Characterization of rare cell phenotypes in peripheral blood and their utilisation as a surrogate tissue in pharmacodynamic biomarker assays

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Abstract

Rare peripheral blood cells are defined as white blood cells (WBC) at a population frequency of less than 1 in 1,000. Because blood is a relatively non-invasive and accessible sample, it has the potential to be exploited as a surrogate tissue for biomarker development. This PhD project therefore focused on the development and validation of pharmacodynamic biomarkers in rare WBCs using flow cytometry techniques. These biomarkers were developed and utilised for use in three clinical studies, the LY3143921 phase I trial, the SIOP Ependymoma II trial and the PROSPECT-NE observational study.

An assay focusing on phosphorylated MCM2 (pMCM2) and ki-67 staining of peripheral blood cells was developed for an early phase clinical trial of LY3143921, a novel CDC7 inhibitor. These proteins were tested *in vitro*, *ex vivo* and *in vivo*. The results indicated a significant association between the number of ki-67 positive cells as a percentage of pMCM2 positive WBCs and drug exposure. Furthermore, an appropriate method was successfully developed to preserve pMCM2 and ki-67 signal in samples being transported from national clinical centres, to facilitate its incorporation in the clinical trial.

An assay for the detection of acetylated histone H4 (acH4) was developed for patients receiving valproic acid, a HDAC inhibitor, as a part of the SIOP Ependymoma II trial. The applicability of the assay was tested *in vitro*, *ex vivo* and in patient samples. AcH4 signal was associated with drug exposure *in vitro* and *ex vivo* but was not associated with dose or concentration of valproic acid WBCs in patient samples. This finding was potentially related to the relatively low plasma drug concentrations observed at the dose levels studied. Additional patients with higher drug exposures need to be recruited to future studies to further investigate this issue.

The protein of interest for the PROSPECT-NE observational study was ki-67, which was detected using the same assay as developed for the LY3143921 trial. This study focussed on the prognostic utility of ki-67, rather than its potential predictive or mechanistic properties. The results suggested significant differences in overall survival

between patients with high and low ki-67 positive WBCs. There was no association observed between ki-67 positive WBCs and plasma ki-67 in this study.

In conclusion, pMCM2 positivity in ki-67 positive cells was identified as a potentially useful pharmacodynamic biomarker, with applicability to an ongoing clinical trial. For acH4 positive cells, their utility as a pharmacodynamic biomarker remains questionable and requires further investigation. Lastly, a high proportion of ki-67 positive WBCs was found to be associated with decreased overall survival in people with cancer recruited to a Phase I clinical trial. These findings highlight the potential utility of peripheral rare WBCs as pharmacodynamic and prognostic biomarkers for clinical studies in an oncology setting.

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Statement of work undertaken

The *in vivo* mouse experiments in Chapter 3 were performed by Huw Thomas, the senior research associate at Northern Institute for Cancer Research, Newcastle University.

Total and free valproic acid concentrations in plasma were measured in the Clinical Biochemistry Unit at the Royal Victoria Infirmary, Newcastle upon Tyne.

Patient information including their survival status in PROSPECT-NE trial (Chapter 6) was provided by Jodie Graham, the NCC project coordinator at Newcastle University.

All other work and analyses were performed by myself.

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5-FU	5-fluorouracil
acH4	Acetylated histone 4
AFP	Alpha fetoprotein
ALL	Acute lymphoblastic leukaemia
ALT	Alanine aminotransferase
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
APRI	Aspartate aminotransferase to platelet ratio index
Ara-C	Cytarabine
ASO	Antisense oligodeoxynucleotide
AUC	Area under the curve
BC	Before Christ
BF	Bright field
BMI	Body mass index
BSA	Bovine serum antigen
CBC	Complete blood count
CCD	Charge coupled device
CDC7	Cell division cycle 7
CDK	Cyclin-dependent kinase
CEA	Carcinoembryonic antigen
CML	Chronic myeloid leukaemia
CNS	Central nervous system
CT	Computerized tomography
CTC	Circulating tumour cell
CTCL	Cutaneous T cell lymphoma
CTLA	Cytotoxic T lymphocyte-associated antigen
CV	Coefficient of variation
DAPI	4',6-Diamidino-2-Phenylindole
DDK	DBF4/DRF1-dependent kinase
DLBCL	Diffuse large B cell lymphoma
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
DRF	Differentiation repressing factor
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EORTC	European Organisation for Research and Treatment of Cancer

ER	Oestrogen receptor
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G6PDH	Glucose-6-phosphate dehydrogenase
GB	Gigabyte
HAT	Histone acetyltransferase
HBV	Hepatitis B virus
HCG	Human chorionic gonadotropin
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HL	Hodgkin lymphoma
HPMC	Hydroxypropyl methylcellulose
HPV	Human papilloma virus
HVA	Homovanillic acid
KD	Knockdown
IHC	Immunohistochemistry
IL	Interleukin
LDH	Lactate dehydrogenase
MCM2	Minichromosome maintenance 2
MRI	Magnetic resonance imaging
NCI	National Cancer Institute
NMR	Nuclear magnetic resonance
NSCLC	Non-small cell lung cancer
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD	Pharmacodynamic
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PK	Pharmacokinetic
pMCM2	Phosphorylated minichromosome maintenance 2
PMT	Photomultiplier tubes
PNA	Peptide nucleic acid
PR	Progesterone receptor
PSA	Prostate specific antigen
RBC	Red blood cell
rcf	Relative centrifugal force
RMH	Royal Marsden Hospital

RNA	Ribonucleic acid
ROC	Receiver operating characteristic
rpm	Round per minute
RSC	Round single cells
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAHA	Suberoylanilide hydroxamic acid
SD	Standard deviation
SSC	Side scatter
TADC	Tumour-associated dendritic cell
TAM	Tumour-associated macrophage
TNF	Tumour necrosis factor
TSA	Trichostatin A
US / USA	United States of America
VEC	Vincristine, Etoposide and Cyclophosphamide
VMA	Vanillymandelic acid
WBC	White blood cell
WHO	World Health Organization

Chapter 1. Introduction

1.1 Cancer

1.1.1 Aetiology of cancer

The term 'cancer' was derived from a Greek word 'karkinos' which was used by Hippocrates of Kos around 400 BC (Faguet, 2015). It was used to describe the appearance of invasive solid tumours with projections penetrating into adjacent tissue macroscopically resembling a crab. It was not until the 19th century, however, that it became commonly accepted that tumours were a disease with cellular aetiology, and only in the 20th century that cancer was recognised as a disease of dysregulated hyper-proliferation of cells. More recently scientists have come to consider cancer as a disease of genetic abnormalities. For instance, mutations of RAS and the RAS-family genes have been found to be associated with lung cancer, colon cancer, pancreatic cancer and acute myeloid leukaemia (Feinberg and Vogelstein, 1983; Radich *et al.*, 1990; Sugio *et al.*, 1994; Nishigaki *et al.*, 2005). Similarly, mutations and deletions of BRCA1 and BRCA2 genes are related to breast and ovarian cancers (Narod *et al.*, 1993; Schmutzler *et al.*, 1997; Tseng *et al.*, 1997) and amplifications of EGFR (HER1) genes can lead to glioblastoma, head and neck cancer, and osteosarcoma (Grandis and Tweardy, 1993; Hayashi *et al.*, 1997; Akatsuka *et al.*, 2002).

These alterations of genes can be inherited, leading to an increased risk of cancer developing in a lifetime, or somatic, appearing due to endogenous or environmental triggers. Many studies have demonstrated associations between environmental factors and cancers. In 1775, an association between scrotal cancer and chimney-sweeping was first observed by Percivall Pott (Brown and Thornton, 1957). Later, a meta-analysis of multiple cohort studies showed that exposure to asbestos increased the risk of developing lung cancer and that the increased risk was still present in non-smokers (Ollier *et al.*, 2014; Paris *et al.*, 2017). Tobacco is another well-known cause of cancer, especially lung cancer (Doll and Hill, 1950). Similarly, the presence of aflatoxins produced by mould in some grains such as corn, peanut and rice is associated with liver cancer (Liu *et al.*, 2012). In addition, infections with certain types of virus such as HPV 16 and 18 are known to be associated with risk of cervical cancer (Cogliano *et al.*, 2005).

1.1.2 Cancer treatment and therapeutics

Since cancer was first identified, numerous treatment strategies, including surgery, medication, radiation and more recently stem cell transplantation, have been developed (DeVita *et al.*, 2018). Focusing on pharmacologic treatment, there are many different types of drugs used to treat cancer patients. These can be divided into two major groups which are small drugs and biologics. Small molecule compounds can be further categorised into cytotoxic and targeted drugs, while monoclonal antibodies (MABs) have until recently resembled small molecule drugs in the pathways they target. MABs can now be further categorised into anti-proliferative and immune checkpoint inhibitors (Reichert, 2001).

1.1.2.1 Traditional cytotoxic drugs

Cytotoxic drugs are drugs that are toxic to cells and mostly this toxicity results in cell death. However, as the drugs are toxic to the vast majority of cells, both cancerous and normal tissues are affected. This means that cytotoxic drugs have many serious side effects related directly to their mechanism of action, such as leukopenia, cardiotoxicity, hepatotoxicity and gastrointestinal toxicity. According to Thirumaran R *et al*, there are five major types of traditional cytotoxic drugs divided by mechanism of action (Thirumaran *et al.*, 2007).

Alkylating agents

The mechanism of alkylating agents is covalent binding to DNA. The alkylating agents contain electron-rich atoms, which form covalent bonds with DNA and create inter- and intra-strand cross-links. When DNA is bound with the drug, cells will detect the damage and start the apoptotic process, consequently causing cell death (Trams *et al.*, 1961).

The first drug in this group and the first cytotoxic drug developed was nitrogen mustard. This was followed ten years later by cyclophosphamide, which is essentially a prodrug of nitrogen mustard (Friedman and Seligman, 1954). These early drugs were derived from mustard gas, a chemical warfare agent used during World War 1. It was observed that in addition to being a strong vesicant, the mustards also had cytotoxic

activity that depleted bone marrow and lymph nodes, consequently reducing the numbers of WBC (Gilman, 1946). This agent was therefore suggested as a new treatment for lymphoma during World War 2 (DeVita and Chu, 2008). Another group of drugs with a very similar mechanism of action to these alkylating agents are the platinum compounds, including cisplatin, carboplatin and oxaliplatin (Lebwohl and Canetta, 1998).

Antimetabolites

Most antimetabolites have similar structures to endogenous substances required for cell division. Therefore, cells will allow antimetabolite agents to substitute these compounds, resulting in DNA synthesis interference (Avendaño and Menéndez, 2008). Tumour cells with high growth rate such as gastrointestinal tumours or cancer in bone marrow are most affected by these drugs (Spooner *et al.*, 1982; Schuetz and Diasio, 1985).

Antimetabolites can be further divided into 3 subclasses. The first group, the folate antagonists, includes methotrexate, a drug first synthesized in 1948 which led to successful remissions in the treatment of leukaemia (Farber *et al.*, 1948). The second group, the purine antagonists, was first developed in 1951 (Hitchings and Elion, 1954). This group includes 6-mercaptopurine and azathioprine. The last group are the pyrimidine antagonists, which includes 5-fluorouracil (5-FU), gemcitabine, capecitabine and cytarabine (Ara-C). The first drug in this group, 5-FU, was first synthesized in 1957 and was later found to exhibit cytotoxic activity against solid tumours (Duschinsky *et al.*, 1957).

Anti-tumour antibiotics

The mechanism of action of most anti-tumour antibiotics is blocking topoisomerase II activity and intercalating into the flat space between the bases of DNA double helix (Sartiano *et al.*, 1979). Most antibiotics in this class contain an anthracycline structure and they were first isolated from the bacteria, *Streptomyces*.

Examples of drugs in this class include doxorubicin, epirubicin, daunorubicin, actinomycin-D and mitoxantrone (Thirumaran *et al.*, 2007). The first drug in this group is actinomycin-D which was developed during World War 2 period and mostly used in paediatric tumours between 1950 and 1960 (Pinkel, 1959).

Topoisomerase inhibitors

The topoisomerase inhibitor group of anticancer drugs consists of some anti-tumour antibiotics such as doxorubicin and mitoxantrone and the plant alkaloids (Pommier, 2006). For example, etoposide is an alkaloid drug extracted from the *Podophyllum* plant. The project that led to the discovery of etoposide started in the 1950s but it wasn't until 1965 that this drug successfully went into cancer clinical trials (Imbert, 1998). Irinotecan and topotecan are isolated from the *Camptotheca* plant species. These two drugs were synthesized based on the discovery of camptothecin in 1966 and were approved by the US FDA in the mid 1990s (Wall *et al.*, 1966).

The mechanism of action of the topoisomerase inhibitors is binding to topoisomerase I and/or II enzymes, resulting in prevention of DNA ligation after cleavage and torsional relief. These irreversible covalent cross-links between the topoisomerase and DNA prevent its replication and thereby cause cell death (Binaschi *et al.*, 1995).

Mitotic inhibitors

Anti-microtubule agents, which were discovered in the 1950s and entered clinical use in the 1960s, work by interrupting the organisation and dynamics of the mitotic spindle within the cell. Consequently, mitosis is prevented and the cell cycle stops (Cheng and Crasta, 2017). Most agents used in this class were originally extracted from two major plants: the periwinkle plant (*Catharanthus roseus* G. Don) and the Pacific yew (*Taxus brevifolia* Nutt) (Eric, 1997).

Vinca alkaloids are a group of anti-cancer drug extracted from the periwinkle plant (Moudi *et al.*, 2013). Vinblastine, vincristine and vinorelbine are examples of

vinca alkaloids. Their mechanism of action is inhibition of tubulin polymerization and prevention of mitotic spindle formation. The other group of antimicrotubules is the taxane derivatives, which were initially extracted from the bark of the Pacific Yew tree (Ojima *et al.*, 2016). In contrast to the vinca alkaloids, the taxanes, including paclitaxel, docetaxel and cabazitaxel, shift microtubules dynamics towards stabilisation and the formation of aberrant intracellular structures, resulting in cell death (Abal *et al.*, 2003).

1.1.2.2 Targeted therapies

With the discovery of multiple oncogenes and proto-oncogenes, the development of anti-cancer drugs has more recently focused on the synthesis of drugs that directly target the cancer genes and proliferative pathways rather than conventional cytotoxic drugs (Sawyers, 2004). Recently there are more than 30 targeted anti-cancer drugs approved by US FDA and more than 20 targets in development processes (National Cancer Institute, 2019).

The RAS-RAF-MEK pathway is one of the most important targets for cancer treatment because upregulation of MAPK cascades were found to be associated with carcinogenesis (McCain, 2013). Examples of drugs approved in this class are dabrafenib (target BRAF), vemurafenib (target BRAF) and trametinib (target MEK). Another critical pathway in cancer is the PI3K-AKT-mTOR pathway (Porta *et al.*, 2014). Anti-cancer drugs targeting this pathway which are approved for clinical use include everolimus and temsirolimus (Ciuffreda *et al.*, 2010).

Based on the hallmarks of cancer, there are several targets for cancer therapeutics being developed and/or FDA approved (Hanahan and Weinberg, 2011). For example, PARP inhibitors such as olaparib target genome instability and mutation (Tangutoori *et al.*, 2015). VEGF signalling inhibitors such as bevacizumab inhibit angiogenesis (Ellis and Hicklin, 2008). EGFR inhibitors such as cetuximab, erlotinib and panitumumab target sustaining cell proliferative signalling (Ono and Kuwano, 2006). Flavopiridol, a cyclin-dependent kinase inhibitor, works by evading growth suppressors of cells (Sedlacek, 2001). The HGF/c-Met pathway is another target to stop cancer invasion and metastasis; rilotumumab, onartuzumab and tivantinib are examples of

drugs inhibiting HGF and/or c-Met (Smyth *et al.*, 2014). In addition, the development of immune checkpoint inhibitors, e.g. CTLA-4 inhibitors, PD-1 inhibitors and PD-L1 inhibitors, is ongoing (Darvin *et al.*, 2018).

1.2 Cancer biomarkers

1.2.1 Definition of a biomarker

The term 'biomarker' or 'biological marker' has been defined by several organisations since 1988. As defined by the National Institutes of Health Biomarkers Definitions Working Group, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). An alternative definition established by WHO in 2001 defines a biomarker as "any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (WHO, 2001).

According to these definitions, biomarkers can be any biological or molecular entity including specific cells, proteins, molecules, genes, gene products or hormones, which can be used to measure a biological process (Huss, 2015). Furthermore, some people may include pulse rate, blood pressure, and routine laboratory tests in their definitions of a biomarker if they use these variables to measure biological processes (Strimbu and Tavel, 2010).

1.2.2 Biomarker classification

There are several categorisation systems of biomarkers currently in use, with biomarkers classified by their intended purposes into six major groups: screening, diagnostic, monitoring, prognostic, predictive and mechanistic.

Screening biomarkers

Several substances have been developed or are being developed as markers for the early detection of cancer (Duffy, 2015). For example, vanillymandelic acid (VMA) and homovanillic acid (HVA) have been used in screening for neuroblastoma in newborn infants (Hanai *et al.*, 1987). Alpha fetoprotein (AFP) is effectively used in screening of hepatocellular cancer, especially in patients infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) (Tsuchiya *et al.*, 2015). CA125 is currently used for screening of ovarian cancer in combination with transvaginal ultrasound (Skates *et al.*, 2017).

Diagnostic biomarkers

Diagnostic biomarkers are used to indicate the existence of pathological changes in tissues or organs, or indicate occurrence of abnormal cells or tissue function (Davis *et al.*, 2013). For example, using aspartate aminotransferase to platelet ratio index (APRI) to indicate liver fibrosis is an example of this type of biomarker (Jin *et al.*, 2012). However, although many biomarkers are currently used for helping diagnosis and/or sub-classification of tumour stage, none are currently recommended in clinical practice guidelines to be used alone for cancer diagnosis.

Monitoring biomarkers

Monitoring biomarkers are characteristics that can be serially measured to assess the status of a disease (Califf, 2018). This type of biomarker commonly overlaps with other categories of biomarkers. Examples include CA15-3, which is used in the therapeutic monitoring of breast cancer and PSA, which can be used to monitor prostate cancer. Similarly, thyroglobulin can be used in the monitoring of thyroid cancer (Nixon *et al.*, 2017).

Prognostic biomarkers

This biomarker class is able to predict the natural course of the disease/effect and inform the long-term outcome. For instance, CA125 half-life has been shown to correlate with survival rate in patients with cancer of the ovary; women with ≤20 days of CA125 half-life had worse overall survival compared to who had a half-life >20 days (Colakovic *et al.*, 2000). Oestrogen receptor (ER) and Progesterone receptor (PR) status can be used to predict the prognosis of breast cancer patients (Yip and Rhodes, 2014).

Predictive biomarkers

Unlike the prognostic biomarkers that predict the natural outcome of the disease, predictive biomarkers predict the outcome of treatment (Mordente *et al.*, 2015). In other words, predictive biomarkers can indicate the probability of a specific response to treatment. KRAS mutation is an example of a predictive biomarker because it can distinguish patients with metastatic colorectal cancer who do not benefit from EGFR inhibiting therapy (Lièvre *et al.*, 2006). In addition, HER2 receptor in patients with breast cancer is used to identify patients likely to respond to treatment with HER2 receptor antagonists such as trastuzumab and pertuzumab (Iqbal and Iqbal, 2014).

Mechanistic biomarker

Mechanistic biomarkers link directly to the modulation of a specific target or signalling pathway (Davis *et al.*, 2013). For example, adipsin is used as a biomarker for disruption of NOTCH-1 signalling and induction of goblet cell proliferation in the small intestine (Searfoss *et al.*, 2003). As this type of biomarker can measure mechanism of action of the drug, it can also be used as a pharmacodynamic biomarker.

1.2.3 Importance of biomarkers in cancer research

As biomarkers can be used for many purposes, their utilities in cancer are numerous, including screening, diagnosis, prognosis, prediction of therapeutic

response, tumour staging and monitoring efficacy of cancer therapy (Henry and Hayes, 2012). Nevertheless, not all biomarkers are currently used clinically as many proposed diagnostic biomarkers provide insufficient sensitivity and/or specificity. Additionally, apart from HCG and AFP, the accuracy of biomarkers for tumour staging remains poor (Kulasingam and Diamandis, 2008).

Many biomarkers with high accuracy and precision are approved by the US FDA for screening and monitoring of cancer therapy (Sauter, 2017). Examples include digital rectal examination (DRE) which is widely used as a screening biomarker for prostate cancer and CEA which is a serum protein effectively used to monitor colorectal cancer (Torosian and Daly, 1991; Jones *et al.*, 2018).

Focusing on the area of cancer drug discovery and development, biomarkers can have several essential roles, ranging from confirmation of the pharmacological mechanism of a drug to the prediction of clinical outcome (Floyd and McShane, 2004). Biomarkers also play important roles in early stage drug development, including therapeutic target identification and validation, candidate drug screening and optimisation, proof-of-concept, and drug combination mechanistic understanding. Furthermore, biomarkers can be used in the prediction of patient response, resistance and toxicity of a drug (Kelloff and Sigman, 2012).

1.3 Techniques for biomarker development

Various techniques have been used for biomarker discovery and development. The development process can be divided into 4 main stages: the discovery of potential biomarkers, biomarker assay development, technical qualification of assays and the clinical validation of new biomarkers (Cummings *et al.*, 2008).

Approaches to biomarker discovery can be considered in terms of the tissue being analysed. These can be described in terms of '-omics' (Davis *et al.*, 2013; Kewal, 2017). Firstly, genomics involve techniques such as DNA sequencing, microarrays, gel electrophoresis and PCR to measure nucleic acid sequences. Proteomics on the other hand are technologies designed to examine protein expression and characterisation.

They have huge potential in biomarker development, especially at the level of diagnostic and predictive biomarkers. Well-known proteomic techniques such as ELISA, mass spectrometry and Western blot are now widely used.

Metabolomics refers to the study of all metabolites found in biological samples. For example, techniques such as high-resolution NMR spectroscopy are effective to quantify *in vivo* metabolites and generate metabolic profiles. Other techniques including mass spectrometry, vibrational spectroscopy and other imaging techniques can also be used (Zhang *et al.*, 2012a). Lastly, analytes in cells and tissues can be measured by 'histocytomic' approaches (Davis *et al.*, 2013). Analysis of cell structure and function can be performed by techniques such as microscopy and flow cytometry. In addition, immunohistochemistry can be applied for phenotypic characterisation of tissues.

Deciding the most suitable approach for assessing any given biomarker depends on many parameters. For example, which tissues express the biomarker or have an active metabolic pathway to be measured. Once tissues have been identified, the most suitable technology for analysis of the marker in that tissue must be decided. In addition, the feasibility and safety of sample collection, cost of the approach, stability of the samples collected and delivery time to the laboratory are factors that need to be concerned in biomarker selection. The projects described in this thesis focus on rare cell populations in peripheral blood samples. The investigation of these populations was initially developed in parallel with establishing CTC detection assays using imaging flow cytometry.

1.3.1 Principles of flow cytometry

Flow cytometry is one of the most commonly used techniques in biomarker discovery and development. It was developed and has been used for over 50 years (Muirhead *et al.*, 1985). This technique is based on the principles of immunofluorescence and cell/particle-analysing technologies. The anatomy of a flow cytometer can be described under 3 main headings, namely fluidics, optical and electronic systems, as shown in Figure 1.1 (BD Biosciences, 2000). Particles are suspended and introduced into the fluidics system focused into single objects. The optics system consists of lasers to excite fluorochromes and filters to direct emitted

light to the electronics of the system, typically photomultiplier tubes (PMT). The digital signals generated by the PMT's are then processed for computer analysis (BD Biosciences, 2000).

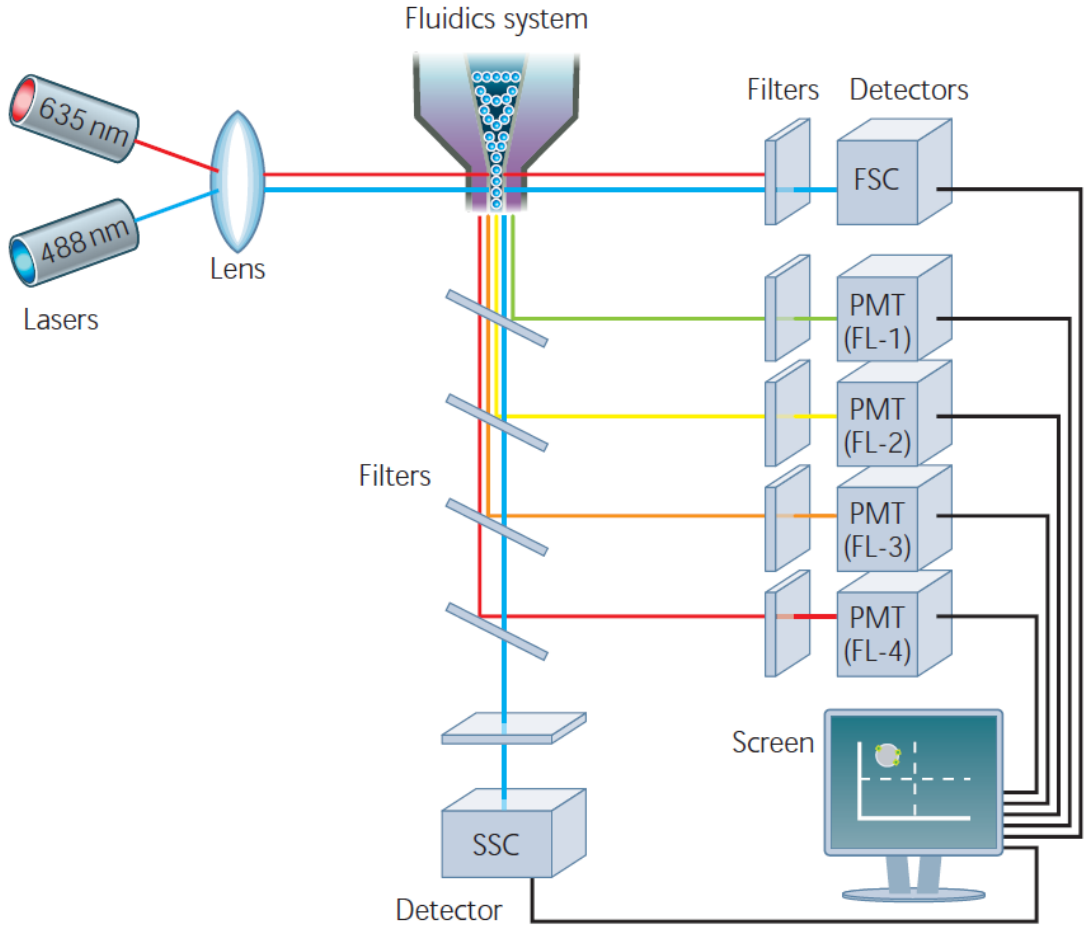


Figure 1.1. The anatomy of a conventional flow cytometer.
Particles are injected into the core where they pass through the path of laser beams. Scattered and emitted light are detected by either silicon photodiodes or PMTs for analysis. Taken from BD Biosciences. Introduction to Flow Cytometry: A Learning Guide Manual. 2000.

Flow cytometry techniques are heavily dependent on immunofluorescence and the appropriate selection of fluorochromes is critical. Fluorochromes are selected based on excitation potential with the wavelengths of lasers in the machine. Knowing the excitation and emission properties of fluorescent compounds is essential for the selection of combination of fluorochromes which optimally work together. Table 1.1 shows examples of fluorochromes that can be used in flow cytometry (Overton, 2006).

Fluorochrome	Excitation Peak (nm)	Emission Peak (nm)	Laser Wavelengths (nm)
DAPI	350	470	405
Hoechst	350	461	405
FITC	490	525	488
Alexa Fluor 488	490	525	488
PE	565	578	488, 561
Cy3	554	568	561
Alexa Fluor 546	556	573	561
APC-Cy7	650	785	658
Alexa Fluor 647	650	665	658

Table 1.1. Examples of fluorochromes for immunophenotyping with excitation and emission peaks at different wavelengths.

Adapted from Overton WR. Guide to Flow Cytometry. Dako; 2006. p. 17-22.

1.3.2 Imaging flow cytometry and its application in biomarker development

Imaging flow cytometry is a technology that combines conventional flow cytometry with microscopy. While conventional flow cytometers capture and convert emitted photons to electrical signals on a photomultiplier tube, imaging flow cytometers are able to capture high resolution magnified images on a charge coupled device (CCD) camera. The CCD cameras in the ImageStream® can collect emitted florescence light that is magnified by a microscopic objective lens and spectrally resolve it into six discrete bandwidths by an array of dichroic filters as shown in Figure 1.2 (Amnis Corporation; Han *et al.*, 2016).

Based on these functions of imaging flow cytometry, high content data can be provided for biomarker development. The unique information given by imaging flow cytometers includes localisation of proteins or particles in cells. For instance, by visual inspection of protein localisation researchers can identify nuclear or cytoplasmic localisation.

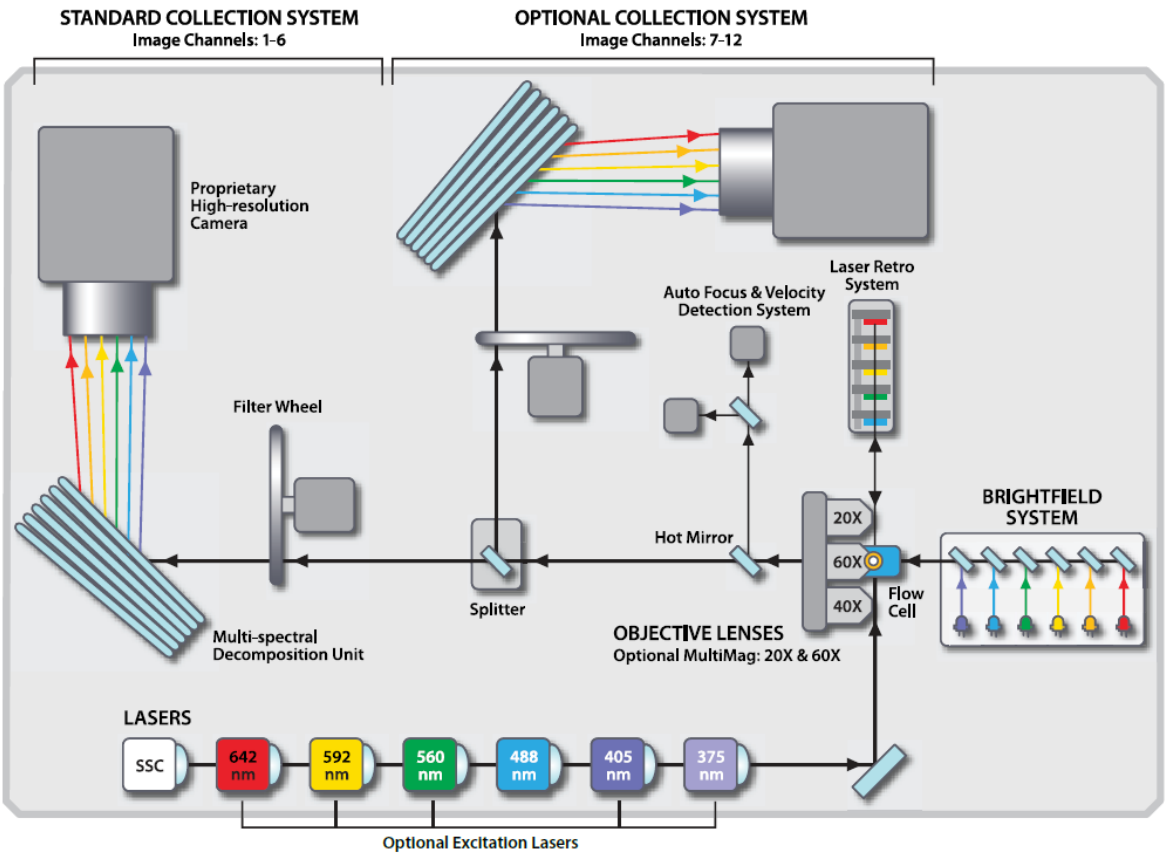


Figure 1.2. The anatomy of an imaging flow cytometer (ImageStream®).
Particles injected through the tube, excited fluorochromes and emitted light at 90° is magnified, spectrally resolved and detected. Taken from Amnis Corporation. The ImageStreamX Mark II Imaging Flow Cytometer

Although imaging flow cytometry provides useful information for research, it also has its drawbacks. A major limitation is that as the machine has to capture so many cell images, the speed of acquisition decreases significantly. Whereas conventional flow cytometers can acquire up to 10,000 events/second, imaging flow cytometers can process less than half this number (Barteneva *et al.*, 2012). For example the ImageStream® system has a maximum acquisition rate of 4,000 cells/second at 20× magnification and only 1,200 cells/sec at 60× magnification (Amnis Corporation). Another challenge is the significant size of the data generated by imaging flow cytometers because each collected particle contains an image file. For a file with 100,000 cells acquired, the size will be approximately 1.5-2.5 GB for example. This may slow down the analysis of data and quickly cause shortage of storage space when many experiments are performed.

1.4 Surrogate tissues used in pharmacodynamic biomarker development

In the area of biomarker development, selection of suitable surrogate tissues is another critical point to be considered, as different tissues provide the researcher with different information. In general an ideal tissue should be non-invasive, relevant to the drug and facilitate measurement of the biomarker of interest. Biopsy samples comprising the target are widely considered to be an ideal tissue, but they are invasive and not always safe to obtain (Floyd and McShane, 2004). Therefore, utilisation of blood derivatives such as white blood cells as a surrogate tissue, is another option to be considered.

1.4.1 Peripheral blood components

Human blood contains numerous components which can be divided into two major groups: a solid part referred to as formed or cellular elements and a liquid part called plasma (Silverthorn, 2013). Plasma consists of water (90% of volume) and more than 100 different solutes including plasma proteins such as albumin (60% of proteins) and globulin (36% of proteins), non-protein nitrogenous substances, organic nutrients, electrolytes respiratory gases and hormones (Marieb and Keller, 2017).

Formed elements in blood consist of erythrocytes, leukocytes, and platelets. Erythrocytes or red blood cells are cells without nuclei or organelles. Their shapes are like biconcave or flattened discs with depressed centres. When looking through a microscope, they appear lighter in colour at the thin centres than at the edges. Red blood cells play an important role in transporting oxygen from the lungs to tissues and carrying carbon dioxide from the tissues back to lungs. They can function for approximately 100-120 days and then are destroyed in the spleens (Crosby, 1959; Jelkmann, 1992).

Platelets are cell fragments of large cells called megakaryocytes. Platelets are instrumental in the coagulation process together with other coagulation factors. Moreover, platelets contain some chemical granules which are important in clotting process such as serotonin, calcium ion, enzymes and platelet-derived growth factor (Marcus, 1969; Harrison, 2005).

Leukocytes or white blood cells are the last formed elements with only 4,800-10,800 cells per microliter of blood (4.8-10.8 × 10^9/L). However, they can be exploited in biomarker development because WBCs contain more particles including a variety of proteins, granules and nuclei. Further details of white blood cells are described below (Feher, 2012; Ashton, 2013).

1.4.2 Characteristics of white blood cells

White blood cells are the only type of blood cell which can be called complete cells as they have nuclei and other organelles. White blood cells can be classified in two different ways (Figure 1.3) (Marieb and Keller, 2017): by expression of granules and by their original stem cells. For the granule classification, there are two types of white blood cells that can be defined as either granulocytes (with granules) or agranulocytes (without granules). Neutrophils, eosinophils and basophils are granulocytes, while lymphocytes and monocytes are agranulocytes. However, when divided by their original stem cell, neutrophils, eosinophils, basophils and monocytes are in the myeloid group, since they are all derived from myeloid stem cells. On the other hand, lymphocytes are classified as lymphoid because of their lymphoid origin.

The functionality of imaging flow cytometry allows granularity to be measured and expressed as side scatter. Since granules contain proteins and enzymes, they are opaque and can refract laser beams, resulting in side scatter. The granularity of white blood cells therefore can be used to differentiate types of white blood cells. Basically granulocytes, i.e. neutrophils, basophils and eosinophils, present with a high side scatter due to their dense granules. On the contrary, lymphocytes with small cytoplasm and no granules exhibit very low side scatter. Monocytes, are typically intermediate in size and density between granulocytes and lymphocytes.

Without additional phenotyping the ImageStream® system allows resolution of lymphocytes and granulocytes. Furthermore, eosinophils are able to directly bind to fluorochromes such as FITC, resulting in false positives (Floyd *et al.*, 1983). This study therefore excluded cells with very high intensity of side scatter which indicate eosinophils.

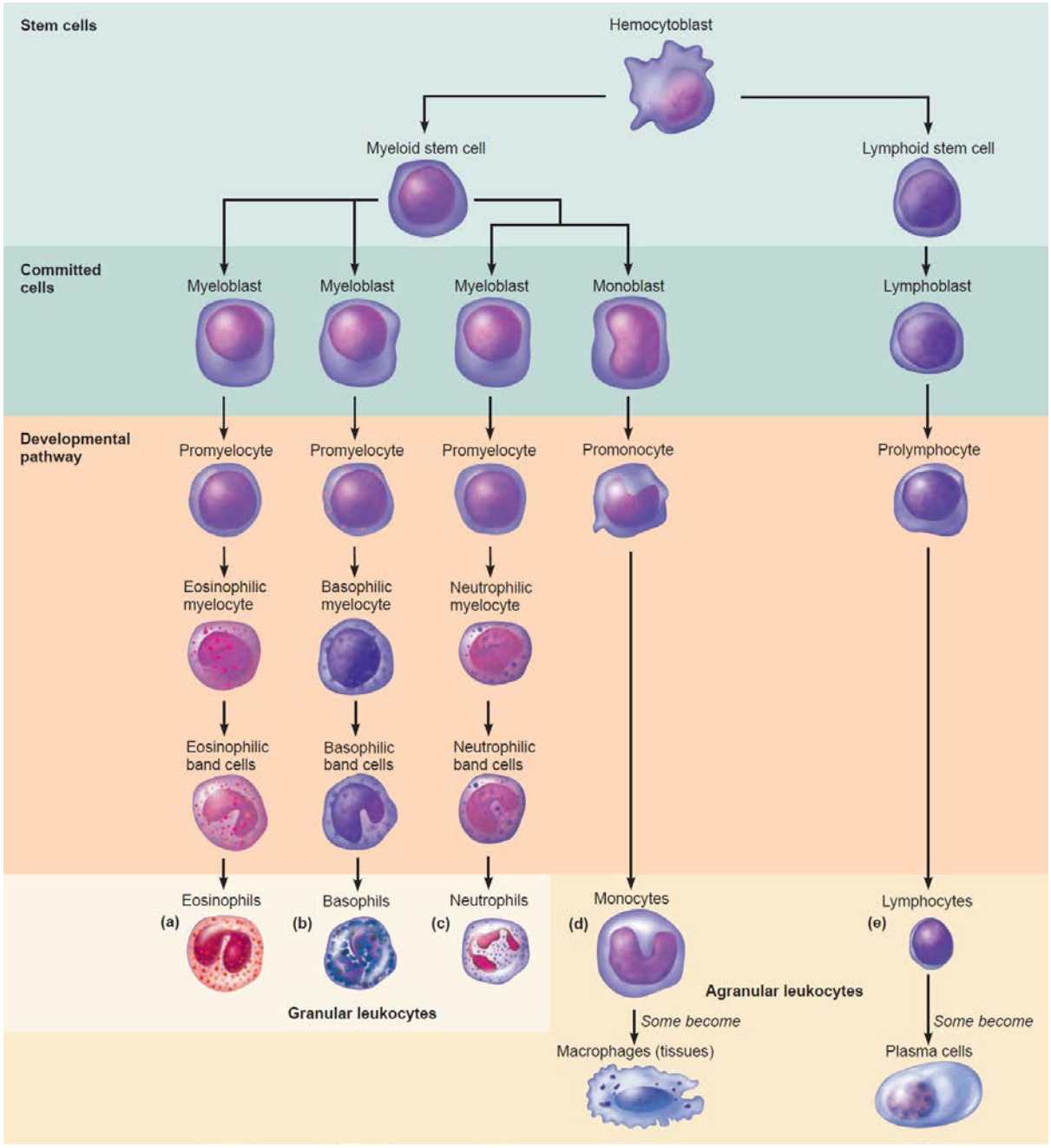


Figure 1.3. Formation of white blood cells from hemocytoblasts to mature cells. White blood cells can be classified either as myeloid and lymphoid cells or granular and agranular leukocytes. Taken from Marieb EN and Keller SM. Pearson Education; 2017. p. 363-81.

In addition to granular expression, myeloid and lymphoid cells exhibit additional characteristics including differences in life span and expression of genes/proteins. Although myeloid cells are the major population of cells comprising >75% of all white blood cells, they generally have a short lifespan, especially in the case of neutrophils (Ashton, 2013). A previously published study showed that

expression of surface markers such as CD45 increased 1.6 times in granulocytes, as compared to 0.9 times in lymphocytes, when left in EDTA tube for 24 hours (Immunicon Corporation). Thus, any assays of myeloid cells should have minimal complexity and rapid data collection; otherwise it may alter the results due to changes inside myeloid cells (Priel and Kuhns, 2019).

Myeloid and lymphoid cells also exhibit differences in gene and protein expression as shown in several studies (Hashimoto *et al.*, 2003; Palmer *et al.*, 2006), with results suggesting that myeloid cells express more genes than lymphoid cells. Moreover, the majority of genes expressed in each cell type have different functions, resulting in different responses to treatment between the two populations. Therefore, WBC type is a key parameter to be considered in pharmacodynamic biomarker development.

Since many characteristics of myeloid and lymphoid cells are variable between cell type, the current studies focused on differences in terms of the expression of biomarkers of interest. Protein expression in each WBC population was measured and compared to the other WBC types, in order to determine the most appropriate cell populations for particular biomarkers of interest.

1.5 Clinical trials related to this study

1.5.1 CDC7 inhibitor LY3143921 - Phase I clinical trial

A Cancer Research UK Phase I Trial of LY3143921 is a first in human trial of a new drug, namely LY3143921, in adult patients with advanced solid tumours. The main aim of this study is to find out the maximum tolerated dose of LY3143921 that can be safely administered to patients. Secondary objectives include assessments of pharmacokinetic and pharmacodynamic properties of the drug.

The clinical trial eligibility criteria includes patients being 18 years or older. Patients must have histological or cytological diagnosis of incurable, advanced/metastatic solid cancer, WHO performance status of 0 or 1, life expectancy of at least 12 weeks, and haematological and biochemical indices within normal ranges.

Patients are excluded from the trial if they received radiotherapy (except for control of symptoms where irradiated lesions will not be followed for response), endocrine therapy, immunotherapy or chemotherapy during the previous 4 weeks. Additionally, if they present grade 2 or greater toxicities due to previous treatments, have symptomatic brain metastases, have hypotension or uncontrolled hypertension, have co-existing active infection, have known to be serologically positive for hepatitis B, hepatitis C or HIV, have a history of allergy or auto-immune disease, or have other serious concurrent medical conditions, they are ineligible for the trial.

LY3143921 hydrate is administered orally to the recruited patients, either once or twice a day depending on the particular patient cohort. Patients have to stay in hospital for 1-3 nights to closely monitor any side effects. Blood samples are taken 9 times over the first 24 hours post-treatment, and once on day 2 for side effect monitoring and PK/PD analyses. Blood samples – 7 blood tests over a period of 24 hours – are collected again 4 weeks later.

As LY3143921 is a CDC7 kinase inhibitor, this PhD project aimed to develop an assay to measure the consequences of CDC7 inhibition. The appropriate candidates for CDC7 inhibitors include phosphorylated MCM2 and ki-67 as downstream proteins in the CDC7 pathway.

1.5.2 SIOP Ependymoma II clinical trial

The SIOP Ependymoma trial is a large clinical trial run by the International Society of Paediatric Oncology to study the diagnosis and treatment of children, adolescents and young adults with ependymoma. This study consists of two main parts; the first part is focused on the results of post-operation MRI or CT scan and the second part is looking at treatment pathways. Patients recruited in the first-part trial would receive MRI or CT scan to observe the existence of tumour and the patients with recurrence might be recruited to the second part of the project. The acH4 analysis project which is the focus of this thesis, is part of the second part of the trial which is divided into 3 strata. Stratum 1 is a phase III randomised study for patients whose age ≥12 months and <22 years at diagnosis and have had a complete resection with no measurable residual disease. The recruited patients will be randomised to receive conformal radiotherapy followed by either 16 weeks of chemotherapy or observation.

Stratum 2 is a randomised phase II study for patients with the same range of age as stratum 1 but have inoperable measurable residual disease. Patients will be randomised to receive two different treatment schedules of chemotherapy either with vincristine, etoposide and cyclophosphamide (VEC) or VEC plus high dose methotrexate.

Stratum 3 involves children <12 months of age or those not eligible to receive radiotherapy, and this is where the acH4 biomarker work is incorporated. Patients who are eligible for this stratum must have newly diagnosed intracranial ependymoma of WHO grade II-III confirmed by central pathological review and be ineligible to receive radiotherapy due to age at diagnosis. In addition, patients must have adequate bone marrow function, liver function and renal function. They must have received no previous chemotherapy (except steroids), no previous radiotherapy, no co-existent unrelated disease, e.g. renal and haematological diseases that would render the patient unable to receive chemotherapy, no medical contraindication to chemotherapy and no signs of infection.

The recruited patients are randomised to receive dose dense chemotherapy, alternating myelosuppressive and relatively non-myelosuppressive drugs at 2 weekly intervals, with or without valproic acid. For valproic acid, the initial dose of 30 mg/kg/day is administered to patients in 2 divided doses (15 mg/kg/dose) for two weeks, with the potential for the dose to be increased weekly (up to 60 mg/kg/day in 2 divided doses), depending on the drug concentrations observed. If therapeutic levels of 100-150 μg/mL are not achieved when giving 60 mg/kg/day, valproic acid can be administered in three divided doses (20 mg/kg/dose twice daily). If therapeutic levels are still not achieved, the dose can be slightly increased up to 70 mg/kg/day in 3 divided doses (23.3 mg/kg/dose).

Blood samples for the pharmacodynamic study are obtained pre-treatment (prior to the first dose of valproate) and at 4-hour post valproate administration following the initial valproate dose. Once the individualised dose resulting in target trough valproate concentrations has been determined, pharmacodynamic samples are again taken at the same time points (4 hours post-treatment and prior to the next

dose). Additional blood samples can be taken at 6 monthly intervals until the end of treatment, whether or not patients achieve the therapeutic target.

Since valproic acid acts as an inhibitor of the HDAC enzyme, acetylation of histone H4 is a result of valproic acid activity and can be developed as a potential marker for measurement of HDAC inhibiting effect. This PhD project therefore aimed to develop an assay to detect acH4 as a pharmacodynamic biomarker of valproic acid.

1.5.3 PROSPECT-NE observational study

PROSPECT-NE (Molecular PROfiling in Early Clinical Trials – North East) is an observational study that primarily aims to determine the range of molecular abnormalities presented in patients with cancer. This trial started in September 2017 but the ki-67 analysis project was only included as part of the study in February 2018. The inclusion criteria are histological or cytological diagnosis of cancer, ECOG performance status of 0-2 and age >16 years old. Patients must not have known HIV, hepatitis B or hepatitis C positive and must have expected life expectancy >8 weeks.

The eligible patients are referred to the Sir Bobby Robson Unit at the Freeman Hospital for potential entry onto a Phase I trial. Whilst awaiting a trial, the recruited patients can donate blood every 4-6 weeks, up to a maximum of four times. These blood samples are then processed in the laboratory for ki-67 analysis. Patients will not receive any chemotherapy or cancer-relating treatment but they can receive palliative care such as corticosteroids.

In addition to blood samples, patients are also subject to other clinical assessments. For instance, performance status, RMH score, and health related quality of life measured with EQ-5D and EORTC QLQ-C30 questionnaires are documented at each visit. Also, patients are asked to perform grip strength test as a biomarker associated with frailty and poor prognosis. Additional blood tests such as CBC, albumin, LDH and C-reactive protein are performed only when necessary.

Ki-67 protein is a well-known proliferation marker and is recently an interest in the field of cancer prognosis (Scholzen and Gerdes, 2000). However, most prognostic studies focused on ki-67 expression in tumour tissue although WBC is considered a safer surrogate tissue. Therefore, this PhD project aimed to investigate the possibility

of utilising ki-67 as a potential prognostic biomarker in patients recruited to the PROSPECT-NE clinical trial.

1.6. Aims and objectives

The primary objective of the work described in this thesis was to investigate the characterization of rare cell phenotypes in peripheral blood, in order to develop them as surrogate tissues for pharmacodynamic biomarker assays. The rare cell phenotypes utilised in this study were divided into three populations depending on the relevant clinical studies. For the LY3143921 clinical trial, CDC7, pMCM2 and ki-67 expression in lymphoid cells were studied. For the SIOP Ependymoma II trial, acH4 expression in myeloid cells was focused on. Lastly, ki-67 expression in lymphoid cells was investigated for the PROSPECT-NE clinical study. The main aims were as follows:

1. To validate imaging flow cytometry assays for CDC7, pMCM2 and ki-67 incorporating assay variability, reproducibility, sample collection and storage experiments.
2. To measure pMCM2 and ki-67 positive lymphoid cells as a PD biomarker as part of a Phase I clinical trial of LY3143921, a novel CDC7 inhibitor.
3. To validate imaging flow cytometry assays for acH4 regarding assay variability and reproducibility.
4. To measure acH4 positive myeloid cells as a PD biomarker as part of a clinical trial in children with ependymoma receiving valproic acid.
5. To characterise the phenotype of a ki-67 cell population in an observational study using imaging flow cytometry and to investigate the prognostic utility of ki-67 in peripheral blood cells.

Chapter 2. Materials and Methods

2.1 Materials and reagents

The CDC7 inhibitor LY3143921 was obtained from Eli-Lilly and Company. Valproic acid sodium salt and DMSO were from Sigma® Life Science. The primary antibody, clone EP4120, against pMCM2 Ser53 was from Abcam® and secondary goat anti-rabbit antibody conjugated to phycoerythrin (PE) was from Life Technologies. CDC7 antibody (polyclonal) conjugated to fluorescein isothiocyanate (FITC) was obtained from Bioss Antibodies. The acH4 antibody (polyclonal) conjugated to PE was from Milli-Mark™. The ki-67 antibody clone B56 conjugated to Alexa Fluor® 647 and Lyse/Fix Buffer were obtained from BD Biosciences. DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) was from BioLegend®.

RPMI-1640 medium with L-glutamine and sodium bicarbonate was from Sigma-Aldrich. Bovine serum antigen (BSA) solution was obtained from Miltenyi Biotec. Foetal bovine serum (FBS) and tablets were from Gibco® by Life Technologies. The ki-67 ELISA kit was from R&D Systems. The ultracentrifugal filters, Centrifree®, were from Merck Millipore.

2.2 Cell culture

HL-60 (Human promyelocytic leukaemia) cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% FBS. Cells were cultured in 75 mm^3 flasks with 1×10^6 cells in 20 mL of media. Flasks were incubated at 37°C in a 5% CO_2 incubator. Sub-culturing was performed every 3-4 days by transferring 1×10^6 cells to a new flask with 20 mL of media. Cells were routinely tested for mycoplasma every 2 months by the institute and disposed after 7 subcultures.

2.3 Whole blood collection and white blood cell extraction

2.3.1 Blood samples from healthy volunteers

Whole blood from healthy volunteers was obtained from students and staff members at the Northern Institute for Cancer Research, Newcastle University. The protocol was approved by the ethics committee of Newcastle University (protocol number ET13-002). All blood samples were taken from volunteer donors by registered doctors and were transferred directly to specific blood tubes depending on the experimental protocol.

To isolate white blood cells, red blood cells in whole blood samples were lysed by BD Lyse/Fix Buffer. This buffer was diluted in distilled water (1:5) and whole blood was added in the proportion of 20 mL of diluted buffer per 1 mL of whole blood. The solution was well mixed and incubated at 37°C for 10 minutes, followed by centrifugation at 500×*g* for 10 minutes. The supernatant was gently poured off and the cell pellet was resuspended in 1 mL of PBS and transferred to an Eppendorf tube. The sample tube was centrifuged at 500×*g* for 5 minutes and the supernatant removed. The cell pellet was again washed with 1 mL of PBS, centrifuged (500×*g* for 5 minutes) and the supernatant removed. Lastly, cells were resuspended in ice cold methanol for permeabilisation. The white blood cell suspension was stored at -20°C for at least 24 hours.

2.3.2 Blood samples from patients in the SIOP Ependymoma trial

Whole blood samples were collected from children with ependymoma being treated with valproic acid as part of the SIOP Ependymoma II clinical trial. The study protocol was approved by the UK East Midlands Multicentre Research Ethics Committee and written informed consent was obtained from patients or parents as appropriate. Participating clinical sites were based in several cities around the UK including Nottingham, Leeds, Sheffield, Oxford and Cambridge. Whole blood was drawn from participants and added directly to the diluted BD Lyse/Fix Buffer, which was prepared using the protocol described above (section 2.3.1), to fix WBCs and lyse RBCs. The blood tubes were frozen on dry ice, and then delivered to the laboratory within 3 days.

When frozen blood samples arrived the laboratory, blood tubes were warmed in a water bath at 37°C for 30 minutes or until the whole sample was defrosted. Samples were then centrifuged at 500×*g* for 10 minutes and the supernatant removed, washed with 1 mL PBS solution and transferred to Eppendorf tubes. The washing step was repeated and cell pellets were resuspended in ice cold methanol. Samples were stored at -20°C until used. This process was performed within 24 hours of the samples arriving in the laboratory.

2.3.3 Blood samples from patients in the PROSPECT-NE trial

Whole blood was taken from cancer patients participating in the PROSPECT-NE clinical trial at the Freeman Hospital, Newcastle upon Tyne. Appropriate ethical approval and consent was obtained. Whole blood was added directly to CellSave® tubes and delivered to the laboratory within 5 days of collection. White blood cells were isolated from patient samples using the protocol described in section 2.3.1.

2.4 Free valproic acid extraction and valproic acid quantification

Plasma samples from patients studied on the SIOP Ependymoma trial were frozen and delivered to the laboratory at the same time as whole blood samples. To obtain free valproic acid, plasma samples were defrosted in a 37°C water bath for 30 minutes, and then centrifuged at 2,000×*g* for 5 minutes. The supernatant was pipetted into the reservoir of an ultrafiltration filter. Samples were centrifuged at 1,500×*g* for 20 minutes following the manufacturer's instruction. The clear filtered solutions were sent to the Department of Blood Sciences laboratory at the Royal Victoria Infirmary, Newcastle upon Tyne, to measure concentrations of valproic acid. These were analysed alongside patient plasma samples, in order to measure free and total valproic acid levels, respectively.

Concentration of valproic acid in plasma, either as free or total drug, was quantified using a Roche/Hitachi™ Cobas C 701 analyser. The assay is based on a homogeneous enzyme immunoassay technique, i.e. the competition between drug in the sample and drug labelled with the enzyme G6PDH for antibody binding sites. The

measuring range of this machine is 2.8-150 μg/mL (19.4-1040 μM) and the repeatability (within-run precision) is 4.8%CV (Roche Diagnostics, 2012).

2.5 CDC7, pMCM2 and ki-67 assay validation experiments

2.5.1 In vitro concentration dependent experiments

HL-60 cells (1×10^6) were seeded in 2 mL RPMI-1640 medium supplemented with 10% FBS in 6-well plates. LY3143921 hydrate powder was dissolved in distilled water (dH_2O) to make up 100 μg/mL of drug solution. The solution was pipetted to the prepared wells at final concentrations of 0.001, 0.01, 0.1, 1 and 10 μg/mL, with dH_2O added to the last well as a negative control. The volume of all wells were adjusted with dH_2O. The plate was then incubated at 37°C, 5% CO_2 for 6 hours.

Cell suspensions were harvested by transfer to Eppendorf tubes and centrifuged at 500×*g* for 5 minutes. The media was aspirated and cell pellets washed twice with 1 mL PBS. Cells were centrifuged at 500×*g* for 5 minutes and supernatants removed. Lastly cells were resuspended in -20°C methanol and stored in a -20°C freezer for at least 24 hours before analysis.

2.5.2 Ex vivo concentration dependent experiments

Whole blood samples from healthy volunteers were collected into EDTA tubes, and then divided into Falcon™ tubes (2 mL of blood/tube). LY3143921 powder was dissolved in dH_2O to obtain a stock concentration of 10 mg/mL. The drug solution was diluted with dH_2O to 1 mg/mL and added to each blood tube to obtain final concentrations of 0.001, 0.01, 0.1, 1, 10 and 100 μg/mL and the final volume adjusted with the solvent. Distilled water was added to the last tube as a negative control. All tubes were incubated in a shaking incubator at 200 rpm, 37°C for 6 hours. Blood samples were collected using the protocol mentioned in section 2.3.1. The experiment was performed three times with three independent blood donors.

2.5.3 Ex vivo time dependent experiments

Whole blood in EDTA tubes collected from healthy volunteers, was divided into two sets of Falcon™ tubes (2 mL of blood/tube). The first set was treated with 10 μg/mL of LY3143921 in dH_2O and the second set was treated with dH_2O at the same volume. Both sets of blood tube were incubated in a shaking incubator at 200 rpm, 37°C. One blood tube in each group was collected at time points of 0, 0.5, 1, 3 and 6 hours. The collected blood samples were immediately processed for RBC lysis and WBC fixation using the protocol mentioned in section 2.3.1. The experiment was performed three times with blood collected from three independent donors.

2.5.4 In vivo experiments

In vivo experiments were performed in 6-8 weeks old female CD1 mice which were between 25-30 g. Eighteen mice were divided into three equal groups of six mice per group. LY3143921 was re-suspended in a vehicle of 5% Ethanol and 95% (1%w/v) HPMC; the final concentrations of the drug were 1.5 mg/mL for 15 mg/kg group and 5 mg/mL for 50 mg/kg group. The first and second groups of mice received 15 mg/kg and 50 mg/kg of LY3143921 suspension by oral gavage, while vehicle was administered to the third group. Blood samples were collected from the tail veins of three mice per group before treatment as baseline control, and then collected at 6 hours. The other three mice in each group were bled at 3 and 24 hours post-treatment and all mice were sacrificed after the second blood collection. All blood samples were processed using the same protocols for human samples as mentioned in section 2.3.1.

2.5.5 Whole blood storage stability tests

2.5.5.1 Whole blood storage in different blood tubes

Different commercially available blood collection tubes including EDTA tubes, CellSave® tubes, TransFix® tubes and Streck® tubes were tested for their ability to preserve CDC7, pMCM2 and ki-67 antigens. Whole blood from one donor was divided equally and added to each blood tube following the manufacturer's instructions. EDTA tubes containing whole blood were stored at 2-8°C while the other tubes were stored at room temperature as recommended by manufacturers. Aliquots of blood (2mL) from each tube were collected to measure CDC7, pMCM2 and ki-67 expression at 0, 6,

24, 48 and 72 hours. RBC lysis and WBC fixation were as described in section 2.3.1. Experiments were performed three times with three independent blood donors.

2.5.5.2 Whole blood storage at -80℃

One part Lyse/Fix Buffer was diluted in 4 parts distilled water to make up a stock solution. Twenty mL of the buffer was added to each of six Falcon tubes. Whole blood from a healthy volunteer was added to the prepared buffer in the proportion of 1 mL of whole blood to 20 mL of buffer. The tubes were gently inverted eight times and immediately frozen at -80°C. Samples were further processed at time points of 0, 1, 2, 3, 4 and 5 days. The collected blood tubes were thawed and WBCs isolated using the protocol for frozen blood as described in section 2.3.2. The experiment was performed three times with three independent blood donors.

2.5.6 CDC7, pMCM2 and ki-67 antibody incubation assays

Aliquots of cells ($1x10^6$) in methanol were pipetted into an Eppendorf tube and centrifuged at 500×*g* for 5 minutes. Methanol was aspirated and the cell pellet was re-suspended in 1 mL of a solution of 5% BSA in PBS. The cell suspension was centrifuged at 500×*g* for 5 minutes, the supernatant removed and the pellet re-suspended in 1 mL of 5% BSA in PBS. The cells were incubated in the solution for 1 hour at room temperature for protein blocking.

Following blocking the cells were centrifuged at 500×*g* for 5 minutes, the supernatant removed and the pellet re-suspended in 1 mL of 5% BSA in PBS with 1 μL of pMCM2 primary antibody. Cells were incubated with the antibody for 1 hour at room temperature and then washed with 1 mL of 5% BSA in PBS. The cells were then incubated in 1 mL of 5% BSA in PBS with 1 μL of secondary antibody conjugated with PE conjugated goat anti-rabbit secondary, 0.5 μL of CDC7 antibody conjugated with FITC and 5 μL of DAPI for 1 hour at room temperature. The antibody solution was then removed and cells washed with 1 mL of 5% BSA in PBS.

Lastly, the cells were centrifuged at 500×*g* for 5 minutes and 90% of the supernatant was aspirated before 1 μL of antibody against ki-67 conjugated with AlexaFluor® 647 was added to the remaining cell suspension. The cells were incubated

with the antibody for 1 hour and washed with 1 mL of 5% BSA in PBS. The supernatant was removed and cells were analysed by an imaging flow cytometer.

For any samples where a CDC7 signal was not required, CDC7 antibody was not added to the sample at the second incubation step. This protocol was applied to all cell types including cell lines, mouse WBCs and human WBCs.

2.6 Acetylated histone H4 assay validation

2.6.1 In vitro concentration dependent experiments

Aliquots of RPMI-1640 medium supplemented with 10% FBS (2mL) were pipetted into each well of a 6-well plate and then one million HL-60 cells were seeded in each well. Valproate sodium powder was dissolved in RPMI-1640 medium to make up a 50 mM stock solution. The solution was pipetted to the prepared wells to yield final concentrations of 0.5, 1, 2, 4 and 8 mM, with medium only added to the last well as a negative control. The volume in each well was adjusted with the medium. The plate was then incubated at 37°C, 5% CO_2 for 6 hours. Cell samples were collected using the protocol for cell lines as described in section 2.5.1. The experiments were repeated three times.

2.6.2 In vitro time dependent experiments

HL-60 cells ($1x10^6$) were seeded in 2 mL RPMI-1640 medium supplemented with 10% FBS in 6-well plates. Valproate sodium powder was dissolved in RPMI-1640 medium to make up a 50 mM stock solution. The solution was then pipetted into all prepared wells to provide a final concentration of 4 mM sodium valproate in each well. The cell suspension in the first well was collected immediately as a baseline control. The plate was then incubated at 37°C with 5% CO_2 for up to 24 hours. The treated cells were harvested at 0.5, 1, 3, 6 and 24 hours post-treatment. Cell samples were collected using the protocol for cell lines as described in 2.5.1. The experiment was repeated three times.

2.6.3 Ex vivo concentration experiments

Whole blood from a healthy volunteer was collected into EDTA tubes and divided into 6 Falcon™ tubes (2 mL of blood per tube). Valproate sodium powder was dissolved in RPMI-1640 medium to obtain a concentration of 50 mM. The drug solution was added to individual blood tubes to generate final concentrations of 0.5, 1, 2, 4 and 8 mM and the final volume was adjusted with RPMI-1640 medium. The medium was added to the last tube as a negative control. All tubes were incubated in a shaking incubator at 200 rpm, 37°C for 6 hours. RBC lysis and WBC fixation were processed following the protocol described in section 2.3.1.

2.6.4 acH4 antibody incubation assay

Aliquots of cells ($1x10^6$) in cold methanol were pipetted into Eppendorf tubes. The cell suspensions were centrifuged at 500×*g* for 5 minutes at room temperature and the methanol was aspirated. Cell pellets were then re-suspended in 1 mL of 5% BSA in PBS and again centrifuged at 500×*g* for 5 minutes at room temperature. The supernatant was aspirated and cells were incubated with 1 mL of 5%BSA in PBS for 1 hour at room temperature, centrifuged and the supernatant again removed. The antibody against acH4 (1 μL) was added to the cell suspensions and incubated overnight at 2-8 °C.

Cells were washed with 1 mL of 5% BSA in PBS, centrifuged and the supernatant removed. DAPI (5 μL) was pipetted into the cell suspensions, which were incubated at room temperature for 1 hour. One mL of 5% BSA in PBS was added to each tube to wash the cells. The tubes were then centrifuged and the supernatants aspirated. Lastly acH4 expression was measured by an imaging flow cytometer as described below.

2.7 Assay for ki-67 detection for the PROSPECT-NE trial

WBCs ($1x10^6$) in methanol, as prepared in section 2.3.3, were pipetted into Eppendorf tubes. Samples were centrifuged at 500×*g* for 5 minutes at room temperature and the methanol was aspirated. The cells were re-suspended in 1 mL 5%

BSA in PBS, centrifuged at the same speed and the supernatants aspirated. The cells were then incubated in 1 mL 5% BSA in PBS at room temperature for 1 hour for protein blocking.

