End of Grant Report

Elisa Izquierdo Delgado report at the end of the grant by Christopher's Smile for the 3 year position of Scientific Officer in the Paediatric Molecular Pathology Team at the Institute of Cancer Research

Christopher's Smile

coz kids get cancer too

Registered Charity 1129906

Contents

1. Position

Title	Scientific Officer in the Paediatric Molecular Pathology Team in the Centre for Molecular Pathology
Role Description	
Start of Grant	January 2012
Period of Grant	3 Years
Name(s) of person in position	Elisa Izquierdo Delgado
Grant funding over period of grant	£155,539.19
Matched funding over period of grant	£43,550.97

Overview of role during grant period

Childhood cancer cure rates have not substantially improved in the last decade, reflecting a lack of new treatment options and limitations of current approaches that employ high-dose chemotherapy, surgery and radiation. To this end delivering precision medicine using next generation sequencing to stratify patients based on their genetic profiling is a key goal for the design of adaptive clinical trials. Although advances in genomics have translated through to many adult cancers, precision medicine trials are still not the standard for children with cancer.

Elisa has focused her work on developing and optimising assays to identify potential prognostic and predictive markers that may aid clinical practise in the context of clinical trials using retrospective and prospective samples in a clinical laboratory.

Elisa has developed a Next Generation Sequencing (NGS) paediatric multi-gene panel for use in formalin fixed paraffin embedded (FFPE) and frozen tissue. The panel can detect recurrent and clinically relevant mutations including 72 genes either altered in paediatric cancers or proven to be clinically actionable in other cancers.

Furthermore Elisa has been working with what is called “liquid biopsies” or circulating DNA. Circulating DNA can be extracted from a blood sample when cancerous cells undergo cell death (apoptosis or necrosis). Elisa has been able to detect circulating DNA from Neuroblastoma patients detecting aberrations in the DNA released from cancerous cells.

We are also delighted to report that we worked exclusively with the BBC to announce the rollout of the new genetic test for children with cancer at 21 hospitals across the UK. Around 400 children with solid tumours will start to receive the new genetic test, as part of a new initiative to begin to personalise children’s cancer treatment. The test could not have been developed without the funding from Christopher’s Smile.

2. Objectives

Year 1

Elisa will develop a clinical assay to detect *ALK* mutations and *ALK* translocations in FFPE clinical samples.

Year 2 and 3

Elisa will search for genes that have been described to be involved in childhood cancer for the development and validation of a paediatric panel using Next Generation Sequencing. She will also work on liquid biopsies and methods to isolate DNA from plasma from Neuroblastoma patients with the possibility of looking for biomarkers in the DNA such as *MYCN* amplification or *ALK* mutations.

3. Work Undertaken

Year 1

Detecting *ALK* abnormalities in Neuroblastoma patients

Elisa designed a test to screen *ALK* mutations by PCR amplification from genomic DNA extracted using Formalin Fixation and Paraffin Embedding (FFPE) followed by Sanger Sequencing. To test the assay 13 samples (4 cell lines and 9 FFPE samples) with known *ALK* mutations were analysed for the validation. All the mutations were identified (Table 1). Electropherograms of samples, 2090, 2180, 916 and 1650 are shown in Figure 1.

ID	Material	Mutation Expected	Mutation observed by Sanger Sequencing
12/9082	cell line	p.F1174L / c.3522C>A	yes
12/9083	cell line	p.F1174L / c.3522C>A	yes
12/9085	cell line	p.F1174L / c.3522C>A	yes
12/9084	cell line	p.R1275Q / c.3824G>A	yes
916	FFPE	p.F1174V / c.3520 T>G	yes
710	FFPE	p.R1275Q / c.3824G>A	yes
771	FFPE	p.R1275Q / c.3824G>A	yes
993	FFPE	p.R1275Q / c.3824G>A	yes
1776	FFPE	p.R1275Q / c.3824G>A	yes
2180	FFPE	p.R1275Q / c.3824G>A	yes
1397	FFPE	p.R1275Q / c.3824G>A	yes
2090	FFPE	p.S1086L / c.3257 C>T	yes
1650	FFPE	p.Y1278S / c.3833A>C	yes

