UNIVERSIDAD AUTÓNOMA DE MADRID

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Development of pharmacodynamic biomarkers of the PI3K pathway for childhood neuroblastoma

TESIS DOCTORAL

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A Maia y Jorge, que son toda mi vida

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Parents/Guardians

Parents/Guardians of children with ne21uroblastoma

Children aged 14 to less than 18

Children aged 8 to 14

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Summary

Introduction: Pharmacodynamic (PD) biomarkers provide proof-of-principle of target modulation and evaluate downstream biological effects of novel targeted therapeutics. PD biomarkers accelerate drug development and the transition of new drugs into the clinic. Neuroblastoma is a high-risk childhood cancer in which bone marrow metastases are frequent. The aim of this study was to develop PD biomarkers of the phosphoinositide-3-kinase (PI3K)/AKT pathway to accompany early clinical trials of molecularly targeted agents. This research included: 1) the adaptation of a surrogate biomarker assay of the PI3K/AKT pathway in platelet-rich plasma (PRP), 2) the development of a technique to isolate tumour cells from bone marrow aspirates for PD biomarker assays, 3) the development of a quantitative ELISA to measure MYCN protein expression (a downstream effector of PI3K) and 4) the development of assays to measure the PI3K/AKT pathway in tumour cells isolated from bone marrow samples.

Material and Methods: MesoScale Discovery (MSD)® Triplex AKT assay (total and phospho protein signals for AKT, GSK3β and p70S6K) was used in PRP and tumour cells, tumour cells were isolated using immunomagnetic separation methods based on MicroBeads from the bone marrow. A Europium-based ELISA was developed to quantify MYCN oncoprotein. Pre-clinical testing was performed with neuroblastoma cell lines spiked in healthy volunteer blood samples. Clinical testing was performed in 14 children with advanced solid tumours, 5 of them with neuroblastoma metastatic to the bone marrow. The study had Royal Marsden Hospital institutional approval.

Results: Detectable signal for all total and phospho proteins was found in all cases with the MSD® Triplex AKT assay in PRP. No differences according to age, diagnosis or stage were found. CD45 depletion was superior to GD2 positive selection in isolating neuroblastoma cells from bone marrow aspirates and obtained cell suspensions with high purity of neuroblastoma cells in spiking experiments in peripheral blood and in bone marrow samples from children with neuroblastoma metastatic to the bone marrow. Cell suspensions contained sufficient amounts of protein for subsequent analyses. A sensitive and specific Europium-based ELISA was developed to quantify total MYCN oncoprotein levels. The MSD ®Triplex AKT assay was able to measure PI3K pathway activation in neuroblastoma cells in the bone marrow in a pre- clinical situation.

Discussion and conclusions: The PRP assay has been validated in the paediatric population and is ready to be implemented in early clinical trials in children as a pharmacodynamic biomarker in the upcoming paediatric *first-in-child* studies of the dual PI3K/mTOR inhibitors BEZ235 and GDC0980. Immunomagnetic separation with CD45 depletion was found to be an effective technique to obtain pure cell suspensions of neuroblastoma cells as a non-invasive source of tumour cells for protein pharmacodynamic biomarker assays. The pre-clinical development phase of the MYCN ELISA has been completed and this will move to clinical development prior to the

implementation of the clinical trials. The pre-clinical development phase of the MSD® PI3K/AKT assay in neuroblastoma cells was completed and this will move to clinical development. The development and implementation of these biomarkers in new biological hypothesis-driven *first-in-child* phase I clinical trials will lead to better drugs taken forward into phase II/III trials and eventually into clinical practice.

Resumen en español

Introducción: Los biomarcadores farmacodinámicos (PD) se utilizan para demostrar inhibición farmacológica de dianas terapéuticas, así como para evaluar los efectos en las cascadas de señalización intracelular de los nuevos tratamientos contra dianas moleculares. Los biomarcadores farmacodinámicos aceleran el desarrollo de nuevos fármacos y la transición de nuevos fármacos hasta su uso clínico. El neuroblastoma es un tumor infantil de alto riesgo en el que las metástasis de médula ósea son frecuentes. El objetivo de este estudio fue desarrollar biomarcadores farmacodinámicos de la vía de señalización fosfoinositil-trifosfato-kinasa (PI3K)/AKT que puedan ser implementados en ensayos clínicos fase I/II de terapias moleculares, incluyendo: 1) la adaptación de un biomarcador indirecto de la vía de señalización PI3K/AKT en plasma enriquecido con plaquetas (PRP), 2) el desarrollo de la metodología para aislar células tumorales de muestras de aspirado de médula ósea para su uso en biomarcadores proteicos, 3) el desarrollo de un ELISA cuantitativo para medir la expresión de la proteína MYCN en neuroblastoma (un efector de PI3K) y 4) el desarrollo de técnicas para medir la vía de señalización PI3K/AKT directamente en células tumorales aisladas de muestras de medula ósea.

Material y métodos: Las señales de proteínas total y fosforilada AKT, GSK3β y p70S6K en plasma enriquecido con plaquetas y células tumorales fueron medidas con la tecnología MesoScale Discovery (MSD) ® Triplex AKT. La separación de células tumorales se realizó con métodos de separación inmunomagnética basados en MicroBeads y se desarrollo un ELISA utilizando Europium para cuantificar la expresión de proteína MYCN total. El desarrollo pre-clínico se realizo en células de neuroblastoma provenientes de líneas celulares diluidas en sangre periférica de voluntarios sanos. El desarrollo clínico se realizó en 14 niños con tumores sólidos avanzados, cinco de ellos con neuroblastoma metastásico en la médula ósea. El estudio se realizo con autorización del Comité de Investigación Clínica del Hospital Royal Marsden.

Resultados: El estudio de los biomarcadores realizados en plasma enriquecido con plaquetas permitió mostrar señales detectables para todas las proteínas totales y fosforiladas en todos los casos. No se encontraron diferencias respecto a edad, diagnostico o estadio. La depleción de células CD45+ fue el método de separación inmunomagnética superior, obteniendo suspensiones celulares con alta pureza en células de neuroblastoma en experimentos con líneas celulares de neuroblastoma en sangre periférica y en muestras de médula ósea de pacientes con afectación metastásica por neuroblastoma. Las suspensiones celulares obtenidas contenían suficiente cantidad de proteína para los biomarcadores requeridos. Se desarrollo un sandwich ELISA basado en Europio para medir la expresión de proteína MYCN total de forma sensible y especifica. Se completo el desarrollo preclínico de la tecnología MSD Triplex AKT para medir la activación de la vía de señalización PI3K/AKT en células de neuroblastoma.

Discusión y conclusiones: los biomarcadores en plasma enriquecido con plaquetas han sido validados en la población pediátrica y están completamente preparados para ser implementados como biomarcadores farmacodinámicos en los ensayos clínicos de fase I en pacientes pediátricos de los inhibidores duales de PI3K y mTOR BEZ235 y GDC0980 de próxima apertura en el Hospital Royal Marsden. La separación inmunomagnética con depleción de células CD45 demostró ser una metodología efectiva para obtener suspensiones celulares purificadas en células de neuroblastoma como una fuente no invasiva de células tumorales para biomarcadores farmacodinámicos proteicos. El desarrollo preclínico del ELISA para MYCN fue completado permitiendo su transición a la fase clínica. Se realizó el desarrollo preclínico piloto de la tecnología MSD para PI3K/AKT en células de neuroblastoma y la tecnología será evaluada en la fase clínica. El desarrollo e implementación de dichos biomarcadores en nuevos ensayos clínicos fase I basados en hipótesis biológicas llevara a una mejoría en la selección de fármacos que se transfieren a ensayos fase II y III y eventualmente a la práctica clínica.

Abbreviations

ALK	Anaplastic lymphoma kinase
APC-H7	Allophycocyanin H7
ASCT	Autologous stem cell transplant
ATP	Adenosine triphosphate
BARQA	British Association on Research Quality Assurance
BCA	Bicinchoninic acid
BM	Bone marrow
CCR	Committee for Clinical Research
CDK	Cyclin dependent kinase
CEC	Circulating Endothelial Cells
CEP	Circulating Endothelial Progenitors
CGH	Comparative genomic hybridisation
CNS	Central nervous system
CTC	Circulating tumour cell
CV	Coefficient of variation
ECL	Electrochemoluminiscence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzime-linked Immunoabsorption assay
EpCAM	Epithelial cell adhesion molecule
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridisation
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FSC-A	FSC – amplitude
FSC-H	FSC – height
GCLP	Good clinical laboratory practice
GCP	Good clinical practice
GLP	Good laboratory practice
GPCR	G protein coupled receptors
HDAC	Histone deacetylase
ICH	International conference on Harmonisation
ICR	The Institute of Cancer Research
IGF-1R	Insulin growth factor-1 receptor
IRS-1	Insulin receptor 1
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MSD	MesoScale Discovery ®
mTOR	Mammalian target of rapamycin
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PARP	poly(ADP)-ribose polymerases

PD	Pharmacodynamic
PDGFR	Platelet derived growth factor receptor
PFH	Preservative-free heparin
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PK	Pharmacokinetic
PNET	Primitive neuroectodermic tumour
PPTP	Pediatric Preclinical Testing Program
PRP	Platelet-rich plasma
QC	Quality control
RCF	Relative centrifugal force
RMH	The Royal Marsden NHS Foundation Trust
RT-PCR	Real time polymerase chain reaction
RTK	Receptor tyrosine kinase
SD	Standard deviation
SOP	Standard operative procedure
SSC	Side scatter
SSC-A	SSC – amplitude
SDS	Sodium dodecyl sulfate
TH	Tyrosine hydroxilase
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

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Introduction

1. Neuroblastoma

1.1 Metastatic and *MYCN* amplified neuroblastoma is a childhood cancer with poor prognosis

Neuroblastoma is a tumour derived from the sympathetic nervous system and is the most common solid tumour in children. The German pathologist R.L.K. Virchow first described the histological appearance in 1864. In 1910 J.H. Wright used the term 'neuroblastoma' [1-2]. A wide clinical spectrum has been described from tumour regression and maturation to ganglioneuroma in localised and infant tumours [3-4] to metastatic and MYCN amplified forms, which although usually are initially chemosensitive eventually recur in a drug resistant form [5-6].

Although the prognosis for paediatric cancer has improved significantly over past decades, childhood cancer is still the principal cause of death from disease between infancy and adulthood in developed countries and 25% of children with cancer die from their disease. According to EUROCARE database, there are approximately 400 cases of neuroblastoma per year across Europe [7].

Around half of cases present at advanced stages or have adverse biologic features. Those patients have a high rate of relapses accounting for 15% of deaths from childhood cancer [8-9]. High-risk neuroblastoma comprises children with stage M (metastatic) disease or *MYCN* amplification [10].

After current first line treatment including multimodal treatment with chemotherapy, surgery, high-dose chemotherapy with haematopoietic stem cell rescue, radiotherapy to the site of the primary tumour and differentiating therapy, more than 70% of patients experience relapse [6]. Recently, the addition of immunotherapy to treat residual disease has increased event free survival and reduced relapse by 20% [11-12]. Outcome after relapse or progression is dismal if the patient has previously received high-dose chemotherapy with haematopoietic stem cell rescue [13-14]. These patients define a cohort of children in whom conventional therapy is not successful with 5-year overall survival below 8%. In a study by the Italian Neuroblastoma Registry, 10-year overall survival was 2% after relapse of high-risk neuroblastoma [15]. Therefore, there is an unmet need to develop new drugs for children with high-risk neuroblastoma.

1.2 *MYCN* amplification in neuroblastoma

Amplification of the *MYCN* oncogenes (*V-myc* myelocytomatosis viral related oncogene, neuroblastoma derived), located on chromosome 2p24 was initially described by Schwab et al. [16]. *MYCN* oncogene amplification is among the best-characterised genomic markers available for risk-based therapeutic classification and in multifactorial analysis identifies patients with neuroblastoma with a poor prognosis [17-18] regardless of their stage.

In addition to human neuroblastoma where it is found in approximately 25% of tumours, *MYCN* amplification has also been reported in a number of other tumours, including alveolar rhabdomyosarcoma (60%), retinoblastoma (20%), medulloblastoma (10%) and in adults, small cell lung cancer (7%) [19]. Aberrations in the expression of *MYC* gene family members (*c-myc* and *MYCN*) contribute also to other adult malignancies (Burkitt's and non-Hodgkin's lymphoma) and several paediatric solid tumours.

Normal *MYCN* expression is restricted to early stages of embryonic development. Targeted homozygous deletion of *MYC* or *MYCN* in mice results in embryonic lethality, while transgenic mouse models of *MYC* and *MYCN* overexpression have been developed harbouring the same genetic profile than human tumours (i.e. 17q). Those mice have been found to develop a range of tumours, suggesting they play a crucial role in both embryonic and oncogenic development [20]. The *MYC* family members are transcription factors. MYC proteins activate the transcription of a number of target genes [19, 21].

1.3 MYCN as a therapeutic target

Due to its central role in neuroblastoma genesis and potent oncogenic stimulus, MYCN is an attractive target for the development of new anticancer drugs.

MYCN amplification is translated into increased levels of MYCN oncoprotein which has effects in cell proliferation, angiogenesis, differentiation and invasive potential. Therefore, *MYCN* and MYCN are potential targets for a number of *MYCN*-driven malignancies. Although it is not clear if a specific MYCN inhibitor would be effective, different approaches at several steps from gene to protein level have been studied and results are encouraging [21].

Several decades ago, attempts of targeting *MYCN* with antisense DNA were successfully performed in pre-clinical models [22-23] although this approach did not succeed in the clinic.

Several candidate genes that are directly or indirectly regulated by MYCN can be targeted with new molecules that are in pre-clinical or clinical development. Examples of these direct or indirect targets are *MDM2* (murine double minute oncogenes, via the p53 pathway), *DKK3* (Dickkopf-related protein 3 via Wnt inhibition), *SKP2* (S-phase kinase-associated protein 2 via proteasome inhibition) and Aurora kinase A [21].

More recently, it has been shown that phosphorylation of MYCN protein causes destabilization and proteosomal degradation of the protein and reversal of its deleterious effects. Several targeted agents inhibiting different pathways such as Phosphoinositide 3-kinase (PI3K), AKT, mammalian target of rapamycin (mTOR), cycline dependent kinase (CDK) and Aurora kinases are able to phosphorylate MYCN and this is a field of very active research at the moment. Chesler et al. showed that pharmacologic inhibition of PI3K in transgenic *TH-MYCN* models decreased the levels of MYCN protein and decreased tumour growth [24]. They were able to demonstrate that PI3K

inhibition with LY294002, a pure PI3K inhibitor blocked MYCN at post-transcriptional level and diminished the levels of MYCN protein. This occurred via activation of GSK3β which phosphorylates MYCN and destabilizes the protein.

Chesler et al. hypothesized that neuroblastoma tumours with *MYCN* amplification are “addicted” to MYCN oncoprotein and that pharmacological blockage of this pathway will result in excellent anti-tumour effects [24]. This hypothesis has been tested in genetically engineered murine models of *TH-MYCN* neuroblastoma, where PI3K inhibition with LY294002 and the dual PI3K-mTOR inhibitor BEZ235 showed antitumour effects via increase of MYCN phosphorylation and proteosomal degradation [25].

Moreover, Otto et al. have shown that Aurora kinase A is a synthetic lethal gene to *MYCN* (synthetic lethality arises when a combination of mutations in two or more genes leads to cell death), and proved that enzyme inhibition stabilizes MYCN [26]. The Aurora kinase A inhibitor MLN8237 showed very good anti-neuroblastoma responses in mouse xenografts when tested by the National Cancer Institute-driven Pediatric Preclinical Testing Program (PPTP), although it is not known whether this is dependent on destabilisation of MYCN [27]. Several clinical trials are evaluating Aurora kinase inhibition in children and there is major interest for its role in neuroblastoma, especially *MYCN* amplified disease [28-29].

Also Molenaar et al. have demonstrated that CDK2 is synthetically lethal to neuroblastoma cells with *MYCN* amplification and increased protein expression [30]. Additionally, treatment with roscovitine (CYC202, a CDK2, 7 and 9 inhibitor) induced MYCN-dependent apoptosis.

2. The PI3K/AKT signalling pathway

The phosphoinositide 3-kinases are enzymes that are primarily involved in the phosphorylation of membrane inositol lipids, mediating cellular signal transduction. Although PI3K was originally characterized two decades ago through its binding to oncogenes and activated receptor tyrosine kinases (RTKs) its association with human cancer was not established until the late 1990s, when it was shown that the tumour suppressor PTEN acts as a phosphatase that is specific for the lipid products of PI3K [31].

PI3Ks are divided into three classes according to their structural characteristics and substrate specificity. Of these, the most commonly studied are the class I enzymes that are activated directly by cell surface receptors. Class I PI3Ks are further divided into class IA enzymes, which are activated by RTKs, G protein coupled receptors (GPCR) and certain oncogenes such as the small G protein RAS, and class IB enzymes, which are regulated exclusively by GPCRs [31-33].

The class I PI3Ks —p110α (also known as PIK3CA), p110β (also known as PIK3CB), p110γ (also known as PIK3CG) and p110δ (also known as PIK3CD) — are best understood, although much remains to be learnt about their coupling to upstream signals and their relative functional output. Class II and III PI3Ks remain enigmatic, with ill-defined roles in signal transduction.

Both RTKs and non-RTKs result in the activation of PI3K, which generates the second messenger Phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3) from Phosphatidylinositol (4, 5)-bisphosphate (PIP2). This recruits pleckstrin homology (PH) domain- containing proteins to the cell membrane, including the AKT/PKB kinases, driving their conformational change and resulting in their phosphorylation by the constitutively active phosphoinositide-dependent kinase 1 (PDK1) at Threonine 308 and by PDK2 [mammalian target of rapamycin complex 2 (mTORC2)] at Serine 473 (Figure 1). Activated AKT translocates to the cytoplasm and nucleus and activates downstream targets involved in survival, proliferation, cell cycle progression, growth, migration, and angiogenesis. AKT is negatively regulated by the tumour suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10), which dephosphorylates PIP3. AKT mediates the phosphorylation and activation of mTOR complex 1 (mTORC1), a serine/threonine kinase that plays critical roles in the regulation of protein translation and synthesis, angiogenesis and cell cycle progression [31-33].

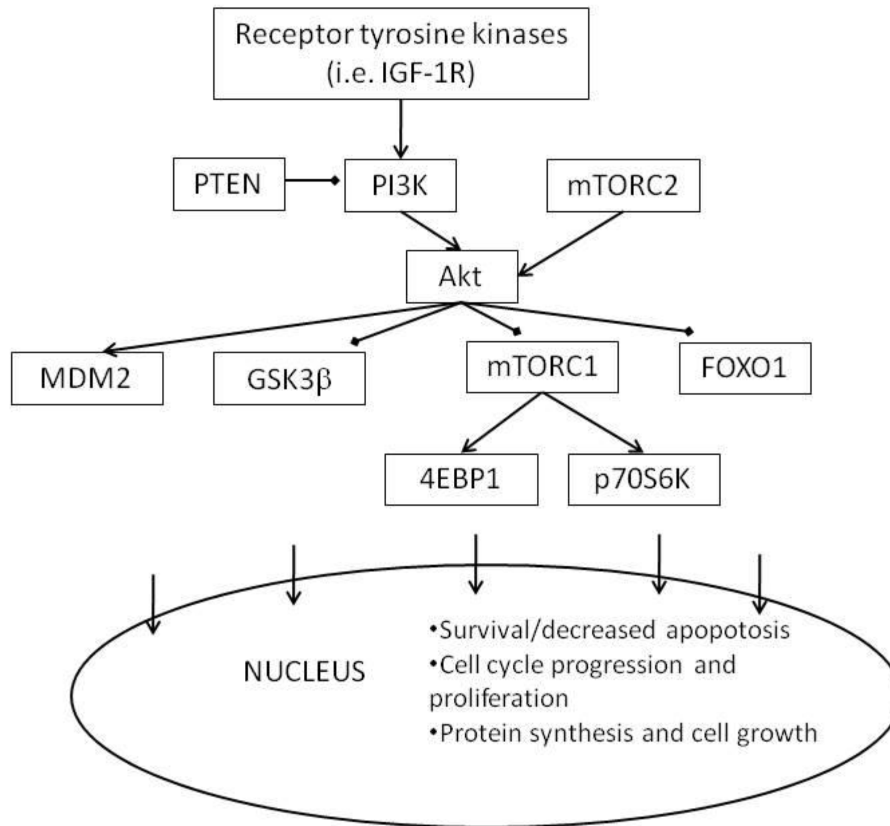


Figure 1, Schematic representation of the phosphorylation cascade through the PI3K/AKT/mTOR pathway. Arrows ending with a square represent inhibitory pathways. Adapted from [31, 34]

PI3K pathway activation by genetic mutation or amplification (for example *PIK3CA* mutations in breast cancer) plays a particularly relevant role in several adult malignancies such as glioblastoma, breast or prostate cancer [35].

3. The PI3K/AKT signalling pathway is activated in neuroblastoma

The PI3K pathway is activated in a variety of human cancers through multiple mechanisms. PI3K plays a crucial role in effecting alterations in a broad range of cellular functions in response to extracellular signals. The pathway is constitutively activated by the loss of the tumour suppressor PTEN, a phosphatase that counteracts the kinase activity of PI3K in many tumour types [36-37]. A key downstream effector of PI3K is the serine-threonine kinase AKT which, in response to PI3K activation, phosphorylates and regulates the activity of a number of targets including kinases, transcription factors and other regulatory molecules. AKT is a downstream target for PI3K and is overexpressed in a variety of cancers [38-40].

Activating mutations within the PI3K subunit p110α (*PIK3CA*) have been detected in many human cancers with the majority of mutations located in the kinase domain [41-44]. These activating mutations promote growth and invasion in cancer cells, which is abrogated by PI3K inhibition [45].

PI3K pathway activation in response to activation of receptor tyrosine kinases such as EGFR, PDGFR and IGF-1R is more common, and increased AKT phosphorylation is independently predictive of poor clinical outcome in neuroblastoma and glioma [46-47].

Opel et al. found increased AKT, S6 and ERK phosphorylation in neuroblastoma samples. AKT activation also correlated with MYCN amplification. They also found how IGF-1R pathway activation is related to tumour apoptosis in a PI3K-dependent manner [47]. Additionally, it has been shown that dual inhibition of IGF-1R and mTOR results in decreased MYCN protein expression, increased MYCN phosphorylation and induced apoptosis in neuroblastoma cell lines [48].

Activating mutations in PI3K pathway components promote growth and invasion of cancer cells, and can be abrogated by PI3K inhibitors. For paediatric solid tumours, inhibition of the PI3K pathway is a promising novel therapeutic approach likely to be efficacious in three groups of paediatric cancers:

- Tumours with PI3K pathway activating mutations (*PI3KCA, AKT, PTEN*) such as medulloblastoma and malignant gliomas.
- Tumours with persistent pathway activation (increased AKT phosphorylation), including malignant central nervous system (CNS) (glioma, medulloblastoma) and non-CNS (neuroblastoma) tumours.
- Tumours with *MYCN* gene amplification (since the MYCN protein is a direct PI3K target) including neuroblastoma, medulloblastoma and alveolar rhabdomyosarcoma.

Tumour mass in *MYCN*-transgenic animals treated with the pure PI3K inhibitor LY294002 was decreased significantly in comparison with vehicle controls. This compound reduced the accumulation of viable cells in both *MYCN* amplified and non amplified cell lines, although this effect was less prominent in the latter group [24].

In *TH-MYCN* murine neuroblastoma models, treatment with the dual PI3K-mTOR inhibitor BEZ235 [49] inhibited tumour growth and the PI3K/AKT/mTOR signalling pathway, reduced neuroblastoma cell growth *in vitro* and destabilised wild-type MYCN and attenuated PI3K/mTOR signalling *in vitro*. Similar effects were found when the mTORC inhibitor AZD8055 was tested [50].

Pharmacologic inhibition of the PI3K/AKT/mTOR pathway is a promising target in adult and paediatric tumours, including neuroblastoma. Pharmacologically viable PI3K, AKT or mTORC inhibitors are currently completing phase I evaluation in adults without significant toxicity [51-52], and trials of those compounds in paediatric patients are under planning.

Potent and isoform-selective PI3K inhibitors with improved pharmacologic properties are now entering clinical trials [53]. Phase I/II studies of pure PI3K (GDC0941, XL147), dual PI3K/mTOR (BEZ235, BGT226, GDC0980), AKT (MK2206, perifosine) and mTORC1 and 2 (OSI027, AZD2014, AZD8055) inhibitors are ongoing in adults [35]. Preliminary results from these studies suggest that these agents are well tolerated and have favourable pharmacokinetic/pharmacodynamic (PK-PD) profiles. Emerging data indicate that isoform selectivity may be important to maximize therapeutic benefit and minimize toxicity, although concerns remain about tumour cell redundancy between different isoforms.

Several AKT inhibitors have been tested in paediatric tumour models showing anti-tumour activity. The evaluation of GSK690693 by the Pediatric Preclinical Testing Program showed survival improvements in osteosarcoma and glioblastoma [54]. Perifosine was shown to be active in neuroblastoma [55], glioma [56], medulloblastoma [57] or sarcomas [58].

Proof of principle that the PI3K pathway can be successfully targeted for clinical use in cancer has been demonstrated by the development of rapamycin analogs (temsirolimus, everolimus, ridaforolimus) that inhibit the mTORC1 kinase. However, preliminary results to date have been disappointing when these analogs have been administered as single agents [59-60]. Superior single-agent activity may potentially be seen with dual mTORC1/mTORC2 ATP-competitive kinase inhibitors, which are currently in clinical development (OSI027, AZD8055, AZD2014). These broader spectrum inhibitors may, however, be more toxic. mTORC1/mTORC2 inhibitors should, nonetheless, abrogate the reported negative-feedback loops associated with rapamycin analog administration, resulting in activation of upstream targets such as IGF-1R and phospho-AKT [61].

Other investigators have found benefit of mTOR inhibition via MYCN degradation using rapamycin for the treatment of neuroblastoma [62] or for the combinations with other agents such as IGF-1R inhibitors [48, 63-64]

Currently, early clinical trials are beginning to evaluate targeted therapies in children with neuroblastoma, including ALK inhibitors [65], Aurora kinase inhibitors [29, 66-67] or IGF-1R inhibitors [68]. It is envisioned that within the next decade, a number of potentially relevant drugs to neuroblastoma will be tested in the paediatric population, including PI3K, AKT, mTORC, CDK inhibitors and other involved signalling pathways. Pharmacodynamic biomarkers will be needed in order to select most promising agents and classes to be taken forward into large phase III clinical trials and eventually into the clinic.

4. Development of pharmacodynamic (PD) biomarkers

Biomarkers have been defined as biological characteristics that can be objectively measured and evaluated as an indicator of normal biological or pathological processes, or pharmacological responses to a therapeutic intervention [69].

4.1 Types and steps of development

Three main types of biomarkers have been defined [70]:

- Prognostic biomarkers, give information on prognostic features helping to distinguish between cases with good or poor outcome (e.g. patients with higher level of circulating tumour cells at diagnosis have worse survival than those with no circulating tumour cells [71].
- Predictive biomarkers, assess the probability that a patient will benefit from a particular treatment (e.g. patients with HER2-positive breast cancer will benefit from treatment with the anti-HER2 monoclonal antibody transtuzumab) [72-73].
- Pharmacological/PD biomarkers, measure effects of the drug on the tumour e.g. the AKT inhibitor MK2206 given at the recommended phase 2 dose inhibits effectively AKT in tumour cells and other surrogate biomarkers [74]. PD biomarkers are of major value in making "go-no go" decisions and assess performance of new drugs.

Four steps are required during biomarker development [75]:

- **Discovery**. In this phase aspects of tumour biology are shown to correlate with clinical endpoints such as survival. Often 'omics' platforms that analyse a large number of biomarkers are used to generate hypothesis which are then refined in further developments.
- **Validation**. This phase is required to determine that the assay is fit-for-purpose for objective measurement. Reproducibility, accuracy, precision and recovery have to be determined.
- **Qualification.** Correlation of biomarkers with clinical endpoint. Sensitivity and specificity of the specific assay are determined.
- **Implementation.** Implementation in the clinic to help clinical decision taking.

4.2 Relevance of biomarkers

The use of pharmacodynamic profiling is essential for the optimal clinical development of targeted anti-cancer agents. These assays are key to demonstrate proof-of-principle for target modulation, to establish optimal drug dosing and schedule, to maximise the extent and duration of target

blockade and to evaluate the downstream biological effects of target modulation on cancer cell proliferation and survival [73]. Several PD biomarkers have been used in adult early clinical trials and need to be adapted to the paediatric population. Additionally, prognostic and predictive biomarkers are needed in children in order to improve risk stratification and to select patients that will benefit most from targeted therapies (i.e. enrich for anaplastic lymphoma kinase (ALK) gene - mutated neuroblastomas in a clinical trial of an ALK inhibitor). So far, early clinical trials in children have lacked PD biomarkers to demonstrate proof-of-principle for target modulation, improve drug dosing and schedule, or assess downstream biological effects of target modulation and in summary, help in the "go-no go" decisions to larger phase III studies.

The objective of the Royal Marsden Hospital – Institute of Cancer Research (RMH/ICR) Paediatric Drug Development Program is to integrate biomarkers in early clinical trials to provide prognostic stratification, selection of appropriate therapy by predictive biomarkers and to measure the effect on pathway targeting by PD biomarkers [76-78].

4.3 Sources of biomarkers

Tumour material is the best tissue in which to develop new biomarkers. However, when translated into human studies, this implies significant logistic and sometimes ethical difficulties, as tumour tissue is often difficult to obtain and often repeated samples are required. This is a key difficulty in the case of childhood malignancies where most tumours are not easily accessible for biopsy and biopsy requires an anaesthetic procedure.

To date, some PD biomarkers have been established in adults at the ICR using surrogate tissues such as peripheral blood mononuclear cells (PBMC), plucked hair follicles or skin fibroblasts for phase I trials.

Due to the specific characteristics of neuroblastoma, especially in the relapsed setting, several sources of tumour material or surrogate tissues can be explored. Primary tumours mostly arise in the suprarenal area or retroperitoneal cavity and therefore, biopsy can be risky and challenging. Most frequent sites of metastases are skeleton and bone marrow and less so lungs or central nervous system (CNS) [2].

4.4 Surrogate biomarkers

Increase in AKT phosphorylation in PBMC or PRP was shown after treatment with the AKT inhibitor MK2206 [52, 79]. Several pharmacodynamic biomarkers of the PI3K/AKT/mTOR pathway have been used in different adult phase I studies including IGF-1R, EGFR, IRS-1, p-AKT, p-MAPK, p-S6 and p-4EBP1 [80-82].

A pharmacological biomarker assay has been developed at ICR to measure PI3K pathway inhibition, using Meso Scale Discovery (MSD®) technology, in adult tumour material or surrogate

PRP samples in accordance with Good Clinical Laboratory Practice (GCLP) on adult trials at The Royal Marsden Hospital (RMH) [83-85]. Those biomarkers have not been used in paediatric clinical trials and its validity has still to be proven.

4.5 Bone marrow samples to develop PD biomarkers

Given that 80% of patients with high-risk neuroblastoma have tumour bone marrow involvement [5, 86], the isolation of tumour material from bone marrow samples, which are otherwise frequently obtained for clinical purposes, is a feasible approach. This degree of involvement ranges from 10 to 99% of nucleated cells and bone marrow aspiration and trephine biopsies are used as part of routine clinical assessments of cases with metastatic disease.

Several FISH (fluorescence *in situ* hybridisation), RT-PCR (real time polymerase chain reaction) or protein phosphorylation assays of different tumour pathways such as PI3K/AKT/mTOR, IGF-1R, EGFR, PDGFR or Aurora Kinase among others to be used in early clinical trials of anticancer drugs require high purity of tumour cells. Therefore, a procedure to isolate and enrich neuroblastoma cells from bone marrow samples of children with neuroblastoma is needed for this purpose.

Bone marrow-derived neuroblastoma cells have been used by the Ghent group to perform genomic analyses. In a very preliminary report, Vandewoestyne et al. were able to use bone marrow neuroblasts for FISH and array CGH (comparative genomic hybridisation) assays, showing similar array profiles of bone marrow samples compared to primary tumours [87]. Bone marrow samples have also been used to detect neuroblastoma mRNA and this assay is widely used across Europe [88].

4.6 Use of biomarkers in paediatric clinical trials

A limited number of phase I/II studies in children have included predictive biomarkers to select patients who would most benefit from targeted therapy.

A study of the PDGFR inhibitor imatinib in children with solid tumours used target expression as a predictive biomarker and included only patients with PDGFR or c-Kit expression. No correlation with clinical response or outcome was found [89].

Several studies of drugs targeting EGFR have also reported results on target expression in tumour samples or mutation analysis. A phase I of erlotinib in children found no correlation between markers of PI3K/AKT pathway activation, including mutational analysis for *PTEN* and *PIK3CA* and response to the current therapy [90]. Likewise, a phase I study of erlotinib with temozolomide for children with solid tumours or a phase I study of cetuximab plus irinotecan in children found pathway activation but no clinical correlation [91-92]. Those studies aimed to identify those patients

that would most benefit from EGFR inhibition based on their EGFR mutational status or pathway activation in samples which were mostly retrospectively collected from diagnosis. However, it was not proved whether the pathway was pharmacologically inhibited at the given doses.

More recently, early clinical trials have also incorporated PD biomarkers. Two studies testing bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF), in children have prospectively investigated PD biomarkers in blood or with new imaging modalities. In the first phase I study, circulating markers including circulating endothelial cells (CEC), circulating endothelial progenitor (CEP) cells and other soluble factors such as VEGF or b-FGF (fibroblast growth factor) showed significant inter-patient variability and no correlation was observed with clinical benefit [93]. However, a second study testing bevacizumab added to irinotecan in children with recurrent malignant glioma or diffuse brainstem glioma found target inhibition showed a reduction in phospho-VEGFR2 levels after treatment. Assessment of VEGFR phosphorylation status in PBMCs showed decreased levels of phosphorylation and target inhibition in a number of patients. However, despite proving effective VEGF inhibition, the trial failed to show any clinical benefit for the patients [94].

A recent paediatric phase I study of the mTOR inhibitor temsirolimus (CCI779) measured p-S6, p-AKT and p-4EBP1 by Western blot in PBMC´s at different time points during therapy [59]. Despite marked inter-patient variability, reductions in all three phospho-proteins were detectable from two hours after temsirolimus dosing and those reductions were more profound from course two onwards. No correlation with PK or patient benefit was found.

PD biomarkers have shown target inhibition and have aided in the dose selection in a phase I trial of vorinostat, an HDAC inhibitor. In this study, accumulation of acetyl H3 histones in PBMCs was detected in patients receiving the highest vorinostat dosages and particularly in patients treated with the higher dose level in whom accumulation of acetyl-H3 persisted for 24 hours. Evidence of a more transient (6 hours) PBMC acetyl H3 accumulation was observed in patients receiving intermediate doses, and no accumulation was found in the lower dose levels [95].

4.7 Good Clinical Practice (GCP) and Good Clinical Laboratory Practice (GCLP) standards

Good Clinical Practice is a set of internationally recognised ethical and scientific quality requirements which must be observed for designing, conducting, recording and reporting clinical trials that involve the participation of human subjects [96].

Compliance with this good practice provides assurance that the rights, safety and well-being of trial subjects are protected, and that the results of the clinical trials are credible and accurate.

The conduct of clinical trials on medicinal products for human use is regulated by the European Clinical Trial Directive 2001/20/EC [97]. The principles of Good Clinical Practice to be followed for the conduct of clinical trials in human subjects are outlined in articles 2 to 5 in the EU Directive

2005/28/EC [98]. There is now extensive regulation and guidance for research assays to be used in clinical trials including recommendations from the European Medicines Agency (EMA) for research involving children [99].

Good Clinical Laboratory Practice (GCLP) provides a bridge between GCP and GLP (Good Laboratory Practice). It provides a framework to organisations on facilities, systems and procedure to ensure the reliability, quality and integrity of the work and results satisfy GCP expectations [100].

It applies to all samples generated during the conduct of a clinical trial and includes

- Organisation and Personnel
- Facilities
- Equipment, materials and reagents
- Standard Operating Procedures (SOPs)
- Planning, conduct and reporting
- Quality Control and Quality audits
- Retention of study records and reports.

Those principles were established by the British Association on Research Quality Assurance (BARQA) in order to ensure the compliance with GCP and GLP in clinical trials conducted in the United Kingdom [100].

The Clinical PD Biomarker Group Laboratory at the ICR is a laboratory specifically designed to adhere to GCP and GCLP in order to provide the highest standard for pharmacokinetic and pharmacodynamic analyses in phase I/II clinical trials of targeted agents carried out at RMH/ICR. In order to facilitate the implementation of all assays developed within this study, all laboratory work was performed at the Clinical PD Biomarker Group Laboratory at the ICR following strict GCP and GCLP standards.

4.8 Development of PD biomarkers of the PI3K pathway in neuroblastoma

The main hypothesis of the Neuroblastoma Drug Development Team at the ICR led by Dr Chesler is that drugs targeting the PI3K/mTOR pathway will have a specific benefit in children with *MYCN* amplified and expressing neuroblastoma by destabilisation of MYCN protein. The upcoming phase I clinical trials of drugs targeting the PI3K/AKT/mTOR pathway need to include pharmacodynamic biomarkers determining total and phospho protein signals targeted by the new agents, the effects upstream and downstream the pathway and functional imaging to evaluate effects on tumour vasculature and perfusion.

If these biomarkers are not implemented, the drug development process for neuroblastoma and other high risk childhood cancers will be empiric and will not be led by scientific hypotheses. There will be substantial risks of withdrawing potentially useful agents because the schedule does not achieve sustained target inhibition, only a small proportion of patients benefit from the drug and allowing ineffective drugs progress into phase II/III trials, implying that a large number of children with advanced cancers will receive ineffective agents.

The incorporation of scientifically and analytically validated biomarkers into rationally designed hypothesis-testing clinical trials offers a promising way forward to increasing the odds for successful and efficient transition of a compounds through the drug development pipeline [77].

Introducción en español

1. Neuroblastoma

1.1 El neuroblastoma de alto riesgo (metastásico o *MYCN*-amplificado) es un cáncer infantil de mal pronóstico

El neuroblastoma es un tumor derivado del sistema nervioso simpático y constituye el tumor sólido más frecuente en la edad pediátrica. El patólogo alemán R.L.K. Virchow lo describió por primera vez en 1864 [1]. El espectro clínico del neuroblastoma es extremadamente amplio, desde tumores que regresan espontáneamente o maduran a ganglioneuroma en formas localizadas y niños menores de 18 meses [2-4] hasta formas metastásicas y *MYCN* amplificadas que si bien inicialmente son quimiosensibles, acaban desarrollando quimio-resistencia [5-6].

Aunque el pronóstico del cáncer infantil ha mejorado significativamente durante las pasadas décadas, todavía es la principal cause de muerte por enfermedad en la edad pediátrica en países desarrollados y un 25% de los niños con cáncer fallecen por la enfermedad. Según los datos recogidos por el registro epidemiológico EUROCARE, hay aproximadamente 400 casos de neuroblastoma al año en Europa Occidental [7].

Alrededor de la mitad de los casos se presentan en estadios avanzados o tienen factores biológicos adversos. Estos pacientes presentan una alta tasa de recaídas y en conjunto representan el 15% de las muertes por cáncer infantil [8-9].

El neuroblastoma de alto riesgo está formado por dos grupos: aquellos pacientes con enfermedad metastásica al diagnostico o aquellos cuyos tumores presentan amplificación del oncogén *MYCN* [10]. A pesar de un tratamiento de primera línea multimodal e intensivo, consistente en quimioterapia, cirugía, megaterapia con transplante autólogo de progenitores hematopoyéticos, radioterapia al lecho tumoral primario y tratamiento diferenciador más del 70% de los pacientes sufren recaídas [6]. Recientemente, la adición de inmunoterapia para tratar la enfermedad residual ha demostrado incrementar en un 20% la supervivencia libre de eventos [11]. El pronóstico tras la recaída es extremadamente pobre para los pacientes que han recibido tratamiento con megaterapia y transplante autólogo [13]. Estos pacientes que ya han agotado el tratamiento convencional definen una cohorte en la que el tratamiento de segunda línea obtiene una supervivencia global menor del 8%. Por tanto hay una necesidad no satisfecha de desarrollar nuevos medicamentos contra el neuroblastoma de alto riesgo.

1.2 Amplificacion de *MYCN* en el neuroblastoma

La amplificación del oncogén *MYCN* es el marcador genómico más relevante de los disponibles para un tratamiento estratificado por categorías de riesgo. En análisis multivariante ha demostrado identificar pacientes con mal pronóstico [17-18] independientemente de su estadio. La amplificación del oncogén *MYCN*, localizada en el cromosoma 2p24fue inicialmente descrita por Scwab y col. en 1983 [16] y se encuentra en aproximadamente un 25% de los casos. Dicha

amplificación del oncogén *MYCN* se encuentra además en otros tumores incluyendo carcinoma microcítico de pulmón (7%), rabdomiosarcoma alveolar (60%) y retinoblastoma (20%) [19]. Aberraciones en la expresión de miembros de la familia de genes *MYC* (*c-myc* y *MYCN*) contribuyen asimismo a otros canceres del adulto (linfoma de Burkitt y linfoma no Hodgkin) y de la edad pediátrica.

Normalmente la expresión de MYCN está restringida a estadios iniciales del desarrollo embrionario. La deleción homocigota de *MYC* o *MYCN* en ratones resulta en letalidad de los embriones, en tanto que modelos transgénicos con sobreexpresión de MYC o MYCN desarrollan una variedad de tumores, lo que sugiere que dicho oncogén juega un papel crucial tanto en el desarrollo embrionario como en la biología tumoral [20]. Actualmente, dichos modelos transgénicos recapitulan la biología tumoral del neuroblastoma humano presentando un perfil genético similar [20]. Los miembros de la familia *MYC* son factores de transcripción, proteínas que activan la transcripción de determinados genes [19, 21].