WBCs were centrifuged again at 500x*g* for 5 minutes and the supernatants removed. Ki-67 antibody (1 μL) and DAPI (5 μL) were added to the samples, which were mixed well and incubated at room temperature for 1 hour. The cells were then washed with 1 mL of 5% BSA/PBS and centrifuged to remove the supernatants. Samples were run on the ImageStream® imaging flow cytometer to measure ki-67 expression.

2.8 Imaging flow cytometer data collection

Cell suspensions as prepared above were analysed on an ImageStream® imaging flow cytometer with the parameters listed below as appropriate:

- To measure CDC7, pMCM2 and ki-67 expression the 405 nm laser was set at 30 mW, The 488 nm laser at 50 mW, the 642 nm laser at 100 mW and the 785 nm laser at 11.83 mW.
- To measure ki-67 expression, the 405 nm laser was set at 30 mW, the 642 nm laser at 100 mW and the 785 nm laser at 11.83 mW.
- To measure acH4 expression, the 405 nm laser was set at 30 mW, the 488 nm laser at 100 mW and the 785 nm laser at 11.83 mW.

The gating used to collect cells of interest is illustrated in Figure 2.1. First, round single cells were gated from area and aspect ratio; particles with 50-200 μm^2 in area and 0.6-1.0 in aspect ratio were gated as round single cells. This population was then screened by raw maximum intensity of DAPI to exclude non-cell particles. Any particles showing expression of DAPI were identified as cells and collected. For cell lines, 5,000 cells were acquired per sample while 100,000 cells were acquired for blood samples.

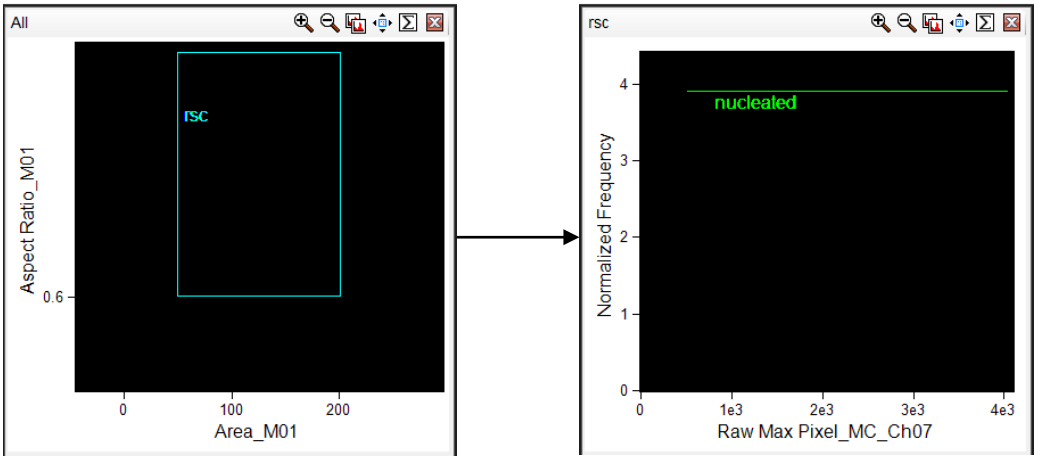


Figure 2.1. Illustration of the imaging flow cytometry gating used to collect the cells of interest in this study.

Round single cells (RSC) were gated from the scatter graph of aspect ratio against area, and then nucleated cells in the RSC population were identified by the histogram of raw maximum intensity of DAPI (Ch07).

2.9 Data analysis by Ideas® software

2.9.1 Analysis of CDC7, pMCM2 and ki-67

The data analysis process is illustrated in Figure 2.2. Collected cells from the imaging flow cytometer were analysed using Ideas® software version 6.2. Cells were first gated as round single cells (RSC) by the area and aspect ratio to reduce non-cell particles and clusters of cells. Of the RSC population only cells in focus were selected for further analysis using a predefined feature in the Ideas® software, gradient root mean squared, of bright field; cells with a value greater than 60 were included in the focused cell group.

Focused cells were divided into two populations – myeloid and lymphoid cells – using intensity of side scatter and area of cells. The expression of CDC7, pMCM2 and ki-67 was assessed in each population based on the intensity of staining and similarity in localisation between protein and nuclear (DAPI) staining. Cells with high intensity and high similarity, with positive signal in the nucleus, were identified as positive. As the majority of CDC7 was cytoplasmic expression, the CDC7 expression was assessed by gating all CDC7 positive cells (nuclear and cytoplasmic positive cells). For pMCM2 and ki-67 expression, only nuclear positive cells were counted.

The results were reported as percentages of CDC7 positive, nuclear pMCM2 positive and nuclear ki-67 positive cells in myeloid, lymphoid and total WBC populations. The percentage of pMCM2 positive cells was also shown as a proportion of ki-67 positive cells. Moreover, the intensity of all three proteins was measured in each cell population by histograms (data not shown) and reported as mean and median intensity of protein expression.

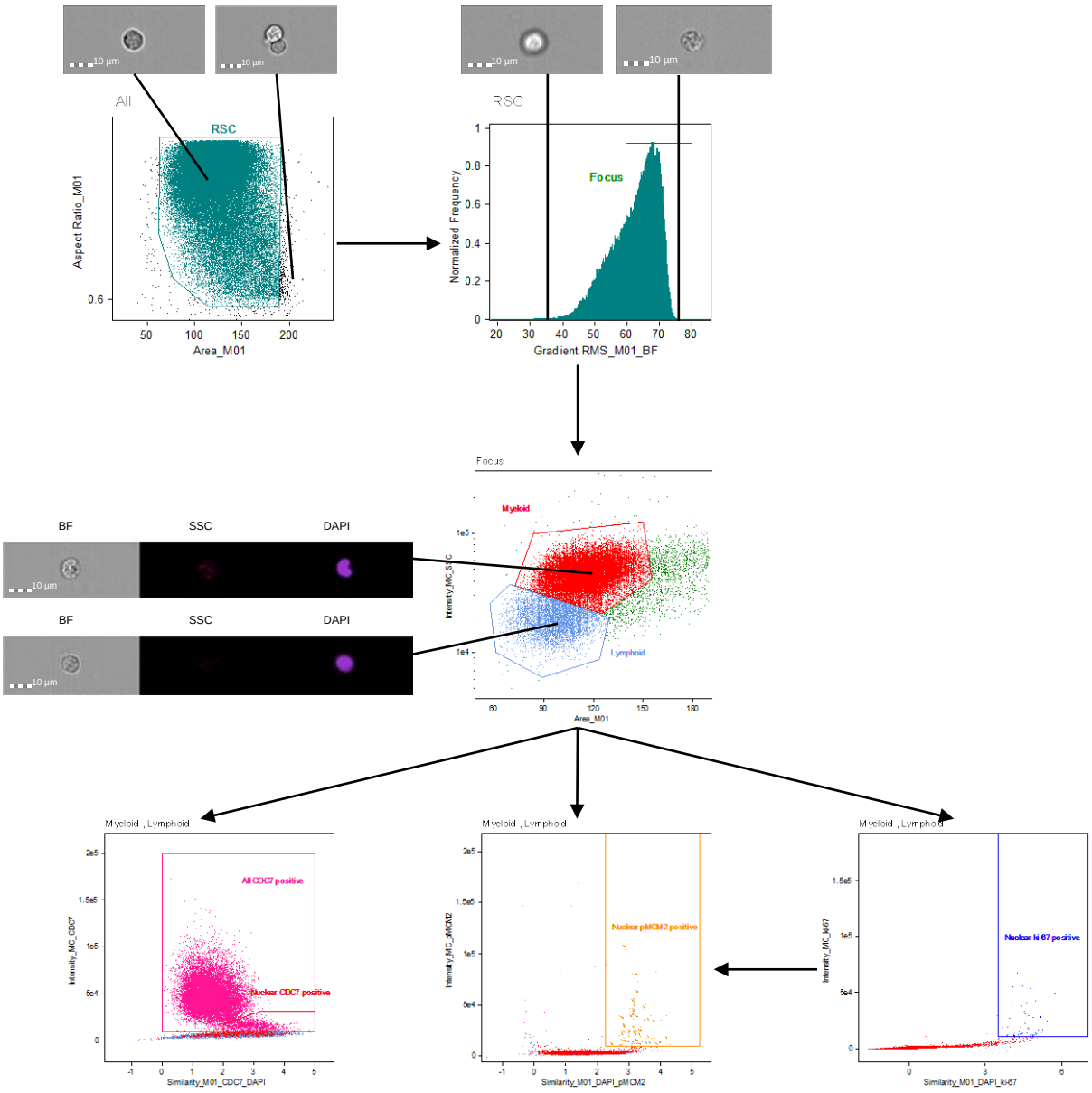


Figure 2.2. The process of data analysis of CDC7, pMCM2 and ki-67.

Round single cells (RSC) were first gated and the focused cells gated out of the RSC population. Cells were then divided into myeloid and lymphoid cells. CDC7, pMCM2 and ki-67 expression levels were determined in each cell type by looking at the similarity between protein and DAPI expression and intensity of protein.

2.9.2 Analysis of acH4

Collected cells from the imaging flow cytometer were analysed using Ideas® software version 6.2. As shown in Figure 2.3, cells were first gated for round single cells, focused cells, and myeloid and lymphoid cells using the same gating as described in section 2.9.1. Myeloid and lymphoid cells were then gated for nuclear acH4 positive cells using a scatter graph between intensity of acH4 and similarity between acH4 and DAPI; cells with high similarity and high intensity of acH4 were identified as nuclear acH4 positive cells.

Results were expressed as percentage of acH4 positive cells in myeloid, lymphoid and total WBC populations. Furthermore, mean and median intensities of acH4 in each population were also reported.

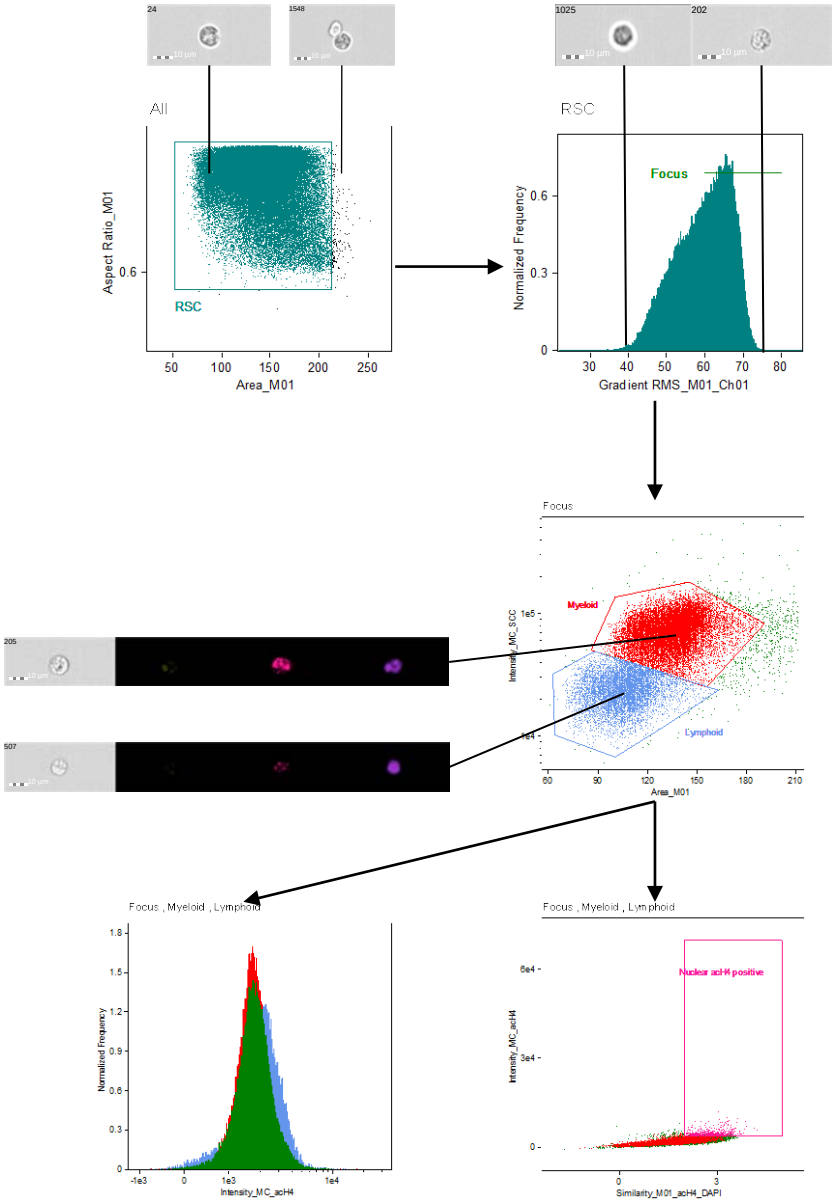


Figure 2.3. Summary of data analysis of acH4.

Round single cells (RSC) were first gated and the focused cells gated out of the RSC population. Cells were then divided into myeloid and lymphoid cells. The level of acH4 expression in each cell type was determined by looking at the similarity between acH4 and DAPI expression and intensity of protein. Additionally a histogram of the acH4 intensity in the three cell populations is shown.

2.9.3 The analysis of ki-67

Cells collected by the imaging flow cytometer were analysed with Ideas® software version 6.2. Round single cells, focused cells, and myeloid and lymphoid cells were gated using the same gating as described in section 2.9.1. In common with the pMCM2 analysis, similarity of ki-67 and DAPI expression alongside ki-67 intensity were applied to identify nuclear ki-67 positive cells as shown in Figure 2.4. The number of ki-67 positive cells was expressed as percentage of positive cells in the myeloid, lymphoid and total WBC populations.

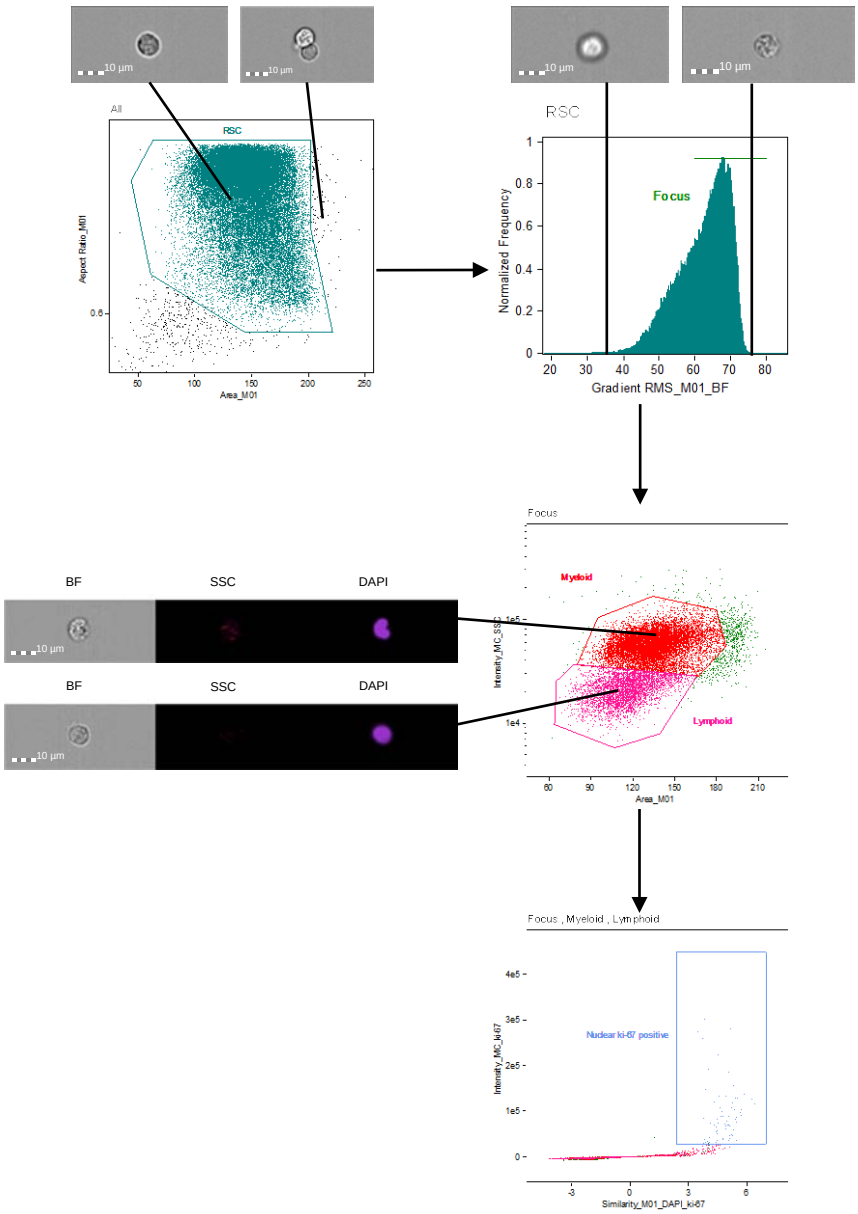


Figure 2.4. Analysis of ki-67 expression in patient samples from the PROSPECT-NE clinical trial.

Round single cells (RSC) were first gated, and then focused cells were gated from the RSC population. Cells were then divided into myeloid and lymphoid cells using side scatter intensity. Each cell type was assessed for ki-67 expression by looking at the similarity between ki-67 and DAPI expression and intensity of the protein.

2.10 ELISA assay for detection of circulating ki-67

When blood samples in CellSave® tubes from patients studied on the PROSPECT-NE trial were delivered to the laboratory for ki-67 cell analysis, plasma was also collected. To obtain plasma, whole blood was centrifuged at 1000×*g* for 10 minutes at 4°C. Plasma was then aliquoted to Eppendorf tubes and immediately frozen at -80°C prior to analysis.

ELISA plates were prepared following the manufacturer's instructions. Briefly, diluted capture antibody (100 μL) was added to each well in a 96-well plate and incubated overnight to coat the wells. The capture antibody was aspirated and each well was washed with 400 μL of wash buffer 3 times. The plates were blocked by adding 300 μL reagent diluent to each well and incubating for 1 hour at room temperature. The plates were then washed 3 times with wash buffer.

One aliquot of plasma sample was thawed in a 37°C water bath for 30 minutes, and then 100 μL of each plasma sample and ki-67 standard solutions, were pipetted to the prepared wells and incubated for 2 hours at room temperature. The plates were then washed twice. The diluted detection antibody (100 μL) was added to each well, incubated for 2 hours and the wells were then again washed twice. The diluted streptavidin-HRP solution (100 μL) was added to each well and incubated in the dark for 20 minutes and the washing step was repeated twice more. Substrate solution (100 μL) was added to each well and incubated in the dark for 20 minutes. The stop solution (50 μL of 2 N Sulphuric acid) was then added to each well and gently mixed.

Lastly, the absorbance of light at 450 nm (A_{450}) was measured for each well using the Omega® microplate spectrophotometer. A standard curve between A_{450} and ki-67 concentration was generated using the four parameter logistic curve-fit function on MS Excel 2013.

2.11 Statistical analysis

All statistical analyses in this PhD project were performed using either GraphPad Prism 6 or IBM SPSS Statistics 24 programmes, depending on the tests being carried out. Comparison tests including one-way and two-way ANOVA and the Mann-Whitney U-test, and correlation tests including Spearman's correlation were performed using GraphPad Prism 6. Survival analysis and the cox proportional hazard model were performed using SPSS Statistics 24. Statistical significance is defined as p-value <0.05.

Chapter 3. Development of an assay for pMCM2 and ki-67 as pharmacodynamic biomarkers for LY3143921, a CDC7 inhibitor

3.1 Introduction

LY3143921 is a novel anti-cancer agent designed to inhibit the cell division cycle 7 kinase (CDC7), an enzyme involved in the initiation of DNA replication cell cycle regulation and found to be over-expressed in cancer cell lines and tumour material. The efficacy of the drug has been tested in both cell lines (*in vitro*) and animals (*in vivo*), and a first-in-human trial to test its safety in man is now ongoing. Development and validation of a robust pharmacodynamic biomarker would be advantageous to demonstrate the mechanism of action of the drug in a clinical setting. Based on its proposed mechanism of action, CDC7, pMCM2 and ki-67 represent potentially useful proteins to focus on for the development of an appropriate biomarker assay for LY3143921.

3.1.1 Physiologic roles of CDC7 in mammalian cells

CDC7 is a serine/threonine kinase protein which was first discovered in *Saccharomyces cerevisiae* yeast in 1974 (Hartwell *et al.*, 1974). This protein, known as hsk1 in yeast, has been shown to have significant importance in yeast budding in many studies (Patterson *et al.*, 1986; Bahman *et al.*, 1988; Buck *et al.*, 1991; Sherlock and Rosamond, 1993). In mammalian cells, numerous studies have suggested crucial functions of CDC7 related to cell division including mitosis, meiotic recombination, checkpoint regulation and DNA damage repair (Jiang and Hunter, 1997; Kumagai *et al.*, 1999).

Cells initiate DNA replication at a particular sequence in a genome called the replication origin (Bousset and Diffley, 1998). There are 2 main steps in DNA replication which are origin licensing and origin activation (Walter, 2000). During the origin licensing process, CDC7 phosphorylates subunits of the MCM2-7 complex including MCM2, MCM4 and MCM6 (Masai *et al.*, 2000). CDC7 also phosphorylates other replication proteins including DNA polymerase alpha p180, CDC45, ORC4 subunit of the ORC1-6 complex, SV40 T antigen and CINP which is a CDK2 interacting protein (Weinreich and Stillman, 1999; Masai *et al.*, 2000; Nougarede *et al.*, 2000).

The DBF4 subunit is another protein which has been reported to interact with CDC7 in S-phase checkpoint activation. These two proteins bind to form a protein complex which is called DBF4-dependent kinase (DDK). DDK was found to control initiation of DNA replication and shown to be essential for phosphorylation of the MCM2-7 complex (Figure 3.1) (Enserink, 2011). This pathway is further explained in section 3.1.2 of this thesis.

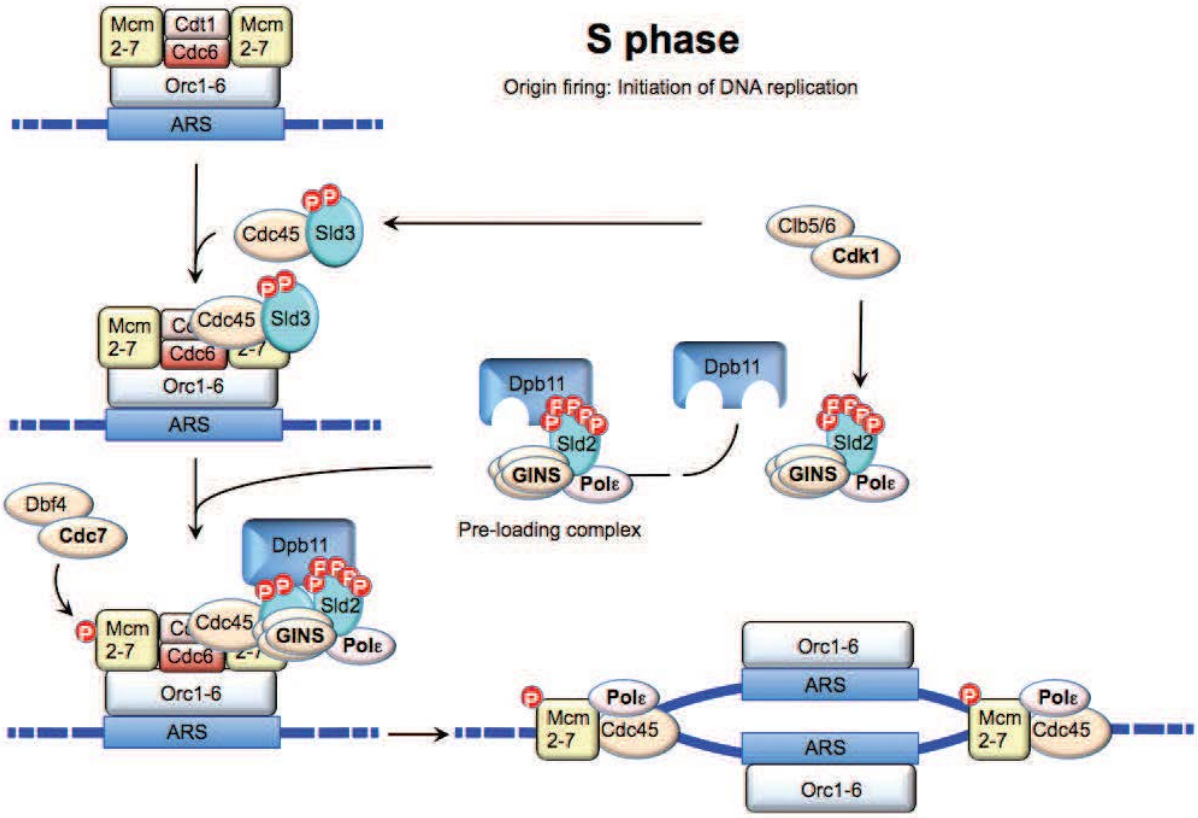


Figure 3.1. Regulation of DNA replication by the cell cycle.
Taken from Enserink JM. InTech; 2011. p. 391-408.

CDC7 was also found to be involved in the meiotic process. Although the roles of CDC7 in pre-meiotic DNA replication remain unclear, its roles in meiotic recombination are clearer. In the same way that MCM2 phosphorylation plays a role in the mitotic pathway, CDC7 acts together with CDK protein to phosphorylate MER2 at Ser 29 (Masai *et al.*, 2000). In addition to studies in yeast indicating the importance of CDC7 during meiosis, studies in CDC7-/- mice also reported disrupted spermatogenesis and infertility (Kim *et al.*, 2003).

3.1.2 Regulation of MCM2 by CDC7

Phosphorylation of MCM proteins has been studied over a number of years. Several *in vitro* studies suggested that, of all seven MCM proteins, MCM2, MCM3, MCM4 and MCM6 subunits are substrates for the CDC7-DBF4 complex (Lei *et al.*, 1997; Sheu and Stillman, 2006); the results have also been confirmed *in vivo*. Among the four MCM proteins mentioned, MCM2 was found to be the most essential substrate for CDC7 (Tsuji *et al.*, 2006; Bruck and Kaplan, 2009).

The pathway of phosphorylation of MCM2 proteins has not yet been fully described. As illustrated in Figure 3.1, it is known that CDC7 works together with DBF4 protein to form a DDK complex. This DDK phosphorylates MCM2 protein which is thought to increase its affinity for CDC45 and GINS (Tanaka and Araki, 2010). This results in formation of a complex between CDC45, GINS and MCM2-7, followed by induction of the unwinding of double-stranded DNA and initiation of DNA replication.

More recently published studies have suggested that only distinct residues of the MCM2 protein are phosphorylated by the DDK complex and that each phosphorylation site of MCM2 has a different affinity to interact with this CDC7 kinase complex (Montagnoli *et al.*, 2006). This study found only minimal changes in levels of pMCM2 at Ser108 but a strong decrease in levels of Ser40/41 and Ser53 MCM2 phosphorylation when CDC7 was depleted.

3.1.3 Association of CDC7, MCM2 and cancer

Associations of cell proteins and cancer have been studied for many decades. Focusing on CDC7 and pMCM2, several preclinical and clinical studies have suggested correlations between these two proteins and several types of cancer. For instance, an *in vitro* study reported overexpression of CDC7 in multiple cancer types including CNS, colon, non-small cell lung and leukaemia cell lines as compared to normal tissue cell lines (Bonte *et al.*, 2008).

These associations have also been shown in clinical studies. For example, a study indicated correlations between high CDC7 expression in diffuse large B-cell lymphoma (DLBCL) tissues measured by IHC and poor prognosis in DLBCL patients (Hou *et al.*, 2012). Similarly in epithelial ovarian carcinoma, patients with higher CDC7

expression in tumour specimens determined by IHC had significant lower overall and disease-free survivals (Kulkarni *et al.*, 2009). Another study also reported overexpression in CDC7 in malignant salivary gland tumours and this was associated with tumour grade (Jaafari-Ashkavandi *et al.*, 2017).

In contrast to the previous findings, a study in colorectal cancer cells found 33.6% negative, 57.2% weak and 9.2% strong CDC7 expression in patient tumour cells measured by immunohistochemistry (Melling *et al.*, 2015). They reported that reduction in CDC7 expression was associated with high tumour stage and tumour grade. Also, patients with stronger CDC7 expression in cancer cells exhibited longer survival times than those with weak or negative CDC7 expression.

Expression of MCM2 protein has also been studied in many types of cancer. A recent meta-analysis from 9 studies suggested that overexpression of MCM2 was associated with poor overall survival (Gou *et al.*, 2018). In patients with lung squamous cell carcinoma, MCM2 was found to be correlated with malignant status and overall survival (Wu *et al.*, 2018). Furthermore, MCM2 showed higher potential to predict recurrence rate in bladder cancer than CK20, ki-67 or histological grade (Burger *et al.*, 2007).

Although there are many publications indicating associations between MCM2 and various parameters of cancers, none of them have addressed the potential application of phosphorylated MCM2 as a predictive or prognostic biomarker for cancer. Instead, pMCM2 has been commonly used as a pharmacodynamic biomarker, especially since CDC7 inhibiting agents have been developed. For instance, pMCM2 was used as a pharmacodynamic biomarker in the development of XL-413 (Koltun *et al.*, 2012) and TAK-931, another novel CDC7-selective inhibitor (Iwai *et al.*, 2019).

3.1.4 Function of ki-67 in man

The ki-67 protein was originally discovered in Kiel city in Germany in 1983 (Gerdes *et al.*, 1984). Since its discovery, however, the biological function of the protein remains unclear (Scholzen and Gerdes, 2000). Early studies suggested that inhibition of ki-67 could inhibit DNA synthesis and result in a decreased rate of cell division. These results highlighted the important roles of ki-67 in cell proliferation.

On the other hand, several recent studies have generated different results. For example, mouse NIH-3T3 cells lacking ki-67 proliferated normally without apparent cell cycle delays. Moreover, it was found that depletion of ki-67 in HeLa and U2OS cell lines did not alter cell cycle distribution or ribosomal RNA synthesis (Sobecki *et al.*, 2016). These findings are challenging the previous perspective of ki-67 as an important factor for cell proliferation.

Even though the importance of ki-67 in cell proliferation remains controversial, ki-67 has been proven to be a promising marker of cell proliferation. Early studies indicated negative expression of ki-67 in peripheral blood leukocytes during G_0 phase but found ki-67 to be highly expressed in S, G_2, and M phase cells (Gerdes *et al.*, 1984). With these findings, ki-67 has been used as marker of cell proliferation for decades especially in cancer research.

3.1.5 ki-67 as a biomarker for cancer

Based on its promising activity as a marker of cell proliferation, ki-67 has been studied in diseases of anomalous proliferation such as cancer for several years. Many papers have reported on various possible utilities for the ki-67 protein in the area of cancer, including its potential use as a diagnostic tool, a prognostic tool and a therapeutic target. Studies in ki-67 as a diagnostic marker, however, remain scarce and this area requires further development.

Unlike the diagnostic biomarker application, use of ki-67 as a prognostic tool has been widely studied in many types of cancer especially breast cancer. A study reported that recurrent breast cancer patients with higher ki-67 expression in tumour biopsies had significantly shorter disease free intervals and overall survival (Nishimura *et al.*, 2014). These results conformed to those obtained in other studies, including studies of triple negative breast cancer patients and African breast cancer patients (Agboola *et al.*, 2013; Mrklić *et al.*, 2013; Pathmanathan *et al.*, 2014).

The utility of ki-67 in other cancers has been much less studied and the results obtained have been ambiguous. A study in prostate cancer reported higher mean and median ki-67 in patients with recurrent prostate cancer (Wilkins *et al.*, 2018). Additionally ki-67 was found to be associated with overall survival in cervical cancer

patients (Pan *et al.*, 2015). In contrast, ki-67 did not provide apparent prognostic effect in non-small cell lung cancer, bladder cancer or gastric cancer (Yang *et al.*, 2006; Czyzewska *et al.*, 2009; Warli *et al.*, 2018).

An alternative application of ki-67 in the field of cancer is as a therapeutic target for therapy. Examples of drug in this group include antisense oligodeoxynucleotides (ASOs), peptide nucleic acids (PNAs) and adenovirus. These agents are generated to have a sequence that specifically targets ki-67 nucleotide sequence. Several studies have been published involving many anti-cancer agents with different mechanisms related to ki-67, with the most promising agents being ASOs. ASOs are a class of RNA interference (RNAi) agents which interfere with ki-67 production and result in specific inhibition of cell proliferation (Yang *et al.*, 2018). Phosphorothioate antisense oligodeoxyribonucleotide, an example of an ASO, provided efficacy in inhibition of cancer cell proliferation and tumour growth *in vitro* and *in vivo* (Kausch *et al.*, 2003). Nevertheless, the clinical application of ASOs has been limited due to their non-specific binding, low affinity and susceptibility to nuclease degradation. PNAs were the second class of RNAi agents synthesized and showed stronger effects in terms of inhibition of proliferation and induction of apoptosis in renal carcinoma cells *in vitro* as compared to ASOs (Zheng *et al.*, 2005). In addition, there are other mechanisms of cancer therapy such as conditionally replicative adenovirus which inhibit ki-67 promoter gene and result in inhibition of cancer cell migration and invasion (Yang *et al.*, 2018).

3.1.6 CDC7 inhibitors as anticancer drug

Inhibition of CDC7 kinase competes for ATP and prevents it from being able to phosphorylate substrates such as MCM2, resulting in potent cell death in cancer cells but minimal inhibitory effects on cell viability in normal cells (Kim *et al.*, 2008; Sawa and Masai, 2009; Montagnoli *et al.*, 2010). Following this pathway, the first ATP-competitive CDC7 inhibitor, (S)-2-(2-aminopyrimidin-4-yl)-7-(2-fluoro-ethyl)-1,5,6,7-tetrahydropyrrolo[3,2-c]pyridin-4-one, was developed in 2008, and several classes of CDC7 inhibitor have since been developed (Menichincheri *et al.*, 2009). The functional structures of CDC7 inhibiting compounds developed include pyrrolopyridinone, indazoles, pyrido-thienopyrimidines, 1H-pyrrolo[2,3-b]pyridines, 5-heteroaryl-3-

carboxamido-2-aryl pyrroles and benzofuropyrimidinone (Montagnoli *et al.*, 2008; Shafer *et al.*, 2008; Ermoli *et al.*, 2009; Zhao *et al.*, 2009; Menichincheri *et al.*, 2010).

Among them, PHA-767491 was the first agent that showed highly potent inhibition of CDC7 activity both *in vitro* and *in vivo* (Montagnoli *et al.*, 2008). Further to this success, several CDC7 inhibiting agents have been developed and entered clinical trials. For example, the orally administered CDC7 inhibitor, NMS-1116354, was studied in a phase I clinical trial setting between 2009 and 2012 (U.S. National Library of Medicine, 2009c). BMS-863233, also known as XL-413, was studied in two clinical trials (phase I/II) in 2009/2010 (U.S. National Library of Medicine, 2009a; U.S. National Library of Medicine, 2009b). Since then, many CDC7 inhibitors including TAK-931, SRA141 and LY3143921 have also been developed (U.S. National Library of Medicine, 2017; Hansen *et al.*, 2018; Toshio *et al.*, 2018).

LY3143921 is a novel CDC7 inhibitor developed by Eli-Lilly and Company. Figure 3.2 illustrates the chemical structure of this agent. The efficacy and toxicity of LY3143921 have been tested in both cell lines, including SW620, A2780, HL-60 and AX521 cells, and animal studies involving mouse, rats, dogs and monkeys. *In vivo* studies demonstrated that administration of 10 mg/kg of the drug to mice resulted in a C_{max} of 1810±350 ng/mL and a T_{max} of 3±1.15 hours. In comparison, beagle dogs receiving 6 mg/kg and monkeys receiving 3 mg/kg of the drug showed a higher C_{max} (4160±1650 and 4450±491 ng/mL, respectively). For pharmacodynamic studies, CDC7 expression in mouse xenograft tumour tissues was potently inhibited by LY3143921 and resulted in a huge decrease in pMCM2 (>70% as measured by IHC) for up to 12 hours after the last dose. Moreover, several studies in xenograft tumour models also reported stable disease and tumour regression in many types of cancer including lung, blood, colon, ovarian and gastric (data from the company – personal communication).

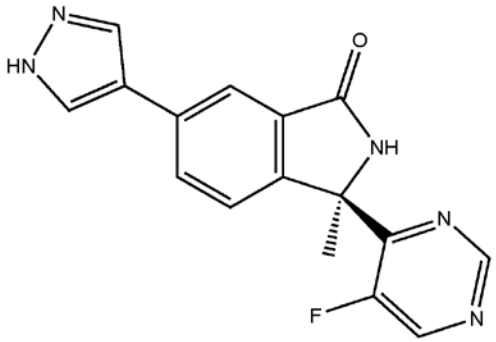


Figure 3.2. Molecular structure of LY3143921

The first phase I clinical trial of LY3143921 started in May 2017. The inclusion criteria were patients with many types of advanced cancer including bladder, colorectal, breast, head and neck and kidney, who had failure from regular treatment (refractory cancer). This trial consisted of two parts; the first part was an optimisation of dose and frequency of LY3143921 and the latter part involved treatment of patients with the drug regimen determined in stage I. Tumour biopsies were taken before treatment and again during the first cycle of treatment. Blood samples were taken regularly and closely monitored to observe any adverse effects of the drug (Cancer Research UK, 2017).

3.1.7 Pharmacodynamic biomarkers for CDC7 inhibitors

Pharmacodynamic biomarkers are increasingly included in clinical trials in oncology, especially in terms of proof-of-mechanism. In this respect, they could be utilised in patient monitoring if correlations are shown between the biomarkers and clinical response. Focusing on CDC7 inhibitors, previously published clinical trials have suggested various different pharmacodynamic biomarkers of relevance. Based on the pathway of CDC7 mentioned above, several downstream proteins could be used as biomarkers including MCM2-7 complex, DBF4 protein or CDC7 itself (Enserink, 2011).

Among them, pMCM2 is considered a promising candidate for development as a CDC7 inhibitor biomarker. However, different phosphorylation sites of MCM2 were found to respond differently to CDC7 inhibitors. Montagnoli et al performed HeLa cell transfection with interference RNA targeting CDC7 for up to 72 hours. The phosphorylation of MCM2 proteins at different subunits was measured using SDS-

PAGE Western Blotting. The results suggested that phosphorylation of MCM2 subunits at Ser40 and Ser53 were completely dependent on CDC7 activity and had the potential to act as pharmacodynamic biomarkers (Montagnoli *et al.*, 2006).

Not only direct CDC7 downstream proteins could be used as biomarkers for CDC7 inhibitors, other indirectly involved proteins could also be utilised. A study of XL413 indicated a decrease in pCHK1 (Ser317) when A431 epidermoid carcinoma cells were treated with a combination of XL413 and etoposide as compared to etoposide alone. (Robertson, 2008). Another study of PHA-767491 showed decreases in several proteins including MCL-1, BCL-A1 and NOXA when CLL cell lines were treated with ≥5 μM of the drug (Natoni *et al.*, 2011). These findings have suggested some potential pharmacodynamic biomarkers for further development of CDC7 inhibitors.

The association between CDC7 and ki-67 proteins has been sparsely studied and there are no publications confirming correlations between CDC7 inhibitors and changes in ki-67. As the mechanism of action of CDC7 inhibition involves inhibition of cell proliferation, ki-67 should be considered as a potential indirect biomarker for CDC7 inhibitors. This study therefore included ki-67 as an indirect biomarker of cell inhibition induced by LY3143921.

3.2 Chapter specific aims

The studies outlined in this chapter primarily aimed to develop and validate an imaging flow cytometry assay for detection of potential pharmacodynamic biomarkers for LY3143921 including CDC7, pMCM2 and ki-67 proteins. The objectives of development included the following:

- Optimisation of antibodies used in the assay including CDC7, pMCM2 and ki-67 antibodies.
- Investigation of changes in proteins of interest in HL-60 cell lines with increasing LY3143921 concentration and duration of treatment.
- Investigation of changes in proteins of interest in human WBCs when treated with increasing concentrations of LY3143921.
- Investigation of changes in proteins of interest in mouse WBCs when animals treated with different doses of LY3143921.
- Optimisation of preservation methods for proteins of interest using different storage conditions including commercial preservative blood tubes and fixed sample freezing at -80°C.

3.3 Results

3.3.1 Antibody validation

3.3.1.1 CDC7 antibody validation

The most suitable volume of antibody against CDC7 protein to be used in the assay was first optimised. HL-60 cells were fixed with ice cold methanol and incubated with different volumes of CDC7 antibody (0.5, 1, 2 and 4 μL) together with 5 μL of DAPI for 1 hour. The cells were analysed for CDC7 expression levels using an imaging flow cytometer. Specificity of CDC7 antibody was determined by comparison of CDC7 expressions in HL-60 cells with/without 1,25-dihydroxyvitamin D_3 treatment in pilot experiments (data not shown), based upon the study by Brackman et al (Brackman *et al.*, 1995).

Figure 3.3 shows the intensity of CDC7 expression in histograms alongside examples of cells at the peak of the graph and scatter plots showing the population of cells with high CDC7 expression in the nucleus, namely nuclear CDC7 positive cells. As compared to incubations with no antibody, cells with 0.5 and 1 μL of antibody provided observable signals. The results suggested that the CDC7 antibody had the required sensitivity to detect signals of CDC7 protein at all antibody dilutions as compared to the control. As shown in Figure 3.4, a background mean intensity of 28,000 was observed, while 0.5 μL of antibody exhibited a mean intensity of 76,000 which increased to 141,000 with 4 μL of antibody.

Although the obtained CDC7 intensity signal was not likely to be saturated at a concentration of 4 μL, the percentages of nuclear CDC7 positive cells at all concentrations were found to be similar (Figure 3.5). With 0.5 μL of antibody, 97% nuclear CDC7 positive cells were detected as compared to 0.72% from the control. Higher volumes of antibody did not generate higher number of positive cells; the percentages of CDC7 positive cells at 1, 2 and 4 μL of antibody were 98.1%, 99.6% and 96%, respectively. The results therefore suggested that 0.5 μL was sufficient to be used in the assay and that increasing the antibody volume did not show conclusive benefits when balanced against increased assay costs.

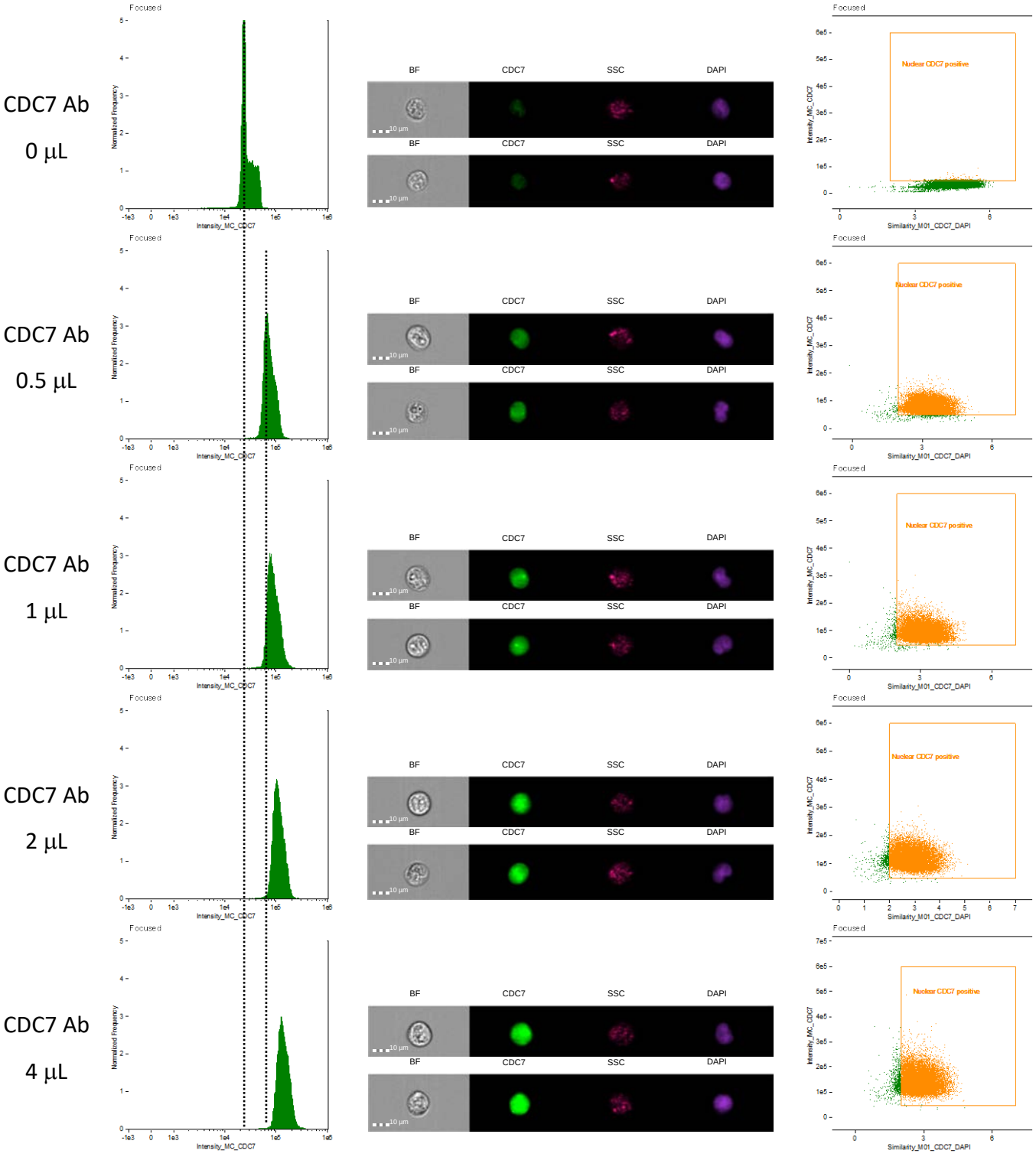


Figure 3.3. Histograms of the intensity of CDC7, examples of cells at the peaks of histograms, and scatter plots between intensity of CDC7 and similarity of CDC7 and DAPI.

Samples were collected from HL-60 cell lines incubated with different volumes of CDC7 antibody for 1 hour.

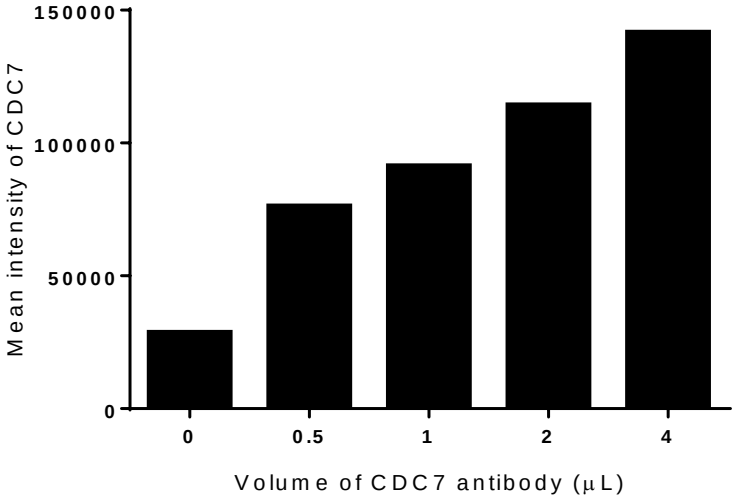


Figure 3.4. Mean intensity of CDC7 expression in HL-60 cells incubated with different volumes of CDC7 antibody (0-4 μL) for 1 hour.

The results shown are from a single experiment. Mean intensity refers to average expression of protein in all cells collected from one sample.

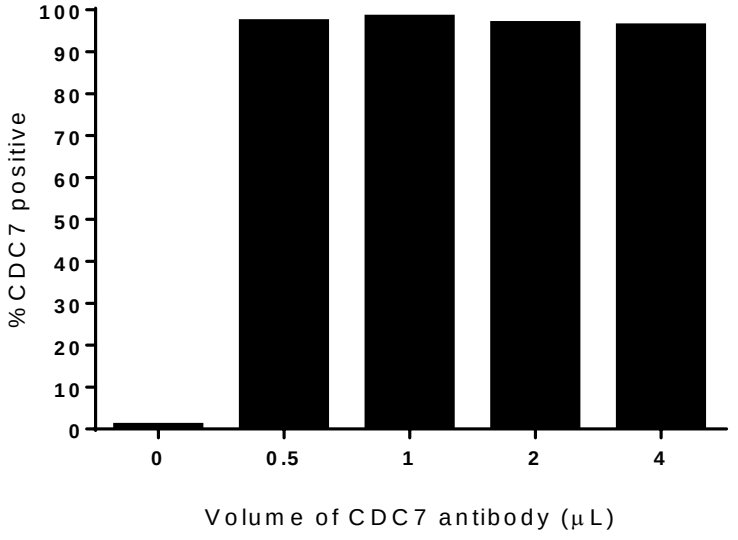


Figure 3.5. Percentage of nuclear CDC7 positive in HL-60 cells incubated with different volumes of CDC7 antibody (0-4 μL) for 1 hour.

The results shown are from a single experiment.

3.3.1.2 pMCM2 antibody validation

The antibody against MCM2 phosphorylation at serine 53 was tested to find the optimal volume for the assay. Different volumes of pMCM2 primary antibody (0, 1, 2.5, 5 and 10 μL) were incubated with HL-60 cell lines for 1 hour. PE secondary antibody (1 μL) and DAPI (5 μL) were then added to all tubes, incubated for 1 hour and the PE signal measured by imaging flow cytometry.

Figure 3.6 shows histograms of pMCM2 intensity and scatter graphs between similarity of pMCM2 and DAPI and intensity of pMCM2 with examples of cells from each antibody volume. As compared to the control (no pMCM2 primary antibody), all dilutions of pMCM2 primary antibody generated detectable signals. In addition, the histograms revealed bimodal curves for antibody volumes of 1, 2.5 and 5 μL but not for 10 μL. Focusing on the mean intensity of pMCM2 expression (Figure 3.7), the values ranged between 23,000 and 27,000 for all concentrations of pMCM2 primary antibody with very low pMCM2 signal (1,200 mean intensity) for samples without pMCM2 antibody.

In agreement with the pMCM2 intensity data, the percentages of nuclear pMCM2 positive cells for all volumes of pMCM2 antibody were observed to be stable (Figure 3.8). The percentages of pMCM2 positive cells were 59.8%, 57.4%, 62.0% and 64.5% for 1, 2.5, 5 and 10 μL of antibody, respectively. There were no pMCM2 positive cells detected in the samples without pMCM2 antibody. These results therefore suggested that 1 μL of pMCM2 primary antibody with 1 μL of PE secondary antibody was suitable for the assay because it generated as strong a signal and number of positive cells as the higher volumes.

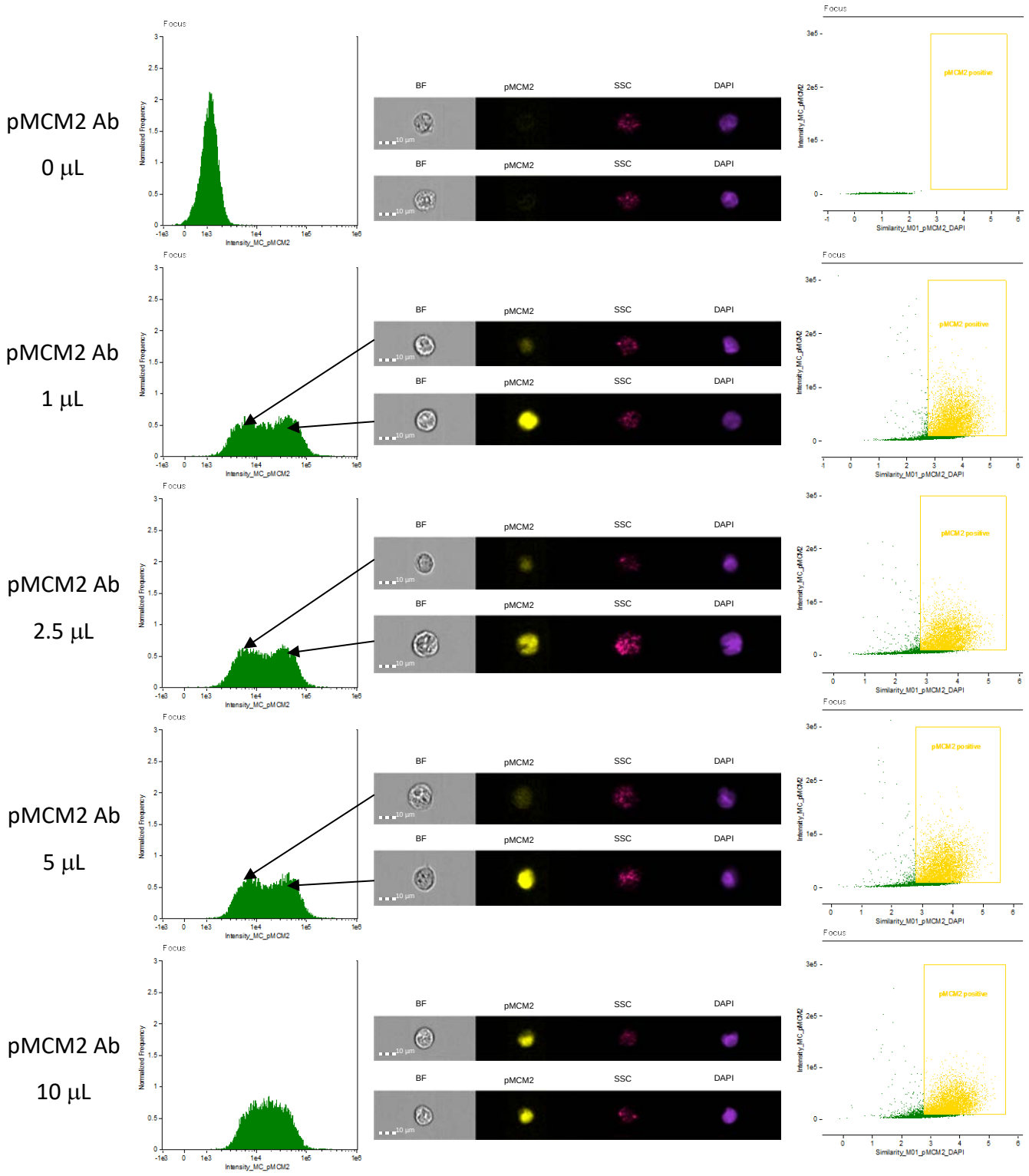


Figure 3.6. Histograms of the intensity of pMCM2, examples of cells at the peaks of histograms, and scatter plots between similarity of pMCM2 and DAPI.

Samples were collected from HL-60 cell lines which were incubated with different volumes of pMCM2 primary antibody for 1 hour. The volume of PE secondary antibody was fixed at 1 µL.

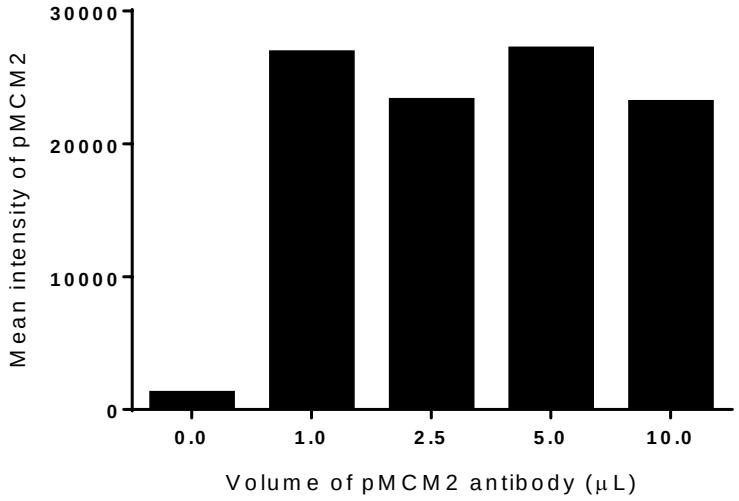


Figure 3.7. Mean intensity of pMCM2 expression in HL-60 cells with different volumes of pMCM2 primary antibody for 1 hour.

PE secondary antibody was 1 µL for all concentrations of primary antibody. The results shown are from a single experiment. Mean intensity refers to average expression of protein in all cells collected from one sample.

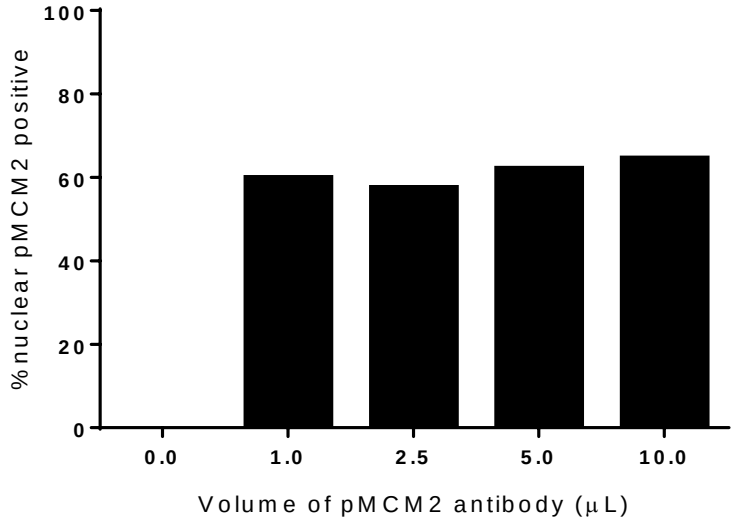


Figure 3.8. Percentage of nuclear pMCM2 positive in HL-60 cells incubated with different volumes of pMCM2 antibody for 1 hour.

PE secondary antibody was 1 µL for all concentrations of primary antibody. The results shown are from a single experiment.

3.3.1.3 ki-67 antibody validation

In order to optimise the volume of FITC-conjugated antibody against ki-67, HL-60 cell lines were incubated with ki-67 antibody at volumes of 1, 2, 3 and 4 μL together with 5 μL of DAPI for 1 hour. Cells were washed with 1 mL of 5% BSA in PBS and ki-67 expression quantified by imaging flow cytometry.

The results shown in Figure 3.9 indicate non-normal distribution curves of ki-67 intensity, so the median was considered instead of mean for ki-67 expression. The representative images of cells for each volume of antibody clearly show detectable signals of ki-67 when incubated with 1 μL of antibody or higher as compared to control cells (no antibody). Figure 3.10 suggests an increase in median intensity of ki-67 expression with increasing amount of ki-67 antibody. Median ki-67 signals of 170,000, 230,000, 277,000 and 313,000 were observed with ki-67 antibody volumes of 1, 2, 3 and 4 μL, respectively; without the antibody added, cells expressed a signal intensity of 14,500.

Figure 3.11 shows high percentages of nuclear ki-67 positive cells with up to 90% positive cells. The numbers of positive cells were similar for all antibody dilutions; there were 85.7%, 88.5%, 87.9% and 90.1% positive cells at volumes of 1, 2, 3 and 4 μL. The control without antibody showed no ki-67 positive cells. Based on the obtained results, 1 μL of ki-67 antibody was efficient for the developed assay and therefore was used for all additional experiments.

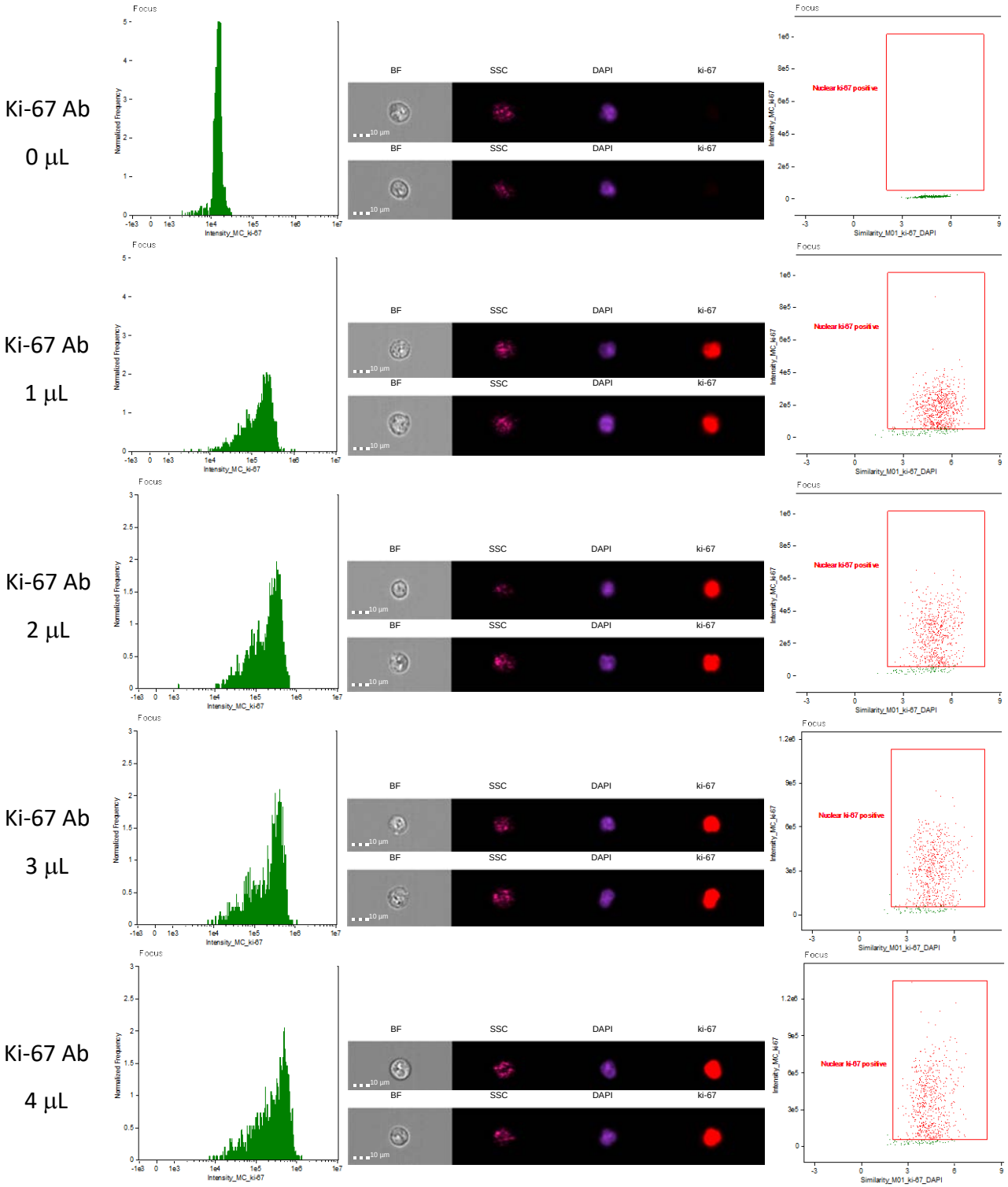


Figure 3.9. Histograms of the intensity of ki-67, examples of cells at the peaks of histograms, and scatter plots between similarity of ki-67 and DAPI.

Samples were collected from HL-60 cell lines that were incubated with different concentrations of ki-67 antibody for 1 hour.

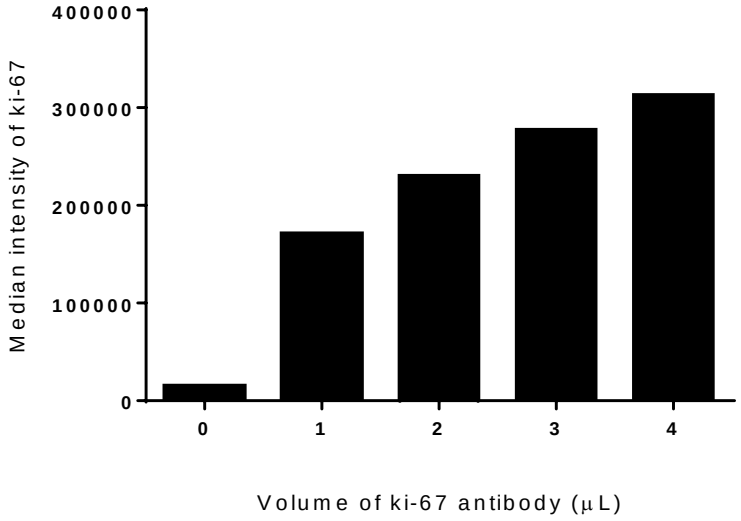


Figure 3.10. Median intensity of ki-67 expression in HL-60 cell lines with different volumes of ki-67 antibody (0-4 μL) for 1 hour.

The results shown are from a single experiment. Median intensity refers to the median expression of protein in all cells collected from one sample.