Table 1. Samples used for *ALK* mutation validation and mutations detected

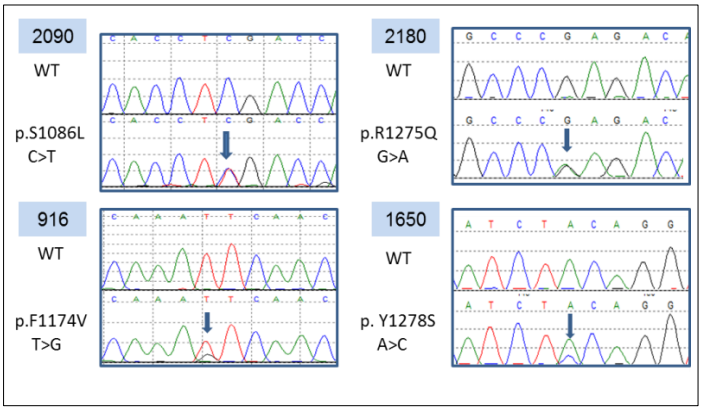

Figure 1. *ALK* mutations. Electropherogram in FFPE neuroblastoma samples using Mutation Surveyor

Once the assay was validated Elisa proceeded to sequence a total of 52 Neuroblastoma clinical samples from 33 different patients (Table 2). Out of the 52 samples 18 failed due to poor quality of DNA, 31 did not have a mutation on *ALK* and 3 samples from different patients were found to have an activating point mutation in the tyrosine kinase domain of *ALK.* Samples that failed were repeated if material was available. Samples where mutations were found were repeated using new FFPE sections to confirm the genetic alteration.

The three mutations identified were present in exon 25. Two patients had a c.3824G>A p.(R1275Q) mutation, resulting in an amino acid substitution at position 1275 in *ALK*, from an arginine (R) to a glutamine (Q). The other sample had a c.3733T>C p.(F1245L) mutation resulting in an amino acid substitution at position 1245 in *ALK,* from a phenylalanine (F) to a leucine (L).

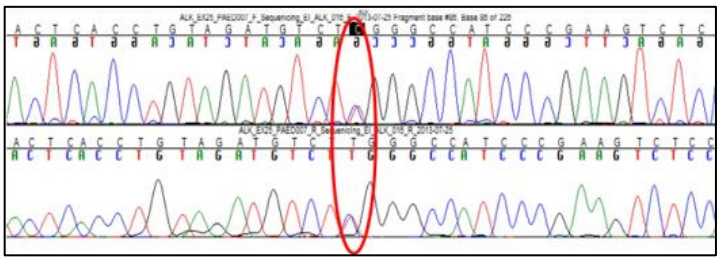

Figure 2. Electropherogram of a FFPE Neuroblastoma sample harbouring an *ALK* mutation

Furthermore, Elisa was trained by a clinical scientist to perform and analyse *ALK* status by *Fluorescence In Situ Hybridisation* (FISH) using the Vysis Dual Colour Breakapart *ALK* FISH probe which detects all variants of the *ALK* gene rearrangement regardless of fusion partners. Once she had completed the training she performed the set up and the analysis for the 52 clinical samples from 33 different patients.

Out of the 52 samples 3 failed due to poor quality sample, 47 were negative for *ALK* translocation and 2 samples (from the same patient) were positive with an unusual pattern for *ALK* rearrangement. *ALK* gene polysomy (3-6 normal *ALK* copies in ≥10% nuclei) was observed in 36 samples (25 patients) and a higher *ALK* copy number (> 6 normal *ALK* copies in ≥ 10% nuclei) was observed in 5 samples (4 different patients). Moreover, one sample had a loss of one copy of *ALK* in 50% of analysed nuclei (Figure 3).