1.3 MYCN como diana terapéutica

Debido a su papel central en la aparición del neuroblastoma y el potente estimulo oncogénico que supone, MYCN es una diana interesante para el desarrollo de nuevos fármacos antitumorales.

La amplificación de *MYCN* se traduce en niveles altos de expresión de la proteína MYCN derivando en efectos en la proliferación celular, angiogénesis, diferenciación y potencial invasivo. Por tanto, *MYCN* y MYCN son dianas terapéuticas atractivas para los tumores dependientes de MYCN. Aunque todavía no se ha establecido si un inhibidor especifico de MYCN seria efectivo, distintas estrategias terapéuticas que van desde el nivel génico a proteico están en estudio y los resultados preliminares son prometedores [21].

En la década de los 90 varios tratamientos dirigidos contra el oncogén *MYCN* con DNA antisentido se llevaron a cabo en modelos preclínicos aunque esta estrategia terapéutica no fue efectivamente trasladada a la clínica [22-23].

Varios genes candidatos que son regulados directa o indirectamente por *MYCN* pueden ser dianas terapéuticas con nuevas moléculas que actualmente están en desarrollo preclínico o clínico, principalmente MDM2 (vía de señalización de p53), DKK3 (inhibidores de WNT), SKP2 (inhibidores del proteosoma) y Aurora Kinasa A [21].

Recientemente, se ha demostrado que la fosforilación de la proteína MYCN produce desestabilización y degradación en el proteosoma de la proteína, frenando así sus efectos deletéreos. Terapias moleculares contra distintas vías de señalización como inhibidores de PI3K/AKT/mTOR, CDK o Aurora Kinasas son capaces de fosforilar MYCN y este es un área donde los esfuerzos investigadores se han intensificado. El grupo de Chesler y col. (Universidad de San Francisco, USA) demostró como la inhibición farmacológica de PI3K en modelos

transgénicos *TH-MYCN* producía un descenso en los niveles de proteína MYCN frenando el crecimiento tumoral [24]. Este grupo ha demostrado que la inhibición selectiva de PI3K con LY294002 bloquea MYCN a nivel post-transcripcional disminuyendo los niveles de proteína MYCN vía activación de GSK3β que fosforila MYCN y desestabiliza la proteína.

Este grupo planteo la hipótesis de que los neuroblastomas con amplificación de *MYCN* son "adictos" a la oncoproteína MYCN y que por tanto, el bloqueo farmacológico de esta vía de señalización resultaría en excelentes efectos antitumorales [24]. Esta hipótesis se ha demostrado en los modelos murinos genéticamente modificados *TH-MYCN* de neuroblastoma donde la inhibición de PI3K con LY294002, con el inhibidor dual de PI3K/mTOR BEZ235 y el inhibidor de mTORC AZD8055 demostró excelentes resultados mediante el aumento de la fosforilación de MYCN resultando en degradación proteosomal [25, 50].

Además, Otto y col. han demostrado como Aurora kinasa A es un gen sintéticamente letal para MYCN y demostrado como la inhibición de dicha enzima estabiliza MYCN [26]. El tratamiento de modelos animales de neuroblastoma (xenoinjertos murinos) con el inhibidor de Aurora kinasas MLN8237 fue muy efectivo en el análisis realizado por el grupo colaborativo PPTP (Pediatric Preclinical Testing Program) en Estados Unidos, aunque este grupo no demostró si el efecto beneficioso se produjo mediante la desestabilización de MYCN [27]. Varios ensayos clínicos fase I/II están evaluando el uso de inhibidores de Aurora kinasas en la población infantil y hay un gran interés en el tratamiento del neuroblastoma, especialmente en los casos de amplificación de MYCN [28-29].

Análogamente, Molenaar y col. han demostrado que CDK2 es sintéticamente letal para las células de neuroblastoma con amplificación y sobreexpresión de MYCN. El tratamiento con roscovitina (un inhibidor de CDK en desarrollo preclínico) indujo apoptosis dependiente de MYCN [30].

2. La vía de señalización PI3K/AKT

La familia de PI3-kinasa (PI3K) está principalmente relacionada con la fosforilación de inositol en la membrana celular, mediando vías de señalización intracelular. Aunque PI3K fue inicialmente caracterizada hace tres décadas mediante su afinidad por oncogenes y receptores de tirosín-kinasas, su relación con el cáncer no se estableció hasta finales de los 90 cuando se demostró que el gen supresor de tumores PTEN actúa como una fosfatasa específica para los productos lipídicos de PI3K [31].

Las PI3-kinasas se dividen en tres clases de acuerdo con sus características estructurales y especificidad por sustratos. De estas, la más conocida es la clase I de enzimas que son activadas directamente por receptores de la superficie celular. Las kinasas de clase I se dividen en: IA, que son activadas por receptores tirosín-kinasas, receptores acoplados a proteína G y determinados oncogenes como la proteína G Ras y IB que están reguladas exclusivamente por receptores acoplados a proteína G [31-33].

Las kinasas de clase I más conocidas son p110α (también conocida como PIK3CA), p110 β (también conocida como PIK3CB), p110γ (también llamada PIK3CG) y p110δ (también llamada PIK3CD). Dichas kinasas son las más estudiadas, aunque todavía quedan por esclarecer los mecanismos de transmisión de las señales y su compleja función. Las kinasas de clase II continúan siendo enigmáticas con roles poco definidos en la transducción de señales. La importancia fisiológica de VPS34 (proteína vacuolar de caracterización 34, también conocida como PIK3C3) es desconocida.

Tanto receptores de tirosín-kinasa como otros receptores activan PI3K lo que genera el segundo mensajero fosfatidilinosidil-(3,5) trifosfato a partir de fosfatidilinosidil (4,5) bifosfato. Esto genera el reclutamiento de proteínas a la membrana celular incluyendo las kinasas AKT/PKB produciendo un cambio conformacional que resulta en su fosforilación por la kinasa PDK1 (kinasa fosfatidilinositol dependiente) en la Treonina 308 y por PDK2 (o mTORC2, [*mammalian target of rapamycin complex 2*]) en la Serina 473. La proteína AKT activada se transloca al citoplasma y el núcleo y activa dianas relacionadas con la supervivencia celular, proliferación, progresión del ciclo celular, crecimiento, migración y angiogénesis. AKT es regulada negativamente por el supresor de tumores PTEN (*phosphatase and tensin homolog*) situado en el cromosoma 10, que desfosforila al inositol tri-fosfato. AKT media la fosforilación y activación del complejo mTOR1 (mTORC1), una serin-treonín kinasa que juega papeles críticos en la regulación de la traducción y síntesis proteica, angiogénesis y progresión del ciclo celular [31-33].

La activación de la vía de señalización celular PI3K mediante mutación genética o amplificación juega un papel especialmente relevante en diversos canceres del adulto, como glioblastoma multiforme, cáncer de mama o próstata [35].

3. La vía de señalización PI3K/AKT esta activada en neuroblastoma

La cascada de PI3K esta activada en diversos canceres humanos mediante múltiples mecanismos. Dicha cascada juega un papel crucial en una amplia variedad de funciones celulares en respuesta a estímulos extracelulares. La vía es constitucionalmente activada por la pérdida del supresor de tumores PTEN, una fosfatasa que frena la actividad kinasa de PI3K en muchos tipos de tumores [36]. El principal efector de PI3K es la Serina-Treonina kinasa AKT que en respuesta a la activación de PI3K fosforila y regula la actividad de varias proteínas incluyendo kinasas, factores de transcripción y otras moléculas reguladoras. Asimismo, AKT esta sobreexpresada en diversos canceres humanos [38-40].

Mutaciones activadoras de la subunidad p110α (*PIK3CA*) se han detectado en canceres humanos. La mayoría de ellas situadas en el dominio kinasa [42-44]. Estas mutaciones que promueven el crecimiento y la invasión de las células tumorales pueden ser contrarrestadas mediante la inhibición farmacológica de PI3K [101].

La activación de la cascada de PI3K en respuesta a la activación de receptores de tirosín-kinasas como EGFR (receptor de factor de crecimiento epidérmico), PDGFR (receptor de factor de crecimiento derivado de plaquetas) e IGF-1R (receptor de factor de crecimiento relacionado con insulina) ocurre con frecuencia. Varios autores han demostrado que un aumento de AKT fosforilada es predictor independiente de mal pronóstico en neuroblastoma y glioma [47].

Opel y col. demostraron la existencia de hiperfosforilación de AKT, S6 y ERK en muestras de neuroblastoma humano, así como una correlación entre la activación de AKT y la amplificación de *MYCN*. También se demostró que la activación de IGF-1R deriva en apoptosis tumoral de manera dependiente de la vía de PI3K [47]. Asimismo, otros autores han demostrado que la inhibición simultanea de IGF-1R y mTOR resulta en una reducción de la expresión de MYCN, aumento de la fosforilación de MYCN y la inducción de apoptosis en líneas celulares de neuroblastoma [48, 63].

Mutaciones activadoras de componentes de PI3K generan el crecimiento y la invasión de células tumorales que pueden ser suprimidas mediante inhibición farmacológica. En el caso de los tumores sólidos pediátricos, la inhibición de PI3K es una diana terapéutica prometedora para tres grupos de canceres pediátricos:

- Tumores con mutaciones activadoras (*PIK3CA*, *AKT*, *PTEN*)
- Tumores con activación de la vía de señalización (hiperfosforilación de AKT), incluyendo tumores cerebrales malignos como glioma de alto grado o meduloblastoma y extracraneales como el neuroblastoma.
- Tumores con amplificación del oncogén *MYCN*, dado que la proteína MYCN es una diana directa de PI3K, incluyendo neuroblastoma, meduloblastoma y rabdomiosarcoma alveolar.

El tratamiento de ratones transgénicos con neuroblastoma (*TH-MYCN*) con el inhibidor de PI3K LY294002 produjo una reducción significativa de la masa tumoral en comparación con animales

tratados con placebo. Este compuesto redujo la acumulación de células viables en líneas celulares con y sin amplificación de *MYCN*, aunque de manera más prominente en el primer grupo [24].

Análogamente, el mismo modelo murino tratado con el inhibidor dual de PI3K y mTOR BEZ235 produjo inhibición del crecimiento tumoral mediante inhibición de la cascada de señalización de PI3K/AKT/mTOR, reducción del crecimiento celular *in vitro*, desestabilización de la proteína MYCN *in vitro*, bloqueo de la activación de la cascada PI3K producida mediante factores de crecimiento, así como el bloqueo del componente mTOR de la vía de señalización en células Kelly *in vitro* resultando en la desestabilización de MYCN y perdida de la viabilidad celular [25].

La inhibición farmacológica de PI3K/AKT/mTOR es una diana altamente interesante en oncología médica y pediátrica, incluyendo al neuroblastoma. Actualmente, existen múltiples inhibidores de PI3K o AKT en desarrollo clínico completando ensayos clínicos de fase I sin encontrar toxicidad significativa [51-52] y los ensayos clínicos pediátricos de dichos compuestos están en preparación. Actualmente múltiples ensayos clínicos fase I/II en adultos están abiertos evaluando inhibidores puros de PI3K (GDC0941, XL147), duales de PI3K y mTOR (BEZ235, BGT226, GDC0980), AKT (MK2206, Perifosine) y mTORC1 y 2 (OSI027, AZD2014, AZD8055).

Inhibidores potentes y selectivos para isoformas especificas de PI3K con altas cualidades farmacológicas están siendo estudiados [53], p.ej. moléculas como XL147 (Exelixis), BEZ235 (Novartis) y GDC0980 (Genentech). Los resultados preliminares de estos estudios sugieren que son fármacos bien tolerados con perfiles farmacocinéticos y farmacodinámicos favorables. Dichos resultados parecen sugerir que la selectividad frente a isoformas puede ser importante para maximizar el beneficio terapéutico y minimizar la toxicidad, aunque el riesgo de redundancia entre isoformas en estas cascadas de señalización podría derivar en falta de eficacia.

Varios inhibidores de AKT han demostrado actividad antitumoral en modelos preclínicos de tumores infantiles. El análisis de GSK690693 por el grupo PPTP mostro mejorías en la supervivencia de xenoinjertos de glioblastoma y osteosarcoma [102]. Otro inhibidor de AKT, perifosina ha demostrado actividad antitumoral en estudios preclínicos en neuroblastoma [55], glioma [56], meduloblastoma [57] y sarcomas [58].

El uso de análogos de rapamicina (temsirolimus, everolimus) en canceres en adultos ha demostrado que es posible inhibir la cascada de PI3-kinasas mediante inhibición de la kinasa mTORC1 en humanos. Sin embargo, los resultados de estos agentes en monoterapia han sido subóptimos y se ha sugerido que la inhibición dual de mTORC1 y mTORC2 kinasas con inhibidores ATP-competitivos podría ser superior, aunque a riesgo de aumentar la toxicidad.

Los inhibidores duales de mTORC1 y mTORC2 kinasas deberían ser capaces de evitar las señales de retroalimentación negativa que se producen con el uso de análogos de rapamicina que llevan a la activación de otras dianas celulares como IGF-1R y AKT.

Grupos de investigación en tumores pediátricos han demostrado que tanto la inhibición de mTOR con rapamicina en monoterapia como en combinación con inhibidores de IGF-1R producen efectos antitumorales mediante la degradación de MYCN en modelos de neuroblastoma [48, 62-64].

Actualmente, diversos ensayos clínicos pediátricos están evaluando terapias moleculares dirigidas, incluyendo inhibidores de ALK (COG-ADVL0912), de Aurora kinasas (COG-ADVL0921) o inhibidores de IGF-1R (R1507)[68]. Durante la próxima década, un gran número de fármacos biológicamente relevantes para el tratamiento del neuroblastoma serán evaluados en la población pediátrica incluyendo inhibidores de PI3K, AKT, CDK y otras cascadas de señalización.

4. Desarrollo de biomarcadores farmacodinámicos

Los biomarcadores se definen como características biológicas que pueden ser medidas objetivamente y evaluadas como un indicador de un proceso biológico normal, de un proceso patológico o respuestas farmacológicas a una intervención terapéuticas [69].

4.1 Tipos y estadios del desarrollo de los biomarcadores

Sawyers y col. han definido tres grandes grupos de biomarcadores:

- Pronósticos, que dan información relativa a la evolución final del paciente, ayudando a diferenciar entre casos con buen y mal pronóstico. Por ejemplo, se ha demostrado que pacientes con mayor número de células tumorales circulantes en sangre al diagnostico tienen una peor supervivencia global que aquellos con menos células tumorales circulantes [71].
- Predictivos, que ayudan a determinar la probabilidad de que un paciente se beneficie de un tratamiento en particular. Por ejemplo, sólo los pacientes con cáncer de mama HER2 positivo se benefician del tratamiento con el anticuerpo monoclonal anti-HER2 transtuzumab [72-73].
- Farmacodinámicos o farmacológicos, que miden los efectos de los fármacos en el tumor. Están dirigidos a ayudar en la toma de decisiones acerca del desarrollo de nuevos fármacos y a evaluar sus efectos en las dianas terapéuticas. Por ejemplo, el inhibidor de AKT MK2206 a la dosis recomendada para ensayos fase II inhibe efectivamente AKT en células tumorales y otros biomarcadores indirectos [74].

Para desarrollar un biomarcador se requieren cuatro fases [75]:

- Descubrimiento. En esta fase, aspectos de la biología tumoral se correlacionan con objetivos clínicos como por ejemplo supervivencia en el contexto de 'estudios de investigación'.
- Validación. En esta fase se evalúa si la técnica utilizada es adecuada para medir el objetivo requerido. Se determinan reproducibilidad, precisión y exactitud.
- Cualificación. Los biomarcadores se correlacionan con un objetivo clínico y se determinan sensibilidad y especificidad.
- Implementación en la clínica para guiar la toma de decisiones clínicas.

4.2 Relevancia de los biomarcadores

El uso de biomarcadores farmacodinámicos es esencial para el desarrollo clínico óptimo de las nuevas terapias moleculares antitumorales. Estas técnicas son cruciales para demostrar que la diana terapéutica es efectivamente inhibida, establecer la dosis y el régimen adecuado, maximizar

la intensidad y la duración de la inhibición de la diana terapéutica y para evaluar los efectos biológicos más abajo en las cascadas de señalización, incluyendo los efectos en proliferación y supervivencia celular [73].

Múltiples biomarcadores farmacodinámicos se están utilizando en los ensayos clínicos fase I y II en adultos y es preciso adaptar dichos biomarcadores para su uso en la población pediátrica. Asimismo, es necesario desarrollar mejores biomarcadores pronósticos y predictivos en niños para obtener el máximo beneficio de los tratamientos moleculares. Por ejemplo, seleccionar pacientes con neuroblastomas con mutaciones de ALK en los ensayos clínicos de inhibidores de ALK. Hasta la fecha, el uso de biomarcadores farmacodinámicos ha sido muy limitado en los ensayos clínicos pediátricos.

El objetivo del programa de desarrollo de nuevos fármacos del Royal Marsden Hospital – Institute of Cancer Research es integrar los biomarcadores en ensayos clínicos fase I/II para mejorar la estratificación de pacientes, incluir biomarcadores de respuesta/predictivos y para medir el efecto de los antitumorales en las dianas terapéuticas [77].

4.3 Tejidos para biomarcadores

Las muestras tumorales constituyen el mejor tejido para desarrollar nuevos biomarcadores. Sin embargo, cuando estas técnicas se trasladan al uso en humanos existen numerosas dificultades logísticas y éticas para obtener tejido tumoral, tanto porque es frecuentemente inaccesible como por la dificultad que representa la necesidad de obtener muestras tumorales seriadas. Esto es particularmente relevante en los canceres pediátricos dado que la mayoría de los tumores no son accesibles y se requerirían complejos procedimientos bajo anestesia general.

En nuestra institución (ICR) se han desarrollado un número de biomarcadores indirectos que utilizan otros tejidos distintos del tumoral como células mononucleares de sangre periférica (PBMC), folículos pilosos o biopsias cutáneas que están siendo utilizados en múltiples ensayos clínicos.

Dadas las características especificas del neuroblastoma, especialmente después de la primera recaída es preciso explorar otras fuentes de tejido para el desarrollo de biomarcadores. El tumor primario suele situarse en el área suprarrenal o la cavidad retroperitoneal y es, por tanto, de difícil acceso. Las localizaciones más frecuentes de metástasis son el esqueleto, la medula ósea y el hígado y con menos frecuencia en los pulmones o el sistema nervioso central (CNS) [2].

4.4 Marcadores indirectos

El grupo de desarrollo de biomarcadores del ICR (Clinical PD Biomarker Group, Division of Cancer Therapeutics, Team Leader Dr. Michelle Garrett) ha demostrado cómo en muestras de células

mononucleares de sangre periférica (PBMC) o plasma enriquecido en plaquetas (PRP) se demuestra un incremento en la fosforilación de AKT relacionada con la inhibición farmacológica de AKT del inhibidor MK2206 [74]. Diversos biomarcadores farmacodinámicos de la cascada de señalización de PI3K/AKT/mTOR se han utilizado en diversos ensayos clínicos de fase I [82].

Específicamente, el grupo del ICR ha desarrollado un biomarcador farmacodinámico de la cascada de señalización PI3K/AKT/mTOR utilizando la tecnología Meso Scale Discovery (MSD ®) en plasma enriquecido con plaquetas que cumple con la normativa de buena práctica de laboratorio clínico (GCLP) y que se utiliza en diversos ensayos clínicos en adultos en el Royal Marsden Hospital [83]. Hasta la fecha, ninguno de estos biomarcadores se ha utilizado en la población pediátrica y su validez no ha sido demostrada..

4.5 Muestras de medula ósea para desarrollar biomarcadores farmacodinámicos.

Dado que el 80% de los pacientes con neuroblastoma de alto riesgo sufren metástasis en medula ósea [5, 86] y que muestras de medula ósea se obtienen rutinariamente como parte del proceso de estadiaje y estudio de la respuesta al tratamiento convencional, parece plausible utilizar dichas muestras como fuente de tejido tumoral para el desarrollo de biomarcadores. El grado de afectación de la medula ósea varía desde lo microscópico detectable solo con las técnicas más sensibles como citometría de flujo hasta altos porcentajes (superiores al 90%) en muchos casos en situación de recaída.

Diversas metodologías como FISH (*Fluorescent In Situ Hybridisation*), RT-PCR (*Real Time Polimerase Chain Reaction*) o análisis de fosforilación de proteínas de diversas cascadas de señalización como PI3K/AKT/mTOR, IGF-1R, EGFR, PDGFR o Aurora kinasas entre otras requieren muestras tumorales de alta pureza. Por tanto, es preciso desarrollar un procedimiento para aislar y purificar células de neuroblastoma obtenidas de aspirados de medula ósea en niños afectos de neuroblastoma metastásico.

4.6 Biomarcadores farmacodinámicos en ensayos clínicos pediátricos

El número de ensayos clínicos pediátricos fase I/II que han incluido biomarcadores farmacodinámicos es muy limitado. El primer estudio utilizando el inhibidor de PDGFR (receptor del factor de crecimiento derivado de las plaquetas) imatinib en niños con tumores sólidos utilizo la expresión de la diana como medio de selección de pacientes (biomarcador predictivo), y solo fueron incluidos niños con alta expresión de PDGFR o c-Kit mediante inmunohistoquímica. No se encontró ninguna correlación con la respuesta al tratamiento [89].

Varios ensayos de fármacos frente a EGFR (receptor del factor de crecimiento epidérmico) han analizado la expresión de la diana terapéutica en tejido tumoral o han realizado estudios

mutacionales. Un ensayo fase I pediátrico de erlotinib no encontró correlación entre biomarcadores de activación de la cascada de PI3K/AKT o mutaciones de *PTEN* y *PIK3CA* y las respuestas al tratamiento antitumoral [90]. Análogamente, otro ensayo de combinación de erlotinib con temozolomida en niños con tumores sólidos refractarios o el ensayo clínico de cetuximab con irinotecan se encontró activación de las vías de señalización pero no una correlación con la respuesta clínica [91]. Dichos estudios intentaron identificar a los pacientes que más se beneficiarían de la inhibición de EGFR basándose en la presencia de mutaciones o activación de la vía de señalización en muestras recogidas al diagnóstico retrospectivamente. Estos estudios no demostraron resultados clínicos suficientemente prometedores para continuar el desarrollo de dichas terapias y la falta de biomarcadores farmacodinámicos nos impide profundizar en las razones de este fallo terapéutico.

Dos estudios utilizando bevacizumab, un anticuerpo monoclonal frente a VEGF (Factor de crecimiento del endotelio vascular) en niños han incorporado de forma prospectiva biomarcadores farmacodinámicos indirectos en muestras de sangre periférica y con nuevas modalidades de imagen funcional. En el primer ensayo clínico fase I en niños, biomarcadores circulantes incluyendo células circulantes endoteliales (CEC) y células progenitoras endoteliales circulantes (CEP) y otros factores solubles como VEGF o b-FGF. El análisis de dichos biomarcadores de angiogénesis mostro una variabilidad inter-paciente significativa y no se observe correlación con el beneficio clínico [93]. Un segundo ensayo estudiando la combinación de bevacizumab con Irinotecan en niños con gliomas de alto grado o gliomas difusos de tronco demostró inhibición de la cascada de señalización de VEGF tras el tratamiento con bevacizumab: el análisis de la fosforilación de VEGFR en células mononucleares de sangre periférica mostro una reducción de la fosforilación e inhibición terapéutica en un grupo de pacientes. A pesar de demostrar inhibición farmacológica de la diana, el ensayo no encontró ningún beneficio clínico en los pacientes [94].

En el caso de vorinostat, un inhibidor de deacetilasas de histona (HDAC) el uso de biomarcadores farmacodinámicos ha servido para demostrar inhibición de la diana terapéutica y establecer la dosis del fármaco para su desarrollo clínico. En el estudio fase I pediátrico de vorinostat [95] se detecto acumulación de acetil-H3-histonas en células mononucleares de sangre periférica en los pacientes recibiendo las dosis más altas. En los pacientes que recibieron el nivel más elevado la acumulación de acetil-H3 persistió más de 24 horas en tanto que en los que recibieron niveles intermedios se observaron efectos transitorios (6 horas). En los niveles más bajos, no se demostró acumulación de acetil-H3.

4.7 Desarrollo de biomarcadores farmacodinámicos de la vía de señalización PI3K en neuroblastoma

Se prevé que los fármacos dirigidos contra la vía de señalización PI3K/AKT/mTOR serán especialmente beneficiosos en los niños con neuroblastoma *MYCN* amplificado, aunque esta hipótesis debe ser testada en la práctica clínica.

En este estudio se llevará a cabo una modificación de la tecnología MSD® para medir la inhibición de la diana PI3K en células de neuroblastoma aisladas de aspirados de médula ósea. Esta técnica será desarrollada antes de su implementación en próximos ensayos clínicos de inhibidores de PI3K/AKT/mTOR. La tecnología será asimismo validada en muestras clínicas de sangre para medir la inhibición de PI3K en plasma enriquecido con plaquetas.

Asimismo, se desarrollará un ELISA basado en Europio para medir los niveles de oncoproteína MYCN total en células de neuroblastoma aisladas de aspirados de médula ósea antes de su implementación en próximos ensayos clínicos de inhibidores de PI3K/AKT/mTOR o de Aurora Kinasas.

4.8 Estándares GCP y GCLP

Los estándares de Buena Práctica Clínica (GCP) son un conjunto de regulaciones y recomendaciones éticas y científicas que se deben seguir para el diseño, conducta, recogida de datos y publicación de ensayos clínicos que incluyen la participación de seres humanos [96].

El cumplimiento de estos estándares asegura que los derechos, la seguridad y el bienestar de los participantes del ensayo clínico están protegidos, y además, que los resultados de los ensayos clínicos son fiables y precisos.

Los principios de Buena Práctica Clínica se establecen en las Directivas Europeas de Ensayos Clínicos (2001) y Buena Práctica Clínica (2005) del Parlamento Europeo.

Los principios de Buena Práctica de Laboratorio Clínico (GCLP) representan el punto de unión entre los sistemas de Buena Práctica Clínica y de Buena Práctica de Laboratorio. Proveen un marco regulador en cuanto a instalaciones, sistemas y procedimientos para asegurar que los análisis de laboratorio realizados en el curso de ensayos clínicos satisfacen el nivel de Buena Práctica Clínica en cuanto a fiabilidad, calidad e integridad del trabajo de laboratorio [100].

Se aplica a todas las muestras manejadas durante la realización de un ensayo clínico e incluye: organización y personal, instalaciones, equipamiento, materiales, reactivos, procedimientos normalizados estándares (SOP), control de calidad, auditoria de calidad y almacenaje y protección de datos.

Estos principios fueron establecidos por la Asociación Británica de Control de Calidad en Investigación (BARQA) para asegurar el cumplimiento de GCP y GLP en ensayos clínicos desarrollados en el Reino Unido [100].

El Clinical PD Biomarker Group del Institute of Cancer Research es un laboratorio específicamente diseñado para cumplir con las regulaciones de GCP y GCLP con el objetivo de garantizar la máxima calidad en los análisis de biomarcadores farmacocinéticos y farmacodinámicos de los ensayos fase I y II de terapias moleculares que se realizan en el Royal Marsden Hospital / ICR.

Hypotheses and objectives

1. Aims

The aims of this study are

- To adapt pharmacodynamic biomarkers of the PI3K signalling pathway
- To develop the methodology to isolate tumour cells from the bone marrow of children with neuroblastoma
- To develop a pharmacodynamic biomarker using MYCN oncoprotein, a downstream key effector of the PI3K signalling pathway to detect target inhibition in children with neuroblastoma

These assays will be of major value in future studies of agents targeting the PI3K/AKT/mTOR pathway and MYCN in neuroblastoma and other MYCN-driven malignancies. These assays will be first implemented in a paediatric phase I trial of the PI3K inhibitor GDC-0980 and a paediatric phase I trial of the PI3K/mTOR inhibitor BEZ235 due to open at The Royal Marsden Hospital and other Children's Cancer and Leukaemia Group (CCLG) phase I centres during 2013.

2. Hypotheses

- Neuroblastoma tumour cells isolated from bone marrow of patients can provide a valuable source of tumour material for pharmacodynamic biomarker assays.
- Changes in phospho- and total protein signals for the PI3K pathway targets AKT, GSK3β and p70S6K in children can be detected in neuroblastoma cells in the bone marrow and in PRP (as a surrogate marker), and can be used as proof of targeted pathway inhibition following administration of PI3K inhibitors.
- Reduction of the PI3K target oncoprotein MYCN in children can be detected using an Europium based technology and can be used as an indicator of downstream PI3K target modulation following administration of PI3K inhibitors.

These hypotheses translate into the following objectives:

3. Objectives

1. Development and standardisation of a procedure for measurement of total and phospho protein signals of the PI3K pathway in children in platelet-rich plasma as a surrogate tissue
2. Development and standardisation of an immunomagnetic separation procedure for isolation of neuroblastoma tumour cells from bone marrow samples
3. Development and standardisation of a procedure for measurement of protein levels of downstream effector MYCN with an Europium-based ELISA in neuroblastoma tumour cells from bone marrow samples

4. Development and standardisation of a procedure for measurement of total and phospho protein signals of the PI3K pathway in children with neuroblastoma in tumour cells from bone marrow samples

Material and methods

1. Reagents

Buffers
PhosSTOP tablets for dilution in 10 ml (Roche Diagnostics,GmbH, Mannheim, Germany)
CST lysis buffer (Cell Signalling Technology, Danvers, USA)
Flow cytometry buffer: MACS BSA and auto-MACS Rinsing Solution (Miltenyi Biotec GmbH, Gladbach, Germany)
Lysis buffer: 100% Cooking buffer (containing hydrochloric acid, sodium orthovanadate, Trizma base, sodium dodecyl sulfate (SDS) and water, The Institute of Cancer Research)
BCA reagent A (Pierce Biotechnology, Rockford, USA)
BCA reagent B (Pierce Biotechnology, Rockford, USA)
Albumin standard (Pierce Biotechnology, Rockford, USA)
Bovine serum albumin BSA (Sigma, Saint Louis, USA)
Delfia ® assay buffer (Perkin Elmer, Turku, Finland)
Delfia ® enhancement solution (Perkin Elmer, Turku Finland)
Phosphate buffered saline (PBS) tablets (Dulbecco 'A' Oxoid, Basingstoke, UK)
20% Cooking buffer: dilute 1:10 10X lysis buffer (Cell Signalling Technology, Danvers, USA) in 100% Cooking buffer (see lysis procedure)
0.1% Tween 20 solution. Dissolve 1mL Tween 20 (Sigma, Saint Louis, USA) into 1 L of water and mix
MSD Blocker A, dry powder (Meso-Scale, Gaithersburg, USA)
MSD Read Buffer T with surfactant (4x) (Meso-Scale, Gaithersburg, USA)
MSD Tris Wash Buffer (10x) (Meso-Scale, Gaithersburg, USA)
MSD Tris Lysis Buffer (1x) (Meso-Scale, Gaithersburg, USA)
MSD Phosphatase inhibitor I (100x) (Meso-Scale, Gaithersburg, USA)
MSD Phosphatase inhibitor II (100x) (Meso-Scale, Gaithersburg, USA)
MSD Protease inhibitor solution (50×) (Meso-Scale, Gaithersburg, USA)
MSD Blocker D-M (Meso-Scale, Gaithersburg, USA)
MSD Blocker D-R (Meso-Scale, Gaithersburg, USA)
Akt1/PKBα active (Upstate) (Meso-Scale, Gaithersburg, USA)
Akt1/PKBα unactive (Upstate) (Meso-Scale, Gaithersburg, USA)
Antibodies and MYCN standards
Anti-human Disganglioside GD2, Mouse IgG2a, Clone 14.G2a (BD Pharmingen, San Jose, USA)
APC-H7 Mouse anti-Human CD45 (BD Pharmingen, San Jose, USA)
Goat polyclonal secondary antibody to Mouse IgG2a – heavy chain FITC (Sigma Saint Louis, USA)
Eu-N1 goat anti-rabbit IgG (Perkin Elmer, Turku Finland)
Anti-Mycn rabbit C-19 policlonal antibody SC-791 (Santa Cruz Biotechnology, Santa Cruz, USA)
Anti-Mycn monoclonal antibody B8.4.B (Santa Cruz Biotechnology, Santa Cruz, USA)
Anti-Mycn monoclonal antibody OP13 (Calbiochem, Merck KGaA, Darmstadt, Germany)
MSD SULFO-TAG anti total Akt Signalling antibodies (50x) (Meso-Scale, Gaithersburg, USA)
MYCN peptide (Abcam, Cambridge, UK)
MYCN lysate (OriGene, Rockville, USA)
MYCN recombinant protein (Abnova, Taipei City, Taiwan)
Immunomagnetic beads
MACS Goat Anti-Mouse IgG2a+b MicroBeads (Miltenyi Biotec GmbH, Gladbach, Germany)
FcR Blocking Reagent (Miltenyi Biotec GmbH, Gladbach, Germany)
CD45 MicroBeads (Miltenyi Biotec GmbH, Gladbach, Germany)
Plates
NUNC 96-well plate (Thermo Scientific, Rochester, USA)
MSD MULTI-SPOTR 96-well Plate with phospho Akt Signalling antibodies attached (Meso-Scale, Gaithersburg, USA)
MSD MULTI-SPOTR 96-well Plate with total Akt Signalling antibodies attached (Meso-Scale, Gaithersburg, USA)
Immulon2HB 96 well polystyrene microtitre plates (Thermo Electron Corp, Thermo Scientific, Rochester, USA)
Other materials
Distilled sterile water, (The Institute of Cancer Research)
Marvel dried skimmed milk, (Premier Brands, UK)

Lymphoprep (Axis-Shield, Oslo, Norway)
MACS BSA (bovine serum albumin) stock solution (Miltenyi Biotec GmbH, Gladbach, Germany)
auto-MACS Rinsing Solution (Miltenyi Biotec GmbH, Gladbach, Germany)
Tubes and columns
BD Vacutainer CPT Tubes (Beckton and Dickinson, Franklin Lanes, USA)
Falcon tubes (The Institute of Cancer Research)
1 mL and 500 μl Eppendorf tubes (The Institute of Cancer Research)
Pasteur pipettes (The Institute of Cancer Research)
MS Columns (Miltenyi Biotec GmbH, Gladbach, Germany)
CellTrics 100 μM filters (Partec GmbH, Görlitz, Germany)
Oncoquick ® (Greiner bio-one, Frickenhausen, Germany)

Table 1, reagents

2. Equipment

Centrifuge, Eppendorf 5417R (Hamburg, Germany)
MiniMACS Separation Unit (Miltenyi Biotec GmbH, Gladbach, Germany)
MACS MultiStand (Miltenyi Biotec GmbH, Gladbach, Germany)
Flow cytometer, BD LSR II flow cytometer, (Beckton and Dickinson, Franklin Lanes, USA)
Heat block (Barloworld Scientific, Stone, UK)
Heidolph Titramax 101 plate shaker (Heidolph Instruments GmbH, Schwabach, Germany)
Wallac EnVision 2103 Multilable Reader (Perkin Elmer, Shelter, USA)
Genlab 50 litre Incubator (Genlab, Widnes, UK)
Mettler Toledo AX5 analytical balance (Mettler-Toledo, Greifensee, Switzerland)
PerkinElmer EnVision 2103 Multilabel counter (Perkin Elmer, Shelter, USA)
MSD SECTOR Imager 6000 (Meso-Scale, Gaithersburg, USA)

Table 2, equipment

3. Cell lines and murine tissue

Kelly, SHEP, SHEP-WT and SHEP-DBL neuroblastoma tumour cell lines were obtained from the Neuroblastoma Drug Development Team at The Institute of Cancer Research (Ms. K. Barker). HeLa cells were used as a negative control for MYCN expression. (Table 3)

Cell line	Clinical Features and Subtype	***MYCN* gene copy number**	MYCN protein status	GD2 expression
Kelly	1 year old boy Neuroblastic subtype	*MYCN* amplified	Very high protein levels	High
SHEP*	4 year old female, thoracic neuroblastoma with bone marrow metastases Substrate-adherent subtype	*MYCN* diploid	No detectable protein expression	Low
SHEP-WT*	4 year old female, thoracic neuroblastoma with bone marrow metastases Substrate-adherent subtype	SHEP cells transfected with murine wild-type *NMYC*	High protein levels	Low
SHEP-DBL*	4 year old female, thoracic neuroblastoma with bone marrow metastases Substrate-adherent subtype	SHEP cells transfected with murine *NMYC* mutated at the S62 and T58 residues: *MYCN* double mutant,	very high protein levels	Low
HeLa	Adult female cervical cancer line	No *MYCN* amplification	No protein expression	Negative

Table 3, details of cell lines used in the study including origin, *MYCN* gene copy number, MYCN protein expression and surface expression of GD2. References [103-104]
* SHEP cell lines are derived from parental SK-N-SH cells

Neuroblastoma frozen tissue was obtained from *TH-MYCN* transgenic mice [20, 24] to be used as positive control for GD2 surface expression. Tumour tissue was obtained surplus to pre-clinical trials carried out by The ICR Neuroblastoma Drug Development team (Ms. Karen Barker). Animal welfare guidance and regulation was followed [105].

4. Patients

Ethical and institutional approval was obtained from the RMH/ICR Committee for Clinical Research (CCR) and Research Ethics Committee (REC), protocol numbers CCR number 3358, REC number 37167/84857/1/333. The study was sponsored by The Royal Marsden Hospital Clinical Research & Development. Patients, parents or legal guardians were given verbal and written information about the study and were asked to participate. Patients (when aged ≥16 years), parents or legal guardians provided written informed consent. See Appendix 1 for Letters of approval of the study and Appendix 2 for Patient and Parent Information Sheets and Informed Consent documents.

The study opened for recruitment in January 2010. Samples were collected from January 2010 until May 2011. Patients were included when aged 1 to 17.99 years, receiving treatment for a solid malignancy at the Children and Young People's Unit of The Royal Marsden NHS Foundation Trust and had a central venous line (to avoid venipunctures). Weight had to be ≥10 kg, Hemoglobin ≥9 g/dl, Platelets ≥100x10^9/L and the patient had to be on a stable condition where blood sampling would not cause additional risks.

One blood sample was taken from fourteen patients. Bone marrow aspirate samples were taken from five patients with metastatic neuroblastoma at different points in treatment. Table 4 summarizes patient demographics of study CCR3358. Blood and bone marrow sample volumes were in accordance with European Medicines Agency guidance for volume of samples for research studies conducted in children [106].

Age (years)	Diagnosis	Stage	Stage in Treatment	Last Chemotherapy	Interval from prior chemotherapy (days)
1.4	Low grade glioma	Localised	Chemo	Vinblastine	7
2.7	Neuroblastoma	Metastatic	Pre ASCT	Busulfan/ Melphalan	>30
2.7	Medulloblastoma	Metastatic	Chemo	Methotrexate	5
2.7	Neuroblastoma	Metastatic	Chemo	Vincristine/ Cyclophosphamide	5
3.7	Wilms	Localised	Chemo	Cyclophosphamide	21
3.9	Neuroblastoma	Metastatic	Post ASCT	Busulfan/ Melphalan	>30
4.1	Medulloblastoma	Localised	Craniospinal radiotherapy	None	>30
4.4	Neuroblastoma	Localised	Chemo	Vincristine/ Doxorubicin	19
4.7	Neuroblastoma	Metastatic	Chemo	Cisplatin/ Vincristine	2
10.3	Medulloblastoma	Localised	Chemo	Cisplatin/ Vincristine / Lomustine	21
12.0	Neuroblastoma	Metastatic	Chemo	Cyclophosphamide	28
13.3	Supratentorial PNET	Metastatic	Post ASCT	Thiotepa	25
15.4	Medulloblastoma	Localised relapse	Chemo	Cisplatin/ Vincristine / Lomustine	2
17.1	Rhabdomyosarcoma	Localised	Chemo	Ifosfamide/ Vincristine/ Actinomycin D	21

Table 4, characteristics of the 14 patients enrolled on the study CCR3358. Chemo – Receiving chemotherapy, ASCT: Autologous Stem Cell Transplant, PNET: Primitive neuroectodermic tumour.

5. Processing of blood samples

Three 2.7 ml tubes were collected in BD Vacutainer CPT ® tubes with sodium citrate and transported to the ICR Clinical PD Biomarker Group Laboratory at one timepoint.

The procedure followed was:

- Immediately after collection, samples were centrifuged at 200 RCF at room temperature for 15 min.
- After centrifugation, 600µl of plasma was extracted from each tube and pooled (Figure 2).
- From this pooled plasma, 350µl aliquots of plasma were pipetted into four separate Eppendorff tubes.
- 150µl of PhosSTOP solution was added.
- 150µl of CST Lysis buffer was added.

Each sample was mixed well and snap frozen at -80°C until processing.