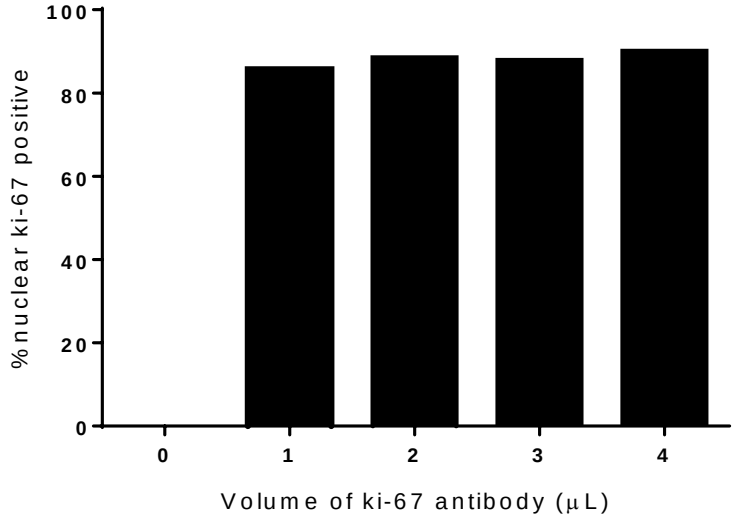


Figure 3.11. Percentage of ki-67 positive cells in HL-60 cell lines incubated with different volumes of ki-67 antibody (0-4 μL) for 1 hour.

The results shown are from a single experiment.

3.3.2 Inter- and intra-staining variation of the developed assay

The precision of the developed assay was tested before embarking on additional experiments with white blood cells. White blood cells obtained from a healthy donor were divided into seven tubes and incubated with pMCM2 and ki-67 antibody at different conditions. Five samples were incubated with the same master mix of antibodies and analysed on the same day, with a single sample measured three times to investigate the accuracy and precision of the imaging flow cytometer. Additional samples were stained 1 week and 2 weeks after the first experiment following the same protocol.

The number of cells with high expression of pMCM2 and ki-67 (pMCM2 positive and ki-67 positive cells) were counted and expressed as a proportion of all white blood cells. The first sample which was measured 3 times generated comparable percentages of pMCM2 and ki-67 positive cells (

Table 3.1) with percentage of coefficient of variation (%CV) less than 10% (8% for pMCM2 and 6.9% for ki-67). The %CVs of five samples incubated in parallel with the same master mix were 12% for pMCM2 and 5.7% for ki-67 as shown in Table 3.2. The %CVs of the sixth and seventh samples which were incubated with different master mix of antibodies were higher (14.4% for pMCM2 and 12.3% for ki-67, Table 3.3). All variation values, however, were less than 20%, thereby indicating acceptable assay precision.

Sample		**%nuclear pMCM2/WBC**	**%nuclear ki-67/WBC**	**%nuclear pMCM2/ki-67**
1/1		0.25	1.13	21.6
1/2		0.27	1.28	20.3
1/3		0.23	1.15	19.8
Flow cytometer precision	Mean	0.25	1.19	20.57
	%CV	8.00	6.86	4.52

Table 3.1. Precision of the imaging flow cytometer in measurement of pMCM2 and ki-67 in the same sample three times.

Sample		%nuclear pMCM2/WBC	%nuclear ki-67/WBC	%nuclear pMCM2/ki-67
Average of sample 1		0.25	1.19	20.57
2		0.25	1.09	21.4
3		0.28	1.19	22.6
4		0.26	1.09	23.5
5		0.33	1.14	28.1
Intra-staining variation	Mean	0.27	1.14	23.23
	%CV	12.27	4.32	12.66

Table 3.2. Intra-staining variation in pMCM2 and ki-67 in 5 samples from the same donor.

All 5 samples were stained in the same experiment using the same master mix of antibodies.

Sample		%nuclear pMCM2/WBC	%nuclear ki-67/WBC	%nuclear pMCM2/ki-67
Average of sample 1-5		0.27	1.14	23.23
6		0.27	1.05	25.4
7		0.21	0.89	23.5
Inter-staining variation	Mean	0.24	0.98	24.11
	%CV	15.16	12.40	4.52

Table 3.3. Inter-staining variation in pMCM2 and ki-67 in 7 samples from the same donor.

Sample 6 and 7 were stained in experiments performed on different days using the same assay as sample 1-5 with different master mixes of antibodies.

3.3.3 LY3143921 concentration dependent in vitro experiments

The developed and validated assay was first tested in HL-60 cell lines in an *in vitro* setting. HL-60 cells were treated with LY3143921 at concentrations ranging from 0.001-10 μg/mL for 6 hours. Cells were incubated with CDC7, pMCM2 and ki-67 antibodies according to the protocol mentioned in Chapter 2 and the results are shown in Figure 3.12 Figure 3.13. Based on the histograms and scatter graphs as compared between 0 and 10 μg/mL, LY3143921 was clearly associated with a decrease in the expression of pMCM2 but not CDC7 or ki-67. Moreover, this changed the pattern of

pMCM2 expression from a single peak to two peaks which distinguish positive and negative cell populations.

Looking at the number of protein positive cells, the percentage of CDC7 positive cells was not affected by the drug, with an average of approximately 90% CDC7 positive cells obtained at all concentrations of LY3143921 (Figure 3.13A). However, significant decreases in nuclear pMCM2 expression were observed following treatment with increasing concentrations of LY3143921 (one-way ANOVA, p-value <0.0001) (Figure 3.13B). Mean percentages of pMCM2 positive cells were 69.2%, 64.6%, 61.4%, 48.3%, 36.2% and 18.2% at concentrations of 0, 0.001, 0.01, 0.1, 1 and 10 μg/mL, respectively. Figure 3.13C shows mean percentages of ki-67 positive cells ranging from 21% to 29.1%; the sample treated with 0.01 μg/mL of the drug had the lowest number ki-67 positive cells (21%) and the control had the highest number (29.1%). These values measured, although were statistically significant different, showed no trend with increasing drug concentrations.

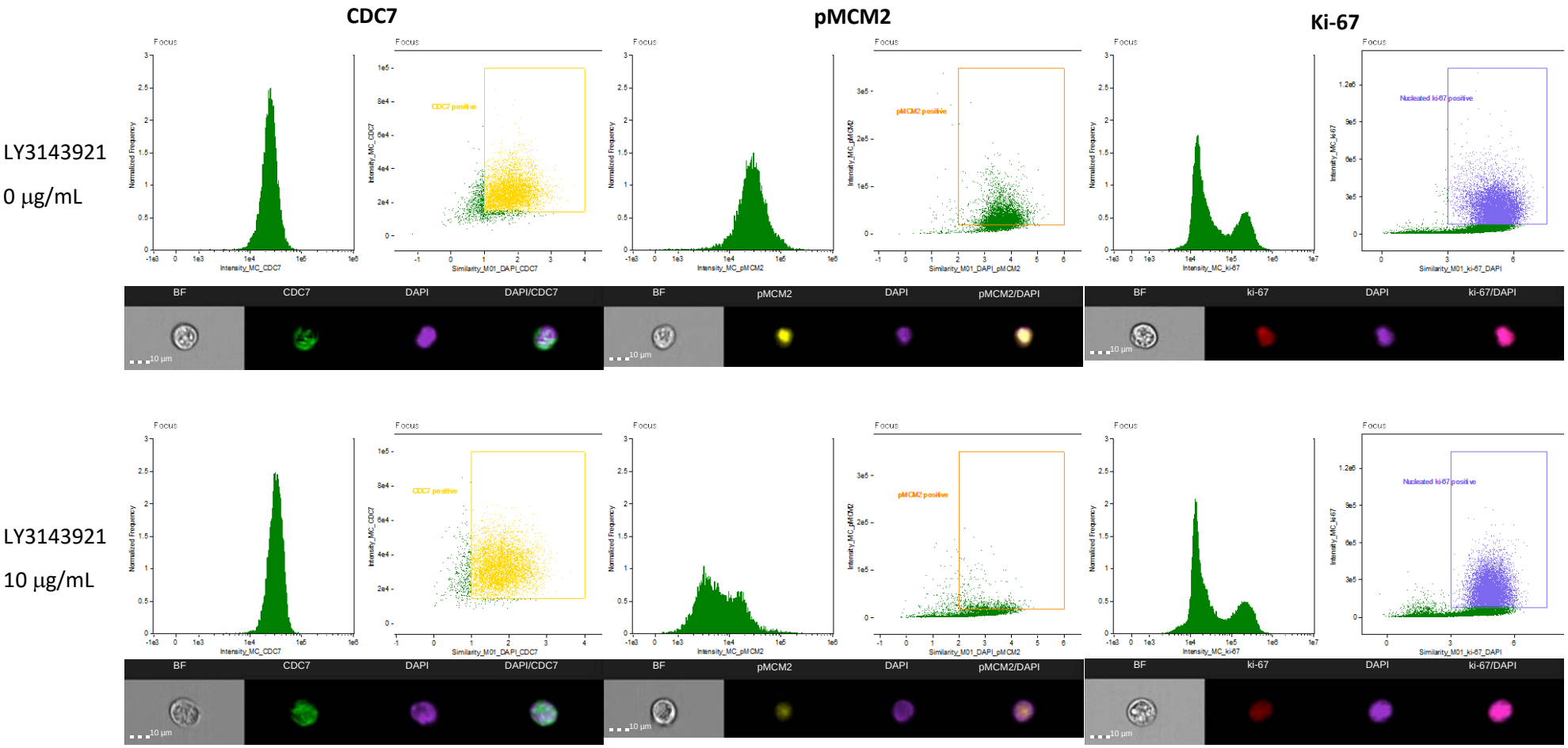


Figure 3.12. Histograms of mean intensity, scatter plots of mean intensity and similarity between protein and DAPI, and examples of cells with protein positivity.

Samples were from HL-60 cell lines treated with 0 and 10 μg/mL of LY3143921 for 6 hours. The proteins shown include CDC7, pMCM2 and ki-67.

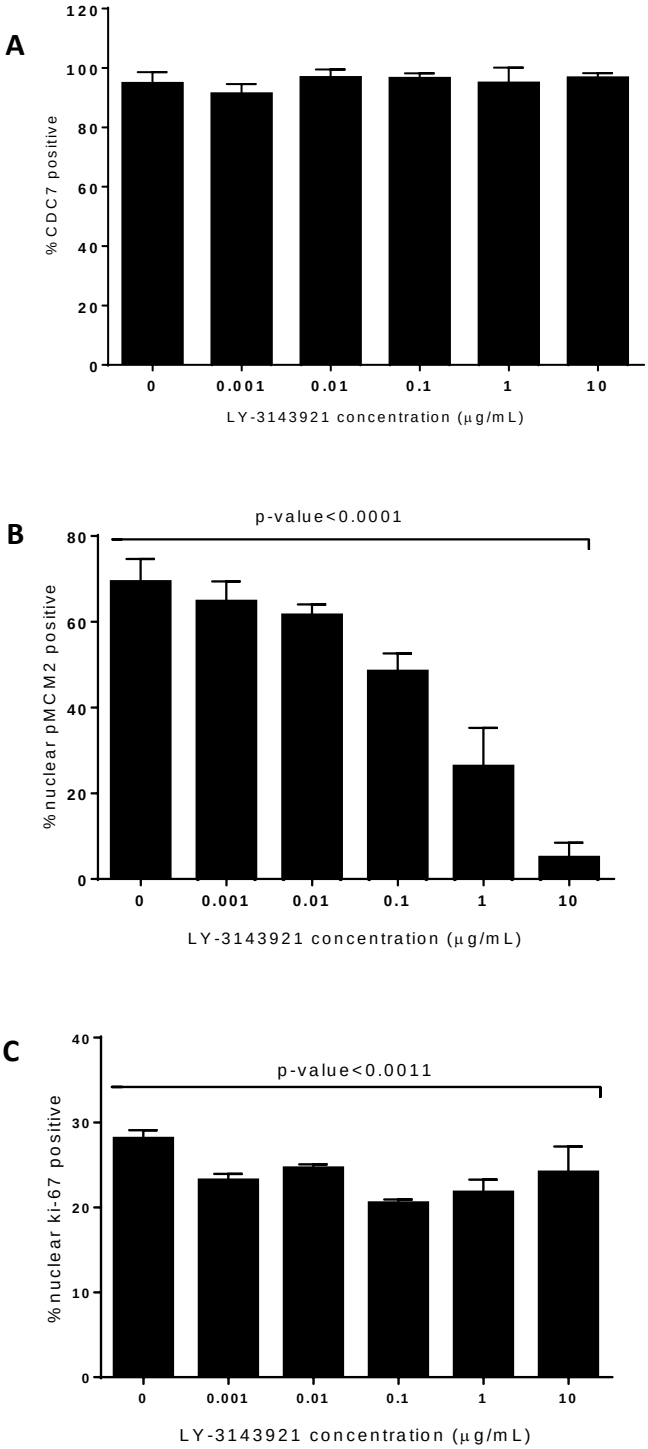


Figure 3.13. Percentages of CDC7 positive (A), pMCM2 positive (B) and ki-67 positive (C) cells in HL-60 cell lines treated with different concentrations of LY3143921 for 6 hours.

Mean and SD shown from 3 separate experiments.

3.3.4 LY3143921 concentration dependent ex vivo experiments

Whole blood samples from three healthy donors were collected, incubated with a range of concentrations of LY3143921 (0.001-100 μg/mL) for 6 hours and then processed and analysed according to the imaging flow cytometry assay procedure described in Chapter 2. Representative images of white blood cells with high levels of expression of CDC7, pMCM2, ki-67 and all three of these proteins are shown in Figure 3.14. As DAPI identified the localisation of the nucleus of the cell, localisation of the proteins being investigated could be estimated using similarity in shape between DAPI and that protein. The CDC7 antibody detected signal outside the nucleus (cytoplasmic expression), while pMCM2 and ki-67 were expressed in the nucleus.

Protein expression could be investigated using several parameters such as mean intensity and number of protein positive (highly expressed) cells. Looking at the intensity of proteins,

Figure 3.15 shows histograms of CDC7, pMCM2 and ki-67 intensity in myeloid and lymphoid cells following an increase in LY3143921 concentrations. The expression of CDC7 in myeloid cells was clearly higher than lymphoid cells. This difference, however, was not found in pMCM2 and ki-67 expression. Moreover, compared to the control cells treated with no drug, neither population showed clear changes in protein expression even when treated with the highest concentration of drug.

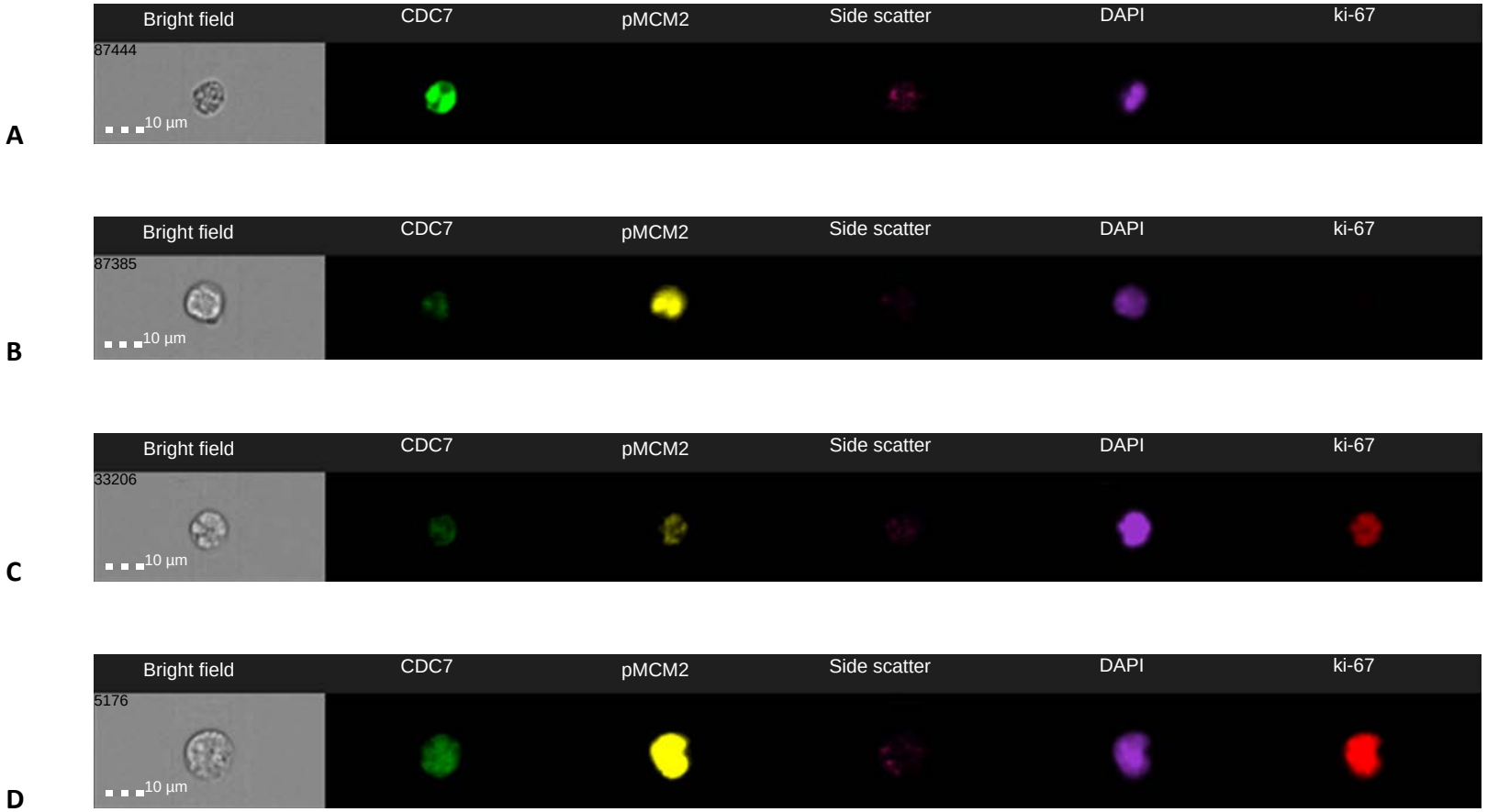


Figure 3.14. Examples of white blood cells with high expression levels of proteins including CDC7 (A), pMCM2 (B), ki-67 (C) and triple protein expression (D).

DAPI locates the nucleus of the cell.

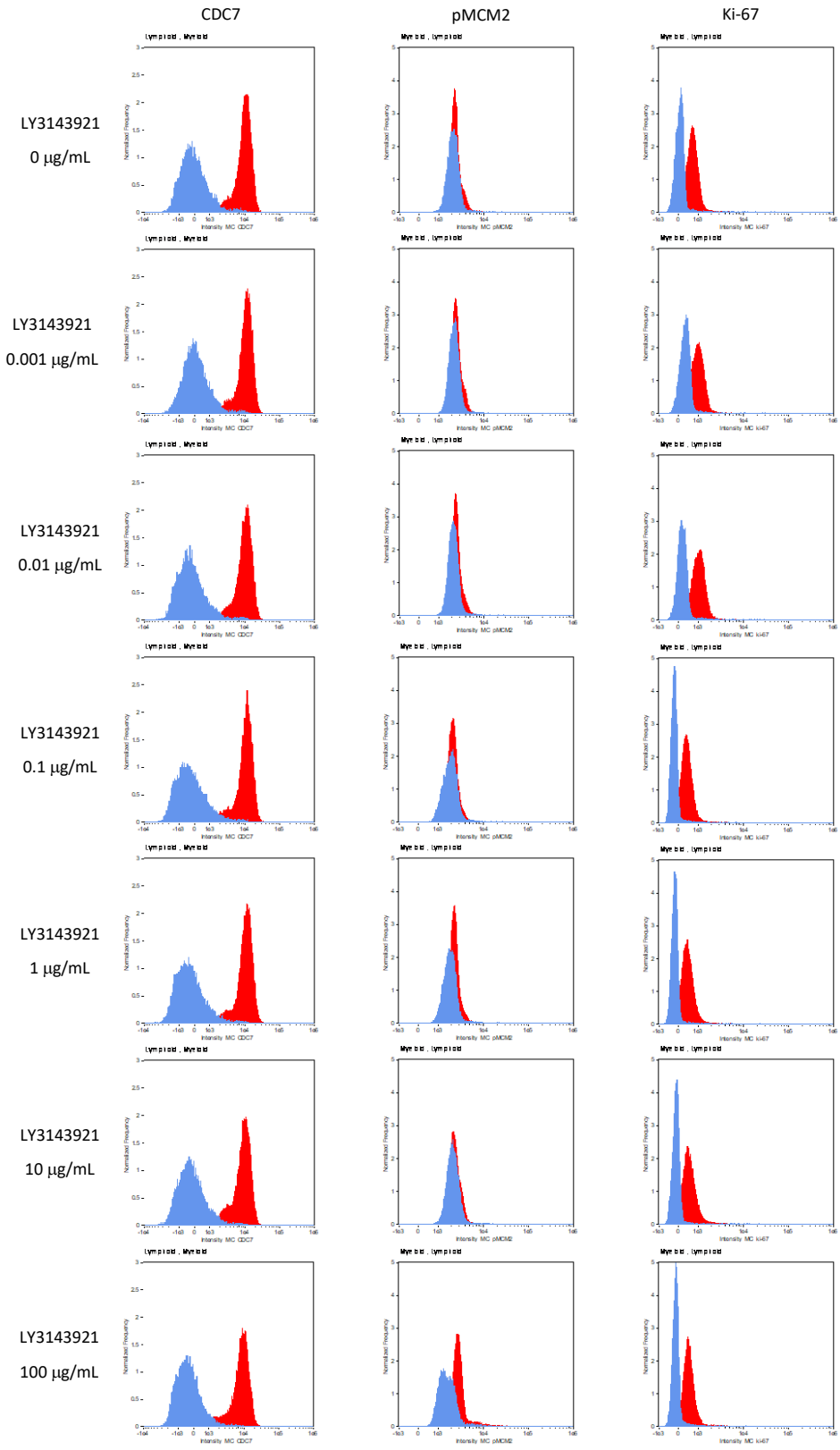


Figure 3.15. Histograms of intensity of CDC7, pMCM2 and ki-67 following different concentrations (0-100 µg/mL) of LY3143921 treatment for 6 hours.

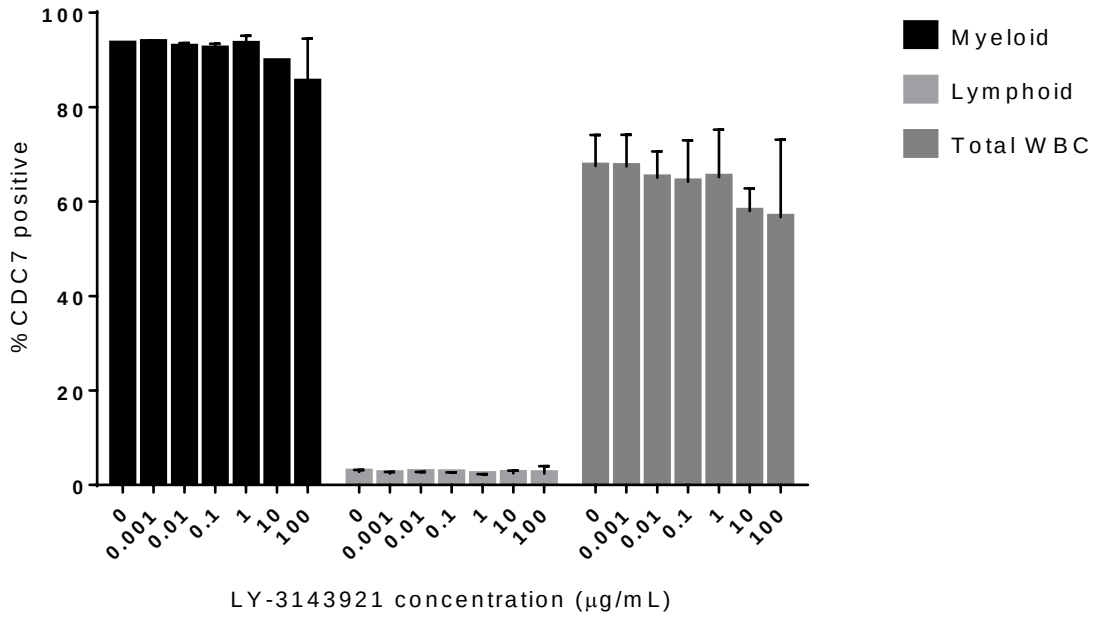
Blue indicates lymphoid population and red indicates myeloid population.

Focusing on numbers of positive cells, the percentages of CDC7 positive myeloid cells, lymphoid cells and total WBCs are shown in Figure 3.16A, percentages of nuclear pMCM2 positive cells are shown in Figure 3.16B, and percentages of nuclear ki-67 positive cells are shown in Figure 3.16C. In agreement with the signal intensity results, percentages of CDC7 positive cells were shown to be markedly different between myeloid and lymphoid cell populations, with >90% of myeloid cells being CDC7 positive, as compared to <5% of lymphoid cells. However, the level of CDC7 expression in either cell type was not significantly changed when treated with LY3143921.

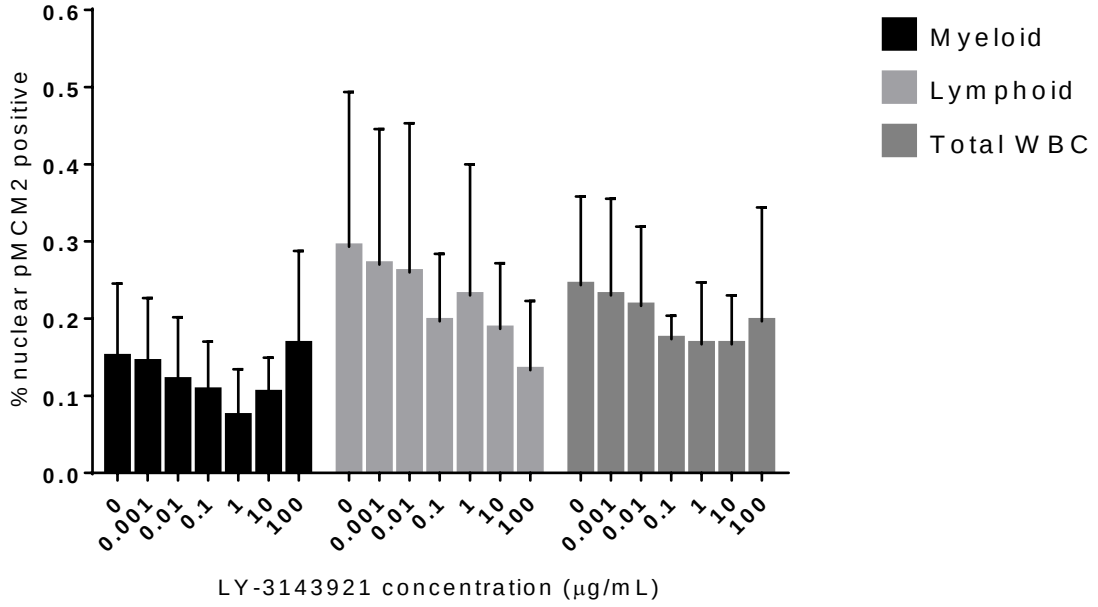
In contrast to the results generated in cell lines, the decrease in percentage of pMCM2 positive WBCs with increasing concentrations of LY3143921 was not significant, although a trend towards a decrease was observed. In the myeloid cell population, percentages of pMCM2 positive cells were 0.15% with no drug treatment and 0.17% at 100 μg/mL of LY3143921. In contrast, percentages of pMCM2 positive lymphoid cells were 0.29% in untreated cells and 0.13% at 100 μg/mL of LY3143921. These results suggest that the trend towards a decrease in pMCM2 positivity was more visible in lymphoid than myeloid cells.

For ki-67 positivity, in agreement with the *in vitro* cell line results, there was no change in either the myeloid or lymphoid populations following incubation of cells with LY3143921. Percentages of ki-67 positive cells at 0, 1 and 100 μg/mL LY3143921 were 0.14%, 0.14% and 0.10% of myeloid and 0.40%, 0.45% and 0.33% of lymphoid cells, respectively.

A


100
80
60
40
20
0
% CDC7 positive
0
0.001
0.01
0.1
1
10
100
LY-3143921 concentration (μg/mL)
Myeloid
Lymphoid
Total WBC

B


0.6
0.5
0.4
0.3
0.2
0.1
0.0
% nuclear pMCM2 positive
0
0.001
0.01
0.1
1
10
100
LY-3143921 concentration (μg/mL)
Myeloid
Lymphoid
Total WBC

C

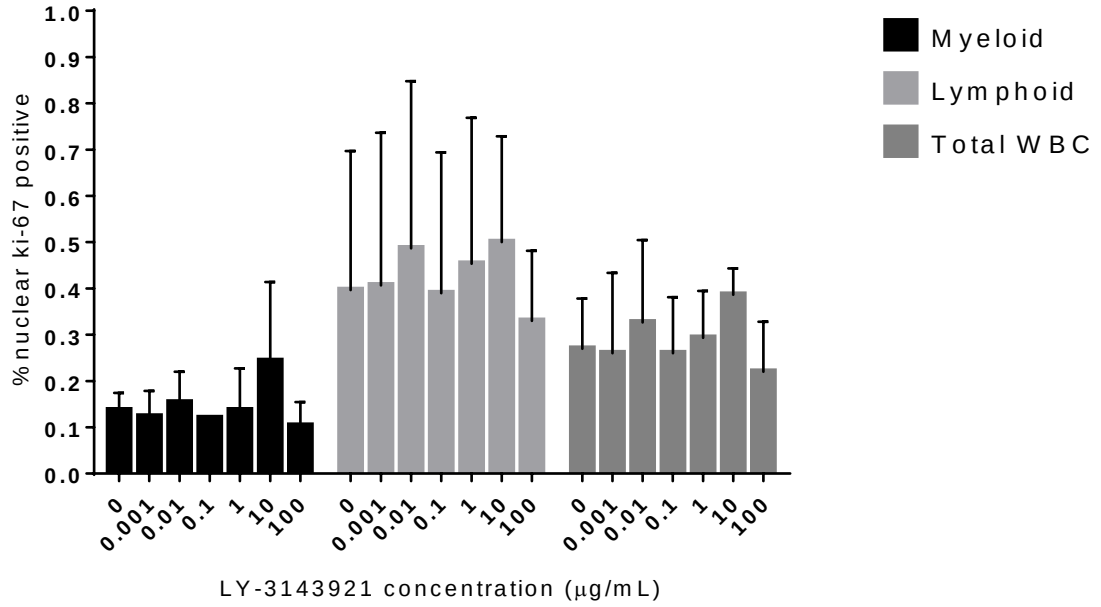


Figure 3.16. Percentages of myeloid, lymphoid and white blood cells with positivity for CDC7 (A), pMCM2 (B) and ki-67 (C) following incubation with LY3143921.

WBCs were collected from whole blood samples treated with different concentrations of LY3143921 (0-100 μg/mL) for 6 hours. A, B and C indicate percentages of CDC7, pMCM2 and ki-67 positivity in myeloid, lymphoid and total WBCs, respectively. Mean and SD shown from 3 independent healthy donors.

Although there were no significant changes in percentages of protein positive cells in WBCs treated with LY3143921, a drug response was observed in a specific population of cells. Focusing on the population of WBCs that were ki-67 positive, a trend towards a reduction in pMCM2 positive cells in this population was clearly observed (Figure 3.17). Mean percentages of pMCM2 positive cells in myeloid cells with ki-67 positivity decreased from 69.1% in untreated cells to 25.5% at a concentration of LY3143921 of 100 μg/mL. Similarly, mean percentages of pMCM2 positive cells in lymphoid cells with ki-67 positivity decreased from 58.5% in untreated cells to 21.5% at a LY3143921 concentration of 100 μg/mL. In both cases a trend for a concentration-dependent effect was observed although this did not reach statistical significance by one-way ANOVA.

Based on the results obtained with CDC7 protein in both cell lines and whole blood treated with LY3143921, CDC7 would not appear to provide a useful biomarker

for this study. Furthermore, the majority of CDC7 positive cells in this study exhibited cytoplasmic expression, which is highly controversial because CDC7 expression is thought to be mainly found only in the nucleus. Only studies in melanoma cells and neuron have reported predominantly cytoplasmic CDC7 expression (Liachko *et al.*, 2013; Gad *et al.*, 2019). However, it is unclear whether the antibody used for CDC7 detection also cross-reacted with some other proteins in the cytoplasm. Therefore, additional experiments focused only on pMCM2 and ki-67 expression.

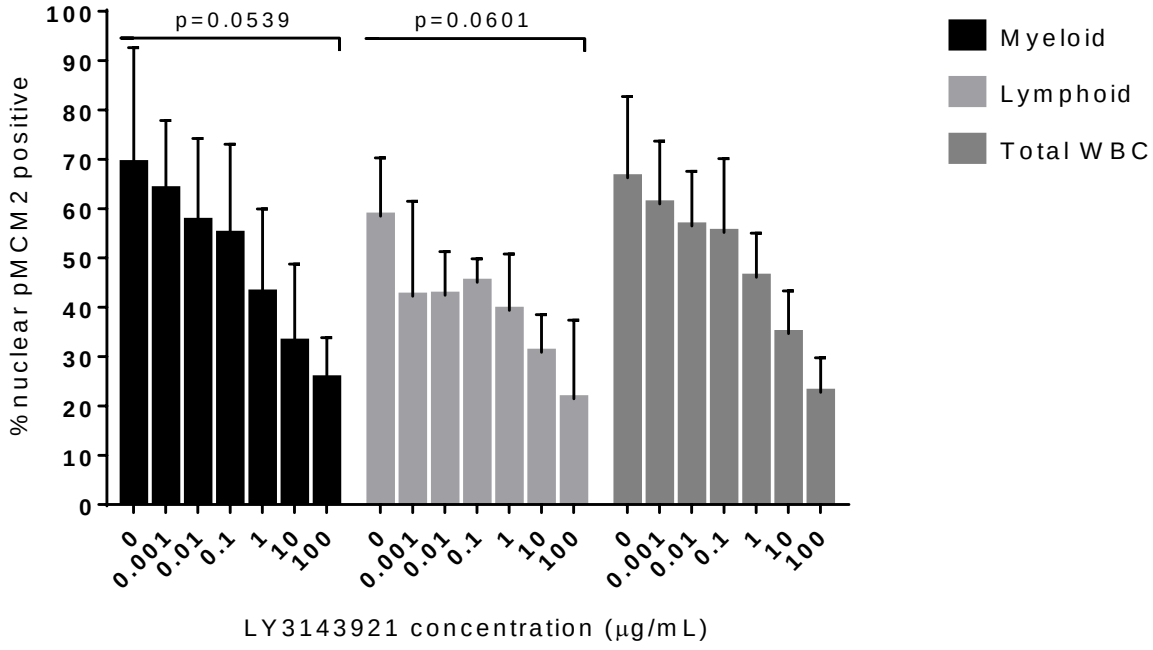
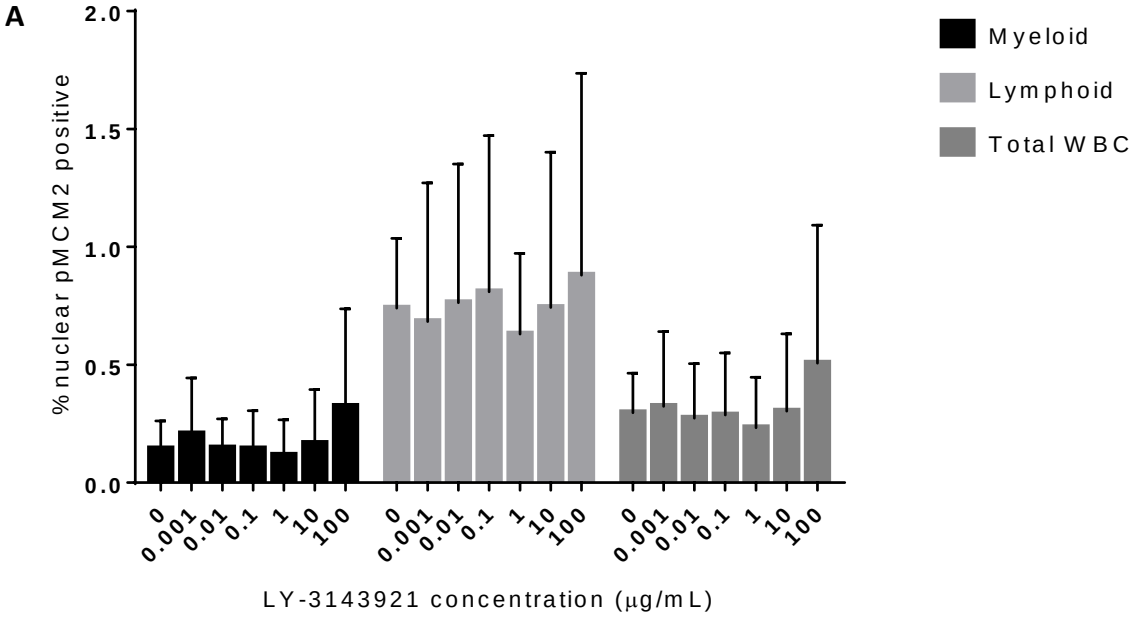


Figure 3.17. Percentages of pMCM2 positive cells in myeloid, lymphoid and white blood cells with ki-67 positivity.

WBCs were collected from whole blood samples treated with different concentrations of LY3143921 (0-100 μg/mL) for 6 hours. Mean and SD shown from 3 independent healthy donors.

The experiments carried out using blood samples collected from healthy donors were next performed using whole blood samples obtained from three cancer patients. The comparable *ex vivo* results are shown in Figure 3.18, with no changes again observed in pMCM2 and ki-67 in the total WBC population following incubation with LY3143921, but a significant decrease in percentage of pMCM2 positive cells in the ki-67 positive cell population. Percentages of nuclear pMCM2 positive myeloid cells

ranged from 0.12 - 0.32% and percentages of nuclear pMCM2 positive lymphoid cells ranged from 0.63 - 0.88% across the range of LY3143921 concentrations investigated (Figure 3.18A). Percentages of nuclear ki-67 positive cells ranged from 0.17 - 0.29% of myeloid cells and 1.1 - 1.6% of lymphoid cells (Figure 3.18B). Taking the ki-67 positive myeloid cell population, percentages of pMCM2 positive cells decreased from 51.8% in untreated cells to 19.8% in cells treated with LY3143921 at a concentration of 100 μg/mL. Similarly, despite non-statistically significant, a reduction in percentage of pMCM2 positive cells from 55.7% to 36.9% was observed in ki-67 positive lymphoid cells following treatment with 100 μg/mL LY3143921 (Figure 3.18C). Concentration-dependent effects of LY3143921 were again observed in myeloid, lymphoid and total WBCs.



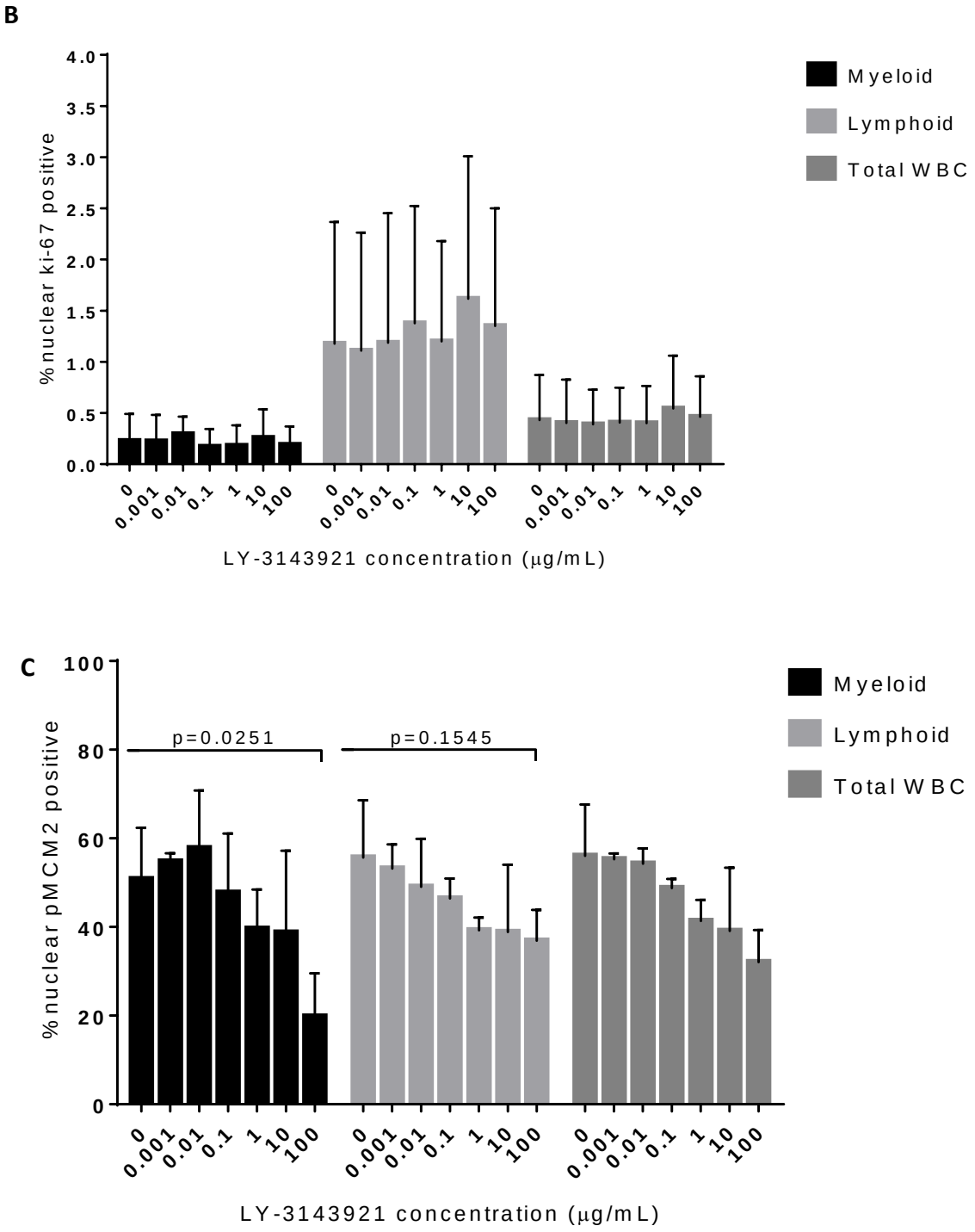


Figure 3.18. Percentages of myeloid, lymphoid and white blood cells with positivity for pMCM2 (A), ki-67 (B) and pMCM2 in a ki-67 positive cell population (C).

Whole blood samples obtained from cancer patients were treated with different concentrations of LY3143921 (0-100 μg/mL) for 6 hours. Mean and SD shown from 3 independent patients.

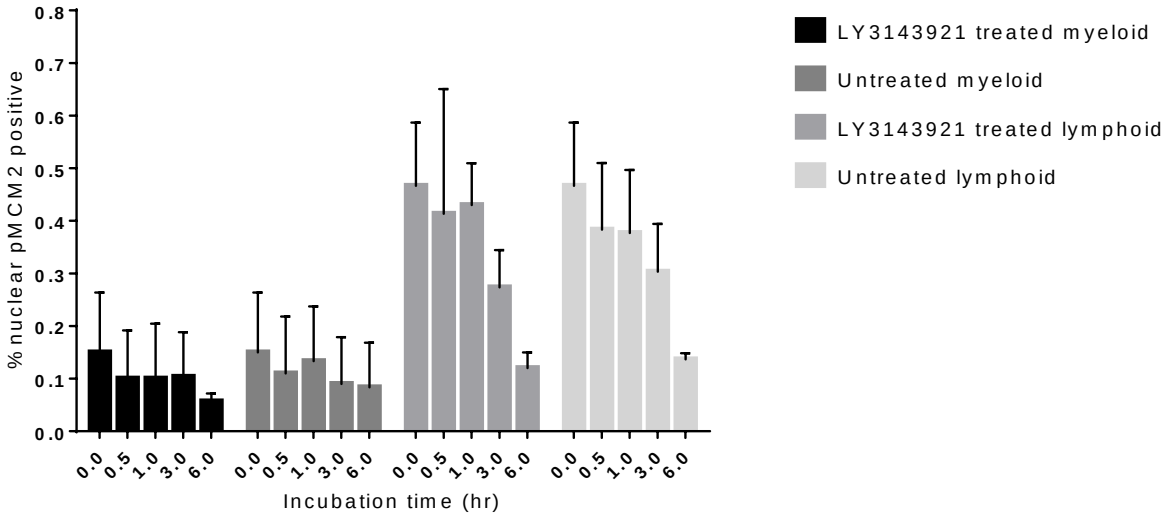
3.3.5 LY3143921 time dependent ex vivo experiments

To confirm that decreases in pMCM2 in ki-67 positive cells resulted from the drug, as opposed to spontaneous degradation of proteins under the experimental conditions, time-dependent *ex vivo* experiments with negative controls were performed as described in Chapter 2. For pMCM2 expression, percentages of pMCM2 positive myeloid cells decreased from 0.15 to 0.06% when treated with LY3143921 for 6 hours. However, these percentages also decreased in the untreated control group from 0.15 to 0.08%. The same trend was observed in the lymphoid cell population, with decreases in pMCM2 positivity from 0.47 to 0.12% in the drug treated group and 0.47 to 0.14% in the control group (Figure 3.19A).

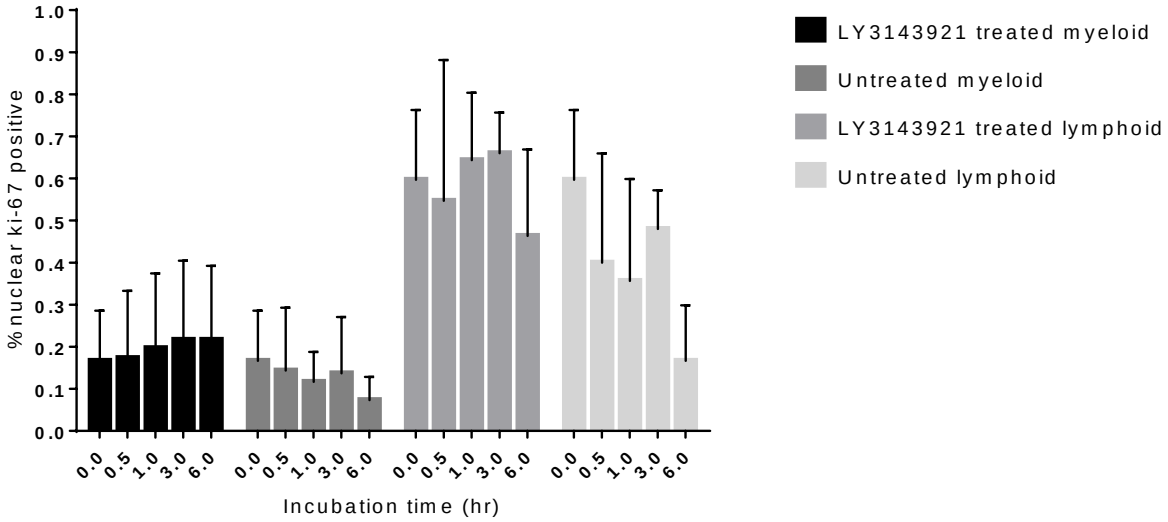
A different pattern of results were observed in percentages of nuclear ki-67 positive cells over time, with less marked reductions over a 6-hour incubation period. The percentages of ki-67 positive myeloid cells increased from 0.17% to 0.22% in the treated group but decreased from 0.17% to 0.07% in the untreated group. Reduced percentages of ki-67 positive cells from 0.60% to 0.46% and from 0.60% to 0.17% were observed in treated and untreated lymphoid cells over this time period, respectively (Figure 3.19B).

Percentages of pMCM2 positive cells in the ki-67 positive population were found to significantly decrease with increasing LY3143921 incubation times for up to 6 hours. Myeloid cells treated with the drug exhibited a decrease in pMCM2 positive cells as a proportion of ki-67 positive cells, from 66.7% to 25.4%, while the untreated cells showed no change (from 66.7% to 66.9%). Similarly, percentages of pMCM2 positive lymphoid cells in the ki-67 positive population decreased from 71.2% to 25.7% when treated with LY3143921, with a less marked decrease observed in untreated cells (Figure 3.19C).

A


% nuclear pMCM2 positive
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.0 0.5 1.0 3.0 6.0
Incubation time (hr)
LY3143921 treated myeloid
Untreated myeloid
LY3143921 treated lymphoid
Untreated lymphoid

B


% nuclear ki-67 positive
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0.0 0.5 1.0 3.0 6.0
Incubation time (hr)
LY3143921 treated myeloid
Untreated myeloid
LY3143921 treated lymphoid
Untreated lymphoid

C

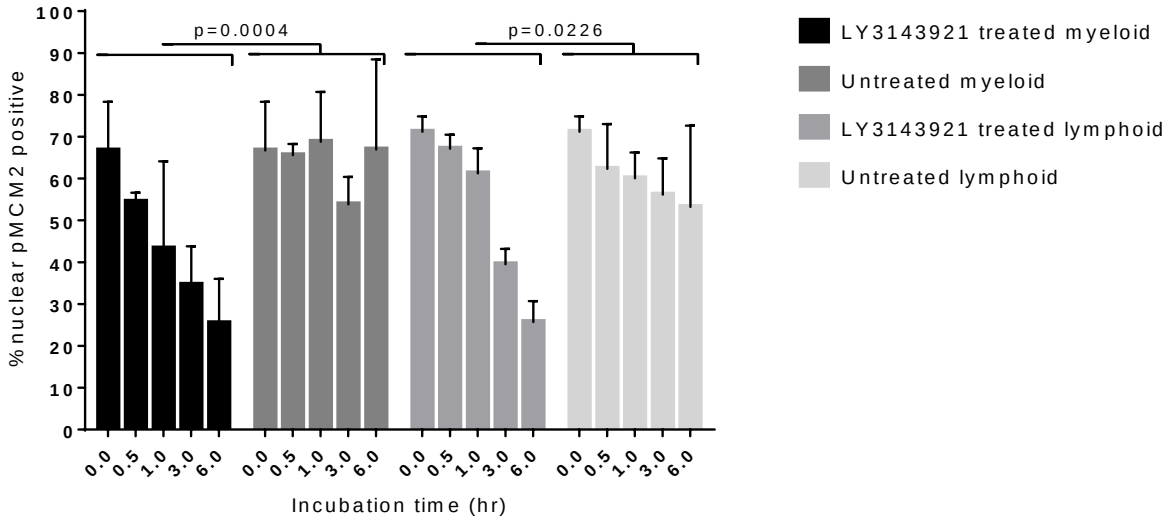


Figure 3.19. Percentages of pMCM2 positive myeloid and lymphoid cells (A), ki-67 positive myeloid and lymphoid cells (B) and pMCM2 positive myeloid and lymphoid cells in the ki-67 positive cell population (C).

Results were obtained from whole blood samples treated with 10 μg/mL LY3143921 for up to 6 hours as compared to non-treated whole blood as a control. Mean and SD shown from 3 independent healthy donors.

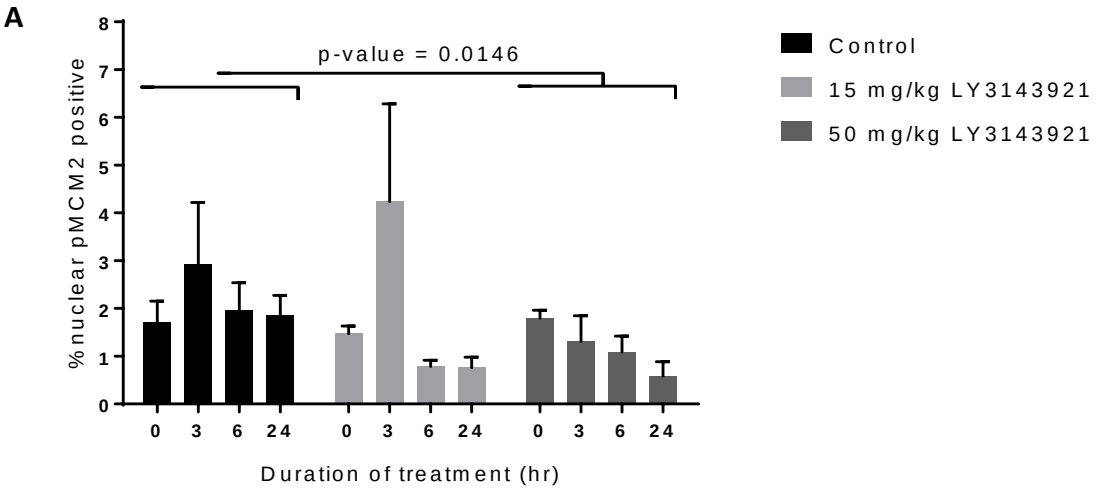
3.3.6 LY3143921 in vivo experiments

The application of the developed assay in an *in vivo* mouse model was next investigated. Eighteen mice were divided to 3 groups of six and each group was administered a different oral dose of LY3143921 (0, 15 or 50 mg/kg). Blood samples were collected from the animals at 0, 3, 6 and 24 hours post-treatment. The same protocol as used for the previously described experiments with human blood was applied to the mouse blood.

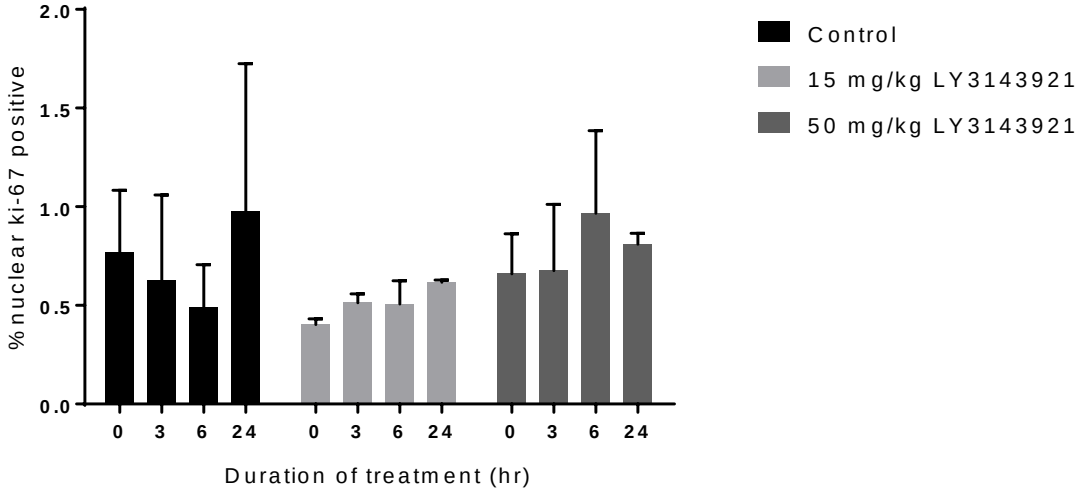
As shown in Figure 3.20A, a significant decrease in percentage of pMCM2 positive WBCs was observed over a period of 24 hours in the 50 mg/kg treatment group as compared to the control untreated group (two-way ANOVA, p-value 0.0146). The mean percentage of pMCM2 positive cells decreased from 1.80% to 0.58% when treated with 50 mg/kg of LY3143921 for 24 hours while there was no observable change in the untreated control group. When treated with 15 mg/kg of the drug, the percentage of pMCM2 positive cells decreased from 1.47% to 0.76% but this decrease was not significant (two-way ANOVA, p-value 0.5513).

In contrast, no significant change in percentage of ki-67 positive cells was observed in any of the treatment or control groups (Figure 3.20B). Mean percentages of ki-67 positive cells ranged from 0.77 – 0.97%, 0.40 – 0.62% and 0.66 – 0.81% in the untreated group, 15 mg/kg and 50 mg/kg treated with the drug for 24 hours, respectively. These changes in percentage of ki-67 positive cells were not statistically significant (two-way ANOVA, p-value >0.5).

Focusing on the ki-67 positive population, significant decreases in percentages of pMCM2 positive cells were observed following treatment with LY3143921 (Figure 3.20C). Mean percentages of pMCM2 positive cells in the ki-67 positive population when treated with 15 mg/kg of the drug for 24 hours decreased from 70.0% to 38.6% and when treated with 50 mg/kg of the drug for 24 hours decreased from 56.6% to 13.9%. P-values of both differences determined by two-way ANOVA were <0.0001.



B



C

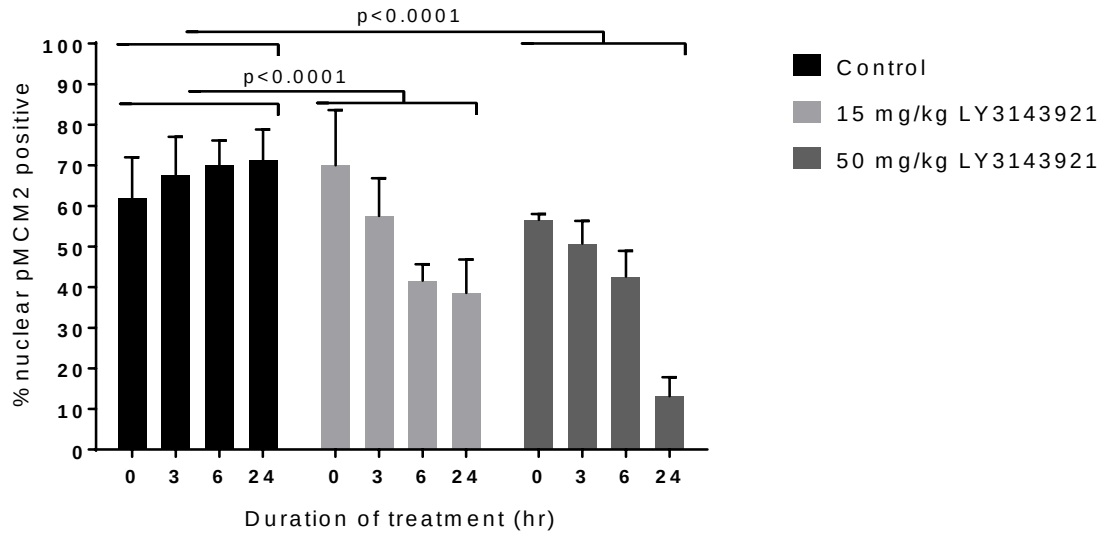


Figure 3.20. Percentages of nuclear pMCM2 positive cells in all WBCs (A), nuclear ki-67 positive cells in all WBCs (B) and of nuclear pMCM2 positive cells in ki-67 positive WBCs (C) observed in an in vivo mouse model.

White blood cells were separated from mice treated with single doses of LY3143921 (15 or 50 mg/kg) and whole blood samples were collected at 0, 3, 6 and 24 hours following administration. Mean and SD shown from 3 sets of mice, 6 mice per group.

3.3.7 Stability tests of pMCM2 and ki-67 in preserved whole blood

In a clinical setting whole blood samples may be taken at different clinical sites and would need to be delivered to the laboratory for analysis. As the shipment of samples may take several days to arrive in the laboratory, a method to reliably preserve pMCM2 and ki-67 protein in clinical samples needed to be developed. Proteins can be preserved using various different methods, with many brands of blood preservative tubes available with the potential to maintain pMCM2 and ki-67 integrity. In addition, freezing fixed WBCs at -80°C may be an effective approach for preservation of pMCM2 and ki-67. These methods were therefore tested in the current study.

3.3.7.1 Stability of pMCM2 and ki-67 in different commercial blood tubes

Four commercial preservative tubes were tested including EDTA tubes, CellSave® tubes, TransFix® tubes and Streck® tubes. Whole blood was collected from healthy donors, added to each type of tube and stored for up to 3 days. EDTA tubes were stored at 2-8°C while other tubes were stored at room temperature according to the manufacturer's instructions. Blood samples were collected from all tube types at 0, 6, 24, 48 and 72 hours after the addition of blood. Samples were processed following the protocol described in Chapter 2.

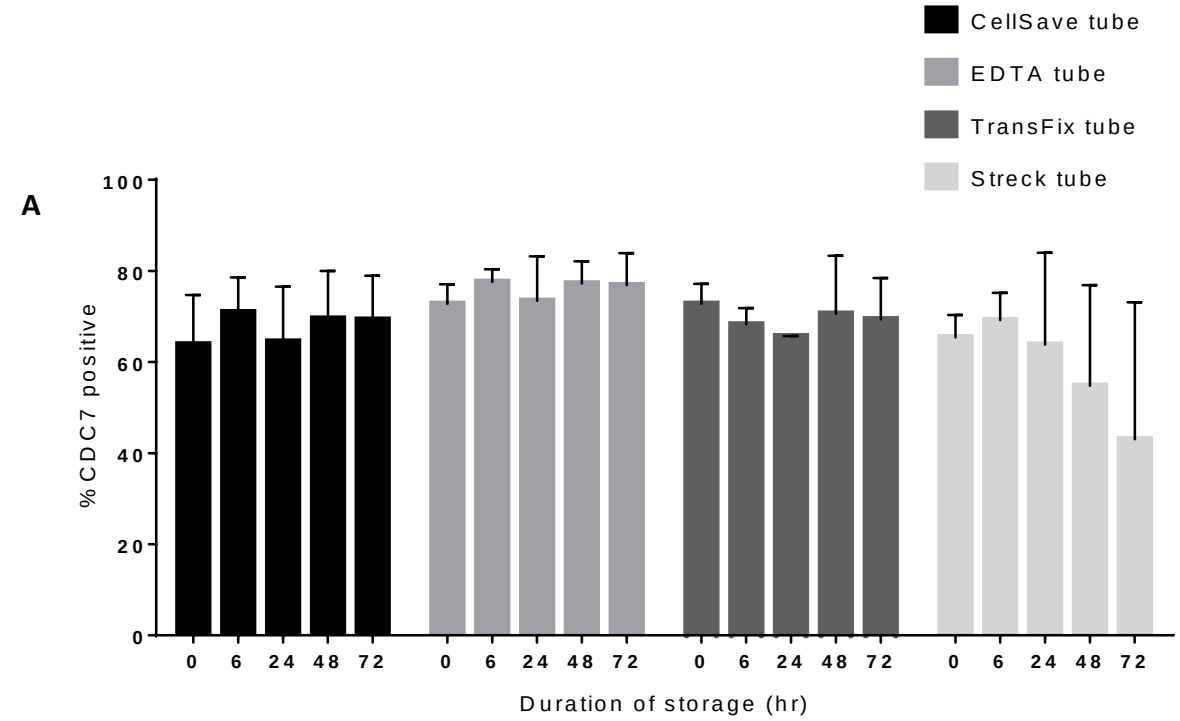
Mean percentages of CDC7 positive WBCs remained stable throughout the 72 hour incubation period for all tubes except Streck® tubes (Figure 3.21A). Average levels of CDC7 measured in CellSave®, EDTA and TransFix® tubes were 67%, 76% and 69%, respectively and did not show any change with time. For the Streck® tubes, the percentage of CDC7 positive cells was observed to decrease from 65% to 43% when stored for 72 hours.

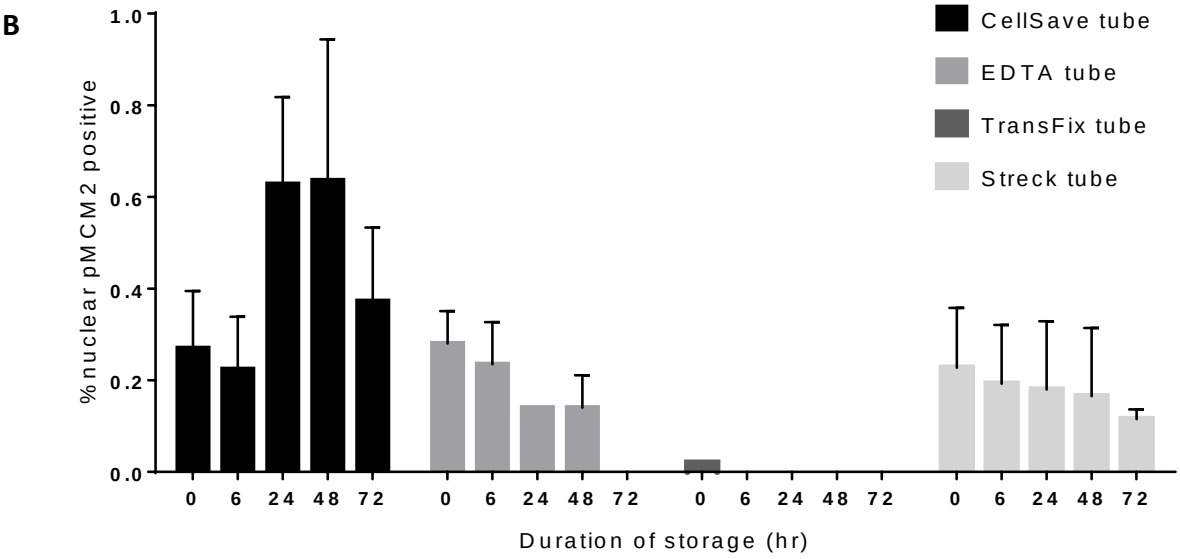
The characteristics of pMCM2 positive WBCs in the various tube types was markedly different from CDC7 (Figure 3.21B). Mean percentages of pMCM2 positive cells from 3 independent experiments increased from 0.27% to 0.63% when stored in CellSave® tube for 24 hours and then decreased to 0.37% at 3 days. Further investigation revealed that this increase mainly resulted from an increase in pMCM2

positivity in myeloid cells; percentages of pMCM2 positive cells in the myeloid cell population increased from 0.09% to 0.61% while in lymphoid cells increased from 0.55% to 0.73% (data not shown on the graph). This pattern of change was not seen in other tubes. Expression of pMCM2 decreased from 0.28% to 0.14% when stored in EDTA tubes and from 0.23% to 0.12% when stored in Streck® tubes. The expression of pMCM2 was undetectable in TransFix® tubes at all time points of storage, so this tube was not suitable for pMCM2 measurement.