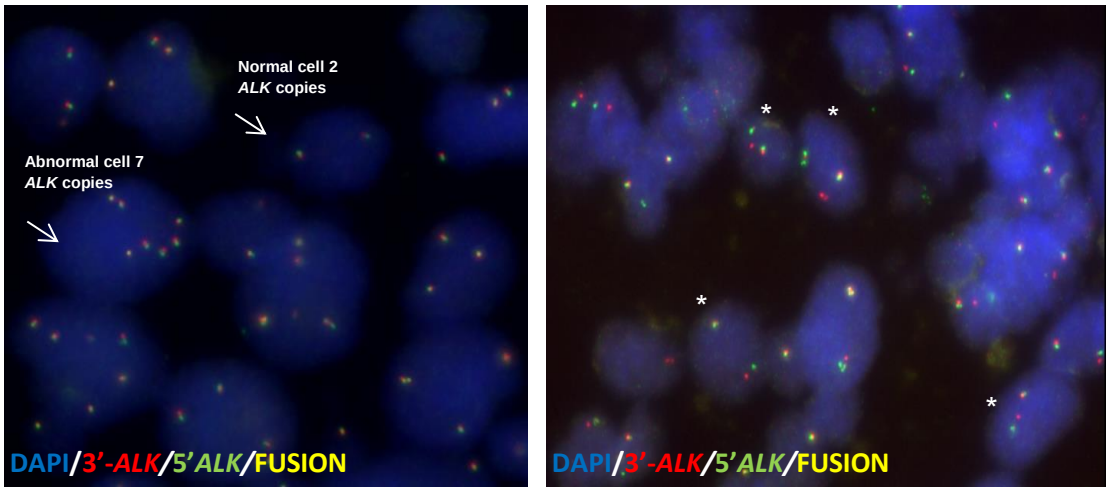

Figure 3. Neuroblastoma samples hybridized with *ALK* dual break apart probe. (A) *ALK*-negative sample for translocation showing some polysomic cells with more than 3 *ALK* copies. (B) *ALK*-positive sample showing some cells with abnormal pattern (*).

Further analysis needs to be done to characterise *ALK* alteration found as it may not translate in the generation of a fusion protein similar to EML4-ALK in lung cancer.

Year 2 and 3

Validation of the paediatric panel using Next Generation Sequencing

Elisa developed a clinically relevant sequencing panel covering 72 genes involved in childhood cancers, for the detection of mutations, amplifications and translocations using FFPE and Fresh Frozen samples (Figure 3). This approach analyses a specific portion of the genome and is called Targeted Next Generation Sequencing. Elisa optimised the protocol for the use of clinical samples.

The panel has been optimised for 200-400 ng of DNA from FFPE or fresh frozen, which are easily available from most paediatric samples. The technology uses DNA to generate NGS library preps using the KAPA HyperPlus kit. These samples are hybridised to a Nimblegen capture panel consisting of 72 genes and regions which are diagnostic, prognostic or predictive in relation to paediatric solid cancer. After hybridisation regions not of interest are washed away and the remaining DNA is sequenced using Illumina technology. The protocol was modified to improve the reads on target and reduce the number of duplicates. Sequence reads are aligned to the genome, metrics determined and somatic SNVs and CNVs called.