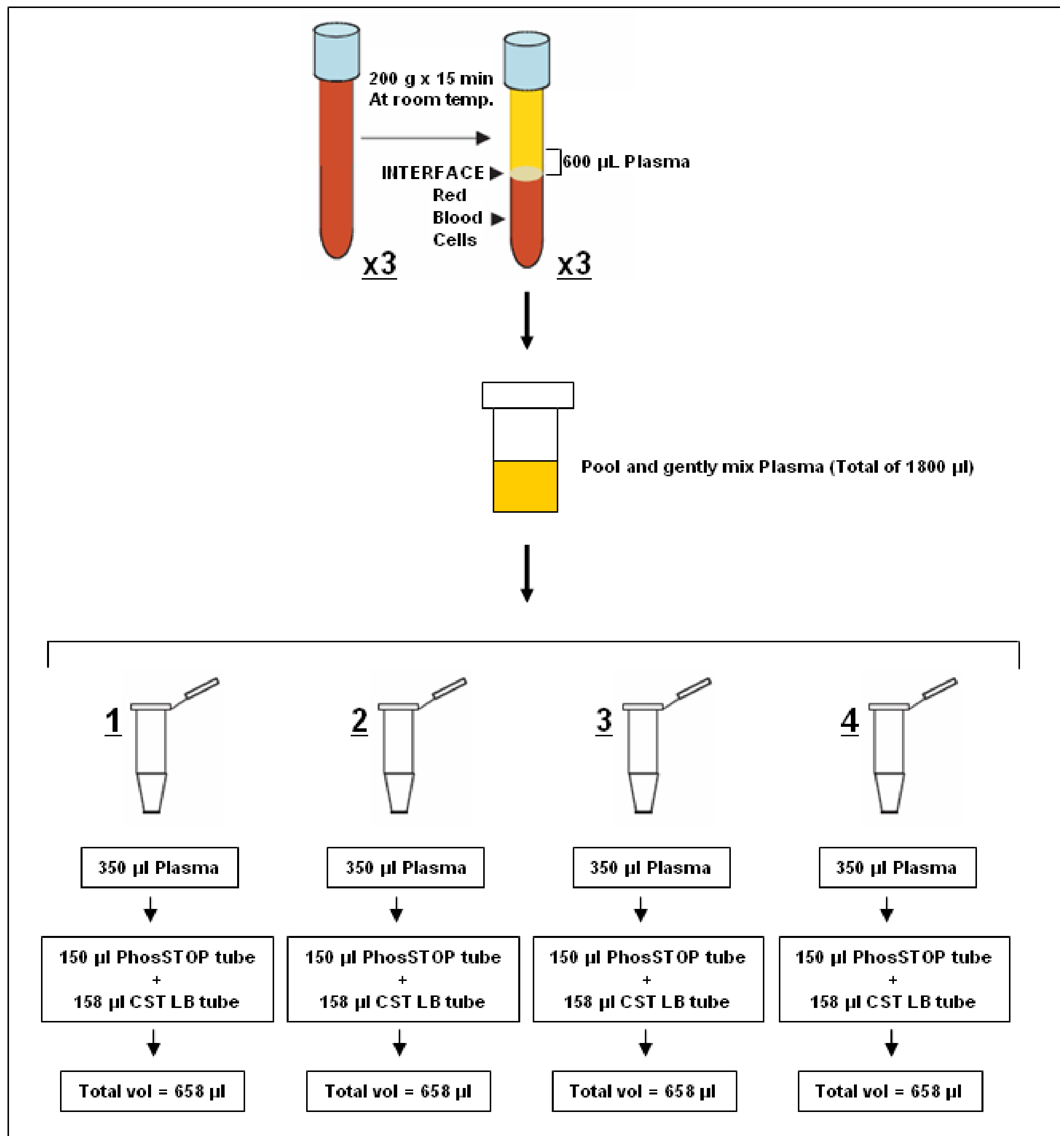


Figure 2, Scheme for peripheral blood sample collection

6. Processing of bone marrow samples

Bone marrow aspirate samples from five patients with confirmed/suspected bone marrow involvement by neuroblastoma undergoing routine clinical bone marrow assessments were obtained. This procedure did not prolong the general anaesthetic times and did not increase the risks to the patient. Two ml sample of bone marrow aspirate sample was collected from each side (right and left iliac crest) on each occasion. EDTA, Preservative-free heparin, Oncoquick and BD Ficoll tubes were used for collection. Results of each collection type were analysed and are shown in "Results". The majority of samples were collected in preservative-free heparin tubes.

After collection, samples were transported to the ICR Clinical PD Biomarker Group Laboratory and Ficoll separation was performed to separate the cellular fraction that includes haematopoietic progenitors and neuroblastoma cells from plasma, red blood cells or platelets.

Ficoll separation was performed:

a) Manually by layering 2ml of the sample to 5 ml of Lymphoprep ® in 15mL Falcon tubes.
b) In pre-prepared BD Ficoll tubes, where 2 to 5 ml of the sample were added directly to commercially available tube that contains a gel phase that separates the cellular fraction after centrifugation.

Tubes were then centrifuged at 400 RCF for 20 minutes at room temperature and the mononuclear cell layer was extracted with a Pasteur pipette and passed through a 100 μM filter.

The cell layer was re-suspended in 10 mL of flow cytometry buffer and centrifuged at 1800 RCF for 10 min.

The cell pellet was re-suspended in 1 mL of flow cytometry buffer.

Following this, procedure for immunomagnetic separation (below) was performed. Those samples were then stored at -80ºC to be used for measurement of the MYCN and PI3K/AKT pathway assays.

7. Immunomagnetic separation

This method uses the Magnetic-activated cell sorting (MACS) technology.

The technology uses MACS MicroBeads which are superparamagnetic particles of approximately 50 nm in diameter. They are composed of a biodegradable matrix which does not alter the function or structure of labelled cells. Different types of magnetic beads bind to specific cell types. The cells are then run through a column placed in a MACS Separator where a high-gradient magnetic field is induced. Labelled cells bound to the MicroBeads are retained in the column while unabelled cells pass through and can be collected. Labelled cells can also be collected after removal of the column from the magnet.

Two strategies can be used for cell separation: positive selection of desired cells or depletion of undesired cells.

In the positive selection method, the MicroBeads are bound to the cell population of interest. Then the cell suspension is run through the column and the labelled cells of interest are bound to the magnet and then collected once the magnet is removed. There are commercially available MicroBeads for a number of cell populations (i.e. for stem cells there are CD133 MicroBeads) although none of them are designed for neuroblastoma, and cannot specifically isolate a population of neuroblastoma cells. There are indirect MicroBeads bound to biotin, fluorochromes or immunoglobulins which are then bound to a secondary antibody specific for the cell of interest. The indirect separation requires additional incubation, wash and centrifugation steps.

In the cell depletion method, the MicroBeads bind to undesired cells. The cell suspension is then run through the column which retains the undesired labelled cells and the desired cells depleted from the unwanted cells are collected (i.e. to remove platelets from a cell suspension, CD61 MicroBeads would be used).

This technology is widely used for: 1) research purposes, such as isolation of circulating tumour cells [107] and 2) in the clinical setting for the separation of products of stem cell harvest for autologous or allogeneic stem cell transplant such as T-cell depletion [108]. Two different approaches were used: positive selection of GD2+ cells and negative depletion of CD45+ cells.

GD2 is a membrane ganglioside present in the surface of neuroblastoma cells. It is expressed in virtually all neuroblastomas [104] and only anecdotal reports have described the loss of GD2 expression in patients treated with anti-GD2 immunotherapy [103]. GD2 is not expressed by any haematopoietic cells [104]. CD45 is a pan-hematopoietic marker present in all haematopoietic cells. It is not expressed by neuroblastoma cells [109].

7.1 Positive selection of GD2-labelled fraction

With this procedure, MACS Anti-Mouse IgG2a+b immunomagnetic beads were added and bound to a monoclonal antibody against GD2 (BD Pharmingen). Samples were run through a magnetic column where cells attached to the MicroBeads (labelled fraction) were retained and non-labelled cells were washed out. Labelled cells were then eluted from the magnetic field and collected as a cell suspension. The MicroBeads are biodegradable and do not alter the biological properties and viability of the neuroblastoma cells. (Figure 3)

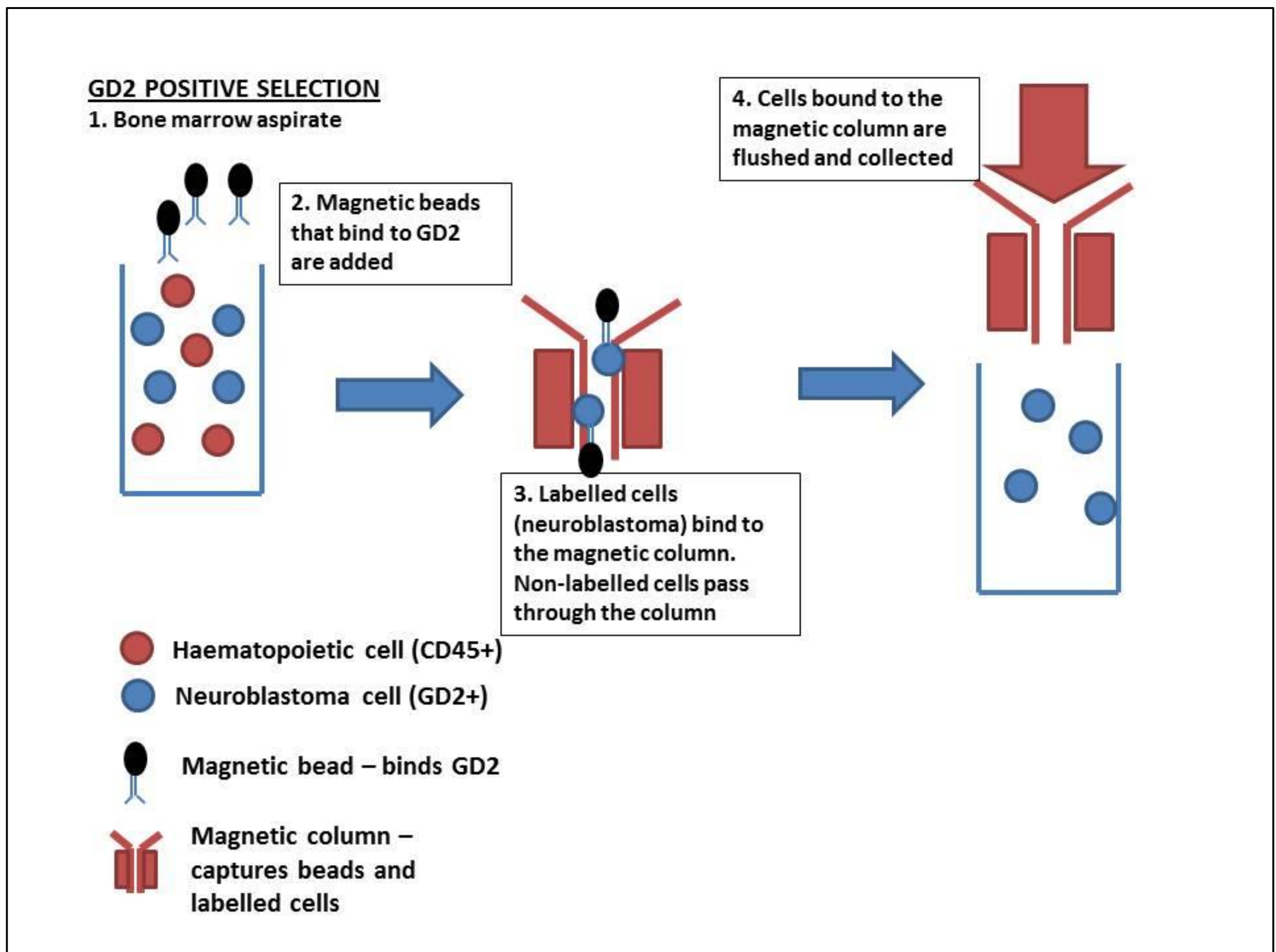


Figure 3, Schematic representation of the GD2 positive selection procedure: 1. the mononuclear cell layer containing haematopoietic nucleated cells and neuroblastoma cells is separated from plasma, platelets and red blood cells, 2. anti-mouse Ig magnetic beads bound to mouse anti-human anti-GD2 monoclonal antibody are added to the cell suspension and bind to neuroblastoma cells, 3. The cell suspension runs through the magnetic column and the labelled cells (GD2+ neuroblastoma cells) bind to the magnetic column. Unlabelled cells pass through and are discarded, 4. the magnet is removed and the labelled cells are collected.

The procedure followed was:

- Sample was centrifuged at 1000 RCF for 10 minutes at 4°C and the supernatant was discarded.
- Sample was then re-suspended in 1mL MACS buffer and 100 µL were separated for flow cytometry (see below procedure for flow cytometry)

- The remaining sample was centrifuged at 1000 RCF for 10 minutes at 4°C and the supernatant was discarded.
- The cell pellet was re-suspended in 60 µL of flow cytometry buffer and 20 µL of Fc blocking reagent and 1µL of anti-GD2 monoclonal antibody were added.
- Sample was incubated for 30 minutes at 4°C and centrifuged at 300 RCF for 10 minutes at 4°C.
- The supernatant was discarded, sample washed with 1 ml of flow cytometry buffer and centrifuged at 300 RCF for 10 minutes at 4°C.
- After discarding the supernatant, the pellet was suspended in 80 µL of flow cytometry buffer and 20 µL of goat anti-mouse IgG2a microbeads were added.
- Sample was incubated for 15 minutes at 4°C, then centrifuged at 300 RCF for 10 minutes at 4°C, supernatant was discarded and cell pellet re-suspended in 1 mL of flow cytometry buffer.
- Sample was then centrifuged at 300 RCF for 10 minutes at 4°C, supernatant discarded and cell pellet re-suspended in 500 µL of flow cytometry buffer.
- The sample was loaded onto a Miltenyi column that had been previously prepared by rinsing with 500 µL of flow cytometry buffer.
- The column was washed with 500 µL flow .cytometry buffer three times.
- The column was removed from the separator. 1 mL of flow cytomtery buffer was then pipetted onto the column and immediately flushed with the plunger.
- 100 µL were separated for flow cytometry analysis.
- The remaining sample was centrifuged at 14000 RCF at 4 ºC for 10 minutes, supernatant discarded and frozen to -80ºC for further analyses.

7.2 Indirect selection by depletion of CD45+ cells

With this procedure, immunomagnetic beads bound to anti-CD45 antibody (CD45 MicroBeads) are added to the sample which is run through the column. The labelled (undesired) cells are bound to the column while the desired cells are collected (CD45-). CD45 is universally present in haematopoietic cells and is not present in the surface of neuroblastoma cells [109]. Figure 4 represents the CD45 depletion procedure.

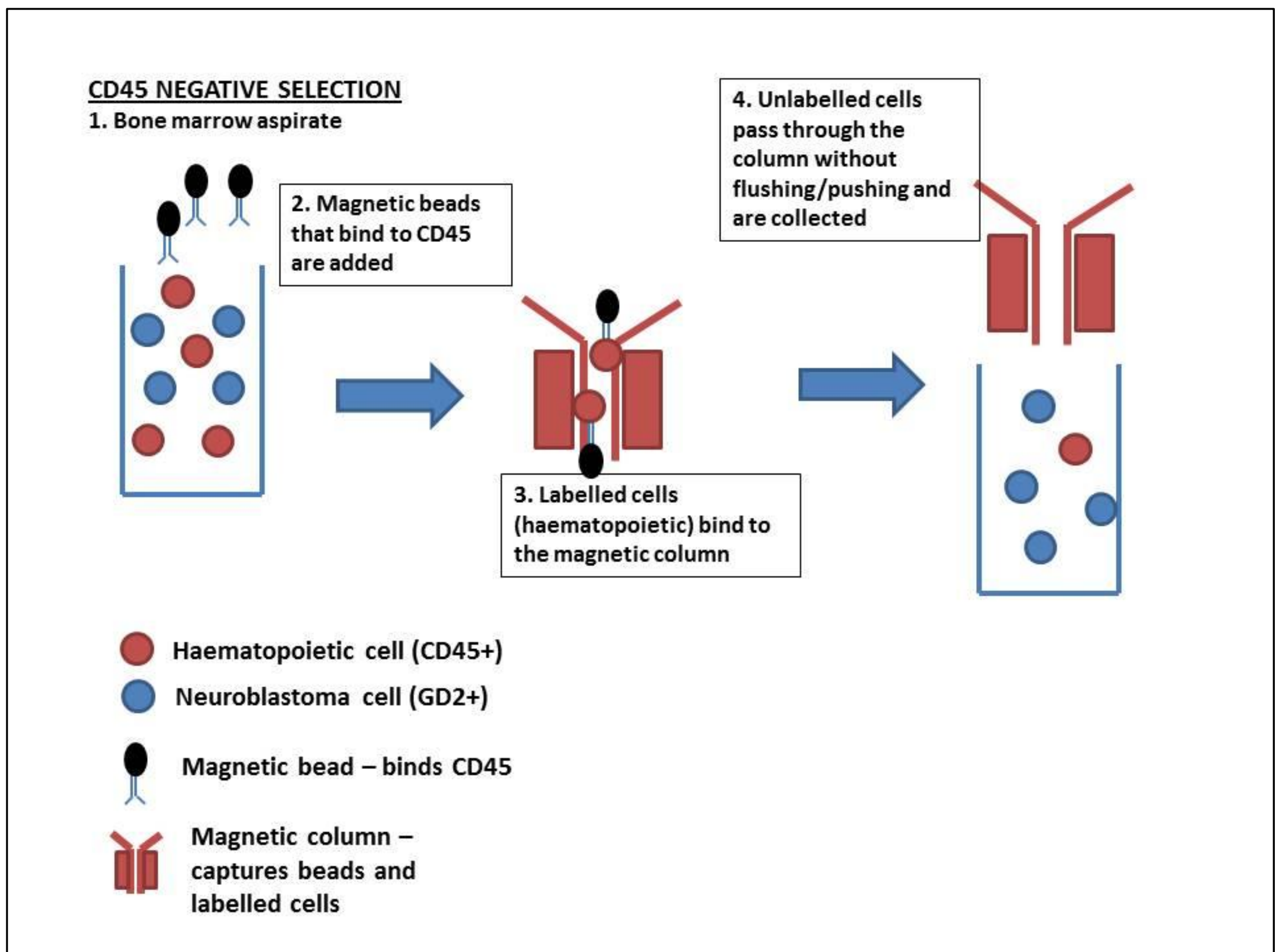


Figure 4, Schematic representation of the CD45 depletion procedure: 1. the mononuclear cell layer containing haematopoietic nucleated cells and neuroblastoma cells is separated from plasma, platelets and red blood cells, 2. CD45-labelled magnetic beads are added to the cell suspension and bind to haematopoietic cells, 3. the cell suspension runs through the magnetic column and the labelled cells (CD45+) bind to the magnetic column, 4. unlabelled cells (CD45-) pass through the column and are collected.

The procedure followed was:

- Sample was centrifuged at 1000 RCF for 10 minutes at 4°C and the supernatant was discarded.
- Sample was then re-suspended in 1mL MACS buffer and 100 µL were separated for flow cytometry (see below procedure for flow cytometry)
- The remaining sample was centrifuged at 1000 RCF for 10 minutes at 4°C and the supernatant was discarded.
- The cell pellet was re-suspended in 80 µL of flow cytometry buffer and 20 µL of CD45 MicroBeads were added.
- Sample was incubated for 15 minutes at 4°C and centrifuged at 300 RCF for 10 minutes at 4°C.
- The supernatant was discarded, sample washed with 1 ml of flow cytometry buffer and centrifuged at 300 RCF for 10 minutes at 4°C.

- Supernatant was then discarded and cell pellet re-suspended in 500 µL of flow cytometry buffer.
- The sample was loaded onto a Miltenyi column that had been previously prepared by rinsing with 500 µL of flow cytometry buffer.
- The unlabelled fraction through the column was collected.
- 100 µL were separated for flow cytometry analysis.
- The remaining sample was centrifuged at 14000 RCF at 4 ºC for 10 minutes, supernatant discarded and frozen to -80ºC for further analyses.

8. Calculation of the purity of neuroblastoma cell suspensions by flow cytometry

In order to establish the performance of each immunomagnetic separation methodology in providing cell suspensions enriched in neuroblastoma cells flow cytometry was used to measure the proportion of neuroblastoma cells out of the total mononuclear cell fraction.

Flow cytometry was performed in 100 μL samples taken before and after the immunomagnetic separation procedures. Flow cytometry was performed in the initial experiments spiking neuroblastoma cell lines in healthy volunteer blood and also in the bone marrow samples involved with neuroblastoma that were collected as part of this study.

Flow cytometry analyses were always performed within 24 hours of the sample collection or immunomagnetic separation.

Two-colour flow cytometry was used to differentiate neuroblastoma cells from haematopoietic cells. A primary monoclonal antibody against GD2 (BD Pharmingen) was incubated with a secondary antibody with FITC (Fluorescein isothiocyanate). A commercially available CD45 monoclonal antibody with APC-H7 (Allophycocyanin H7) was used to evaluate CD45 expression. Flow cytometry including both CD45 and GD2 has been previously described in the literature [110]. Although combinations of more colours have been reported to be more specific, for the purpose of this study, out of the clinical diagnostic and minimal residual disease settings, only these two antibodies were used [110].

Compensation controls were performed for every analysis for GD2-FITC and CD45-APC-H7. No unstained control was used. Titration experiments were performed for GD2-FITC. For CD45 manufacturer´s recommendations were followed. Neuroblastoma Kelly cell lines, and neuroblastoma cells suspended from murine neuroblastoma tumours were used as positive controls. HeLa cell lines were used as negative controls.

For the flow cytometry analysis, 100 μL samples were taken from the cell suspensions before and after the immunomagnetic separation. They were incubated for 20 minutes with 5 μL of anti-GD2 mouse anti-human monoclonal antibody and 5 μL of goat anti-mouse FITC at 4°C. Samples were then washed with flow cytometry buffer, centrifuged at 300 RCF at 4ºC for 5 minutes and supernatant discarded. The cell pellet was then resuspended in 100 μL of flow cytometry buffer and 3 μL of APC-H7 anti-CD45 and incubated for 20 minutes at 4°C. The cells were then washed with flow cytometry buffer, centrifuged at 300 RCF at 4ºC for 5 minutes and supernatant discarded. Cell pellets were then resuspended in 500 μL flow cytometry buffer and kept at 4ºC until taken to the flow cytometer.

Samples were analysed with the BD LSR II flow cytometer, (Beckton and Dickinson, USA) and at least 10,000 events were counted using the FACSDiva® software. Data was analysed with WinMDI® flow cytometry software (version 2.8). Neuroblastoma cells were positive for GD2 and

negative for CD45. Therefore, percentages of GD2+/CD45- and GD2-/CD45+ cells were calculated by using the following gating strategy.

On a forward scatter (FSC-A)/side scatter (SSC-A) dot plot, the mononuclear cells were identified by region R1. In all other dot plots, only the cells lying in R1 were represented. On a forward scatter amplitude (FSC-A)/height (FSC-H) dot plot, the Region R2 was determined aiming to exclude non cellular events. Region R3 defined the GD2-/CD45+ cells (haematopoietic) and region R4 defined the GD2+/CD45- cells (neuroblastoma). Figure 5 represents the gating strategy used in this study.

'**Purity'** was defined as the proportion of neuroblastoma events (R4 = GD2+/CD45-) out of the total gated cellular events (R3 + R4).

$$\boldsymbol{Purity} = \frac{\mathbf{R4\ events}}{\mathbf{R3 + R4\ events}} * \mathbf{100}$$

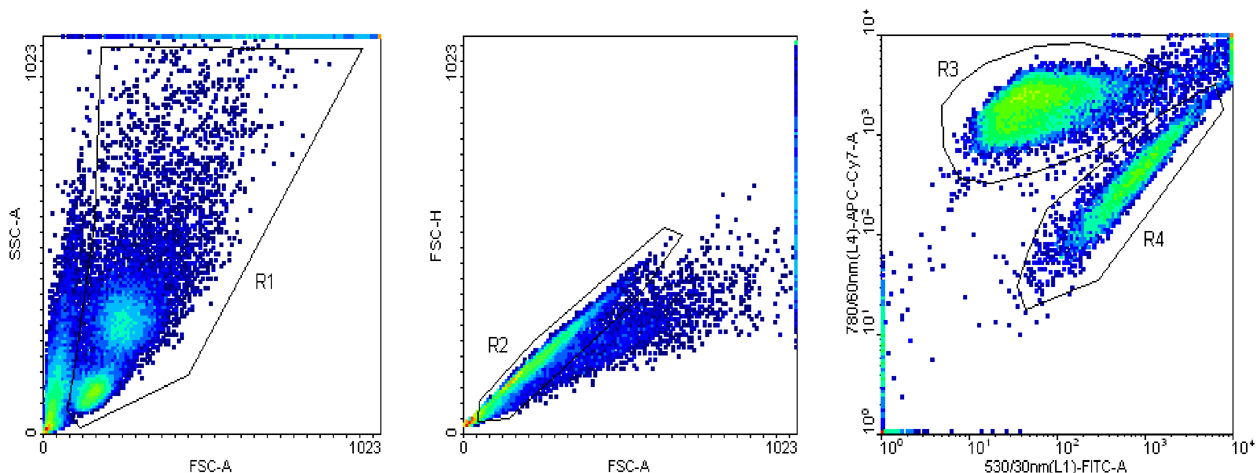


Figure 5, Gating strategy for flow cytometry. The mononuclear cells were gated as R1 on a FSC-A/SSC-A density plot. Then the R2 gate aimed to include only cellular events on a FSC-A/FSC-H density plot (centre). On a CD45-APC-H7 (Y axis) / GD2-FITC (X axis) plot the two populations were gated as R3 (GD2-/CD45+ corresponding to haematopoietic cells) and R4 (GD2+/CD45- corresponding to neuroblastoma cells).

Estimation of cell recovery with flow cytometry

'Recovery' was defined as the proportion of cells that can be identified after the immunomagnetic separation procedure out of the initial total cells (i.e. the cells that are not lost during the procedure). It was not possible to evaluate recovery with a cell counter and for this reason, an estimation of recovery was obtained from the flow cytometry data.

For this estimation, we acquired flow cytometry data during 60 seconds in the samples before and after the immunomagnetic separation. The total number of gated events (R3+R4) after immunomagnetic separation divided by the total number of gated events before the immunomagnetic separation was used to provide an estimation of the cell recovery and hence, cell loses.

$$\boldsymbol{Recovery} = \frac{\textbf{R3 + R4 events (after separation)}}{\textbf{R3 + R4 events (before separation)}} * \mathbf{100}$$

9. Cell lysis

Prior to performing any protein assay, samples were thawed and lysed. The lysis of PRP samples followed the manufacturer's instructions (MesoScale Discovery) for the triplex AKT assay and used MSD lysis buffer.

For the MYCN ELISA, pellets of cell lines and BM samples stored at -80ºC were thawed and lysed according to our institutional SOP for PBMC samples/cell suspensions. This lysis procedure had been optimised at our lab for several ELISA assays including the determination of HSP72 or 4EBP1 and was therefore deemed suitable for the MYCN ELISA. During the optimisation phase of this process, it was found that the best results were achieved with the in-house lysis buffer (data not shown, unpublished data, Simon Heaton and Michelle Garrett, Clinical PD Biomarker Group at ICR). Details of the composition of this buffer ("cooking buffer") are given in Table 1.

First, samples were defrosted on ice, 100 µL of lysis buffer (100% cooking buffer) were added to each sample and left at room temperature for 15 minutes. Then, samples were put on the heat block at 100ºC for 15 minutes, mixed well and centrifuged at 14000 RCF at 4ºC for 10 minutes. Finally, supernatant was collected, 4µL were spared for BCA analysis and the remaining sample was frozen at -80ºC.

10. BCA analysis

After samples were lysed, the bicinchoninic acid (BCA) protein assay was performed to assess the concentration of total protein present in the samples. This would allow: 1) to assess the exact amount of total protein obtained after the immunomagnetic separation procedures, 2) to evaluate the losses in total protein caused by the immunomagnetic separation and 3) to normalize the protein assays to equal protein concentrations in order to achieve comparable results.

The BCA assay is a sensitive assay for protein determination that takes advantage of the biuret reaction to form a coloured solution in the presence of protein that can subsequently be detected at 562nm in a plate reader. The biuret reaction occurs when a blue coloured complex is formed when a peptide containing three or more amino acid residues chelates copper ions in an alkaline environment. The complex of Cu^{1+} bound with four to six peptide bonds exhibits light absorbsion proportional to the number of bonds. The assay intensifies the signal 100 fold through the binding of BCA to the Cu^{1+} ion to produce a purple colour and a linear colorimetric absorbsion at 562nm.

To perform this assay, the BCA reagent was prepared at 50 parts (reagent A) with 1 part (reagent B). A 1:10 dilution of samples was then prepared (4 µL sample + 36 µL distilled water in a 500 µL Eppendorf tube). Then, a 1:100 dilution of Quality Control bovine serum albumin solution to a final concentration of 500mg/ml (2 µL QC stock + 198 µL distilled water in a 500 µL Eppendorf tube) was performed together with a 1:2 serial dilution (50µL of standard + 50µL distilled water) in 500 µL Eppendorf tubes from a 100 µL bovine serum albumin stock (50 mg/ml). For the blank wells, distilled water was used.

10 µL of standards, QCs and Blanks were added to a 96-well plate, as shown in Figure 6. For each lysed sample 10 µL were added to the first two wells, 5 µL to the next two and 2.5 µL to the final two for the 1/10, 1/20 and 1/40 dilutions. Then 200 µL of the prepared BCA reagents (A+B) was added and the plate was incubated at 37ºC for 30 minutes. The plates were read on the Wallac EnVision 2103 Multilable Reader following protocol for BCA analysis. Figure 7 shows an example of the data generated calculating the total amount of protein for each lysate.

Date:

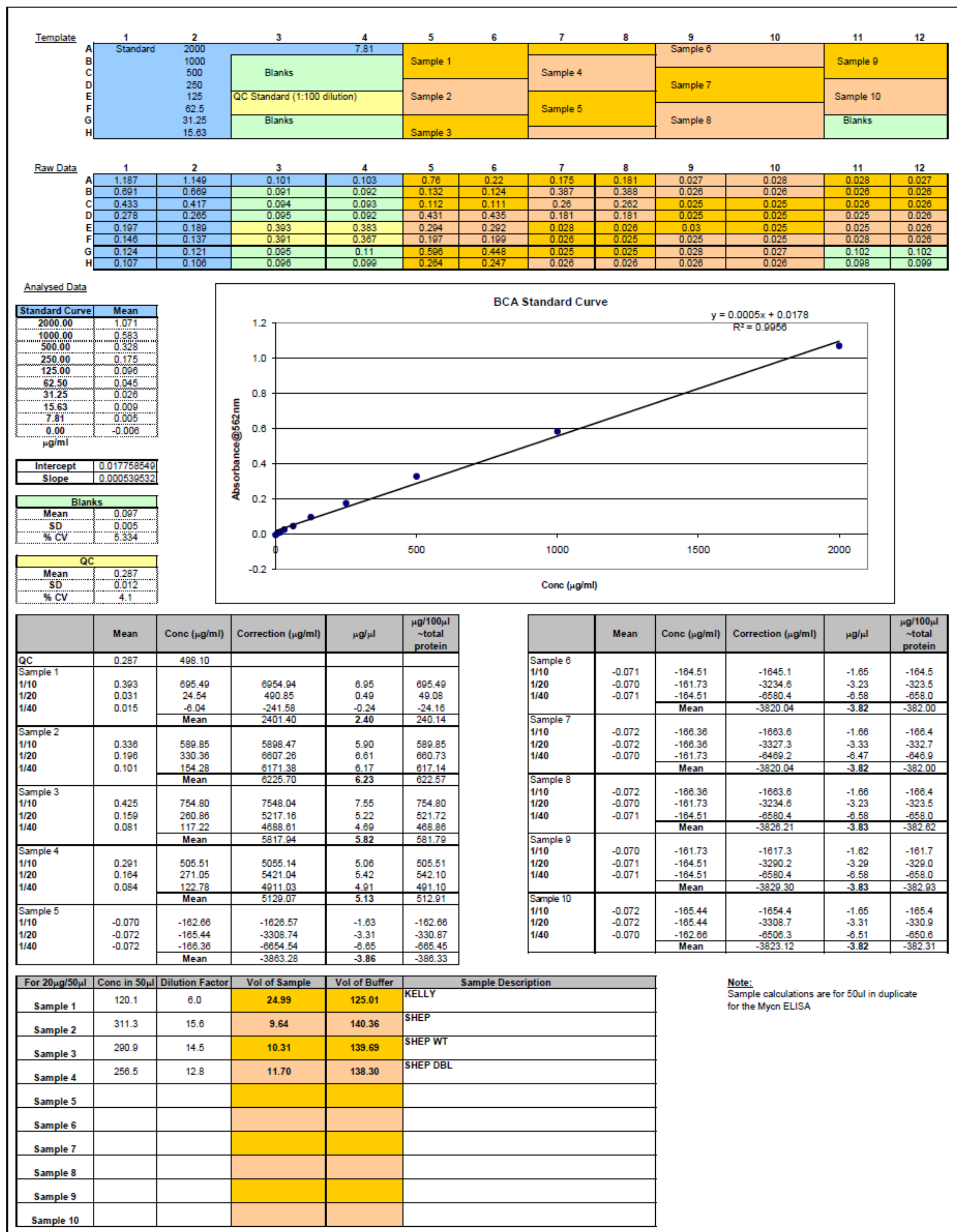
Assay:

Assay Details:

PlateID:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard Curve (µg/ml)	2000	7.8		Sample 1	1/10		1/40		1/20	Sample 9	1/10
B	1000		Blanks			1/20	Sample 4	1/10		1/40		1/20
C	500					1/40		1/20	Sample 7	1/10		1/40
D	250				Sample 2	1/10		1/40		1/20	Sample 10	1/10
E	125		QC's			1/20	Sample 5	1/10		1/40		1/20
F	62.5					1/40		1/20	Sample 8	1/10		1/40
G	31.3				Sample 3	1/10		1/40		1/20		
H	15.6					1/20	Sample 6	1/10		1/40		

Figure 6 shows the plate lay-out for BCA analysis. The blue wells correspond to the nine standard albumin solution dilutions, the yellow wells to the Quality Control (QC) solution, the green wells to the blank (distilled water) wells and orange and purple to the different analysed samples (up to 10).



Template

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard	2000		7.81					Sample 6			
B		1000			Sample 1						Sample 9	
C		500	Blanks				Sample 4					
D		250							Sample 7			
E		125	QC Standard (1:100 dilution)		Sample 2						Sample 10	
F		62.5					Sample 5					
G		31.25	Blanks						Sample 8		Blanks	
H		15.63			Sample 3							

Raw Data

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.187	1.149	0.101	0.103	0.76	0.22	0.175	0.181	0.027	0.028	0.028	0.027
B	0.691	0.669	0.091	0.092	0.132	0.124	0.387	0.388	0.026	0.026	0.026	0.026
C	0.433	0.417	0.094	0.093	0.112	0.111	0.26	0.262	0.025	0.025	0.026	0.026
D	0.278	0.265	0.095	0.092	0.431	0.435	0.181	0.181	0.025	0.025	0.025	0.026
E	0.197	0.189	0.393	0.383	0.294	0.292	0.028	0.026	0.03	0.025	0.025	0.026
F	0.146	0.137	0.391	0.367	0.197	0.199	0.026	0.025	0.025	0.025	0.028	0.026
G	0.124	0.121	0.095	0.11	0.596	0.448	0.025	0.025	0.028	0.027	0.102	0.102
H	0.107	0.106	0.096	0.099	0.264	0.247	0.026	0.026	0.026	0.026	0.098	0.099

Analysed Data

Standard Curve	Mean
2000.00	1.071
1000.00	0.583
500.00	0.328
250.00	0.175
125.00	0.096
62.50	0.045
31.25	0.026
15.63	0.009
7.81	0.005
0.00	-0.006
µg/ml	

Intercept	0.017758549
Slope	0.000539532

Blanks	
Mean	0.097
SD	0.005
% CV	5.334

QC	
Mean	0.287
SD	0.012
% CV	4.1

	Mean	Conc (µg/ml)	Correction (µg/ml)	µg/µl	µg/100µl ~total protein
QC	0.287	498.10			
Sample 1					
1/10	0.393	695.49	6954.94	6.95	695.49
1/20	0.031	24.54	490.85	0.49	49.08
1/40	0.015	-6.04	-241.58	-0.24	-24.16
		Mean	2401.40	2.40	240.14
Sample 2					
1/10	0.336	589.85	5898.47	5.90	589.85
1/20	0.196	330.36	6607.26	6.61	660.73
1/40	0.101	154.28	6171.38	6.17	617.14
		Mean	6225.70	6.23	622.57
Sample 3					
1/10	0.425	754.80	7548.04	7.55	754.80
1/20	0.159	260.86	5217.16	5.22	521.72
1/40	0.081	117.22	4688.61	4.69	468.86
		Mean	5817.94	5.82	581.79
Sample 4					
1/10	0.291	505.51	5055.14	5.06	505.51
1/20	0.164	271.05	5421.04	5.42	542.10
1/40	0.084	122.78	4911.03	4.91	491.10
		Mean	5129.07	5.13	512.91
Sample 5					
1/10	-0.070	-162.66	-1626.57	-1.63	-162.66
1/20	-0.072	-165.44	-3308.74	-3.31	-330.87
1/40	-0.072	-166.36	-6654.54	-6.65	-665.45
		Mean	-3863.28	-3.86	-386.33

	Mean	Conc (µg/ml)	Correction (µg/ml)	µg/µl	µg/100µl ~total protein
Sample 6					
1/10	-0.071	-164.51	-1645.1	-1.65	-164.5
1/20	-0.070	-161.73	-3234.6	-3.23	-323.5
1/40	-0.071	-164.51	-6580.4	-6.58	-658.0
		Mean	-3820.04	-3.82	-382.00
Sample 7					
1/10	-0.072	-166.36	-1663.6	-1.66	-166.4
1/20	-0.072	-166.36	-3327.3	-3.33	-332.7
1/40	-0.070	-161.73	-6469.2	-6.47	-646.9
		Mean	-3820.04	-3.82	-382.00
Sample 8					
1/10	-0.072	-166.36	-1663.6	-1.66	-166.4
1/20	-0.070	-161.73	-3234.6	-3.23	-323.5
1/40	-0.071	-164.51	-6580.4	-6.58	-658.0
		Mean	-3826.21	-3.83	-382.62
Sample 9					
1/10	-0.070	-161.73	-1617.3	-1.62	-161.7
1/20	-0.071	-164.51	-3290.2	-3.29	-329.0
1/40	-0.071	-164.51	-6580.4	-6.58	-658.0
		Mean	-3829.30	-3.83	-382.93
Sample 10					
1/10	-0.072	-165.44	-1654.4	-1.65	-165.4
1/20	-0.072	-165.44	-3308.7	-3.31	-330.9
1/40	-0.070	-162.66	-6506.3	-6.51	-650.6
		Mean	-3823.12	-3.82	-382.31

For 20µg/50µl	Conc in 50µl	Dilution Factor	Vol of Sample	Vol of Buffer	Sample Description
Sample 1	120.1	6.0	24.99	125.01	KELLY
Sample 2	311.3	15.6	9.64	140.36	SHEP
Sample 3	290.9	14.5	10.31	139.69	SHEP WT
Sample 4	256.5	12.8	11.70	138.30	SHEP DBL
Sample 5					
Sample 6					
Sample 7					
Sample 8					
Sample 9					
Sample 10					

Note:
Sample calculations are for 50ul in duplicate for the Mycn ELISA

Figure 7 shows the example of the excel worksheet generated after BCA analyses of four samples of lysates of Kelly, SHEP, SHEP WT and SHEP DBL cell lines. A standard curve is performed with the serial albumin dilutions and R^2 is calculated. Also, the coefficient of variation is calculated for the Quality Controls (QC). The concentration in micrograms per 50 µl of lysate is plotted in the table at the bottom and a calculation of the volume of sample required for protein analysis normalized to 20 µg per 50 µl well of total protein.

11. MYCN ELISA

This Enzyme Linked Immunoabsorption Assay (ELISA) uses Delfia technology. Sample, standard and quality controls are coated directly onto the plate. A primary antibody to the MYCN protein is then bound to the captured MYCN protein. Europium labelled goat anti-rabbit IgG is then bound to complete the sandwich. Excess label is washed off and an enhancement solution releases the captured europium. The free europium is highly fluorescent in the 615nm range and is the assay output. (Figure 8)

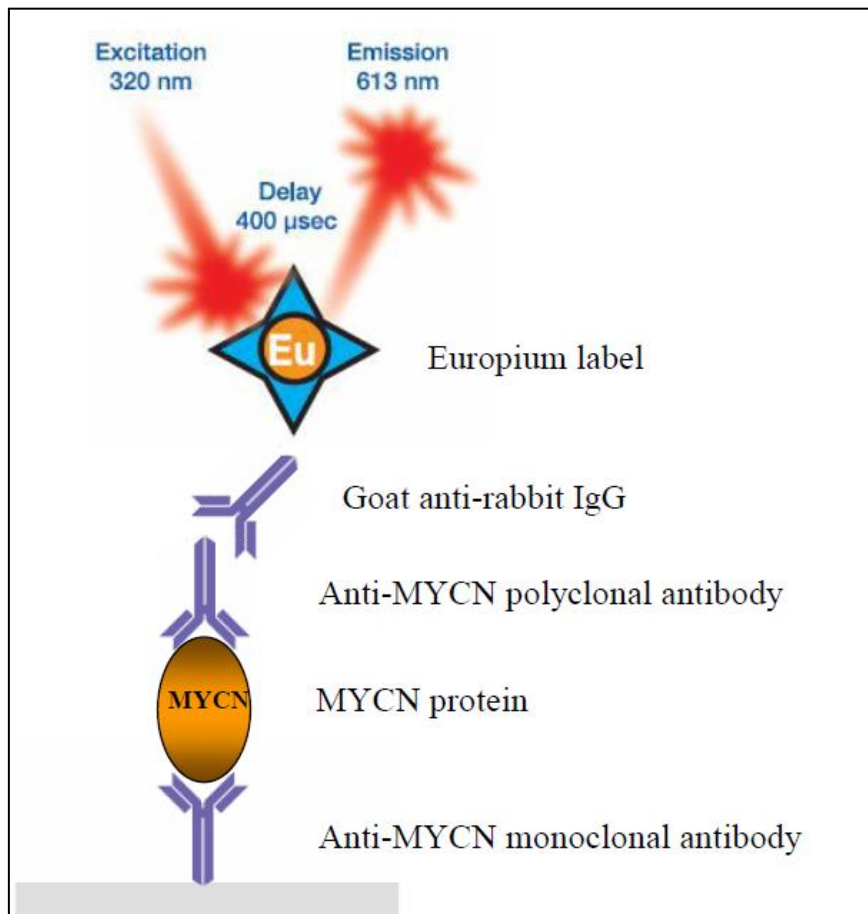


Figure 8 shows a diagram representing the sandwich two-phase ELISA for MYCN protein. The plate is coated with the capture anti-MYCN monoclonal antibody (B8.4) and the lysates are added (yellow oval 'MYCN protein'). The liquid phase antibody is an anti-MYCN polyclonal antibody (SC-791) that is bound to Europium in a two-step process.