The expression of ki-67 is shown in Figure 3.21C. After storage in CellSave® tube for 24 hours, the percentage of ki-67 positive cells increased from 0.23% to 0.46% and then slightly decreased to 0.41% at 3 days. This fluctuation was predominately due to variation of ki-67 positivity in myeloid cells which increased from 0.05% to 0.17% at 1 day and then decreased to 0.09% at day 3 (data not shown on the graph). For EDTA and Streck® tube, percentages of ki-67 positive WBCs remained stable throughout 72 hour of storage. No ki-67 expression was detected in WBC stored in TransFix® tubes at any of the time points investigated.

The percentage of pMCM2 positive cells measured in the ki-67 positive population was observed to decrease with time in CellSave®, EDTA and Streck® tubes (Figure 3.21D). Although the percentage of pMCM2 positive cells was found to be increased at 24-48 hours after storage in CellSave® tubes, it still decreased when considered as proportion of ki-67 positive cells. Mean percentages of pMCM2 in ki-67 positive cells decreased from 79.4% to 27.6% in CellSave® tubes, from 24.9% to 4.9% in EDTA tubes and from 28.4% to 16.9% in Streck® tubes.


A
CellSave tube
EDTA tube
TransFix tube
Streck tube
% CDC7 positive
100
80
60
40
20
0
0 6 24 48 72 0 6 24 48 72 0 6 24 48 72 0 6 24 48 72
Duration of storage (hr)


B
CellSave tube
EDTA tube
TransFix tube
Streck tube
% nuclear pMCM2 positive
1.0
0.8
0.6
0.4
0.2
0.0
0 6 24 48 72 0 6 24 48 72 0 6 24 48 72 0 6 24 48 72
Duration of storage (hr)

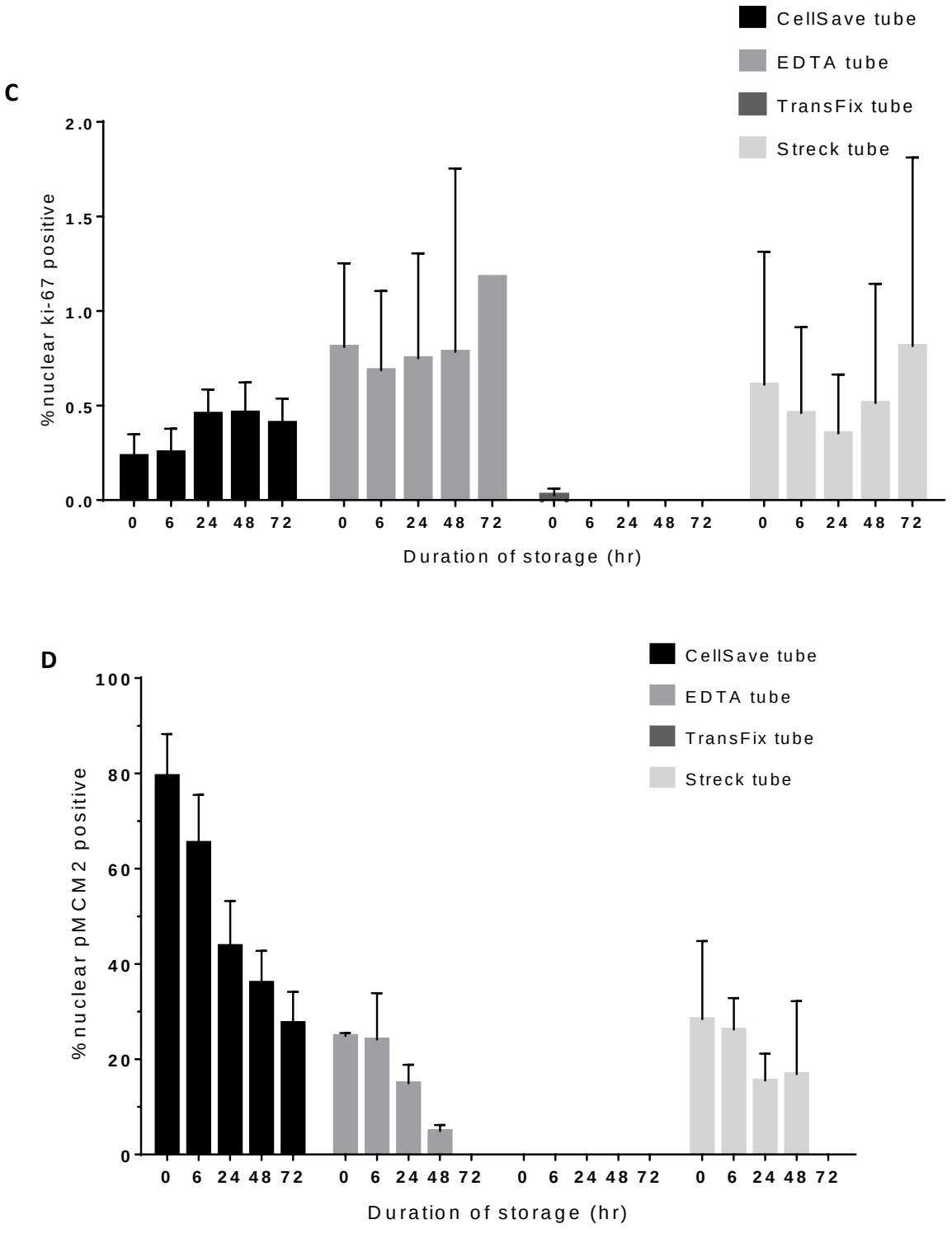


Figure 3.21. Percentages of CDC7 positive cells in all WBCs (A), nuclear pMCM2 positive cells in all WBCs (B), nuclear ki-67 positive cells in all WBCs (C) and percentage of nuclear pMCM2 positive cells in ki-67 positive WBCs (D).

WBCs were separated from whole blood samples stored in different types of blood tubes for up to 3 days. Mean and SD shown from 3 independent donors.

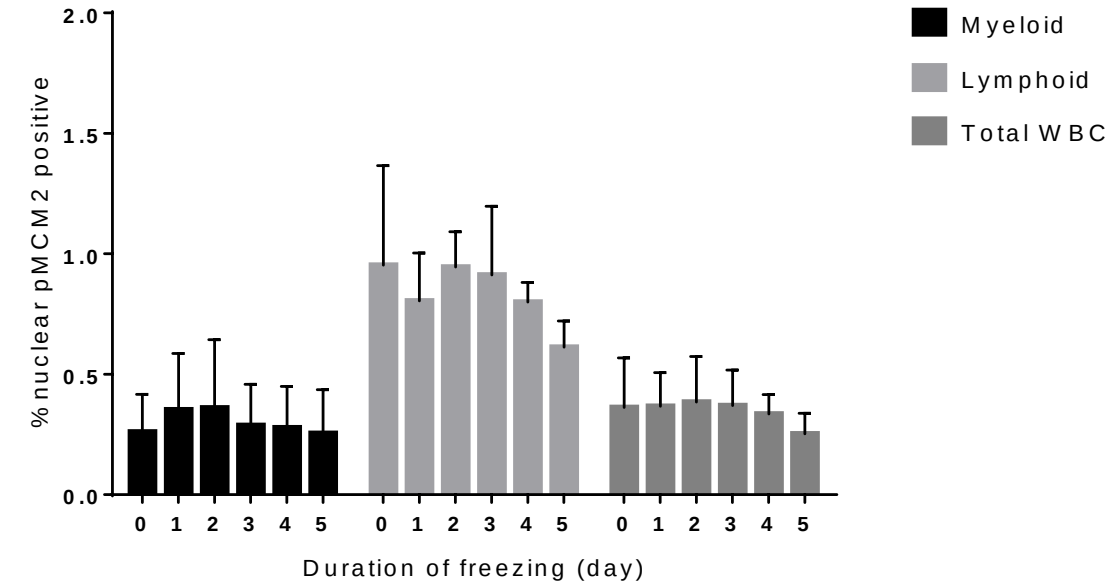
3.3.7.2 Stability of pMCM2 and ki-67 when fixed with Lyse/Fix buffer and stored at -80℃

An alternative method to preserve pMCM2 and ki-67 involved freezing the fixed samples at -80°C. To investigate this approach, whole blood was collected from healthy donors and added to freshly prepared Lyse/Fix buffer solution (6 tubes per donor). The first tube was further processed following the protocol immediately, while the other tubes were frozen at -80°C. A tube of sample was analysed at time points of 1, 2, 3, 4 and 5 days after freezing. Blood tubes were thawed in a 37°C water bath for 30 minutes and then processed using the protocol described in Chapter 2.

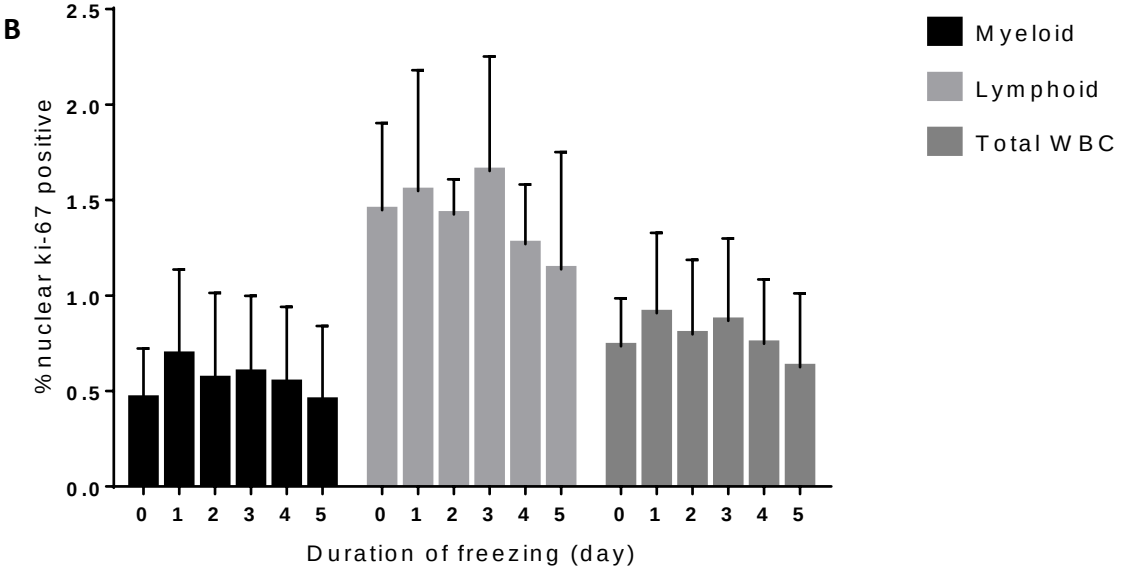
The results obtained from this set of experiments showed that sample stability was maintained for measuring percentages of positive cells for both pMCM2 (Figure 3.22A) and ki-67 (Figure 3.22B) for up to 5 days. Mean percentages of pMCM2 positive myeloid, lymphoid and total white blood cells were 0.26%, 0.95% and 0.46% at time zero and 0.26%, 0.61% and 0.36% at the 5-day time point, respectively.

Similarly, average percentages of ki-67 positive cells at time zero and when frozen for 5 days were 0.46% and 0.45% for myeloid cells, 1.45% and 1.14% for lymphoid cells, and 0.74% and 0.63% for total WBCs, respectively. The percentages of pMCM2 positive cells in a ki-67 positive WBC population ranged between 45-60% for all three types of cell and showed no trend with time of storage (Figure 3.22C).

A


2.0
1.5
1.0
0.5
0.0
% nuclear pMCM2 positive
0 1 2 3 4 5 0 1 2 3 4 5 0 1 2 3 4 5
Duration of freezing (day)
Myeloid
Lymphoid
Total WBC

B


2.5
2.0
1.5
1.0
0.5
0.0
% nuclear ki-67 positive
0 1 2 3 4 5 0 1 2 3 4 5 0 1 2 3 4 5
Duration of freezing (day)
Myeloid
Lymphoid
Total WBC

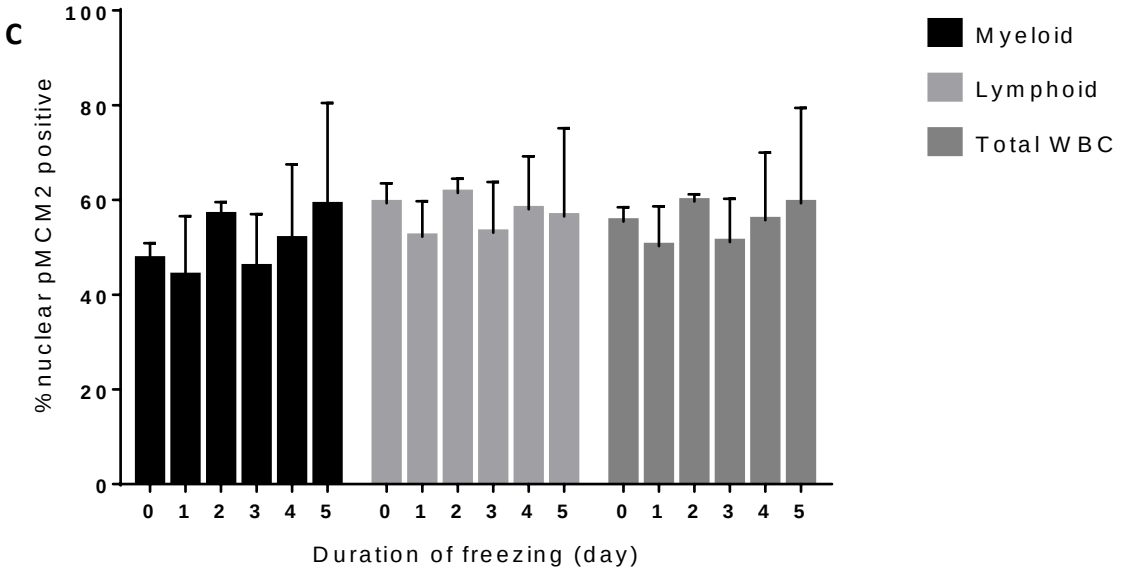


Figure 3.22. Percentages of nuclear pMCM2 positive cells in all WBCs (A), nuclear ki-67 positive cells in all WBCs (B) and nuclear pMCM2 positive cells in ki-67 positive WBCs (C).

White blood cells were separated from whole blood samples, fixed in Lyse/Fix buffer and frozen at -80°C for up to 5 days. Mean and SD shown from 3 independent experiments.

3.4 Discussion

An assay for detection of potential pharmacodynamic biomarkers of a CDC7 inhibitor including CDC7, pMCM2 and ki-67 proteins has been developed and validated in cell lines (*in vitro*), white blood cells (*ex vivo*) and in a mouse model (*in vivo*). In addition, the most appropriate method of collection and storage of clinical samples was investigated to find the optimal method for ensuring the integrity of the data following delivery to the laboratory for analysis.

LY3143921 is a novel anti-cancer agent developed with the proposed mechanism of CDC7 inhibition. However, there is currently no pharmacodynamic biomarker for this agent developed for use in clinical trials. As CDC7 phosphorylates the MCM2 protein, inhibition of CDC7 by the drug should result in a reduction of pMCM2 expression. In addition, as the CDC7 pathway is essential for cell proliferation and division, ki-67, a well-known proliferation marker, was also investigated as a potential biomarker for LY3143921.

The results of antibody optimisation indicated good potency of all antibodies used in this study including antibody against CDC7, pMCM2 and ki-67. However when HL-60 cell lines were treated with LY3143921, no differences were observed in CDC7 expression or ki-67 expression at concentrations of LY3143921 up to 10 μg/mL. According to pre-clinical data from the company, drug peak concentrations in rats and dogs ranged between 0.2-8 μg/mL with the highest tolerated dose up to 20 mg/kg. These concentrations were similar to the concentrations used in this study but decreases in ki-67 were not observed as expected.

These findings are in contrast to previous studies of similar drugs such as PHA-767491 and XL413 (Montagnoli *et al.*, 2008; Sasi *et al.*, 2014). Montagnoli, et al. reported a decrease in CDC7 expression in HeLa cells treated with 5 μM of PHA-767491 for 24 hours measured by Western blot (Montagnoli *et al.*, 2008). Sasi, et al. presented reductions in CDC7 kinase activity in bacteria cells treated with different concentrations of PHA-767491 and XL413 with IC_{50} values of 18.6 nM and 22.7 nM, respectively (Sasi *et al.*, 2014). The difference might result from cytoplasmic CDC7 expression observed in this chapter which was potentially caused by differences in antibodies, methods and different cell lines used. Given our results involved CDC7

cytoplasmic staining, more comparable data may have been generated with an assay that had improved specificity and quantified nuclear CDC7 expression.

In contrast to CDC7 and ki-67, the percentage of pMCM2 positive HL-60 cells was significantly decreased over the concentration range of LY3143921 investigated. This conformed to the proposed mechanism of action of LY3143921 as a CDC7 kinase inhibitor. The drug inhibits functions of CDC7 and therefore affect its downstream proteins instead of directly depleting CDC7.

Ex vivo experiments with whole blood showed comparable results as generated *in vitro*, with no changes in percentages of CDC7 or ki-67 positive cells but a trend towards a decrease in pMCM2 positive cells following treatment with LY3143921. Following analysis of these data, there were no further investigations carried out into the measurement of CDC7 protein as a potential biomarker for LY3143921 activity. Although no changes were observed in the percentage of ki-67 positive cells following treatment with LY3143921, a potentially important relationship was observed when focusing on the percentage of pMCM2 positive cells measured within the ki-67 positive cell population. Indeed, the significant decrease in pMCM2 observed in this cell population following treatment with LY3143921 was found to be more robust than the decrease in pMCM2 in the whole WBC population. However, according to the results, the concentration of LY3143921 in patient samples should be higher than 10 μg/mL in order to observe comparable pMCM2 decreases.

These findings were confirmed with the results obtained from *in vivo* mouse experiments. According to the LY3143921 pharmacokinetic profile in mouse, the dose of 20.8 – 10.4mg/kg was identified as midrange dose, while over 30 mg/kg was high dose (data from the company – personal communication). Therefore the doses used in this chapter were reasonable to classify mice into high-dose and moderate-dose groups. In addition, the pharmacokinetic profile indicated that t_{max} of the 10 mg/kg drug was 3±1.15 hours which was comparable with the percentage of pMCM2 positive cells shown in Figure 3.20A. As given the dose of 10 mg/kg resulted in a detectable decrease in pMCM2, the doses of 15 mg/kg and 50 mg/kg used in this study were expected to generate higher significant decreases. Although using pMCM2 alone provided the required level of sensitivity to detect the effect of LY3143921 in mouse

WBCs, measuring the percentage of pMCM2 in a ki-67 positive population provided a greater power of detection.

To the best of our knowledge, studies investigating associations between pMCM2 and ki-67 are very limited. There was only one published study which previously mentioned a significant correlation between CDC7, pMCM2 and ki-67 (Krawczyk *et al.*, 2009). This study was investigating the prognostic effects of several proliferative markers including CDC7, pMCM2 and ki-67 in invasive breast cancer and the results suggested significant correlations among these three proteins. Nonetheless, this study did not propose a rationale for the correlations observed, so could not provide any insights to explain our findings. A possible explanation is that cells with a higher proliferation rate (high ki-67 expression) may respond to the drug better than normal cells. Another possible reason is that as LY3143921 causes cell cycle arrest, the ki-67 level was stable while pMCM2 levels decreased. These hypotheses, however, need to be further investigated.

The results generated in the current chapter suggest a potential benefit in using two proteins of interest in combination to provide a robust biomarker. While pMCM2 itself was sensitive enough to detect the effect of LY3143921, its use in combination with ki-67 allowed us to focus on a specific population of proliferating cells. Although pMCM2 expression decreased with increasing concentrations of LY3143921, the overall effects on cancer cells and clinical effects in patients need to be further evaluated in the clinic.

With regard to storage of clinical samples, our results suggested that none of the commercial blood tubes tested including CellSave™, TransFix™ and Streck™ tubes were suitable for preservation of the three proteins of interest (CDC7, pMCM2 and ki-67) as well as EDTA tubes stored at 2-8 °C. However, it was found that freezing fixed samples in Lyse/Fix buffer at -80°C was able to preserve pMCM2 and ki-67 proteins for up to 5 days. This preservation method can therefore be applied to clinical samples in a clinical trial setting.

In conclusion, a promising assay for detection of pMCM2 and ki-67 as a combined pharmacodynamic biomarker of LY3143921 has been developed and validated. This assay has the potential to be applied to the analysis of patient samples

following treatment with LY3143921 in a clinical trial setting. Furthermore, the applicability of this assay for use with other CDC7 inhibitors such as PHA-767491 could be tested to expand the future utility of the assay.

Chapter 4. Development and pre-clinical application of an assay for detection of histone H4 acetylation as a pharmacodynamic biomarker for valproic acid

4.1 Introduction

4.1.1 Histones, histone acetylation and histone acetylation enzymes

Histones are a group of proteins isolated through acid-extractions and named by Albrecht Kossel in 1884 (Kossel, 1884). Studies of histone structure have revealed two parts of chromatin structure: a core histone octamer and a tetramer portion. Each octamer consists of two nucleosomal histones which are H3 and H4 while each tetramer is composed of H2A and H2B dimers (Kornberg, 1974).

The acetylation of histones was discovered nearly a century after their initial isolation (Allfrey *et al.*, 1964). The addition of an acetyl moiety to the ε-amino group of a lysine residue which was later defined as N^{ε}-acetylation was revealed (Gershey *et al.*, 1968). This pathway involves two essential groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Discovery of HAT enzymes in the mid-1990s led to a surge of interest in histone acetylation. HATs are classified into three major groups including the GNAT-family, MYST-family and CBP/p300 family. Gu, et al identified their association with tumour suppressor p53, identifying histones as proteins of interest in cancer research (Gu and Roeder, 1997).

While studies investigating the impact of HATs on cancers are ongoing, HDACs are currently considered to represent a more relevant drug target in relation to their association with cancers (Cress and Seto, 2000; Mahlknecht and Hoelzer, 2000). This contributed to the discovery of the first naturally extracted HDAC inhibitor, Trichostatin A (Yoshida *et al.*, 1987). Since then, a number of HDAC inhibitors have been discovered and developed which will be discussed later.

4.1.2 Classification of histone deacetylases

Histone deacetylase (HDAC) enzymes have been extensively studied alongside the development of HDAC inhibitors. These enzymes are classified into 4 major classes as described in Table 4.1 (West and Johnstone, 2014). Class I includes HDAC1, HDAC2,

HDAC3 and HDAC8. Class II includes HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10. HDAC4, HDAC5, HDAC7 and HDAC9 are in subclass IIa while HDAC6 and HDAC10 are in subclass IIb. Class III is called the SIRTUIN class because these enzymes contain a SIRTUIN catalytic domain instead of a HDAC catalytic domain. This class consists of SIRT1 to SIRT7, which can be subdivided to 4 minor classes. Lastly, class IV includes only HDAC11.

Several studies have suggested a correlation between specific HDACs and certain cancer types as highlighted in Table 4.1 (West and Johnstone, 2014; Patra *et al.*, 2019). For instance, all HDACs in class I have been shown to be deregulated in many cancers, with HDAC1 overexpressed in gastric, lung and breast carcinomas (Choi *et al.*, 2001; Zhang *et al.*, 2005; Minamiya *et al.*, 2011). Overexpression of HDAC1, HDAC2 and HDAC3 has also been found in renal cell, colorectal and gastric cancer as well as in classical Hodgkin's lymphoma (Fritzsche *et al.*, 2008; Weichert *et al.*, 2008; Adams *et al.*, 2010). Class IIa HDACs have also been linked to several types of cancer. A study reported that HDAC4 expression was upregulated in renal, bladder and colorectal cancer (Ozdağ *et al.*, 2006). HDAC5 and HDAC9 were overexpressed and have been linked to poor survival in high-risk medulloblastoma patients (Milde *et al.*, 2010). HDAC6 in class IIb was found to be overexpressed in oral squamous cell carcinoma (Sakuma *et al.*, 2006).

HDAC	HDAC protein associations	Expression in cancer	Examples of functional involvement
Class I (homologous to RDP3 yeast protein, nuclear location, ubiquitous tissue expression)			
HDAC1	HDAC2	Elevated in gastric, breast, colorectal, Hodgkin lymphoma, lung, liver	KD induced growth arrest, decreased viability, increased apoptosis and induced genomic instability in colon, breast, and osteosarcoma cancer cell lines
HDAC2	HDAC1	Elevated in gastric, prostate, colorectal, Hodgkin lymphoma, cutaneous T-cell lymphoma	KD induced growth arrest, decreased viability, and increased apoptosis in colon and breast cancer cell lines, and induced apoptosis and decreased lung cancer in vivo
HDAC3	HDAC4, HDAC5, HDAC7	Elevated in gastric, breast, ALL, colorectal, Hodgkin lymphoma Decreased in liver	KD decreased viability and increased apoptosis in colon cancer cell lines
HDAC8		Elevated in neuroblastoma	KD reduced proliferation of lung, colon, and cervical cancer cells
Class IIa (homologous to Hda1 yeast protein, shuttle between nucleus and cytoplasm, tissue-restricted expression			
HDAC4	HDAC3-NCor		KD increased VEGF expression and reduced growth in chondrosarcoma cell lines KD induced apoptosis and decreased cell viability of colon and glioblastoma tumours in vivo
HDAC5	HDAC3-NCor	Elevated in medulloblastoma; decreased in lung	KD decreased cell growth and viability in medulloblastoma
HDAC7	HDAC3-NCor	Elevated in ALL; decreased in lung	KD induced growth arrest in colon and breast cancer cells
HDAC9		Elevated in ALL, medullablastoma	KD inhibited homologous recombination, increased sensitivity to DNA damage and decreased medulloblastoma cell growth and viability

HDAC	HDAC protein associations	Expression in cancer	Examples of functional involvement
Class IIb (homologous to yeast protein Hda1, mostly cytoplasmic location, tissue-restricted expression)			
HDAC6	HDAC11	Elevated in breast, cutaneous T-cell lymphoma; decreased in lung	KD decreased VEGF expression and decreased cell viability
HDAC10			KD inhibited homologous recombination, increased sensitivity to DNA damage and decreased VEGF expression
Class IV (unknown yeast protein homology, cytoplasmic location, tissue-restricted expression)			
HDAC11	HDAC6	Elevated in breast, renal, liver	KD induced apoptosis in colon, prostate, breast and ovarian cancer cell lines
KD = knockdown (gene silencing by siRNA). ALL = acute lymphoblastic leukaemia			

Table 4.1. The association of HDAC in cancer.
Adapted from West AC, et al. J Clin Invest. 2014;124(1):30-39. and Patra S, et al. Cell Mol Life Sci. 2019;76(17):3263-3282.

Although proteins in the SIRTUIN class do not contain HDAC catalytic domains and are occasionally not classified as HDACs, these proteins were found to have important roles in cancer. For example, upregulation of SIRT1 has been reported in AML, prostate cancer and non-melanoma. In contrast, downregulation of SIRT1 has been reported in colon cancer (Barneda-Zahonero and Parra, 2012), while levels of SIRT3 and SIRT7 expression were increased in breast cancer (Ashraf *et al.*, 2006). Some studies have reported downregulation of SIRT2 in gliomas and gastric carcinoma and have suggested that this protein might acts as a tumour suppressor (Hiratsuka *et al.*, 2003; Inoue *et al.*, 2007). Additionally dysregulation of SIRT3 was reported in many types of breast cancer (Ashraf *et al.*, 2006).

Studies of class IV HDAC and cancer are limited. Overexpression of HDAC11 has been found in several carcinoma cell lines including colon, prostate, breast and ovarian cells (Deubzer *et al.*, 2013). However, the translation and clinical relevance of these findings are currently lacking and the expression of this protein in tumour tissue requires further investigation.

4.1.3 Histone deacetylase inhibitors as anticancer drugs

Since the association between HDAC and cancer was discovered, many anticancer drugs based on this mechanism of action have been discovered, developed and clinically tested. HDAC inhibitors can be classified based on their chemical structures or their targets. In terms of their chemical structures, HDAC inhibitors are classified into three major groups: short chain fatty acids, hydroxamic acids and miscellaneous agents (Manal *et al.*, 2016). Examples of chemical structure of HDAC inhibitors are illustrated in Figure 4.1.

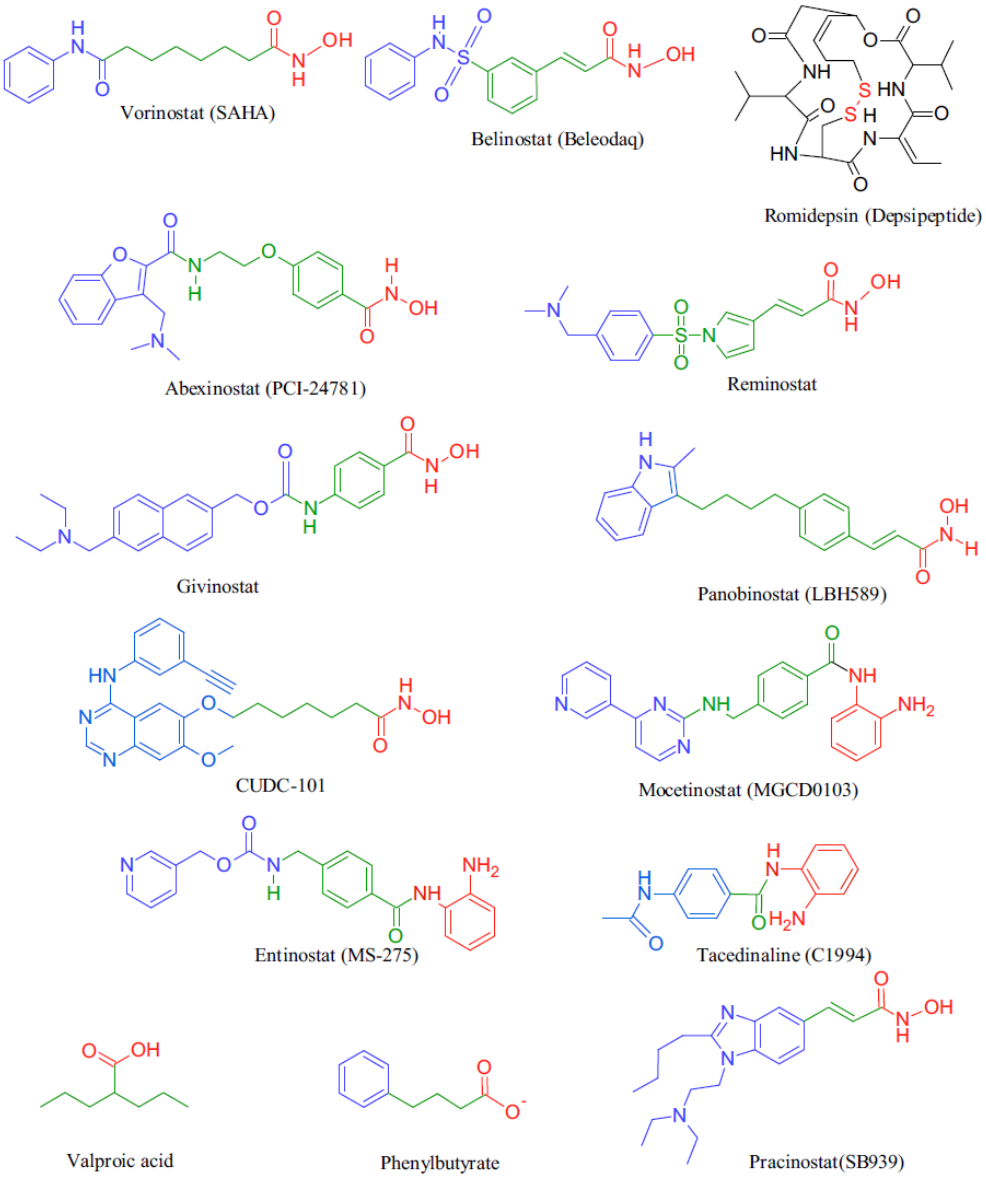


Figure 4.1. Chemical structures of selective HDAC inhibitors. The cap, linker and the Zn^{2+} binding group are represented in blue, green and red, respectively.

Taken from Manal M, et al. Bioorganic Chemistry. 2016;67:18-42.

The derivatives of short chain fatty acids include valproic acid and butyric acid. Valproic acid has been studied in cancer for several decades and been shown to have potency against both solid tumours and leukaemia in preclinical studies (Göttlicher *et al.*, 2001; Shabbeer *et al.*, 2007). Phenyl butyrate also achieved effective results in preclinical trials (Warrell *et al.*, 1998; Iannitti and Palmieri, 2011). These findings contributed to the development of several butyrate-based anticancer prodrugs including tributyrin, AN-1, AN-7, 3,4,6-OBu3GlcNAc, PMX 550B and PMX 550D (Manal *et al.*, 2016).

The other backbone structure of HDAC inhibitors is hydroxamic acid, which was first reported to exhibit HDAC inhibitory effects in 2001, subsequently a number of hydroxamate-based anticancer drugs have been developed (Lavoie *et al.*, 2001). Vorinostat or suberoylanilide hydroxamic acid (SAHA) was the first US FDA approved hydroxamic acid based HDAC inhibitor for the treatment of cutaneous T-cell lymphoma (Mann *et al.*, 2007). Other approved drugs in this class include abexinostat, reminostat, panobinostat, mocetinostat, pracinostat and givinostat.

Focusing on the target sites of HDAC inhibitors, it is known that different drugs target different types of HDAC (Table 4.2) (Eckschlager *et al.*, 2017). Most hydroxamate-based agents inhibit almost all HDACs except SIRTUIN (pan HDAC inhibitor) while drugs in the short chain fatty acid class target only class I and IIa HDAC. Benzamide class drugs bind mainly to class I HDAC while the cyclic tetrapeptide romidepsin, inhibits only HDAC1 and HDAC4. Drugs which target class III or SIRTUINs include nicotinamide (all SIRTs), sirtinol (SIRT1 and 2) and cambinol (SIRT1 and 2). The classification by target sites, however, was varied depending on the definition of inhibition and availability of information (Bolden *et al.*, 2006; Thomas *et al.*, 2011; West and Johnstone, 2014; Li and Seto, 2016).

Class	Drug name	HDAC target	Clinical status
Hydroxamic acids	Trichostatin A	Pan	Phase I clinical trial
	Vorinostat	Pan	Approved for cutaneous T-cell lymphoma
	Belinostat	Pan	Approved for peripheral T-cell lymphoma
	Panabiostat	Pan	Approved for multiple myeloma
	Givinostat	Pan	Phase III clinical trial
	Resminostat	Pan	Phase II clinical trial
	Abexinostat	Pan	Phase III clinical trial
	Quisinostat	Pan	Phase II clinical trial
	Rocilinostat	Class II	Phase II clinical trial
	Practinostat	Class I, II and IV	Phase III clinical trial
Short chain fatty acids	Valproic acid	Class I and IIa	Phase III clinical trial for cancer treatment
	Butyric acid	Class I and IIa	Phase II clinical trial
Benzamides	Entinostat	Class I	Phase III clinical trial
	Tacedinaline	Class I	Phase III clinical trial
	Mocetinostat	Class I and IV	Phase II clinical trial
Cyclic tetrapeptides	Romidepsin	Class I	Approved for cutaneous T-cell lymphoma
SIRTUINs inhibitors	Nicotinamide	Class III	Phase III clinical trial
	Sirtinol	SIRT1 and 2	Preclinical
	Cambinol	SIRT1 and 2	Preclinical

Table 4.2. Classification of histone deacetylase inhibitors and their clinical status.
Data from www.ClinicalTrials.gov (Access on 28 October 2019).

4.1.4 Pharmacodynamic biomarkers for histone deacetylase inhibitors

Recently, a number of HDAC inhibitors have been approved for clinical use and many more agents are being developed (Manal *et al.*, 2016). Consequently, a number of robust biomarkers have been developed in order to evaluate the effect of these developed agents, with pharmacodynamic biomarkers required to prove the mechanism of action of the drug during development. Based on the histone acetylation pathway being targeted, either HDAC enzyme expression or histone acetylation have the potential to provide appropriate surrogate biomarkers.

As HDAC inhibitors inhibit the deacetylase activity of these target proteins, measurement of enzyme activity is the most direct approach for the assessment of HDAC inhibitor efficacy. In order to determine the enzyme activity, measurement of histone acetylation as downstream proteins of HDAC activity is selected. Histone acetylation of histones H3 and H4 represent the most extensively used biomarkers in preclinical and clinical trials involving HDAC inhibitors (Shi and Xu, 2013). These two proteins, especially histone H4, have become potential biomarkers since the loss of acetylation at Lys16 of histone H4 was found to be a common hallmark of human cancer (Fraga *et al.*, 2005). Furthermore, a previously published study indicated that HDAC inhibitors globally enhance H3/H4 tail acetylation (Drogaris *et al.*, 2012). Measurement of histone acetylation provides two key advantages compared to HDAC measurement. Firstly, histone acetylation is a modification catalysed by HDACs and HATs, so the level of acetylation reflects the enzyme activity of HDACs and HATs. Secondly, histone acetylation can be measured in white blood cells from peripheral blood which allows non-invasive procedures to be utilised in cancer patients (Plumb *et al.*, 2003).

The utility of histone acetylation as a pharmacodynamic biomarker became well-known following publication of results from the SAHA study. Histone hyperacetylation in peripheral blood mononuclear cells was reported to be rapidly induced by 2-3 fold in patients receiving vorinostat, regardless of the drug level or tumour response (Garcia-Manero *et al.*, 2008). However, using histone acetylation as a predictive or prognostic biomarker has been questioned due to its unpredictable response. For example, a study showed that the level of histone H4 acetylation in patients receiving belinostat returned to normal within 2 hours following drug

administration; this decrease was faster than the elimination rate of the drug (Steele *et al.*, 2008). Moreover, an increase in acH4 was only observed within a certain dose range in the same study. These issues limit the applicability of histone acetylation as a predictive biomarker and need to be resolved in order to utilise these proteins in a clinical setting.

4.2 Chapter specific aims

The main objective of this study was to develop and validate an assay for detection of acH4 using an imaging flow cytometer. In particular, this study aimed to:

- Optimise the concentration of acH4 antibody used in the developed assay by evaluation of acH4 expression in cells, and by measurement of mean acH4 intensity and percentage of acH4 positive white blood cells.
- Assess the precision of the flow cytometer used and determine inter-assay variability of the developed assay.
- Test the applicability of the developed assay in HL-60 cell lines by measurement of mean and median intensity of acH4 expression as well as percentage of acH4 positive cells following concentration- and time-dependent valproic acid treatment.
- Test the applicability of the developed assay in WBCs treated with valproic acid outside the body (*ex vivo*) by measurement of mean and median intensity of acH4 expression as well as percentage of acH4 positive WBCs.
- Investigate the characteristics of acH4 expression in myeloid and lymphoid cell populations following treatment with valproic acid.

4.3 Results

4.3.1 Antibody validation

The antibody against histone H4 acetylation was tested to determine the optimal conditions for the assay. Whole blood samples from healthy volunteers were divided into two tubes; one was treated with 4 mM of valproic acid for 6 hours and the other was stored at room temperature for 6 hours. The condition of treatment was based on the concentration of valproic acid (>2 mM) that is known to inhibit HDACs and increase acH4 expression, and the treatment duration (≤6 hours) that does not change the phenotypic proteins in WBC. The processes of red blood cell lysis, antibody staining and imaging flow cytometry are described in Chapter 2.

Intensity histograms of acH4 expression at different concentrations of acH4 antibody are illustrated in Figure 4.2, together with representatives of cell at the peaks of the histograms. The results suggest an increase in acH4 signal with increasing acH4 antibody volumes (Figure 4.3); the mean acH4 intensity in untreated cells increased from 8,400 to 32,100 and in treated cells increased from 16,300 to 59,500 for 1 μL and 4 μL of acH4 antibody used, respectively.

Focusing on cells with highly expressed acH4 intensity, namely acH4 positive cells, the percentage of acH4 positive cells increased with increasing volumes of acH4 antibody (Figure 4.4). An increase in acH4 antibody volume from 1 μL to 4 μL resulted in an increase of acH4 positive cells from 3.75% to 74% for untreated cells and from 19.7% to 91.6% for valproic treated cells, respectively.

Although these results suggest that 4 μL of the antibody did not result in saturation of acH4 intensity, nor the percentages of acH4 positive cells, the greatest ratio of acH4 signal over background noise was observed in 1 μL of antibody. Looking at fold change in acH4 positive cells between treated and untreated cells, an antibody volume of 1 μL generated a 5.25-fold increase while 2, 3 and 4 μL antibody volumes provided 1.69-, 1.43- and 1.24-fold increases, respectively. Therefore, 1 μL of antibody was considered to be suitable to use in the developed assay.

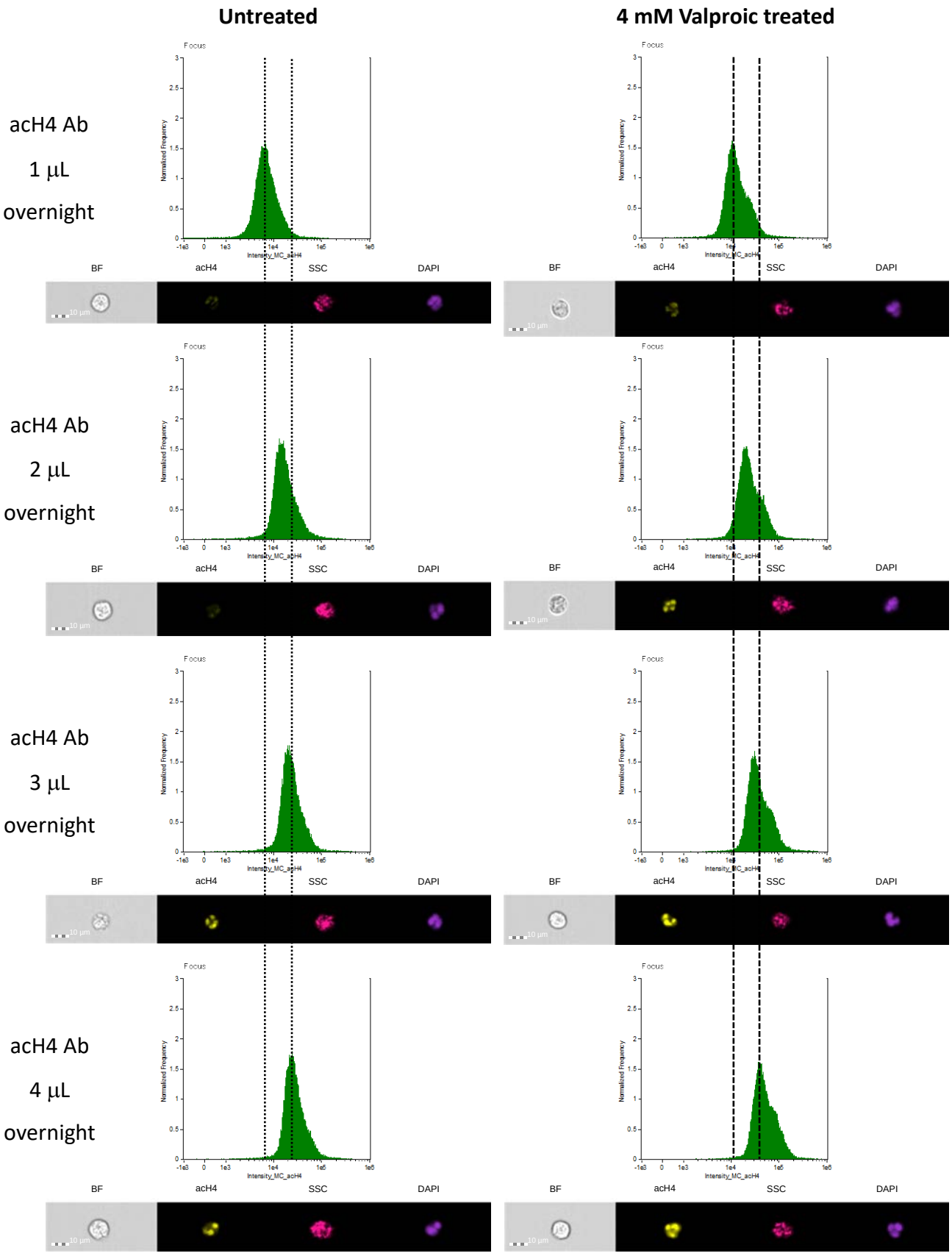


Figure 4.2. Histograms of acH4 intensity and examples of cells at the peaks of histograms.

WBCs shown were separated from whole blood either treated with 4 mM valproic acid for 6 hours or stored in EDTA tubes for 6 hours at room temperature. Cells were incubated with different volumes of acH4 antibody (1-4 µL) overnight.

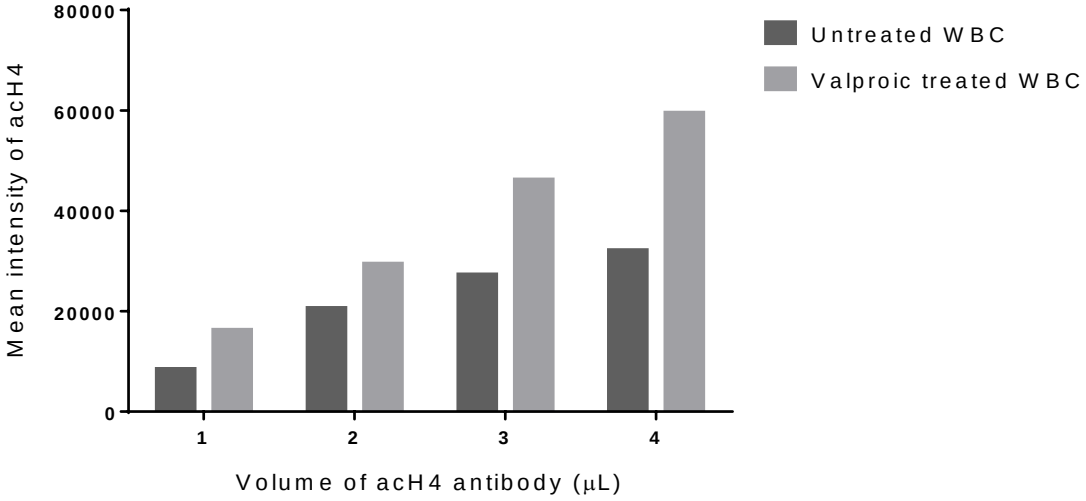


Figure 4.3. Mean intensity of acH4 expression in WBCs incubated with different volumes of acH4 antibody (1-4 μL) overnight.

WBCs were separated from whole blood samples treated with 4 mM valproic acid for 6 hours and untreated blood at the same condition. The results shown are from a single experiment. Mean intensity refers to average expression of protein in all cells collected from one sample.

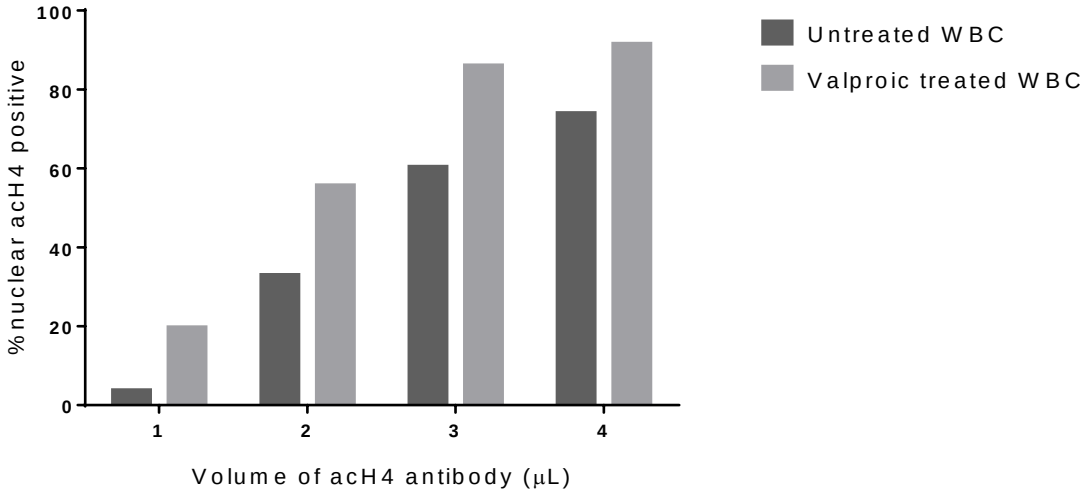


Figure 4.4. Percentages of nuclear acH4 positive WBCs following incubation with different volumes of acH4 antibody (1-4 μL) overnight.

WBCs were separated from whole blood samples treated with 4 mM valproic acid for 6 hours and untreated blood under the same conditions. The results shown are from a single experiment.

4.3.2 Intra- and Inter-assay variation

The precision of the developed assay was tested prior to the analysis of clinical samples. Whole blood from a healthy donor was treated with 4 mM valproic acid for 6 hours and then processed following the protocol described in chapter 2. White blood cells were divided into three tubes and each tube was incubated with 1 μL of acH4 antibody overnight on three different days. The acH4 intensity was determined three times on day 1 to investigate the accuracy and precision of the imaging flow cytometer and the other two were measured once each on different days in order to investigate inter-assay variability.

The precision of imaging flow cytometer and variation of the assay for measuring acH4 positive cells are summarised in Table 4.3 and Table 4.4. For the first sample which was measured three times, the coefficient of variation (%CV) was 17.1% for myeloid cells, 18.2% for lymphoid cells and 17.8% for total WBCs. For inter-staining variation, %CVs in myeloid, lymphoid and total white blood cells were 14.2%, 13.3% and 12.8%, respectively. These findings suggested high precision (<20 %CV) of the imaging flow cytometer used and the developed assay.

Sample		**%nuclear acH4/myeloid**	**%nuclear acH4/lymphoid**	**%nuclear acH4/WBC**
1/1		14	21.3	19.4
1/2		11.8	16.7	15.4
1/3		9.92	15.1	13.8
Flow cytometer precision	Mean	11.91	17.70	16.20
	%CV	17.15	18.18	17.81

Table 4.3. Precision of imaging flow cytometer by measurement of acH4 positive cells in the same sample three times.

The sample was stained in one experiment and acH4 measured three times.

Sample		%nuclear acH4/myeloid	%nuclear acH4/lymphoid	%nuclear acH4/WBC
Average of sample 1		11.91	17.70	16.20
2		10.50	17.50	15.60
3		10.70	19.30	16.70
Inter-staining variation	Mean	11.04	18.17	16.17
	%CV	6.90	5.43	3.41

Table 4.4. Inter-staining variation in acH4 positive cells in 7 samples from the same donor.

All three samples were stained using the same assay at three different days.

4.3.3 Valproic acid concentration dependent in vitro experiments

HL-60 cell lines were incubated with different concentrations of valproic acid (0.5, 1, 2, 4 and 8 mM) for 6 hours. Culture media without the drug was used as a control. Cells were fixed and incubated with acH4 antibody following the protocol described previously (section 2.6.1) and the intensity of acH4 expression was measured by an imaging flow cytometer.

Examples of acH4 intensity histograms, scatter plots between similarity of acH4 and DAPI and acH4 intensity, and representative cells showing acH4 and DAPI expression are illustrated in Figure 4.5. These results indicate a stronger intensity of acH4 expression and higher percentage of acH4 positive cells following an increase in valproic acid concentration. Average values together with SD of three repeats including mean intensity, median intensity and percentages of acH4 positive cells are shown in Figure 4.6.

Mean and median intensity of acH4 expression in HL-60 cell lines were found to be increased with increasing valproic acid concentrations (Figure 4.6A and Figure 4.6B). Both parameters provided comparable trends of increase with an average intensity of 5,000 at control and 13,000 at a valproic acid concentration of 8 mM. Statistical analysis (One-way ANOVA) revealed significant increases for both mean and median acH4 expression (p-value <0.0001). Also, a multiple comparison approach indicated that there was no significant difference between valproic acid concentrations of 4 mM

and 8 mM (Mean difference -600, 95%CI -3066 to 1848) which suggested that 4 mM could generate a maximum signal for this assay.

Focusing on number of highly expressed acH4 cells (acH4 positive cells), the average percentage of acH4 positive cells was found to be increased with increasing concentrations of valproic acid (Figure 4.6C). The results showed that percentage of acH4 positive cells increased from 0.51±0.65% in control cells to 30.6±4.16% at a valproic acid concentration of 8 mM (One-way ANOVA, p-value <0.0001). No significant difference between 4 mM and 8 mM was observed (mean difference -5%, 95%CI -16.44% to 6.37%).

All *in vitro* experiments investigating acH4 intensity and percentage of acH4 positive cell suggested that 4 mM should be the highest concentration used for the developed assay, as higher concentrations did not generate higher signals. This concentration therefore was used in further experiments throughout this study.

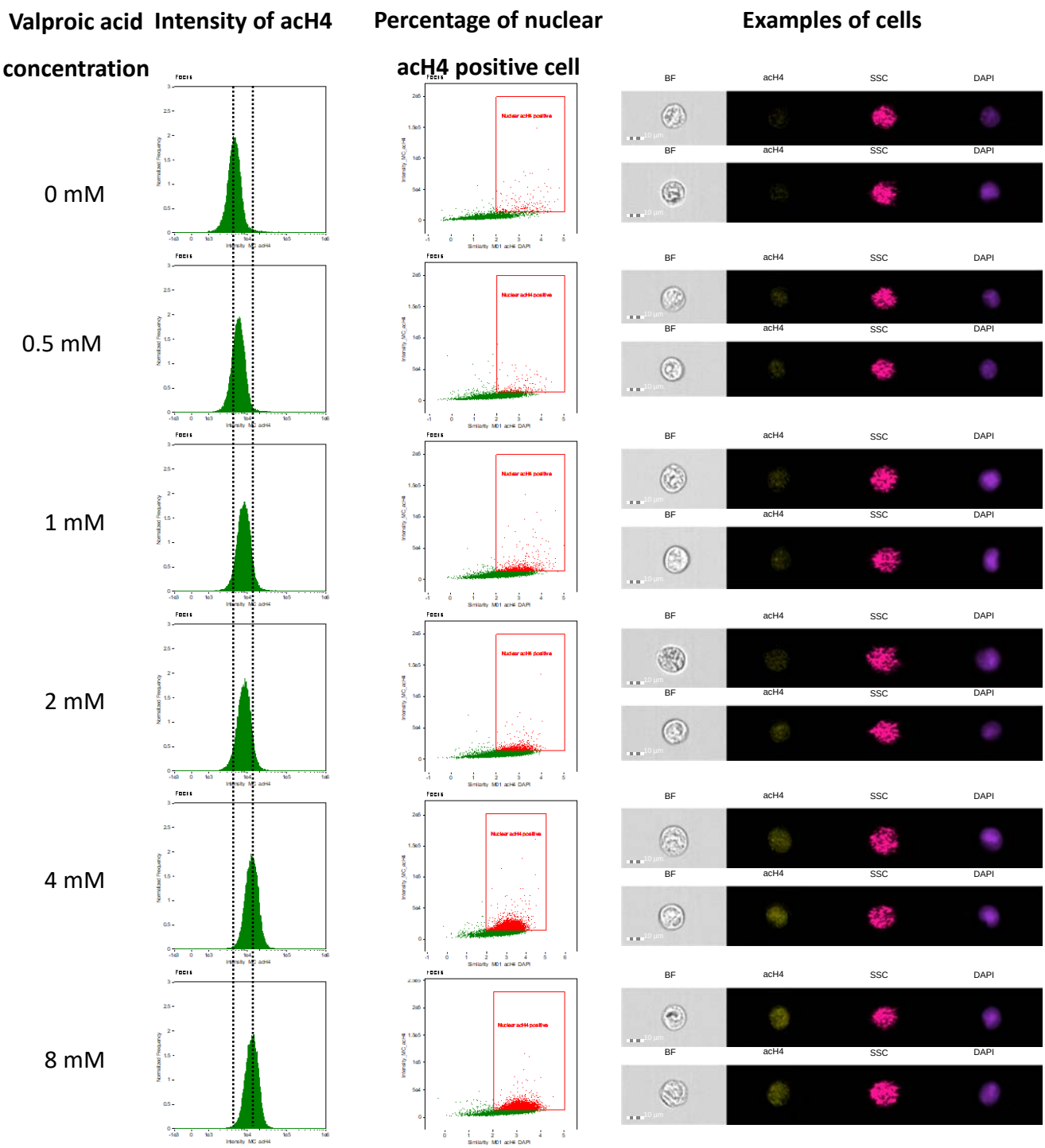
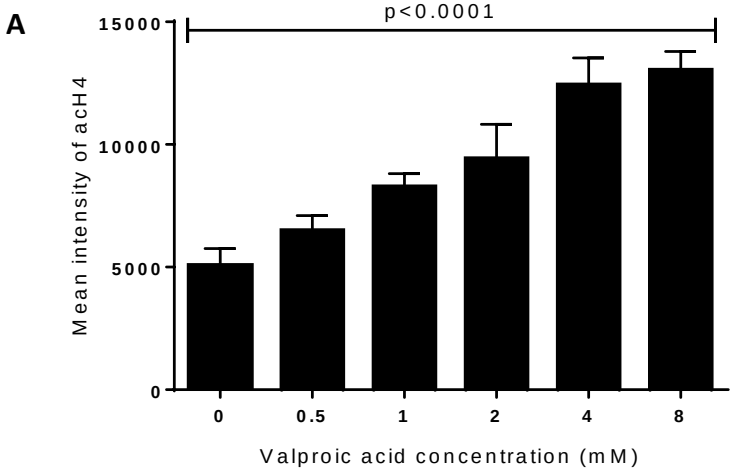
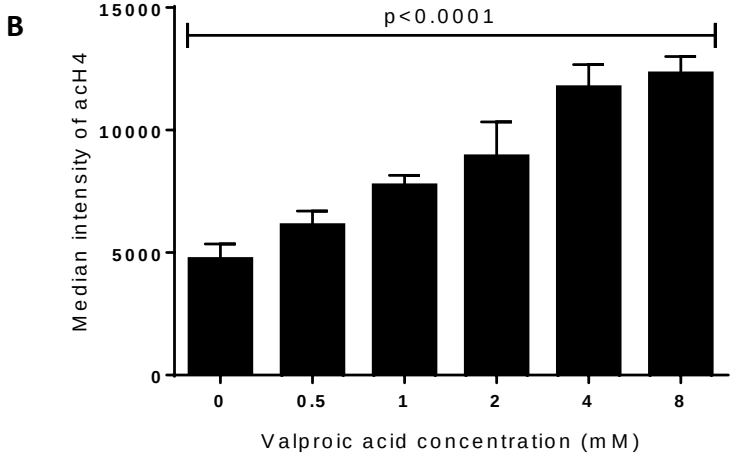


Figure 4.5. Intensity histograms of acH4 expression, scatter plots between similarity of acH4 and DAPI and intensity of acH4, and examples of HL-60 cell lines showing acH4 expression at the peaks of histograms.

Samples were separated from HL-60 cells treated with valproic acid at concentrations of 0.5-8 mM for 6 hours. The control is untreated (0 mM valproic acid) HL-60 cells under the same conditions.





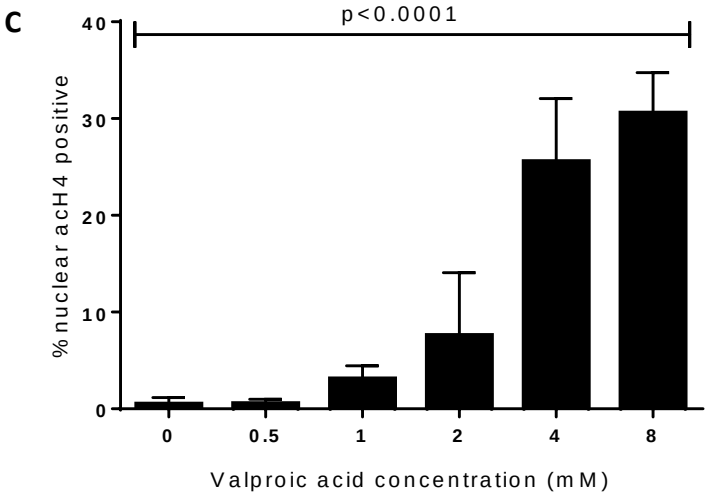


Figure 4.6. Expression of acH4 in HL-60 cells treated with increasing concentrations of valproic acid (0-8 mM) for 6 hours.

Figure 4.6A and Figure 4.6B indicate mean and median fluorescence intensity of acH4, respectively, and Figure 4.6C indicates average percentage of acH4 positive cells. Mean and SD shown from 3 separate experiments.

4.3.4 Valproic acid time dependent in vitro experiments

In order to investigate the effect of duration of valproic acid treatment, HL-60 cell lines were incubated with 4 mM valproic acid for up to 24 hours. The processes of cell fixation, antibody incubation and imaging flow cytometry were the same as previously described. The histograms of acH4 intensity, scatter plots between similarity of acH4 and DAPI and intensity of acH4, and examples of cells at the peaks of histogram showing the expression of acH4 are shown in Figure 4.7.

Data relating to average mean intensity of acH4, median intensity of acH4 and percentage of acH4 positive cells are summarised in Figure 4.8, with all three parameters exhibiting the same trend of change. For mean and median intensity (Figure 4.8A and Figure 4.8B), the expressions of acH4 were stable at 0.5 and 1 hour of drug incubation as compared to baseline; mean intensities of 3,500 at baseline and 4,600 at 1 hour (one-way ANOVA, 95%CI of difference -3887 to 1565). Significant increases in acH4 expression were observed when duration of treatment was longer than 1 hour and peaked at 6 hours, with an intensity of 9,800 observed (one-way ANOVA, p-value <0.0001). However, the acH4 expression then decreased to 7,500 at 24 hours of treatment.

The mean percentage of acH4 positive cells followed the same trend as intensity with no significant changes observed compared to baseline following incubation times of <3 hours (Figure 4.8C). Statistical analysis (one-way ANOVA and multiple comparison analysis) revealed that the mean difference between 0 hour and 3 hours was -5% (95%CI -11.82% to 1.743%) and between 0 hour and 6 hours was -9.5% (95%CI -16.29% to -2.724%). A reduction in percentage of acH4 positive cells at 24 hours was also observed but this was not statistically significant (Mean difference 6.8%, 95%CI -0.75% to 14.41%). However, the overall increase determined by one-way ANOVA was significant (p-value 0.0032).

These results suggested that a 6-hour duration of treatment was suitable for further experiments due to a decrease in acH4 signal observed following an incubation period of 24 hours.