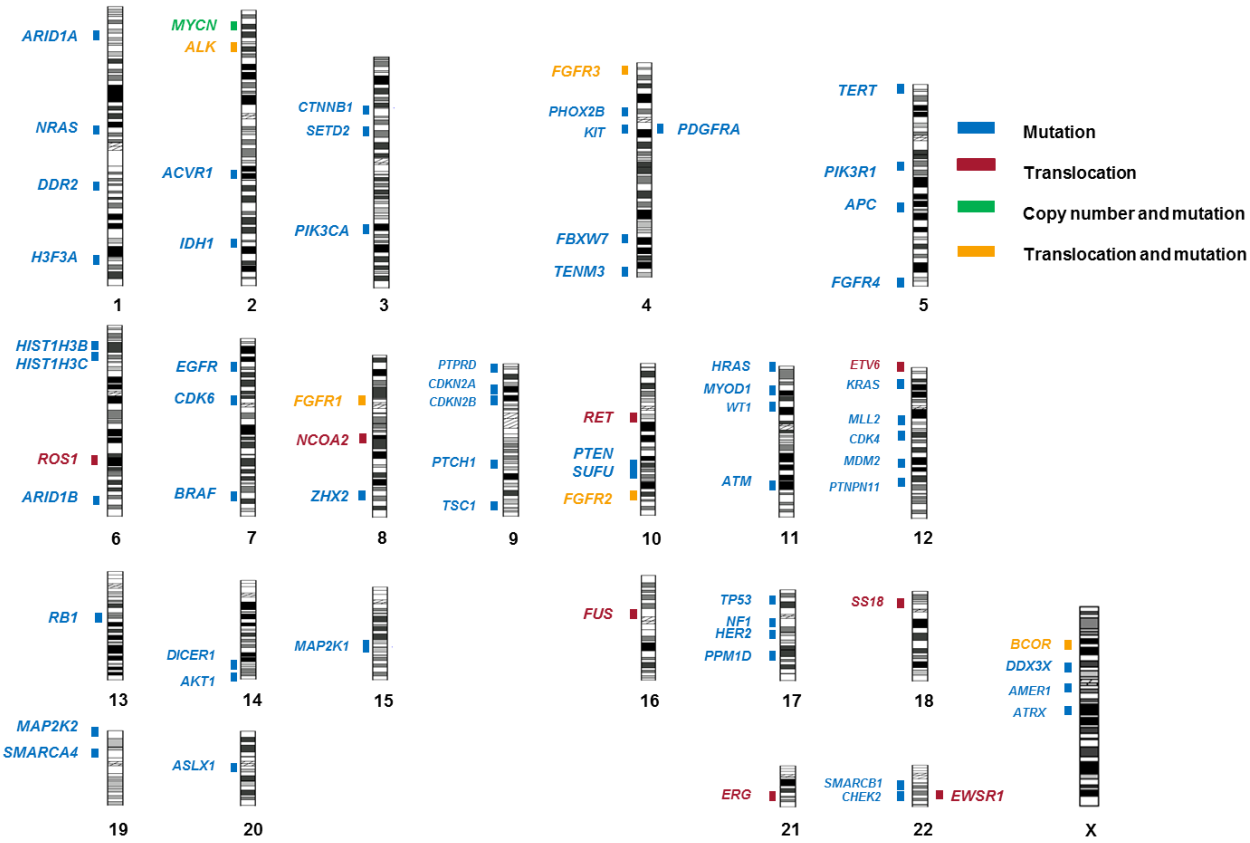

Figure 4 . Diagram of genes analysed by the NGS panel.

For the validation of the technique she used 19 cell lines with known single nucleotide variants, insertions, deletions and copy number variations in order to determine overall performance as well as sensitivity, specificity, repeatability and reproducibility. She compared the variants identified through our pipeline to those previously identified by other methodologies such as Sanger Sequencing, droplet digital PCR (*polymerase chain reaction*) and *FISH (Fluorescence in situ hybridization)*.

Furthermore Elisa used a total of 74 FFPE and 15 Fresh Frozen paediatric tumour samples to determine if the panel design was adequate to detect mutations and copy number variations in clinical tumour samples. She also compared the performance of FFPE against Fresh Frozen samples in paired samples.

The results have been very successful obtaining a very high correlation within the alterations expected to be found with a true allele frequency giving a great sensitivity (>99% for SNVs and >83% for indels [95%CI]) and specificity (>98% for SNVs and >83% for indels [95%CI] Figures 5, 6 and 7). Cell lines were compared against target sequencing data from the Cancer Cell Line Encyclopaedia (CCLE) and we were able to identify all the variants in the regions interrogated by our panel (Table 4).

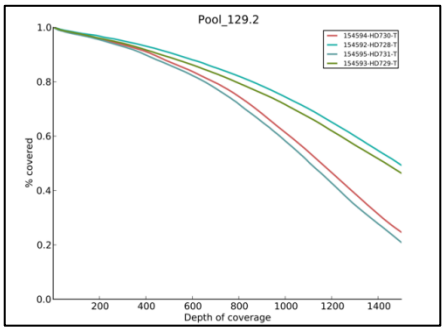

Figure 5. Proportion of panel covered at specified depth in the cell blends. The mean depth was 1285.8 ± 204.2 for the cell blends.