Prior to the analysis all neuroblastoma samples (cell lines and bone marrow cell suspensions) required isolation and lysis (described above). Initially, a single phase ELISA was tested although further development showed lack of specificity and for this reason a two-phase (sandwich) ELISA was further developed as described in the 'Results' (figure 8). The procedure described in this section is the two-phase ELISA after all steps of optimisation. For the initial one-phase ELISA only the SC-791 polyclonal anti-MYCN antibody was used. Other modifications such as the choice of monoclonal antibody are discussed in the 'Results' section.

To perform the sandwich MYCN ELISA a 1:1000 dilution of the anti-MYCN monoclonal antibody (B8.4) was prepared in PBS and 100 μL were applied per well. The plate was covered and kept overnight at 4ºC. In the following morning, the plate was washed four times with Tween 20 (0.1%), blocked with 200 μL per well of Marvel 5% (powder milk) and incubated for 1 hour at 37 ºC. A standard solution of the MYCN commercial recombinant protein was prepared and dilutions were made (240, 200, 160, 120, 90, 60, 45 and 0 pg/μl of MYCN recombinant protein). For Quality Control, an in-house lysate of Kelly cells was used. Samples previously lysed were thawed and the required amount diluted in 20% cooking buffer following the BCA spreadsheet (Figure 7). 50 μL of the Standard, QC and samples were added to the plate in duplicate. Figure 9 shows a typical plate layout. The plate was then incubated at 37ºC for 2 hours and washed four times with Tween 20. To each well, 50 μL of anti-MYCN rabbit antibody (1:1000) were added, incubated at 37ºC for 2 hours and washed four times with Tween 20. Then, 50 μL of Eu-N1 goat anti-rabbit IgG (1:500 dilution) were added to each well, incubated 37ºC for 1 hour and washed four times with Tween 20.

50 μL enhancement solution were added to each well, the plate was shaken for 5 minutes on shaker at room temperature and the plate was read on the Envision machine at 615 nm. Figure 10 gives an example of the standard curve generated with the serial dilutions of the MYCN recombinant protein.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	HQC	PS2	PS6	PS10	PS14	PS18	PS22	PS26	PS30	PS34
B	S2	S2	HQC	PS2	PS6	PS10	PS14	PS18	PS22	PS26	PS30	PS34
C	S3	S3	MQC	PS3	PS7	PS11	PS15	PS19	PS23	PS27	PS31	PS35
D	S4	S4	MQC	PS3	PS7	PS11	PS15	PS19	PS23	PS27	PS31	PS35
E	S5	S5	LQC	PS4	PS8	PS12	PS16	PS20	PS24	PS28	PS32	PS36
F	S6	S6	LQC	PS4	PS8	PS12	PS16	PS20	PS24	PS28	PS32	PS36
G	S7	S7	PS1	PS5	PS9	PS13	PS17	PS21	PS25	PS29	PS33	PS37
H	S8	S8	PS1	PS5	PS9	PS13	PS17	PS21	PS25	PS29	PS33	PS37

Standard solutions
High Quality Control
Medium Quality Control
Low Quality Control
Patient Samples

Figure 9 shows typical plate layout where the standard MYCN recombinant protein dilutions are represented in yellow, the quality controls in orange (high, medium and low quality controls are represented as HQC, MQC and LQC) and the patient samples in blue (up to 37 samples in duplicate).

24/06/2011

Conc/well pg/ul	Standard 1	Standard 2	Standard 3	Standard 4	Average	SD	%CV
240.0	7987	8369	9475	9018	8712	663	7.6
200.0	7291	7034	7334	6930	7147	196	2.7
160.0	5998	6004	5391	5636	5757	299	5.2
120.0	4313	4124	4022	4001	4115	143	3.5
90.0	2899	3059	3021	2682	2915	170	5.8
60.0	1902	1980	1566	1795	1811	180	9.9
45.0	1547	1628	1592	1527	1574	45	2.9
0.0	397	367	394	363	380	18	4.7

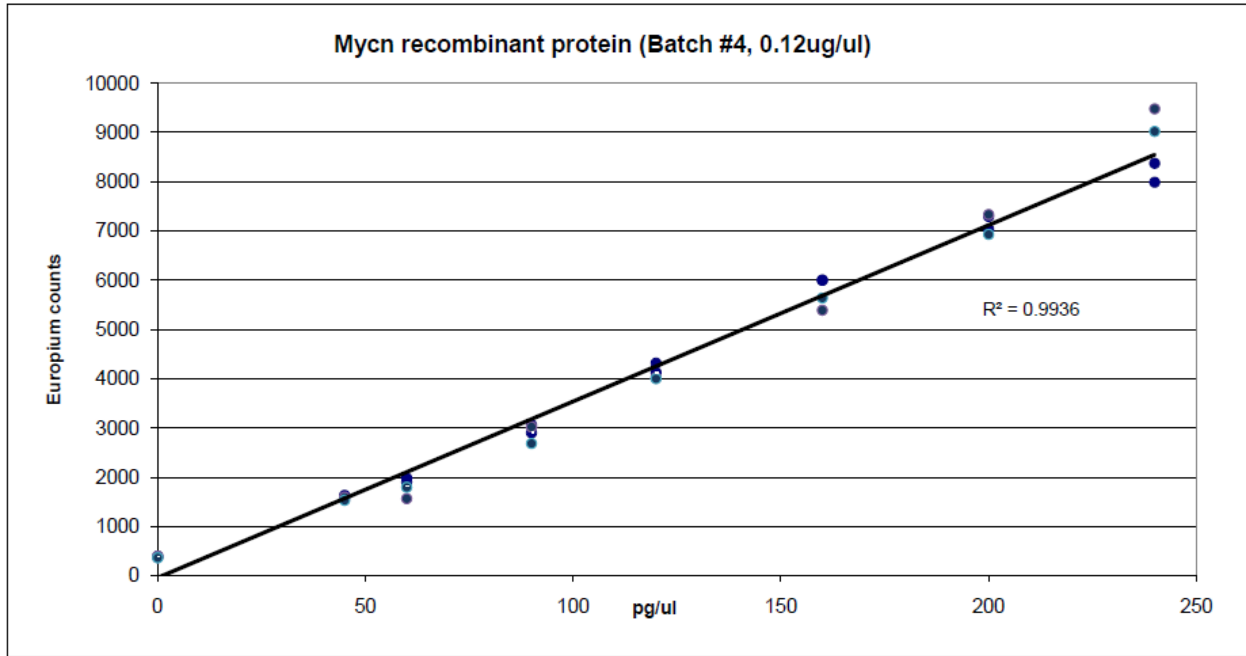


Figure 10 shows the standard curve generated after analysing serial dilutions of MYCN recombinant protein with the sandwich ELISA. In this case, each dilution was analysed in four wells. Average, standard deviation (SD), coefficient of variation (CV) and the value of R^2 are calculated for quality control purposes.

12. MSD triplex assay for the PI3K pathway

In previous decades pharmacodynamic (protein) biomarker analyses were performed by Western blotting, and this technique has been found to have several disadvantages, including the laborious procedure that is very operator-dependent, the lack of reproducibility as well as the significant volumes of sample required for single protein analysis. The development of new technologies that allow to study multiple analytes in the same sample in a more reliable and reproducible way have revolutionised the field of pharmacodynamic biomarker assays and after thorough validation processes are now used in clinical trials [84, 111]. Meso Scale Discovery® (MSD®) has developed a number of kits where plates are pre-coated with multiple antibodies for several cell signalling pathways.

For the MSD AKT triplex assay two plates (phospho and total signalling) that have been pre-coated with the capture antibodies for AKT, GSK3β*, and p70S6K* (*MSD kit specific) are provided. The sample and a solution containing the labelled detection antibodies (an anti-total AKT, GSK3β and p70S6K antibody with an electrochemiluminescent compound, MSD SULFO-TAG® label attached) are then added.

Phosphorylated and total AKT, GSK3β and p70S6K present in the sample binds to the capture antibodies immobilized on the working electrode surface; recruitment of the labelled detection antibody by bound AKT, GSK3β and p70S6K completes the sandwich.

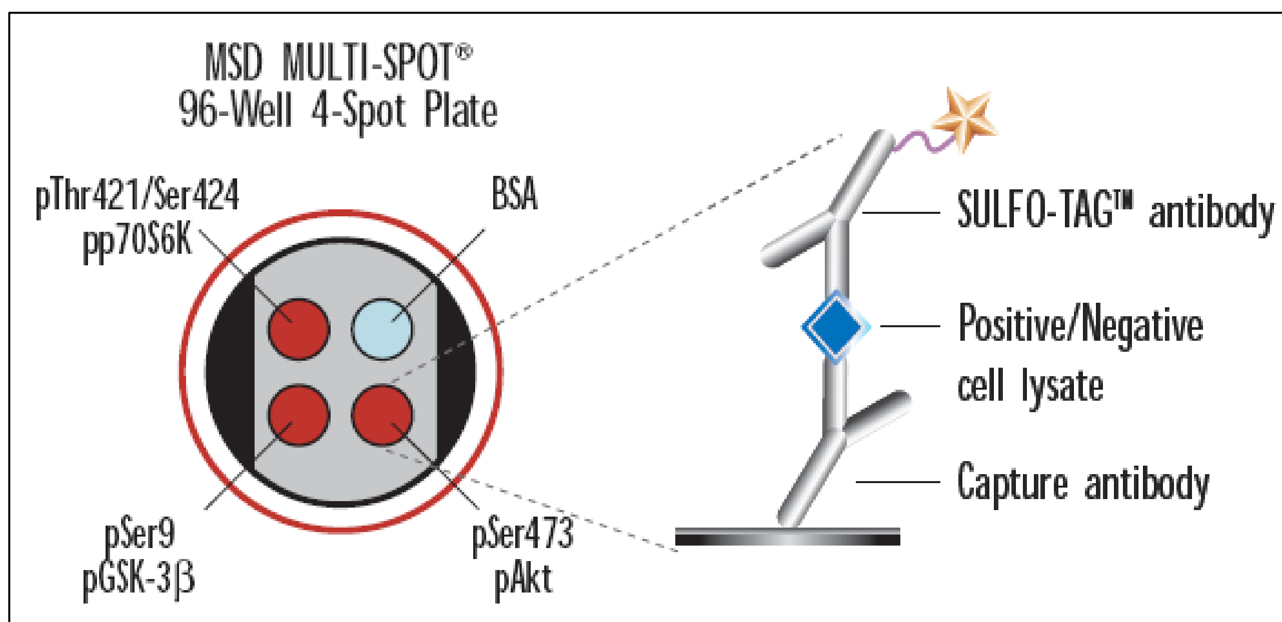


Figure 11, the diagram on the left represents a well from the MSD phospho-triplex AKT plate where there are four spots, each coated with a phospho-antibody (pp70S6K, pGSK3β, pAKT) or blank (BSA). The diagram on the right represents the sandwich formed for the analysis of the lysates with the capture antibody coated to the plate and the SULFO-TAG® antibody bound to the cell lysate.

A MSD® Read Buffer that provides the appropriate chemical environment for electrochemiluminescence (ECL) is added and the plate is then loaded into an MSD® SECTOR®

Imager for analysis. Inside the SECTOR Imager, a voltage is applied to the plate electrodes that cause the labels bound to the electrode surface to emit light. The instrument measures intensity of the emitted light to produce a quantitative measure of the amount of phosphorylated and total AKT present in the sample.

The MSD total and phospho-triplex analyses were carried out on lysates from the PRP samples collected from children with solid tumours and in lysates from neuroblastoma cell lines. The procedure was performed in parallel for phospho- and total triplex analyses: The lysates were divided in two, then two different plates were used (phospho- and total) and two antibody kits (Anti-phospho Akt Antibody solution and Anti-total Akt Antibody solution). The required volume of each lysate was determined with the BCA assay as described before and normalised for a protein concentration of 20 µg of total protein per well. For this, the plates were blocked with 150 µL per well of Blocking Solution (600 mg Blocker A added to 20 mL Tris Wash Buffer) and incubated with shaking for 1 hour at room temperature. The plates were then washed 4 times with Tris Wash Buffer. 25 µL of each sample (lysate) was added and plates were incubated with shaking for 1 hour at room temperature. Antibody Dilution Buffer (1mL of Blocking Solution to 2mL of Tris Wash Buffer) was prepared. The plates were washed 4 times with Tris Wash Buffer and 25 µL per well of Detection Antibody Solution (60 µL 50x SULFO-TAG Anti-Total or Anti-Phospho Akt Antibody to 2.94 mL of Antibody Dilution Buffer) were added, plates were incubated with shaking for 1 hour at room temperature and washed 4 times with Tris Wash Buffer. Read Buffer (5mL of 4X Read Buffer T with surfactant to 15mL of deionized water) was added and plates were imaged within 5 minutes in the SECTOR imager. Figure 12 shows an example of the results of the MSD analysis of PRP samples.

Clinical Trial	
Patient number	Paed Samples

Patient Details:	
Initials	
Date of Birth	
Schedule Description	03/11/2010

Total Akt

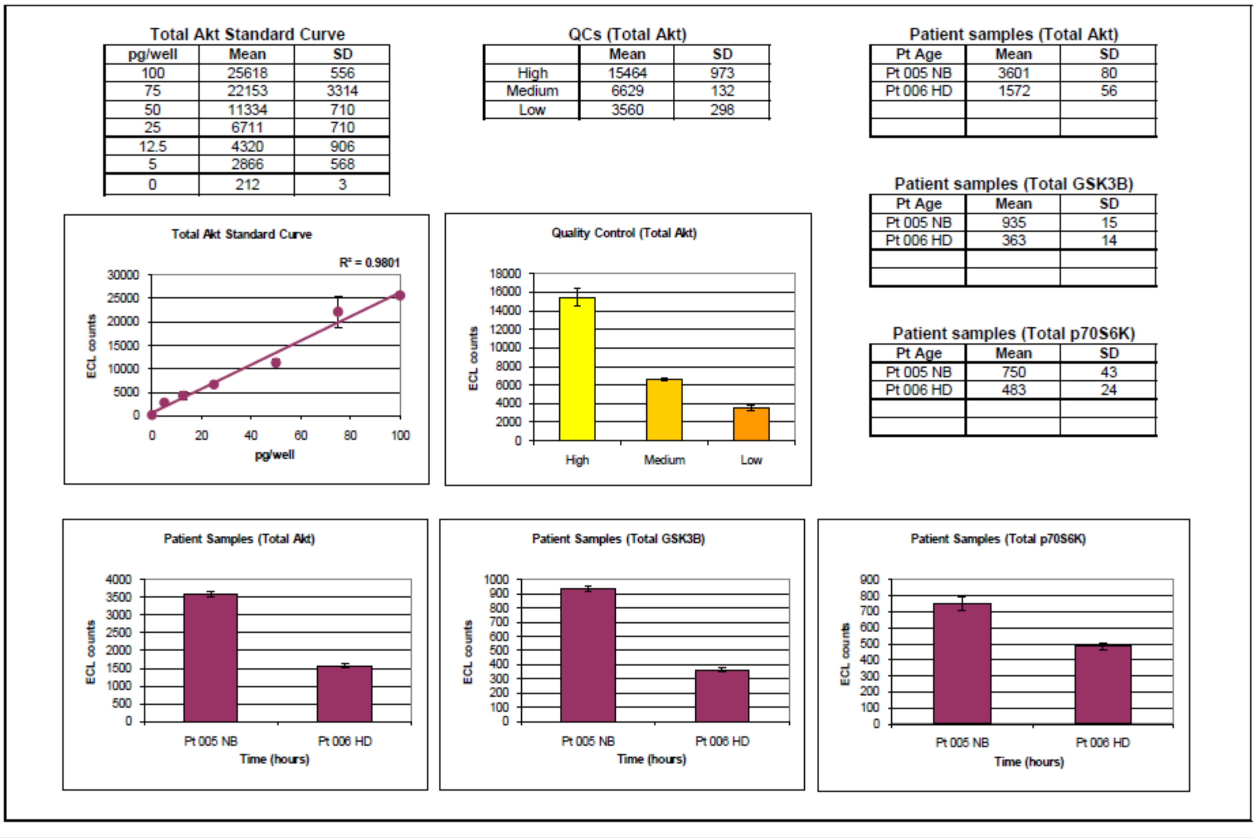
	1	2	3	4	5	6	7	8	9	10	11	12
A (E)	26016	20015	11228	3785	2629	209	1508	180	203	14433	6531	3736
B (F)	24983	20474	6366	3809	3514	3683	1613	178	211	16365	6779	187
C (G)	25856	11046	7527	5366	214	3524	1596	162	237	15594	3728	159
D (H)	25970	11728	6239	2454	213	3595	178	184	216	6576	3216	177

Total GSK3B

	1	2	3	4	5	6	7	8	9	10	11	12
A (E)	269	272	225	216	224	193	379	177	156	965	485	142
B (F)	280	266	221	190	326	952	359	151	167	145	158	124
C (G)	475	244	246	295	169	930	352	155	165	151	128	140
D (H)	367	212	203	194	174	923	170	169	164	147	132	119

Total p70S6K

	1	2	3	4	5	6	7	8	9	10	11	12
A (E)	349	374	326	323	281	309	460	311	285	238	227	218
B (F)	384	401	333	306	285	799	507	245	323	242	229	181
C (G)	376	369	310	305	291	734	482	301	303	255	254	206
D (H)	390	311	307	320	297	718	293	268	319	200	218	221



Total Akt Standard Curve

pg/well	Mean	SD
100	25618	556
75	22153	3314
50	11334	710
25	6711	710
12.5	4320	906
5	2866	568
0	212	3

QCs (Total Akt)

	Mean	SD
High	15464	973
Medium	6629	132
Low	3560	298

Patient samples (Total Akt)

Pt Age	Mean	SD
Pt 005 NB	3601	80
Pt 006 HD	1572	56

Patient samples (Total GSK3B)

Pt Age	Mean	SD
Pt 005 NB	935	15
Pt 006 HD	363	14

Patient samples (Total p70S6K)

Pt Age	Mean	SD
Pt 005 NB	750	43
Pt 006 HD	483	24

Figure 12 shows the results of the MSD total triplex analysis of PRP samples from patients 005 and 006 with raw data, the standard curve for total AKT, Quality Controls and the results of Total AKT, GSK3β and p70S6K.

13. Statistical methods

The following statistical calculations were performed

1. PRP data
    a. Median, range, mean and standard deviation of total and phosphorylated AKT, GSK3β and p70S6 were calculated for the two groups of adult and paediatric patients.
    b. The levels of each of the total and phosphorylated proteins were compared between children and adults, age (children vs. adolescents), histological diagnosis (neuroblastoma vs. other), time from prior chemotherapy (less than 20 days vs. ≥20 days) and stage (localised vs. metastatic disease) using the t-Student test.
2. Immunomagnetic separation
    a. Purity was calculated before and after immunomagnetic separation with flow cytometry as described in section 8 of the Methods [R3 events/(R3+R4 events)]. Median and ranges of purity were calculated for the different immunomagnetic separation procedures but no statistical comparisons were made.
    b. Recovery was estimated with flow cytometry as described in section 8 of the Methods [(R3+R4 events after separation) / (R3+R4 events before separation)]. Median and ranges of the recovery were calculated but no statistical comparisons were made.
3. MYCN ELISA
    a. Sensitivity: using MYCN recombinant protein (Abnova®), a standard curve was established to determine the minimal level of MYCN protein that could be detected with the ELISA assay. Additionally, the minimal amount of total protein (measured by BCA analysis) and the minimal number of neuroblastoma cells required to obtain a detectable signal was established.
    b. Specificity: Positive and negative controls were used to establish specificity of the assay. Positive controls included: Kelly and SHEP-DBL cells. Negative controls included: HeLa and SHEP cells, peripheral blood mononuclear cells and bone marrow samples clear of neuroblastoma involvement. Those analyses were performed in parallel with Western blotting to confirm the results.
4. MSD in neuroblastoma cells. For the characterization of assay signal ranges in neuroblastoma cell lines, descriptive statistics were used. Median, range, mean and standard deviation of total and phosphorylated AKT, GSK3β and p70S6 were calculated in cell lines and in spiking experiments in peripheral blood and bone marrow samples. Signal ranges before and after immunomagnetic separation were compared using the t-Student test.

For all values, 95% confidence intervals were calculated when appropriate. All p values were two sided. A p value <0.05 was considered statistically significant. All statistical calculations were performed using the SPSS 17.0 ® statistical software (Chicago, IL, USA).

14. Good Clinical Practice and Good Clinical Laboratory Practice compliance

The study was conducted according to Good Clinical Practice (GCP) guidelines and regulations that apply to the United Kingdom. The research followed practice according to International Conference on Harmonisation (ICH), Good Clinical Practice (GCP), the Declaration of Helsinki, Research Governance Framework for Health and Social Care 2005, and the Human Tissue Act 2004. All staff involved in the study abided with the Data Protection Act 1998 and also with the Confidentiality Code of Practice and Data Protection Policy and Procedure. Laboratory experiments were conducted following the guidelines for Good Clinical Laboratory Practice (GCLP).

Results

1. Measurement of total and phospho protein signals of the PI3K pathway in children in platelet rich plasma as a surrogate tissue

Previous work performed at the ICR Clinical PD Biomarker Group has used the MSD triplex panel to measure total and phospho signals for the PI3K/AKT signalling pathway in platelet-rich plasma. This assay has been developed and validated pre-clinically [83, 85, 112] and clinically at our lab [74] and has been implemented in several adult phase I trials, some of which have already been finalised [74]. The choice of the MSD triplex assay offers a strong and comprehensive evaluation of the PI3K/AKT/mTOR pathway from AKT to downstream effectors mTORC1 and mTORC2.

Validation work performed in our lab prior to this study included: a) Surrogate tissue optimisation that found that PRP was superior to PBMC as a surrogate biomarker for the PI3K pathway, b) Proof-of-principle *in vitro* experiments that showed how phospho AKT decreased and the phospho-AKT/total-AKT ratio increased after treating U87MG malignant glioma cells with an AKT inhibitor, c) Proof-of-principle *ex vivo* experiments that showed that these changes can be also detected in PRP incubated with an AKT inhibitor, d) establishment of Quality Controls, Sensitivity and Specificity and e) Assay fit-for-purpose in a clinical trial, optimisation of sample collection, stability and lysis that led to an SOP to GCLP standards.

In the first-in-man study of the AKT inhibitor MK2206 an AKT inhibitor [74], a dose dependency was observed and the PD assay results were used to guide dose selection and scheduling in the trial.

Therefore the goal was to develop the MSD technology to assess the PI3K/AKT signalling pathway in PRP from children with solid tumours in order to implement this assay in the forthcoming paediatric trials of PI3K/AKT/mTOR inhibitors.

As discussed in the Methods section, PRP was collected from 14 children with solid tumours within the ethically and institutionally approved RMH CCR 3358 single-centre study. For comparison, baseline measurements from 24 adults with advanced solid cancers treated at the Drug Development Unit of RMH were used.

The results of the MSD AKT Triplex total and phospho-proteins in children and adults are shown in Figure 13 and Table 5.

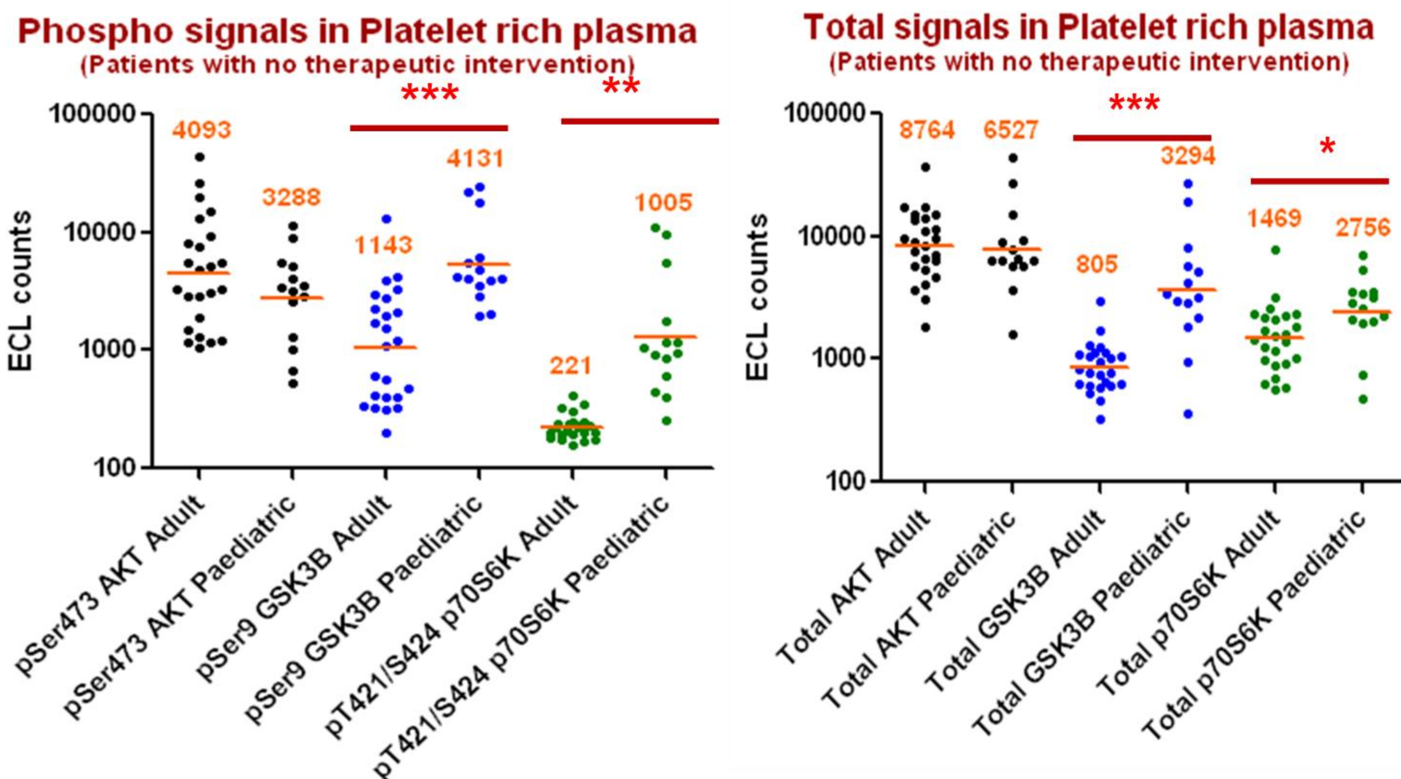


Figure 13, Results of MSD Phospho and Total Triplex AKT assay. Figure on the left shows phospho-protein signals in paediatric vs. adult patients for pAKT, pGSK3β and pp70S6K. * p<0.05, ** p<0.01, *** p<0.001

		Age Group	Mean	Standard deviation	p value
Phospho protein signals	pAKT	Children	3963.85	3269.547	0.186
		Adult	7853.96	10056.473	
	pGSK3B	Children	8109.69	7833.582	0.001
		Adult	1940.79	2703.307	
	pp70S6K	Children	2697.15	3675.442	0.002
		Adult	231.62	61.120	
Total protein signals	AKT	Children	11435.50	11736.476	0.695
		Adult	10197.20	7357.307	
	GSK3B	Children	6512.62	7945.476	0.001
		Adult	949.08	537.102	
	p70S6K	Children	2995.31	1743.713	0.03
		Adult	1783.88	1445.649	

Table 5 summarizes mean and standard deviation of protein signals using the MSD multiplex total and phospho-AKT panel in children (n=14) vs. adults (n=24). Means were compared using the t-Student test.

Total and phospho-AKT signals in children are within similar ranges compared to signals in adults. Phospho protein signals for GSK3β and p70S6K were 4.2-fold and 11.6-fold higher in children respectively (p=0.001 and p=0.002). Total protein signals for GSK3β and p70S6K were 6.9-fold and 1.7-fold higher in children (p=0.001 and p=0.03).

There was no statistically significant differences between signals (for all total and phospho-proteins analysed) when the children data were grouped according to histological diagnosis (neuroblastoma vs. non-neuroblastoma), stage (localised vs. metastatic), age group (children vs. adolescents older than 10 years) and the time interval from previous chemotherapy (greater compared vs. less than 20 days from last chemotherapy).

2. Isolation of neuroblastoma cells from bone marrow samples

2.1 Establishment of adequate methodology, standards and controls to assess enrichment methods

2.1.1 Expression of GD2 in neuroblastoma with flow cytometry: positive and negative controls

The membrane ganglioside GD2 has been consistently reported to be expressed in neuroblastoma cells [103-104]. The objective was to ensure that flow cytometry using the monoclonal antibody 14.G2a (BD Pharmingen) bound to FITC is able to detect expression of GD2 membrane ganglioside on neuroblastoma cells form Kelly cell lines or neuroblastoma tumours from the *TH-MYCN* transgenic mouse murine tumours [20].

Using flow cytometry, cells from the Kelly cell line and neuroblastoma cells suspended from tumour samples of *TH-MYCN* mice expressed GD2 on 94.3 and 83.6% of the total cellular events respectively (Figures 14 to 16 and Table 6). On the contrary, GD2 expression was much lower in unstained Kelly cells and HeLa carcinoma cells (0.1 and 5.9% respectively).

	Events gated	GD2+ events	%
Unstained Kelly	7681	6	0.1
Kelly	7530	7099	94.3
HeLa	9245	542	5.9
TH-MYCN	3464	2895	83.6

Table 6 shows the raw data acquired in the flow cytometry experiments depicted in Figures 14, 15 and 16. Kelly cells stained and unstained with GD2-FITC, HeLa cells and neuroblastoma cells obtained from tumours in *TH-MYCN* mice [20] were analysed. 30000 events were analysed for each experiment. Events gated represents the 'R1' gate shown in Figure 14 (corresponding to the neuroblastoma cells).

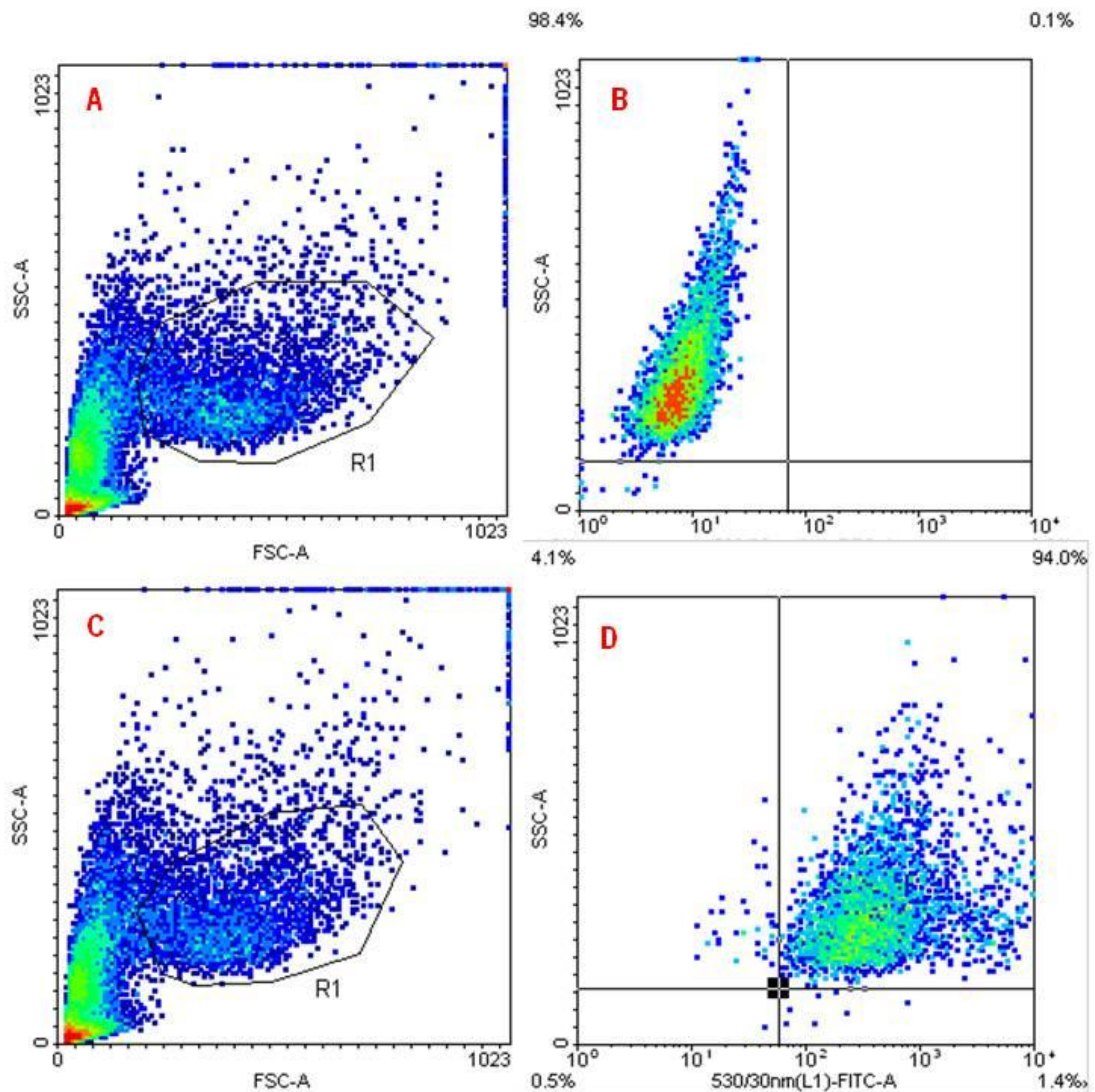


Figure 14 shows the flow cytometry experiments to determine the expression of GD2 on Kelly cell lines. A and B represent the unstained Kelly cell suspension, C and D represent the Kelly cell suspension stained with GD2-FITC.

A and C represent the FSC (forward scatter) / SSC (side scatter) density plot where the R1 gate corresponds to the population of neuroblastoma cells excluding dead cells and debris. In B and D, the expression of GD2-FITC is charted on the X axis and the quadrant quantifies the proportion of GD2 positive events. Without the GD2-FITC staining, only 0.1% of events were GD2+ (panel B). With the GD2-FITC staining, 94.0% of Kelly neuroblastoma cells in a cell suspension showed surface expression of GD2 (panel D).

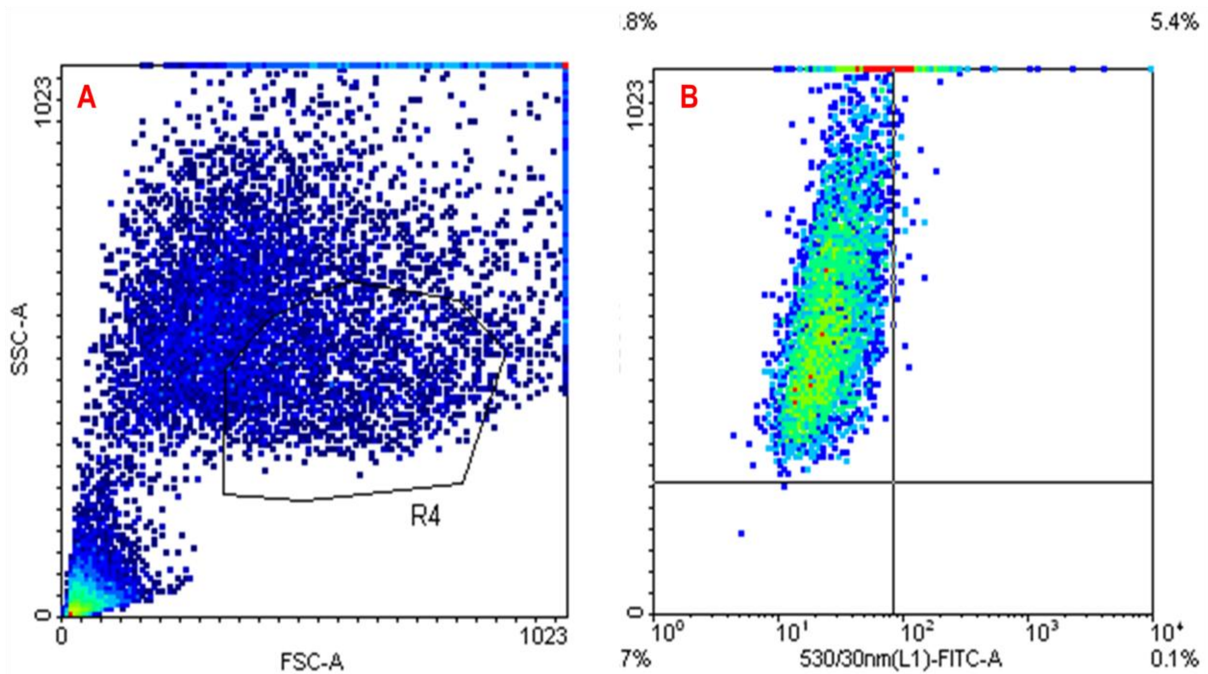


Figure 15 shows the flow cytometry experiments to determine the expression of GD2 in HeLa cells. Panel A shows the FSC/SSC scatter plot where the same population used in the previous experiment (Figure 14) was gated in R4. Panel B shows the GD2-FITC expression in the X axis. Only 5.4% of the HeLa cells expressed GD2 in their surface.

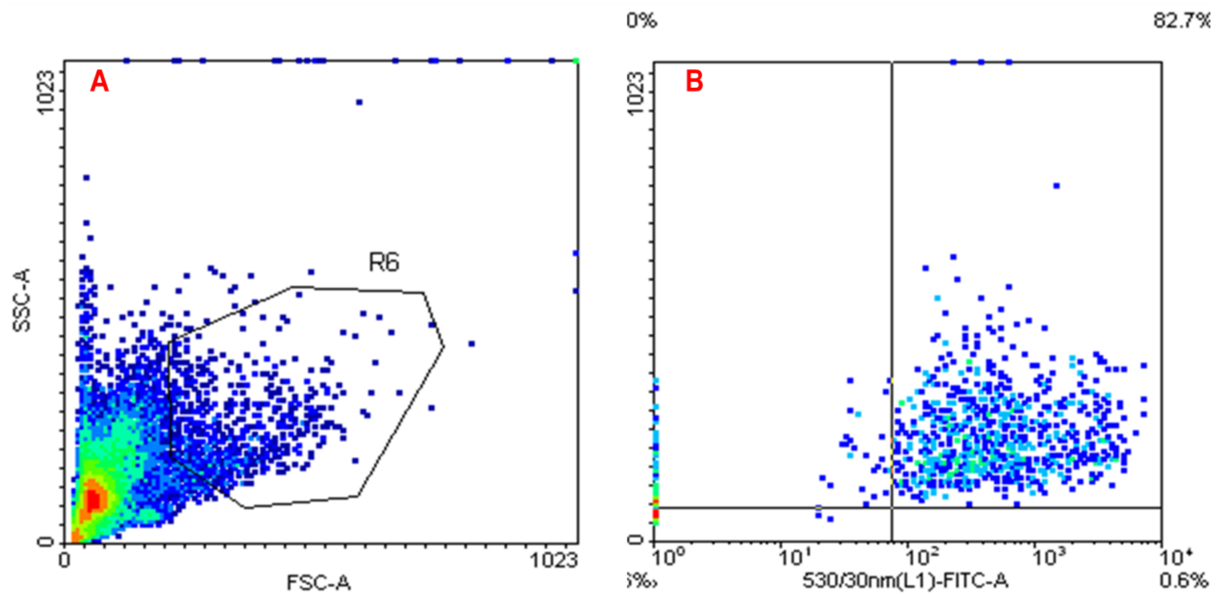


Figure 16 shows the flow cytometry experiments to determine the expression of GD2 in single cell suspensions from neuroblastoma tumours that develop in the *TH-MYCN* transgenic murine model [20]. Panel A shows the FSC/SSC scatter plot where the same population used in the previous experiments (Figures 14 and 15) was gated in R6. Panel B shows the GD2-FITC expression in the X axis. 82.7% of the tumour cells showed expression of GD2 in the surface..

Diluting the anti-GD2 14.G2a monoclonal antibody, GD2 expression progressively decreased with serial dilutions (from 91.8% for the pure antibody to 68.4% for the 1:20 dilution (Figure 17 and Table 7 for raw data). Therefore undiluted antibody was required to maximize the signal for the detection of GD2 expression.