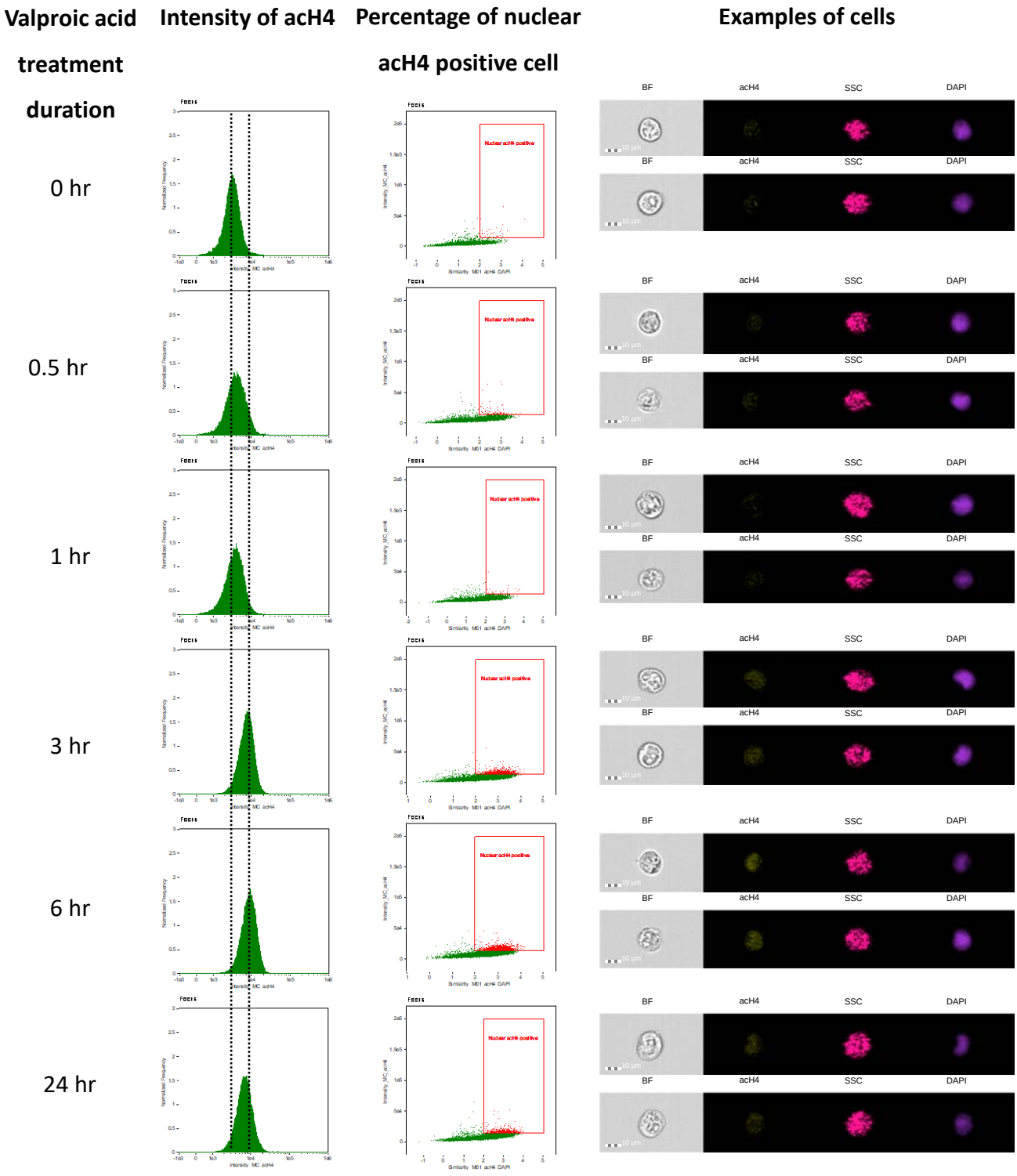
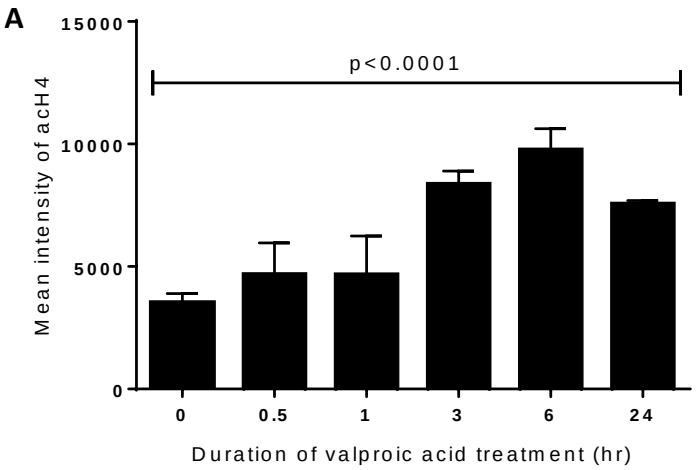
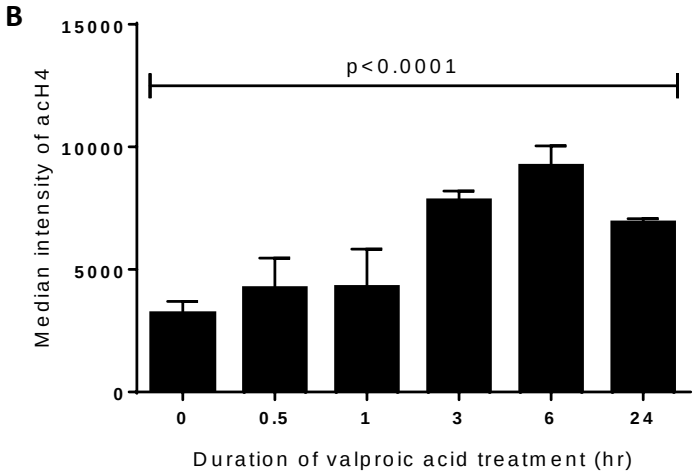


Figure 4.7. Intensity histograms of acH4 expression, scatter plots between similarity of acH4 and DAPI and intensity of acH4, and examples of HL-60 cell lines showing acH4 expression at the peaks of histograms.

Samples were separated from HL-60 cells treated with 4 mM valproic acid for up to 24 hours. The control is cells treated with 4 mM valproic acid and harvested immediately.





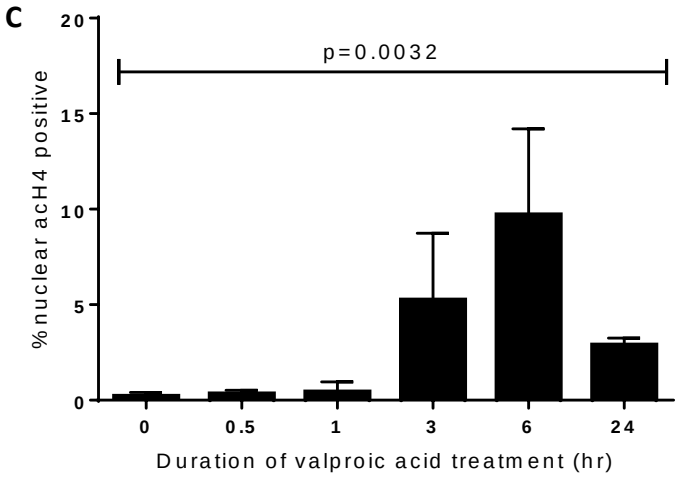


Figure 4.8. Expression of acH4 in HL-60 cells treated with 4 mM of valproic acid for up to 24 hours.

Figure 4.8A, B and C indicate mean fluorescence intensity, median fluorescence intensity and average percentage of acH4 positive cells, respectively. Mean and SD shown from 3 separate experiments.

4.3.5 Valproic acid whole blood ex vivo experiments

The applicability of the developed assay to *ex vivo* incubations of human white blood cells was investigated by conducting concentration-dependent experiments in whole blood from three independent healthy donors. Whole blood samples were treated with increasing concentrations of valproic acid (0, 0.5, 1, 2, 4 and 8 mM) for 6 hours. Protocols for red blood cell lysis, acH4 antibody incubation and imaging flow cytometry were described in Chapter 2.

Examples of white blood cells with and without acH4 expression are shown in Figure 4.9; Figure 4.9A and Figure 4.9B represent myeloid cells while Figure 4.9C and Figure 4.9D represent lymphoid cells with nuclear acH4 positivity and negativity, respectively. Moreover, Figure 4.9E shows clear cytoplasmic expression of acH4. Examples of acH4 intensity histograms and scatter graphs between similarity of acH4 and DAPI and intensity of acH4 are illustrated in Figure 4.10 and Figure 4.11, respectively.

Based on the results shown, a difference in pattern of change between acH4 expression in myeloid and lymphoid cells was observed. At baseline, both cell types expressed a single peak of acH4 intensity (Figure 4.10). After treatment with 8 mM valproic acid for 6 hours, the acH4 expression curve in myeloid cells became bimodal while in lymphoid cells it remained unimodal. These results conformed to the scatter graphs showing cells with high and low similarity between acH4 and DAPI which indicated nuclear and cytoplasmic expression of the protein.

As illustrated in Figure 4.11, the number of myeloid cells with cytoplasmic acH4 expression increased when treated with 8 mM valproic acid compared to no treatment. Further analysis suggested that the second peak in the myeloid histogram was the population of cells with cytoplasmic acH4 expression (data not shown on the graph). This phenomenon, however, was less obvious in lymphoid cells and did not appear when cells were treated with lower concentrations of the drug (<4 mM).

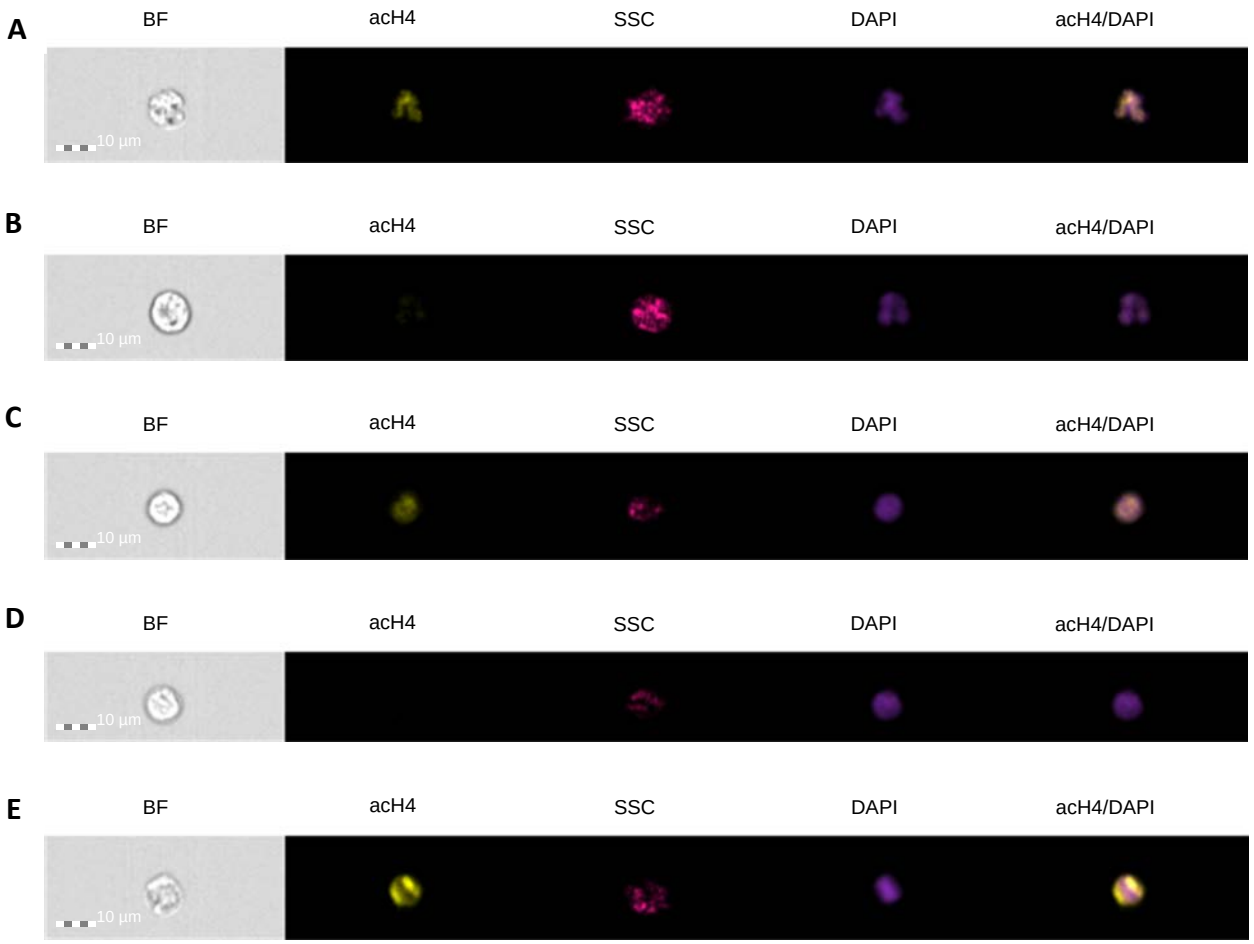


Figure 4.9. Representative images of myeloid cells with nuclear acH4 positivity (A) and negativity (B), lymphoid cells with nuclear acH4 positivity (C) and negativity (D), and myeloid cells with cytoplasmic acH4 positivity (E).

Samples were separated following incubation of WBCs with 8 mM sodium valproate for 6 hours.

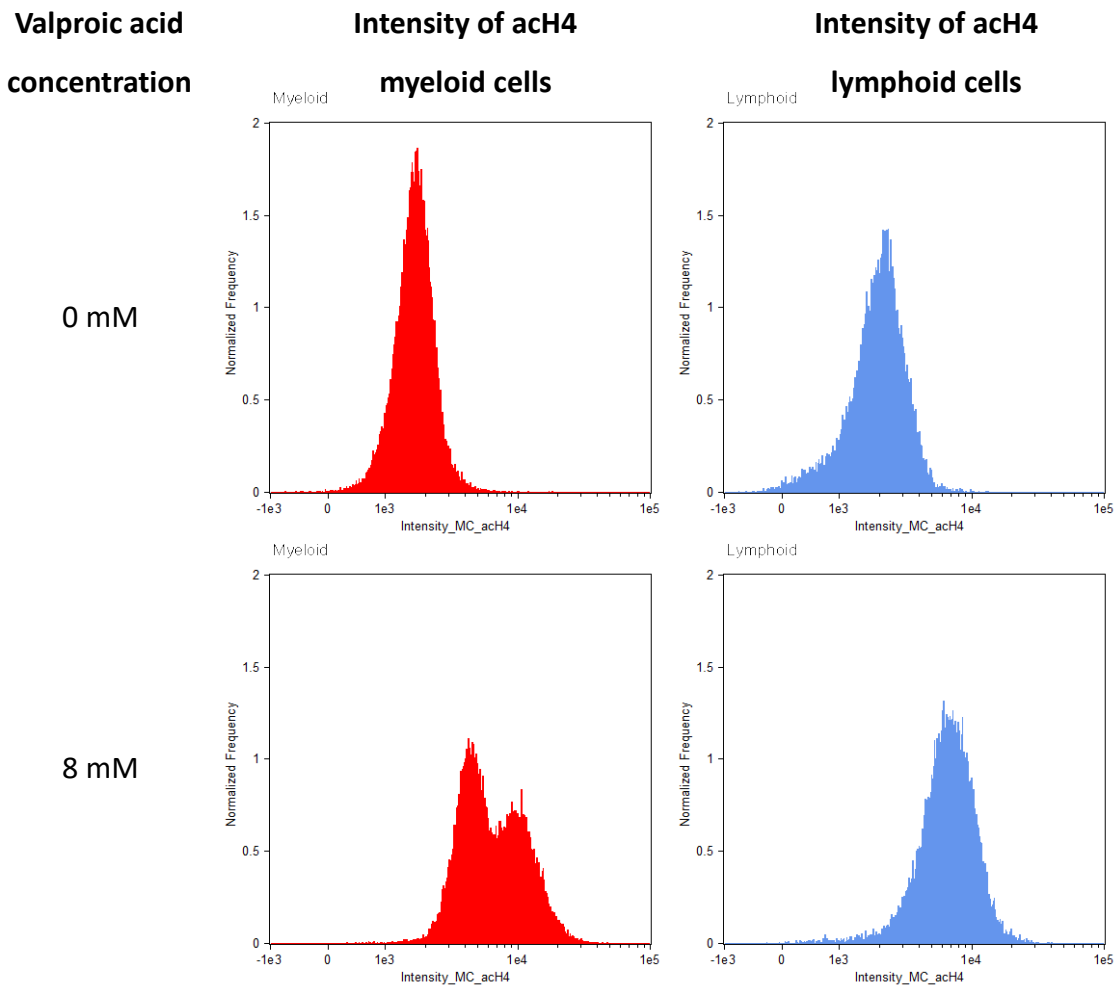


Figure 4.10. Examples of acH4 intensity histograms of myeloid and lymphoid cells either untreated or treated with 8 mM valproic acid for 6 hours.

Red colour indicates myeloid and blue colour indicates lymphoid populations.

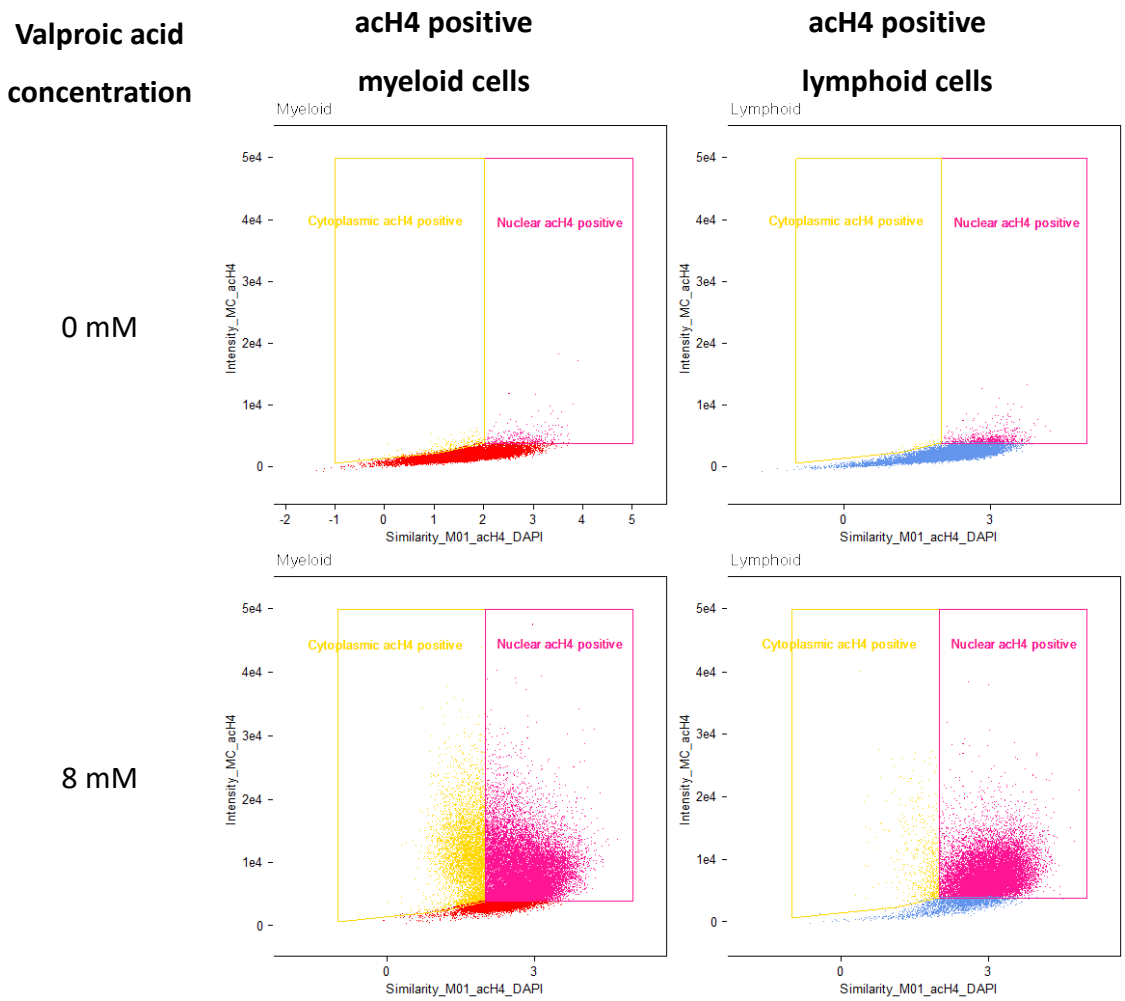


Figure 4.11. Examples of scatter plots of myeloid and lymphoid cells showing nuclear and cytoplasmic acH4 positive cells.

WBCs were separated from whole blood either untreated or treated with 8 mM valproic acid for 6 hours.

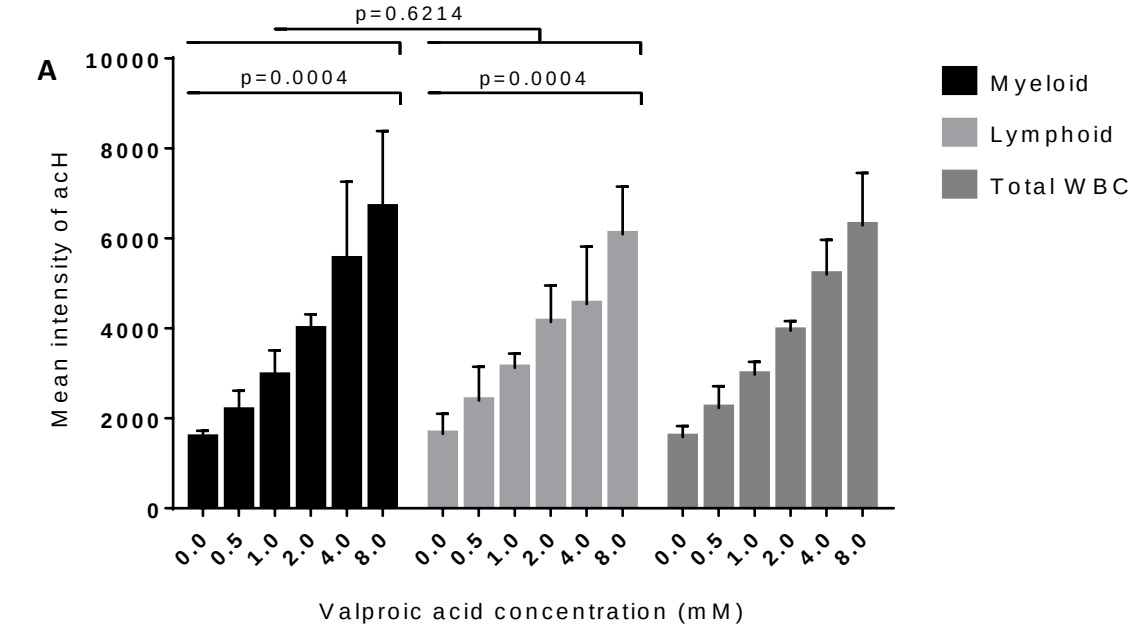
A summary of data showing average mean intensity and median intensity of acH4 expression and average percentage of acH4 positive cells together with SD from 3 independent healthy donors are presented in Figure 4.12. These three parameters showed significant increases following incubation with increasing concentrations of valproic acid. However, the difference between myeloid and lymphoid cells was not significant when looking at mean or median intensity.

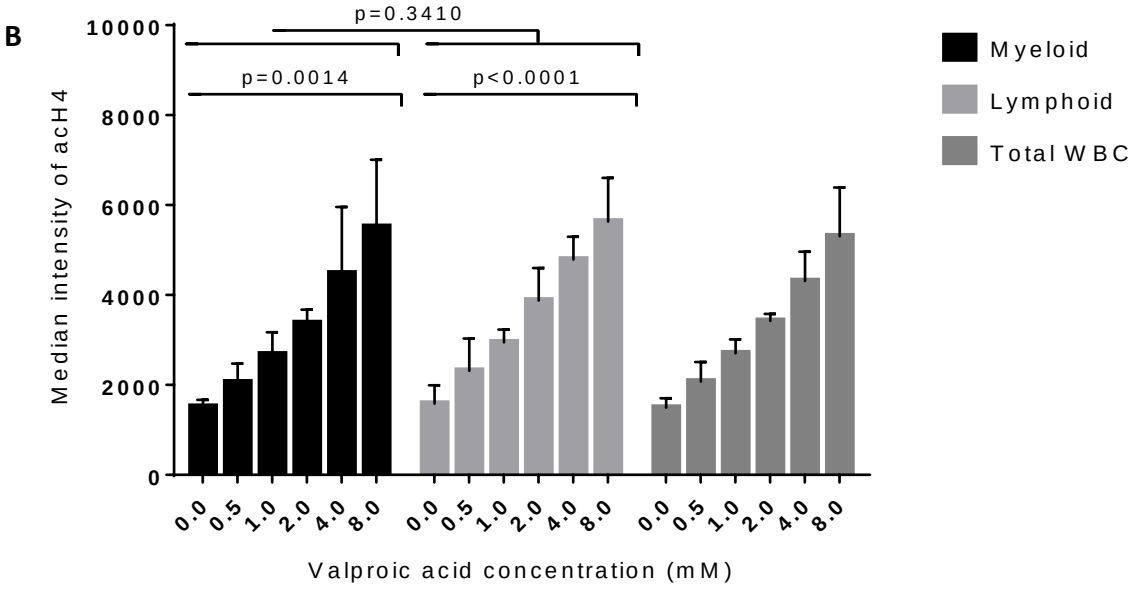
When the concentration of valproic acid increased from 0 to 8 mM, the mean intensity of acH4 increased from 1,500 to 6,700 (4.5-fold) for myeloid cells and from 1,600 to 6,100 (3.8-fold) for lymphoid cells, and median intensity of acH4 increased from 1,500 to 5,500 (3.7-fold) and from 1,600 to 5,600 (3.5-fold) for myeloid and lymphoid cells, respectively. A one-way ANOVA indicated significant increases for both mean and median intensity in both myeloid and lymphoid cells with p-values <0.01 (Figure 4.12A and Figure 4.12B). However, when the increases observed in myeloid cells were compared with the increases in lymphoid cells, a two-way ANOVA analysis revealed that there was no significant difference between cell types for either mean or median intensity (p-values of 0.62 and 0.34).

In contrast, comparison of the change in percentage of acH4 positive cells showed significant differences between myeloid and lymphoid cells with a greater fold-increase than observed with intensity. An increase in valproic acid concentration from 0 to 8 mM resulted in increases in percentage of acH4 positive cells from 0.6% to 55% (92-fold) for myeloid cells and from 2.1% to 70% (33-fold) for lymphoid cells; both increases were statistically significant with one-way ANOVA p-values <0.0001 (Figure 4.12C). Moreover, two-way ANOVA indicated a significant difference between the two type of cells in terms of increases in myeloid and lymphoid cells (p-value <0.0001).

These *ex vivo* findings highlighted two important issues. Firstly, fold-increases observed in percentage of acH4 positive cells were higher than mean and median intensity of acH4 expression, so the percentage of acH4 positive cells is a better representative of the magnitude of change than acH4 intensity. Secondly, considering the increases in acH4 positive cells, the myeloid population provided a 3 times greater fold-change than lymphoid cells (92-fold vs. 33-fold). Together these results suggest

that the myeloid population may be more suitable than lymphoid cells for the detection of acH4 changes in clinical samples.





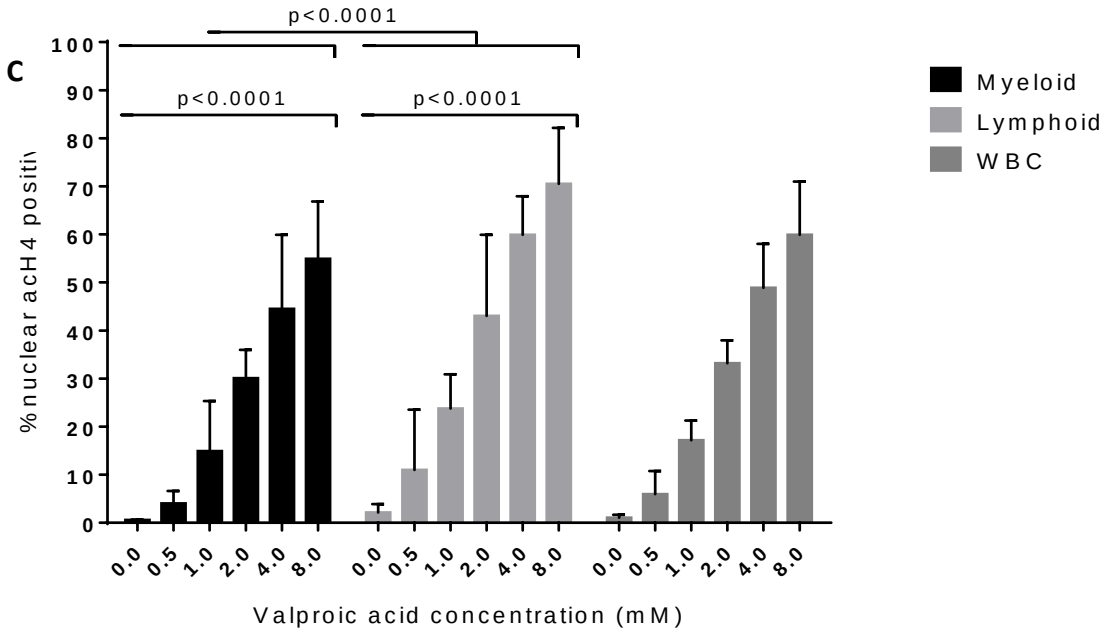


Figure 4.12. Expression of acH4 in myeloid, lymphoid and total white blood cells from whole blood samples treated with increasing concentrations of valproic acid (0-8 mM) for 6 hours.

Expression is shown as mean intensity (A), median intensity (B) and percentage of acH4 positive cells (C); mean and SD shown from 3 independent donors.

4.4 Discussion

An assay for detection of acH4 expression as a pharmacodynamic biomarker for valproic acid was developed and validated in cell lines (*in vitro*) and white blood cells (*ex vivo*). The assay was developed using an imaging flow cytometry based technique and the assessment of drug efficacy using percentage of acH4 overexpressed myeloid white blood cells. Results obtained in both cell lines and white blood cells showed similar responses of acH4 to the drug. The results generated have been published in the *European Journal of Clinical Investigation* (Uitrakul *et al.*, 2019).

Since an interest in using acetylation of histone H3 and H4 as a potential biomarker has increased over recent years, various assays have been developed to measure these proteins including Western blot, immunohistochemistry, immunofluorescence and conventional flow cytometry (Chung *et al.*, 2005; Ronzoni *et al.*, 2005; Rigby *et al.*, 2012). Although the current study was based on the principles of flow cytometry, it was imaging flow cytometry which provided additional information and facilitated a more detailed analysis. For instance, localisation of nucleus and acH4 identification using the imaging flow cytometry software (Ideas® 6.2) indicated which cells had nuclear or cytoplasmic acH4 expression without the need for additional antibodies.

It should be noted that the assays mentioned above only measured histone acetylation in PBMCs based on the results of a pioneering study which showed an increase in acH4 protein in mouse PBMCs treated with the HDAC inhibitor PXD101, measured by Western blot analysis (Plumb *et al.*, 2003). PBMCs therefore have become the primary type of WBCs for measurement of histone acetylation. However the findings of this chapter indicate that myeloid cells, i.e. granulocytes and monocytes, provided a higher dynamic range than lymphocytes. This was related to the fact that myeloid cells had lower acH4 expression at baseline than lymphocytes, so any changes in myeloid cells could be more clearly observed than in lymphoid cells. Therefore these findings suggest a potential new surrogate cell type for the measurement of histone acetylation.

Another novel parameter which has not previously been investigated was the percentage of acH4 positive cells. All recent assays have measured the overall

expression of acetylated histones but none has focused on the overexpressed cells. Qualitative techniques such as Western blot are unable to quantify these cells, but with flow cytometry this is feasible. A study by Ronzoni, et al quantified mean fluorescence intensity of histone acetylation using conventional flow cytometry and reported the results as fold changes compared to control (Ronzoni *et al.*, 2005). In addition, Rigby et al also reported mean and median fluorescence intensity of acH3 and acH4 measured by conventional flow cytometry (Rigby *et al.*, 2012). However, the findings of the current chapter suggest that counting the number of overexpressed cells provides a greater dynamic magnitude of change than fluorescence intensity approaches. This finding is supported by the fact that histone H4 and acH4 are proteins found in normal cells, so histone H4 acetylation can be observed even in untreated normal cells (Allfrey *et al.*, 1964). Consequently, increases in acH4 levels were less pronounced when treated with HDAC inhibitors. Similarly, the concept of rare cells states that not every cell has the same response to a particular drug, so specifically considering the responsive cells might be more appropriate than considering the whole cell population (De Biasi *et al.*, 2017). This conformed to the results in this chapter, which showed some cells with overexpression and some cells with normal expression of acH4, despite treatment with the same drug under the same conditions. Hyperacetylated cells, namely acH4 positive cells, were therefore measured in the developed assay instead of average fluorescence intensity.

A number of *in vitro* experiments included in published studies have investigated the hyperacetylation of histone H4 by valproic acid treatment measured by Western blot analysis. Results by Gottlicher et al showed a clear expression of acH4 when cells were treated with $\geq$0.5 mM concentrations of valproic acid for a minimum of 4 hours (Gottlicher *et al.*, 2001). Eyal et al showed acH4 accumulation at 0.5 and 1 mM of valproic acid following a 1 hour incubation (Eyal *et al.*, 2004) and Pheil et al reported a significant increase in acH4 expression when cells were treated with 2 and 5 mM of valproic acid for 24 hours (Phiel *et al.*, 2001). Also, Gurvich et al indicated increases in acH4 expression when cells were treated with 2 mM of valproic acid for 24 hours (Gurvich *et al.*, 2004). These previous findings are in agreement with results generated in HL-60 cell lines in the current study, which showed an increase in acH4 expression at 0.5 mM of valproic acid and a more marked increase at 1 mM. Also, the

results in this chapter suggest that mean and median intensity of acH4 were increased over the concentration of valproic acid until 4 mM and become stable at higher concentrations.

Furthermore, a significant increase in percentage of acH4 positive cells was observed in this study when valproic acid incubation time was ≥3 hours. This increase was most marked at 6 hours, and then declined although cells were still incubated with the drug. This trend was also noticed in a study by Eyal et al, who noted a decline in accumulated acH4 in HeLa cell lines after 16 hours of valproic acid incubation (Eyal *et al.*, 2004). This study also reported the same trend towards a decrease in signal when cells were treated with topiramate and 2-pyrrolidinone-n-butyric acid. There were 2 possible suggestions to explain this phenomenon.

Firstly, this was possibly caused by an apoptotic effect of valproic acid when cells were treated for a long enough duration. Cells with overexpression of acH4 are more affected by the drug, resulting in greater instances of cell death than non-responsive cells. As mentioned previously, however, almost all studies focused on overall expression, so apoptotic response of H4 hyperacetylated cells needs to be investigated further. For instance, apoptotic markers such as BID, BAX and cleaved Caspase-3 could be measured in acH4 positive cells as well as culture media. Another explanation relates to the potential for intracellular compensation to decrease the levels of acH4 in drug stimulating conditions; longer incubation times may trigger the reverse reaction, such as histone acetylation by HAT enzymes to maintain a normal acH4 level. However, this hypothesis also needs to be further studied.

The *ex vivo* results generated in the current chapter were comparable with cell line results. Increases in mean and median intensity of acH4 as well as percentage of acH4 positive cells were observed following an increase in valproic acid concentration. The magnitude of change was also in parallel with the *in vitro* results, with greater increases in percentage of acH4 positive cells from baseline than the increase in fluorescence intensity of acH4. Moreover, not only a change in the magnitude of acH4 was observed, but a different pattern of increase in acH4 was also noted in this study, with greater cytoplasmic acH4 expression observed in myeloid than in lymphoid cells.

This phenomenon was detected only when cells were treated with high concentrations of valproic acid of 4-8 mM.

Histones are known to be present in both the nucleus and cytoplasm of cells but have to be transported into the nucleus to be acetylated (Apta-Smith *et al.*, 2018). Therefore acH4 theoretically should not be detected in the cytoplasm. Results from Uchida et al, however, reported cytoplasmic acH4 expression when NIH3T3 cell lines were treated with 50 ng/mL trichostatin A for 12 hours (Uchida *et al.*, 2007). This study suggested that cytoplasmic acH4 expression could be observed although, unlike nuclear expression, cytoplasmic acH4 did not respond to drug treatment. In the work presented in this chapter, only nuclear acH4 was measured as the importance of cytoplasmic acH4 has not been well characterised and its relevance as a biomarker for HDAC inhibitors is unclear. Additionally, cytoplasmic expression was only detected at high concentrations of valproic acid (>4 mM, Figure 4.11), which is unlikely to have clinical relevance.

In conclusion, the *in vitro* and *ex vivo* results summarised in this chapter show that the novel assay developed can be used to detect histone H4 acetylation in human white blood cells. These results suggested a novel parameter for acH4 assessment, i.e. percentage of acH4 positive cells, rather than acH4 fluorescence intensity. In addition, myeloid cells were shown to express a greater magnitude of change in acH4 than lymphoid cells. This assay has been applied to clinical blood samples from patients with ependymoma being treated with valproic acid, with the results presented in the next chapter.

Chapter 5. Clinical application of a developed assay for detection of histone H4 acetylation as a pharmacodynamic biomarker for valproic acid

5.1 Introduction

Since the HDAC inhibitory effects of valproic acid were first discovered, use of the drug has been investigated in many areas including cancer. Successful preclinical studies showing the potential efficacy of valproic acid in various cancer cell lines have been conducted, as previously described (Göttlicher *et al.*, 2001; Phiel *et al.*, 2001; Gurvich *et al.*, 2004; Eyal *et al.*, 2005). These studies contributed to a growing in clinical trials of valproic acid as a repositioned drug. Although a number of studies have shown clinical efficacy of valproic acid either alone, or in combination with other drugs in various types of cancer, relatively few studies have monitored the mechanistic effects of the drug on changing histone acetylation (Iwahashi *et al.*, 2014; Issa *et al.*, 2015; Krauze *et al.*, 2015; Fushida *et al.*, 2016; Nilubol *et al.*, 2017). This was partly related to the publication of data from a clinical study that suggested no association between histone hyperacetylation and dose or concentration of belinostat as well as clinical response (Steele *et al.*, 2008).

Furthermore, only a small number of studies have investigated the use of valproic acid in CNS tumours (Wolff *et al.*, 2008; Su *et al.*, 2011; Weller *et al.*, 2011; Krauze *et al.*, 2015). Most clinical studies of valproic acid have been carried out in solid tumours or leukaemia (Iwahashi *et al.*, 2014; Issa *et al.*, 2015; Fushida *et al.*, 2016). Since this study focused on acH4 expression as a pharmacodynamic biomarker rather than predictive or prognostic biomarker, we are interested in published clinical studies of valproic acid involving the expression of histone acetylation regardless of disease type.

5.1.1 Clinical studies of valproic acid as an HDAC inhibitor.

5.1.1.1 Clinical cancer studies

Although associations between acetylated histone levels and dose and concentration of valproic acid have to date been somewhat unclear, approaches to measure histone acetylation have been incorporated into several clinical trials. Arguably the most relevant study was conducted by the Children's Oncology Group in the USA (Su *et al.*, 2011). Childhood cancer patients with refractory solid or central nervous system tumours were treated with oral valproic acid twice daily, with a view to maintaining trough concentrations of 100 to 150 μg/mL (0.7-1 mM). Acetylation of histones H3 and H4 in PBMCs was measured pre- and post-treatment using commercial ELISA kits. The expressions of acH3 and acH4 were assessed and matched with total and free drug concentrations (Figure 5.1). The results indicated that 4 out of 7 patients had lower acH3 and acH4 as compared to pre-treatment levels, despite the fact that only 2 of the 4 had achieved the target trough drug levels. Moreover, patients who had post/pre acH4 ratios higher than 1 still had progressive disease. Only one patient, who had an extremely high post/pre acH4 ratio (14.7-fold), exhibited a partial response.

Another phase I clinical trial in patients with refractory advanced cancers revealed similar results (Atmaca *et al.*, 2007). Each patient received intravenous valproic acid starting at a dose level of 30 mg/kg, with a standard dose-escalating three-patient cohort design. Blood samples were taken at baseline, 6-hr, 24-hr and 48-hr following administration of doses from 30-120 mg/kg. The results showed no relationship between acetylated histone levels measured in peripheral blood lymphocyte cell lysates and valproic acid dose, although an increase in histone hyperacetylation was observed in 12/16 (75%) patients. Four patients reported undetectable acH3 and acH4 by Western blot throughout the study, despite receiving a valproic acid dose of 120 mg/kg. Moreover, a patient who received 30 mg/kg exhibited a more marked acH4 expression than a second patient who received 60 mg/kg. Although the study mentioned an increase of the acetylation status of peripheral blood lymphocytes measured by flow cytometry, no results were presented.

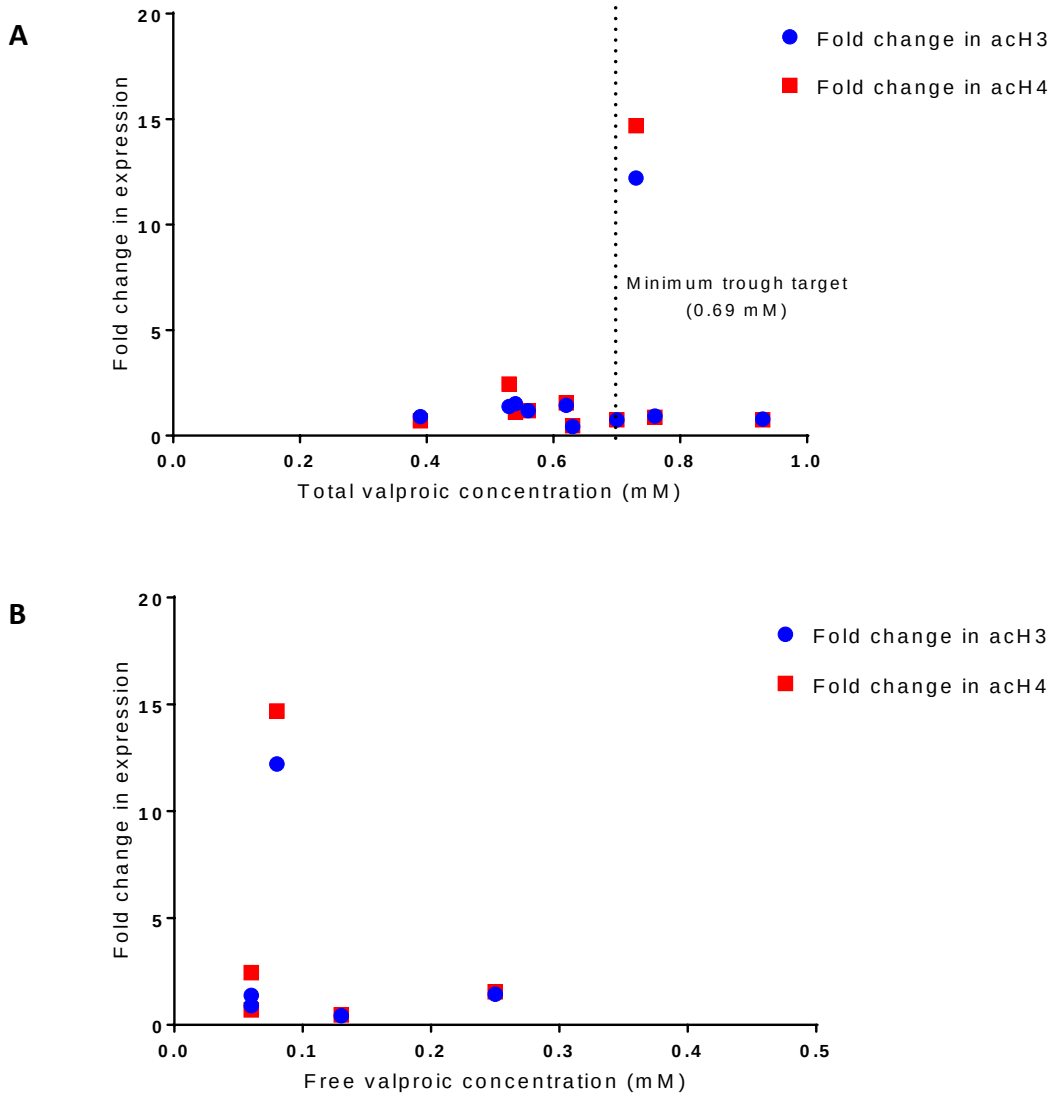


Figure 5.1. Association between fold-change in acH3/acH4 expression and concentration of total valproic acid (A) and free valproic acid (B).

Fold change was determined by dividing the histone expression observed at steady state by the expression determined pre-treatment. No target concentration for free valproic acid was set. Adapted from Su JM, et al. Clin Cancer Res. 2011;17(3):589-97.

These results conformed to the findings of three other clinical trials in prostate cancer, advanced solid tumours and leukaemia (Garcia-Manero *et al.*, 2006; Sharma *et al.*, 2008; Wheler *et al.*, 2014), suggesting that valproic acid is unable to consistently cause histone acetylation in lymphocytes. In the same way, no association between histone acetylation and response was consistently observed across these studies. However, several clinical studies have provided conflicting observations (Munster *et al.*, 2007; Daud *et al.*, 2009; Munster *et al.*, 2009; Rocca *et al.*, 2009). A phase I clinical

study in patients with advanced solid tumours who received valproic acid and epirubicin reported a significant correlation between histone H4 acetylation and valproic acid dose as well as valproic acid concentration (Munster *et al.*, 2007). Unlike the studies mentioned above, this study indicated increases in acH4 expression in patients who received a valproic acid dose of 30 mg/kg/day and had plasma concentrations of >50 μg/mL (0.35 mM). These increases were observed between baseline and 3 days after treatment. Also, patients with acH4 increases achieved partial responses at the end of study. Similar results from the same cohort were presented by the same authors a year later (Munster *et al.*, 2009). Additionally, this study indicated that there was a correlation between acH4 levels in PBMCs and valproic concentration, but not acH4 levels in tumour tissues.

A phase I-II clinical study of valproic acid plus chemo-immunotherapy in patients with advanced melanoma also showed positive results (Rocca *et al.*, 2009). Patients were treated with 10 mg/kg/day valproic acid in three divided doses and the dose was increased weekly by 10 mg/kg/day, until 90 mg/kg/day or maximum tolerated dose for 6 weeks. Dacarbazine and interferon-α were then administered at week 7 in combination with valproic acid. Histone H4 acetylation in PBMCs was measured at the end of the second week of treatment, at the beginning of the combination phase and weekly thereafter using cytofluorimetric techniques developed by Ronzoni, et al (Ronzoni *et al.*, 2005). The results showed that only 5 of 27 patients did not achieve acH4 increases (>1 Post/Pre ratio). Some patients with increases in acH4 had plasma valproic acid concentrations <100 μg/mL. The authors, however, highlighted that these values referred to the maximum values of plasma valproic acid concentrations and the maximum fold increase in histone acetylation reached during treatment at the maximal dose of valproic acid. They reported that patients who had more than a 4-fold acH4 increase did not maintain this increase at repeated measurements, notwithstanding maintenance of the valproic acid dose.

Another phase I/II clinical trial suggested similar results (Daud *et al.*, 2009) following treatment of patients with valproic acid and karenitecin, a novel topoisomerase I inhibitor. Valproic acid dose was escalated until the occurrence of dose-limiting toxicities and acetylation of histone H3 and H4 in PBMCs was investigated by Western blot analysis. The fold changes between 3-day post-treatment

and baseline at doses of 60, 75 and 90 mg/kg/day were higher than 45 mg/kg/day. This increase, however, was not statistically significant and there was no difference between the three dose levels.

Western blot results from a phase I study in patients with cervical cancer also questioned the utility of acH4 expression in tumour cells as a biomarker (Chavez-Blanco *et al.*, 2005). Among tumour samples from 10 patients, 7 had clear hyper-acetylation of histone H4 on day six after the five days of magnesium valproate treatment; the effects of the remaining patients were minor or non-existent. The magnitude of change, however, did not follow serum drug concentrations. The authors also mentioned heterogeneity in degree of baseline acH4, i.e. some patients had very strong pre-treatment acH4 while others had minimal levels.

5.1.1.2 Clinical studies of non-cancer diseases

Discovery of the HDAC inhibitory effects of valproic acid is not only relevant to cancer research, but also other areas including epilepsy, migraine and bipolar mania, where valproic acid has been used for decades. Historically valproic acid has been used to treat these diseases without a full understanding of the potential role of histone acetylation, but recent discoveries suggest that histone acetylation may be relevant to many disease types.

A clinical study in patients with spinal muscular atrophy recently reported a significant increase in histone H4 acetylation with valproic acid treatment (Renusch *et al.*, 2015). This study measured the expression of acH4 in patient PBMCs using a liquid chromatography/mass spectrometry technique. The AUC showed a significant increase in acH4 expression when treated with valproic acid for 6 months (from 0.599 to 0.683) and decrease when valproic acid treatment was stopped for 6 months (from 0.695 to 0.616). Also, the authors indicated that approximately 71% of their subjects were 'responders' to valproic acid, defined as those patients whose AUC increased by at least 15% after 6 months of treatment. However, this study did not conclude an association between acH4 and clinical outcome because of insufficient evidence to suggest a treatment effect.

Another study reported measurement of acH3 and acH4 expression in lymphocytes from schizophrenic and bipolar patients using Western Blot analysis (Sharma *et al.*, 2006). The results showed that acH3, but not acH4, significantly increased from baseline (p-value <0.03) when treated with valproic acid for 4 weeks. Moreover, acH4 expression was not found to be correlated with valproic acid levels or behavioural rating scores, while acH3 correlated with both valproic acid levels and the symptom severity of patients with schizophrenia. This contributed to a surge in using acH3 as a biomarker of HDAC inhibiting activity in the area of neurology, as opposed to acH4 (Gavin *et al.*, 2009; Tremolizzo *et al.*, 2012).

In addition, there were significant numbers of pre-clinical trials that investigated various effects of valproic acid as a HDAC inhibiting agent, in disease areas including autism, retinal ischemia injury, acute lung ischemia injury and acute central nervous system injury (Zhang *et al.*, 2012b; Kataoka *et al.*, 2013; Chen *et al.*, 2014; Wu *et al.*, 2015). Although the relationship between histone acetylation and clinical response is not clear, this emphasises the important role for robust biomarker development to allow the detection of histone acetylation as surrogate biomarkers for forthcoming clinical trials.

5.2 Chapter specific aims

This project primarily aimed to investigate the expression of histone H4 acetylation (acH4) in patients receiving valproic acid as part of the SIOP Ependymoma clinical trial. The level of acH4 expression was measured using the assay developed and validated in the previous chapter. Specifically the objectives of this chapter were:

- To measure mean acH4 intensity, median acH4 intensity and percentage of acH4 positive WBCs in patients receiving valproic acid in the SIOP Ependymoma trial.
- To evaluate differences in acH4 expression between myeloid and lymphoid cell populations following administration of valproic acid.
- To compare changes in percentage of acH4 positive myeloid cells between patients treated with valproic acid over several courses of treatment.
- To investigate an association between changes in percentage of acH4 positive cells and valproic acid dose.
- To investigate relationships between total and free valproic acid plasma concentrations and valproic acid dose.
- To investigate associations between percentage of acH4 positive cells and both total and free valproic acid plasma concentrations.

5.3 Results

5.3.1 Patient characteristics

From October 2016 to December 2018, there were seven patients recruited onto the study. One patient had started valproic acid treatment before the first samples were collected for analysis of histone acetylation, therefore no pre-treatment sample was available for this patient. Table 5.1 describes the valproic acid dose that patient received at each visit. Doses were calculated by dividing the actual administered dose by the patient's weight. One patient provided blood samples for analysis from one visit only (pre-treatment and 4-hour post-first dose). Baseline characteristics of all patients at the start of their treatment are described in Table 5.2. For the patient who started treatment before the first sample was collected for analysis, the data shown are from the first time that samples were sent to the laboratory.

The median age of the seven patients recruited was 11 years old. Numbers of male and female patients were similar (4:3). The highest creatinine value was 25 μmol/L at the start of the clinical trial. Patient 5 presented the highest ALT enzyme (35 IU/L) and patient 6 presented the highest WBC count (17.62 $\times 10^9$/L). Due to limitations in data availability, numbers of neutrophil shown were from only four patients.

Patient	Valproic acid dose (mg/kg/dose twice daily)						
	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7
1	23*	28.9	35	40.5	42	45	29+
2	15	15	20	25	30	33	35
3	15	15	19.5	20			
4	15	35.5	29.2				
5	15						
6	15	20					
7	15	19	28.5				

* Visit 1 of this patient was not the first dose of treatment

+ Patient received this dose three times a day

Table 5.1. Summary of valproic acid dosage at patient sample collection date

Patient	Age (month)	Gender	Weight (kg)	WBC ($\times 10^9$/L)	Neutrophil ($\times 10^9$/L)	ALT (IU/L)	Creatinine (μmol/L)
1	11	Male	9.1	2.33	N/A	22	22
2	36	Male	13.8	2.20	N/A	13	25
3	7	Female	6.9	12.1	N/A	10	11
4	5	Male	6.8	7.18	1.08	13	17
5	11	Female	8.7	15.7	11.2	35	19
6	19	Male	12.5	17.6	11.6	9	16
7	19	Female	11.9	4.10	1.75	18	<18

Table 5.2. Baseline characteristics of patients at the start of SIOP Ependymoma II trial until December 2018.

Figure 5.2 represents the WBC count of all patients at each visit. Most patients had values of WBC count within the normal range, except patient 1 who reached leukopaenic levels several times, including visits 2, 4 and 6. Also, clinical data indicated that patient 1 had neutropenia at visits 4 and 6. Figure 5.3 shows different patterns of results analysed in neutropaenic samples and non-neutropenic samples obtained from patient 1. The percentage of myeloid cell in neutropenic samples decreased to 22.5% as compared to 62.3% in non-neutropaenic samples. Since the developed assay mainly measures acH4 expression in myeloid cells, the proportion of neutrophils markedly affects interpretation of the results which will be discussed later.

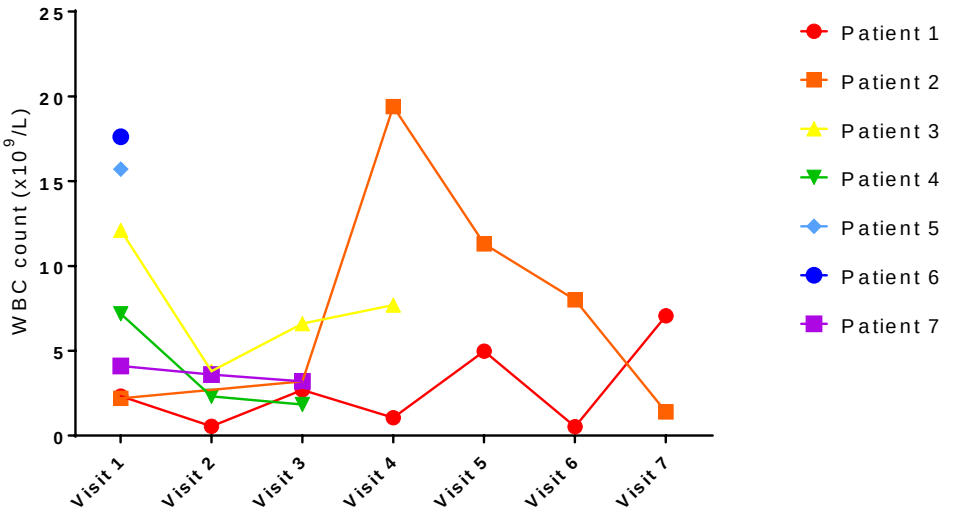
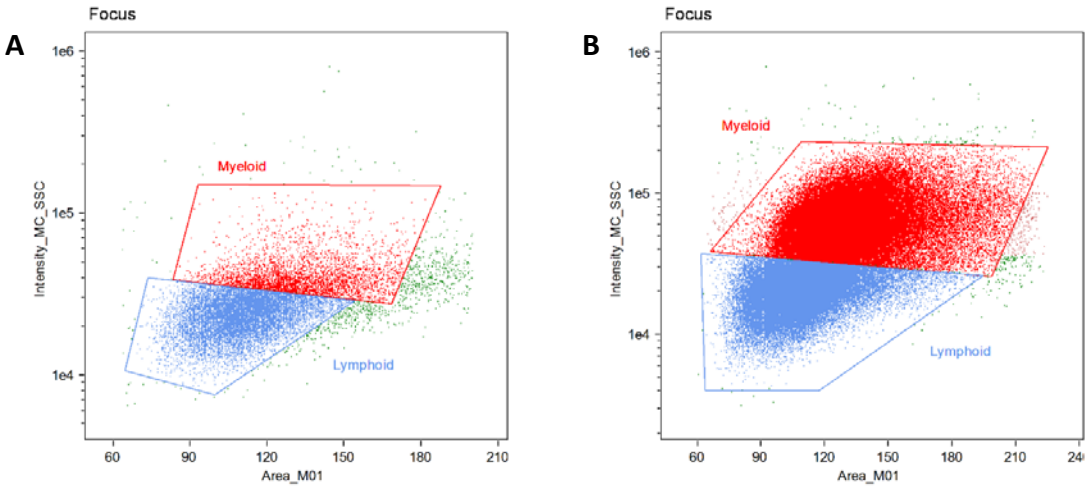


Figure 5.2. WBC counts measured at all visits of seven patients studied on the Ependymoma trial.

WBC counts were determined in hospital before patients received valproic acid. Note that the interval between consecutive visits were not the same and could be several weeks or months.



Area_M01, Intensity_MC_SSC

Population	Count	%Gated
Focus & RSC	17131	100
Myeloid & Focus & RSC	3857	22.5
Lymphoid & Focus & RSC	12341	72

Area_M01, Intensity_MC_SSC

Population	Count	%Gated
Focus & RSC	133155	100
Myeloid & Focus & RSC	82955	62.3
Lymphoid & Focus & RSC	48947	36.8

Figure 5.3. Representative samples from patient 1 with (A) and without (B) neutropaenia detected by imaging flow cytometry with gating between side scatter (SSC) and area of cell.

The neutropaenic sample shown is from visit 4 and the normal sample shown is from visit 1. Red colour indicates myeloid and blue indicates lymphoid populations.

All patients had received not only valproic acid, but also a number of concomitant medicines as listed in Table 5.3. The drugs patients received included antibiotics, analgesics, antihistamines and antiemetics as well as chemotherapeutic drugs. The chemotherapy most frequently administered to patients were platinum-based (four patients) and vincristine-based regimens (three patients). Several classes of antibiotic were used in multiple patients including cephalosporins, fluoroquinolones, sulfonimides, aminoglycosides and carbapenems. Other drugs commonly used in these patients were metoclopramide and ondansetron which are antiemetic drugs.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5*	Patient 6	Patient 7
Chemotherapy	Cisplatin	Vincristine	Carboplatin	Vincristine		Vincristine	Cisplatin
		Methotrexate	Cisplatin	Cyclophosphamide		Cyclophosphamide	
		Cisplatin					
		Carboplatin					
Anti-infective agents	Piperacillin / Tazobactam	Co-trimoxazole	Meropenem			Flucloxacillin	Co-trimoxazole
	Co-amoxiclav	Tazocin	Ceftriaxone			Ciprofloxacin	Piperacillin / Tazobactam
	Meropenem	Gentamicin	Gentamicin			Co-amoxiclav	
	Tobramycin		Teicoplanin				
	Aciclovir		Co-trimoxazole				
			Anidulafungin				
Analgesics	Oxycodone	Morphine					
Antihistamines		Cetirizine					
Others	Ranitidine	Loperamide	Domperidone	Metoclopramide		Metoclopramide	Ondansetron
	Metoclopramide	Metoclopramide	Ondansetron	Ondansetron		Ondansetron	
	Ondansetron	Ondansetron				Glycopyrronium	
		Folic acid					

* Patient 5 received unknown chemotherapeutic drugs following the protocol.

Table 5.3. List of medications that patients received alongside valproic acid not including topical medicines.

Not all medicines were administered at the same visit and some medicines were administered at more than one visit.

5.3.2 Phenotyping of WBCs and changes in acH4 expression

The developed assay was applied to measured acH4 positive cells in patients with ependymoma. In order to investigate the potential significance of the myeloid population observed in *ex vivo* results, WBCs from patient samples pre- and post-treatment were incubated with acH4 antibodies and analysed using imaging flow cytometry. The intensity of acH4 expression and percentage of acH4 positive cells were then assessed for each type of WBC.

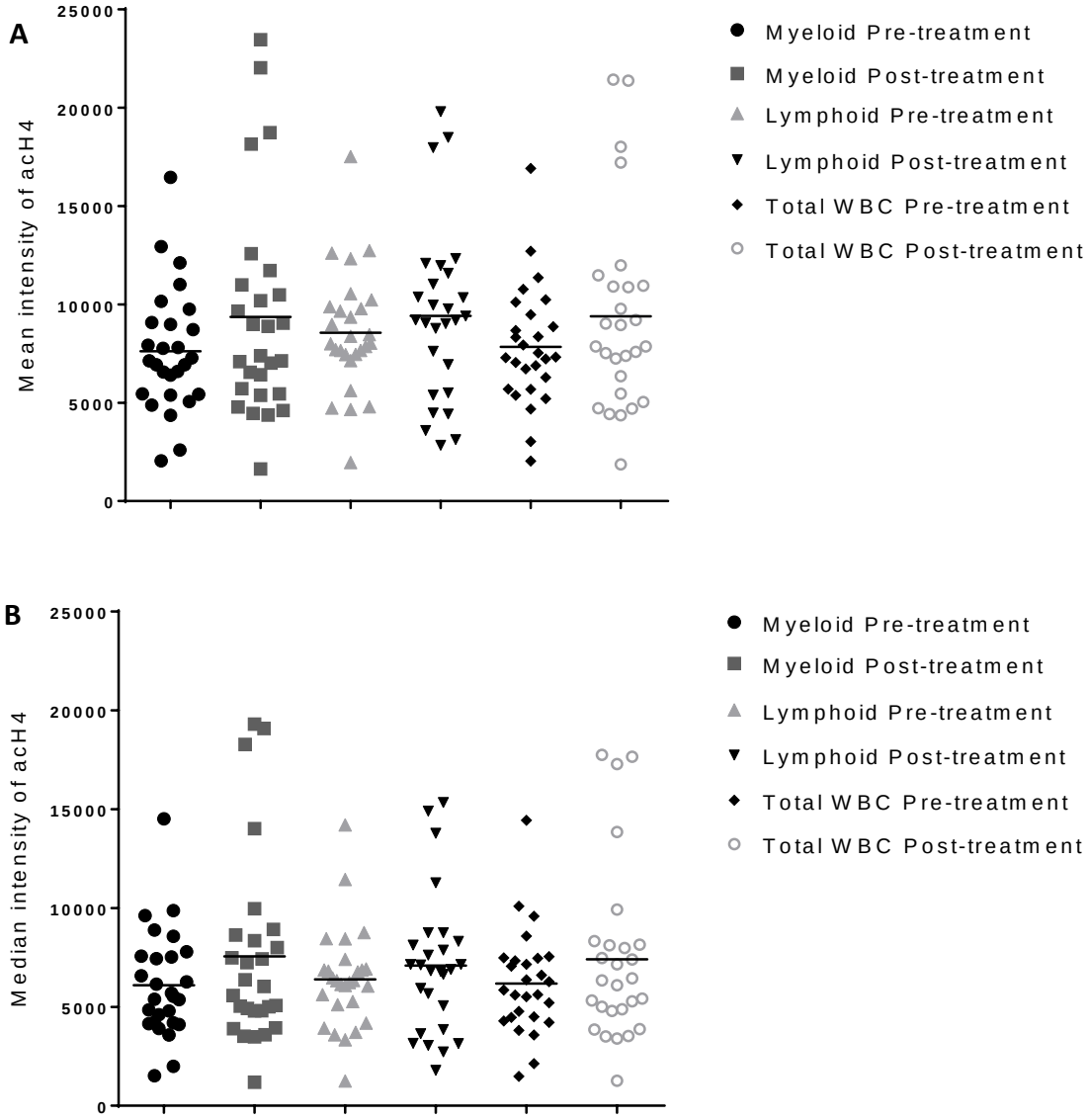
Figure 5.4 illustrates changes in mean and median intensity of acH4 expression, and percentage of acH4 positive WBCs between pre- and post-dose of valproic acid. Focusing on fluorescence intensity (Figure 5.4A and Figure 5.4B), average values for the mean intensity were higher than the median for all three populations (myeloid, lymphoid and total WBCs). Averages values for pre-treatment mean intensity of myeloid, lymphoid and total WBCs were 7600, 8600 and 7800 while median intensity values were 6100, 6400 and 6200, respectively. Similarly, averages in post-treatment mean intensity were 9400 for all three populations while median intensity values were 7600, 7000 and 7400 in myeloid, lymphoid and total WBCs, respectively. The Wilcoxon matched-pairs signed rank test revealed significant differences between pre- and post-treatment in myeloid cells for both mean and median acH4 expression, with p-values of 0.0059 and 0.0069, respectively. In lymphoid cells, however, neither parameter showed statistical significance between pre- and post-treatment samples (p-values of 0.1482 for mean and 0.1111 for median intensity).

Mean percentages of nuclear acH4 positive cells post-treatment showed increases in myeloid, lymphoid and total WBCs as compared to pre-treatment: 0.70% to 1.44% in myeloid, 1.39% to 1.88% in lymphoid and 0.85% to 1.63% in total WBCs (Figure 5.4C). These increases were not significant in either myeloid or lymphoid cells with p-values of 0.0721 and 0.2660, respectively, measured by Wilcoxon matched-pairs signed rank test.

Considering fold-changes in all parameters, mean intensity of acH4 provided lower average fold-changes with 1.2, 1.1 and 1.2-fold changes for myeloid, lymphoid and total WBCs, respectively. Similarly, average fold-changes in median intensity of

acH4 were 1.2, 1.1 and 1.2-fold for myeloid, lymphoid and total WBCs, respectively. The greatest average fold-increases were percentages of acH4 positive cells, with 2.7, 2.5 and 2.3-fold increases observed in myeloid, lymphoid and total WBCs, respectively.

The results of fold-changes alongside significant values conformed to the previous *ex vivo* results in Chapter 4 and provide two conclusions. Firstly, percentage of acH4 positive cells was a more sensitive method for detection of acH4 changes than fluorescence intensity. Secondly, a higher dynamic change was observed in myeloid as compared to lymphoid cell populations. The percentage of acH4 positive myeloid cells was therefore used in further analyses.