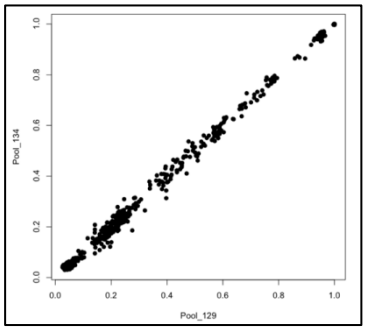

Figure 6. Overall correlation between two runs by different users in different instruments was r^r >0.996 for SNPs and r^r>0.83 for insertions-deletions [95%CI]

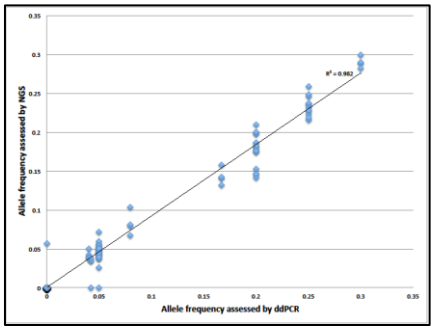

Figure 7. Correlation of variant allele frequencies obtained by NGS and ddPCR for all cancer variants.

For FFPE samples we compared variants against those identified in *ALK* and *H3F3A* using Sanger sequencing and again obtained fully concordant results. Elisa's work was also able to detect amplification of *MYCN* and *CDK4* on the paediatric panel and this was confirmed using SNP6.0 array data from CCLE on the cell lines and FISH for FFP

The comparison between Fresh Frozen and FFPE samples showed that despite the large degree of FFPE sample quality, the correlation of the variant allele frequencies between the

two types of samples was greater than 0.99 concluding that the method can accurately detect variants and copy number changes in FFPE samples as well as in Fresh Frozen samples (Figure 8 and 9).

CELL LINE	GENE	ALTERATION	DETECTED	Allele frecuency expected	Allele frecuency observed
SKNSH	*NRAS*	p.Gln61Lys	YES	15%	23%
RD	*ATM**	p.D273N	YES	17%	2%
SKNSH	*SMARCA4*	p.Arg973Trp	YES	32%	45%
KELLY	*ALK*	p.Phe1174Leu	YES	39%	32%
SK-N-AS	*NRAS*	p.Gln61Lys	YES	45%	46%
SK-N-AS	*RB1*	p.Leu477Pro	YES	47%	31%
KELLY	*MAP2K1*	p.Ala390Thr	YES	48%	47%
RD	*NF1*	p.Glu977Ter	YES	56%	59%
SKNSH	*CHEK2*	p.Thr410MetfsTer15	YES	59%	44%
IMR32	*ATM*	p.Val2716Ala	YES	59%	59%
RH-41	*APC*	p.Met526Leu	YES	60%	59%
RD	*NRAS*	p.Gln61His	YES	68%	61%
KELLY	*TP53*	p.Pro177Thr	YES	93%	99%
RD	*TP53*	p.Arg248Trp	YES	100%	100%
RH-41	*TP53*	p.P152fs	YES	100%	100%
LAN1	*ALK*	p.Phe1174Leu	YES	no data available	47%
LAN1	*TP53*	p.Cys182Ter	YES	no data available	99%
LAN5	*ALK*	p.Arg1275Gln	YES	no data available	50%
Be(2)C	*TP53*	p.Cys135Phe	YES	no data available	100%
RMS559	*FGFR4*	p.Val582Leu	YES	no data available	76%
CCA	*KRAS*	p.Gln61Leu	YES	no data available	29%
SKNSH	*ALK*	p.Phe1174Leu	YES	no data available	36%
KELLY	*MYCN*	AMPLIFICATION	YES	no applicable	no applicable
IMR32	*MYCN*	AMPLIFICATION	YES	no applicable	no applicable
LAN1	*MYCN*	AMPLIFICATION	YES	no applicable	no applicable
LAN5	*MYCN*	AMPLIFICATION	YES	no applicable	no applicable
Be(2)C	*MYCN*	AMPLIFICATION	YES	no applicable	no applicable
RH30	*CDK4*	AMPLIFICATION	YES	no applicable	no applicable
SK-N-AS	*TP53*	DEL exons 10,11	YES	no applicable	no applicable

***ATM mutation in this cell line is subclonal and variation in AF is expected with on-going passages**

Table 4. Known variants in paediatric cell lines were compared against capture sequencing from the Cancer Cell Line Encyclopaedia and other published data. All the variants interrogated by our panel, including mutations, deletions and amplifications, were detected.

