples in relation to BM involvement (P<0.0001- Mann-Whitney-test). 

j. Scatter plot showing the association between numbers of DTCs in 35 BM samples and BM involvement (P<0.0001- Mann-Whitney-test). 

k. Scatter plot showing the number of CTCs/ml blood in 19 newly diagnosed high-risk NB patients with BM involvement at diagnosis comparing those who achieved a BM complete response (CR) after first line induction therapy versus patients whose BM was not in CR. 

l. Scatter plot showing the number of DTCs/ml in BM aspirates from 18 patients with BM involvement who achieved a CR v those who did not after first line induction therapy (mean ±
SD), BM +ve = BM involvement, BM –ve = Bone marrow not involved, Horizontal lines = median with range.

**Figure 2:** Ploidy determination from CTCs in NB blood samples. **a** DNA histogram showing the DNA content of residual WBCs and CTCs from a blood sample (Case-No-15) following WBC depletion. The histogram shows an overlay of the DNA content of CTCs (red peak) and WBCs (black peak) to determine the ploidy status of CTCs. The gatings were DNA 1 (single CTCs/WBCs) and DNA 2 (CTC/WBC doublets), but for ploidy determination, only single CTC gatings were used and compared with a WBC DNA ratio of 1. From the histogram, Case-No-15 was considered diploid as CTC DNA was < 1.25× WBC DNA ploidy. **bi** SNP array log R ratio track of Case-No-15 primary tumour at diagnosis showing a diploid tumour and multiple segmental chromosomal abnormalities (SCA) (2p, 4q with hyper-rearrangement, 6p, 9p, 11q, 12pq, 17q gain and 1p, 3p, 4p, 11q loss). **bii** B allele frequency **c** DNA histogram from Case-No-33 blood sample at first relapse with DNA ploidy of CTCs determined from WBC DNA ratio found to be hyperdiploid with CTC DNA >1.25× WBC DNA ploidy. **di** Log R ratio track of Case-No-33 BM aspirate at further relapse showing near-triploidy (whole chromosomal abnormalities-chr7, chr12, chr18, chr20, chr22 gain and chr9, chr10, chr11, chr14, chr16, chr19 loss, and SCA-loss of 1p and 6q). **dii** B allele frequency. The profile also shows amplification of MYCN and ALK on chromosome 2p which were also present in the primary tumour at diagnosis when the tumour was diploid. Yellow straight line on the SNP log R ratio indicates 0, normal diploid copy number and the green data points indicate the log R ratio of all individual SNPs. An increased or decreased log R ratio indicates gained and deleted regions of chromosomes respectively.

**Figure 3:** a & b Comparison of copy number profiles from corresponding cfDNA from blood and BM (Case-No-23). **ai** SNP array log R ratio track and (aii) B allele frequency plot log R
ratio from Oncoscan FFPE array of cfDNA from blood and (bi) and (bii) from BM showing the SCAs including +2p, -3q, +6p, -6q, -9p, +11p, -11q, -17p, +17q and WCAs such as -5, +7, -10, -19, +18 which are identical in cfDNA from both sites. The primary tumour was not biopsied in this case at diagnosis so unavailable for comparison c & d Comparison of copy number profiles from Case 11-diagnostic primary tumour and Case 11-R cfDNA from blood at relapse ci & di) SNP array log R ratio track and (cii & d ii) B allele frequency plot log R ratio c) Illumina array showing ALK and MYCN amplification together with -1p, -10q, +11q and +17q. d) Oncoscan array of Case 11-R cfDNA showing ALK and MYCN amplification -1p and +17q and additional gains and losses including +1q, -5p, -18p, +18q. Interestingly 10q loss and 11q gain were not detected in Case 11-R cfDNA.