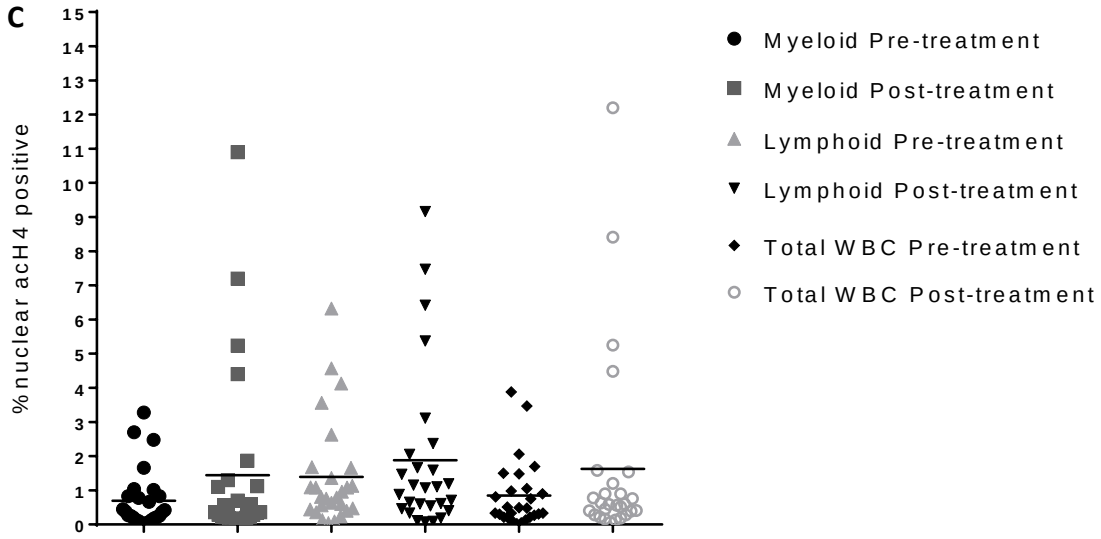


Figure 5.4. Mean (A) and median (B) fluorescence intensity of acH4, and percentage of acH4 positive cells (C) in myeloid, lymphoid and total WBCs from patients pre- and post-administration of valproic acid.

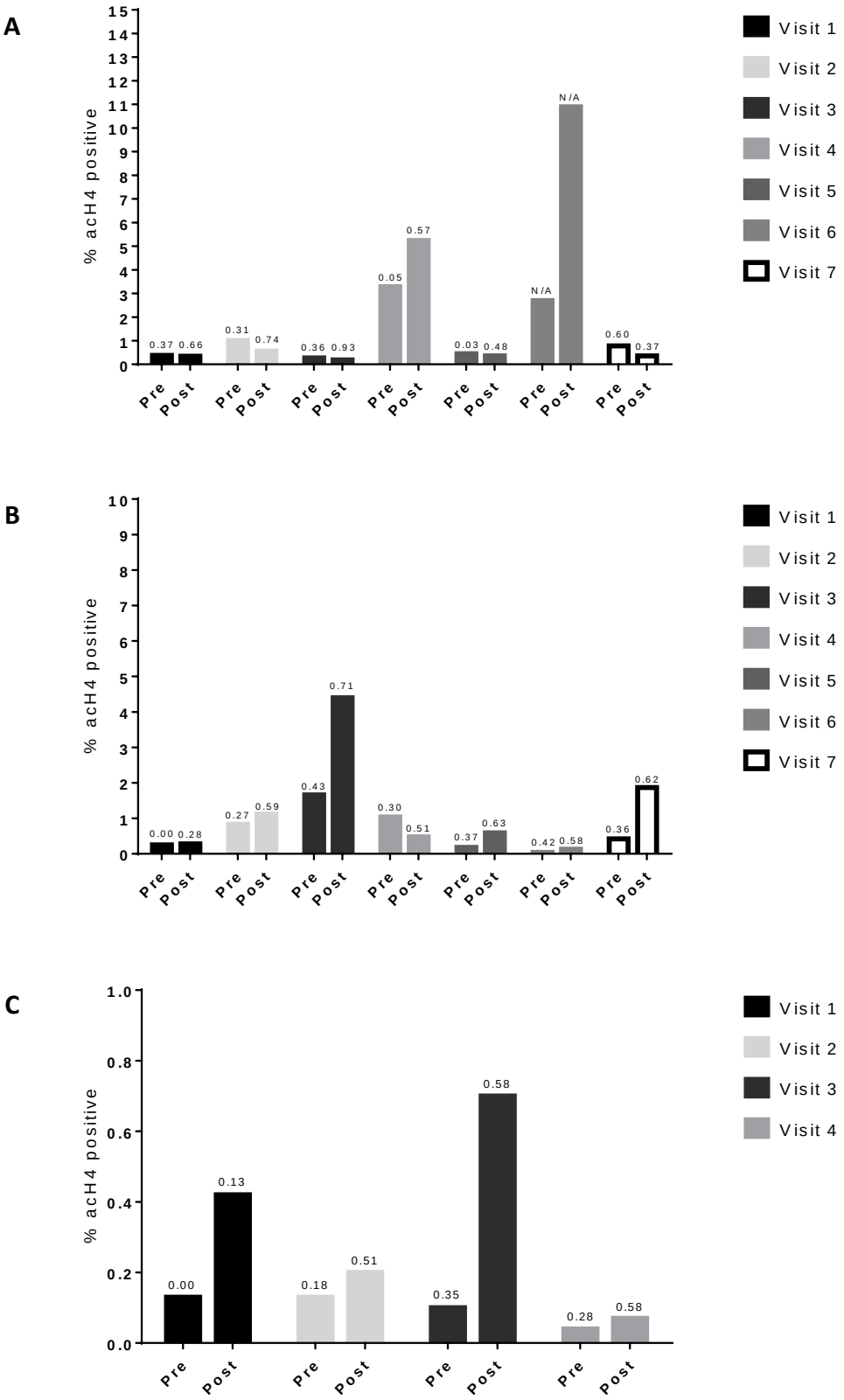
The marked lines indicate mean values.

5.3.3 Individual percentages of acH4 positive myeloid cells pre- and post-valproic acid treatment in patients with ependymoma

For each study visit, blood samples were generally taken from the patient before and 4-hour after receiving valproic acid treatment. For patient 1 samples were taken before and 2-hour after receiving the drug. Processed blood plasma samples were delivered to the laboratory on dry ice to measure acH4 expression using the developed assay. Percentages of acH4 positive myeloid cells determined for each patient at each visit are shown in Figure 5.5A-G.

According to the figures shown, there was marked heterogeneity in degree of baseline acH4 as well as magnitude of change in acH4 expression following treatment with valproic acid. Most patients presented with less than 1% of acH4 positive myeloid cells at baseline (pre-treatment of the first visit) whilst patient 7 expressed 2.5% of acH4 positive cells. Changes in acH4 positive cells at 4-hr post-treatment were inconsistent but the majority showed relatively modest increases. The highest increase was from the third visit of patient 3, with a 7-fold increase from pre-treatment observed. Furthermore, decreases in acH4 post-treatment were observed in several samples such as the second visit of patient 7 (3.6-fold decrease) and the first visit of

patient 5 (2.3-fold decrease). The actual values of decrease, however, were less than 1%.



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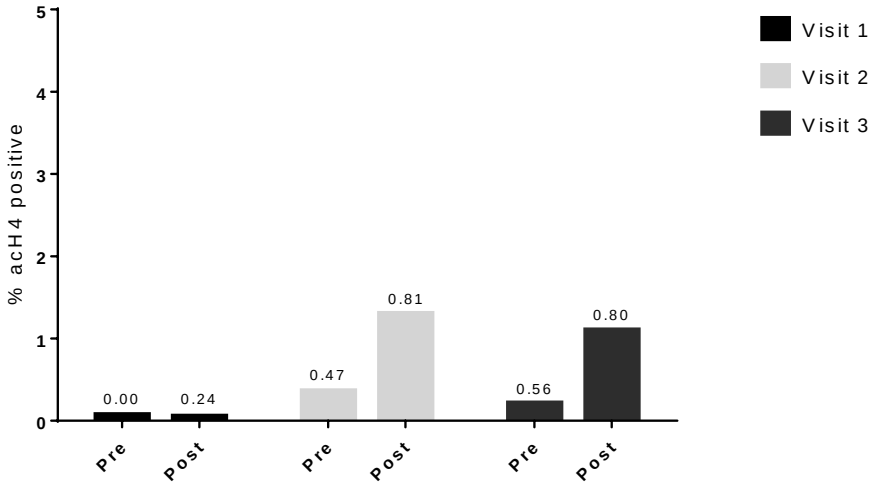
% acH4 positive
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2
1
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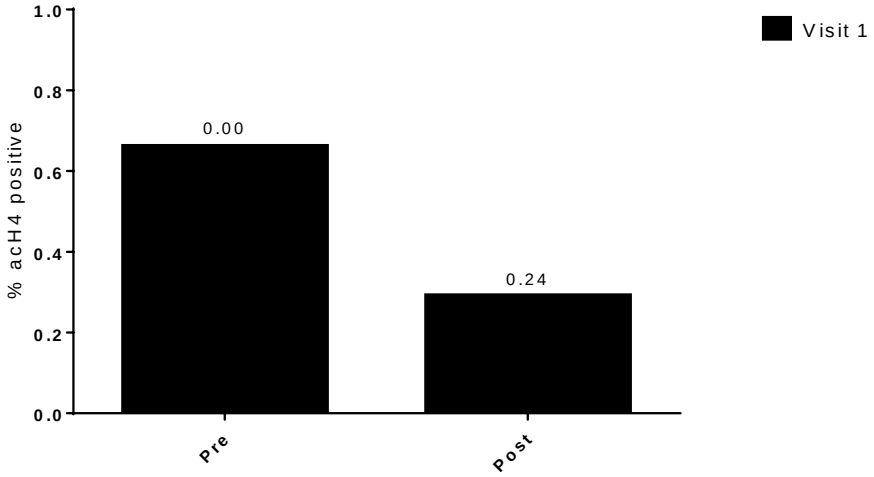
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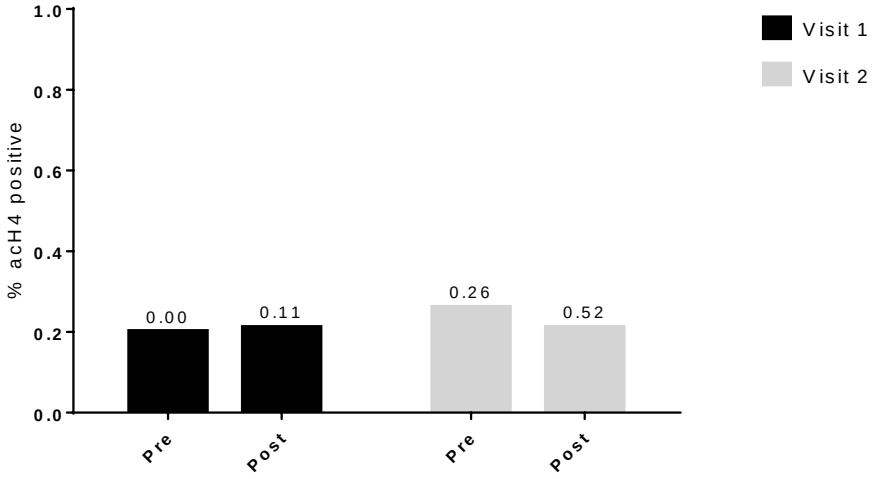
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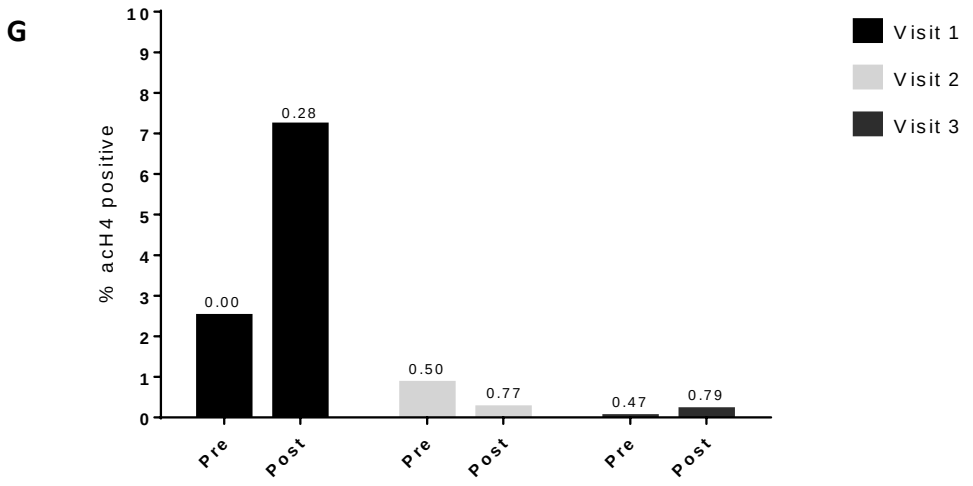


Figure 5.5. Percentages of acH4 positive myeloid cells collected from patients 1-7 (A-G) with concentrations of valproic acid at each visit.

Blood samples were taken pre- and 4-hr post valproic acid treatment, except patient 1 where a 2-hr post treatment was collected, at different dose levels. Visit 1 indicates the first time that each patient received valproic acid, except patient 1 who began treatment before samples were collected for acH4 analysis. The concentrations of valproic acid shown on top of the bars are in mM. N/A indicates no data available. Note that the intervals between consecutive visits were not consistent between patients.

Focusing on each patient, most of the samples from patient 1 showed slight decreases following treatment with valproic acid (Figure 5.5A). There were two samples with extremely high increases in acH4 positivity. This patient, however, had a history of leukopenia and neutropenia (Figure 5.2) which could affect the measurement of acH4 positive myeloid cells. These increases were therefore questioned and will be discussed in the discussion part of this chapter. Patients 2, 3 and 4 had similar profiles with increased acH4 positive myeloid cells observed post-treatment on almost all study days, although the degree of increase was variable (Figure 5.5B-Figure 5.5D). Patients 5 and 6 had too few samples to draw any real conclusions from the results obtained, but there were no observed increases in acH4 positivity in post-treatment samples (Figure 5.5E-Figure 5.5F). The response observed in patient 7 was variable, with a marked increase in acH4 following the first administration of drug but small changes observed at the second and third visits (Figure 5.5G).

5.3.4 Associations between percentage acH4 positive myeloid cells and valproic acid dose

Patients recruited to the SIOP Ependymoma II trial were given oral valproic acid at a starting dose of 15 mg/kg/dose twice daily. Blood samples were taken to measure acH4 expression and valproic acid concentrations in plasma. The valproic acid dose was then adjusted based on the trough drug concentrations observed. Since there is no standard to measure change in acH4 positive cells, Figure 5.6 and Figure 5.7 summarise the association between dose of valproic acid and change in acH4 positive myeloid cells calculated by two methods: absolute change (Difference) and fold change, respectively.

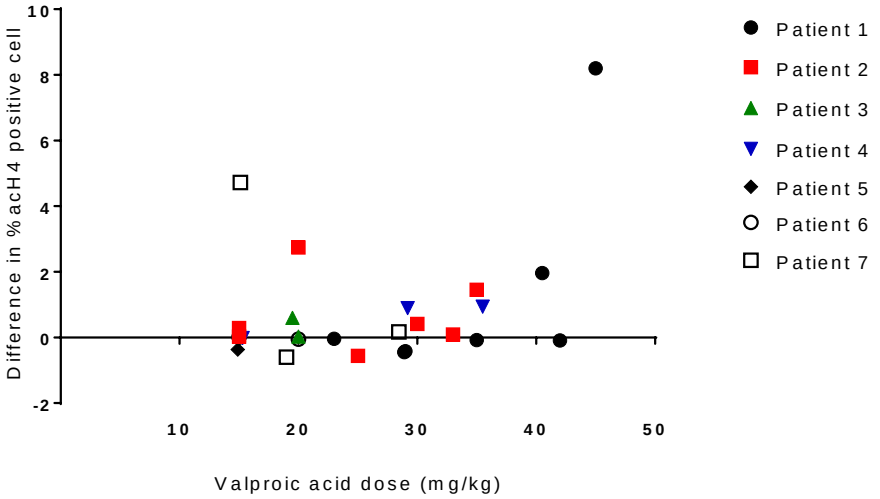


Figure 5.6. Association between valproic acid dose and difference in percentage of acH4 positive myeloid cells between post- and pre-valproate dose.

Difference was determined by post-treatment value minus pre-treatment value.

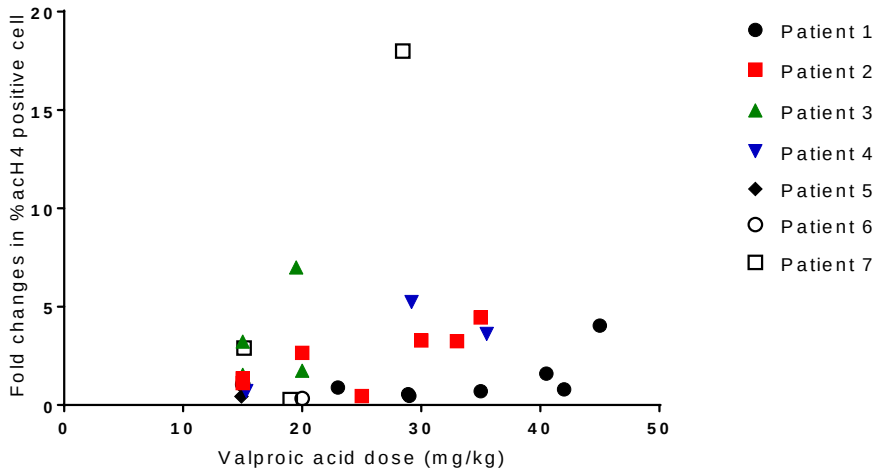


Figure 5.7. Association between valproic acid dose and fold change in percentage of acH4 positive myeloid cells as compared to pre-valproate dose.

Fold change was determined by dividing post-treatment value by pre-treatment value.

Analysis of the results by Spearman correlation analysis showed no significant correlation between valproic acid dose and fold change (p-value 0.1538), or difference (p-value 0.3012) in acH4 positive myeloid cells. However, the different patterns of change between the two graphs were highlighted. The fold change observed at the highest dose of valproic acid for patient 7 was exceptionally high, whilst the actual change of acH4 at the same dose was close to zero. In contrast, the fold change at the highest dose of patient 1 was much lower than the difference at the same dose. As there are clear differences in the interpretation of the data depending on the method of analysis used, both approaches were used for further analyses.

5.3.5 Associations between percentage acH4 positive myeloid cells and total valproic acid concentrations

Total valproic acid concentrations were measured using the homogeneous enzyme immunoassay technique performed by the Royal Victoria Infirmary. Details of the machine and protocol used for drug measurement are described in section 2.4. The results were reported to the lab and used to investigate potential relationships between drug concentration and percentage of acH4 positive myeloid cells.

There are several ways to investigate potential associations between valproic acid concentration and acH4 expression; the most commonly used method is an

assessment of fold change in acH4 expression and trough valproic acid concentration at steady-state as compared to pre-treatment. In the current study samples from each patient were not taken at the same time points, e.g. every 2 weeks or every month, so the comparison between samples at particular time point of all patients was not possible. Instead, the drug concentrations and acH4 positive cell results at all valproate administrations were used to investigate associations between these variables.

Figure 5.8 shows the relationship between total valproic acid concentration and percentage of acH4 positive myeloid cells in all patients regardless of pre- and post-treatment. The overall results suggested no significant correlation between these two parameters (Spearman, p-value 0.171). Although subgroup analysis for each patient revealed a significant correlation for patient 4 with a p-value 0.033, this correlation was heavily biased by two data points and might not be of biological significance.

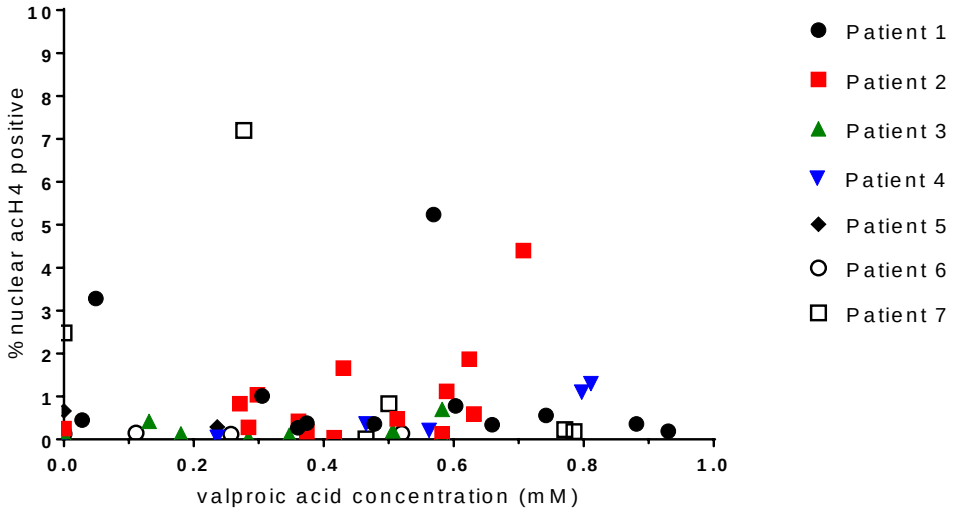


Figure 5.8. Relationship between total valproic acid concentration and percentage of acH4 positive myeloid cells for all patients.

Note that these values represent both pre- and post-treatment values.

A comparison of difference in valproic acid concentration and percentage of acH4 positive cells was analysed by two aspects; the fold change in acH4 positive cells is shown in Figure 5.9 and the actual difference in acH4 positive cells is shown in Figure 5.10. Unlike the dose results, the patterns of fold change and difference in acH4 positivity were similar. For instance, patient 3 showed the highest values for both

difference and fold change in acH4 positive cells when the concentration increased by 0.235 mM. Patient 1 had the highest acH4 change at the valproic acid concentration of 0.52 mM. Interestingly, for patient 4 who presented a correlation between acH4 positive cells and valproic acid concentration, neither fold change nor difference in acH4 positive cells significantly correlated with increase in valproic acid concentration (Spearman, p-value 0.999 and 0.667, respectively). The results of all patients also indicated no statistical significance in either fold change or difference (Spearman, p-value 0.594 and 0.935, respectively).

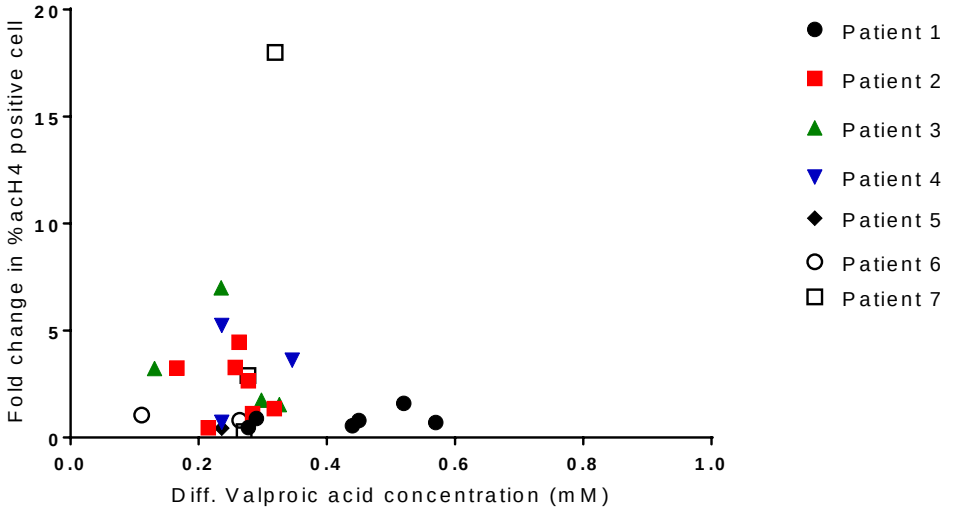


Figure 5.9. Association between difference in total valproic acid concentration and fold change in percentage of acH4 positive myeloid cells.

Difference was determined by post-treatment values minus pre-treatment values and fold change was determined by dividing post-treatment values by pre-treatment values.

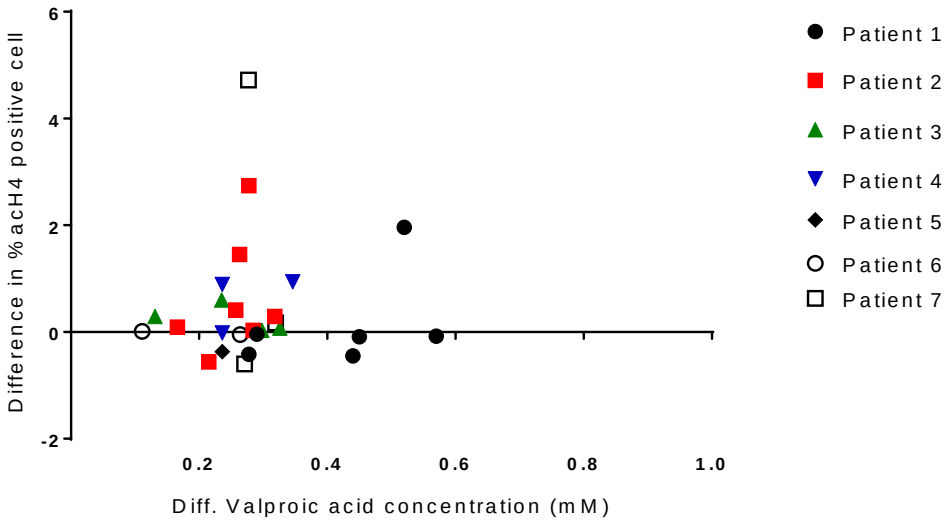


Figure 5.10. Association between difference in total valproic acid concentration and difference in percentage of acH4 positive myeloid cells.

Difference was determined by post-treatment values minus pre-treatment values.

5.3.6 Association between increase in acH4 positive myeloid cells and free valproic acid concentrations

Patient plasma samples were filtered through Centrifree® ultrafiltration devices to obtain the ultrafiltrate with free valproic acid. The samples were then sent to the Royal Victoria Infirmary to measure the concentration of free valproic acid using the homogeneous enzyme immunoassay technique as described in Chapter 2. The results were compared with percentages of acH4 positive myeloid cells to investigate potential relationships.

Figure 5.11A shows a significant correlation between total and free valproic acid concentrations in clinical samples analysed (Spearman, p-value <0.0001). Regression analysis indicated a y-intercept of 0.144 mM, thus the concentration of total valproic acid needs to be higher than 0.144 mM to be able to detect free valproic acid. However, this number increased to 0.251 mM when analysed without the four free concentration values of 0 mM (data not shown). The slope of the graph was 0.299 and the r^2 value was 0.7923.

For the fraction of free valproic acid, Figure 5.11B illustrates the relationship between this parameter and total valproic acid, with a significant correlation between the two variables (Spearman, p-value <0.0001). The linear regression analysis indicated

a y-intercept of 0.062 mM, slope of 0.267 and r^2 value of 0.712. Based on the graph shown, the free valproic acid fraction was found to be greater at higher concentrations of total valproic acid. For example, a sample with 0.257 mM of total valproic acid had 8.1% free valproic acid detected, while another sample with 0.811 mM of total valproic acid had 39.3% free valproic acid detected.

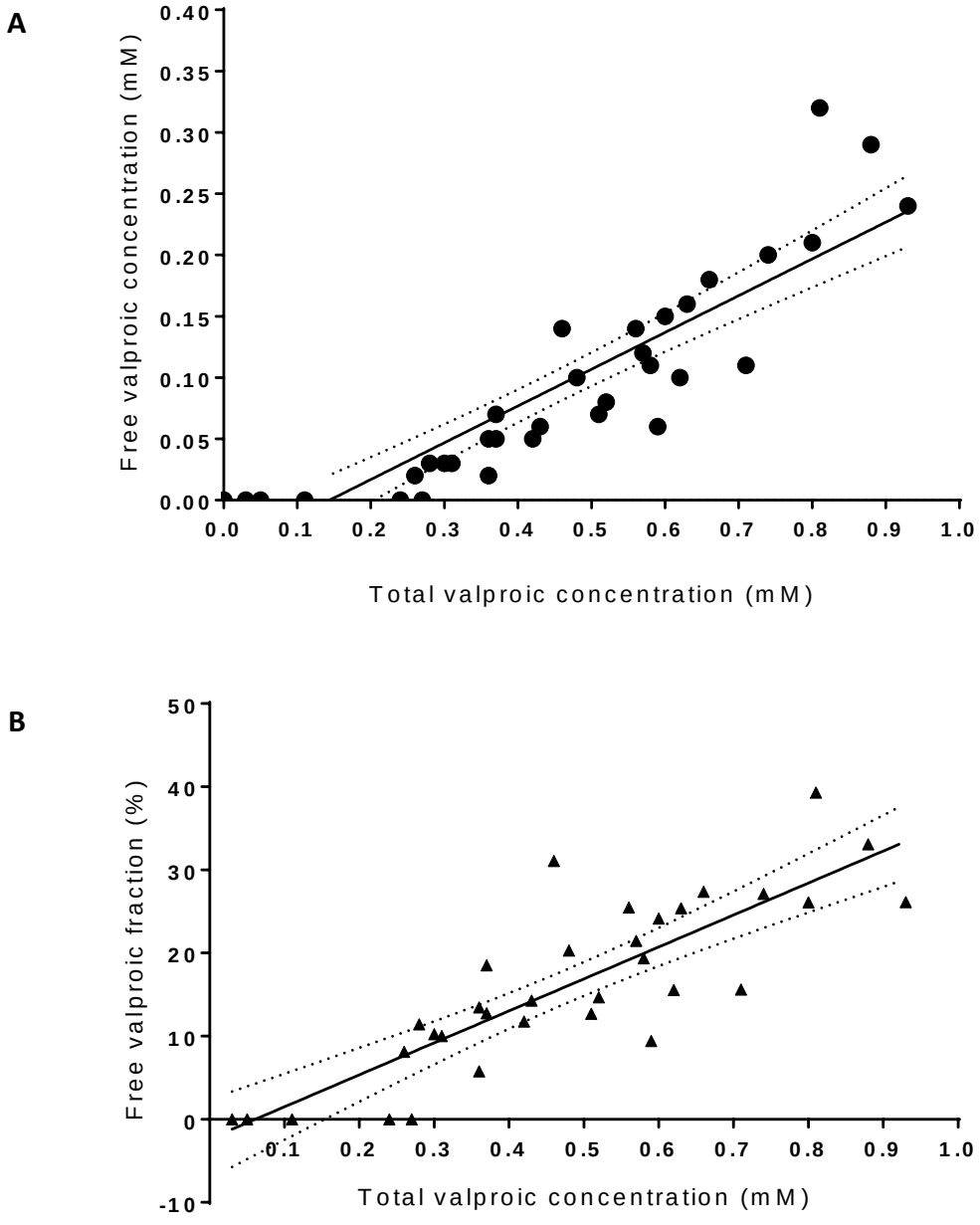


Figure 5.11. Correlation between total valproic acid and free valproic acid concentrations (A) and fraction of free valproic acid (B) in patients received different doses of valproic acid.

Free valproic acid was analysed from the ultrafiltrates of patient 1, 2, 4 and 6. The line represents linear regression analysis with 95% confidence interval.

The relationship between percentage acH4 positive myeloid cells and free valproic acid concentration was determined in the same way as total valproic acid, and the results were comparable to total valproic acid results in all dimensions (Figure 5.12-Figure 5.14). Only patient 4 showed a significant correlation between free valproic acid concentration and percentage of acH4 positive cells (Spearman, p-value 0.0056, Figure 5.12). This significance, however, disappeared when absolute change and fold change were determined instead of actual acH4 values. Patient 2 presented a trend towards an increase in fold change in acH4 positive cells with increasing free drug concentrations, although this increase was not significant (Spearman, p-value 0.0773, Figure 5.13).

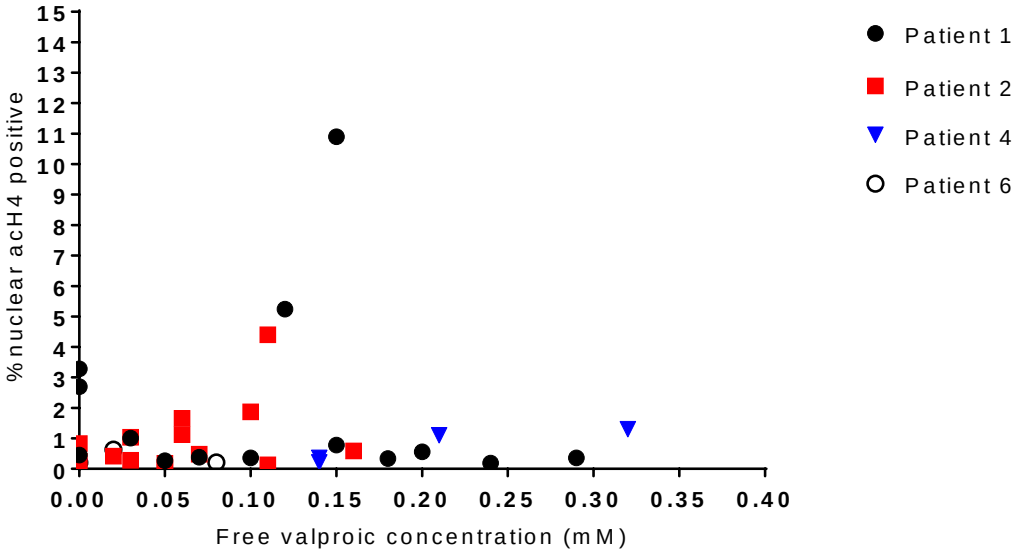


Figure 5.12. Association between free valproic acid concentration and percentage of acH4 positive myeloid cells in all samples of patient 1, 2, 4 and 6 regardless of pre- or post-treatment.

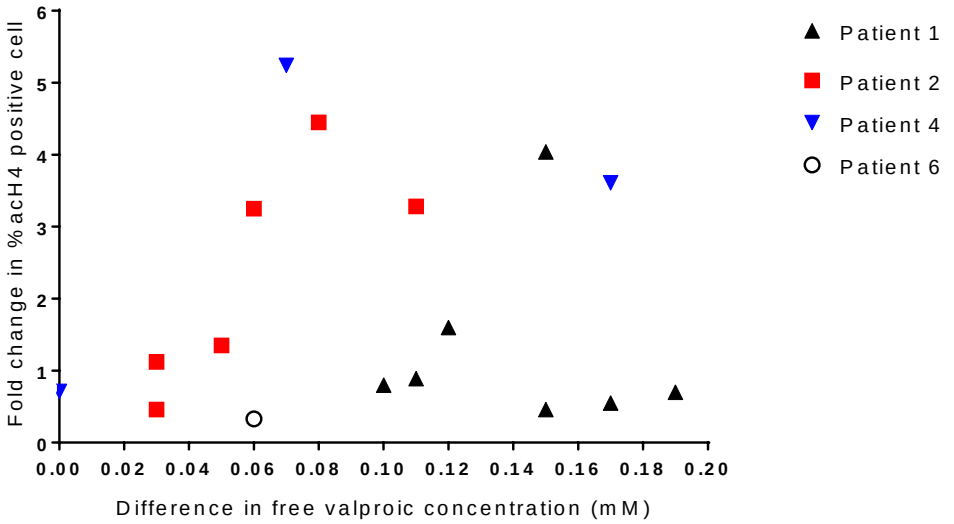


Figure 5.13. Association between difference in free valproic acid concentration and fold change in percentage of acH4 positive myeloid cells.

Free valproic acid was analysed from the ultrafiltrates of patient 1, 2, 4 and 6. Difference was determined by post-treatment values minus pre-treatment values and fold change was determined by dividing post-treatment values by pre-treatment values.

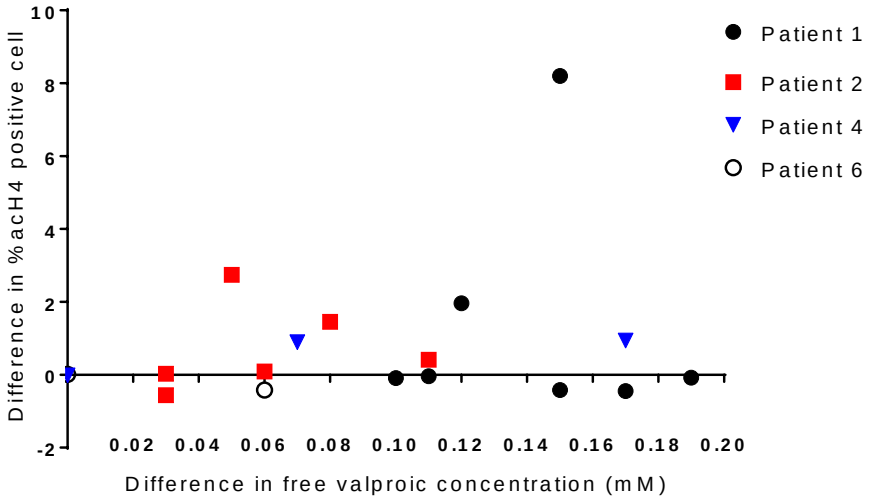


Figure 5.14. Association between difference in total valproic acid concentration and difference in percentage of acH4 positive myeloid cells.

Free valproic acid was analysed from the ultrafiltrates of patient 1, 2, 4 and 6. Difference was determined by post-treatment values minus pre-treatment values.

5.4 Discussion

The developed imaging flow cytometry assay for detection of acH4 expression in myeloid cells was used to analyse clinical samples collected from patients studied on the SIOP Ependymoma II trial. Seven patients were recruited onto the trial and blood samples were collected pre- and 4-hour post-administration of valproic acid on multiple visits to measure acH4 expression. Plasma concentrations of the drug were also measured and used to adjust subsequent doses. Expression levels of acH4 analysed by this assay were compared to dose and concentration of drug in order to investigate potential relationships between these parameters. In addition, the association between total and free valproic acid concentrations was investigated as well as associations between free valproic acid concentrations, dose and acH4 cell positivity. Preliminary results from this study were published in the *European Journal of Clinical Investigation* in 2019 (Uitrakul *et al.*, 2019).

5.4.1 Histone H4 acetylation in human white blood cells

The acH4 analysis results showed modest differences in acH4 expression, i.e. mean intensity, median intensity and percentage acH4 positive cells, between pre- and post-treatment as shown in Figure 5.4. In particular, as compared to pre-treatment, the average percentage of acH4 positive cells increased more than mean and median fluorescence intensity of acH4. Regarding cell type, increases observed in all values were greater in myeloid as opposed to lymphoid cells. These findings therefore supported the use of percentage of acH4 positive myeloid cell as a surrogate tissue for acH4 measurement.

According to patient data obtained from the SIOP Ependymoma trial, some patients had very low WBC counts to the point of leukopenia and some also had neutropenia, which theoretically could result in changes in the number of acH4 positive myeloid cells. Basically, identification of myeloid and lymphoid cells in the developed assay is based on gating by size of cell and intensity of side scatter which reflects cell granularity. This method sometimes provides overlap between the two populations. If there are fewer myeloid cells, it is possible to have more lymphocytes in the myeloid population, resulting in a higher number of acH4 positive cells because acH4 expression is stronger in lymphocytes.

The results shown in this chapter, however, suggested that not all samples with neutropenia presented high numbers of acH4 positive myeloid cells. In fact, only the sixth visit of patient 1 provided extremely high acH4 positive myeloid cells (>10%) together with neutropenia. Other samples with neutropenia, such as the fourth visit of patient 1 and the second visit of patient 6, did not show such high acH4 positive myeloid cells. The overall results therefore were not changed, although the neutropenic samples were removed from the analysis. Since neutrophils or myeloid cells are rarely cells of specific interest in this type of clinical trial, the correlation between acH4 positive myeloid cell and neutropenia condition has not previously been shown and needs more investigation.

The heterogeneity of acH4 expression in human cells was observed in the current study. Differences in baseline acH4 expression were found in recruited patients; patient 7 presented higher than 2% of acH4 positive myeloid cells while others had lower numbers at the beginning of the study. This variation was mentioned in two phase I clinical trials which showed obvious differences in baseline acH4 expression between their cohorts measured by Western blot (Chavez-Blanco *et al.*, 2005; Atmaca *et al.*, 2007). These findings caused difficulty for interpersonal comparison of acH4 since the same magnitude of change in one patient could not be compared with others.

A possible covariate which might cause these markedly different acH4 baselines in patients was co-medication. The inclusion criteria of the trial mention that patients must have no previous chemotherapy and no co-existent unrelated disease. It does not state that patients must not have received any other medication prior to the trial. Thus it is likely that patients received drugs before and during the valproic acid treatment which may influence baseline acH4 levels. While it is impractical to investigate all medication that patients received before valproate treatment, this possibility should be taken into account.

Percentages of acH4 positive myeloid cells suggested a large variation – both intra-patient and inter-patient variation – in histone acetylation when treated with valproic acid. Marked increases in numbers of acH4 positive cells were found in small numbers of samples, while the majority of samples exhibited more moderate changes.

Focusing on intra-patient variation, the results suggested that an increase in percentage of acH4 positive myeloid cells did not correlate with duration of valproic acid treatment; the percentages of acH4 positive cells of a patient measured at later visits could be fewer than the first visit, regardless of the drug exposure in the individual patient. To the best of our knowledge, this is the first study reporting acH4 expression in patients at multiple time points. Other studies have presented results at baseline and steady state, so the intra-patient variation during chronic treatment could not be assessed.

Inter-patient variation in acH4 positive cells following treatment with valproic acid was also observed in this study. Intuitively patients who continuously received valproic acid for longer durations would have higher acH4 expression than those treated with the drug for shorter periods. However, it was found that patients who had received valproic acid for longer time periods and at higher doses could have fewer acH4 positive cells than those who had received the drug for first time. This demonstrated differences in drug response between patients which could reflect the utility of histone acetylation as a predictive biomarker. On the other hand, it could be argued that these results may limit the applicability of acH4 as a pharmacodynamic biomarker, due to high variation of acH4 changes between patients.

5.4.2 Associations between acH4 expression and valproic acid dose

Associations between dose of valproic acid and increases in acH4 expression have been widely studied and the results remain controversial, as previously mentioned (Chavez-Blanco *et al.*, 2005; Atmaca *et al.*, 2007; Munster *et al.*, 2007). However, correlations between increases in acH4 positive myeloid cells, analysed as actual change and fold change, and administered dose of valproic acid were not observed in this study. Two possible explanations should be discussed.

First, the pharmacodynamic behaviour in children may be different from adults. Although several studies have suggested correlations between acH4 expression and valproic acid dose, all of them have studied adult patients. The published valproate study in children did not mention associations between dose and acH4 expression but reported no correlation between valproic acid dose and concentration (Su *et al.*, 2011). Based on no correlation between fold-change in acH4 and drug concentration shown

in Figure 5.1, it could be assumed that valproic acid dose did not relate to acH4 change. This finding suggested that changes in acH4 in children might not be comparable to the results in adults.

Secondly, patients studied in the SIOP Ependymoma study might have received too low doses of valproic acid, which insufficiently impacted on histone acetylation levels in the blood. According to the study in adults that found a correlation, patients were given valproic acid up to a dose of 80 mg/kg, while patients in the SIOP Ependymoma trial mostly received doses of 15-30 mg/kg and a maximum of 45 mg/kg (Munster *et al.*, 2007). On the other hand, another phase I study administered doses up to 120 mg/kg of valproic acid to adult patients, but found no dose-dependent acH4 expression (Atmaca *et al.*, 2007). According to the US FDA recommendation, valproic acid at doses above 60 mg/kg/day is not recommended in children due to safety concerns (US FDA, 2011). It is known that the pharmacokinetics of children are different from adults and pharmacokinetic studies of valproic acid in paediatrics are limited as well as pharmacodynamic studies. A pharmacokinetic study, for example, indicated a lower volume of distribution and lower clearance of valproic acid in children as compared to adults (Cook *et al.*, 2016). This therefore makes the estimation of effective valproic acid dose in children more difficult than adults.

5.4.3 Associations between acH4 expression and valproic acid concentration

Associations between valproic acid concentration (both total and free concentrations) and acH4 expression were not found in this study, despite being reported previously (Munster *et al.*, 2007; Munster *et al.*, 2009; Rocca *et al.*, 2009). Concentration of valproic acid was investigated in different aspects including original values, fold changes and absolute changes between post- and pre-treatment, but none of them showed significant correlations with percentage of acH4 positive myeloid cells. These results were comparable with a previous clinical trial in children which reported that only half of the patients had increased acH4 at the steady-state valproic acid concentrations (Su *et al.*, 2011). Furthermore, a phase I study in adults indicated no correlation between acH4 expression and serum concentrations of valproic acid (Chavez-Blanco *et al.*, 2005). This highlights current controversies in potential associations between histone acetylation and concentration of valproic acid, although

this association was noted in the previous *ex vivo* results. Focusing on this difference, there are some factors to be discussed.

First, the concentration of valproic acid which significantly increased acH4 positive cells in *ex vivo* studies was 1 mM or higher. This effective concentration was similar to most pre-clinical and clinical studies mentioned above. The lowest concentration associated with significant acH4 increases *in vitro* was 0.5 mM, similar to a study in children with solid tumours that suggested at least 70 μg/mL (~0.5 mM) was required to inhibit HDAC activity in PBMCs (Göttlicher *et al.*, 2001; Eyal *et al.*, 2004; Coulter *et al.*, 2013). As this level was higher than the differences in concentration observed in SIOP Ependymoma patients (Figure 5.9 and Figure 5.10), increases in acH4 level should not necessarily be expected in this study.

Secondly, the concentrations measured in published clinical studies were concentrations at baseline and trough steady-state, not a 4-hour post-treatment as in the SIOP Ependymoma study (Munster *et al.*, 2009; Su *et al.*, 2011). The effective concentrations of valproic acid reported (~0.5 mM) therefore were the trough levels. However, the levels of acH4 expression in the Ependymoma samples at 4-hour post-treatment were expected to be peak concentrations. If peak concentrations in Ependymoma samples were similar to trough concentrations in the above-mentioned studies, it could be assumed that their peak concentrations in published studies were much higher than in the Ependymoma patients. This therefore could be one of the reasons for differences in the results in this chapter and previously published results.

Thirdly, focusing on the *ex vivo* experiments, WBCs have been continuously treated with the same concentrations of valproic acid for 6 hours. This condition is totally different from the real pharmacokinetics in the human body, where drug concentrations are always changing. The time-dependent *in vitro* results in Chapter 4 suggested that acH4 levels significantly changed when treated with 4 mM valproic acid for longer than 1 hours, in agreement with other pre-clinical studies (Eyal *et al.*, 2004). However, there is no clinical pharmacodynamic publication stating an effective time of valproic acid treatment in humans, especially at low concentrations (<1 mM), so comparison between pre- and 4-hour post-treatment possibly was not enough to observe changes in acH4 expression.

Fourthly, as explained earlier, almost all patients in this clinical trial had received co-medication such as antibiotics, chemotherapeutics and opioids. Although there is no strong information available for histone-relating effect of these drugs, drug repositioning studies hypothesise this possibility. For instance, sodium phenylbutyrate was registered for treatment of urea cycle disorders before the discovery of its class I and class IIa HDAC inhibiting effects. Nicotinamide was first used to treat acne vulgaris or other inflammatory skin disorders, but was recently found to exhibit a class III HDAC inhibitory effect (Elvir *et al.*, 2017). Moreover, associations between morphine and histone acetylation have been studied for over a decade and results have shown morphine-induced histone acetylation in murine models (Koo *et al.*, 2015). These studies suggest that there might be many available drugs with unknown histone-related mechanisms of action. Furthermore, drug interactions with valproic acid may impact on acH4 expression. For example, a clinical study reported significant increases in histone acetylation after treatment with both valproic acid and temsirolimus, but not valproic acid alone (Coulter *et al.*, 2013). In case that some patients received valproic-interacting drugs, the levels of acH4 would change regardless of valproic acid concentration. This could then result in a lack of a correlation between acH4 expression and valproic acid concentrations whether or not it exists.

With respect to free valproic acid levels, a significant correlation between free and total valproic acid concentrations was observed in this study. The linear regression analysis revealed that no free valproic acid was detected if the concentration of total valproic acid was less than 30 μg/mL (Figure 5.11). Moreover, the results suggested an increase in fraction of free valproic acid following an increase in total valproic acid concentration. This trend was similar to a study in children with cancer which reported a positive relationship between free valproic acid fraction and total valproic acid concentration (Su *et al.*, 2011). With total valproic acid concentrations less than 50 μg/mL, the fraction of free valproic acid was 12.9%. When the concentration of total valproic acid was 50-100 μg/mL, free valproic acid fraction increased to 16.8% and when total drug concentrations were 100-125 μg/mL, the free drug fraction increased to 21.3%. These findings are similar to other published valproic acid studies (Otten *et al.*, 1984; Herngren *et al.*, 1991). The results of free valproic acid analysis suggest that samples need to have high enough concentrations of total valproic acid to

be able to detect free drug content. Moreover, similar to total valproic acid concentrations, no correlations between free drug concentrations, dose and percentage of acH4 positive cells were observed.

Regarding the use of valproic acid in epileptic patients in clinical settings, US FDA recommends the therapeutic range of valproic acid for adults and children >10 years old of 50-100 μg/mL (0.35-0.69 mM) (US FDA, 2011). However, there is no information of valproic acid therapeutic range in neonate patients published. Focusing on the target concentrations in adults, valproic acid concentrations recommended for seizures are lower than the concentrations used in cancer studies and are too low to detect acH4 changes using the developed assay (Atmaca *et al.*, 2007; Munster *et al.*, 2007; Su *et al.*, 2011). Therefore use of the developed assay in patients receiving valproic acid for non-cancer treatments may not be appropriate until the sensitivity of the assay is improved.

In summary, the developed assay was applied to measure histone H4 acetylation in patients in the SIOP Ependymoma II clinical trial and the results showed both high intra-patient and inter-patient variability in acH4 expression. There were also inconsistent changes in percentages of acH4 positive myeloid cells when treated with valproic acid as compared to before treatment. These changes did not correlate with dose, total concentration or free concentration of the drug, nor the duration of treatment. However, there were only seven patients recruited and none of them had achieved target trough concentrations, which is seen as the minimum effective concentration for histone acetylation. More patients with higher drug concentrations therefore may be expected to experience greater changes in acH4 and to confirm the applicability of the developed pharmacodynamic biomarker.

Chapter 6. Clinical application of an assay for detection of ki-67 expression in lymphoid cells as a potential prognostic biomarker

6.1 Introduction

6.1.1 Characteristics of ki-67

Ki-67 function in human cells and its potential use as a biomarker for cancer have been described in Chapter 3, section 3.1.5. High expression of ki-67 protein has been found in S, G_2 and M phase cells, but not G_0 (Gerdes *et al.*, 1984). In G_1 stage, ki-67 concentrations are controlled by two mechanisms and its protein levels can vary (Sun and Kaufman, 2018). Although its function in human cells has not been fully elucidated, ki-67 is widely used as a marker of cell proliferation (Scholzen and Gerdes, 2000). In cancer research, ki-67 has been studied both as a potential prognostic/predictive biomarker and as a therapeutic target.

Focusing on the prognostic utility of ki-67, associations between high levels of ki-67 positive cancer cells and poor prognosis have been reported in various types of cancer such as breast cancer (Agboola *et al.*, 2013; Inwald *et al.*, 2013; Mrklić *et al.*, 2013; Nishimura *et al.*, 2014), nasopharyngeal carcinoma (Zhao *et al.*, 2018), gastric cancer (Ko *et al.*, 2017) and prostate cancer (Wilkins *et al.*, 2018). The level of ki-67 expression reported in these papers has been measured using IHC, which is a qualitative technique, and the assessment of percentage of ki-67 positive cells can be subjective. High variability of ki-67 expression measured by IHC techniques in different laboratories has been shown in several studies, resulting in no consensus being reached in terms of a cut-off value for ki-67 positivity (Mengel *et al.*, 2002; Focke *et al.*, 2017).

6.1.2 Cancer immune response

Associations between cancer and the immune system were first studied in 1863 by Rudolf Virchow (Balkwill and Mantovani, 2001). He reported the prevalence of WBCs in tumour tissues and suggested linkage between cancer and chronic inflammation. Later, this association became one of the hallmark concepts for cancer prevention and treatment.

The pathology of host immune response involves the presence of inflammatory cells and proinflammatory cytokines. When there are cancer cells in the body, macrophages are the major cell type that initiate response to the tumour cells in the form of tumour-associated macrophages (TAMs) (Mantovani *et al.*, 1992a). These TAMs are led to cancer cells and activated by chemokines. The activated TAMs play a crucial role in killing cancer cells; however, they can also produce growth factors, angiogenic factors and protease enzymes which may help stimulate tumour cell proliferation (Mantovani *et al.*, 1992b). Dendritic cells, also called tumour-associated dendritic cells (TADCs), have important roles in the activation of antigen-specific immunity and the maintenance of adaptive immunity. It was found that TADCs could stimulate T-cell activation in breast cancer (Allavena *et al.*, 2000). When lymphocytes such as T-cells and B-cells are activated by inflammatory cytokines, they secrete additional cytokines that further support the activation of immunity (Finn, 2012).

The other major immune response to the presence of cancer cells is the release of proinflammatory cytokines such as tumour necrosis factor (TNF), interleukins (IL) 1 and 6, and chemokines by both activated leukocytes and tumour cells (Hendry *et al.*, 2017). For instance, $CD8^+$ cytotoxic T-cells and T-helper 1 (Th1) cells can produce interferon-γ. Similarly, Th2 cells, myeloid derived suppressor cells and $FOXP3^+$ regulatory T-cells (Treg) can produce IL-10 and TGFβ.

6.1.3 Prognostic utility of white blood cells in cancer

The prognostic impacts of WBCs in a cancer setting have been widely studied, mainly focusing on the total number of WBCs and the presence of tumour-relating WBCs. For WBC count, it was found to be associated with patient prognosis in several cancer studies. Hao *et al* reported poor prognosis in patients with non-small cell lung cancer who had WBC counts >7,000 cell/mL (Hao *et al.*, 2018). This was in agreement with the results of Tibaldi *et al* who suggested an association between elevated WBC and poor prognosis (Tibaldi *et al.*, 2008). Feng *et al* also reported that in patients with advanced pancreatic cancer, WBC counts >5,800 cell/mL were associated with a poorer overall survival (Feng *et al.*, 2018). Moreover, in cases where patients presented with tumour-related leucocytosis, they had poorer prognosis than ones who showed leucocytosis by a known cause (Kasuga *et al.*, 2001).

Regarding tumour-infiltrating lymphocytes, these WBCs detected in tumour tissues were not only a focus for the development of cancer therapeutics, but were also found to be associated with prognosis in many cancer types (Gooden *et al.*, 2011). A meta-analysis study in colorectal cancer reported that $CD8^+$ and $FOXP3^+$ infiltrating cells, but not $CD3^+$ T-cells, were prognostic markers for overall survival (Zhao *et al.*, 2019). A meta-analysis in hepatocellular carcinoma showed significant associations of overall survival and lymphocytes with $CD8^+$, $FOXP3^+$, $CD3^+$ and Granzyme B^+ (Ding *et al.*, 2018). In patients with oesophageal cancer, T-cells with $CD8^+$, $FOXP3^+$ and $CD57^+$ were associated with overall survival while $CD3^+$, $CD4^+$ and $CD45RO^+$ T-cells were not (Zheng *et al.*, 2018).

These findings highlighted the potential for using protein expression in WBCs as a prognostic biomarker. The prognostic efficacy was observed when looking at the WBC count and the expression of some specific proteins in WBCs. This project therefore focused on the assessment of ki-67 expression in peripheral WBCs in order to investigate its potential to act as a prognostic biomarker in cancer patients. The project focused on patients in PROSPECT-NE study because these patients had end-stage cancer diseases and received no anti-cancer therapy. Therefore the possibility of medication affecting ki-67 measurement was minimized.

6.2 Chapter specific aims

This study aimed to investigate ki-67 expression in peripheral WBCs in cancer patients recruited to the PROSPECT-NE clinical study. The primary objective was to investigate the potential for using ki-67 expression in WBCs as a prognostic biomarker. Secondary objectives included the following:

- To determine potential associations between ki-67 expression and patient clinical data and markers of frailty (including grip strength, gender, BMI and routine blood test).
- To observe changes in ki-67 expression in patients with time (between hospital visits).
- To compare ki-67 expression in WBCs measured by imaging flow cytometry and ki-67 expression in plasma measured by ELISA. Associations between ki-67 expression in plasma and patient data were also investigated alongside ki-67 expression in WBCs.

6.3 Results

6.3.1 Patient characteristics

The PROSPECT-NE clinical trial is an observational study in patients with end stage cancer diseases. Patients recruited in the trial will be collected blood samples and other clinical data for further molecular analysis without receiving any anti-cancer therapy. This trial started in September 2017, with the ki-67 analysis project beginning in February 2018. From February to December 2018, a total of 43 patients were recruited onto the ki-67 analysis project and were studied until the project finished. Among them, 15 patients entered the PROSPECT-NE trial before the ki-67 analysis project started, leaving 28 patients whose blood samples were collected at the first visit. At the end of the ki-67 project, 22 out of 43 patients had died, with 14 of these 22 deceased patients having had blood samples collected at the first visit.

Baseline characteristics of patients at recruitment are shown in Table 6.1. Of 43 patients recruited, 25 patients were male (58%). The median age and SD of all patients was 61 $\pm$ 11.4 years. The average BMI of all patients was 27 kg/m^2 and all patients had performance status of 0-1 at the first visit. No patients with leukopenia or neutropenia were observed in this study. The mean right hand and left hand grip strengths at the first visit were 29.5 and 27.2 kg, respectively. A Z-score assessment of grip strength was calculated via the online calculator (https://stuartbman.github.io/grip_strength/index.html), which was developed within Newcastle University. This online tool adjusts the Z-score of a patient using age and gender as compared to the normative values from a meta-analysis of 12 British grip strength studies (Dodds *et al.*, 2014). The mean $\pm$ SD of patients' grip strength was -0.83$\pm$1.01 for right hand and -1.15$\pm$0.80 for left hand. The majority of cancer type in this study was colorectal (27.9%) and breast (16.3%) cancer. Examples of other cancer types in this study included mesothelioma, prostate cancer, lung cancer, gastric cancer and sarcomas.

Parameter	Value
Age (year) (Median±SD)	61±11.4
Male (n) (%)	25 (58%)
Weight (kg) (Mean±SD)	77.5±14.9
BMI (kg/m^2) (Mean±SD)	27.2±4.4
Performance status 1 (%)	24 (56%)
LDH (U/L) (Mean±SD)	295±157
Albumin (g/L) (Mean±SD)	42.3±4.3
WBC count (×10^9/L) (Mean±SD)	6.8±3.0
Neutrophil count (×10^9/L) (Mean±SD)	4.6±2.7
Grip strength right hand (kg) (Mean±SD)	29.5±9.5
Grip strength right hand (Z-score, Mean±SD)	-0.83±1.01
Grip strength left hand (kg) (Mean±SD)	27.2±8.3
Grip strength left hand (Z-score, Mean±SD)	-1.15±0.80

* Z-score is calculated using the online system via https://stuartbman.github.io/grip_strength/index.html

Table 6.1. Baseline characteristics of patients recruited in PROSPECT-NE trial (n=43).

6.3.2 ki-67 expression in patient WBCs

Percentages of ki-67 positive white blood cells were measured in all patient samples sent to the lab using the developed assay as described in Chapter 2. The gating and analysis used in this project were the same as used to measure ki-67 expression in Chapter 3. Representative images of myeloid and lymphoid cells with and without ki-67 positivity are illustrated in Figure 6.1. The percentages of ki-67 positive cells in myeloid, lymphoid and total WBCs from all patient samples are shown in Figure 6.2. The median percentage of ki-67 positive myeloid cells was 0.45% while lymphoid cells was 1.93% (Mann-Whitney U-test, p-value <0.0001). Lymphoid cells were selected for further analysis of ki-67.

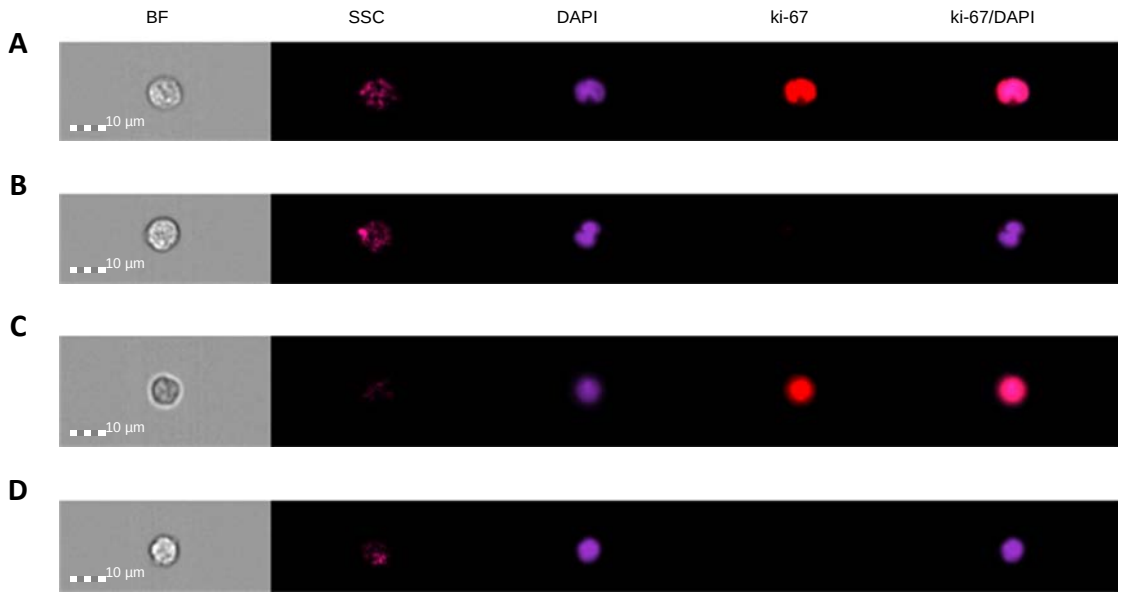


Figure 6.1. Examples of myeloid cells with ki-67 positivity (A) and ki-67 negativity (B), and lymphoid cells with ki-67 positivity (C) and ki-67 negativity (D).

Samples were obtained from patients studied on the PROSPECT-NE clinical trial.

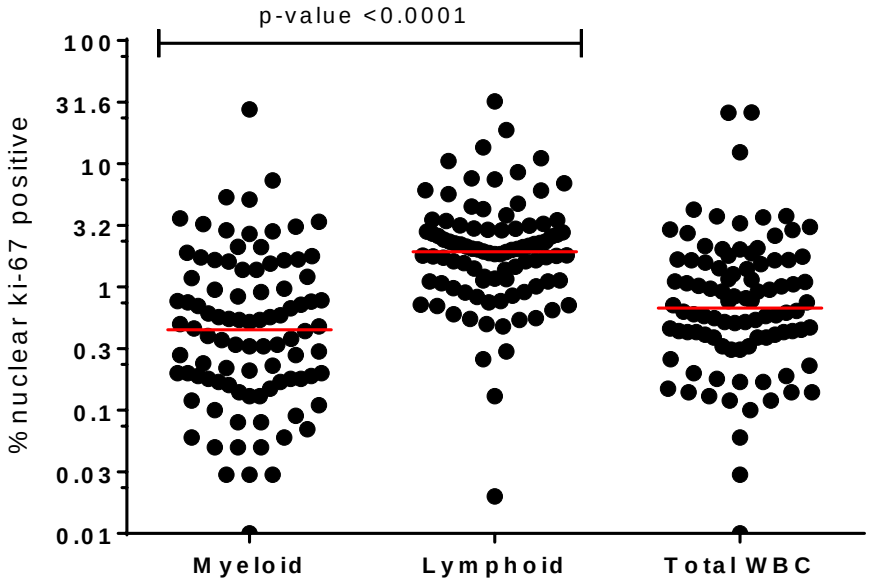


Figure 6.2. Percentages of ki-67 positive myeloid, lymphoid and total white blood cells in all cancer patients in PROSPECT-NE clinical trial.

Y-axis data shown on a logarithmic scale; red lines indicate median values.

In order to confirm that the gating used in this study designated a lymphoid cell population without monocyte contamination, 9 patient samples were incubated with antibodies against ki-67 and CD14. WBCs were first gated to myeloid and lymphoid populations using side scatter and area, and then CD14 positive cells (which indicate monocytes) were removed from the lymphoid population. The percentages of lymphoid cells gated by only side scatter and by side scatter plus removing CD14 positive cells were seen to be comparable (Figure 6.3A). The percentages of ki-67 positive cells measured in these two populations were also comparable (Figure 6.3B). Additionally, the percentage of ki-67 and CD14 positive cells ranged between 0.1% to 14.3%. Therefore, lymphoid gating by side scatter was suitable and could be used for this ki-67 analysis.