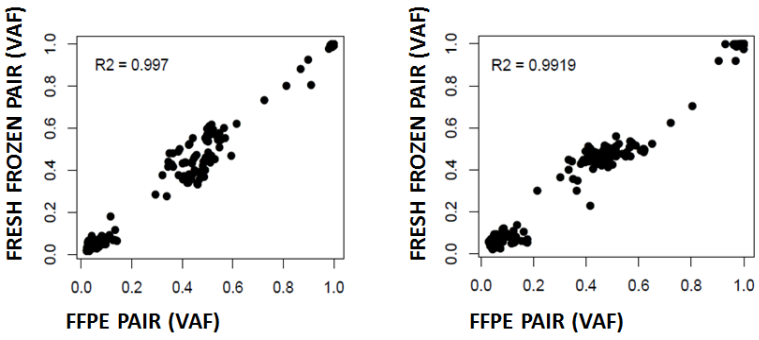

Figure 6. Correlation of the VAFs found between FFPE

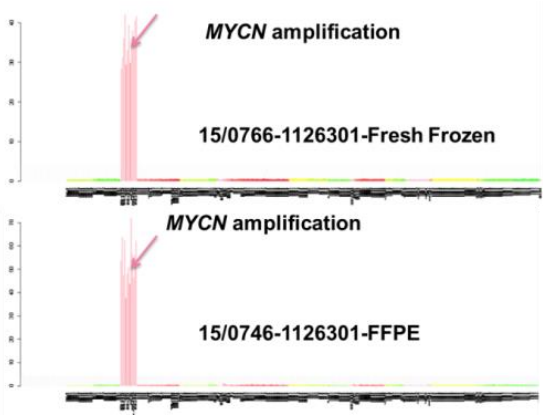

Figure 9. Detection of *MYCN* amplification in neuroblastoma FFPE-Fresh Frozen paired samples previously identified by FISH.

According to these validation results the panel can detect variants with high sensitivity, specificity, reproducibility and repeatability as well as accurate quantification of the variant allele frequency. The panel can detect variants and copy number variation in FFPE samples as well as Fresh Frozen.

Liquid biopsy

Elisa has been working on the detection of circulating DNA from blood samples of patients with Neuroblastoma. She has been able to identify tumour derived genetic alterations analysing the DNA isolated from the blood samples comparing against alterations found in their tumour biopsy.

4. Key Achievements

Year 1

Development of a clinical test for the detection of ALK abnormalities.

Year 2 and 3

Optimisation and validation of the paediatric NGS panel in cell lines, FFPE and Fresh Frozen clinical samples.

The results of the validation have been presented at several meetings including European meetings (SIOPEN, EpSSG). Furthermore Elisa submitted a poster at the Neuroblastoma Research Symposium in Newcastle last November and her work was awarded as the best poster in the category of translational research over 72 posters.

These developments have led to the team being granted a BRC 3-year project award. With this funding, knowledge from the validation will be translated into prospective samples at the Royal Marsden and Great Ormond Street Hospital providing NGS analysis for all paediatric solid tumours within a clinically relevant timeframe. Additionally plasma from the patients will be collected at different stage of disease trying to correlate the presence of genetic alteration detected in the circulating DNA with the tumour tissue.

5. Objectives not met during grant period

All objectives for this project have been met or will be addressed further through future research work.