Figure 4: The effect of Nutlin-3 treatment on p53 wt SHSY5Y cells, SHSY5Y cells spiked into healthy volunteer blood, CD45+ve WBCs (blood and BM), CTCs and DTCs (Case-No-12). ISx immunofluorescence images of (a & b) p53 wt SHSY5Y neuroblastoma cells and spiked cells following treatment with DMSO or 10µM Nutlin-3 for 24h showing increased p53 and p21 expression following Nutlin-3 treatment. (c & d) Case-No-12 Blood and BM WBCs (CD45+ve/GD2-ve) showing increased nuclear p53 and p21 expression in Nutlin-3 treated cells compared with controls. (e & f) Case-No-12 CTCs and DTCs showing increased nuclear p53 and p21 expression following Nutlin-3 treatment compared with DMSO control.

Figure 5: The effect of Nutlin-3 on p53 and p21 expression and the cell cycle in DTCs & BM CD45+ve cells from Case No-12. a & c Histograms showing increased p53 expression in DTCs and BM CD45+ve cells when treated with 10µM Nutlin-3 for 24 h compared with DMSO. b & d Histograms showing increased p21 expression after Nutlin-3 treatment compared with DMSO controls in DTCs and BM CD45+ve cells. e Histograms showing cell cycle analysis of DTCs after Nutlin-3 treatment showing an increased G1 population. f Bar chart showing cell
cycle analysis of Be2C cells (p53 mutant), spiked (SPIK) Be2C cells, SHSY5Y cells, spiked SHSY5Y cells (SPIK), WBC from healthy volunteer blood samples (HV) (n=3), WBCs from Case-No-12 blood and BM samples, and DTCs from Case-No-12 following Nutlin-3 treatment, showing a G1 arrest in SHSY5Y cells and spiked SHSY5Y cells and a partial G1 arrest compared with DMSO controls in all other samples except mutant Be2C cells and spiked mutant Be2C cells. **g and h** Bar charts showing the percentage of cells expressing p53 and p21 in Be2C cells (n=3), spiked Be2C cells (n=3), SHSY5Y cells (n=3), spiked SHSY5Y cells (n=3), HV WBC (n=3), Case-No-12 blood and BM WBC and DTCs (n=3 for Case-No-12; error bars represent analysed data from three different files using IDEAS software) following Nutlin-3 treatment compared with DMSO controls. In SHSY5Y cells, spiked SHSY5Y cells, HV WBCs, Case-No-12 blood and BM WBCs and DTCs increased p53 and p21 expression was observed following Nutlin-3 compared with DMSO controls, but not in p53 mutant Be2C cells and spiked mutant Be2C cells. For SHSY5Y cells, spiked SHSY5Y cells, Be2C cells, spiked Be2C cells, healthy volunteer WBC (n=3), Case-No-12 CD45+ve, blood and BM cells and DTCs, a minimum of = 1000 cells were analysed. Paired t test, p<0.05*, p<0.01** and p<0.001***).
Figure 2

a) CTCa (hypodiploid) overlay on WBCa

Case-No-15

b)  

bi

bii

C

Case-No-33

c) CTCa (hypodiploid) overlay on WBCa

Case-No-33

d)  

di

dii

MYCN
ALK

-1p -11q
-1p -12q
+7
+12

-10 -11
-14 -15
+17q
+12pq
+11q

+1p +4p +6p +9p +11q +17q
-1p -3p -4p

+2p +4p +6p +9p +11q +12pq
-1p -6q -9
+7
-10 -11
+12
-14 -16
+18
-19
+20 +22
Figure 4

a

SHSY5Y-DMSO

SHSY5Y-Nutlin-3

b

Spiked SHSY5Y-DMSO

Spiked SHSY5Y-Nutlin-3

c

Case-No-12-Blood-CD45-DMSO

Case-No-12-Blood-CD45-Nutlin-3

d

Case-No-12-BM-CD45-DMSO

Case-No-12-BM-CD45-Nutlin-3

e

Case-No-12-CTCs-DMSO

Case-No-12-CTCs-Nutlin-3

f

Case-No-12-DTCs-DMSO

Case-No-12-DTCs-Nutlin-3

35