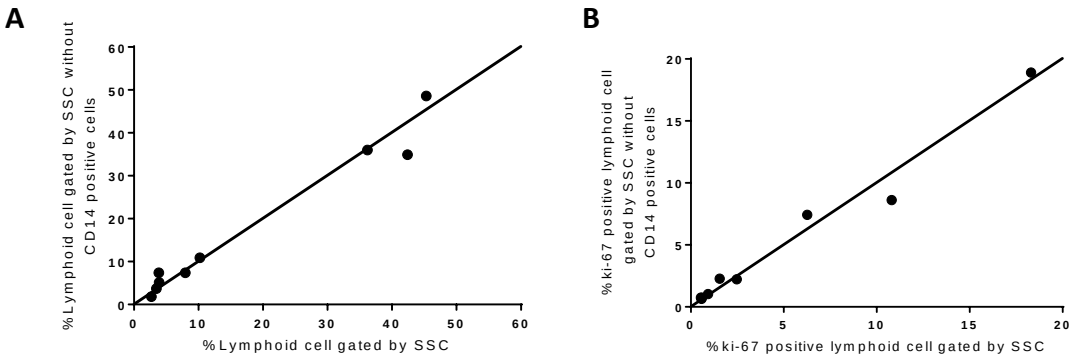


Figure 6.3. Percentages of lymphoid cells gated by only side scatter and by side scatter plus removal of CD14 positive cells (A), and percentages of ki-67 positive cells in lymphoid cell population gated by both methods (B).

The comparison of ki-67 positivity between cancer patients recruited to the PROSPECT-NE trial and healthy donors was investigated. Figure 6.4 shows the difference in ki-67 positive lymphoid cells between people with cancer and people not known to have cancer (Mann-Whitney U-test, p-value <0.0001). The median percentage of ki-67 positive lymphoid cells was 1.16% for healthy volunteers, with a maximum of 3%, and 1.93% for cancer patients, with the maximum of 32%. These data indicate that cancer patients have higher percentages of ki-67 positive lymphoid cells compared to a control population not known to have cancer. It should be noted that this comparison was not adjusted by patient age and gender due to no strong evidence

of association between ki-67, age and gender. Moreover, further results in this study showed no correlation between these three parameters.

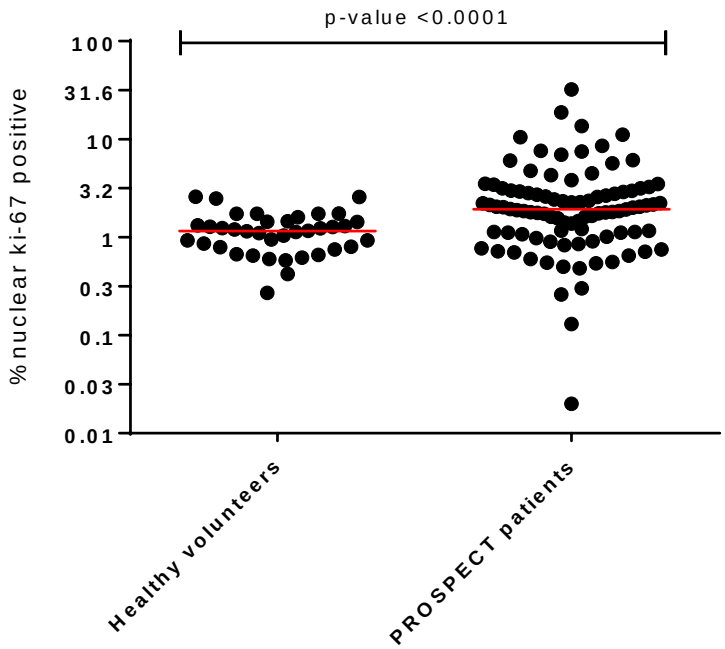


Figure 6.4. Comparison of percentages of ki-67 positive lymphoid cells in healthy donors and cancer patients recruited to the PROSPECT-NE trial.

Healthy samples were collected from 8 independent donors and PROSPECT samples were from all study patients. Y-axis data shown on a logarithmic scale; red lines indicate median values.

Regarding subgroup analysis of cancer type, there was no significant difference in percentages of first-visit ki-67 positive lymphoid cells between cancer types (Figure 6.5). The median percentage of ki-67 positive cells in all cancer types ranged between 0.54 – 5.7%. The two samples with the highest proportion of ki-67 positive cells (8.5% and 6.1% positive cells) were from breast cancer and colorectal cancer patients. The median percentage of ki-67 positive cells in breast cancer was 1.79%, comparable to other cancer types. Patients with sarcoma had the highest percentages of ki-67 positive lymphoid cells, with 5.7% positive cells. The number of patients in this subgroup, however, was too low to determine statistical significance.

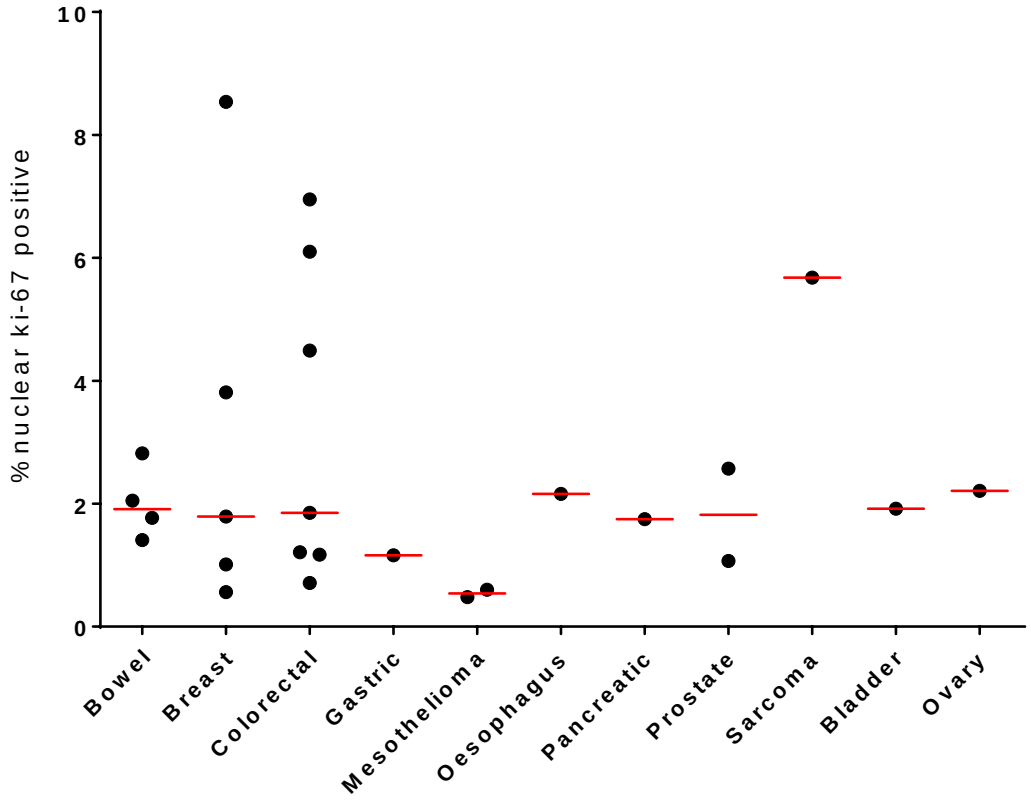


Figure 6.5. Percentages of ki-67 positive lymphoid cells in PROSPECT-NE patients according to cancer type.

Ki-67 values were from the first visit of the patients. Red lines indicate median values.

6.3.3 Variation of ki-67 levels in patients throughout the study

Variations in percentages of ki-67 positive lymphoid cells were observed throughout the trial. Figure 6.6A suggests fluctuation in ki-67 expression in all patients and Figure 6.6B shows the same data in deceased patients only. Four patients had %CV over 100%, with the highest value of 150%. The greatest absolute change was 18.5%, observed in the patient with highest percentage of ki-67 positive cells.

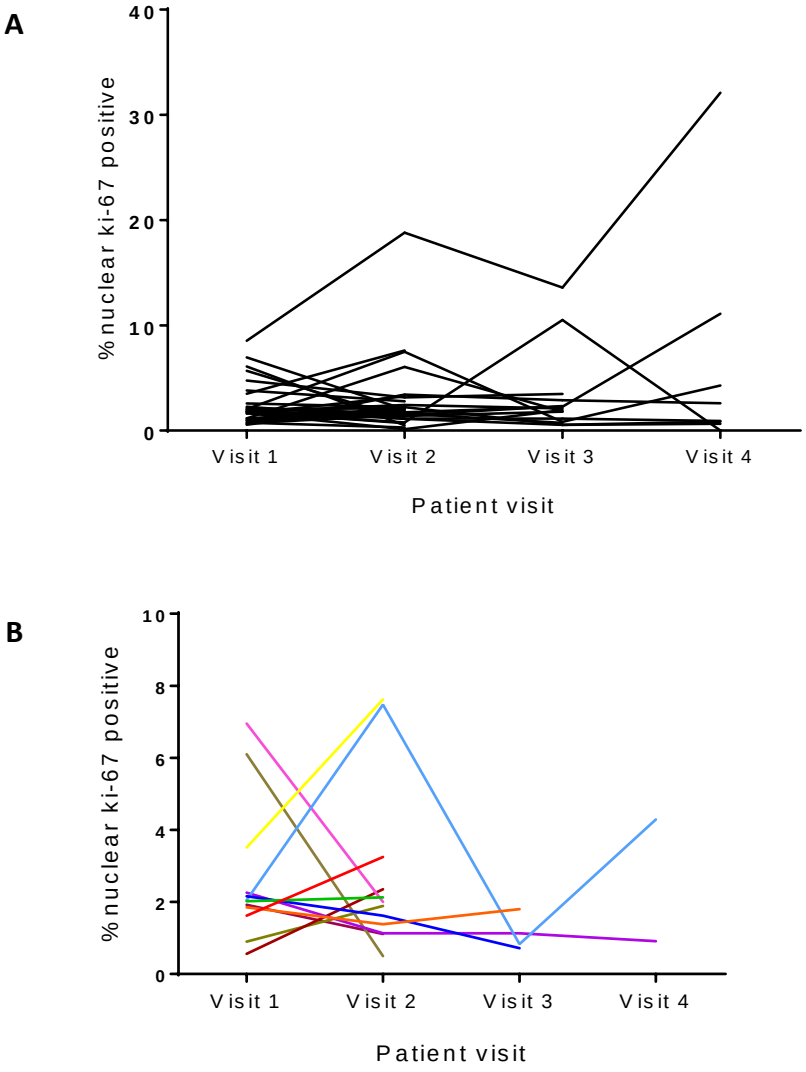


Figure 6.6. Changes in percentage of ki-67 positive lymphoid cells in all patients studied (A) and in deceased patients (B) throughout the study.

Only patients who had more than 1 visit were included in the graphs.

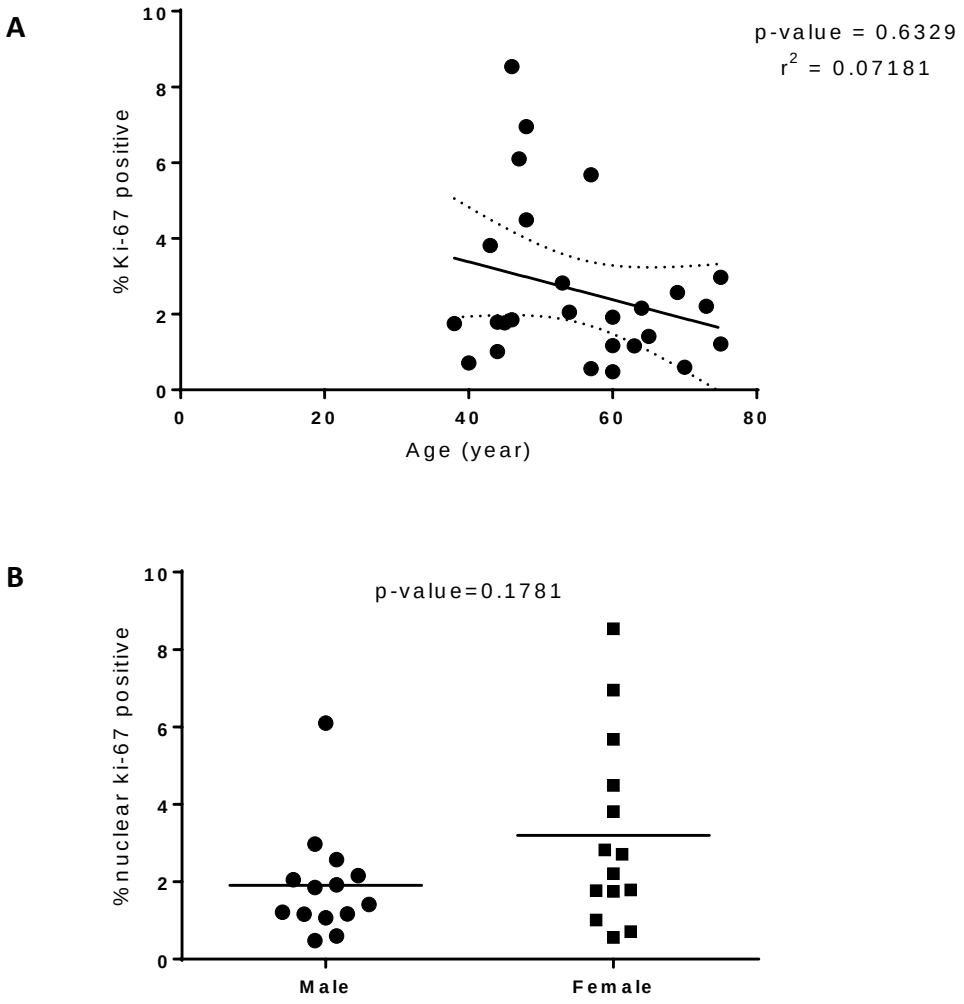
Focusing on the deceased patients, there was also a marked fluctuation in ki-67 positive lymphoid cells observed throughout the study (Figure 6.6B). Two patients expressed clear decreases in ki-67 at their last visits whilst four patients had marked increases. The other patients showed minimal changes in ki-67 positive cells until their last visits. These results suggest no association between ki-67 expression and terminal stage of cancer patients.

6.3.4 Association between ki-67 positive lymphoid cells and patient data

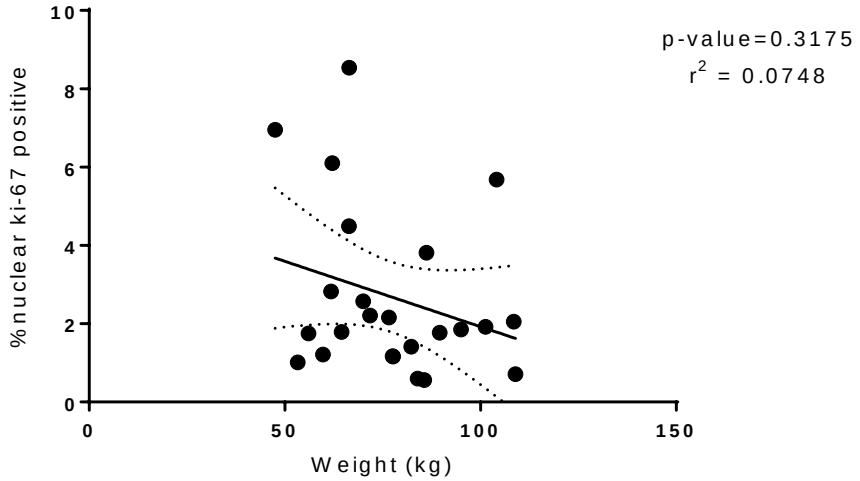
The association between percentages of ki-67 positive lymphoid cells and patient data at the first patient visit was investigated. There were two analyses used in

this study depending on variable type. A Spearman correlation analysis was used for continuous variables including percentage of ki-67 positive lymphoid cells, age, weight, BMI, LDH, albumin, WBC count, and Z-score of grip strength (right hand and left hand). The Mann-Whitney U-test was used for non-parametric categorical variables including gender and performance status.

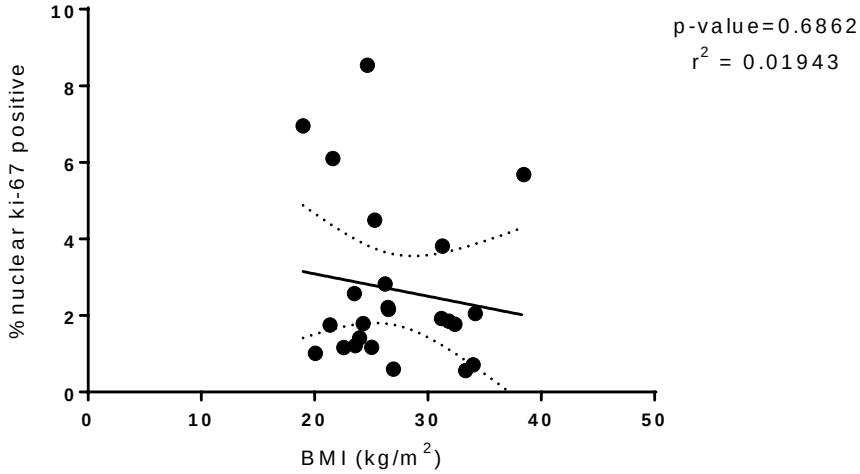
The correlation analysis graphs showing percentage of ki-67 positive cells and each parameter alongside p-values, linear regression and 95% confidence intervals (95%CI) are illustrated in Figure 6.7. The correlation coefficients (r) of other continuous variables analysed by Spearman test are presented in Table 6.2 and p-values of other categorical variables analysed by Mann-Whitney U-test are presented in Table 6.3.



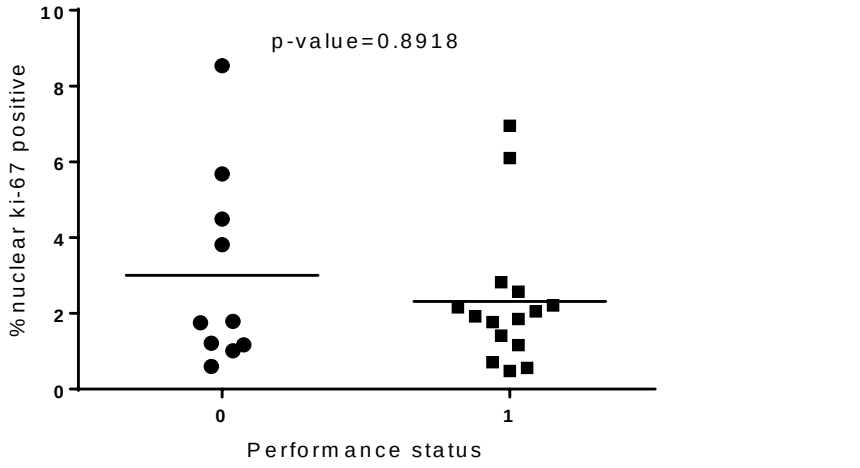
C


p-value=0.3175
r2 = 0.0748
% nuclear ki-67 positive
0
2
4
6
8
10
0
50
100
150
Weight (kg)

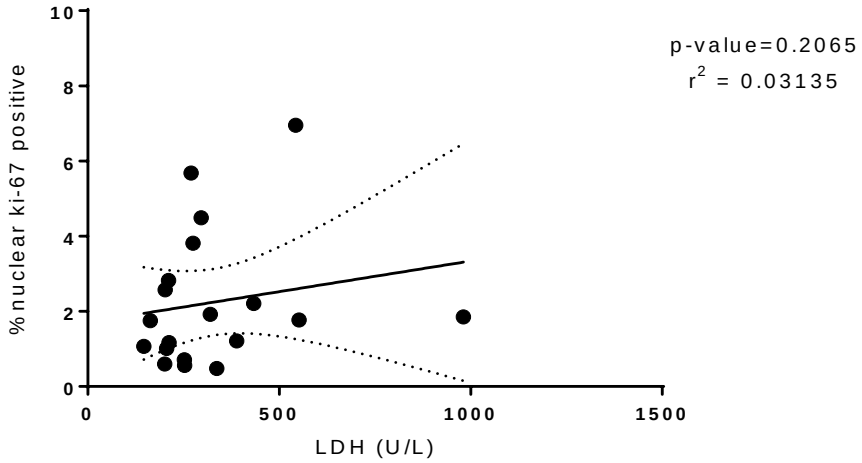
D


p-value=0.6862
r2 = 0.01943
% nuclear ki-67 positive
0
2
4
6
8
10
0
10
20
30
40
50
BMI (kg/m2)

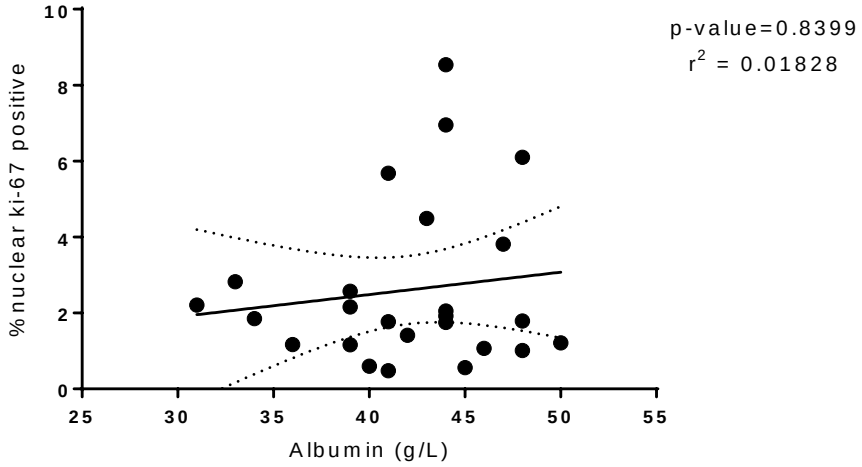
E


p-value=0.8918
% nuclear ki-67 positive
0
2
4
6
8
10
0
1
Performance status

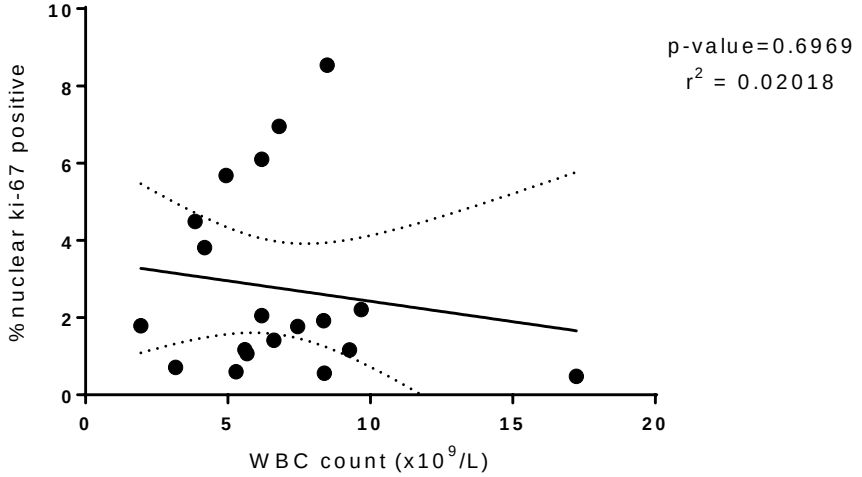
F


p-value=0.2065
r2 = 0.03135
% nuclear ki-67 positive
LDH (U/L)
0
2
4
6
8
10
0
500
1000
1500

G


p-value=0.8399
r2 = 0.01828
% nuclear ki-67 positive
Albumin (g/L)
0
2
4
6
8
10
25
30
35
40
45
50
55

H


p-value=0.6969
r2 = 0.02018
% nuclear ki-67 positive
WBC count (x10^9/L)
0
2
4
6
8
10
0
5
10
15
20

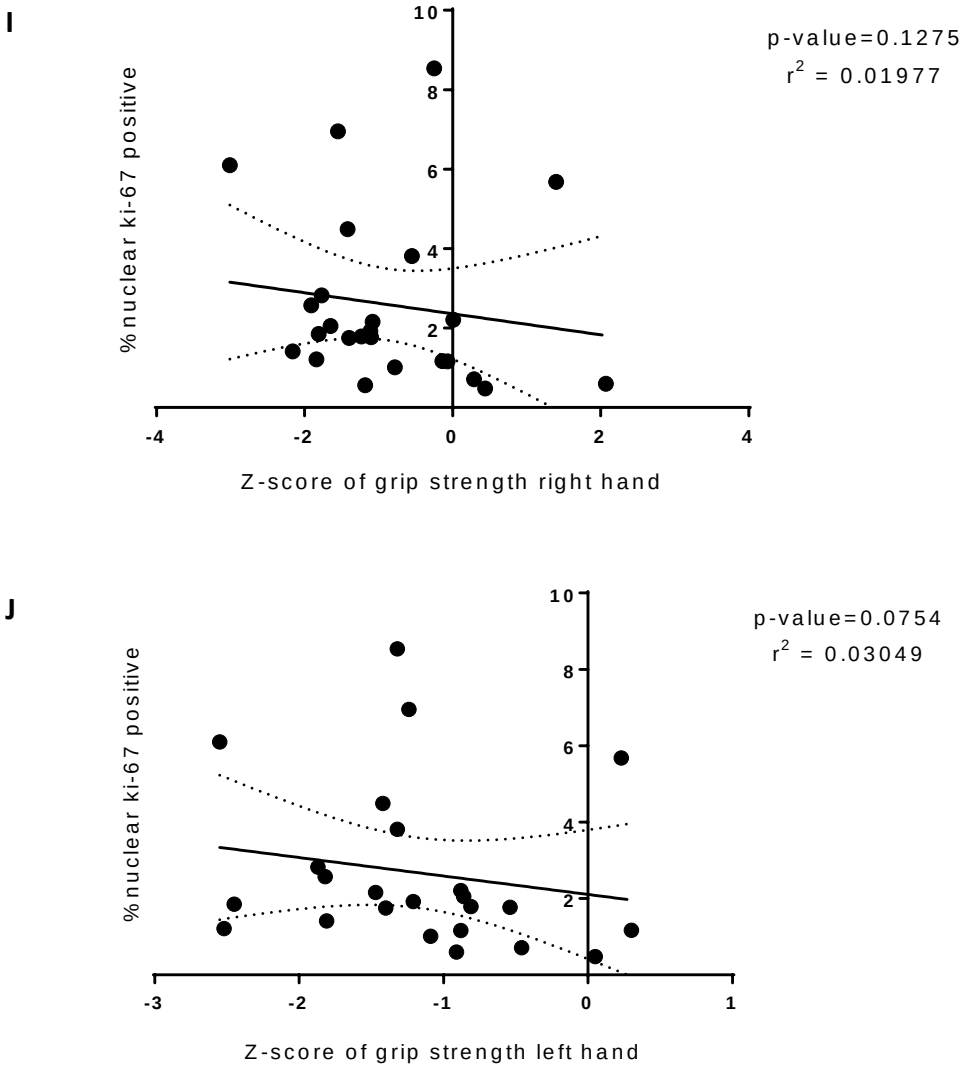


Figure 6.7. Association between percentage of ki-67 positive lymphoid cells and patient data including age (A), gender (B), weight (C), BMI (D), performance status (E), LDH (F), albumin (G), WBC count (H), Z-scores of grip strength right hand (I) and left hand (J).

Data shown were collected at the first visit after patients were recruited to the clinical trial. Continuous parameters were analysed using the Spearman correlation test with linear regression and 95%CI shown; categorical parameters were analysed using the Mann-Whitney U-test with black lines indicating the median values.

	Age	Weight	BMI	LDH	Albumin	WBC	Z-score of grip strength right hand	Z-score of grip strength left hand
Age	1.000	0.045 (0.834)	-0.015 (0.945)	0.088 (0.728)	**-0.409 (0.047)**	**0.509 (0.031)**	0.025 (0.905)	-0.103 (0.632)
Weight		1.000	**0.866 (<0.001)**	0.235 (0.362)	-0.273 (0.207)	-0.110 (0.671)	0.356 (0.088)	**0.447 (0.032)**
BMI			1.000	0.262 (0.308)	-0.254 (0.242)	-0.211 (0.414)	0.360 (0.084)	0.392 (0.064)
LDH				1.000	-0.148 (0.557)	0.456 (0.120)	-0.146 (0.565)	-0.047 (0.861)
Albumin					1.000	-0.341 (0.166)	-0.262 (0.216)	-0.130 (0.556)
WBC						1.000	0.104 (0.680)	-0.038 (0.884)
Z-score of grip strength right hand							1.000	**0.764 (<0.001)**
Z-score of grip strength left hand								1.000

Table 6.2. Correlation matrix of patient continuous data including age, weight, BMI, LDH, albumin, WBC count, and grip strength (of right and left hand) at the first patient visit.

Correlation coefficients (r) are presented with p-values shown underneath. Data were analysed using the Spearman correlation test and significance (p-value <0.05) is shown in bold.

	Gender	Performance status
Age	**0.001**	0.291
Weight	0.361	0.192
BMI	0.733	0.472
LDH	0.778	0.101
Albumin	0.467	0.082
WBC	0.388	**0.019**
Z-score of grip strength right hand	0.320	0.216
Z-score of grip strength left hand	0.325	0.575

Table 6.3. P-values of difference in patient continuous data parameters including age, weight, BMI, LDH, albumin, WBC, grip strength (of right and left hand) at the first patient visit separated by patient gender and performance status.

Data were analysed using the Mann-Whitney U-test and significance (p-value <0.05) is shown in bold.

For percentage of ki-67 positive cells (Figure 6.7), the results indicated no correlation with any variables. However, the analysis of covariates revealed some significance (Table 6.2 and Table 6.3). As expected, weight correlated with BMI and grip strength of right hand correlated with left hand. Patients with lower body weight had lower grip strength (left hand). This study found that older patients had lower albumin and higher WBCs. In addition, male patients in the study population were significantly older than the female patients and patients with higher performance status at recruitment had significantly higher WBC counts.

As presented above (section 6.3.3), variability in percentages of ki-67 positive lymphoid cells measured in multiple visits to the clinic was observed and associations between changes in ki-67 level and some parameters were investigated. A Spearman correlation analysis was performed to investigate associations between percentage of ki-67 positive cells and high-variability parameters including, LDH, albumin and WBC count from all patient visits. The results indicated no correlation between ki-67 expression and the levels of the variables analysed (Figure 6.8).

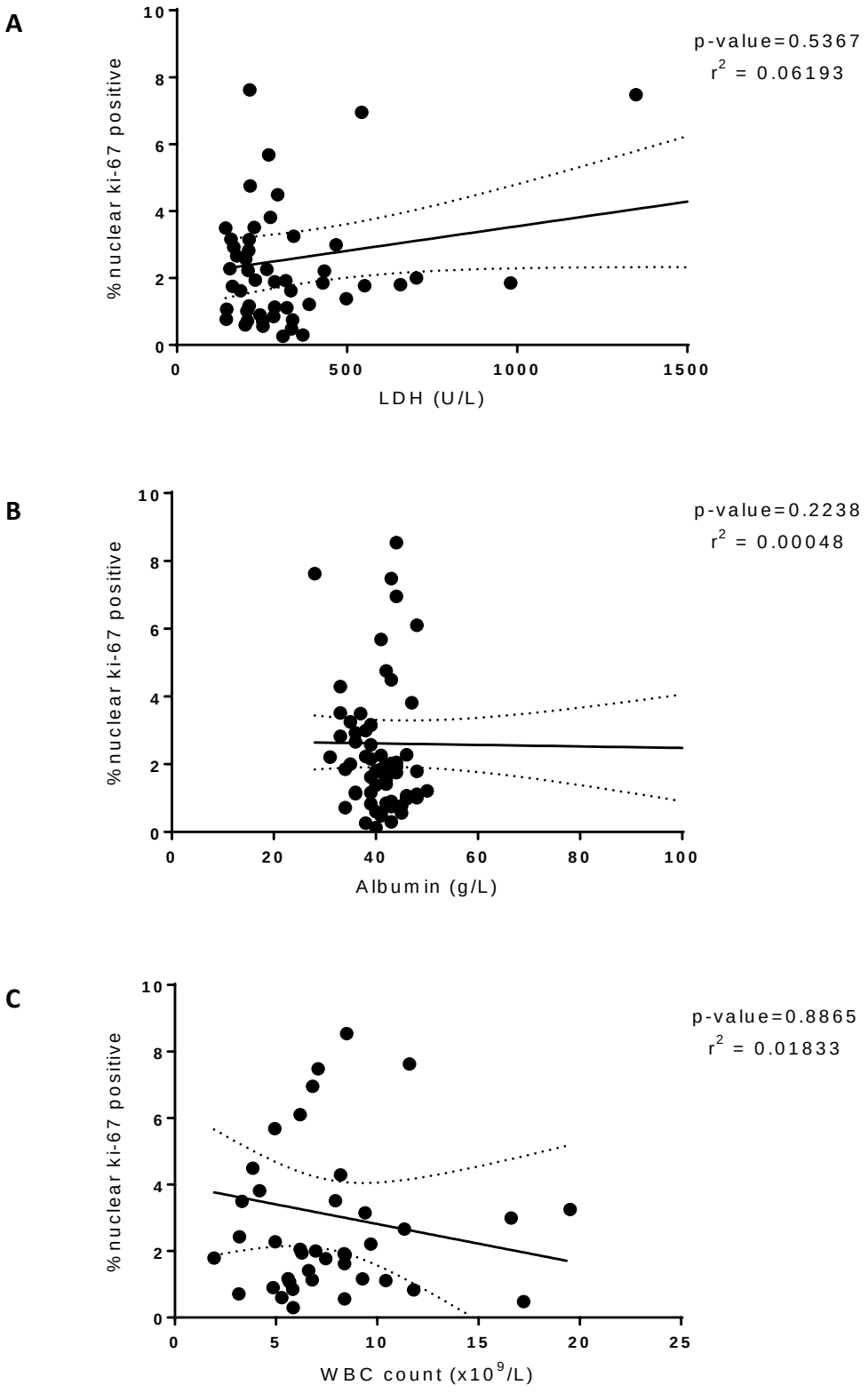


Figure 6.8. Associations between percentage of ki-67 positive lymphoid cells and LDH level (A), albumin level (B), and WBC count (C).

Data shown were collected from all patient visits and analysed using the Spearman correlation test with linear regression and 95%CI shown.

6.3.5 Prognostic impact of ki-67 positive lymphoid cells on patient survival

Correlations between percentage of ki-67 positive lymphoid cells and patient survival status were determined using two analyses in this study: log rank test and Cox's proportional hazards model (Cox regression). The first approach tests the difference between survival times of different groups of a variable while the second test is able to test the correlation of more than two variables (Bewick *et al.*, 2004). Moreover, the Cox regression analysis allows testing of continuous variables whereas the log rank test uses only categorical variables.

Since the log rank test allows only categorical variables, the percentage of ki-67 positive cells had to be categorised. Thus, the threshold of ki-67 level was developed to classify patients into high and low ki-67 expression groups. Figure 6.9 shows two receiver operating characteristic (ROC) curves analysed using percentage of ki-67 positive cells from the first visit of each patient (A) and by the median percentage of all visits of each patient (B). The median value of all visits from one patient was also used as the representative of all ki-67 values for that patient in the ROC analysis because of no standard guideline suggesting which value of ki-67 should be used. Furthermore, using median values was expected to increase the sample size of the analysis because patients who were recruited into the study before the ki-67 project started could provide median ki-67 values but no first-visit ki-67 positive cells was collected from this population.

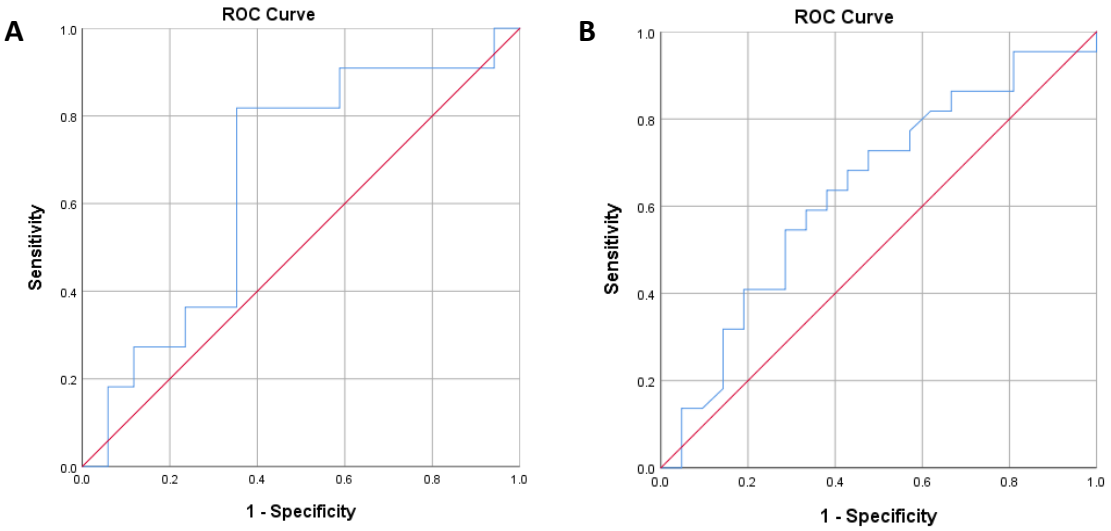


Figure 6.9. The ROC performed to calculate the optimal cut-off values of (A) percentage of ki-67 positive cells at the first visit and (B) the median percentage of ki-67 positive cells of all visits for overall survival.

Both ROC curves suggested neither significant difference between survival status and percentage of ki-67 positive cells at the first visit (AUC 0.658, p-value 0.165) nor median percentage of ki-67 of all visits (AUC 0.636, p-value 0.126). Using the Youden index method, a cut-off value for first-visit ki-67 positivity of 1.82%, with sensitivity of 0.818 and specificity of 0.647, was determined (Figure 6.9A). The cut-off value for the median of all visits was 1.61% with sensitivity of 0.727 and specificity of 0.524 (Figure 6.9B). Based on the threshold of 1.82%, there were 13 patients classified as low ki-67 including 2 deceased patients and 15 patients classified as high ki-67 with 9 deceased patients (Figure 6.10A). With the threshold of 1.61%, there were 17 patients and 26 patients in low and high ki-67 groups, respectively. Only 6 patients in the low ki-67 group died while 16 patients died in the high ki-67 group (Figure 6.10B). The full details of patient categorisation are described in Appendix A.

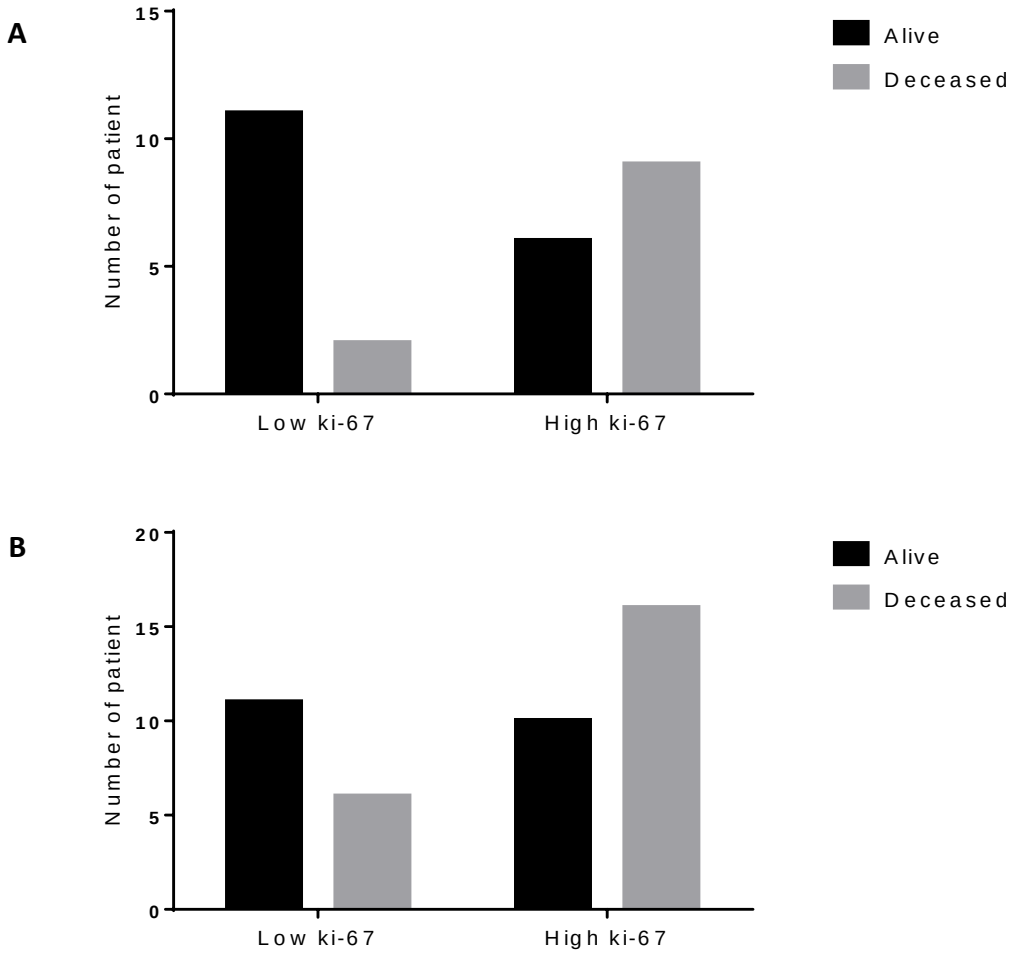


Figure 6.10. The numbers of alive and deceased patients at the end of study categorised into four groups.

Figure 6.10A categorised using the percentage of ki-67 positive cells at first visit and Figure 6.10B using the median percentage of ki-67 positive cells of all visits.

Survival analysis was performed using the percentage of ki-67 positive cells at the first visit for two reasons. Firstly, ROC analysis suggested that the first-visit values provided higher sensitivity and specificity than the median values to predict mortality of patients. Secondly, using the first-visit values strengthened the predictive impact of ki-67 since there was no effect of intra-personal variability observed in other visits. This study therefore analysed the association between overall survival and percentage of ki-67 positive cells at the first visit.

The log rank test was first performed to determine differences in mortality between patients with high and low ki-67 positivity. A Kaplan-Meier curve of overall survival and percentage of ki-67 positive cells at first visit is shown in Figure 6.11. Patients who presented high ki-67 positivity at the first visit had mean survival time of

186 days (95%CI 121-250 days) while mean survival time of patients with low ki-67 positivity was 271 days (95%CI 233-308 days); the p-value for the difference between the two groups was 0.011. Median survival time for the high ki-67 population was 136 days (95%CI 99-174 days). However, there were too few deceased patients in the low ki-67 group to determine a meaningful median survival time. The longest survival time in this study was 151 days and the shortest survival time observed was 45 days after recruitment. Patients who were alive at the end of the project had a duration of observation ranging from 126 to 344 days.

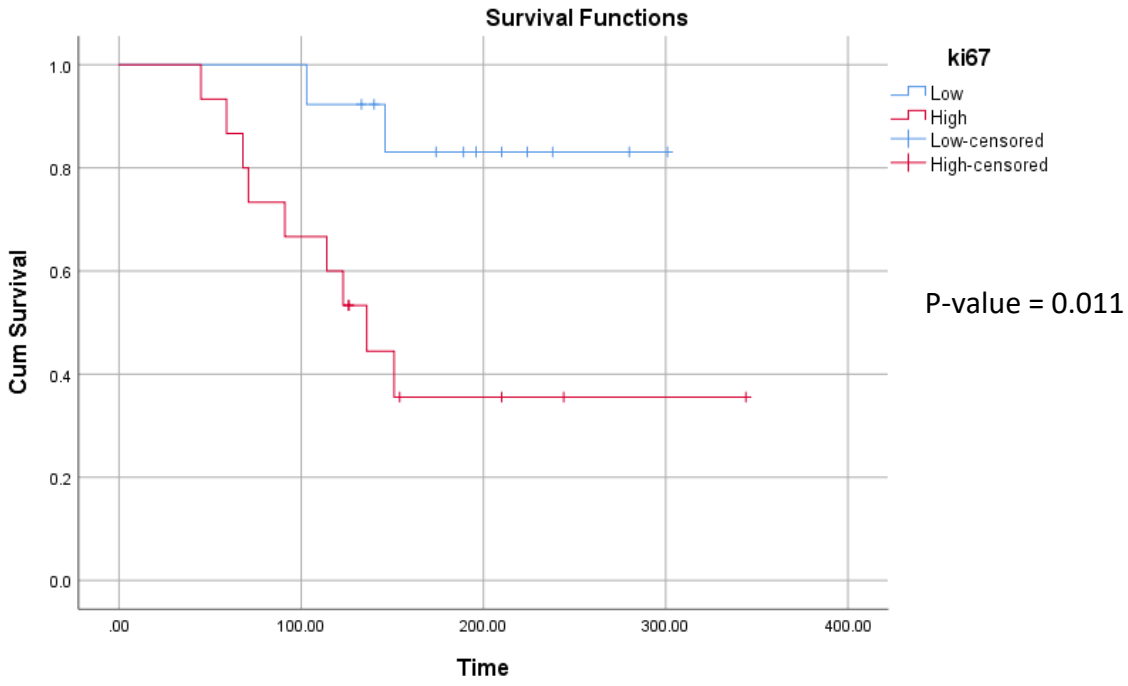


Figure 6.11. Kaplan-Meier curve of overall survival according to the percentage of ki-67 positive lymphoid cells at first visit.

Cox's proportional hazard model was next performed to test for correlations of overall survival and other variables, as well as percentage of ki-67 positive lymphoid cells. All continuous variables were analysed as continuous parameters except for the percentage of ki-67 positive cells, which was analysed as both continuous and categorical variables using the cut-off value from the ROC analysis (1.82%). The categorical variables included gender and performance status.

The Cox regression analysis results are shown in Table 6.4. Univariate analysis revealed significant correlations between overall survival and LDH level, Z-score of grip strength right hand and left hand with the hazard ratios of 1.006, 0.341 and 0.258, respectively. These variables were considered as covariates in the multivariate analysis of ki-67. Other variables, i.e. age, gender, weight, BMI, performance status, albumin level and WBC count, did not show correlations with overall survival and were not analysed further.

For ki-67 analysis, the results indicated a significant correlation between survival status and categorical ki-67 (p-value 0.025) but not continuous ki-67 values (p-value 0.363). The hazard ratio observed for categorical ki-67 was 5.86. However, when the three additional covariates were added to the multivariate equation, the association between categorical ki-67 and overall survival became non-significant, whilst LDH level remained as a significant variable.

Variable	**Exp(B)**	**SE**	**Sig.**	**95.0% CI for Exp(B)**	
				Lower	**Upper**
Univariate analysis					
Continuous ki-67 alone					
Continuous ki-67	1.120	0.125	0.363	0.877	1.432
Categorical ki-67 alone					
Categorical ki-67	5.860	0.786	**0.025**	1.255	27.357
Age alone					
Age	1.012	0.027	0.660	0.960	1.066
Gender alone					
Gender	1.190	0.607	0.774	0.362	3.913
Weight alone					
Weight	0.991	0.018	0.592	0.957	1.026
BMI alone					
BMI	1.000	0.058	0.995	0.893	1.121
Performance status alone					
Performance status	0.222	0.786	0.056	0.048	1.037

Variable	Exp(B)	SE	Sig.	95.0% CI for Exp(B)	
				Lower	Upper
LDH alone					
LDH	1.006	0.002	**0.007**	1.002	1.010
Albumin alone					
Albumin	0.918	0.073	0.243	0.796	1.060
WBC count alone					
WBC count	1.028	0.101	0.786	0.843	1.253
Grip strength right hand alone					
Grip strength right hand	0.341	0.427	**0.012**	0.147	0.787
Grip strength left hand alone					
Grip strength left hand	0.258	0.476	**0.004**	0.101	0.656
Multivariate analysis					
Ki-67, LDH and grip strength right hand					
Ki-67	4.931	0.905	0.078	0.836	29.08
LDH	1.005	0.003	**0.039**	1.000	1.011
Grip strength right hand	0.453	0.587	0.176	0.143	1.429
Ki-67, LDH and grip strength left hand					
Ki-67	24.53	1.669	0.055	0.931	646.2
LDH	1.009	0.004	**0.042**	1.000	1.018
Grip strength left hand	0.155	1.201	0.121	0.015	1.634

Note: categorical ki-67, gender and performance status were analysed as categorical variables; ki-67 was categorised by 1.82% cut-off value. Grip strength indicates Z-score of raw grip strength value which was calculated online via https://stuartbman.github.io/grip_strength/index.html

Table 6.4. Regression analysis of ki-67 expression in lymphocytes

The table shows coefficient value (Exp(B)), standard error (SE), p value (Sig) and 95%CI of all variables. The univariate analysis was performed to find significant variables and then multivariate analysis of those variables was performed. Significant value (p-value <0.05) is shown in bold.

6.3.6 Associations between ki-67 expression in patient's lymphocytes and plasma

Associations between percentages of ki-67 positive lymphoid cells and circulating ki-67 plasma concentrations were investigated. Whole blood samples from patients in the PROSPECT-NE trial were centrifuged to obtain plasma using the protocol mentioned in Chapter 2. Ki-67 concentrations in plasma were measured by a

commercial ELISA kit. An example standard curve generated with the ELISA kit is shown in Appendix A. The results obtained were compared to percentages of ki-67 positive lymphoid cells measured previously as well as overall patient survival data. Since the plasma ki-67 analysis project started after the ki-67 positive cells analysis, circulating ki-67 in plasma concentrations were only obtained for 26 out of 43 patients. In addition, of these 26 patients, only 16 provided samples at the first visit. The median circulating ki-67 concentration at the first visit determined from these 16 patients was 82.2 ng/mL (range: 19 – 346 ng/mL), while the median of all visits was 57.5 ng/mL (range: 9 – 346 ng/mL).

Concentrations of circulating ki-67 values measured in samples collected from the 26 patients were compared with the percentage of ki-67 positive lymphoid cells. The correlation analysis results suggested a significant correlation between the percentage of ki-67 positive cells and mean circulating ki-67 in plasma (Spearman, p-value 0.0141, Figure 6.12). As was observed with the percentage of ki-67 positive cells, high intra-assay variability with CV values up to 104% were observed in plasma ki-67 concentrations. High variability in the plasma ki-67 of each patient was also observed throughout the study with no clear trend of increase or decrease over time (Figure 6.13). The largest absolute increase was 52.9 ng/mL and largest decrease was 44.3 ng/mL. This very high intra-assay variability observed requires a better assay to be developed for future clinical use.

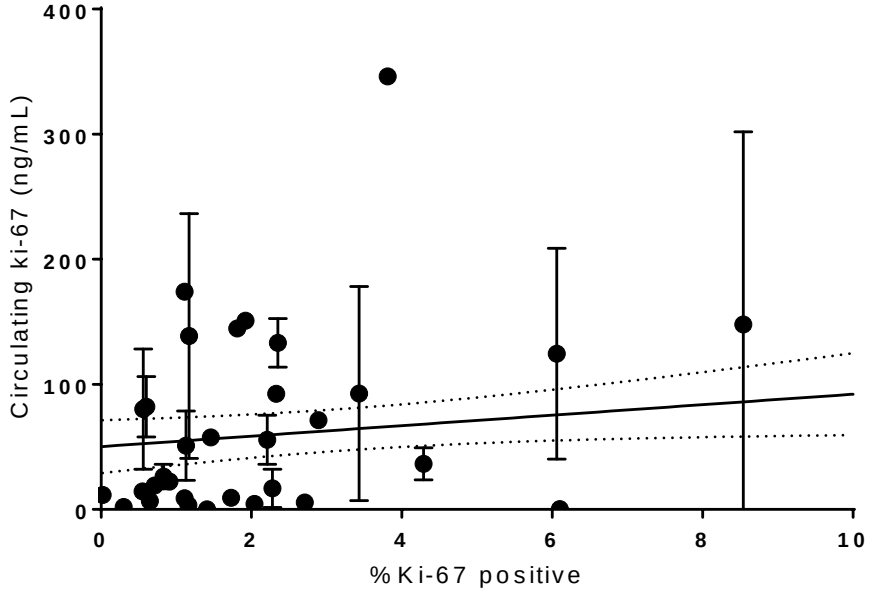


Figure 6.12. Association between percentage of ki-67 positive lymphoid cells and circulating ki-67 expression in plasma.

For samples where plasma ki-67 expression was measured three times, the mean of three values were used to correlate with ki-67 positive cells from the same blood sample.

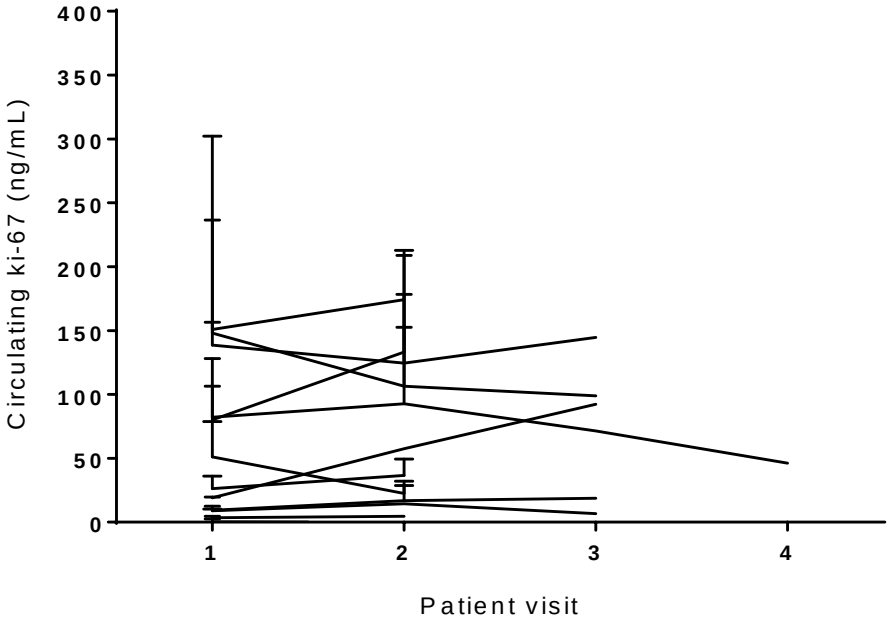


Figure 6.13. Changes in mean circulating ki-67 expression in plasma for all patients measured throughout the study.

Only patients who had more than one visit were included in the graphs.

Survival analyses were also performed to determine potential correlations between circulating ki-67 in plasma and overall survival. To investigate prognostic effect, circulating ki-67 values at the first visit of each patient were used. A histogram showing data for all 16 patient is shown in Figure 6.14, and the cut-point of 250 ng/mL was selected to categorise patient samples into low and high ki-67 groups based on this graph.

After categorisation, 58% of patients who were alive had low circulating ki-67 plasma concentrations as compared with 86% of deceased patients. The Kaplan-Meier curve indicated no significant difference between patients with high and low first-visit circulating ki-67 expression (log rank, p-value 0.229; Figure 6.15). The mean survival time for patients with high plasma ki-67 was 373 days (95%CI 314-434 days) while the comparable value for patients with low plasma ki-67 was 286 days (95%CI 211-361 days). Median survival times could not be estimated in either group due to the limited numbers of deceased patients at the end of the study.

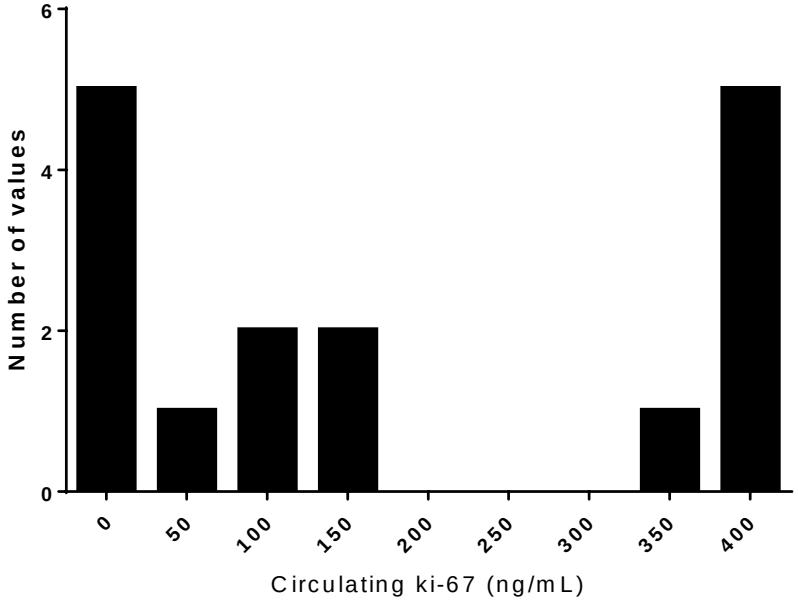


Figure 6.14. Histogram showing two distinct populations of circulating ki-67 in plasma from all patients.

The value shown for each patient was plasma ki-67 from the first visits of the particular patient.

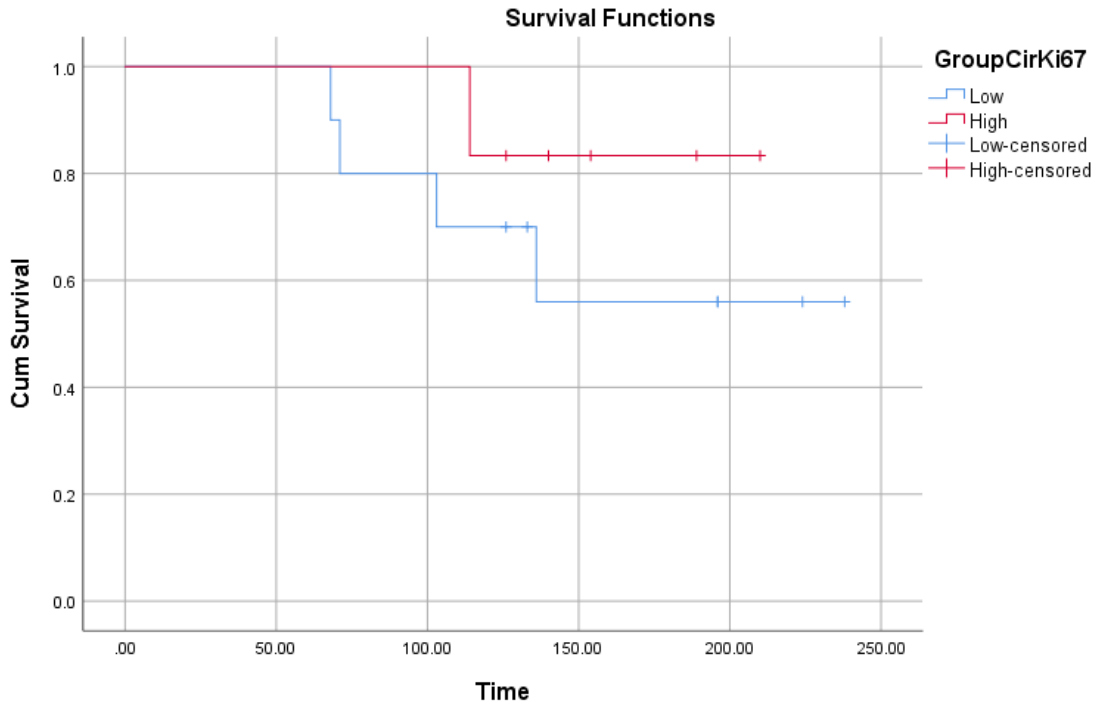


Figure 6.15. Kaplan-Meier curve of overall survival according to the circulating ki-67 plasma concentrations measured on the first patient visits.

Cox's proportional hazard model was also performed to confirm the log rank results. In contrast to the percentage of ki-67 positive cells, neither continuous nor categorical ki-67 expression in plasma correlated with overall survival (Table 6.5). Furthermore, univariate analysis revealed no significance between overall survival and variables including LDH and Z-score of grip strength. Therefore, multivariate analysis was not performed for circulating ki-67 in plasma.

Variable	Exp(B)	SE	Sig.	95.0% CI for Exp(B)	
				Lower	Upper
Continuous ki-67 alone					
Continuous ki-67	0.999	0.002	0.408	0.995	1.002
Categorical ki-67 alone					
Categorical ki-67	2.930	1.119	0.337	0.327	26.286
Age alone					
Age	1.056	0.046	0.231	0.966	1.156
Gender alone					
Gender	1.502	0.916	0.657	0.249	9.051
Weight alone					
Weight	0.965	0.039	0.355	0.895	1.041
BMI alone					
BMI	1.003	0.114	0.981	0.803	1.253
Performance status alone					
Performance status	0.302	1.121	0.285	0.034	2.714
LDH alone					
LDH	1.019	0.010	0.067	0.999	1.040
Albumin alone					
Albumin	0.986	0.106	0.898	0.801	1.215
WBC count alone					
WBC count	1.035	0.104	0.737	0.845	1.269
Grip strength right hand alone					
Grip strength right hand	0.348	0.558	0.058	0.117	1.038
Grip strength left hand alone					
Grip strength left hand	0.106	1.152	0.051	0.011	1.014

Table 6.5. Univariate analysis of circulating ki-67 in plasma.

The table shows coefficient value (Exp(B)), standard error (SE), p-value (Sig) and 95%CI of all variables. The variables included the first-visit circulating ki-67 expression in plasma (continuous and categorical variables), age, gender, weight, BMI, performance status, LDH, albumin, WBC count, and Z-score of grip strength right and left hand. ki-67 was analysed as both continuous and categorical variables; ki-67 was categorised by 250 ng/mL cut-off value. Grip strength indicates Z-score of raw grip strength value, which was calculated online via https://stuartbman.github.io/grip_strength/index.html. Significant value (p-value <0.05) is shown in bold

6.4 Discussion

The developed assay for the detection of ki-67 expression in lymphoid cells was applied to patient samples from the PROSPECT-NE clinical trial. The analysis of percentage of ki-67 positive WBCs, variability of ki-67 expression throughout the study, associations between ki-67 expression in lymphoid cells and patient clinical data including overall survival, and associations between circulating ki-67 in plasma and overall survival were investigated.

6.4.1 Percentage of ki-67 positive WBCs and its variability

The first observation in this study was the difference in ki-67 positivity between myeloid and lymphoid WBCs. The results indicated significantly higher numbers of ki-67 positive lymphoid cells than myeloid cells. The appropriate gating of lymphoid cells was confirmed using an antibody against CD14 to ensure monocytes were not being captured in the lymphoid gate on a FSC/SSC plot. As higher ki-67 expression was observed in lymphoid WBCs, the lymphoid population was selected for further study.

The percentages of ki-67 positive lymphoid cells in blood collected from cancer patients were significantly higher than observed in blood from healthy volunteers. Ki-67 expression in tumour tissues has been widely studied by IHC; in contrast, not many studies have assessed this parameter in WBCs (Todorov *et al.*, 1998; Uchikawa *et al.*, 2003; Bai *et al.*, 2016; Sobecki *et al.*, 2017; Ragab *et al.*, 2018). A study in patients with multiple myeloma reported higher percentages of ki-67 positive cells in T and B lymphocytes than in healthy adult volunteers (Miguel-Garcia *et al.*, 1995). A positive correlation between percentage of ki-67 positive cells and stage of multiple myeloma was also observed. Moreover, a pilot study revealed that the mean frequency of ki-67 positive WBCs in patients with hepatocellular carcinoma was significantly higher than in healthy volunteers (Hutton *et al.*, 2016). These differences therefore support the potential utility of ki-67 in peripheral white blood cells as a biomarker in cancer patients.

Although differences in ki-67 expression between different types of WBC have been observed in some studies as noted above, no single study has compared differences between cancer types. Comparison of ki-67 expression across cancer types

may suggest the application of this protein in some specific cancers. However, the results of this study were not able to identify differences in percentages of ki-67 positive lymphoid cells between cancer types, arguably due to the limited sample size for each cancer type. More patients in each cancer type should be recruited to future studies in order to further investigate the application of ki-67 positive cells as a prognostic biomarker more widely.

A challenging limitation of ki-67 as a prognostic/predictive biomarker may result from the high variability of ki-67 observed throughout the study. These results suggested marked differences in ki-67 expression between each visit for one particular patient, with up to a 15-fold change in ki-67 positive cells observed. These results demonstrate the highly dynamic modulation of ki-67 in peripheral WBCs, potentially relating to parameters which have not yet been elucidated. A plausible cause of ki-67 change is the medication that patients receive during visits to an oncology clinic, such as corticosteroids, hormonal steroids and steroid-like drugs. The efficacy of steroid receptor agonists including sex steroids in terms of inhibition of ki-67 expression has been widely studied (Wagner *et al.*, 2013; Urata *et al.*, 2014; Zhao *et al.*, 2017). For example, a study reported a significant decrease in ki-67 positive nasal epithelial cells when treated with oral methylprednisolone and nasal fluticasone for 3 weeks (Zhao *et al.*, 2017). The negative correlation between ki-67 in cancer specimens and vitamin D_3 or calcitriol levels in cancer patients has been demonstrated in two studies (Wagner *et al.*, 2013; Urata *et al.*, 2014). Administration of the aromatase inhibitor exemestane, has been found to be associated with a significant reduction in ki-67 index in breast cancer patients, whereas tamoxifen treatment resulted in the opposite effect (Hachisuga *et al.*, 1999; Toi *et al.*, 2011). Moreover, ki-67 expression has been shown to be affected by menstrual cycle phase; the same patients could have different ki-67 levels if the protein was measured at different phases of menstruation (Horimoto *et al.*, 2015).