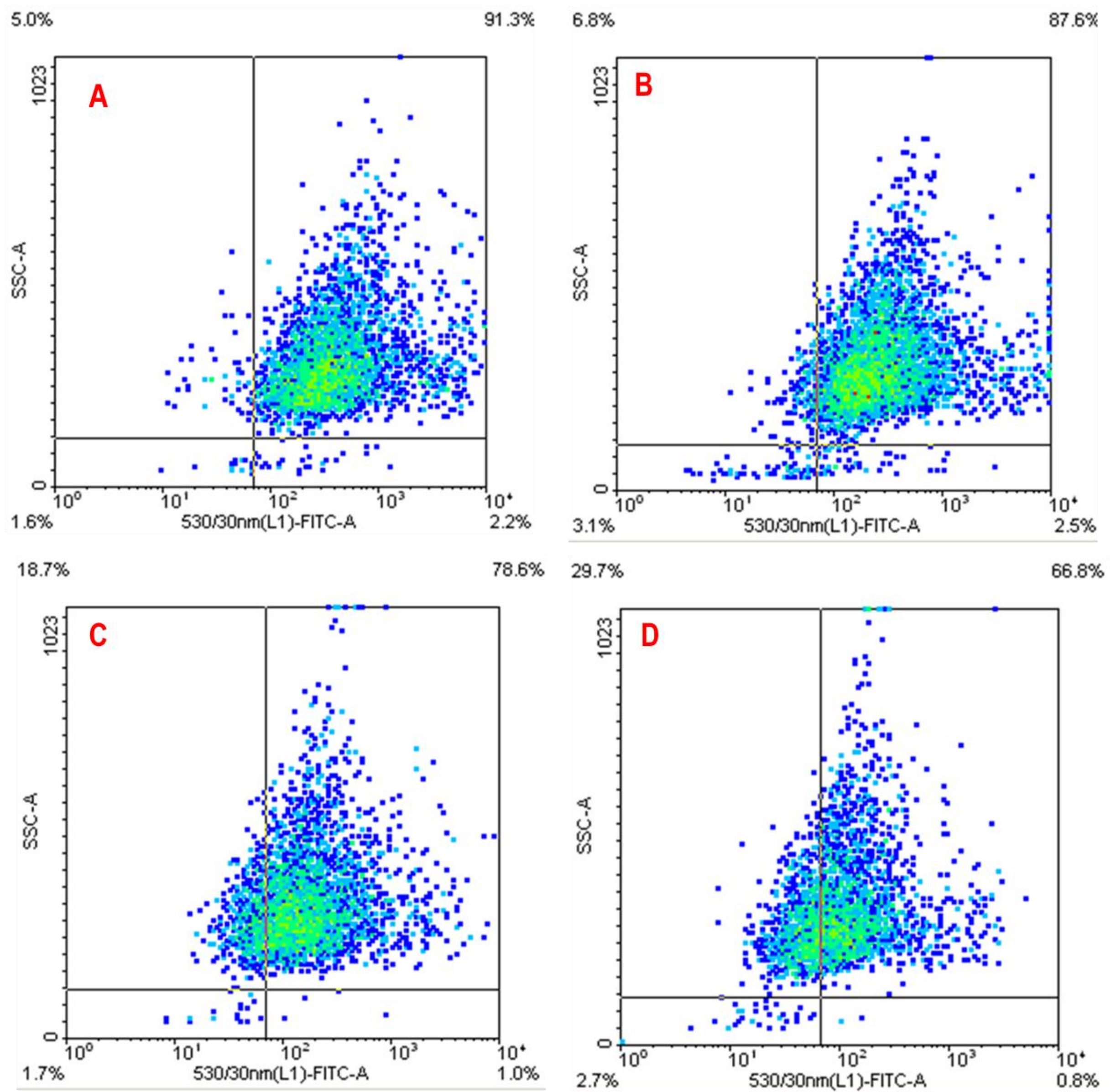


Figure 17 shows the flow cytometry experiments to determine the expression of GD2 in Kelly cells using decreasing dilutions of the GD2-FITC antibody. The anti-GD2 antibody was used pure (5µL, panel A), and in 1:5 (panel B), 1:10 (panel C) and 1:20 (panel C) dilutions. The figures show the expression of GD2-FITC in the right upper quadrant. The expression of GD2 decreased with the dilutions (91.3%, 87.6%, 78.6% and 66.8%) and in all future experiments pure undiluted anti-GD2 antibody was used.

Dilutions	Events gated	GD2+ events	%
1:1	7686	7058	91.8
1:5	8888	7885	88.7
1:10	7778	6255	80.4
1:20	6896	4714	68.4

Table 7 shows the raw data acquired in the flow cytometry experiments depicted in Figure 17. Kelly cell suspensions were stained with decreasing concentrations of anti-GD2 antibody (titration). 30000 events were analysed for each experiment.

2.1.2 CD45 expression in Peripheral blood mononuclear cells (PBMC)

CD45 reacts with the isoforms of the leukocyte common antigen present on all human white blood cells, including lymphocytes, monocytes, granulocytes, eosinophils, and thymocytes whereas it is not expressed in neuroblastoma cells [110]. CD45 is a pan-haematopoietic cell marker.

Peripheral blood was obtained from three adult healthy volunteers on four separate occasions.

The expression of CD45 in peripheral blood mononuclear cells isolated by Ficoll separation with CD45-H7 (BD Pharmingen) by flow cytometry was determined. Haematopoietic cells derived from peripheral blood mononuclear cells showed high expression of CD45 (69.8%) and no expression of GD2 (0%), (Figure 18). A cell suspension of neuroblastoma cells from the Kelly cell line showed no expression of CD45 (0 out of 19489 events).

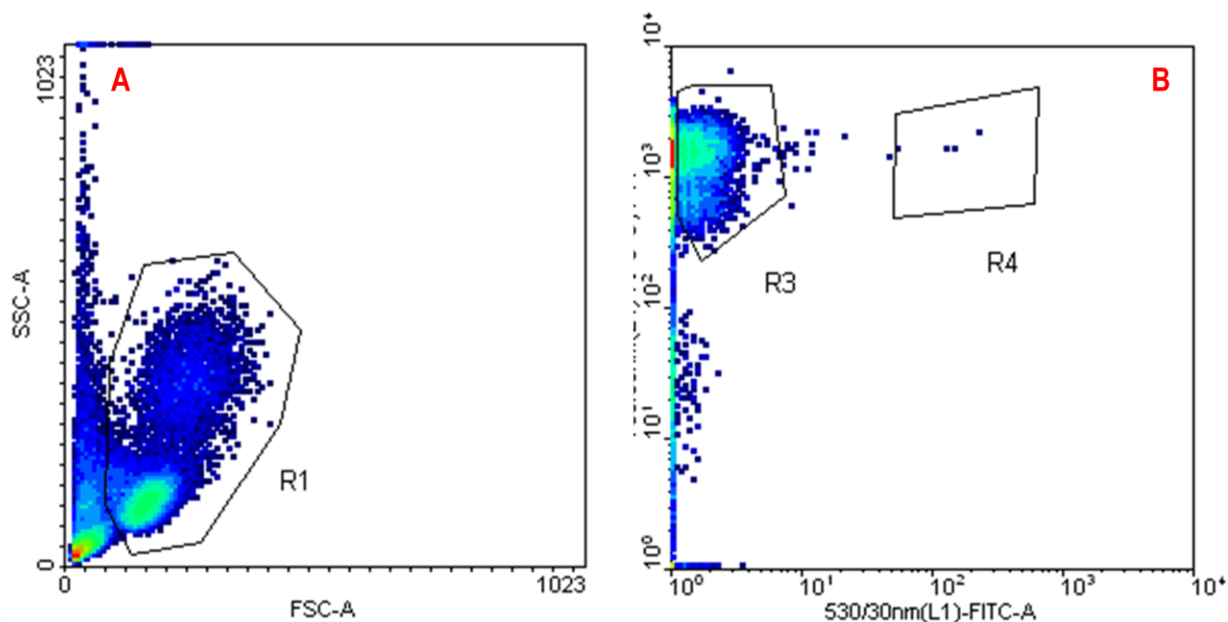


Figure 18, shows the flow cytometry experiments to determine the expression of CD45 and GD2 in healthy volunteer haematopoietic cells. Panel A shows the FSC/SSC scatter plot where population of viable haematopoietic cells was gated in R1. Panel B shows the GD2-FITC expression in the X axis and the CD45-APCH7 in the Y axis. 69.8% of isolated cells in R1 showed positivity for CD45. Total events 6974, gated (R1) 6734. R3 gate (CD45 expressing cells) 4702 events, R4 gate (GD2 expressing cells) 4 events.

2.1.3 Identification of neuroblastoma and haematopoietic populations with flow cytometry

Spiking experiments of peripheral blood with neuroblastoma cells (Kelly cell line) were performed at different levels of involvement by neuroblastoma cells to mimic the clinical scenario of clinical bone marrow samples.

Aliquots with 0.2, 0.5, 1 and 2 ml of Kelly cell suspension (10^6 cells/ml) were added to 5 ml of healthy volunteer peripheral blood.

Flow cytometry analysis of neuroblastoma cells spiked in peripheral blood showed two populations which were clearly differentiated (Figure 19). This served as a baseline to evaluate the two different immunomagnetic separation methods.

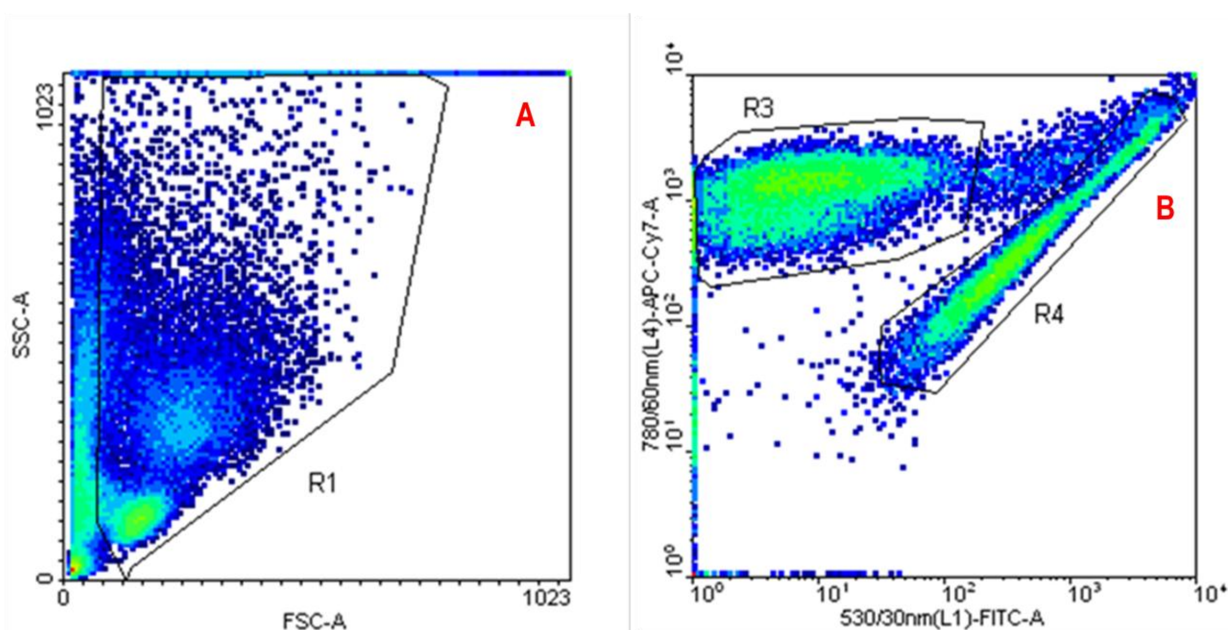


Figure 19, shows the flow cytometry experiments to determine the expression of CD45 and GD2 in healthy volunteer peripheral blood spiked with Kelly cell lines (1:5 dilution). Panel A shows the FSC/SSC scatter plot where population of viable cells was gated in R1. Panel B shows the GD2-FITC expression in the X axis and the CD45-APCH7 in the Y axis. Two populations are identified. R3 represents the population of CD45+/GD2- cells (haematopoietic) and R4 represents the population of CD45-/GD2+ cells (neuroblastoma). In this figure, the R3 population (haematopoietic) comprises 61.9% of the total gated events and the R4 population (neuroblastoma) comprises 36.7% of the total gated events. Total events 63473, gated events (R1) 30231. R3 events (CD45+/GD2-) 23448, R4 events (CD45-/GD2+) 8809.

2.2. Identification of the optimum immunomagnetic separation method: purity and recovery of isolated cell suspensions

Several technologies have been used in adult cancer to identify circulating tumour cells and there are currently several very successful automated systems such as CellSearch® by Veridex [71, 113] or microchip technology [114]. However those methods are based on the selection of antigens, such as epithelial cell adhesion molecule (EpCAM) or cytokeratines 8, 18 and 19 present on adult carcinoma cells, which are not present in childhood malignancies. There are currently no commercially available kits that are able to separate/enrich neuroblastoma cells and therefore we aimed to develop a specific neuroblastoma assay that could be used in bone marrow and blood samples.

The membrane ganglioside GD2 is strongly expressed by neuroblastoma cells and not by haematopoietic cells. In normal tissues it is only expressed in neural tissues (cerebellum and peripheral nerves) [104].

The objective was to achieve the maximum cell purity (% of neuroblastoma cells) and recovery (minimal cell losses) in an assay with the minimal possible complexity. This was important as ultimately the assay would need to be validated for use in clinical trials across multiple centres.

2.2.1 GD2 positive selection

Given the lack of commercially available MicroBeads for GD2 positive selection, an indirect methodology was used, by a two-step process adding the anti-human GD2 mouse monoclonal antibody (BD Pharmingen) to anti-mouse MicroBeads (Miltenyi Biotech).

2.2.2 CD45 depletion

Due to the availability of commercial CD45-labelled immunomagnetic beads, CD45 depletion is a quicker procedure as several steps are eliminated. In addition, with this methodology, the tumour cells (neuroblastoma cells) remain untouched. Hence, this procedure would be more suitable and reproducible for protein assays.

2.2.3 CD45 depletion compared to GD2 positive selection in peripheral blood samples

CD45 depletion, in spiking experiments of Kelly cells in healthy volunteer peripheral blood achieved purity above 90% in 4 out of 5 experiments, whereas none one out of the 4 experiments with GD2 positive selection achieved purity above 90%. (Table 8 and Figure 20)

Average±SD post-separation purity was 84.88%±0.14 for CD45 depletion compared to 51.48%±0.26 for GD2 positive selection (t Student, $p=0.04$).

CD45 depletion achieved a median 6.2-fold (range 2.0-33.8) improvement in the purity of the neuroblastoma cell suspensions. GD2 positive selection achieved a median 5.1-fold (1.2-22.4) improvement in purity.

Therefore CD45 depletion achieved higher purity and more consistent results.

	Pre separation	Post separation	-x fold increase
CD45 depletion (n=5)	2.4%	82.6%	33.8
	9.7%	61.3%	6.3
	14.7%	91.1%	6.2
	27.3%	97.0%	3.6
	46.7%	92.4%	2.0
GD2 positive selection (n=4)	2.4%	54.7%	22.4
	9.7%	84.2%	8.7
	14.7%	22.0%	1.5
	37.2%	45.0%	1.2

Table 8 shows the proportion of neuroblastoma cells determined by flow cytometry (GD2+/CD45- events) out of the total gated events before and after the inmunomagnetic separation with CD45 depletion and GD2 positive selection in peripheral blood samples from healthy volunteers spiked with different concentrations of neuroblastoma cells from the Kelly cell line. CD45 depletion showed achieved better purity in the cell suspensions.

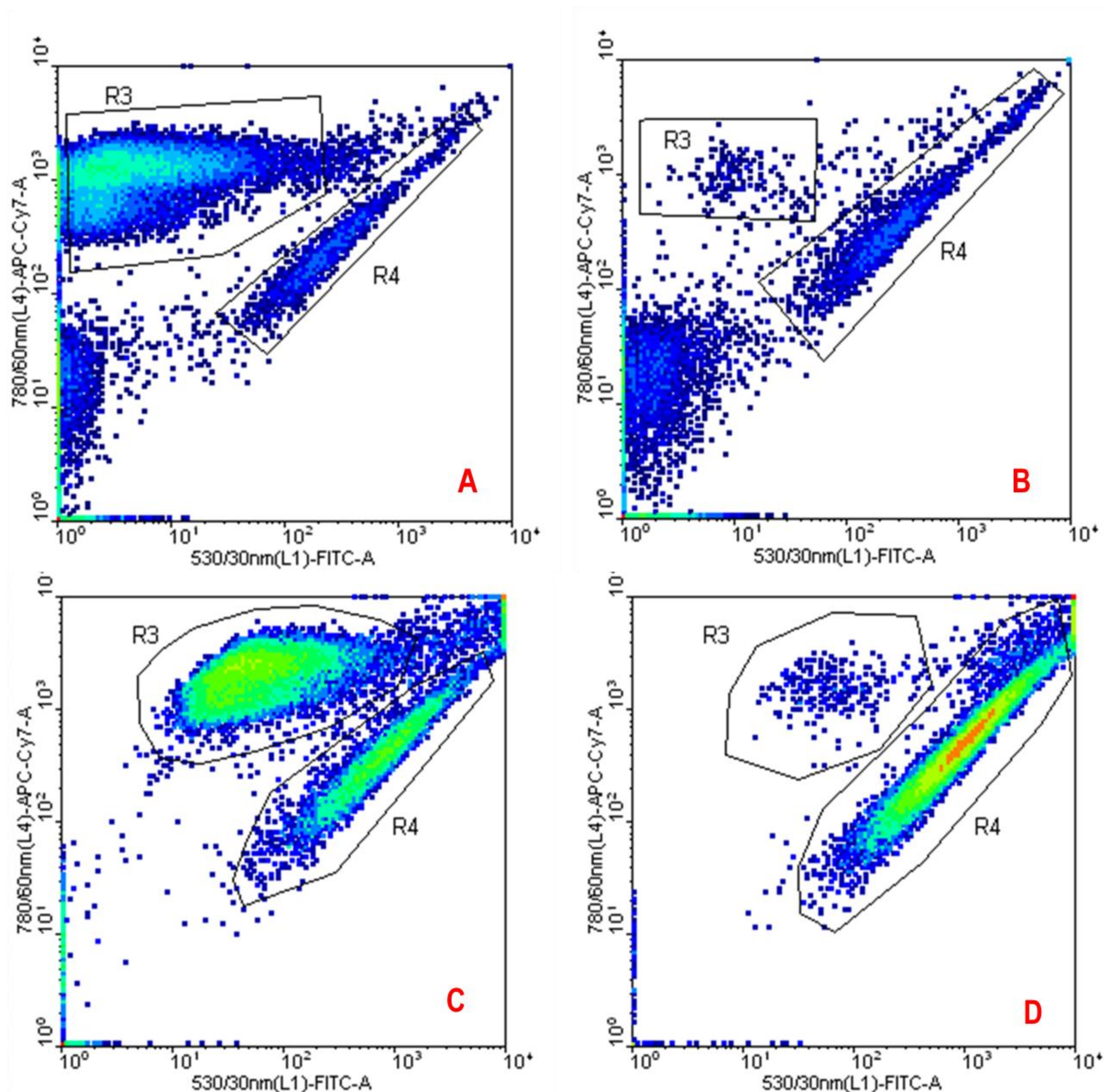


Figure 20, shows the flow cytometry experiments to determine the best immunomagnetic separation methodology (GD2 positive selection vs. CD45 depletion). In all panels, the GD2-FITC expression is plotted in the X axis and the CD45-APCH7 in the Y axis. Two populations are identified, R3 represents the population of CD45+/GD2- cells (haematopoietic) and R4 represents the population of CD45-/GD2+ cells (neuroblastoma). Panel A shows a 1:10 cell suspension of Kelly cells into healthy volunteer peripheral blood before the GD2 positive selection and panel B shows the cell suspension after the GD2 positive selection. Before the separation, in panel A, neuroblastoma cells (R4) comprise 9.7% (2697 events) of the gated events (27686). After the GD2 positive selection, in panel B, neuroblastoma cells (R4) comprise 84.2% (3559 events) of the gated events (4225 events).

Panel C shows a 1:5 cell suspension of Kelly cells into healthy volunteer peripheral blood before the CD45 depletion and panel D shows the cell suspension after the CD45 depletion. Before depletion, in panel C, neuroblastoma cells (R4) comprise 27.3% (8809 events) of the gated events (32257 events). After depletion, in panel D, neuroblastoma cells (R4) comprise 97.0% (30774 events) of the gated events (31712 events).

2.2.4 CD45 depletion in bone marrow aspirates

CD45 depletion was also performed in bone marrow samples spiked with neuroblastoma cells from the Kelly cell line (n=3) and clinical bone marrow samples (n=4). In bone marrow aspirates, CD45 depletion achieved 2.5-fold (2.0-11.0) and 6.1-fold (2.8-9.3) improvement in purity of neuroblastoma cells in spiked BM and clinically involved BM samples respectively. (Table 9 and Figures 21 and 22)

Average±SD post-separation purity was 64.77% ±0.05 for BM samples spiked with Kelly neuroblastoma cells and 76.85%±0.10 for BM samples from patients with neuroblastoma bone marrow metastases. (Figure 22)

	Pre separation	Post separation	-x fold increase
BM spiked with Kelly (n=3)	6.4%	70.6%	11.0
	25.9%	63.6%	2.5
	29.8%	60.1%	2.0
BM with neuroblastoma (n=4)	17.0%	47.8%	2.8
	10.1%	94.0%	9.3
	12.2%	86.7%	7.1
	14.7%	78.9%	5.4
Negative BM's (n=4)	No population identified		

Table 9 shows the proportion of neuroblastoma cells determined by flow cytometry (GD2+/CD45- events) out of the total gated events before and after the inmunomagnetic separation with CD45 depletion in normal bone marrow samples spiked with neuroblastoma cells from the Kelly cell line (n=3 experiments), clinical bone marrow aspirates involved with neuroblastoma cells (n=4) and clinical bone marrow aspirates not involved with neuroblastoma (n=3). CD45 depletion achieved great improvements in purity (above 60%) in all but one samples.

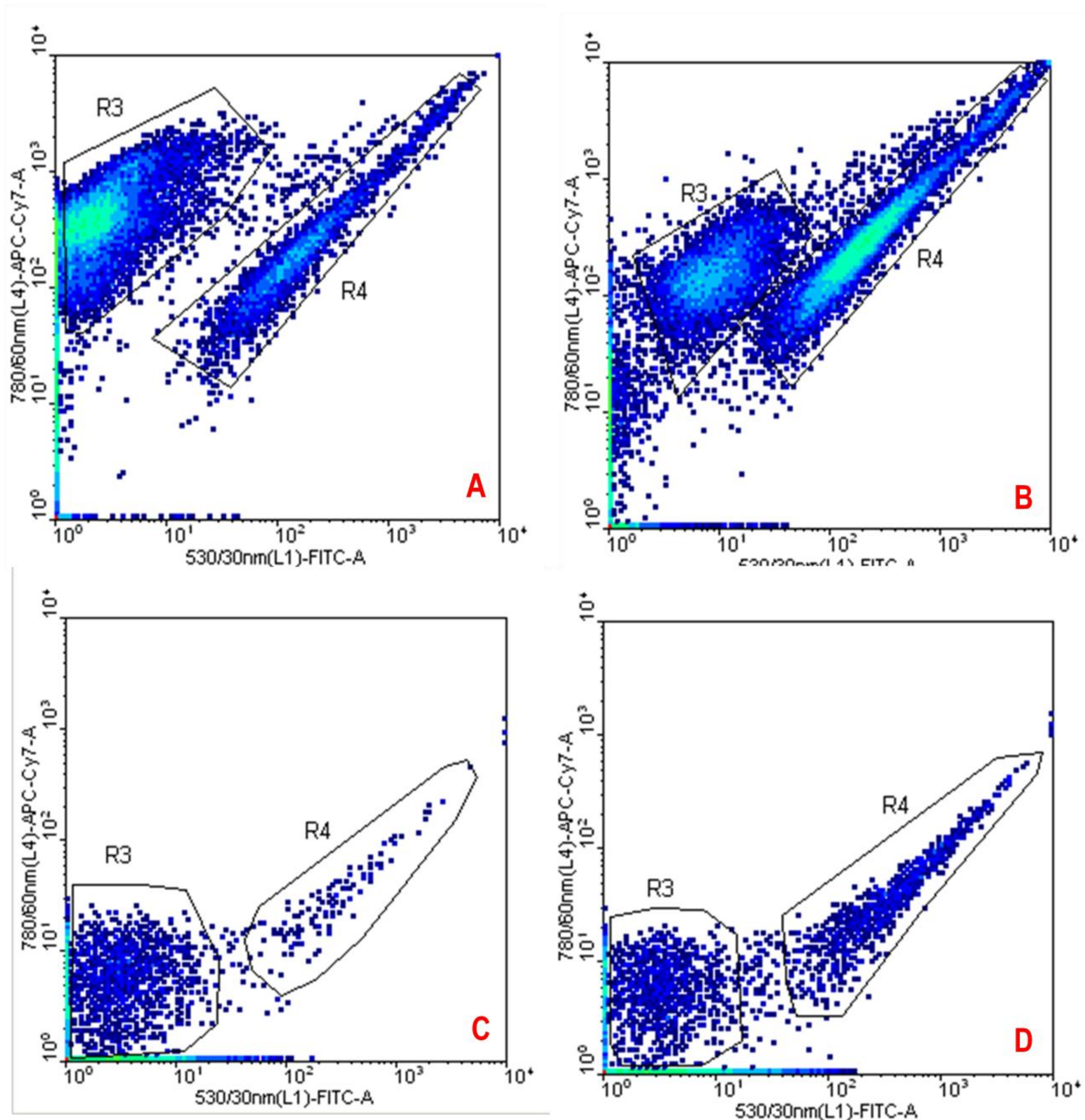


Figure 21, shows the flow cytometry experiments using CD45 depletion in bone marrow aspirates spiked with Kelly cells 1:4 (Panels A and B, before and after, respectively) and clinical bone marrow samples from a patient with neuroblastoma metastatic to the bone marrow (panels C and D, before and after, respectively). In all panels, the GD2-FITC expression is plotted in the X axis and the CD45-APCH7 in the Y axis. Two populations are identified, R3 represents the population of CD45+/GD2- cells (haematopoietic) and R4 represents the population of CD45-/GD2+ cells (neuroblastoma).

Before the CD45 depletion in the bone marrow sample spiked with Kelly cells, in panel A, neuroblastoma cells (R4) comprise 25.9% (5598 events) of the gated events (21572 events). After the depletion, in panel B, neuroblastoma cells (R4) comprise 63.6% (15115 events) of the gated events (23766 events).

In a sample from a patient with metastatic neuroblastoma, before depletion, in panel C, neuroblastoma cells (R4) comprise 17.0% (579 events) of the gated events (3400 events). After depletion, in panel D, neuroblastoma cells (R4) comprise 47.8% (2059 events) of the gated events (4310 events).

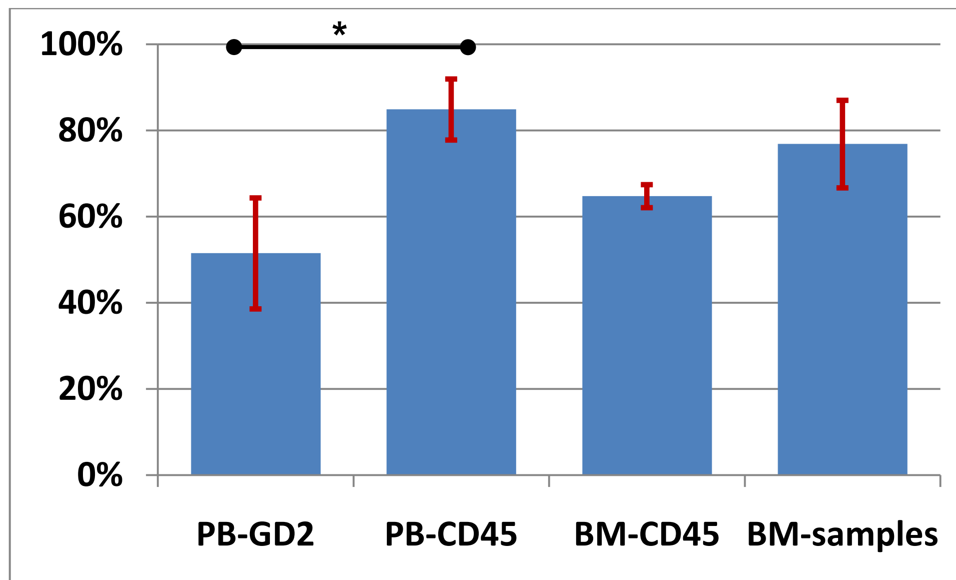


Figure 22, represents the purity of neuroblastoma cells achieved after immunomagnetic separation using GD2 positive selection in peripheral blood spiked with neuroblastoma cells (PB-GD2) and CD45 depletion in peripheral blood spiked with neuroblastoma cells (PB-CD45), healthy bone marrow spiked with neuroblastoma cells (BM-CD45) and clinical bone marrow samples (BM-samples). Blue bars represent the average of all experiments in that category and red bars represent standard deviation. * $p<0.05$

2.2.5 Recovery

In order to estimate recovery with flow cytometry, GD2 positive selection and CD45 depletion were performed in two blood samples spiked with Kelly neuroblastoma cells with a 15% and 2.5% concentration of neuroblastoma cells. The total gated events collected over 60 seconds before and after the immunomagnetic separation were used to estimate recovery as per the formula described in the 'Methods' section.

For the spiking experiment with 15% concentration of neuroblastoma cells, 1.5 ml of Kelly cell suspension (containing 2.7×10^6 cells) were added to 5 ml of blood (containing 25×10^6 white blood cells). Using CD45 depletion 29.3% of cells were recovered compared to only 6.4% of cells recovered using GD2 positive selection.

An estimation of total cell numbers that would be available for lysis and further protein assays was performed. Using CD45 depletion it was estimated that 7.9×10^5 viable cells could be isolated whereas only 1.7×10^5 viable cells could be obtained after GD2 positive selection. (Figure 23)

For the spiking experiment with 2.5% concentration of neuroblastoma cells, 0.4 ml of Kelly cell suspension (containing 0.7×10^6 cells) were added to 5 ml of blood (containing 25×10^6 white blood cells). Using CD45 depletion 6.9% of cells were recovered compared to only 2.1% of cells recovered using GD2 positive selection.

An estimation of total cell numbers that would be available for lysis and further protein assays was then performed. Using CD45 depletion it was estimated that 0.5×10^5 viable cells could be isolated whereas only 0.15×10^5 viable cells could be obtained after GD2 positive selection. (Figure 23)

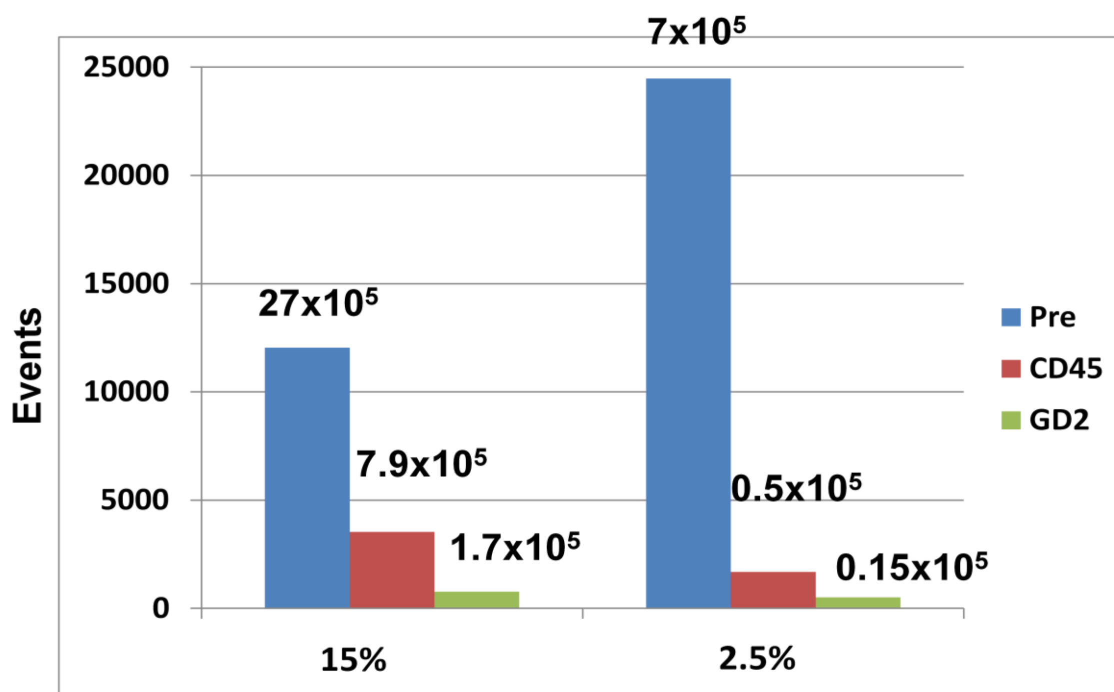


Figure 23 shows an estimation of cell recovery with CD45 depletion (depicted 'CD45' in red) and GD2 positive selection (depicted 'GD2' in green) in the two experiments spiking neuroblastoma cells in peripheral blood at two different concentrations, 15% and 2.5%. Each of the experiments with 15% and 2.5% purity (pre-separation) is represented in the X axis. The number of flow cytometry events (R3+R4) as described in the 'Methods' is shown in the Y axis. Blue bars represent the sample before the immunomagnetic separation, red bars the samples after CD45 depletion and green bars the samples after GD2 positive selection. In the experiment using 15% purity as baseline, 29.3% of cells were recovered with CD45 depletion vs. 6.9% of cells recovered with GD2 positive selection in the experiment using 2.5% as baseline purity, 6.9% of cells were recovered with CD45 depletion compared to 2.1% of cells recovered with GD2 positive selection. Taking into account the known cell numbers used to set the experiments, the estimated cell numbers after CD45 depletion and GD2 positive selection are plotted on top of the bars. Mean±SD recovery was 18.1%±0.16 for CD45 depletion vs. 4.5%±0.03 for GD2 positive selection (p=0.35).

Despite significant cell loses using both methods which were attributable to the manipulations of the procedure, CD45 depletion showed to be superior to GD2 positive selection. The estimated number of cells isolated after CD45 depletion was sufficient for protein analyses. Protein signals for the PI3K/AKT pathway and MYCN protein expression were detected in the lysates generated after CD45 depletion as is described in the relevant sections of the 'Results' below. (Table 12 and Figures 28, 40 and 43)

The GD2 positive selection required an additional step pushing the plunger manually to extract the labelled neuroblastoma cells fraction. This introduced a significant amount of variability. This was shown in an experiment with neuroblastoma Kelly cells only, where the same methodology was followed three times and the step where the plunger is pushed to detach the labelled cells from the column was performed differently: a hard push, a soft push and no push, just allowing dripping. Significant variability was seen with this step. (Figure 24)

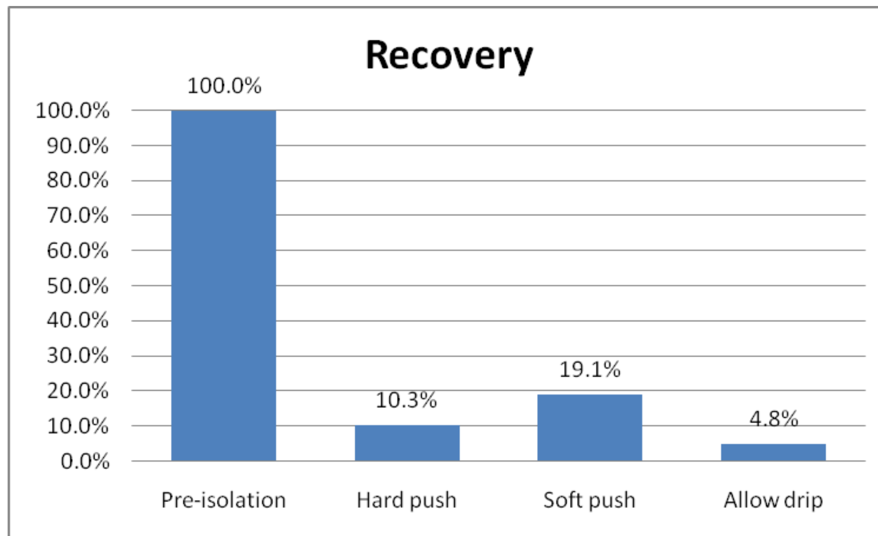


Figure 24, shows an experiment with Kelly cell suspensions only before ('Pre-isolation') and after GD2 positive selection. The same GD2 positive selection was performed three times, only differing on the step that manually plunged the labelled cells from the magnetic column: the plunger was pushed hardly ('Hard push'), softly ('Soft push') and the plunger was not used allowing the sample to drip ('Allow drip'). Significant variability in the recovery is seen with the three steps. The percentages shown are the average of five measurements of cell numbers.

2.2.6 Linearity

Previous reports developing methodologies for the detection of circulating tumour cells in peripheral blood demonstrated linearity although this had never been shown in tumour cells isolated from bone marrow samples [71, 107]. No linear relationship was found between the purity of neuroblastoma cells before and after the CD45 depletion procedure. (Figure 25)

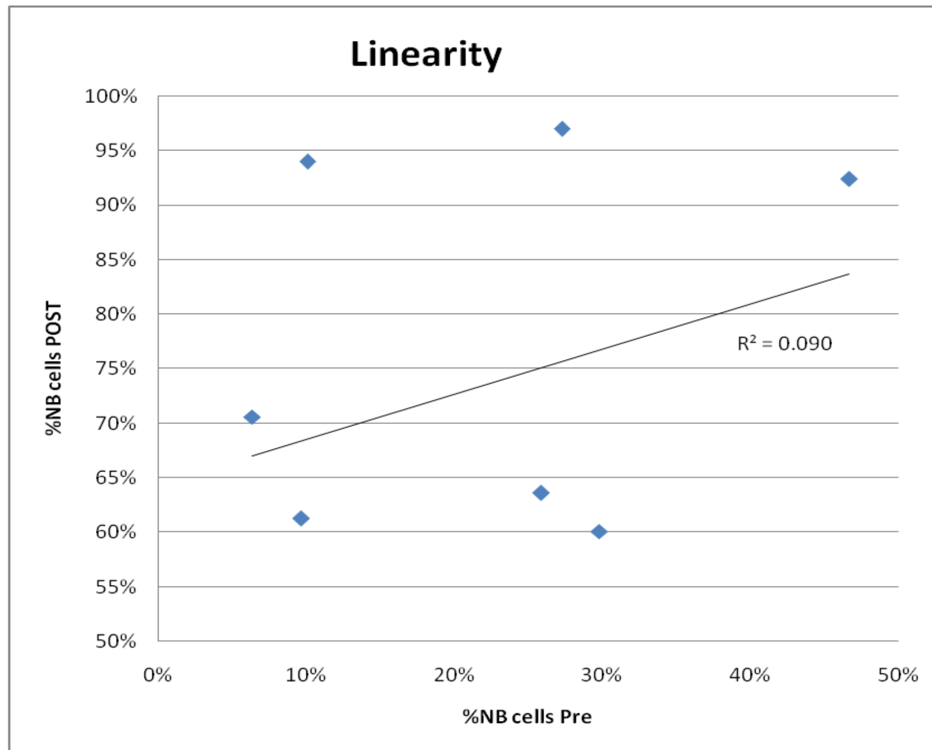


Figure 25, shows the linearity of the purity of neuroblastoma cells before and after CD45 depletion. The X axis represents the purity of the samples before the immunomagnetic separation and the Y axis the purity of the samples after the separation. No linear relation was found, R^2 = 0.09, not significant.

In summary, the immunomagnetic separation experiments showed that in peripheral blood spiked with neuroblastoma cells, CD45 depletion achieved superior purity and better recovery compared to GD2 positive selection. Moreover, CD45 depletion was able to achieve high purity in bone marrow samples spiked with neuroblastoma cells and bone marrow samples from patients with involvement by metastatic neuroblastoma.

2.3. Optimization of sample collection and processing procedures

In order to optimise the assay, several issues related to collection and processing of samples were studied.

2.3.1 Collection tubes

The performance of three methods to separate mononuclear cell fraction was compared:

- A commercially available Ficoll collection tube (BD Vacutainer CPT®) where the Ficoll reagent is a solid gel permitting a 1-step Ficoll separation: once the blood or bone marrow is collected into the heparinised tube, the centrifugation is done immediately and the PBMC layer can be collected easily as is separated by the solid gel. (Figure 26)
- A commercially available Oncoquick® tube which is a variation of a Ficoll separation method where the density gradient is optimised to obtain the best purity of tumour cells (as they have a specific density) [115-116]. (Figure 27)
- Collection in heparinised tubes (preservative-free heparin) and manual Ficoll separation [117].

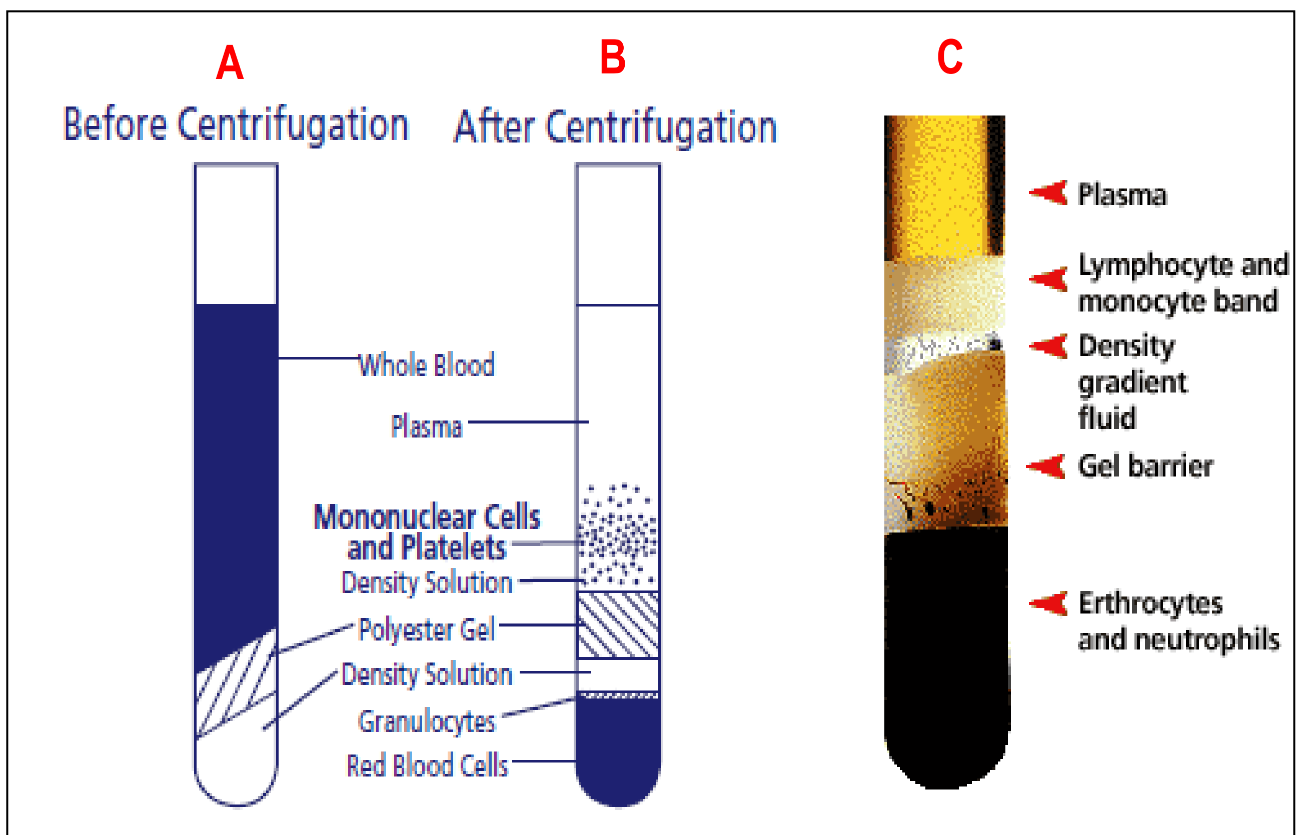


Figure 26 shows the BD Vacutainer CPT® Ficoll separation collection tubes. Panel A shows a schematic representation of the collection of blood before centrifugation. From bottom to top, the first layer is the density solution (Ficoll), and then a layer of Polyester gel and the blood collected. Panel B shows the tube after one step of centrifugation where (from bottom to top) the first layer is the red blood cells, followed by the granulocytes and the density solution, then the Polyester gel, density solution and the plasma with the mononuclear cell, platelets and tumour cells. Panel C shows a picture of the blood tube after centrifugation with the same layers than B.