6. Contribution to published, peer reviewed scientific paper(s)

Poster presented at 4th Neuroblastoma Research Symposium 26-27 November 2015 (Elisa obtained a prize for the best poster in translational research category):

Development of a clinical grade targeted sequencing panel capable of detecting prognostic, predictive and diagnostic markers in patients with Neuroblastoma. Elisa Izquierdo Delgado[1], Sally George[1], Chris Jones[1], Janet Shipley[1], Caedyn Stinson[2], Andrew Moore[2,3], Lynley Marshall[1], Lucas Moreno[1], Louis Chesler[1], Andrew Pearson[1], Lina Yuan[1], Brian A Walker[1] and David Gonzalez De Castro[1] on behalf of the Children's Cancer and Leukaemia Group (CCLG)

[1]The Royal Marsden NHS Foundation Trust & The Institute of Cancer Research, London, United Kingdom [2]The University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia [3]Childrenersity of Queensland Diamantina Institute, Translational Research Institute of Cancer Research

Abstract submitted for the Childhood Cancer Conference 5-7th September 2016:

Validation of a Next-Generation Sequencing assay for detecting actionable mutations in Paediatric Solid Tumours. Elisa Izquierdo Delgado[1,2], Lina Yuan[1], Sally George[3,4], Chris Jones[2], Janet Shipley[5], Susanne A Gatz[4,5], Caedyn Stinson[6], Andrew Moore[6,7], Steven C. Clifford[8], Debbie Hicks[8], Janet Lindsey[8], Rebecca Hill[8], Thomas Jacques[9], Jane Chalker[9], Khin Thway[10], Lynley Marshall[3,4], Lucas Moreno[3,4], Andrew Pearson[3,4] Louis Chesler[3,4], Brian A Walker[1], David Gonzalez De Castro[1]

[1]Centre for Molecular Pathology, The Royal Marsden NHS Foundation Trust & The Institute of Cancer Research, London, United Kingdom. [2]Division of Molecular Pathology and Cancer Therapeutics, The Institute of Cancer Research, London, United Kingdom. [3]Division of Clinical Studies, The Institute of Cancer Research, London, United Kingdom. [4]Paediatric Drug Development Team, Children & Young People's Unit, The Royal Marsden Hospital NHS Foundation Trust, London, United Kingdom. [5]Sarcoma Molecular Pathology Team, Division of Molecular Pathology and Cancer Therapeutics, The Institute of Cancer Research London, United Kingdom. [6]The University of Queensland Diamantina Institute, Translational Research Institute, Brisbane, Australia. [7]Children's Health Queensland Hospital and Health Service, Brisbane, Australia. [8]Northern Institute for Cancer Research, Newcastle University, United Kingdom. [9]Great Ormond Street Hospital for Children NHS Foundation Trust, London, United KingdoM. [10]Sarcoma Unit, Royal Marsden Hospital, London, United Kingdom

Manuscript including all the validation data under preparation -aim for submission by summer 2016.

7. Overview by Dr David Gonzalez de Castro: Team Leader/Department Head -achievements during grant period

Over the last three years Elisa has shown a great enthusiasm to learn and develop new procedures focused on paediatric cancer, obtaining a better understanding of molecular profiling in children with cancer. She has acquired many technical skills, developing tests and following instructions timely and efficiently. She has also developed training experience by supervising and supporting other members of the team.

Elisa's work has progressed significantly, starting with the development of a single gene test to the optimisation and validation of a clinical Next Generation Sequencing paediatric panel which covers 72 genes. Her results have been presented at several meetings including all

the UK paediatric leads (i.e. ECMC network) as well as in European meetings (i.e. SIOPEN, EpSSG) and regulatory meetings (i.e. European regulatory authorities). The NGS panel has now become the standard for paediatric clinical profiling in the UK. The developments that Elisa has achieved in the last 3 years have led to the team being granted with a 3-year project through RMH/ICR BRC. Thanks to the grant and the successful validation prospective molecular profiling in paediatric solid cancers is now implemented at the Royal Marsden providing NGS analysis for all paediatric solid tumours.

She has been very well supported by Dr Brian Walker to develop new skills and supervising her work daily and she is now in position where she has started a PhD with Dr Chris Jones who is a team leader at the ICR internationally recognised for his work in paediatric brain tumours. Thanks again to Christopher's Smile, the opportunity of doing a PhD is expected to contribute significantly to Elisa's support in paediatric oncology from a scientific point of view.

The constant support and motivation from Kevin and Karen have made this work possible and it is very much appreciated.