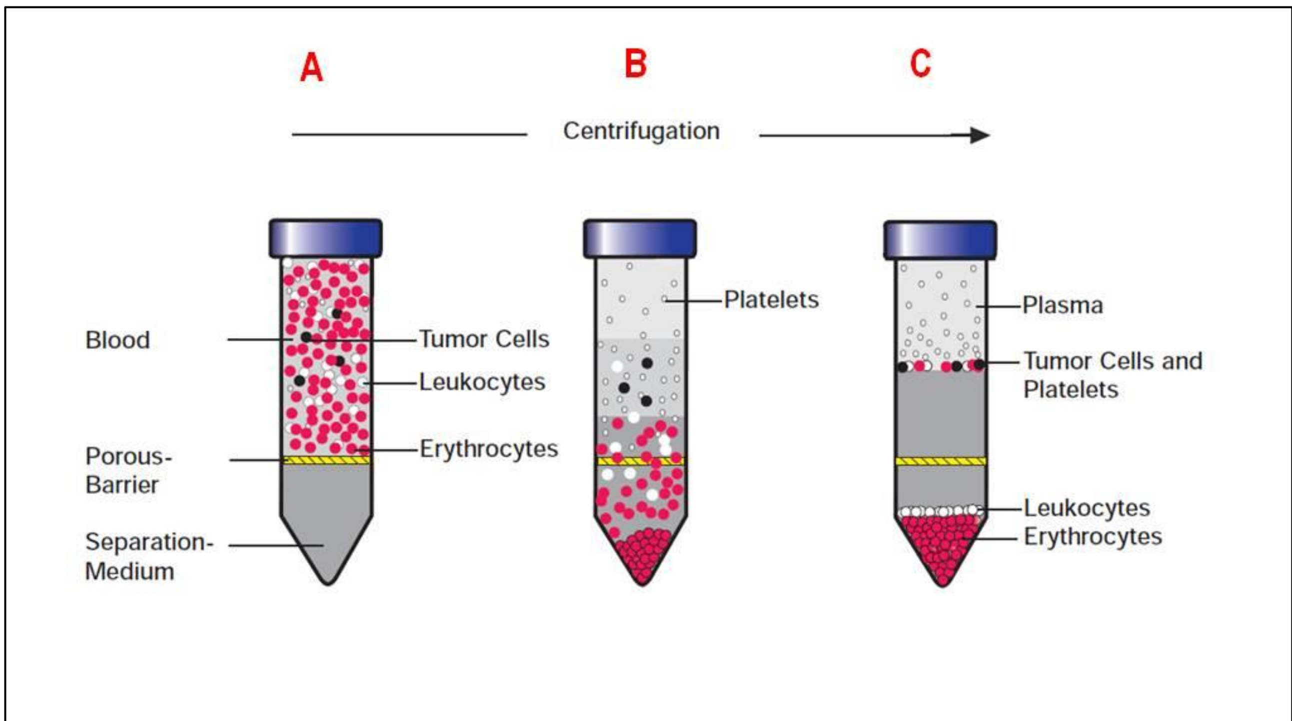


Figure 27 shows a representation of blood with circulating tumour cells collected with Oncoquick tubes. A represents the sample before centrifugation, where blood is mixed with the tumour cells and separated by a porous barrier from the separation medium. B shows an intermediate snapshot during centrifugation where the red blood cells and leukocytes are accumulating in the bottom of the tube and C shows the final result after centrifugation where the fraction containing tumour cells and platelets is separated of the leukocytes and red blood cells by the separation medium.

For this experiment, Kelly cells were spiked in peripheral blood with 25% purity. The collection with BD Ficoll tubes reproduced this level of purity achieving 25.9% purity (5598/15974 events) whereas Oncoquick tubes achieved 34.0% purity (7966/15473 events). In spiking experiments in peripheral blood it was found that Oncoquick tubes were able to provide a moderate improvement in the proportion of neuroblastoma cells within the same sample.

However, when those tubes were tested with BM samples, it was found that clots occurred very frequently in this type of sample with both Oncoquick and BD Vacutainer CPT tubes. It was believed that the cause of this was the small amount of anticoagulant in these commercial tubes, as they are designed for whole blood and not for bone marrow collection. The production of clots had two major disadvantages: - clots blocked the magnetic column, therefore hampering the immunomagnetic separation procedure and also increased the contamination by red cells which then interferes other protein assays. (Table 10)

Collection tubes	Observations
BD Vacutainer CPT tubes (n=4)	Frequent clots, unable to process the sample
Oncoquick (n=1)	Frequent clots made unable to process the sample
PFH tube, manual Ficoll (n=4)	No clots, adequate cell suspension, longer processing time

Table 10 shows clinical observations made when performing collection of bone marrow samples with BD Vacutainer CPT, Oncoquick and PFH tubes with a manual (2-step centrifugation) Ficoll separation. PFH: Preservative-free heparin.

Therefore the finally proposed collection method consisted of tubes with 1 ml of preservative-free heparin and a manual Ficoll separation given that the increase in anticoagulant would avoid clots and provide better cell suspensions.

2.3.2 MS compared to LD columns

One potential disadvantage of using the CD45 depletion procedure is that the amount of CD45 positive events in a blood/bone marrow sample is very large and hence the column could be saturated impairing the ability to enrich the samples. This has been particularly relevant for the detection of methods to isolate circulating tumour cells which are very rare events [107]. Therefore, MS columns (standard) with LD columns (suitable for larger amounts of events) were compared in a peripheral blood spiking experiment with 2 ml samples (same volume as clinical BM samples).

The same sample with a 36.5% neuroblastoma cells (1573/4308 events) was ran through two different columns. CD45 depletion achieved a purity of 77.0% (3904/4540 events) using the MS column and 57.0% purity using the LD column (538/944 events).

For this volume of blood/bone marrow, the use of LD columns did not improve the purity or recovery of the experiment, therefore MS columns were chosen.

2.3.3 Sample stability

The ratio of the events that were gated in R1 (events that correspond to live cells that will be analysed with CD45 and GD2 to determine purity) and the total number of events detected by the system was compared. This ratio would estimate the proportion of 'live' cells that are suitable for analysis and not dead cells or cellular debris. (Table 11)

This ratio was compared between samples analysed within four hours on the same day and samples stored in the fridge at 4°C for 24 hours. The average ±SD ratio of R1 gated cells was 51.7% ± 22.3 for samples processed in the same day and 11.1% ± 1.3 for those processed after 24 hours (t Student, $p=0.01$). This demonstrated that samples can be processed up to 24 hours, but the proportion of cell losses significantly increases with time.

	Total events	Events gated (R1)	% of total events
Processing in the same day within 4 hours (n=4)	10000	6915	69.2%
	72032	52419	72.8%
	27345	9636	35.2%
	34711	10357	29.8%
Processing after 24 h (n=4)	261101	26739	10.2%
	265646	26249	9.9%
	234293	28782	12.3%
	240247	29200	12.2%

Table 11, shows the ratio between the events gated (R1) for analysis (viable cells) and the total events detected by the flow cytometer, reflecting the proportion of events that are cellular. The significant decrease in this ratio when processing was delayed 24 hours reflects significant cell loses.

2.4. Quantification of protein content in isolated samples

Pellets from cell lines with high cellular content were lysed up to a total volume of 200 µL of lysis buffer (100% 'Cooking buffer'). PB and BM samples provided smaller pellets and were lysed up to a volume of 100 µL of lysis buffer.

BCA analysis was performed in all samples and the amount of total protein and volumes required from each sample was calculated to ensure that the immunomagnetic separation procedure was able to obtain sufficient amounts of tissue to carry out protein analyses.

Mean, SD, median and range of total protein concentration for the BCA analyses performed in cell lines, spiked samples before and after the separation are shown in Table 12. Volume of lysate required for each of those categories is shown in Table 12 as well.

	Total protein concentration in the lysates (µg/µL)		Sample volume		Suitable for 4X protein analyses
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	
Cell lines (n=19)	7.6 ± 4.3	6.3 (2.4 – 17.4)	10.2 ± 5.2	9.6 (3.5 – 25.0)	19/19 (100%)
Samples Pre (n=7)	9.1 ± 6.5	8.4 (1.1 – 20.8)	10.3 ± 8.6	7.2 (2.9 – 28.3)	6/7 (85.7%)
Samples Post (n=4)	5.7 ± 3.2	5.4 (2.4 – 9.7)	13.7 ± 8.3	11.7 (6.2 – 25.1)	3/4 (75%)

Table 12, shows the results of BCA analysis on lysates obtained on cell lines and on samples before (Pre) and after (Post) immunomagnetic separation with CD45 depletion. Cell line lysates had a total volume of 200 µL and Pre- and Post- lysates had a total volume of 100 µL The total protein concentration (2nd and 3rd columns) in the 100 µL was calculated as well as the sample volume (4th and 5th columns) that would be required to perform a protein analysis using 20 µg per 50 µl well of total protein which is our standard for protein assays. The column on the right (6th column) shows the proportion of the samples that would have been suitable to perform four analysis, i.e. allow to perform two different protein assays in duplicate.

There were no statistically significant differences in protein concentration and sample volume between cell line lysates compared to spiked samples: mean ± SD total protein concentration was 7.6 ± 4.3 for cell lines vs. 7.9 ± 5.6 µg/µL for spiked samples (p=0.88) and mean ± SD sample volume was 10.2 ± 5.2 for cell lines vs. 11.5 ± 8.2 µL for spiked samples (p=0.59).

There was a decrease in protein concentration (as well as an increase in required sample volume) in spiked samples post-separation compared to samples pre-separation, although those differences were not statistically significant: mean ± SD total protein concentration was 9.1 ± 6.5 for samples pre-separation vs. 5.7 ± 3.2 µg/µL for samples post-separation (p=0.36) and mean ± SD sample volume was 10.3 ± 8.6 for samples pre-separation vs. 13.7 ± 8.3 µL for spiked samples (p=0.59). (Table 12 and Figure 28)

All lysates were suitable to perform up to three protein assays (i.e. ELISA, MSD and the repeat of one of them), but samples would not allow to perform both assays in duplicate in all cases. (Table 12)

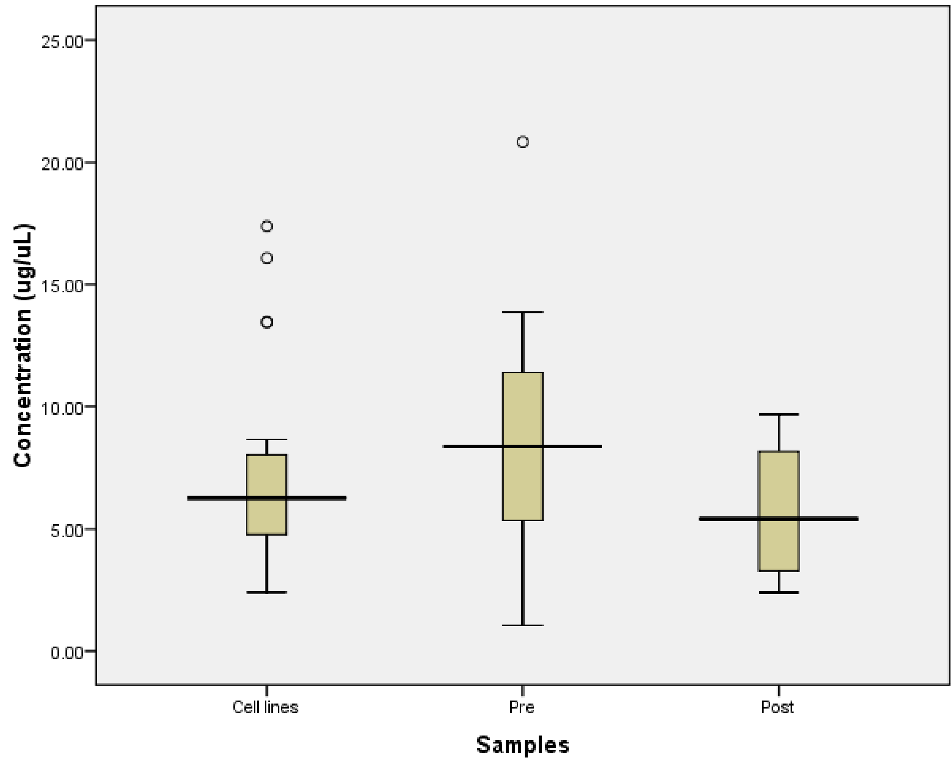


Figure 28 shows the results of BCA analyses performed in lysates from cell lines (left), samples before the immunomagnetic separation (centre) and after the immunomagnetic separation (right) corresponding to the data detailed in Table 12. The Y axis represents the total protein concentration in µg/µL for the lysates obtained (200 µL for cell lines and 100 µL for the Pre- and Post- lysates). The box plot shows how the total protein concentration was lower (Table 12) in the samples after the immunomagnetic separation.

3. Development of a quantitative ELISA to measure total MYCN protein expression in neuroblastoma tumour cells from bone marrow samples

The aim was to develop a quantitative/semiquantitative methodology to measure total MYCN protein levels in neuroblasts isolated from bone marrow samples. There are no commercially available technologies for this purpose and published reports on MYCN protein expression have used non quantitative methods – immunohistochemistry (IHC) or Western blotting (WB) [118-119]. There is a number of existing commercial polyclonal and monoclonal antibodies against MYCN.

The objectives were:

1. To initially test a one-phase ELISA (with a MYCN polyclonal antibody)
2. To establish a two-phase sandwich ELISA (combining a MYCN monoclonal with a polyclonal antibody) addressing issues with specificity with the one-phase ELISA.
3. To establish sensitivity, standard curves and correlation with cell numbers
4. To optimise of the assay by :
    - Determining the best protein concentration
    - Establishing adequate standards and quality controls
    - Determining the impact of the immunomagnetic separation procedure in the assay results

3.1. Testing of SC-791 alone

Initially an indirect ELISA approach using the MYCN polyclonal antibody SC-791 was tested as primary antibody against serial dilutions of MYCN recombinant protein (Abnova) and neuroblastoma cell lines and the results were compared to Western blotting.

Western blotting with SC-791 showed that this antibody was be able to detect MYCN protein expression in those cell lines with known MYCN expression (Kelly and SHEP-DBL) and did not detect the protein in those with no expression of MYCN (SHEP and HeLa). (Figure 29)

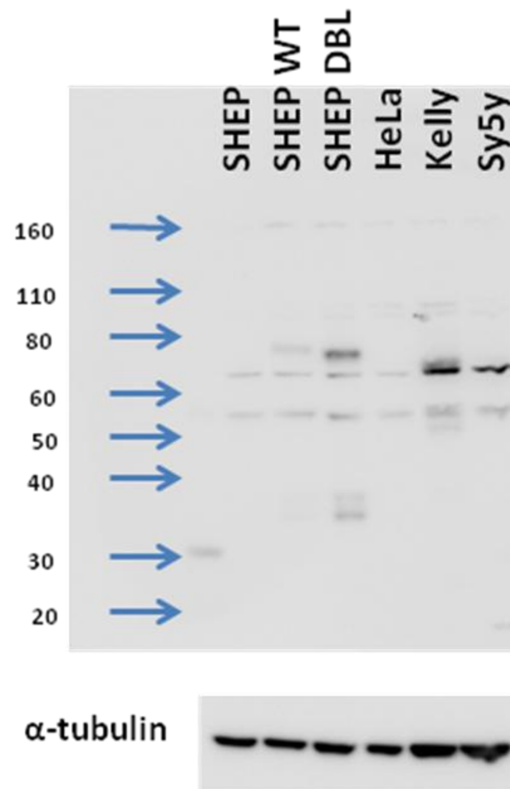
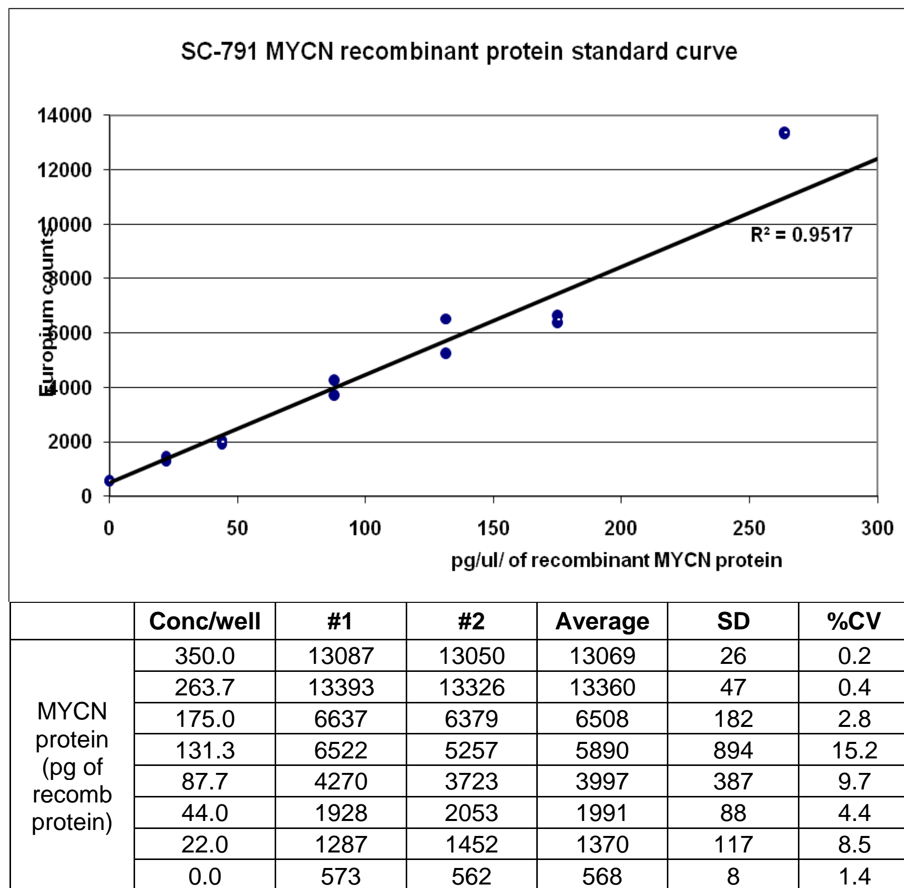


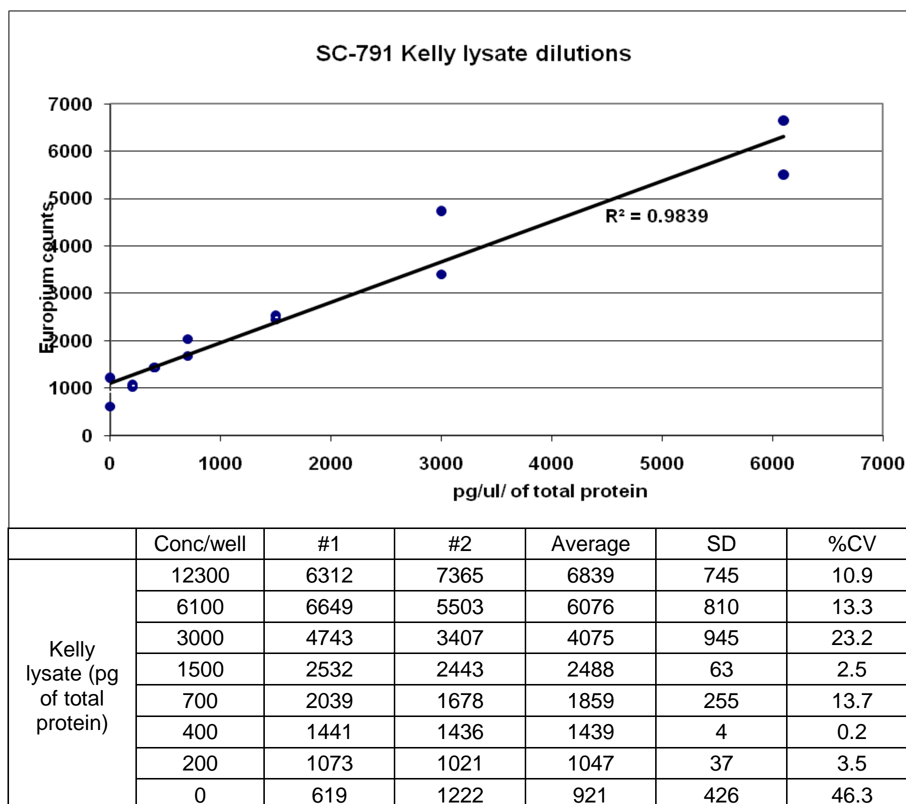
Figure 29 shows a Western blotting for MYCN expression using the anti-MYCN polyclonal antibody SC-791. Five neuroblastoma cell lines and the ovarian cancer cell line HeLa were tested. The arrows represent the molecular weight bands (kDa). MYCN appears in two bands (two isoforms) between 60 and 80. As reported previously, Kelly, SY5Y and SHEP DBL cell lines showed high expression of MYCN whereas SHEP and HeLa cell lines were negative. Protein expression was weakly seen in SHEP WT.

Therefore, the indirect ELISA on a Europium-based platform was used to measure MYCN recombinant protein (Abnova) and lysates of neuroblastoma cell lines. Although the standard curve using the recombinant protein showed a linear curve (R^2=0.95, Figure 30) and a signal was detectable at small amounts of protein (Figure 31), the assay proved to lack specificity (Figure 32).



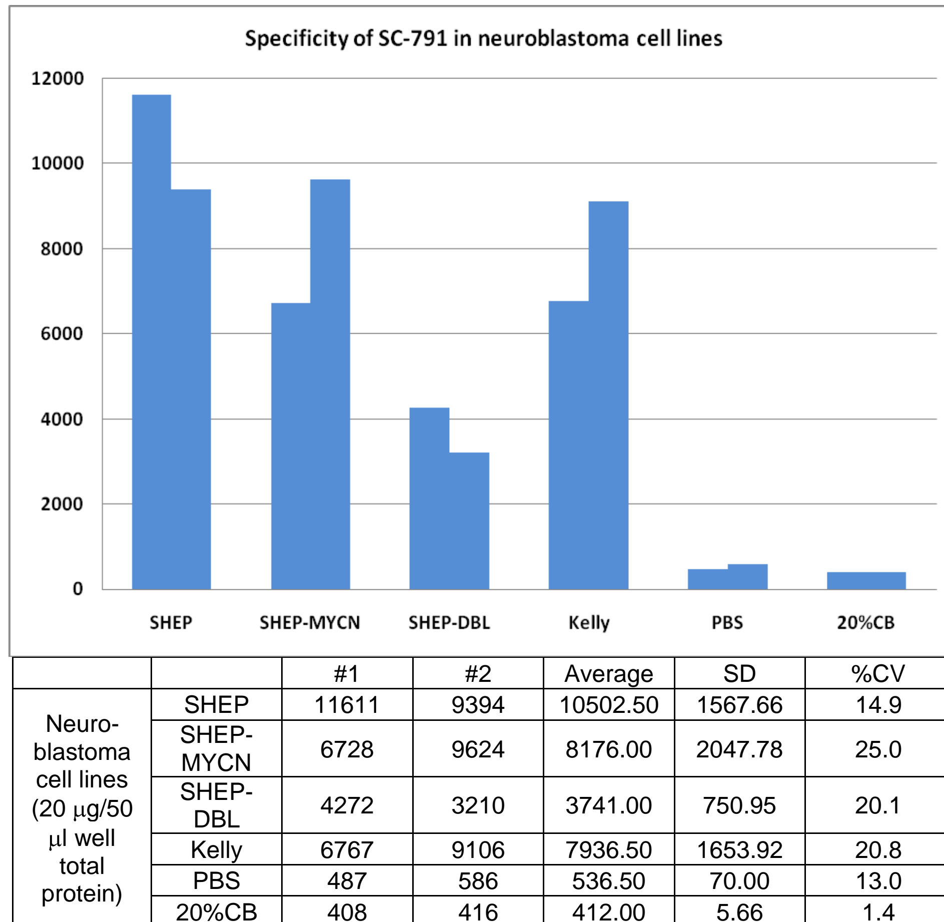
	Conc/well	#1	#2	Average	SD	%CV
MYCN protein (pg of recomb protein)	350.0	13087	13050	13069	26	0.2
	263.7	13393	13326	13360	47	0.4
	175.0	6637	6379	6508	182	2.8
	131.3	6522	5257	5890	894	15.2
	87.7	4270	3723	3997	387	9.7
	44.0	1928	2053	1991	88	4.4
	22.0	1287	1452	1370	117	8.5
	0.0	573	562	568	8	1.4

Figure 30, shows the standard curve for the one-phase ELISA performed with the anti-MYCN polyclonal antibody SC-791 with the raw data in the table below the figure (experiments in duplicate). Dilutions of the MYCN recombinant protein are shown in the X axis and Europium-counts on the Y axis.



	Conc/well	#1	#2	Average	SD	%CV
Kelly lysate (pg of total protein)	12300	6312	7365	6839	745	10.9
	6100	6649	5503	6076	810	13.3
	3000	4743	3407	4075	945	23.2
	1500	2532	2443	2488	63	2.5
	700	2039	1678	1859	255	13.7
	400	1441	1436	1439	4	0.2
	200	1073	1021	1047	37	3.5
	0	619	1222	921	426	46.3

Figure 31 shows serial dilutions of a Kelly cell line lysate for the one-phase ELISA performed with the anti-MYCN polyclonal antibody SC-791 with the raw data in the table below the figure (experiments in duplicate). Serial dilutions of the Kelly cell line lysate are shown in the X axis and Europium-counts on the Y axis.



		#1	#2	Average	SD	%CV
Neuro-blastoma cell lines (20 µg/50 µl well total protein)	SHEP	11611	9394	10502.50	1567.66	14.9
	SHEP-MYCN	6728	9624	8176.00	2047.78	25.0
	SHEP-DBL	4272	3210	3741.00	750.95	20.1
	Kelly	6767	9106	7936.50	1653.92	20.8
	PBS	487	586	536.50	70.00	13.0
	20%CB	408	416	412.00	5.66	1.4

Figure 32, shows the specificity of the one-phase MYCN ELISA performed with the anti-MYCN polyclonal antibody SC-791. Four different cell lines were tested (X axis), two with high known expression of MYCN (Kelly and SHEP-DBL), one with low expression (SHEP-MYCN) and one with no expression of MYCN (SHEP), as well as two buffers, PBS and 20% ‘Cooking Buffer’ (‘20% CB’). The Y axis represents Europium counts. Raw data for the experiments in duplicate are shown below. The one-phase ELISA was not specific and was not able to differentiate the MYCN-expressing cell lines from the non-MYCN expressing ones.

3.2. Testing of a sandwich ELISA

Given that there was lack of specificity of the one-phase ELISA the assay was modified to a sandwich ELISA combining a monoclonal and a polyclonal antibody for the detection of MYCN.

B8.4 monoclonal antibody produced stronger signals on Western blotting than the OP13 monoclonal antibody. (Figure 33)

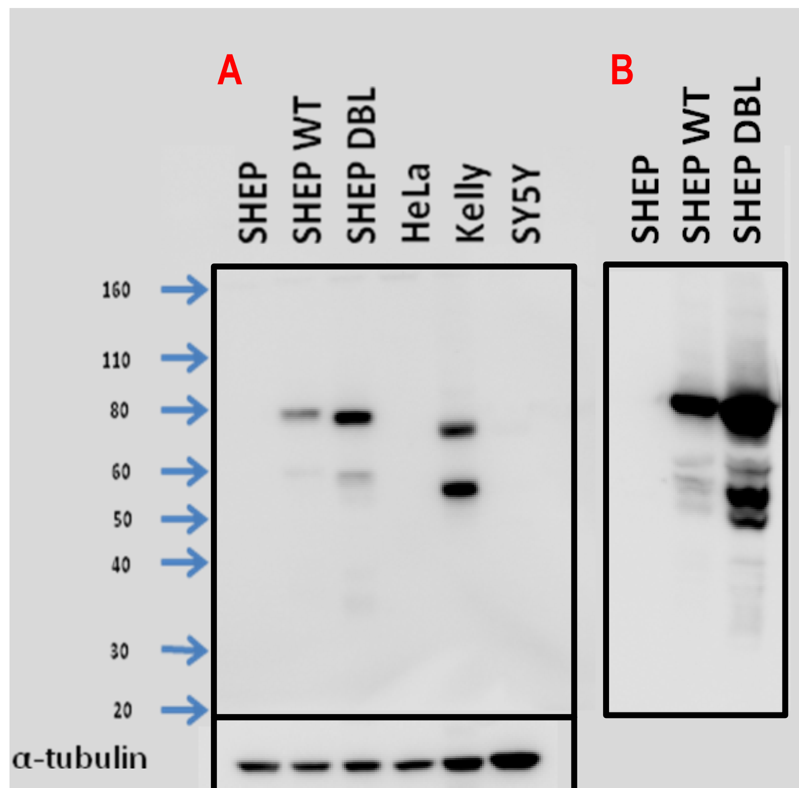
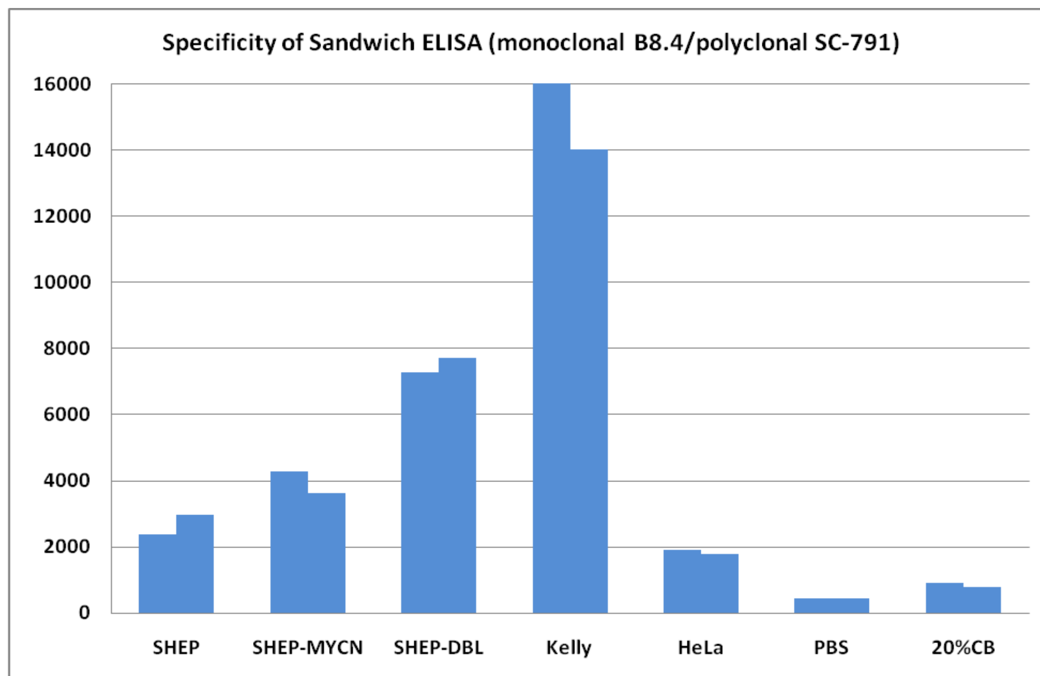


Figure 33, shows a Western blotting for total MYCN expression comparing the anti-MYCN monoclonal antibodies OP13 (A) and B8.4 (B). In both cases, the secondary anti-MYCN antibody was the polyclonal antibody SC-791. Five neuroblastoma cell lines and the ovarian cancer cell line HeLa were tested. The arrows represent the molecular weight bands (kDa). MYCN appears in two bands (two isoforms) between 60 and 80. Non-expressing MYCN cell lines (SHEP and HeLa) were negative for expression and MYCN-expressing cell lines (SHEP WT, SHEP DBL and Kelly) were positive.

With the sandwich ELISA (Figure 34) cell lines with high expression of MYCN protein (Kelly) showed a 5.65-fold increase in signals compared to non-MYCN expressing cells (SHEP). A level of ~2000 Europium counts was defined as background noise signal.

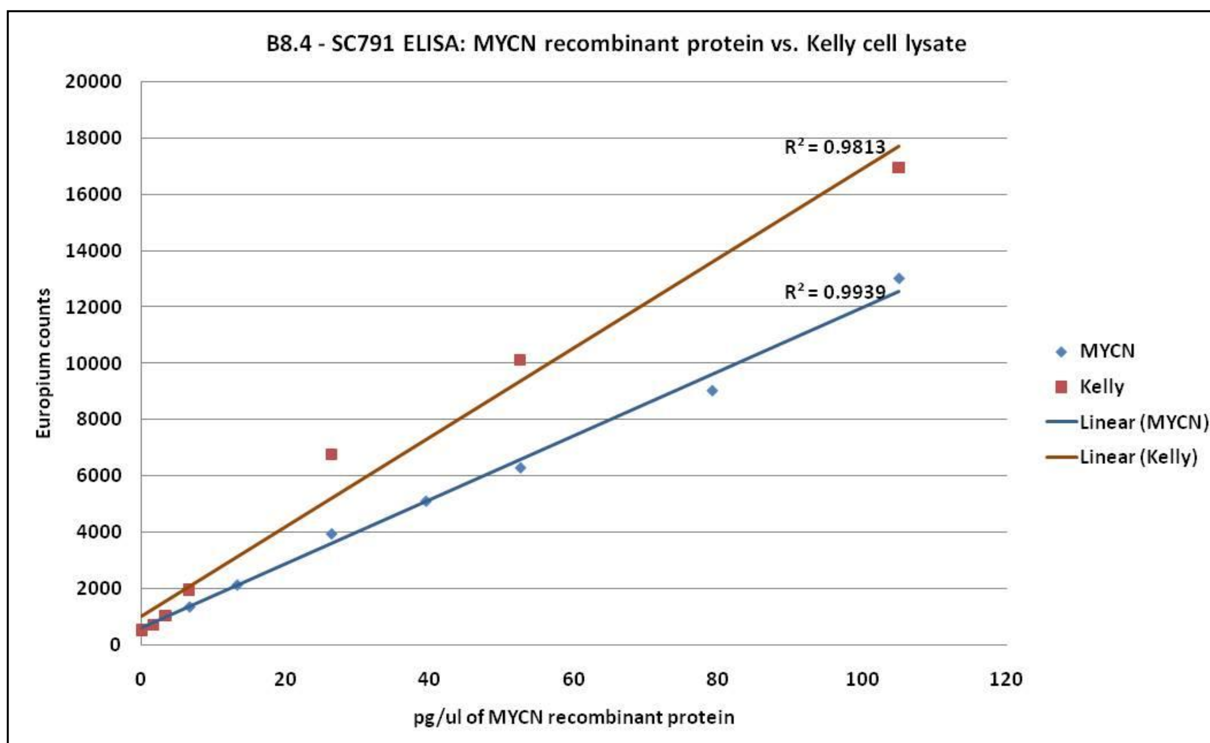


	#1	#2	Average	SD	%CV
SHEP	2396	2967	2681.50	403.76	15.1
SHEP-MYCN	4279	3629	3954.00	459.62	11.6
SHEP-DBL	7268	7711	7489.50	313.25	4.2
Kelly	16293	14031	15162.00	1599.48	10.5
HeLa	1914	1777	1845.50	96.87	5.2
PBS	439	456	447.50	12.02	2.7
20%CB	920	802	861.00	83.44	9.7

Figure 34 shows the specificity of the sandwich MYCN ELISA performed with the anti-MYCN monoclonal antibody B8.4 and the polyclonal antibody SC-791. Five different cell lines were tested (X axis), two with known high expression of MYCN (Kelly and SHEP-DBL), one with low expression (SHEP-MYCN) and two with no expression of MYCN (SHEP and HeLa), as well as two buffers, PBS and 20% ‘Cooking Buffer’ (‘20%CB’). The Y axis represents Europium counts. Raw data for the experiments in duplicate are shown below. The sandwich ELISA showed specificity, where signals for Kelly cells (MYCN expressing) were 5.65-fold higher than SHEP cells (MYCN non-expressing).

3.3 Sensitivity, standard curves and correlation with cell numbers

The lower level of sensitivity of the ELISA assay to detect MYCN protein was then assessed, and the signals obtained with MYCN recombinant protein were correlated with those obtained in Kelly cell lysates (Figure 35). This would allow an estimation of the minimum number of neuroblastoma cells required for a valid analysis of MYCN protein expression.



Raw data MYCN standard Curve					
Conc/well	**Standard**	**Standard**	**Average**	**SD**	**%CV**
pg/µL	**1**	**2**			
105.0	11174	14867	13021	2611	20.1
79.1	8917	9164	9041	175	1.9
52.5	6638	5973	6306	470	7.5
39.4	5457	4791	5124	471	9.2
26.3	3651	4272	3962	439	11.1
13.2	2159	2138	2149	15	0.7
6.6	1225	1519	1372	208	15.2
0.0	487	586	537	70	13.0
Raw data Kelly lysate dilutions					
Conc/well	**Standard**	**Standard**	**Average**	**SD**	**%CV**
pg/µL	**1**	**2**			
20.0	17950	15928	16939	1430	8.4
10.0	10377	9839	10108	380	3.8
5.0	5857	7660	6759	1275	18.9
2.5	2335	1614	1975	510	25.8
1.3	1091	993	1042	69	6.7
0.0	695	753	724	41	5.7
0.0	484	608	546	88	16.1

Figure 35, shows the standard curve for the sandwich ELISA performed with the anti-MYCN monoclonal antibody B8.4 and the polyclonal antibody SC-791. Serial dilutions of MYCN recombinant protein (blue dots and line) and Kelly cell lysate (red dots and line) are plotted with their respective R^2. The Y axis represents Europium (Eu) counts and the X axis increasing concentrations of MYCN recombinant protein. The raw data for both standard curves is shown in the table below the figure (experiments in duplicate).

Table 13 shows the levels of MYCN protein expression correlated with the detected signals for Kelly cell lysates with different cell numbers.

A level of 2000 Europium counts was considered background noise signal, as this was detected with lysates from cell lines without MYCN expression (HeLa or SHEP), (Figure 34). Therefore, detectable and distinguishable MYCN expression levels were detected from Kelly cells when 3.07 $x10^4$ cells (or more) were present per 50 µL well.

30.000 cells was established as the minimum number of neuroblastoma cells required for analysis of MYCN protein expression with this sandwich ELISA. This is equivalent to the signal generated by a 50 μL solution containing 39.4 pg/μL of MYCN recombinant protein alone (i.e. 1.97 ng of MYCN protein).

MYCN protein concentration	**MYCN protein signals**	**Kelly lysate signals**	**Total protein concentration per well**	**Amount of cells per well**
pg/μL	Europium counts	Europium counts	μg per 50 μL well	$x10^4$
		16939	**20**	**12.18**
105	**13021**			
79.1	**9041**	**10108**	**10**	**6.14**
52.5	**6306**			
39.4	**5124**	**6759**	**5**	**3.07**
26.3	**3962**			
13.2	2149	1975	2.5	1.54
6.1	1372	1042	1.25	0.77
0	537	724	0	0

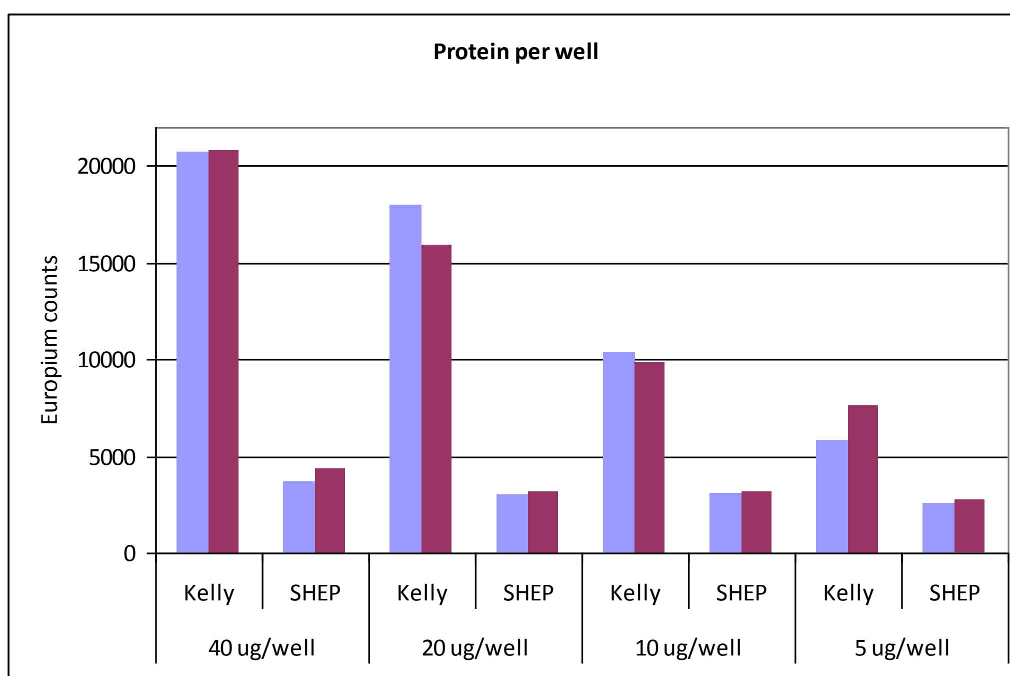
Table 13, shows a numeric comparison between Europium signals of the serial dilutions of MYCN recombinant protein with known concentration and Kelly cell lysates with known cell numbers shown in Figure 35. Given that the background signal was considered to be 2000 Europium counts (Figure 34), the following level where Europium signals were detectable above background was the 3.07×10^4 cells per well (minimum level of sensitivity for cell lines).

3.4 Optimisation of the assay

3.4.1 Ideal protein concentration

The next aim was to determine the level of total protein that maximises the differentiation between MYCN expressing and non-expressing cells. Therefore, signals obtained at 40, 20, 10 and 5 micrograms per well for Kelly and SHEP cells were compared.

The best protein concentration was 20 micrograms per well of total protein. At this level, the signal for MYCN positive cells was maximized while the background generated by SHEP levels was kept stable around 2000 Europium counts. (Figure 36)



		#1	#2	Average	SD	%CV	Kelly/SHEP ratio
40 µg/well	Kelly	20738	20820	20779	58	0.3	5.14
	SHEP	3722	4365	4044	455	11.2	
20 µg/well	Kelly	17950	15928	16939	1430	8.4	5.48
	SHEP	2974	3210	3092	167	5.4	
10 µg/well	Kelly	10377	9839	10108	380	3.8	3.23
	SHEP	3121	3134	3128	9	0.3	
5 µg/well	Kelly	5857	7660	6759	1275	18.9	2.56
	SHEP	2572	2711	2642	98	3.7	

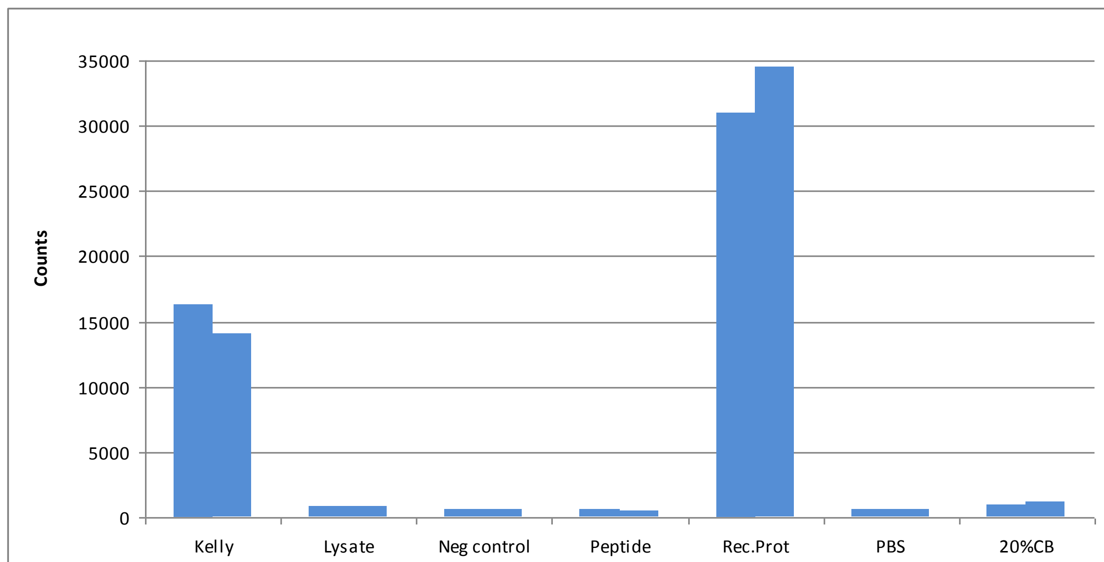
Figure 36 shows the results of a MYCN sandwich ELISA (B8.4 monoclonal / SC791 polyclonal) to determine the best total protein concentration that maximises the signal difference between MYCN expressing and non-expressing cells. Lysates expressing (Kelly) and non-expressing (SHEP) MYCN protein were tested at four different concentrations of total protein (5, 10, 20 and 40 µg per 50 µL well). Raw data is in the table below the figure, the column in the right shows the ratio between the average signal for Kelly lysate and SHEP lysate for each total protein concentration category. Experiments were performed in duplicate. The Y axis represents Europium counts. The figure shows how the concentration of 20 µg of total protein per 50 µg well (used for other ELISA in our lab) gives the maximum signal for Kelly cells (5.48-fold) while maintaining a lower background noise signal for SHEP.

3.4.2 Establishing the best control for standard curve and quality control

Several commercially available products were evaluated: Abnova MYCN recombinant protein, Abcam MYCN peptide and OriGene MYCN lysate to identify the best products to generate a standard curve and a robust quality control for further clinical development of the assay.

The sandwich ELISA combining B8.4 and SC791 antibodies was not able to generate signals for the MYCN peptide or the MYCN lysate. However, MYCN recombinant protein gave strong signals and therefore was chosen to be used as the standard curve. (Figure 37)

Additionally lysates from Kelly cell lines produced robust and constant signals at the concentration of 20 µg/50 µl well of total protein determined by BCA analysis. (Figure 39)



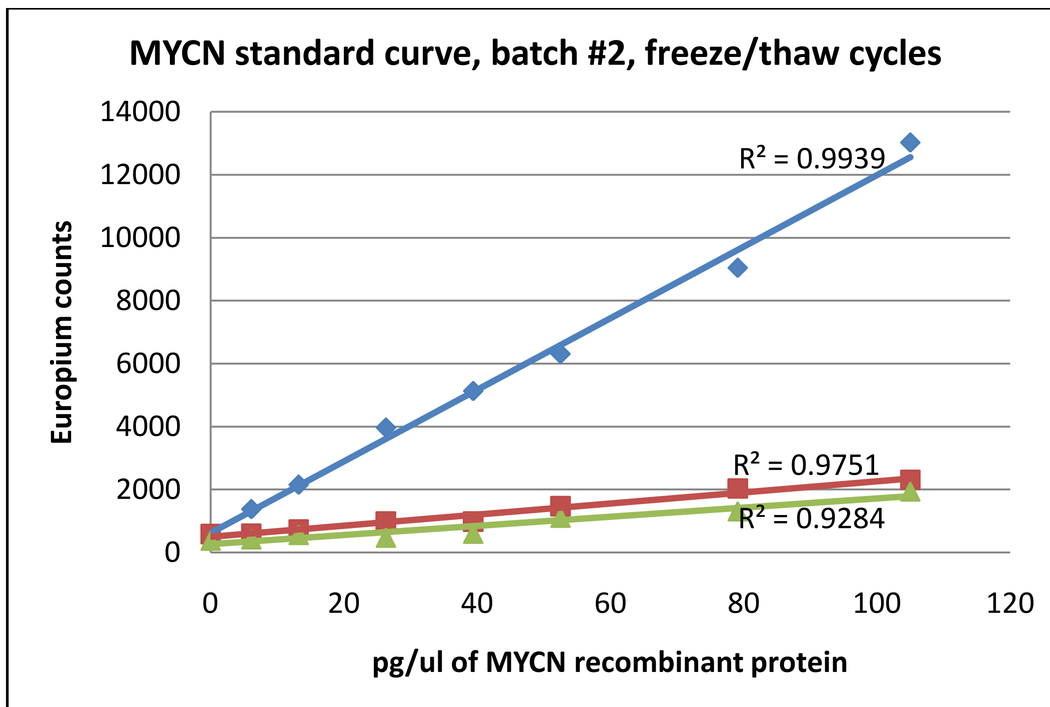
	#1	#2	Average	SD	%CV
Kelly lysate	16293	14031	15162.00	1599.48	10.55
MYCN Lysate	820	741	780.50	55.86	7.16
Negative control for the lysate	564	542	553.00	15.56	2.81
Abcam Peptide	540	497	518.50	30.41	5.86
Abnova recombinant protein (Rec.Prot)	31020	34537	32778.50	2486.89	7.59
PBS	540	567	553.50	19.09	3.45
20%CB	893	1125	1009.00	164.05	16.26

Figure 37, shows the results of a MYCN sandwich ELISA (B8.4 monoclonal / SC791 polyclonal). Different commercially available MYCN products were tested to be used as controls for the ELISA: Origene MYCN lysate ('lysate' and negative control 'neg.control'), Abcam MYCN peptide ('peptide') and Abnova MYCN recombinant protein ('Rec.Prot'), and compared to an in-house Kelly cell lysate ('Kelly'). Raw data is in the table below the figure. Experiments were performed in duplicate. The Y axis represents Europium counts. It was found that the commercial lysate and the peptide were not recognised by the pair of antibodies in this ELISA and only MYCN recombinant protein gave detectable signals.

3.4.3 Quality of the MYCN standard protein

Unfortunately it was found that the signals obtained from MYCN recombinant protein experienced great variability after several cycles of freeze/thawing. (Figure 38)

It was established that upon reception of the new batch of MYCN recombinant protein, aliquots of 20 μL would be performed and stored at -80°C. Prior to each analysis an aliquot is defrosted and 4 μL are required and diluted in 196 μl of PBS. The remaining recombinant protein in the aliquot must be discarded. When smaller aliquots are made, the signals detected fall significantly and this justified the amount of recombinant protein required for each aliquot.



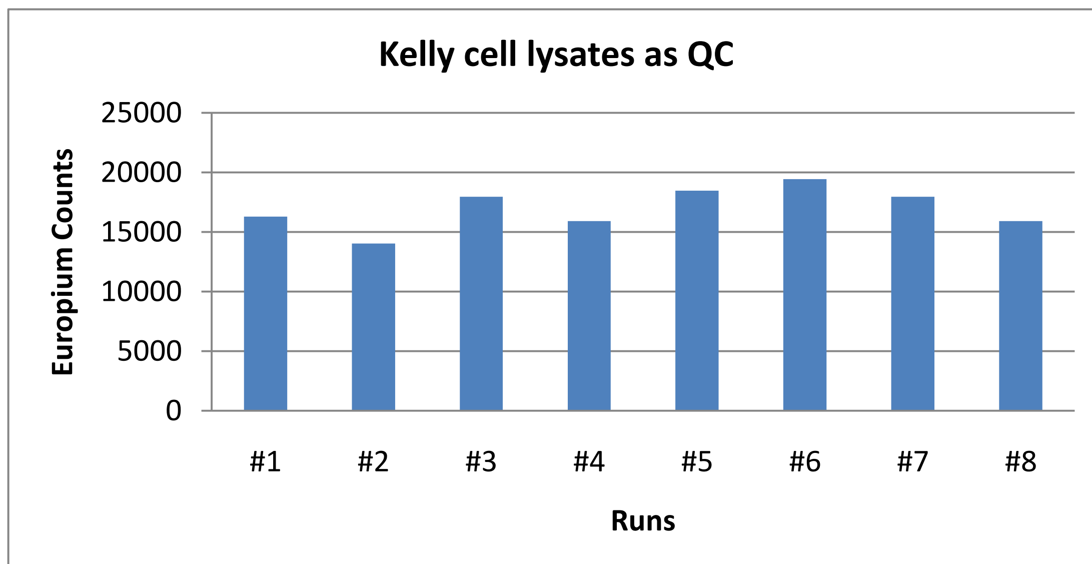
MYCN protein concentration	Freeze/Thaw cycles		
pg/ul	1st	2nd	3rd
105	13020.5	2305.5	1938
79.1	9040.5	2031.5	1308
52.5	6305.5	1471	1105
39.4	5124	982.5	595
26.3	3961.5	980	471
13.2	2148.5	733	557
6.1	1372	597.5	418
0	536.5	586	375

Figure 38 shows the result of a MYCN sandwich ELISA (B8.4 monoclonal / SC791 polyclonal). A standard curve using the same batch of MYCN recombinant protein was performed after one (blue dots and line), two (brown dots and line) and three (green dots and line) freeze/thaw cycles. This experiment showed that signals from Abnova MYCN recombinant decrease significantly after more than one freeze/thaw cycles and therefore after one use, this product should not be used for the standard curve after one freeze/thaw cycle. Values plotted in the line are the average of the duplicate analyses for each standard curve. Raw data (average of the duplicate analysis) is in the table below the figure. The Y axis represents Europium counts and the X axis increasing concentrations of MYCN recombinant protein.

3.4.4 Reproducibility of the QC using Kelly lysates

We analysed MYCN protein expression for four different batches of in-house Kelly lysates (in duplicate, all at 20 µg/50 µL well of total protein concentration) and obtained consistent results (average 16996.1, standard deviation 1750.34, coefficient of variation 10.3%). (Figure 39)

Therefore, it was decided that in-house lysates from Kelly cell lines would be used as quality controls. Each time a new batch was lysed, a level for high, medium and low quality controls would be established de novo.



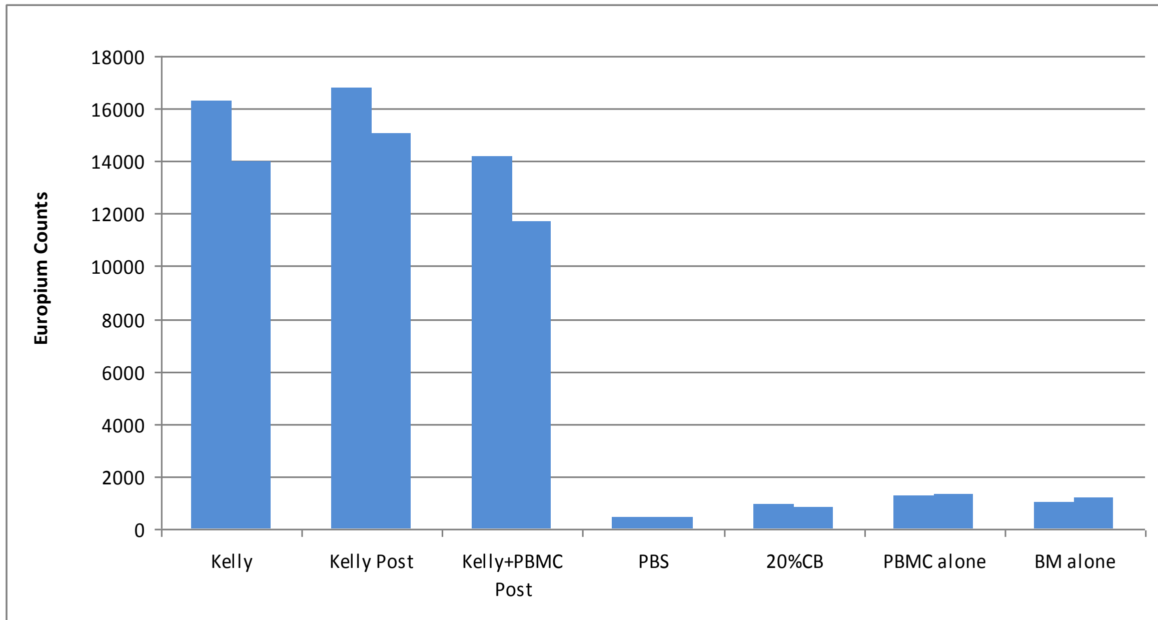
Batch	1		2		3		4	
Bar	#1	#2	#3	#4	#5	#6	#7	#8
Europium count	16293	14031	17950	15928	18462	19427	17950	15928

Figure 39 shows the results of a MYCN sandwich ELISA (B8.4 monoclonal / SC791 polyclonal) performed using in four different batches of lysates (in duplicate, plotted in the X axis) from Kelly cell lines: 1 (#1 and #2), 2 (#3 and #4), 3 (#5 and #6) and 4 (#7 and #8). Y axis represents Europium counts. All four lysates showed similar range of signals. Average ± SD = 16996.13 ± 1750.34.

3.4.5 Effect of the immunomagnetic separation in the detection of signals

Finally, the aim was to demonstrate that the immunomagnetic separation procedure did not alter total MYCN protein signals.

MYCN protein signals were not significantly altered after the immunomagnetic separation procedure using CD45 depletion (15162 ± 1599.48 compared to 15934.50 ± 1262.19, p=0.65). Additionally, signals were conserved when cells were spiked into peripheral blood and were different from peripheral blood (10.3-fold) alone [12950 ± 1736.55 for Kelly+peripheral blood vs. 1254.5 ± 50.20 for peripheral blood alone (p=0.01)] or bone marrow alone (11.9-fold) [12950 ± 1736.55 for Kelly+peripheral blood vs. 1083.50 ± 127.99 for bone marrow alone (p=0.01)]. (Figure 40



	#1	#2	Average	SD	%CV
Kelly	16293	14031	15162.00	1599.48	10.55
Kelly Post	16827	15042	15934.50	1262.19	7.92
Kelly+PBMC Post	14178	11722	12950.00	1736.65	13.41
PBS	439	456	447.50	12.02	2.69
20%CB	920	802	861.00	83.44	9.69
PBMC alone	1219	1290	1254.50	50.20	4.00
BM alone	993	1174	1083.50	127.99	11.81

Figure 40 shows the result of a sandwich MYCN ELISA (B8.4 monoclonal / SC791 polyclonal) performed for Kelly cells ('Kelly'), Kelly cells after the immunomagnetic separation with CD45 depletion procedure ('Kelly Post') and Kelly cells spiked in peripheral blood ('Kelly+PBMC') after the CD45 depletion procedure. Signals for peripheral blood alone ('PBMC') and bone marrow clear of neuroblastoma metastases ('BM alone') are shown as well. PBS and 20% 'cooking buffer' ('20%CB') were used as negative controls. Raw data is in the table below the figure. Experiments were performed in duplicate. The Y axis represents Europium counts. The figure shows how total MYCN protein signals were not affected by the immunomagnetic separation procedure.

4. Measurement of total and phospho-protein signals of the PI3K pathway in children with neuroblastoma

The MSD triplex AKT panel for total and phospho-protein signals has been used both in tumour tissues as well as surrogate tissues such as PBMC's or PRP at the ICR Clinical PD Biomarker Group [74, 83, 85, 112]. The present study aimed to measure total and phospho protein signals of the PI3K/AKT pathway in neuroblastoma cell lines as the first pre-clinical step to implement this methodology to measure pathway activation in bone marrow-derived neuroblastoma cells as a pharmacodynamic biomarker for early clinical trials of PI3K/AKT/mTOR inhibitors. This, in conjunction with MYCN protein expression would allow studying in humans the hypothesis that tumours expressing MYCN protein are extremely sensitive to PI3K/AKT/mTOR inhibition [24-25, 120].

Therefore, the objectives for this initial step of biomarker development were to demonstrate that:-

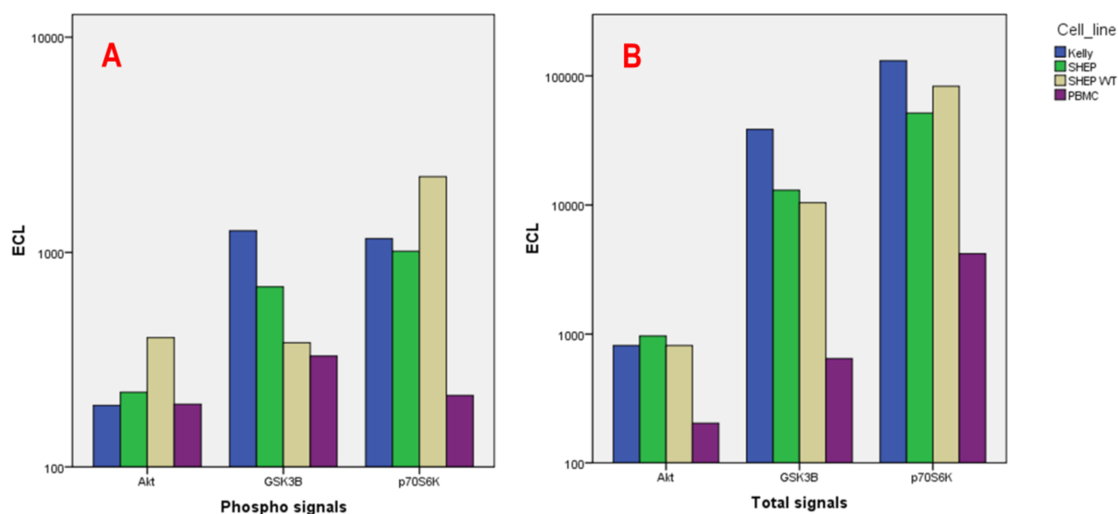
- MSD technology is able to quantify total and phospho protein signals in neuroblastoma cell lines
- MSD technology is able to quantify total and phospho protein signals in neuroblastoma cell lines spiked in peripheral blood and bone marrow samples and that signals are detectable and above background caused by PB and BM alone.
- Immunomagnetic separation procedure does not significantly alter the total and phospho protein signals for the PI3K/AKT pathway detected with MSD technology.

4.1 MSD analysis of neuroblastoma cell lines

MSD multiplex Total and Phospho AKT Panel were performed in lysates from several neuroblastoma cell lines with high (Kelly and SHEP-WT) and no expression of MYCN protein (SHEP) using peripheral blood mononuclear cells as a negative control.

The MSD platform was able to detect total and phospho signals for the three proteins (AKT, GSK3β and p70S6K).

Signals for phospho-AKT (average Kelly 194 ECL counts, SHEP 223.5 ECL counts compared to 196.5 for PBMC's) were within the background signal range. These results concur with previous published data by Opel et al. where a low phosphorylation of AKT had been previously described in neuroblastoma cell lines that are unstimulated (without IGF-1) using Western blotting [47]. (Figure 41)

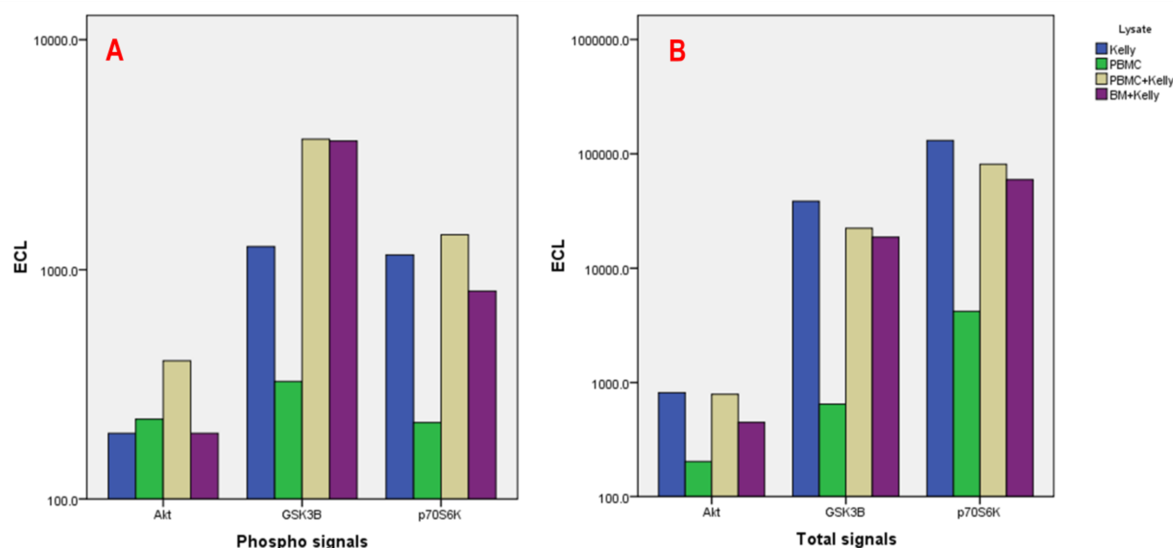


		Kelly	SHEP	SHEP-WT	PBMC
Phospho protein signals	pAKT	194	223.5	401.5	196.5
	pGSK3B	1261.5	692	380.5	326.5
	pp70S6K	1160.5	1013	2251	216
Total protein signals	AKT	816	968	817	203.5
	GSK3B	38519	13027.5	10413	648
	p70S6K	131017.5	51562	83109.5	4201.5

Figure 41, shows the analysis of total and phospho protein signals of the PI3K/AKT pathway using MesoScale Discovery Triplex AKT kit in neuroblastoma cell lines. Electrochemoluminiscence counts are shown in the Y axis. Panel A shows phospho-signals for AKT, GSK3β and p70S6K and Panel B shows the total protein signals for AKT, GSK3β and p70S6K. Three cell lines (Kelly in blue, SHEP in green, SHEP-WT in beige) were analysed and compared to Peripheral Blood Mononuclear Cell lysates ('PBMC' in purple). Experiments were run in duplicate and the table below the figure shows the raw data for the average of the two signals. Low phospho-AKT signals were found for the three unstimulated cell lines.

4.2 MSD analysis of neuroblastoma cell lines spiked in peripheral blood and bone marrow samples

Signals of total and phospho proteins of the PI3K/AKT pathway from lysates containing neuroblastoma Kelly cell lines (lysates of Kelly cells only and Kelly cells spiked into peripheral blood and bone marrow) were superior to signals from lysates of peripheral blood alone in all cases excepting for phospho-AKT. Phospho-AKT signals are known to be low in unstimulated neuroblastoma cells [47] and signals were similar to background (194 ECL for Kelly cells, 293 ECL for Kelly cells spiked in PB, 188 ECL for Kelly cells spiked in BM vs. 196.6 ECL for PBMC alone) and therefore could not be distinguished from those of healthy volunteer PBMC (Figure 42)



		Kelly	PBMC	PBMC+Kelly	BM+Kelly
Phospho protein signals	pAKT	194	196.5	293	188
	pGSK3B	1261.5	326.5	3693.5	3626
	pp70S6K	1160.5	216	1420.5	1227.5
Total protein signals	AKT	816	203.5	791.5	448.5
	GSK3B	38519	648	22415	18699
	p70S6K	131017.5	4201.5	81166.5	59625

Figure 42, shows the analysis of total and phospho protein signals of the PI3K/AKT pathway using MesoScale Discovery Triplex AKT kit in Kelly cells ('Kelly' in blue), peripheral blood ('PBMC' in green), peripheral blood spiked with Kelly ('PBMC+Kelly' in beige) and bone marrow spiked with Kelly ('BM+Kelly' in purple). Electrochemoluminiscence counts are shown in the Y axis. Panel A shows phospho-signals for AKT, GSK3β and p70S6K and Panel B shows the total protein signals. Experiments were run in duplicate and the table below the figure shows the raw data for the average of the two signals. For all total protein analyses, signals of Kelly cells and Kelly cells spiked in peripheral blood and bone marrow were clearly above background level of the peripheral blood alone. Also, for phospho-GSK3β and phospho-p70S6K lysates containing neuroblastoma cells had superior signals to peripheral blood alone. Only phospho-AKT signals of all the lysates were within the background noise level and hence no valuable results could be obtained for the analysis of phospho-AKT.

4.3 Effect of the immunomagnetic separation on the MSD analysis

Kelly cell lines spiked into peripheral blood and bone marrow samples underwent CD45 depletion. Lysates obtained before and after the CD45 depletion were analysed with MSD to assess the effect of the separation in total and phospho protein signals.

There was a decrease in the total protein signals (average±SD 39300±44386 ECL counts before vs. 22800±27063 ECL counts after the immunomagnetic separation), reflected on a -0.24 log difference (p=0.36).

There was an increase in the phospho protein signals (average±SD 1404±1360 ECL counts before vs. 1924±1870 ECL counts after the immunomagnetic separation) reflected on a +0.14 log difference (p=0.51) for the PI3K pathway after the separation (Figure 43). The immunomagnetic separation procedure impacted the total and phospho-protein signals for the PI3K/AKT pathway but the signals were detectable and consistent.

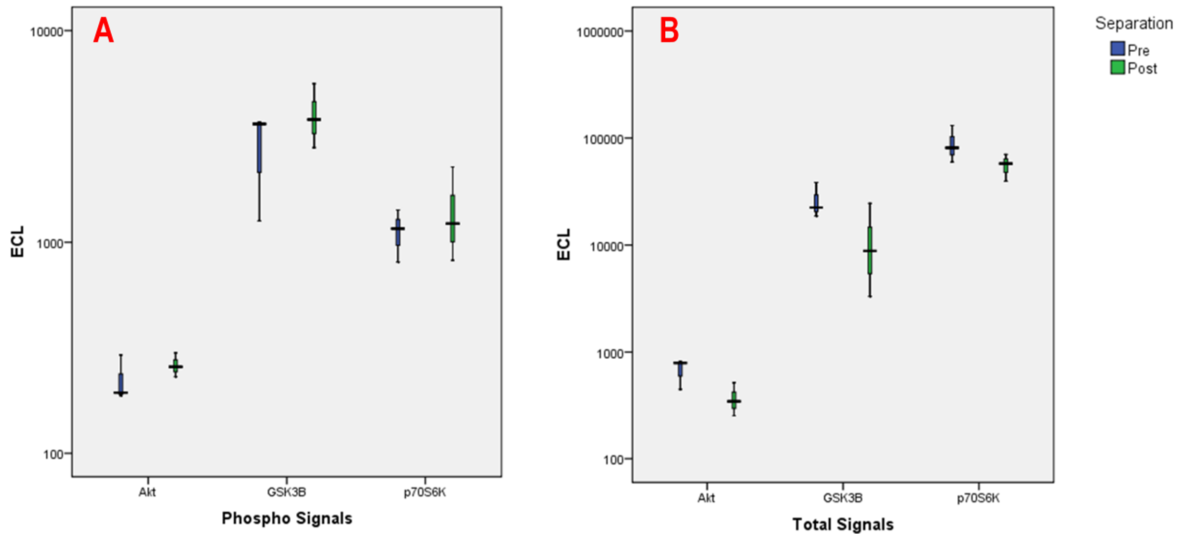


Figure 43, shows the analysis of total and phospho protein signals of the PI3K/AKT pathway using MesoScale Discovery Triplex AKT kit in Kelly cells spiked in peripheral blood before and after CD45 depletion. Electrochemoluminiscence counts are shown in the Y axis. Panel A shows phospho-signals for AKT, GSK3β and p70S6K and Panel B shows the total protein signals. An increase in the mean phospho-protein signals and a decrease in the total protein signals were documented although none reached statistical significance.

These results showed that it is possible to use MSD technology to quantitatively assess the PI3K signalling pathway in bone marrow-derived neuroblastoma cells

Once this initial work had been completed, the next steps would include MSD analysis of bone marrow samples involved with neuroblastoma to establish baseline signals in children with neuroblastoma. However, due to the staggered approach of this project, this part of the validation work has not been completed yet and hence is not presented.

Discussion

Despite advances in multimodal treatment of children with neuroblastoma, the long-term outcome is still poor and half of the children with high-risk disease will experience relapse and succumb [6, 9]. Due to the recent increase in knowledge of neuroblastoma biology, novel drug targets have been identified [121]. In order to select the best agents to be taken forward for large multinational phase III clinical trials and eventually into the clinic, carefully conducted early clinical studies incorporating pharmacodynamic biomarkers are essential.

Pharmacodynamic biomarkers of the PI3K pathway have shown proof of target inhibition in a number of early clinical trials [82, 122]. The model of the "updated pharmacological audit trail" [77] provides a conceptual and practical framework to relate clinical observations with measurable end points. In the updated pharmacological audit trail biomarker assays are designed to test biological hypotheses in the pre-clinical and clinical settings. Each successive question when answered appropriately increases the likelihood of success of a clinical trial of a particular drug.

Some examples of successful early clinical trials in adults with the use of pharmacodynamic biomarkers are the Phase I study of olaparib, a poly(ADP)-ribose polymerase (PARP) inhibitor [123], ARQ197, a MET inhibitor [111], MK2206, a pan-AKT inhibitor [74] or the combination study of MK8669 and MK0646 (an mTOR and an IGF-1R inhibitor respectively) [82].

The use of pharmacodynamic biomarker assays in paediatric phase I studies poses several challenges: - the relatively rare frequency of childhood cancer requires that phase I studies are conducted in several sites (or even countries); the specific aberrations of childhood tumours which are not present in adult cancers, such as MYCN and the limitations of the procedures to obtain tissues that can be safely performed without compromising the children's wellbeing.

The present study:

a) Adapted a MSD ® assay employed in adult clinical trials for use in paediatrics using a surrogate tissue (platelet-rich plasma) that requires small amounts of blood and avoids invasive procedures to obtain tumour tissue. This assay is ready to be implemented in clinical trials.
b) Developed an immmunomagnetic separation strategy that is able to isolate neuroblastoma cells from bone marrow aspirates with a high purity that is suitable for pharmacodynamic biomarker assays. This technique is ready for clinical use.
c) Completed the pre-clinical development of a sandwich ELISA which will allow the quantification of total MYCN protein. This assay is at a clinical development phase.
d) Completed the pre-clinical development of a MSD ® assay to measure total and phospho signals of the PI3K pathway in neuroblastoma cells. This assay is at a clinical development phase.

The first part of the study aimed to adapt an existing pharmacodynamic biomarker assay, the MSD triplex AKT in PRP for use in children. Total and phospho signals of AKT, GSK3β and p70S6K

were analysed and results showed that the assay is fit-for-purpose. No differences were observed in AKT or phospho-AKT between a group of fourteen children with solid tumours receiving treatment at our institution and a group of 24 adults with advanced malignancies. The baseline signal levels for GSK3β, phospho- GSK3β, p70S6K and phospho-p70S6K were significantly higher in children than in their adult counterparts.

This study was not designed to investigate the reasons for differences in baseline values in adults compared to children. The aim was to demonstrate that these proteins can be detected in the paediatric population, hence that the assay is fit-for-purpose for the population of children with solid tumours. Additionally, it was noted that for adults, the phospho-p70S6K signal was below the level of background detectable by the assay and it was not possible to use the pair phospho/total p70S6K for biomarker assessment. In the contrary, we have shown that both total and phospho-p70S6K are detectable in children and therefore the assay could potentially offer a more thorough assessment of the pathway which will become relevant when assessing combination therapies

This triplex assay provides a comprehensive assessment of the PI3K pathway including biomarkers of PI3K, AKT and mTOR activity and therefore will be useful to be included in a number of early clinical studies of drugs targeting PI3K, AKT, mTOR, TORC1 and TORC2 and for the study of feedback loops in the occurrence of resistance.

This assay will be implemented as a tertiary endpoint in the upcoming phase I paediatric dose escalation studies of BEZ235 and GDC0980 (dual PI3K/mTOR inhibitors).

Secondly, this study aimed to develop and validate methodology for the isolation of neuroblastoma tumour cells from bone marrow aspirates of patients with metastatic involvement by neuroblastoma. In experiments spiking neuroblastoma cells into peripheral blood, several immunomagnetic enrichment methods and sample collection procedures were evaluated. CD45 depletion was the best procedure to ensure cell suspensions highly rich in neuroblastoma cells. The procedure has been validated in a subset of clinical bone marrow samples where cell suspensions highly rich in neuroblastoma cells have been obtained. Moreover protein content obtained after the immunomagnetic separation was found to be sufficient to perform several pharmacodynamic analyses and the pre-clinical development of the other two biomarkers in this study showed that this approach would not affect the results in total and phospho signals for MYCN and the PI3K pathway.

Although similar enrichment approaches have been piloted to obtain tumour material for genomic studies in neuroblastoma [87], this is the first study to show a comprehensive pre-clinical and clinical assessment of the different enrichment methodologies, aimed to obtain sufficient cell numbers for pharmacodynamic analyses.

This assay has multiple applications. It will be routinely used in our institution to obtain and store neuroblastoma cells at different time points in treatment (i.e. at diagnosis, at relapse, during

molecularly targeted therapies and at the development of resistance) for different genomic, expression and pharmacodynamic analyses and will also be implemented in the upcoming phase I-II studies enrolling children with neuroblastoma. Future developments will include the automatisation of the immunomagnetic separation procedure with the auto-MACS technology that will improve reproducibility and reduce processing times. This technology will be implemented as an ancillary biomarker sub-study of the Merck-led phase I paediatric clinical trials of MK8669 and MK0646 (mTOR and IGF-1R inhibitor respectively) and will be used as a source of PD biomarkers in the upcoming paediatric phase I trials of BEZ235 and GDC0980.

The third part of the study aimed to develop a quantitative ELISA for total MYCN oncoprotein. There are no commercially available assays and MYCN is currently detected with Western blotting, a technique that is not quantitative, is time consuming, requires large volumes of samples and is operator dependant. Very few technical improvements in the detection of MYCN protein have occurred since the initial publications assessing MYCN protein expression in neuroblastoma [118-119].

A sandwich ELISA that combines a mouse anti-MYCN monoclonal antibody for the solid-phase capture and a rabbit anti-MYCN polyclonal antibody for detection of antibody complexes has been developed using a Europium-based platform.

The assay has been optimised and provided a specific and sensitive detection of MYCN protein. MYCN recombinant protein is used for the standard curve and lysates from Kelly cell lines will be used as Quality Controls.

Future work will include the clinical validation of the assay in clinical samples and the comparison versus the currently established standard Western blotting that will be performed at a further stage of development in our institution. It is envisaged that this assay could also be tested as a predictive biomarker to select those patients with higher levels of MYCN protein expression that could potentially benefit more from MYCN-targeted therapies. Future work will also include the finalisation of Standard Operating Procedure (SOP), reproducibility and Good Clinical Laboratory Practice standards. This technology will be implemented as an ancillary biomarker sub-study of the Merck-led phase I paediatric clinical trials of MK-8669 and M-0646 (mTOR and IGF-1R inhibitor respectively) and will be used as a tertiary biomarker endpoint in the upcoming paediatric phase I trials of BEZ235 and GDC0980.

Finally, this study aimed to adapt the MSD triplex AKT assay for use in bone marrow-derived neuroblastoma tumour cells. The methodology has been used in pre-clinical and clinical testing of molecularly targeted therapies of the PI3K/AKT pathway in adult cancers [83]. The initial pre-clinical development of the assay for neuroblastoma cells was undertaken. This methodology was able to detect signals of the PI3K/AKT pathway in neuroblastoma cell lines and showed that the immunomagnetic separation procedure did not have a significant impact on the detection of total and phospho-protein signals.

Future work will be directed towards the validation of the methodology in human neuroblastoma cells obtained from bone marrow aspirates prior to the implementation in early clinical trials.

Steps of biomarker development	Before	Now	Next
PI3K in PRP	Fully developed in adults None in children	Ready to use in children	Implementation in a clinical trial of a PI3K/AKT/mTOR inhibitor (BEZ235 and GDC0980)
Immunomagnetic isolation of neuroblasts from bone marrow samples	None	Discovery Pre-clinical and pilot clinical development	Validation and optimisation within a clinical trial (MK8669 and MK0646)
MYCN ELISA in bone marrow-derived neuroblasts	None	Discovery Pre-clinical development	Clinical development in patient samples Validation and optimisation
PI3K in bone marrow-derived neuroblasts	None	Pilot pre-clinical development	Further pre-clinical development Pilot clinical development Validation and optimisation in patient samples.

Table 14, Steps of biomarker development achieved with the current study

Discusión en español

A pesar de numerosos avances en el tratamiento multidisciplinar del neuroblastoma, la supervivencia a largo plazo es todavía muy baja y más de la mitad de los niños con neuroblastoma de alto riesgo sufrirán recaídas y fallecerán por su enfermedad. Gracias a los nuevos avances en el conocimiento de la biología del neuroblastoma, se han encontrado nuevas dianas terapéuticas. La incorporación de biomarcadores farmacodinámicos en los ensayos clínicos fase I/II es esencial para seleccionar racionalmente los mejores fármacos a desarrollar y ser evaluados en grandes ensayos clínicos fase III que eventualmente serán utilizados en la práctica clínica.

El uso de biomarcadores farmacodinámicos de la vía de señalización PI3K en ensayos clínicos fase I de fármacos dirigidos contra dianas terapéuticas de dicha vía ha permitido demostrar qué fármacos y en qué regímenes son capaces de inhibir de manera efectiva las dianas terapéuticas relevantes. El marco conceptual y práctico en el que las observaciones clínicas se correlacionan con objetivos concretos para desarrollar nuevos fármacos ha sido descrito en el concepto "The Pharmacologic Audit Trail" (traducido como *la ruta de auditoría de la investigación farmacológica*) desarrollado por los profesores Paul Workman, Alan Ashworth y Johann De Bono en el Institute of Cancer Research de Reino Unido [77]. En este modelo, los biomarcadores se utilizan para demostrar hipótesis biológicas tanto en la investigación preclínica como clínica. De este modo, para un determinado fármaco o diana terapéutica, cada hipótesis biológica que se comprueba positivamente mediante los biomarcadores incrementa las posibilidades de éxito de un ensayo clínico de un medicamento determinado. El objetivo principal es, por tanto, que los biomarcadores ayuden a seleccionar los mejores fármacos en fases precoces, evitando así la administración de medicamentos ineficaces a pacientes que padecen cáncer en grandes ensayos clínicos.

Algunos ejemplos de ensayos clínicos de medicamentos anticancerosos que han sido un éxito gracias al uso de biomarcadores farmacodinámicos han sido los ensayos fase I de olaparib, un inhibidor de PARP [123], ARQ197, un inhibidor de MET, MK2206, un inhibidor de AKT [74] o la combinación de MK8669 y MK0646 (inhibidores de mTOR y IGF-1R respectivamente) [82].

El uso de biomarcadores farmacodinámicos en ensayos fase I pediátricos supone varios retos debidos a la baja frecuencia del cáncer infantil que requiere que dichos estudios se lleven a cabo en múltiples centros (o incluso países), las aberraciones moleculares o genéticas que son distintas en los tumores infantiles (p.ej. la amplificación del oncogén *MYCN*) y las limitaciones éticas y logísticas con los procedimientos que se pueden realizar de forma segura en la población pediátrica.

En el estudio actual

a) Se ha adaptado una tecnología utilizada en ensayos clínicos de adultos para su uso pediátrico utilizando un tejido de fácil acceso (plasma enriquecido en plaquetas). Dicha tecnología se encuentra preparada para ser implementada en ensayos clínicos pediátricos.

b) Se ha desarrollado una estrategia de separación inmunomagnética mediante la cual se pueden obtener suspensiones de células tumorales neuroblásticas de alta pureza aptas para ser utilizadas para analizar biomarcadores farmacodinámicos. Esta técnica esta lista para su uso clínico
c) Se ha completado el desarrollo preclínico de un ELISA de dos fases para cuantificar la oncoproteína MYCN. Esta técnica está actualmente en fase de desarrollo clínico.
d) Se ha completado el desarrollo preclínico de la tecnología MesoScale Discovery para medir señales de proteína total y fosforilada de la vía de señalización PI3K en células de neuroblastoma. Esta técnica está actualmente en fase de desarrollo clínico.

La primera parte de este estudio estaba dirigida a adaptar un biomarcador farmacodinámico existente, usando el kit Triplex AKT de MSD® en plasma enriquecido en plaquetas para su uso en la población infantil. Se analizaron las señales de proteínas totales y fosforiladas de la vía de señalización de PI3K, incluyendo AKT, GSK3β y p70S6K, y los resultados han demostrado que la técnica es adecuada para este propósito y puede ser implementada en la población infantil. No se observaron diferencias en AKT o fosfo-AKT entre un grupo de catorce niños con tumores sólidos tratados en nuestra institución y un grupo de 24 pacientes adultos con tumores sólidos avanzados. Las señales basales de GSK3β, fosfo- GSK3β, p70S6K y fosfo-p70S6K fueron significativamente mayores en niños que en adultos.

Este estudio no fue diseñado para investigar las razones que explican las diferencias entre los valores basales en niños comparados con adultos. El objetivo era demostrar que estas proteínas pueden ser detectadas en la población pediátrica y, por tanto, esta técnica es adecuada (*fit-for-purpose*) para ser utilizada en dicho grupo.

Además, los datos en adultos mostraron una señal de fosfo-p70S6K muy baja que impedía su uso como biomarcador. Por el contrario, en la población pediátrica, tanto fosfo-p70S6K como la proteína total fueron detectables en niños y por ello este análisis ofrece una evaluación más detallada y de la vía de señalización. Esto será especialmente relevante cuando se estudien combinaciones de terapias moleculares como un inhibidor de PI3K con in inhibidor de MEK.

Esta técnica será utilizada en diversos ensayos clínicos de moléculas dirigidas contra PI3K, AKT, mTOR, TORC1 y TORC2, así como para estudiar las señales de retroalimentación negativa en caso de resistencia farmacológica.

Esta técnica será implementada como un objetivo terciario en los ensayos clínicos de fase I de BEZ235 y GDC0980, ambos inhibidores duales de PI3K y mTOR que comenzarán en RMH/ICR durante 2013.

En segundo lugar, este estudio fue diseñado para desarrollar y validar la metodología para aislar células tumorales de neuroblastoma de aspirados de medula ósea con afectación metastásica. En experimentos diluyendo células de neuroblastoma en sangre periférica, se compararon diversos

métodos de enriquecimiento inmunomagnético y se evaluó la metodología de colección de las muestras. La depleción de CD45 fue el mejor procedimiento para asegurar la mayor pureza de células de neuroblastoma en las suspensiones celulares. El procedimiento ha sido validado en una muestra piloto de aspirados de medula ósea donde se han obtenido muestras altamente purificadas con células de neuroblastoma. Además, el contenido proteico obtenido tras la separación inmunomagnética fue suficiente para la realización de diversos análisis farmacodinámicos. El desarrollo preclínico de otros dos biomarcadores durante este estudio demostró que esta estrategia de separación inmunomagnética no afectaría significativamente las señales de proteína total y fosforilada para la vía de señalización de PI3K y los niveles de MYCN.

En un estudio piloto belga [87] se utilizaron métodos de separación inmunomagnética para estudios genómicos en neuroblastoma, pero este es el primer estudio que realiza un análisis minucioso comparando diversas estrategias de separación inmunomagnética dedicado a obtener muestras para análisis proteicos de biomarcadores farmacodinámicos.

Esta técnica por tanto, tendrá diversas aplicaciones. Será utilizada rutinariamente en nuestra institución para obtener y almacenar células de neuroblastoma en diferentes puntos del tratamiento de los pacientes (por ejemplo, al diagnostico, durante la recaída y durante tratamientos con terapias moleculares dirigidas) para realizar análisis genómicos, de expresión y farmacodinámicos. Asimismo será implementada en los próximos ensayos clínicos fase I y II que incluyan pacientes con neuroblastoma.

La fase de desarrollo inmediata a este estudio se centrara en la automatización del procedimiento con la tecnología auto-MACS que mejorara la reproducibilidad reduciendo los tiempos de procesamiento de las muestras.

Esta tecnología será implantada en los sub-estudios de biomarcadores (objetivos terciarios) de los ensayos fase I de MK8669 y MK0646 (inhibidores de mTOR e IGF-1R respectivamente) esponsorizados por Merck que comenzaran a comienzos de 2012 y en los ensayos fase I pediátricos de BEZ235 y GDC0980.

La tercera parte del estudio estaba dirigida a desarrollar un ELISA cuantitativo para cuantificar el contenido de la oncoproteína MYCN total. Actualmente, no hay técnicas comercialmente disponibles y se utiliza el Western blotting, que es una técnica no cuantitativa, laboriosa y muy dependiente del operador. Desde que los estudios iniciales cuantificando la proteína MYCN en neuroblastoma fueron publicados [118-119] no se han llevado a cabo mejorías en la tecnología de detección de MYCN.

Durante este estudio, se ha desarrollado un ELISA tipo sándwich que combina un anticuerpo monoclonal anti-MYCN murino para la fase solida y un anticuerpo policlonal anti-MYCN de conejo para la fase liquida, mediante la utilización de una plataforma basada en Europio para la detección lumínica.

La técnica se ha optimizado hasta ofrecer una detección sensible y especifica de la proteína MYCN. Se utilizará proteína recombinante MYCN para realizar la curva estándar y lisados de células Kelly para realizar el control de calidad.

Los planes de desarrollo en nuestra institución incluyen la validación clínica de la técnica en muestras clínicas y la comparación frente al patrón oro (*gold standard*) actual, el Western blotting. Además de su potencial como biomarcador farmacodinámico, esta tecnología se podría validar como biomarcador predictivo para seleccionar los pacientes con niveles muy elevados de expresión de proteína MYCN que podrían potencialmente beneficiarse selectivamente de tratamientos moleculares contra MYCN. Asimismo, se completara el procedimiento normalizado estándar (PNE). Esta tecnología será implantada en los sub-estudios de biomarcadores (objetivos terciarios) de los ensayos fase I de MK8669 y MK0646 (inhibidores de mTOR e IGF-1R respectivamente) esponsorizados por Merck que comenzaran el reclutamiento a comienzos de 2012 y los ensayos fase I pediátricos de BEZ235 y GDC0980 durante 2012-2013.

El objetivo final de este estudio fue la adaptación del MSD triplex AKT assay para su uso en células de neuroblastoma derivadas de muestras de médula ósea. Esta metodología se ha utilizado en el desarrollo preclínico de terapias dirigidas moleculares de la vía de señalización PI3K/AKT [83]. Se llevo a cabo el desarrollo preclínico inicial de dicha tecnología en células de neuroblastoma. Este método fue capaz de detectar señales de activación de la vía PI3K/AKT en líneas celulares de neuroblastoma y se demostró que los procedimientos de separación inmunomagnética no tenían un impacto significativo en la detección de señales de proteína total y fosforilada.

La tecnología se continuara desarrollando la metodología en células humanas de neuroblastoma obtenidas de aspirados de medula ósea previamente a la incorporación en ensayos clínicos fase I/II.

Fases del desarrollo	Previamente	Actualmente	Desarrollo Futuro
PI3K en plasma enriquecido con plaquetas	Completamente validado e implementado en adultos. Ningún dato pediátrico	Preparado para su uso en ensayos clínicos pediátricos	Implementación en los próximos ensayos clínicos de inhibidores de PI3K/AKT/mTOR BEZ235 y GDC0980 (2012-2013)
Separación inmunomagnética de células de neuroblastoma en medula ósea	Ningún dato en neuroblastoma para biomarcadores proteicos	Descubrimiento Fase preclínica Fase clínica piloto	Validación y optimización durante el primer ensayo clínico (MK8669 y MK0646)
ELISA para MYCN en células de neuroblastoma derivadas de aspirados de medula ósea	Ningún dato pediátrico	Descubrimiento Fase preclínica	Desarrollo clínico en muestras de pacientes Validación y optimización
PI3K en células de neuroblastoma derivadas de medula ósea	Ningún dato pediátrico	Desarrollo preclínico piloto	Continuación del desarrollo preclínico Desarrollo clínico piloto Validación y optimización en muestras clínicas

Tabla 14. Fases del desarrollo de los diversos biomarcadores en estudio

Conclusions

1. This study developed pharmacodynamic biomarkers to accompany early clinical trials in childhood cancers and specifically for neuroblastomas driven by MYCN.
2. The MesoScale Discovery® Triplex AKT assay was fit-for-purpose to measure the PI3K/AKT pathway in platelet-rich plasma of children with solid tumours
3. Depletion of CD45+ cells was the best immunomagnetic separation procedure to isolate neuroblastoma cells from bone marrow aspirates
4. CD45 depletion produced cell suspensions highly enriched in neuroblastoma cells that were suitable for protein assays
5. A quantitative assay to assess levels of total MYCN protein in bone marrow-derived neuroblastoma cells has been developed
6. The MesoScale Discovery® Triplex AKT assay can be used to evaluate the PI3K/AKT pathway in neuroblastoma cells
7. This study has developed several biomarkers that will be able to provide a comprehensive assessment of pharmacodynamic changes after PI3K/AKT targeted therapy, to show proof of target modulation for PI3K, AKT and mTOR and downstream effects in MYCN protein levels for children with neuroblastoma.

Conclusiones en español

1. Este estudio ha desarrollado biomarcadores farmacodinámicos para acompañar ensayos clínicos fase I/II en neuroblastoma derivado por MYCN
2. Se demostró que la tecnología MesoScale Discovery Triplex® AKT es adecuada para su propósito (fit-for-purpose) y permitió medir la vía de señalización de PI3K/AKT en plasma enriquecido con plaquetas en niños con tumores sólidos
3. La depleción de células CD45+ fue el mejor procedimiento de separación inmunomagnética para aislar células de neuroblastoma de aspirados de medula ósea
4. La depleción de células CD45+ fue capaz de producir suspensiones celulares altamente enriquecidas en células de neuroblastoma que son adecuadas para análisis proteicos.
5. Se ha desarrolló una tecnología cuantitativa para medir los niveles de proteína MYCN total en células de neuroblastoma derivadas de medula ósea
6. Se demostró como la tecnología MesoScale Discovery Triplex® AKT puede ser utilizada para evaluar la vía de señalización de PI3K/AKT in células de neuroblastoma.
7. Este estudio ha desarrollado diversos biomarcadores que proveerán un análisis comprehensivo de los cambios farmacodinámicos producidos con el tratamiento con inhibidores de PI3K/AKT, servirá para demostrar prueba de inhibición de la diana terapéutica para PI3K, AKT y mTOR así como efectos subsecuentes en los niveles de proteína MYCN en pacientes pediátricos afectos de neuroblastoma.

Appendices

Appendix 1, Institutional letters of approval for patient sample collection (CCR3358 study)

THE ROYAL
MARSDEN

Prof David Dearnaley (Chairman)
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Research & Development Office
West Wing, Downs Road,
RMH Sutton

Tel: 020 8661 3047
Fax: 020 8915 6700

Committee for Clinical Research

Prof ADJ Pearson
Downs Road
Sutton
SM2 5PT

17 March 2010

Prof ADJ Pearson

ID: CCR3358 **Adaptation of an existing meso scale discovery (MSD) pharmacodynamic assay measuring signalling on the P13-kinase pathway for use in children's cancer**

Thank you for your letter dated 01.12.09 in response to Committee for Clinical Research (CCR) outcomes letter dated 12.11.09. The documentation and information provided was reviewed and approved by the CCR Chair on 02.12.09

I am pleased to inform you that the R&D Office has received all outstanding documentation with regard to the above project as follows:

- CCR approval of protocol version 1.0 dated 23.10.09
- CCR approval of Patient Information Sheet and Consent form version 1.0 dated 23.10.09
- Sponsorship details as outlined in the agreement dated 10.03.10
- REC approval letter dated 03.02.10

We have activated your study on the Hospital Information System (HIS). Please ensure that you have fulfilled all sponsor requirements before recruiting patients.

Please note that the patient participation in this study should be recorded on the "Maintain CCR Protocols (CCRPAT)" computer system on the HIS and the original completed consent form should be kept in the trial master file, a copy should be given to the patient and a copy should be kept in the patient's notes.

Please be aware that the R&D Office must be notified of the following as they arise:

- Serious Adverse Events/SUSARs in line with guidance on the intranet
- Amendments
- Progress Reports
- Closure of study
- Planned Audits by Sponsor

Please ensure that you notify the main REC where necessary with regards to the above.

Please note, in order to publish the results of clinical trials in an ICMJE journal, your trial must be registered on either www.controlledtrials.com or www.clinicaltrials.gov before your first patient is recruited. For further information, please refer to http://www.dh.gov.uk/assetRoot/04/11/42/45/04114245.doc or contact the R&D Office.

You are reminded that your project must be conducted in accordance with the Research Governance Framework for Health and Social Care (2nd edition 2005) and that all members of the research team must be aware of and understand their responsibilities under this Framework.

Tissue studies are to be conducted in accordance with the Human Tissue Act 2004 and the Codes of Practice issued by the Human Tissue Authority. Where tissue is used for human application please make sure that you abide by the requirements of the Human Tissue (Quality and Safety for Human Application) Regulations 2007, especially with regards to reporting of serious adverse events.

Please note that the most up to date guidance and forms are available on the R&D section of the RMH intranet site available at CLINICAL: Clinical Research & Development.

If there is any way in which the R&D Office can assist your research, please don't hesitate to contact us.

Yours sincerely

Ann Gandolfi

Research Governance Coordinator

cc: Andrea Boast

Copy to: Dr Lucas Moreno,

National Research Ethics Service

The Royal Marsden Research Ethics Committee
St Georges University of London
South London REC Office (1)
Room 1.13
1st Floor - Jenner Wing
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SW17 0RE
Telephone: 020 8725 0252
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03 February 2010

Professsor Andrew DJ Pearson
Head of Children's Unit
Royal Marsden Hospital
Department of Paediatrics
Royal Marsden Hospital
Downs Road
Sutton, Surrey
SM2 5PT

Dear Professor Pearson,

Study Title:	**Adaptation of an existing Mesoscale DiscoveryB® (MSDB®) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer.**
REC reference number:	**10/H0801/4**

The Research Ethics Committee reviewed the above application at the meeting held on 18 January 2010. Thank you for attending to discuss the study.

Ethical opinion

- The Committee asked that you make provision for the study information to be translated for non English speaking participants

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should

be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter		09 December 2009
REC application	37167/84857/1/333	22 December 2009
Protocol	1.0	23 October 2009
Chief Investigator CV		
Participant Information Sheet: Parent Info - PI3K biomarkers in children	1.0	23 October 2009
Participant Information Sheet: Parent info - PI3K biomarkers in neuroblastoma	1.0	23 October 2009
Participant Information Sheet: For patients over 14 yrs	1.0	23 October 2009
Participant Information Sheet: For patients aged 8-14 yrs	1.0	23 October 2009
Participant Information Sheet: For children aged under 8 yrs	1.0	23 October 2009
Participant Consent Form: For patients over 14 yrs	1.0	23 October 2009
Participant Consent Form: Parent consent - PI3K biomarkers in neuroblastoma	1.0	23 October 2009
Participant Consent Form: Parent consent - PI3K biomarkers in children	1.0	23 October 2009
Letter to GP	1.0	23 October 2009
Letter from Sponsor		12 November 2009
Case Report Form	1.1	20 November 2009
Confirmation of Lead CLRN	email	10 December 2009

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

This Research Ethics Committee is an advisory committee to London Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H0801/4	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Ms Shelley Dolan
Chair

Email: royalmarsden.rec@stgeorges.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers"

Copy to: *Ms Andrea Boast, RMH*
Ms Jane Lawrence, R&D, RMH

This Research Ethics Committee is an advisory committee to London Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

The Royal Marsden Research Ethics Committee

Attendance at Committee meeting on 18 January 2010

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mr Roger A'Hern	Senior Statistician	Yes	
Ms Shelley Dolan	Chief Nurse	Yes	
Reverend Thomas Gillum	Church of England Priest	Yes	
Mrs Susan Hamlyn	Lay Member	No	
Ms Jane Ashton	GI Specialist Pharmacist	Yes	
Dr Alexandra Leary	SpR Medical Oncologist	Yes	
Mrs Patricia Pank	Lay Member	No	
Ms Cate Savidge	CT Scanning Superintendent	Yes	
Dr Mary Taj	Consultant Paediatric Oncologist	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Ms Simone Morris	Acting Co-ordinator
Dr Derek Gibson	Consultant Cardiologist & Chair – Brompton, Harefield & NHIL REC

This Research Ethics Committee is an advisory committee to London Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

MHRA CT Authorisation				

Please note: Each participating site is responsible for ensuring insurance and indemnity arrangements are in place to cover the liability of the Principal Investigator.

SIGNATURES

Unit Head Approval

I approve submission of this application to the CCR

Signature Date 23.10.09

Unit head / Medical Director (as appropriate)

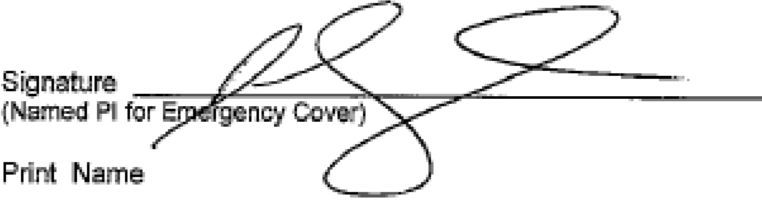
Print

Study Personnel

Signature Date 23.10.09

(Principal Investigator)

Print Name

Signature  Date 10/11/09

(Named PI for Emergency Cover)

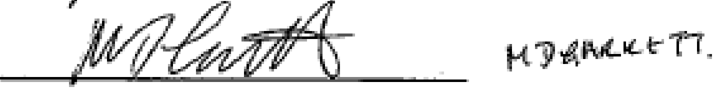
Print Name

Signature ____________ Name Date ________

(All other members of RMH / ICR Research Team)

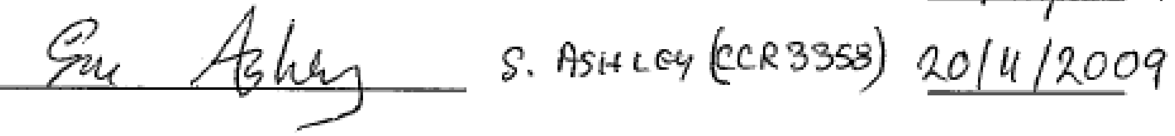
LUCAS MORENO 20.10.2009

A J. BOATT 21/10/2009



M D BARKETT. 22/10/2009.

L. CHESLER 10/11/2009.



S. ASHLEY (CCR3358) 20/4/2009

Appendix 2, Patient information sheets and informed consent documents (CCR3358 study)

The Royal Marsden NHS
NHS Foundation Trust

Downs Road, Sutton
Surrey
SM2 5PT
Switchboard: 020 8642 6011
Direct Dial: 020 8 ______

INFORMATION FOR PARENTS

Adaptation of an existing Mesoscale Discovery (MSD) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer

Information Sheet
Version 1.0: October 2009

We are asking you to consider the possibility of allowing your child to take part in a research study involving taking a blood sample.

Before you decide it is important for you to understand why the research is being done and what is involved. Please take time to read the following information carefully and discuss it with friends, relatives, doctors and nurses if you wish. Ask us if there is anything that is not clear or if you would like more information. Take the time to decide whether or not you wish your child to take part.

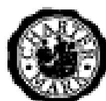
1. What is the purpose of the study?

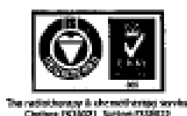
At the moment, we are developing tests to assess if new drug treatments are working adequately using blood samples (these are called pharmacodynamic biomarkers). These tests have been used for years in adults and proved to be helpful in certain types of cancer. By using them, you can find out if a determinate drug is functioning and doing its job properly just with a blood sample.
The PI3K signalling pathway is involved in the majority of childhood cancers and there are new drugs that can modify this pathway. Now we want to find out which children will benefit from these drugs by developing a blood test. For this reason we need one blood sample from children diagnosed of any form of cancer.
This will help us to learn more about the most effective ways to choose new treatments to children and adolescents in the future.

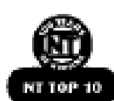
2. Why has my child been chosen?

All children and adolescents who attend Children and Young People's Unit of The Royal Marsden NHS Foundation Trust for diagnosis/treatment of any form of solid tumour are eligible to take part in this study.

3. Does my child have to take part?







Chairman: Mrs Tessa Green
Chief Executive: Miss Cally Palmer CBE
www.royalmarsden.nhs.uk

No. Participation in the study is entirely voluntary. If you agree to your child taking part and then later change your mind, you are still free to withdraw at any time without giving a reason. This will not affect the standard of care received by your child.

4. Consent

If you agree to take part in this study we will need you to sign a consent form. You will be given a copy of the consent form and this information sheet to keep. If you do consent initially and subsequently change your mind that won't be a problem, but if consent is withdrawn then the data that has already been collected cannot be deleted from the database and will still be processed.

5. What will happen if my child takes part?

A blood sample will be taken from your child's long line just after other bloods are drawn. Your child will not have to have a cannula for this. We only need one 10 mL sample (2-3 teaspoons). The amount of blood taken should not cause your child any problems. The samples taken will allow us to measure different proteins related to the tumour relevant to show if one group of new drugs might be used.
Any samples remaining following analysis will be destroyed.

6. Are there any disadvantages or risks involved in my child's participation in the study?

Any risks associated with one isolated sampling from the long line are minimised by following the established aseptic (sterile or germ-free) guidelines practiced by the paediatric oncology unit. Different studies have shown that the amount of blood that we are taking can be safely drawn without causing symptoms to your child.

7. What are the possible benefits of taking part?

The information gained from the blood samples taken will not be of direct benefit to your child at the moment but may improve the way we use new drugs for the treatment of children and adolescents with poor prognosis cancers in the future.

8. Confidentiality

Information needed for this study will only be collected and stored in The Royal Marsden NHS Foundation Trust where it will be retained for the duration of the study and will be kept strictly confidential. Your child's medical records and all data obtained from this study will be made available to

representatives of the study Sponsor and regulatory authorities (in the UK this is the Medicines and Healthcare products Regulatory Agency). This is to make sure that the information collected is an accurate reflection of the study.

This information collected will be stored on a database and analysed. The information will be published in medical and scientific journals, and may also be presented at international conferences. If you withdraw your consent for your child to continue in the study then the information provided will not be used, but the paper records will not be destroyed and the information recorded in the database cannot be deleted. Your child's name will not be used in any publication. All of the information which can be traced back to your child will remain confidential. Your rights under the Data Protection Act 1998 remain at all times.

9. What if something goes wrong or I am unhappy about any aspect of the study?

If your child is harmed by taking part in this research project, there are no special compensation arrangements. If your child is harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or are unhappy about any aspect of the way you have been approached or treated during the course of this study, in the first instance please contact your child's consultant. If you are still unhappy, you can contact the hospital complaints department.

10. What will happen to the results of the study?

The study will run for approximately twelve months. Analysis will be carried out and the results published in medical journals and possibly used to modify future treatments. Your child will not be identified in any report or publication.

11. Who is organising and funding the research?

The study is organised by The Royal Marsden NHS Foundation Trust and funded by Cancer Research UK. The study is being chaired by Prof. Andrew DJ Pearson, Head of Children's Unit.

12. Contact for further information

If you require any further information please contact the consultant who is looking after your child.

Name:

Telephone Number:
Thank you for reading this information sheet.

The Royal Marsden NHS
NHS Foundation Trust

Downs Road, Sutton
Surrey
SM2 5PT
Switchboard: 020 8642 6011
Direct Dial: 020 8 ______

Patient Identification Number for this trial:

PARENT CONSENT FORM
(Version 1.0: October 2009)

Title of Project: Adaptation of an existing Mesoscale Discovery (MSD) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer

Name of Researcher:

Name of patient:

Please initial box

1. I confirm that I have read and understand the information sheet dated. (Version 1.0, October 2009) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my child's participation is voluntary and that I am/he/she is free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected. ☐

3. I understand that my child's medical notes may be made available to responsible individuals from The Royal Marsden NHS Foundation Trust, the research group and regulatory authorities where it is relevant to my child's taking part in research. I give permission for these individuals to have access to my child's medical records and to collect, store, analyse and publish information obtained from this research. I understand that my child's name will be kept confidential. I understand that my child's General Practicioner will be informed. ☐

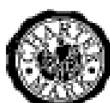
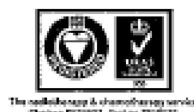
4. I understand that a blood sample will be taken for analysis of proteins that reflect the mechanisms of action of new drugs that will be used in the future. ☐

5. I agree to my child taking part in the above study. ☐

______________________	____________	
Name of Parent/Guardian	Date	Signature
Name of Person taking consent (if different from Principal Investigator)	Date	Signature
Name of Principal Investigator	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

PI3K biomarkers in children /Parent Information Sheet/Consent Form/Version 1.0 October 2009

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Chairman: Mrs Tessa Green
Chief Executive: Miss Cally Palmer CBE
www.royalmarsden.nhs.uk

Patron:
Her Majesty The Queen
President:
Prince William

The Royal Marsden NHS
NHS Foundation Trust

Downs Road, Sutton
Surrey
SM2 5PT
Switchboard: 020 8642 6011
Direct Dial: 020 8 ______

INFORMATION FOR PARENTS
(Neuroblastoma)

Adaptation of an existing Mesoscale Discovery (MSD) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer
Information Sheet
(Version 1.0: October 2009)

We are asking you to consider the possibility of allowing your child to take part in a research study involving taking a blood and bone marrow sample.

Before you decide it is important for you to understand why the research is being done and what is involved. Please take time to read the following information carefully and discuss it with friends, relatives, doctors and nurses if you wish. Ask us if there is anything that is not clear or if you would like more information. Take the time to decide whether or not you wish your child to take part.

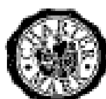
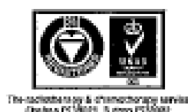
1. What is the purpose of the study?

At the moment, we are developing tests to assess if new drug treatments are working adequately using blood samples (these are called pharmacodynamic biomarkers). These tests have been used for years in adults and proved to be helpful in certain types of cancer. By using them, you can find out if a determinate drug is functioning and doing its job properly just with a blood sample.
The PI3K signalling pathway is involved in the majority of childhood cancers but is especially important in neuroblastoma. Now there are new drugs that can modify this pathway. We want to find out which children will benefit from these drugs by developing a blood and bone marrow test. For this reason we need one blood and bone marrow sample from children diagnosed of neuroblastoma.
This will help us to learn more about the most effective ways to choose new treatments to children and adolescents in the future.

2. Why has my child been chosen?

All children and adolescents who attend Children's Department of The Royal Marsden NHS Foundation Trust for diagnosis/treatment of any form of solid tumour are eligible to take part in this study.

3. Does my child have to take part?

Chairman: Mrs Tessa Green
Chief Executive: Miss Cally Palmer CBE
www.royalmarsden.nhs.uk

No. Participation in the study is entirely voluntary. If you agree to your child taking part and then later change your mind, you are still free to withdraw at any time without giving a reason. This will not affect the standard of care received by your child.

4. Consent

If you agree to take part in this study we will need you to sign a consent form. You will be given a copy of the consent form and this information sheet to keep. If you do consent initially and subsequently change your mind that won't be a problem, but if consent is withdrawn then the data that has already been collected cannot be deleted from the database and will still be processed.

5. What will happen if my child takes part?

A blood sample will be taken from your child's long line just after other bloods are drawn. Your child will not have to have a cannula for this. We only need one 10 mL sample (2-3 teaspoons). The amount of blood taken should not cause your child any problems. When your child undergoes a bone marrow aspirate, an additional 5ml sample will be drawn. This will not prolong the time of the general anaesthetics and should not cause your child any problems. The samples taken will allow us to measure different proteins related to the tumour relevant to show if one group of new drugs might be used. Any samples remaining following analysis will be destroyed.

6. Are there any disadvantages or risks involved in my child's participation in the study?

Any risks associated with one isolated sampling from the long line or bone marrow are minimised by following the established aseptic (sterile or germ-free) guidelines practiced by the paediatric oncology unit. Different studies have shown that the amount of blood that we are taking can be safely drawn without causing symptoms to your child.

7. What are the possible benefits of taking part?

The information gained from the blood and bone marrow samples taken will not be of direct benefit to your child at the moment but may improve the way we use new drugs for the treatment of children and adolescents with poor prognosis cancers in the future.

8. Confidentiality

Information needed for this study will only be collected and stored in The Royal Marsden NHS Foundation Trust where it will be retained for the duration of the study and will be kept strictly confidential. Your child's medical

records and all data obtained from this study will be made available to representatives of the study Sponsor and regulatory authorities (in the UK this is the Medicines and Healthcare products Regulatory Agency). This is to make sure that the information collected is an accurate reflection of the study.

This information collected will be stored on a database and analysed. The information will be published in medical and scientific journals, and may also be presented at international conferences. If you withdraw your consent for your child to continue in the study then the information provided will not be used, but the paper records will not be destroyed and the information recorded in the database cannot be deleted. Your child's name will not be used in any publication. All of the information which can be traced back to your child will remain confidential. Your rights under the Data Protection Act 1998 remain at all times.

9. What if something goes wrong or I am unhappy about any aspect of the study?

If your child is harmed by taking part in this research project, there are no special compensation arrangements. If your child is harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or are unhappy about any aspect of the way you have been approached or treated during the course of this study, in the first instance please contact your child's consultant. If you are still unhappy, you can contact the hospital complaints department.

10. What will happen to the results of the study?

The study will run for approximately twelve months. Analysis will be carried out and the results published in medical journals and possibly used to modify future treatments. Your child will not be identified in any report or publication.

11. Who is organising and funding the research?

The study is organised by The Royal Marsden NHS Foundation Trust and funded by Cancer Research UK. The study is being chaired by Prof. Andrew DJ Pearson, Head of Children's Unit.

12. Contact for further information

If you require any further information please contact the consultant who is looking after your child.

Name:
Telephone Number:

Thank you for reading this information sheet.

Patron: Her Majesty The Queen
President: Prince William

The Royal Marsden NHS
NHS Foundation Trust

Patient Identification Number for this trial:

Downs Road, Sutton
Surrey
SM2 5PT
Switchboard: 020 8642 6011
Direct Dial: 020 8 ______

PARENT CONSENT FORM (Neuroblastoma)
(Version 1.0: October 2009)

Title of Project: Pilot study for development of pharmacodynamic biomarkers of the PI3K signalling pathway for early clinical trials in children with advanced tumours

Name of Researcher:

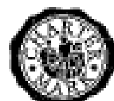
Name of patient:

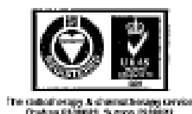
Please initial box

1.	I confirm that I have read and understand the information sheet dated. (Version 1.0, October 2009) for the above study and have had the opportunity to ask questions.	☐
2.	I understand that my child's participation is voluntary and that I am/he/she is free to withdraw at any time, without giving any reason, without my child's medical care or legal rights being affected.	☐
3.	I understand that my child's medical notes may be made available to responsible individuals from The Royal Marsden NHS Foundation Trust, the research group and regulatory authorities where it is relevant to my child's taking part in research. I give permission for these individuals to have access to my child's medical records and to collect, store, analyse and publish information obtained from this research. I understand that my child's name will be kept confidential. I understand that my General Practicioner will be informed.	☐
4.	I understand that a blood and bone marrow samples will be taken for analysis of proteins that reflect the mechanisms of action of new drugs that will be used in the future.	☐
5.	I agree to my child taking part in the above study.	☐

Name of Parent/Guardian	Date	Signature
Name of Person taking consent (if different from Principal Investigator)	Date	Signature
Name of Principal Investigator	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes





Chairman: Mrs Tessa Green
Chief Executive: Miss Cally Palmer CBE
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Patron: Her Majesty The Queen
President: Prince William

The Royal Marsden NHS
NHS Foundation Trust

Downs Road, Sutton
Surrey
SM2 5PT
Switchboard: 020 8642 6011
Direct Dial: 020 8 ———

INFORMATION FOR PATIENTS AGED OVER 14 YEARS

Adaptation of an existing Mesoscale Discovery (MSD) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer

Information Sheet
(Version 1.0: October 2009)

We are asking you whether or not you would like to take part in a research study involving taking a blood sample.

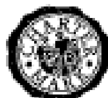
Before you decide it is important for you to understand why the research is being done and what is involved. Please take time to read the following information carefully and discuss it with friends, relatives, doctors and nurses if you wish. Ask us if there is anything that is not clear or if you would like more information. Take the time to decide whether or not you wish to take part.

1. What is the purpose of the study?

At the moment, we are developing tests to assess if new drug treatments are working adequately using blood samples (these are called pharmacodynamic biomarkers). These tests have been used for years in adults and proved to be helpful in certain types of cancer. By using them, you can find out if a determinate drug is functioning and doing its job properly just with a blood sample.
The PI3K signalling pathway is involved in the majority of childhood cancers and there are new drugs that can modify this pathway. Now we want to find out which children will benefit from these drugs by developing a blood test. For this reason we need one blood sample from children diagnosed of any form of cancer.
This will help us to learn more about the most effective ways to choose new treatments to children and adolescents in the future.

2. Why have I been chosen?

All children and adolescents who attend Children and Young People Unit of The Royal Marsden NHS Foundation Trust for diagnosis/treatment of any form of solid tumour are eligible to take part in this study.



Chairman: Mrs Tessa Green
Chief Executive: Miss Cally Palmer CBE
www.royalmarsden.nhs.uk

3. Do I have to take part?

No. Participation in the study is entirely voluntary. If you agree to take part and then later change your mind, you are still free to withdraw at any time without giving a reason, this will not affect the standard of care you receive.

4. Consent

If you agree to take part in this study we will need you/your parents to sign a consent form. You will be given a copy of the consent form and this information sheet to keep.

5. What will happen if I take part?

A blood sample will be taken from your long line just after other bloods are drawn. You will not have to have a cannula for this. We only need one 10 mL sample (2-3 teaspoons). The amount of blood taken should not cause you any problems. The samples taken will allow us to measure number of tumour-related proteins relevant to show if new drugs might be used. Any samples remaining following analysis will be destroyed.

6. Are there any disadvantages or risks involved in my participation in the study?

Any risks associated with one isolated sampling from the long line are minimised by following the established aseptic (sterile or germ-free) guidelines practiced by the paediatric oncology unit. Different studies have shown that the amount of blood that we are taking can be safely drawn without causing symptoms to you.

7. What are the possible benefits of taking part?

The information gained from the blood samples taken will not be of direct benefit to you at the moment but may improve the way we use new drugs for the treatment of children and adolescents with poor prognosis cancers in the future.

8. Confidentiality

Only information needed for this study will be collected and stored in The Royal Marsden NHS Foundation Trust where it will be retained for the duration of the study and will be kept strictly confidential. Your medical records and all data obtained from this study will be made available to representatives of the study Sponsor and regulatory authorities (in the UK this is the Medicines and Healthcare products Regulatory Agency). This is to make sure that the information collected is an accurate reflection of the study.

This information collected will be stored on a database and analysed. The information will be published in medical and scientific journals, and may also

be presented at international conferences. If you withdraw your consent to continue in the study then the information provided will not be used, but the paper records will not be destroyed and the information recorded in the database cannot be deleted. Your name will not be used in any publication. All of the information which can be traced back to you will remain confidential. Your rights under the Data Protection Act 1998 remain at all times.

9. What if something goes wrong or I am unhappy about any aspect of the study?

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or are unhappy about any aspect of the way you have been approached or treated during the course of this study, in the first instance please contact your consultant. If you are still unhappy, you can contact the hospital complaints department, (insert local contact details).

10. What will happen to the results of the study?

The study will run for approximately twelve months. Analysis will be carried out and the results published in medical journals and possibly used to modify future treatment. You will not be identified in any report or publication.

11. Who is organising and funding the research?

The study is organised by The Royal Marsden NHS Foundation Trust and funded by Cancer Research UK. The study is being chaired by Prof. Andrew DJ Pearson, Head of Children's Unit.

12. Contact for further information

If you require any further information please contact the consultant who is looking after you.

Name:

Telephone Number:

Thank you for reading this information sheet.

Patient Identification Number for this trial:

The Royal Marsden NHS
NHS Foundation Trust

Downs Road, Sutton
Surrey
SM2 5PT
Switchboard: 020 8642 6011
Direct Dial: 020 8 ______

PATIENT CONSENT FORM (above 16 years)
(Version 1.0: October 2009)

Title of Project: Adaptation of an existing Mesoscale Discovery (MSD) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer

Name of Researcher:

Name of patient:

Please initial box

1. I confirm that I have read and understand the information sheet dated. (Version 1.0, October 2009) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that my medical notes may be made available to responsible individuals from The Royal Marsden NHS Foundation Trust, the research group and regulatory authorities where it is relevant to my child's taking part in research. I give permission for these individuals to have access to my medical records and to collect, store, analyse and publish information obtained from this research. I understand that my name will be kept confidential. I understand that my General Practicioner will be informed. ☐

4. I understand that a blood sample will be taken for analysis of proteins that reflect the mechanisms of action of new drugs that will be used in the future. ☐

5. I agree to taking part in the above study. ☐

_______________________ _______________

Name of Patient **Date** **Signature**

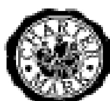
_______________________ _______________

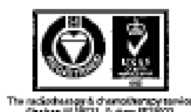
Name of Person taking consent Date Signature
(if different from Principal Investigator)

_______________________ _______________

Name of Principal Investigator Date Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes





PI3K biomarkers in children /Patient Information Sheet 14+/Consent Form/Version 1.0 October 2009

- 4 -

Chairman: Mrs Tessa Green
Chief Executive: Miss Cally Palmer CBE
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The Royal Marsden NHS
NHS Foundation Trust

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INFORMATION FOR PATIENTS AGED 8 - 14 YEARS

Adaptation of an existing Mesoscale Discovery (MSD) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer

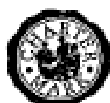
Version 1.0: October 2009

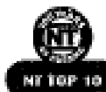
You are having treatment with us for some form of lump in your body and we were wondering if you would be happy to take part in a research study.

Please read this information sheet and talk about it with your mum and dad, your friends, and the doctors and nurses if you want. Ask us if there is anything that you are not sure about. Take plenty of time to decide whether or not you want to take part.

1. Why are you doing this study?

We use different medicines to treat children like you, but at the moment we cannot be completely sure of which medicine is best for each child. By taking one sample from your blood we can try to find out how different drugs might work in your body and then select the best for each situation.





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Chief Executive: Miss Cally Palmer CBE
www.royalmarsden.nhs.uk

2. Why have I been chosen?

Everyone with similar illnesses attending The Royal Marsden can take part in this study.

3. Do I have to take part?

No. It is up to you and your mum and dad to decide if you want to take part. If you take part but then change your mind that is okay and we will not be upset with you.

4. What will happen if I take part?

We will take 1 blood from your long-line. The amount of blood we take is small and will not do you any harm.

5. Confidentiality

All information collected about you for this study is confidential and will be stored securely in locked filing cabinets.

6. What if something goes wrong?

We are not expecting anything to go wrong but if there are any problems please tell us. If you are still unhappy in any way about the study your Mum and Dad can contact the hospital complaints department on your behalf.

7. What will happen to the results of the study?

The results of the study will be printed in medical magazines and may be used to change the future treatment of children with similar illnesses. Your name will not be mentioned.

8. Who is organising the study?

The study is organised by a group of experts who work together to improve treatment for children with cancer.

9. Who can I ask if I want to know more?

If you want to know anything else about this study please ask your doctor or the research nurses and they will be happy to talk to you again.

Thank you for reading this information sheet.

The Royal Marsden **NHS**
NHS Foundation Trust

Downs Road, Sutton
Surrey
SM2 5PT
Switchboard: 020 8642 6011
Direct Dial: 020 8 ______

INFORMATION FOR CHILDREN AGED UNDER 8 YEARS

(to be read by parent or guardian)

Adaptation of an existing Mesoscale Discovery (MSD) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer

(Version 1.0: October 2009)

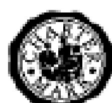
We have spoken to your mum and dad about some of the chemo drugs that we will use in the future in other patients. We are very interested in these drugs and were wondering if you would mind helping us learn more about them. We will tell you what we want to do and then you can tell us yes or no.

We are asking all of the children that come to our hospital if they will let us take some bloods from their long-line.

These bloods will tell us how other drugs might work in your body.

If you are happy for us to take these bloods but then don't want to anymore, it is OK to tell us to stop. We won't be upset or cross with you.

There is nothing for you to worry about and we'll be very happy to talk to you about this again if you want.



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Adaptation of an existing Mesoscale Discovery (MSD) pharmacodynamic assay measuring inhibition of the PI3-kinase pathway to use in children's cancer

Downs Road, Sutton
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Version 1.0, October 2009

Letter to GP

Department Of Paediatrics
Royal Marsden Hospital
Downs' Road
Sutton
SM2 5PT

Tel: 0208 6613603

Dear Doctor

Pharmacodynamic Research at the Royal Marsden Hospital, Sutton

Re: ______________________________________

____

The above mentioned is currently undergoing treatment at the Royal Marsden Hospital in Sutton and has agreed to take part in a pharmacodynamic biomarker research study.

The aim of this study is to validate adult assays in children with cancer to measure the PI3K molecular pathway targeted by new anticancer agents. No specific drug treatment is being investigated.

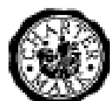
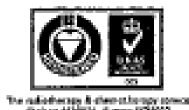
The child will undergo one blood extraction before treatment to validate these assays in paediatric population. An additional bone marrow sample will be drawn in patients with neuroblastoma. The results of the study will not alter the patient treatment plan.

If you need to seek further clarifications, please do not hesitate to contact us. Please ring the number above and ask to speak to Dr.Moreno or the Paediatric Research Nurses team

With best wishes,

Prof. ADJ Pearson
Head of Children's Unit

PI3K biomarkers in children /Letter to GP/Version 1.0 October

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