This information highlights a limitation of the current study as there were no patient medication profiles available and the above-mentioned drugs, especially corticosteroids, could conceivably have been given to these patients. High intra-personal variation found in this study might result from a change in

medication/hormonal status at each visit. If patient medication profiles were available, this hypothesis could be investigated.

6.4.2 Associations between percentage of ki-67 positive lymphoid cell and clinical data

The level of inter-patient variation in percentage of ki-67 positivity observed in these studies led to an interest in utilising this parameter as a prognostic biomarker. In order to investigate the prognostic impact, correlations between ki-67 expression and other variables including key clinical parameters should be tested. Associations between ki-67 positive lymphoid cells at first patient visit, overall survival and other variables including patient age, weight, BMI, performance status, LDH, albumin, WBC count, Z-score of grip strength (of right hand and left hand) were analysed using different tests depending on the variable type.

Firstly, correlations between ki-67 expression and the above-mentioned parameters were analysed by Spearman correlation analysis for continuous variables and the Mann-Whitney U-test for categorical variables (gender and performance status). No correlations between ki-67 positive cells and any variables studied were observed (Figure 6.7). This contrasted with results obtained from a previous preliminary study performed locally, which observed significant negative correlations between ki-67 positive lymphoid cells and grip strength and between ki-67 positive lymphoid cells and age (Talks *et al.*, 2017).

Further analyses of all variables revealed a negative correlation between age and albumin level, and a positive correlation between age and WBC count. Based on previous studies, older patients were found to have lower albumin levels (Gom *et al.*, 2007; Weaving *et al.*, 2016) and WBC counts (Kubota *et al.*, 1991). An increase in WBC counts in older patients found in this study might suggest infectious conditions due to the frailty of old cancer patients. Similarly, the significant association between performance status and WBC count indicated that patients with higher performance status had higher WBC count and therefore higher chance to be infected patients. In addition, the analysis revealed that recruited male patients were significantly older than female, which conformed to studies showing a generally higher incidence of cancer in males across the older age ranges (White *et al.*, 2010; Torre *et al.*, 2016).

Cox regression analysis was performed to investigate associations between overall survival and other variables. Two variables that showed significant correlations with overall survival were LDH level and Z-score of grip strength. The hazard ratio indicated that higher level of serum LDH significantly correlated with patient survival status. This conformed to previously published studies which suggested positive correlations between LDH level and mortality rate in cancer patients (Zhang *et al.*, 2016a; Zhang *et al.*, 2016b; Deng *et al.*, 2018). For example, Liu *et al* reported very short overall survival time (1.7 months) in patients who had non-specific cancer with LDH >1,000 U/L and a significant increase in overall survival when serum LDH was decreased (Liu *et al.*, 2016). Moreover, several studies have reported a predictive effect of grip strength to all-cause mortality in old people; poorer grip strength being associated with a significantly increased risk of mortality including from cancer (Gale *et al.*, 2007; Chaturvedi, 2015; Oksuzyan *et al.*, 2017; Wu *et al.*, 2017). Therefore, LDH level and grip strength should be considered as covariates for survival analysis of ki-67.

Regarding the percentage of ki-67 positive cells, an association between the percentage of ki-67 positive cells and overall survival was investigated using the log rank test and Cox regression analysis. To perform the log rank test, percentages of ki-67 positive lymphoid cells were categorised into high and low populations based on the ROC curve. It was found that percentage of ki-67 positive cells at the first visit for each patient provided higher sensitivity and specificity to overall survival, as compared to the median number of ki-67 positive cells across all visits, so only the first-visit values were used in survival analysis.

Significant differences in survival rates between high and low ki-67 groups were observed following the log rank test analysis. However, Cox regression analysis revealed a significant correlation of overall survival with only categorical ki-67 (categorised into high and low levels) but not with continuous ki-67. Furthermore, categorical ki-67 became a non-significant parameter when adjusted for the two covariates above. These findings suggested that categorical ki-67 cell positivity might not be a strong prognostic indicator, especially when other variables are taken into account. Moreover, an optimal cut-off value is necessary to categorise ki-67 into positive and negative groups. Since there is no such gold standard cut-off value to

categorise ki-67, the utilisation of categorical ki-67 in clinical settings may be limited. This issue will be discussed later.

With regards to the fluctuation of percentages of ki-67 positive cells throughout the study, a feasible hypothesis was that changes in ki-67 positive cells were related to some unknown parameters in the blood. Due to the limited availability of blood tests in the PROSPECT-NE trial, only WBC count, albumin levels and LDH levels obtained on each visit were tested for their associations with ki-67 expression. The results suggested no correlation between ki-67 expression and these three variables. Consequently, causes of the high variability in ki-67 positive cells needed further investigation. Potentially levels of ki-67 protein were variable as a result of spontaneous physiologic changes or a response to medication that patients received. The most pragmatic experiment is testing the association between ki-67 expression in WBC and commonly administered drugs such as corticosteroids.

Several flow cytometry assays for measurement of ki-67 in white blood cells have been developed over many years, but rarely have they been used in clinical practice (Vukmanovic-Stejic *et al.*, 2008; Sanvito *et al.*, 2011; Marcondes *et al.*, 2018), thus the availability of data on prognostic impact of ki-67 expression in WBCs is very limited. All published studies have shown correlations between patient survival and ki-67 expression measured in tumour tissues by IHC. However, several studies have also shown the prognostic impacts of protein expression such as CTLA-4 and FOXP3 in WBCs, although their prognostic effects were first observed in tumour tissues (Shang *et al.*, 2015; Hu *et al.*, 2017). Furthermore, a study reported correlations between PD-L1 in tumour tissues and circulating WBCs which could be used to predict survival in patients with advanced-stage NSCLC (Ilié *et al.*, 2017). These studies suggested a possibility of associations between protein expression in tumour tissues and WBCs, including ki-67 expression.

Focusing on ki-67 expression in tissues, its prognostic impact has been well documented as previously described. According to two large meta-analysis studies, ki-67 expression in breast tumour tissues appeared to be a promising marker for overall survival and disease free survival; high expression of ki-67 was found to be correlated with shorter overall survival and disease free survival (de Azambuja *et al.*,

2007; Stuart-Harris *et al.*, 2008). Prognostic values of ki-67 were also shown in gastric, bladder and prostate cancer (Tian *et al.*, 2016; Berlin *et al.*, 2017; Luo *et al.*, 2017). There were two issues, however, that needed to be addressed before clinical application of these data.

Firstly, the thresholds of ki-67 positivity used in previous studies were variable. This reflected inconsistency of ki-67 expression between assays, and resulted in difficulty in compilation of all study data as well as difficulty in application of the results obtained from individual studies. The 2011 St.Gallen's International Expert Consensus firstly recommended a ki-67 cut-off point of 14% measured by IHC, but this value has not been widely used (Goldhirsch *et al.*, 2013). Another study, however, suggested that a cut-off value of 20% was the most effective prognostic factor (Tashima *et al.*, 2015). While unresolved, the American Society of Clinical Oncology (ASCO) guidelines do not include ki-67 expression as a biomarker for cancers (Harris *et al.*, 2007; Van Poznak *et al.*, 2015). Standardization of the most appropriate ki-67 positive threshold is needed to support the routine clinical application of ki-67 as a prognostic biomarker. In order to standardise a positive threshold for ki-67, a larger sample size of well-controlled patients is required, as well as a more reproducible method for ki-67 measurement.

Secondly, the compiled studies in those meta-analyses contained high heterogeneity in the assays used. Although all of them have involved immunohistochemistry, there were dissimilarities in clones of antibody, patient treatments and types of result presented between the compiled studies. It was found that clones of antibody used in the assays affected the signal of ki-67 expression (Lindboe and Torp, 2002; Sun *et al.*, 2016). The compiled studies in the above-mentioned meta-analysis included anti-ki-67, anti-MIB-1, anti-ki-s5 and anti-ki-s11 antibody clones which expectedly provided different values. Those values were then reported in diverse types such as mean, median, proportion, tertile distribution and arbitrary units. Furthermore, patients in some studies had been treated with hormonal therapy, some had been treated with only chemotherapy and some were untreated. As previously mentioned, some drugs could result in changes in ki-67 and the ki-67 levels measured might not reflect the actual prognosis of patients. This inconsistency means the optimal cut-off value for ki-67 still remains controversial.

Although there is no published study showing a correlation between ki-67 expression in WBC and cancer prognosis, the results of this study might suggest a feasibility of using ki-67 expression in WBC as a prognostic biomarker in solid cancers. More information between ki-67 expression and immune contexture in WBCs needs to be investigated. Moreover, higher number of patient samples in PROSPECT-NE trial as well as longer observational times should be considered for future work. Full patient profiles including all medication patients received should be collected to reduce any confounding factors to ki-67 expression.

6.4.3 Association between percentage of ki-67 positive lymphoid cells and circulating ki-67 in plasma

The hypothesised association between percentage ki-67 positive WBCs and circulating ki-67 expression in plasma was based on the results of two studies by Bruey *et al*, which reported an association between elevated plasma ki-67 and shorter survival time in both ALL and CLL patients (Bruey *et al.*, 2010a; Bruey *et al.*, 2010b). This chapter therefore investigated concentrations of ki-67 in plasma using ELISA techniques, as compared to percentages of ki-67 positivity in lymphoid cells measured by imaging flow cytometry.

The results indicated a significant positive correlation between ki-67 expression in plasma and percentage of ki-67 positive lymphoid cells. However, high intra-assay variability in plasma ki-67 was observed. Moreover, high intra-patient variability in plasma ki-67 levels at different visits were observed throughout the study, although it was less marked than the variability in percentages of ki-67 positive lymphoid cells. In contrast to percentage of ki-67 positive cells, a log rank test analysis indicated that plasma ki-67 did not correlate with overall survival. Also, cox-regression analysis suggested that neither categorical nor continuous circulating ki-67 expression in plasma was significantly correlated with overall survival. Nonetheless, it was noticeable that patients with high ki-67 expression in plasma tended to have higher survival rates (Figure 6.15).

These findings contrast to the previously mentioned studies (Bruey *et al.*, 2010a; Bruey *et al.*, 2010b). The difference observed in this study might result from two factors. Firstly, there were no patients with haematologic malignancies in the

PROSPECT-NE trial, so different types of cancer were studied between Bruey's work and this study. As Bruey's studies were performed in patients with leukaemia, with WBCs highly proliferated, they tended to have higher ki-67 expression levels in plasma than patients with solid tumours. The only study of serum ki-67 in solid tumour was from Ragab *et al*, but this study did not investigate the association between circulating ki-67 in serum and survival rate (Ragab *et al.*, 2018). It was a study in breast cancer patients and reported significant differences in plasma ki-67 expression between patients with breast tumours and healthy people, but no difference between benign and malignant tumours. Another possible explanation was that too few patients were studied and the observational time was too short in this survival analysis of circulating ki-67.

As a result of the limited number of studies conducted and the inconsistent data generated, the potential relevance of circulating ki-67 expression in cancer patient plasma or serum needs further investigation. Similar to ki-67 expression in tumour tissues and WBCs, a precise assay, an optimal cut-off value and drug-interaction data are necessary for further investigation of correlations of ki-67 expression in plasma. Furthermore, larger sample size and controlled patients such as comparable medication received are needed for future studies.

In conclusion, the developed imaging flow cytometry assay was applied to measure ki-67 positive white blood cells in cancer patients in the PROSPECT-NE clinical trial. The percentage of ki-67 positive lymphoid cells was found to be higher than the percentage of ki-67 myeloid cells and was higher in cancer patients than in people not known to have cancer. A correlation between overall survival and percentage of ki-67 positive lymphoid cells at first patient visit was observed when patients were categorised into high and low ki-67 groups. Furthermore, correlations between overall survival, LDH level and grip strength were observed in this study. However, only LDH level showed significance by multivariate analysis of three variables, so the prognostic effect of LDH should be studied in future works. Moreover, no significant correlations between ki-67 expression in plasma and overall survival were observed in this study. Larger sample sizes, longer observation times and more thorough patient profiles are recommended to confirm these findings in future studies.

Chapter 7. General discussion

The main objective of this PhD project was to investigate characteristics of protein expression in peripheral blood cells for the development of potential pharmacodynamic biomarkers for use in clinical trials in a cancer setting. The proteins of interest included CDC7, pMCM2, ki-67 and acH4. CDC7, pMCM2 and ki-67 were investigated as potential pharmacodynamic biomarkers for a phase I clinical trial (LY3143921), while acH4 was studied as a pharmacodynamic biomarker being utilised for a childhood cancer trial (SIOP Ependymoma II). An additional objective of the studies carried out was an investigation into the potential prognostic significance of the ki-67 protein within an early phase patient population as part of the PROSPECT-NE clinical study.

In order to investigate the pharmacodynamic properties of the proteins of interest, suitable assays were developed. The assay development processes for the clinical trials of interest included antibody optimisation, precision testing, and protein detection in cell lines and human WBCs. Lastly the applicability of the developed assays were assessed in patient samples participating in clinical trials. In the case of the LY3143921 trial, blood samples from mice treated *in vivo* were analysed ahead of future clinical sample analysis on an expansion phase of the trial which is yet to begin.

Patient samples from the PROSPECT-NE observational study were analysed for expression of the ki-67 protein using the previously developed assay. Correlations between protein expression and patient clinical data, including survival status, were investigated. Suitable statistical analyses, i.e. the Mann-Whitney U-test, Spearman correlation test and survival analysis, were performed as determined by the type of parameter being measured, the setting and the objectives of study.

7.1 Development of an assay for detection of CDC7, pMCM2 and ki-67

An imaging flow cytometry assay for detection of CDC7, pMCM2 and ki-67 has been developed for use in an early phase clinical trial of LY3143921, a novel CDC7 inhibitor. In addition to CDC7, pMCM2 was also chosen to be studied as it is a known

downstream protein of CDC7. Ki-67 protein, which is a well-known marker of cell proliferation, was included in this project since preliminary results showed cell proliferation inhibitory effects of the CDC7 inhibitor.

In order to develop an imaging flow cytometry assay, the volumes of antibody against CDC7, pMCM2 and ki-67 were first optimised. Precision of the assay including intra-assay and inter-assay precision, as well as precision of the imaging flow cytometry machine was tested. The results suggested less than 15% CV for all parameters investigated. This assay therefore offered the appropriate level of precision to measure protein expression in further samples.

Expression of the three proteins was first investigated in HL-60 cell lines, using an imaging flow cytometry technique. When cells were treated with different concentrations of LY3143921, only pMCM2 expression decreased in parallel with increasing concentrations of the drug. Neither CDC7 nor ki-67 showed any change following treatment. Similar to the cell line results, only pMCM2 expression decreased when healthy whole blood was treated *ex vivo* with different concentrations of the drug.

Although the CDC7 protein did not show any significant response to LY3143921 and was excluded from the assay, the unique characteristics of this protein were of interest. While pMCM2 and ki-67 proteins showed slightly higher expressions in lymphoid as opposed to myeloid cell populations, CDC7 proteins showed very low expression in lymphoid cells as compared to very high expression in myeloid cells. Moreover, it was found that the majority of CDC7 expression observed in this study was cytoplasmic expression, which was in contrast to pMCM2 and ki-67. As the developed assay may not be effective for detecting nuclear CDC7, this protein was not further investigated using this assay.

Another critical finding in this study was a new parameter for protein monitoring, i.e. percentage of protein positive cells based on the principal of rare cell populations. This study suggested that counting percentages of overexpressed cell showed greater dynamic changes than measuring intensity across all cells. As CDC7, pMCM2 and ki-67 are common proteins in normal cells, there will be basal expression levels of these proteins in all cells. These baseline expression levels could blind or

diminish a change in protein expression by drug treatment. Therefore, specifically looking at cells with overexpressed protein may provide better results, especially in comparison of changes.

Focusing on the percentage of protein positive cells, both *in vitro* and *ex vivo* results in this study suggested a decrease in percentage of pMCM2 positive cells in WBCs following an increase in drug concentration. Although the decrease in percentage of pMCM2 positive cells was not significant in all WBCs, a significant decrease was observed when assessed as a proportion of ki-67 positive cells. In the same way, *in vivo* results showed a significant decrease in pMCM2 positive cells at a lower dose of LY3143921 (15 mg/kg) when specifically considering the ki-67 positive population. These findings highlight the importance of utilising ki-67 and pMCM2 as a dual pharmacodynamic biomarker for CDC7 inhibition. Moreover, the results suggest a potential mechanism of the drug involving cell cycle arrest, which resulted in steady levels of ki-67 in *ex vivo* experiments.

With regard to the storage of samples, clinical blood samples need to be processed and transported from clinical sites to the laboratory, which can take up to 3 days. Although four commercial preservative-containing tubes including EDTA, CellSave®, TransFix® and Streck® tubes were examined, none of them were shown to be suitable, in terms of maintaining the percentage of pMCM2 positive in ki-67 positive cells for 3 days. However, it was found in this study that freezing WBCs in diluted Lyse/Fix buffer at -80°C was effective in terms of preserving the cell population for up to 5 days. This approach will therefore be adopted at allow delivery of clinical samples to the analysing laboratory, without impacting on the validity of the data generated following analysis.

In conclusion, this PhD project has developed and validated an assay for detection of pharmacodynamic biomarkers for a new CDC7 inhibitor, LY3143921, by measuring the number of cells with overexpression of pMCM2 in the population of ki-67 positive cells. Furthermore, the freezing method for preservation of proteins during delivery has been validated. The assay is therefore ready to be applied to clinical sample analysis when the expanded phase of the clinical trial starts. Also, the applicability of this assay to other CDC7 inhibitors should be assessed in future work.

7.2 Development of an assay for detection of acH4

An imaging flow cytometry assay for detection of acetylated histone H4 has been developed and validated for the analysis of clinical samples collected from patients on the SIOP Ependymoma II clinical trial. Patients in this clinical trial receive valproic acid which acts as an HDAC inhibitor. Since acH4 was considered as a potential pharmacodynamic biomarker for HDAC inhibitors, it was selected as a protein of interest in this study. The acH4 assay validation included antibody volume optimisation, intra- and inter-assay precision, and application in cell lines (*in vitro*) and human WBCs (*ex vivo*) before applying the assay to actual clinical samples.

The acH4 assay was developed appropriately and exhibited intra- and inter-assay precision of <20% CV for both parameters. The *in vitro* results revealed increases in acH4 expression following increases in both valproic acid concentration and duration of treatment. Based on the results obtained, the minimum effective valproic acid concentration in these studies was 1 mM and the maximum effective concentration was 4 mM. Moreover, the peak expression of acH4 was observed at 6 hours of drug treatment (4mM) with longer durations of treatment than 6 hours reduced the expression of acH4 in cell lines. The reason for this decrease has not been investigated in this project but should be further studied in order to more clearly understand the PK/PD of the drug.

Focusing on the expression of acH4 protein, this study compared changes in acH4 by monitoring two different parameters, i.e. mean/median intensity of acH4 expression and percentage of acH4 positive cells. A greater dynamic change (higher fold-increase) was noted in percentage of acH4 positive cells compared to mean intensity. In agreement with the CDC7, pMCM2 and ki-67 assays, percentage of cells with protein overexpression was considered to be a more informative parameter than overall protein intensity, especially when used for comparison of change. Moreover, higher expression of acH4 was observed in lymphoid than in myeloid WBCs. This supported the use of PBMCs as a surrogate cell for measurement of acH4 in previous studies. However, the results in chapter 4 revealed higher fold-increases in the myeloid population following an increase in valproic acid concentration, as compared to

lymphoid cells. Based on these findings, this study focused on changes of acH4 in myeloid cells rather than lymphoid cells.

Another characteristic of acH4 expression in myeloid cells noted in this study was an increase in cytoplasmic acH4 expression. Based on the similarity between acH4 and DAPI (nuclear) expression, more cytoplasmic acH4 positive cells were observed in myeloid WBCs treated with 8 mM of valproic acid as compared to lymphoid population with the same condition. Furthermore, the majority of cytoplasmic acH4 positive myeloid cells showed higher acH4 expression than nuclear acH4 positive cells. To date, there is only limited information relating to expression of cytoplasmic acH4 protein, although it has been reported in a previous study (Uchida *et al.*, 2007). The characteristics and importance of this cytoplasmic acH4 should be investigated in future studies.

To conclude, an imaging flow cytometry assay for detection of acH4 expression in WBCs was validated in both cell lines and WBCs. The results obtained resulted in the proposal of myeloid WBCs as a novel cell population for acH4 monitoring. Also, the results suggested that percentage of acH4 positive cells, rather than overall acH4 intensity, should be utilised to measure acH4 changes induced by valproic acid. Altogether this assay was suitable to be applied to patient samples from the Ependymoma trial.

7.3 Measurement of acH4 positivity in Ependymoma patients and its pharmacodynamic significance

Whole blood samples from seven patients recruited to the SIOP Ependymoma clinical trial were analysed in terms of measurement of acH4 expression using the developed assay described in 7.2. Patient samples were collected pre- and 4-hour post-valproic acid treatment at different dose levels. The percentage of acH4 positive myeloid cells was analysed and compared both between and within patients. Moreover, associations between the percentage of acH4 positive cells and dose and concentration of valproic acid were examined. Lastly, the association between free and total valproic acid concentrations in patient samples was also studied.

Both intra- and inter-patient results revealed high heterogeneity in terms of percentage of acH4 positive cells. Within a patient, not all post-treatment samples showed increases in percentages of acH4 positive cells compared to pre-treatment. Also, the increase in acH4 positive cells did not follow the duration of treatment; patients who had been treated for longer periods of time possibly had lower acH4 positive cells than those who received valproic acid for the first time. However, when comparing the results of the seven patients, no clear trends were observed. When comparable doses of valproic acid, e.g. 15 mg/kg were administered to patients, the post-treatment acH4 responses were different between each patient. Furthermore, patients who had been treated with the same dose for the same duration showed different levels of change in acH4 positive cells. These findings conformed to the results from several published clinical studies as discussed in Chapter 5.

With regards to observed trends in acH4 positive cells, the results showed no associations between percentage of acH4 positive cells and valproic acid dose, nor total drug concentration. No associations were observed in terms of actual values, absolute change (difference) and fold change. These findings were supported by other clinical studies showing no correlation of acH4 protein with valproic acid dose, concentration or clinical outcome (Garcia-Manero *et al.*, 2006; Atmaca *et al.*, 2007; Sharma *et al.*, 2008; Su *et al.*, 2011; Wheler *et al.*, 2014). However, it should be noted that the concentrations of valproic acid in all patients in this study were much lower than the effective concentrations found in the pre-clinical results as discussed in chapter 4. These low concentrations may be associated with only modest changes in acH4 positivity and result in no correlations being observed with any of the parameters investigated.

Correlations between total and free valproic acid concentrations were observed in this study as shown in previously published studies (Otten *et al.*, 1984; Herngren *et al.*, 1991; Su *et al.*, 2011). Furthermore, the free valproic acid fraction was found to be increased following an increase in total valproic acid concentration and no associations were observed between free valproic acid concentrations and parameters including percentage of acH4 positive cells.

Overall, the developed assay was applied to clinical samples from the SIOP Ependymoma II trial and percentages of acH4 positive myeloid cells in all 7 patients were presented and discussed. No trends or changes in acH4 positivity between pre- and post-treatment were observed, or between the first dose and later doses in each patient. Also, there were no associations between the percentage of acH4 positive cells and parameters including dose, total concentration or free concentration of valproic acid. The limitations of this study included too few patient samples and low drug concentrations in plasma samples obtained from patients on the study.

7.4 Measurement of ki-67 positivity in PROSPECT-NE patients and its prognostic impact

The assay for detection of ki-67 expression in WBCs was adapted from the combined assay developed for CDC7, pMCM2 and ki-67. This assay was applied to measure ki-67 positive cells in patient samples from the PROSPECT-NE observational study. The ki-67 results were studied in association with patient data and survival status to investigate its potential prognostic significance. Moreover, the potential prognostic impact of circulating ki-67 in plasma was also investigated in this study.

The patient results obtained indicated higher percentages of ki-67 positive cells in lymphoid as compared to myeloid WBCs. Unlike the SIOP Ependymoma clinical trial, PROSPECT-NE is an observational study with no defined treatment protocol. Lymphoid cells were considered to be a suitable surrogate population due to their relatively high level of ki-67 expression. Focusing on ki-67 positive lymphoid cells, the results in cancer patients from the PROSPECT trial showed higher ki-67 positivity than in healthy volunteers. However, no significantly high ki-67 expression was observed in any specific type of cancer.

Marked variation in ki-67 expression levels in some patients was highlighted, and this contributed to limitations in terms of interpretation of the results. Some patients had fluctuations of ki-67 throughout the study without any treatment, so the cause of change was unclear. Regarding the deceased patients in this study, no clear trend in ki-67 levels was observed, thus ki-67 was unlikely to change following patient

illness such as performance status and tumour stage. Correlation analysis performed in this study also showed no correlation of ki-67 with patient data including age, weight, BMI, LDH, albumin, WBC count or Z-score of grip strength (right hand and left hand). Based on these results, it is currently unclear what caused the high variability of ki-67 levels observed in this study.

Although fluctuations in percentage of ki-67 positive cells were observed throughout the study, the percentage of ki-67 positive cells at the first visit of each patient appeared to be correlated with patient survival status. The survival analysis suggested that patients with a high percentage of ki-67 positive cells had significantly lower mean survival times as compared to those with low percentages of ki-67 positive cells. However, there was no association with overall survival when actual numbers of ki-67 positive cells, not categorised into high and low groups, were analysed. Furthermore, when ki-67 positive cell numbers were adjusted based on LDH and Z-score of grip strength, it lost the significance in survival analysis. The actual relationship between percentage of ki-67 positive cells and prediction of patient prognosis therefore needs further investigation.

Moreover, based on the published results by Bruey et al (Bruey *et al.*, 2010a; Bruey *et al.*, 2010b), this study investigated associations between circulating ki-67 in patient plasma and overall survival. In contrast with the mentioned studies, no correlation between the two parameters was observed. Also, no association between plasma circulating ki-67 expression and percentage of ki-67 positive cell was observed in the PROSPECT-NE study. However, it was found that patients with high circulating ki-67 expression generally had longer mean survival times than patients with low ki-67 expression. The prognostic impact of circulating ki-67 expression in plasma needs to be further studied.

The results obtained in this study suggest a potential prognostic impact of ki-67 positive cells. However, due to the unavailability of complete patient datasets and low sample size, this effect should be analysed with more detailed patient information and a larger number of patients in the future. Also, a longer observation time is needed in order to fully investigate the relationship between ki-67 and patient survival.

7.5 Conclusion

This PhD project mainly aimed to develop imaging flow cytometry assays for detection of proteins as potential pharmacodynamic biomarkers for three particular studies, i.e. the LY3143921 phase I clinical trial, the SIOP Ependymoma II clinical trial and the PROSPECT-NE observational study. The protein characteristics and associations of protein expression with relevant clinical parameters were studied.

The assay for LY3143921 clinical study has been developed and validated to measure the expressions of CDC7, pMCM2 and ki-67 in WBCs. The results showed high precision of the assay and capability to detect changes in pMCM2 with LY3143921 drug treatment, most noticeably within a ki-67 positive cell population. Moreover, a clinically relevant freezing method for storage of proteins during delivery was validated. The clinical trial is currently recruiting patients to the dose escalation phase of the study, with the developed pharmacodynamic assay proposed to be used for the analysis of samples in the planned expanded phase of the study.

A pharmacodynamic assay for the SIOP Ependymoma trial has been developed and validated to measure acH4 expression in myeloid WBCs. *In vitro* and *ex vivo* results indicated high precision of the assay as well as a potential to detect protein changes following drug treatment. However, clinical results from seven patients studied to date have shown marked intra- and inter-patient variability and no associations have been observed between percentage of acH4 positive cells and either dose or concentration of valproic acid. Low concentrations of valproic acid in patient plasma alongside small numbers of patient samples represent clear limitations to this study and future work should take these issues into account.

For the PROSPECT-NE study, an assay was applied to measure ki-67 expression in lymphoid WBCs. The results showed no correlations with the available patient data, nor the patient overall survival. Although when ki-67 positivity was categorised into high and low groups, it showed a potential prognostic impact, multivariate analysis revealed no significance for ki-67 expression when adjusted with LDH and Z-score of grip strength. The prognostic effect of percentage of ki-67 positive cells should be further investigated, with larger numbers of patient samples studied and more detailed patient information made available.

In summary, three assays for detection of proteins of interest including CDC7, pMCM2, ki-67 and acH4 were developed and validated and their clinical utility shown. A number of important lessons have been learnt which have applicability to the development of similar assays in the future, and the practical aspects of developing assays for use in clinical trials involving the analysis of clinical samples in real-time. Future studies should include more patient samples, longer observational times and more complete patient datasets.

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Appendix A. Association between percentage of ki-67 positive lymphoid cells and patient data

Since this study analysed both percentage of ki-67 positive lymphoid cells at the first visit and the median value of all visits, the difference in ki-67 categorisation by different values is promising. The table A1 compares classification of each patient using percentage of ki-67 positive cells at the first visit, the median percentage of ki-67 positive cells of all visits. Difference in ki-67 values determined by different parameters is also presented.

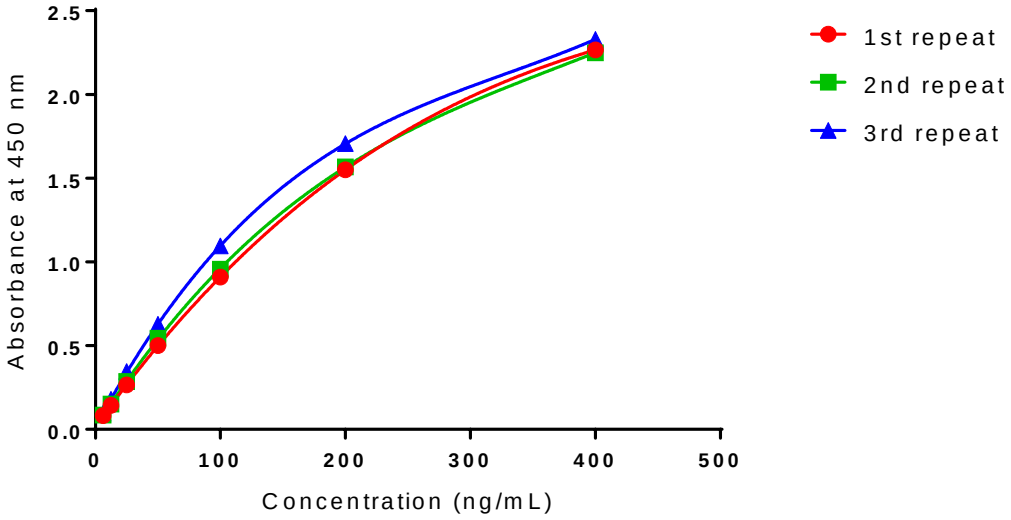
%ki-67 at 1st visit		Median ki-67 of all visits		
Value	**Classification (Cut-off 1.82%)**	**Value**	**%difference from 1st visit**	**Classification (Cut-off 1.61%)**
0.48	Low	0.48	0.00	Low
1.07	Low	1.07	0.00	Low
1.21	Low	1.21	0.00	Low
1.77	Low	1.24	-30.23	Low
1.41	Low	1.41	0.00	Low
0.56	Low	1.46	159.82	Low
0.71	Low	1.46	105.63	Low
2.16	High	1.62	-25.00	High
1.92	High	1.52	-21.09	Low
2.57	High	1.54	-40.08	Low
1.16	Low	1.60	37.93	Low
1.85	High	1.85	0.00	High
5.68	High	0.88	-84.51	Low
1.79	Low	2.11	17.88	High
2.21	High	2.21	0.00	High
0.60	Low	2.75	357.50	High
2.71	High	2.71	0.00	High
2.82	High	2.82	0.00	High
2.97	High	2.97	0.00	High
1.17	Low	1.81	54.70	High

%ki-67 at 1st visit		Median ki-67 of all visits		
Value	**Classification (Cut-off 1.82%)**	**Value**	**%difference from 1st visit**	**Classification (Cut-off 1.61%)**
1.75	Low	1.26	-28.00	Low
3.81	High	3.30	-13.52	High
6.10	High	3.30	-45.90	High
2.05	High	3.17	54.63	High
1.01	Low	2.01	98.51	High
6.95	High	4.48	-35.61	High
4.49	High	4.49	0.00	High
8.54	High	16.20	89.70	High

Table A1. Difference in classification of patients using different values and different cut-points

The table shows difference between percentage of ki-67 positive lymphoid cells at the first visit with cut-off value 1.82% and median percentage of ki-67 positive lymphoid cells of all visits of a patient with cut-off value 1.61%.

The standard curves used for the analysis of absorbance by ELISA tests are showed in figure A1 together with three equations from three repeats. Four parameter logistic regression was used as recommended by the manufacturer. The standard curves ranged from 6.25 – 400 ng/mL. The concentration of sample which was lower than 6.25 ng/mL was identified as less than the lower limit of quantification (<LLOQ) and the value was set to 0 ng/mL. The concentration of sample which was higher than 400 ng/mL was identified as greater than the upper limit of quantification (>ULOQ) and the value was set to 400 ng/mL. Both <LLOQ and >ULOQ values were not compared with percentages of ki-67 positive cells but were still used in the survival analysis.



Equation 1st repeat: $y = -1E\text{-}10x^4 + 9E\text{-}08x^3 - 3E\text{-}05x^2 + 0.0115x$ $R^2 = 1$

Equation 2nd repeat: $y = -1E\text{-}10x^4 + 9E\text{-}08x^3 - 4E\text{-}05x^2 + 0.0125x$ $R^2 = 1$

Equation 3rd repeat: $y = -1E\text{-}10x^4 + 1E\text{-}07x^3 - 5E\text{-}05x^2 + 0.0148x$ $R^2 = 1$

Figure A1. Standard curves of the absorbance and concentration of ki-67 measured by a commercial ELISA kit along with the curve-fit equations calculated using the MS Excel software.

Samples were analyzed three times, so three different equations were used.

Appendix B. Conference abstracts

Postgraduate Cancer Conference 2017, Newcastle University, Newcastle upon Tyne, UK – Oral presentation

Characterisation of rare cell phenotypes in peripheral blood and utilisation as a surrogate tissue in pharmacodynamic biomarker assays

Suriyon Uitrakul

Introduction: LY3143921 is novel small molecule CDC7 inhibitor being investigated in early phase trials. To demonstrate mechanism of action pharmacodynamic biomarker assays are required. MCM2 is phosphorylated by CDC7 to yield pMCM2 which is detectable by a phosphospecific antibody. Ki-67 is a marker of proliferation.

Objective: To develop and validate an imaging flow cytometry assay to detect pMCM2 and ki-67 in circulating haematopoietic cells as a surrogate tissue to demonstrate LY3143921 mechanism of action.

Method: Eighteen mice were divided into 3 equal groups and received 15 or 50 mg/kg LY3143921 p.o. or vehicle. Blood was taken from 3 mice per group pre-administration and at 3, 6, and 24h post-administration. Red blood cells were lysed and white blood cells (WBCs) fixed in formaldehyde before storage in methanol at -20°C. WBCs were incubated with antibodies to pMCM2, ki-67 and DAPI for 1h. Expression was measured by imaging flow cytometry.

Result: The percentage of pMCM2 positive cells in the ki-67 positive WBC population at 0, 3, 6 and 24 hours post-LY3143921 treatment were 70.0%, 57.5%, 41.6% and 38.6% (15 mg/kg group); 56.6%, 50.8%, 42.6% and 13.9% (50 mg/kg group) and 62.0%, 67.5%, 70.0% and 71.3% for control animals. Two-way ANOVA showed significance in both duration of treatment and dose (p-values 0.035 and 0.024, respectively). The frequency of ki-67 positive cells was not affected by LY3143921 treatment.

Conclusion: The assay is appropriate for analysis of changes in frequency of pMCM2 positivity as a percentage of ki-67 positive cells over time, following administration of LY3143921.

American Association for Cancer Research (AACR) Annual Conference 2018, Chicago, Illinois, USA – Poster presentation

Imaging flow cytometry assay development and validation for the detection of histone H4 acetylation in white blood cells

Suriyon Uitrakul, Gareth James Veal, Claire Hutton, David Jamieson

Introduction: Histone deacetylases have been identified as oncogenes in several cancer types, providing an attractive target for anticancer treatment. In this respect, the histone deacetylase inhibitor valproic acid has been shown to inhibit the growth of multiple paediatric tumour types and is well tolerated in children with refractory solid or CNS tumours.

Objective: The aim of the current study was to develop and validate a novel imaging flow cytometry method for the detection of histone H4 acetylation in lymphoid and myeloid cell populations, and to assess the applicability of the method to a clinical trial setting.

Method: HL-60 cells and whole blood samples from healthy volunteers were incubated with valproic acid (0.5-8 mM) for 0.5-24 hours, followed by RBC lysis for the whole blood samples and fixed with cold methanol. Additional blood samples were collected from patients with ependymoma who were receiving valproic acid as part of the SIOP Ependymoma II clinical trial. An imaging flow cytometry method was developed using an ImageStream$^{\chi}$ flow cytometer, collecting WBCs with excitation of PE conjugated acH4 antibody and DAPI. Data were collected using Inspire™ software and further analysed by Ideas™ software 6.2. Both in vitro and ex vivo experiments were repeated on at least 3 occasions.

Result: In the HL-60 cell line the mean percentage of acH4 positive cells was 1.98% in the vehicle control sample, increasing to 10.9-77.9% when treated with 0.5-8 mM valproic acid for 6 hours, with percentages of 8.7-49.0% observed following incubation with 4 mM valproic acid for 0.5-24 hours. Comparable data were generated in lymphoid and myeloid WBC populations following ex vivo incubation of whole blood samples with valproic acid. Increases in the percentage of acH4 positive cells were observed in samples collected at 4 hours post-administration in patients receiving valproic acid as compared to pre-treatment samples. Myeloid cells appeared to have a smaller proportion of acH4 positive cells than observed in the lymphoid population but a greater fold increase above basal levels.

Conclusion: A new assay for detection of histone H4 acetylation in WBCs by imaging flow cytometry has been developed and optimised. The assay showed increases in acH4 positivity in both in vitro and ex vivo experiments following exposure to valproic acid. The method can be used for the measurement of acH4 as a pharmacodynamic biomarker for histone deacetylase inhibitors in drug development and monitoring of drug efficacy in clinical trials.

Appendix C. Publication

Uitrakul, S., Hutton, C., Veal, G.J. and Jamieson, D. (2019) 'A novel imaging flow cytometry method for the detection of histone H4 acetylation in myeloid cells', European Journal of Clinical Investigation, 49(7), p. e13115.

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ORIGINAL ARTICLE

WILEY

A novel imaging flow cytometry method for the detection of histone H4 acetylation in myeloid cells

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Abstract

Background: The histone deacetylase inhibitor (HDACI) valproic acid has been shown to inhibit the growth of multiple paediatric tumour types and is well tolerated in a childhood cancer setting. The current study was designed to develop a novel imaging flow cytometry method for the detection of histone H4 acetylation in white blood cells obtained from childhood cancer patients treated with valproic acid.

Materials and methods: HL-60 cells and whole blood samples from healthy volunteers were incubated with valproic acid (0-8 mM) for 0-24 hours, with additional blood samples collected from ependymoma patients receiving valproic acid on the SIOP Ependymoma II clinical trial. An imaging flow cytometry method was developed using an ImageStream$^{®X}$ flow cytometer, collecting 100 000 images per sample following excitation of PE tagged acH4 antibody and DAPI.

Results: The mean percentage of acH4-positive cells increased to a greater extent than increases in mean and median fluorescence intensity following incubation with valproic acid. Comparable results were observed for in vitro and ex vivo experiments, and the assay was shown to be appropriate for clinical sample analysis. Myeloid cells exhibited a smaller proportion of acH4-positive cells than the lymphoid population, but a greater fold increase above basal levels.

Conclusions: The percentage of acH4-positive myeloid cells has the potential to be used as a robust pharmacodynamic biomarker for the measurement of acH4 for HDACIs. The developed assay is now being utilised in a clinical trial involving the treatment of childhood ependymoma patients with valproic acid.

KEYWORDS
biomarkers, cancer, histone H4 acetylation, imaging flow cytometry, valproic acid

1 | INTRODUCTION

Histone deacetylase inhibitors (HDACIs) represent a novel type of anticancer drug which have been studied over a number of years since the association between HDAC expression and cancer was first observed.[1,2] This class of drugs includes valproic acid, a well-known anticonvulsant, which has been shown to exhibit HDAC inhibitory activity in a cancer setting.[4] In the modern era of cancer therapy, pharmacodynamic biomarkers play increasingly important roles in furthering our understanding of drug efficacy, with the development of novel and informative biomarker assays representing a key area of research.

Many proteins can be used as biomarkers of drug activity and histone H4 acetylation (acH4) has become a commonly used biomarker for measurement of HDACI activity in preclinical and clinical studies.[5,6] Modulation of acH4 has been measured by many techniques including Western

Blot, ELISA, immunofluorescence and flow cytometry, but no imaging flow cytometry methods have been published to date.[7,8] Furthermore, published assays have focused on the populations of peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leucocytes (PMNLs), but none have investigated differences in acH4 expression between these populations.

The current study therefore aimed to develop and validate a novel imaging flow cytometry protocol for the detection of histone H4 acetylation by quantitative image analysis of nuclear acH4 signal, including studies to investigate differences in acH4 expression between myeloid and lymphoid WBC populations.

2 | MATERIALS AND METHODS

2.1 | Reagents

Valproic acid sodium salt and RPMI-1640 medium were from Sigma® Life Science. The acH4 antibody conjugated to PE was from Merck Millipore. DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) was from BioLegend® (California, United States). BD Phosflow™ Lysis/Fix Buffer was obtained from BD Biosciences. Bovine serum antigen (BSA) MACS® buffer was provided by Miltenyi Biotec Ltd. PBS was supplied by Thermo Fisher Scientific.

2.2 | Cell lines

Authenticated HL-60 (Human promyelocytic leukaemia) cells were cultured routinely in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% foetal bovine serum (FBS). Cells were grown in 75 mm^2 flasks, incubated at 37°C in a 5% CO_2 incubator.

2.3 | Blood samples

Whole blood from healthy volunteers was obtained from staff members in the Northern Institute for Cancer Research (Newcastle University) following standard operating procedures. All blood samples were drawn from volunteer donors by registered doctors and were transferred directly to blood tubes. Additional blood samples were collected from patients with ependymoma who were recruited as part of the SIOP Ependymoma II clinical trial (ClinicalTrials.gov identifier: NCT02265770). This trial is designed to investigate the efficacy of combined chemotherapy including valproic acid, for children and young adults with ependymoma. Valproic acid (15 mg/kg) was administered orally to randomised patients two or three times daily, with doses adjusted in individual patients to achieve target trough plasma concentrations of 100-150 μg/mL (690-1040 μM). Appropriate ethical approval and written informed consent for study patients were obtained. Samples for analysis of acH4 were collected immediately before the initiation of valproic acid treatment (pretreatment baseline), at 4 hours post-administration and at trough levels during continuous therapy across a range of valproic acid dose levels. Whole blood samples were taken from patients and directly added to prepared Lyse/Fix Buffer. Samples were then frozen and transported on dry ice to the Northern Institute for Cancer Research, Newcastle University, within 2 days.

2.4 | In vitro valproic acid treatment

HL-60 cells (1×10^6) were seeded in 2 mL RPMI-1640 medium supplemented with 10% FBS in 6-well plates. Valproic acid was dissolved in RPMI medium to make a stock solution of 100 mM. The stock solution was diluted with RPMI medium by serial dilution to obtain final valproic acid concentrations of 0.5, 1, 2, 4 and 8 mM, with RPMI alone included as a negative control, and the plate was incubated at 37°C, 5% CO_2 for 6 hours. Additional experiments investigated the effect of a fixed concentration of valproic acid (4 mM) at 0.5, 1, 3, 6 and 24 hours. Collected samples were washed with PBS and fixed in −20°C methanol overnight. Experiments were repeated three times.

2.5 | Ex vivo valproic acid treatment

Whole blood samples from healthy volunteers were collected in EDTA tubes, then divided into Falcon™ tubes (2 mL blood/tube). Valproic acid sodium salt was dissolved in RPMI-1640 medium to obtain a final concentration of 100 mM. The drug solution was added to each blood tube to generate final concentrations of 0.5, 1, 2, 4 and 8 mM with RPMI included as negative control. Tubes were incubated in a shaking incubator at 200 rpm, 37°C for 6 hours. All blood samples were lysed and fixed using the protocol described below. This experiment was repeated three times with three independent donors.

2.6 | Red blood cell lysis

Red cells in whole blood samples were lysed with Lyse/Fix Buffer, diluted in distilled water (1:5) and added directly to the whole blood tube (20 mL of diluted lysis buffer per 1 mL of blood). The solution was mixed by inversion, incubated at 37°C for 10 minutes and centrifuged at 500 g for 10 minutes. The supernatant was gently poured off and the cell pellet re-suspended in 5% BSA in PBS for 30 minutes. The tube was centrifuged again at 500 g for 5 minutes and the cell pellet re-suspended in methanol for permeabilisation. The white blood cell suspension was stored at −20°C for at least 24 hours.

2.7 | Flow cytometry

Cell pellets were re-suspended in PBS at a density of 1×10^6 cells and incubated in 1 mL of 5% BSA in PBS for 1 hour. Cells were then centrifuged at 500 g for 5 minutes and 90% of the supernatant aspirated. Approximately 100 µL of each cell suspension was incubated with 1 µL of acH4 antibody at 2-8°C overnight. Samples were washed with 1 mL of 5% BSA in PBS, re-suspended in 100 µL of 5% BSA in PBS with 5 µL of DAPI and incubated at room temperature for 1 hour. Cells were washed with 1 mL of 5% BSA in PBS and the acH4 signal measured by imaging flow cytometry.

2.8 | Imaging flow cytometry analysis

Imaging flow cytometry was performed using an ImageStream$^{®\chi}$ flow cytometer (Amnis), collecting 100 000 images per sample. The instrument was set to excite the PE tagged acH4 antibody and DAPI by 488 nm (100 mW) and 405 nm (30 mW) lasers, respectively. Data were collected using Inspire software and further analysed by Ideas software 6.2. The process of data collection and gating used in this study is illustrated in Figure S1 (supplementary data).

2.9 | Statistical analysis

Mean and standard deviation (SD) values were calculated for all experiments. One-way ANOVA was performed to compare results obtained from in vitro and ex vivo experiments. A paired *t* test was used for the comparison of myeloid and lymphoid acH4 positivity. All statistical data were generated using Prism 6.0 software.

3 | RESULTS

3.1 | Assay validation in cell lines

In order to investigate acH4 expression using the proposed method, HL-60 cells were treated with valproic acid across a range of concentrations and time points and the percentage of acH4-positive cells was determined. Figure 1 provides examples of acH4-positive (1A) and acH4-negative (1B) cells as visualised on the imaging flow cytometer. The expression of acH4 was quantified using three parameters as shown in Figure 2.

The first and second parameters, mean and median intensity of fluorescence, have previously been used to measure changes in acH4. An average mean intensity of acH4 in control cells of 5051 increased to 13 044 when treated with 8 mM of valproic acid (Figure 2A). Increase in median

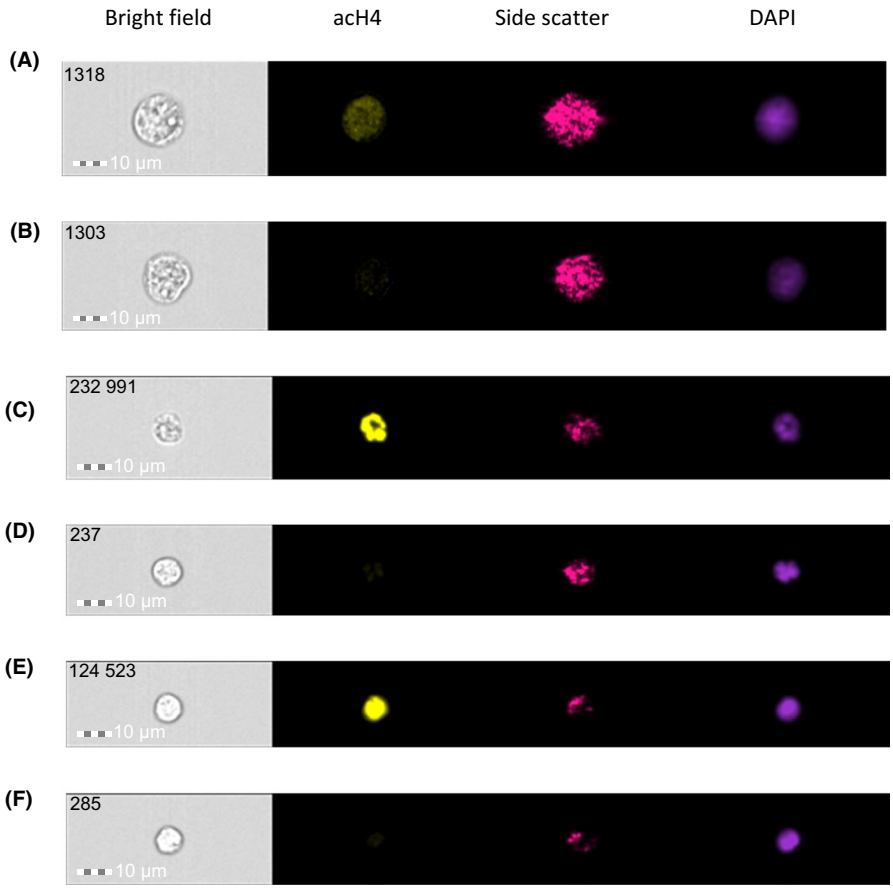

FIGURE 1 Representative images of HL-60 cells with nuclear acH4 positivity (A) and negativity (B), myeloid cells with acH4 positivity (C) and negativity (D), and lymphoid cells with acH4 positivity (E) and negativity (F). Samples were separated following incubation of cells with 4 mM sodium valproate at 37°C for 6 h

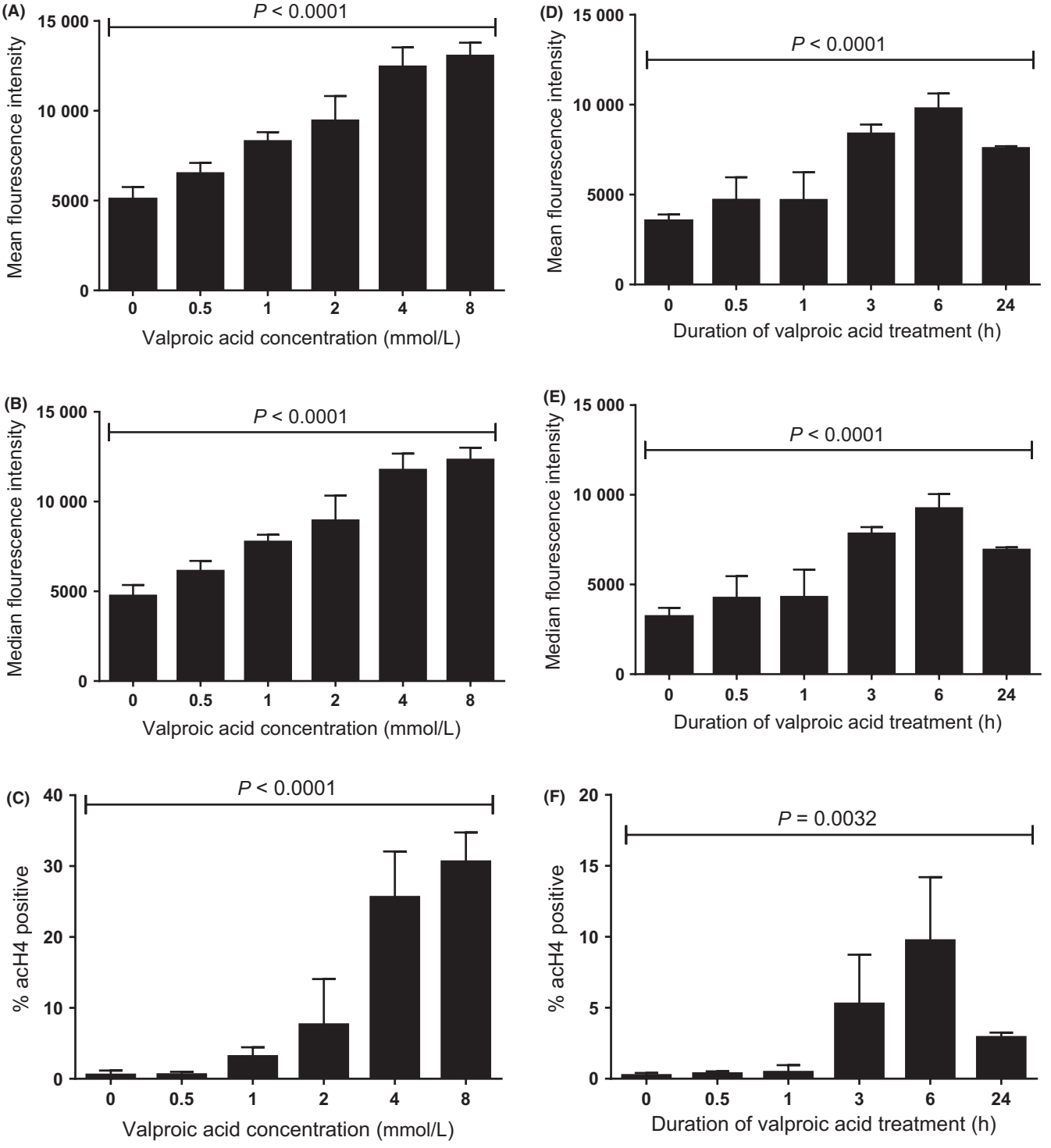

FIGURE 2 Expression of acH4 in HL-60 cells treated with valproic acid (0-8mM) for up to 24 hours. Figure 2A and 2B indicate mean and median fluorescence intensity of acH4, respectively, and Figure 2C indicates average percentage of acH4-positive cells following treatment for 6 hours. Figure 2D, 2E and 2F indicate mean fluorescence intensity, median fluorescence intensity and average percentage of acH4-positive cells, respectively, when treated with 4 mM valproic acid for up to 24 hours. Mean and SD shown from three separate experiments. Level of significance from one-way ANOVA statistical analysis is shown

intensity of acH4 was similar to the increase in mean intensity (4730 and 12 311, respectively; Figure 2B). The third parameter reported was the percentage of nuclear acH4-positive cells, developed based on the functionality of the imaging flow cytometer. Cells with ≥2 in similarity between DAPI and PE and ≥45 000 in intensity of PE were identified as nuclear acH4-positive cells and expressed as a percentage of the total number of cells present. The average percentage of

acH4-positive cells was 0.51% in the vehicle control sample and increased to 30.6% when treated with 8 mM valproic acid for 6 hours (Figure 2C).

Figure 2D-F show the expression of acH4 following different incubation times with valproic acid at a concentration of 4 mM. The average mean and median fluorescence increased approximately twofold (Figure 2D-E), while the percentage of acH4-positive cells increased from 0.22% to 9.7% (>10-fold) following incubation of HL-60 cells with 4 mM valproic acid for 6 hours, respectively (Figure 2F). All increases shown in Figure 2 were statistically significant ($P < 0.001$).

3.2 | Assay validation in white blood cells

The method developed in HL-60 cells was applied to the measurement of acH4 expression in WBCs following incubation of whole blood from healthy volunteers ex vivo with valproic acid. The process of red blood cell lysis and cell staining was the same as HL-60 cells. Example images of four types of white blood cell are shown in Figure 1, namely acH4-positive myeloid (1C), acH4-negative myeloid (1D), acH4-positive lymphoid (1E) and acH4-negative lymphoid (1F).

Levels of acH4 detected in WBCs were increased following exposure to valproic acid, with the proportion of acH4-positive cells in the untreated WBC population markedly lower than in cells treated with valproic acid and a clear concentration-dependent effect observed in all three parameters measured (Figure 3 and supplementary Figure S2). Fold changes in mean fluorescence, median fluorescence and nuclear acH4-positive WBCs over a concentration range of 0-8 mM valproic acid were 3.9-, 3.5- and 57-fold, respectively. Measurement of the percentage of acH4-positive cells was therefore selected to measure dynamic change in acH4 in clinical samples.

Looking at the data generated in different types of WBC in Figure 3C, percentages of acH4-positive myeloid cells increased from 0.60% to 54.9% (92-fold) with a valproic acid concentration of 8 mM, as compared to untreated cells. In comparison, percentages of acH4-positive lymphoid cells increased from 2.2% to 70.5% (33-fold) under the same conditions. Fold changes in mean and median acH4 signal exhibited the same trend. These increases between myeloid and lymphoid cells were significantly different ($P = 0.03$). Lymphoid cells therefore exhibited a higher percentage of acH4-positive cells than myeloid cells but a lower magnitude of increase when treated with drug.

3.3 | Clinical sample analysis

Blood samples from four patients receiving valproic acid treatment on the SIOP Ependymoma II trial were analysed to examine applicability of the assay to a clinical setting.

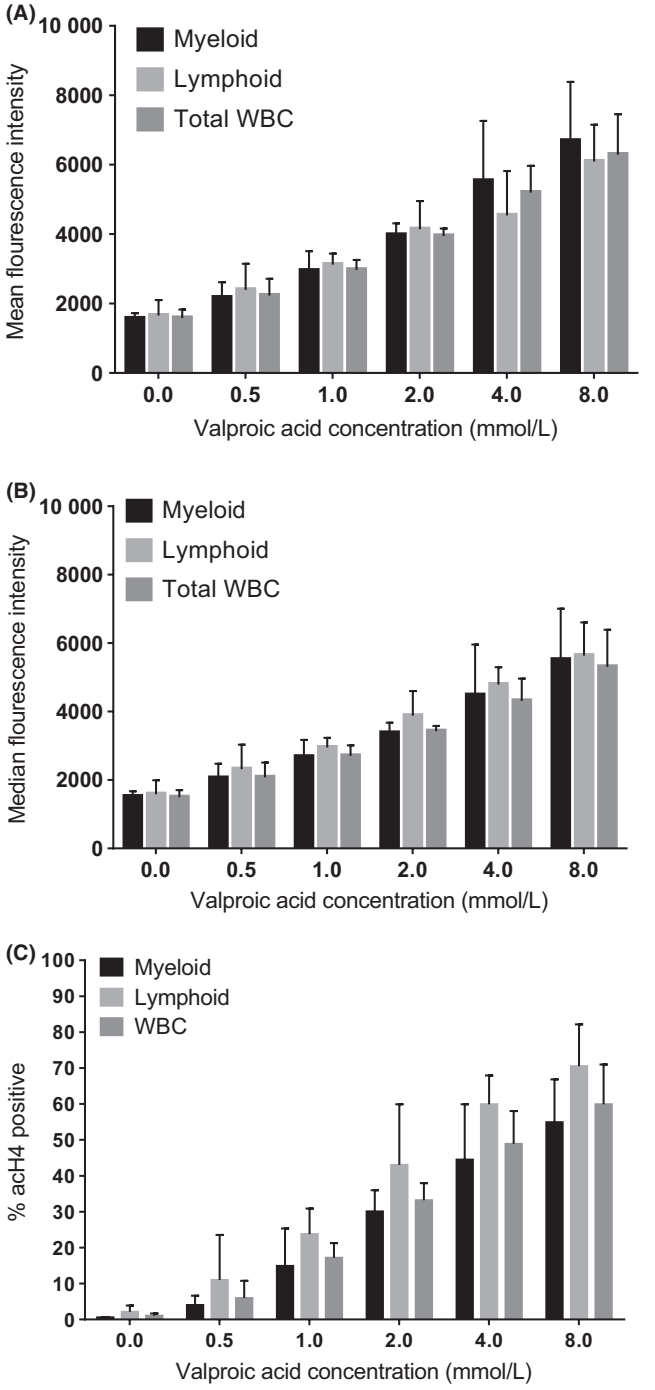

FIGURE 3 Expression of acH4 in myeloid, lymphoid and total white blood cells from whole blood samples treated with different concentrations of valproic acid (0-8 mM) for 6 hours. Expression is shown as mean fluorescence (A), median fluorescence (B) and percentage of acH4-positive cells (C); mean data shown from three independent donors. Significant differences between acH4 changes in myeloid and lymphoid cells were observed in Figure 3C (ANOVA, $P = 0.0313$). Significant changes in acH4 with increasing valproic acid concentrations were observed in all cell populations (ANOVA, $P < 0.01$)

Whole blood samples collected pre-administration and at 4 hours post-administration of valproic acid were processed as described.

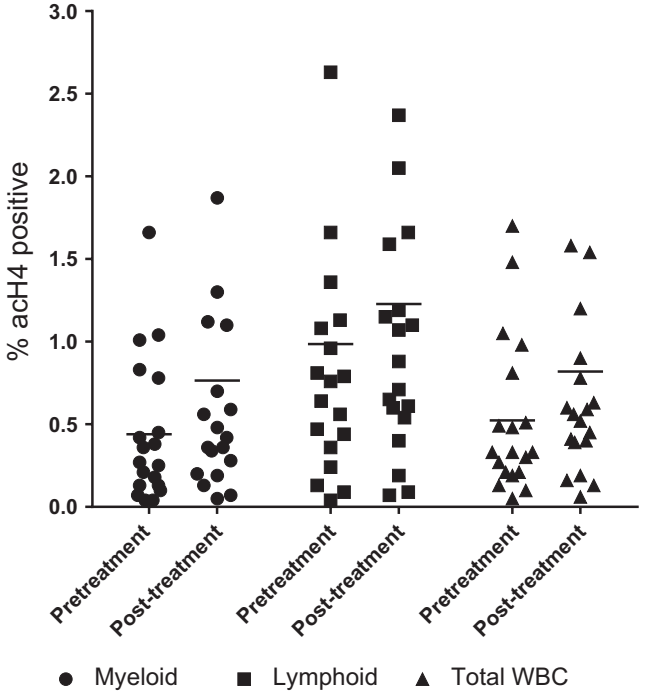

FIGURE 4 Percentage of acH4-positive cells in cell populations isolated from patient samples collected pretreatment and 4-h post-treatment with valproic acid. The bars indicate mean values

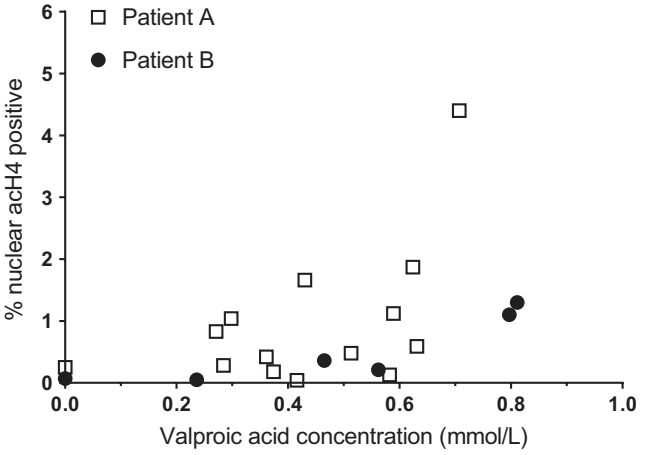

FIGURE 5 Association between valproic acid concentration and percentage acH4-positive myeloid cells in two patients treated with increasing doses of valproic acid on the SIOP Ependymoma study. Samples were collected at 4 h following drug administration or 12 h following drug administration (trough level before next dose) over a range of doses of valproic acid (from 15-35 mg/kg)

Figure 4 indicates the percentage of acH4-positive cells in 38 samples collected from four ependymoma patients receiving valproic acid, suggesting a trend towards an increase in acH4-positivity post-treatment. In agreement with the ex vivo results, myeloid cells appeared to have lower acH4 expression but a greater increase in percentage of acH4-positive cells after valproic acid treatment than lymphoid cells. These data confirm that myeloid cells represent the preferred population of interest for the developed assay.

The association between percentage acH4-positive myeloid cells and valproic acid concentrations measured in plasma from two patients where sequential blood samples were collected is shown in Figure 5. For each patient, samples were collected before the initiation of valproic acid (treatment naïve), at 4 hours following drug administration and at 12 hours following drug administration (trough level before next dose) over a treatment period of up to 3 months and across a range of doses of valproic acid (from 15-35 mg/kg). Increases in valproic acid concentrations between pre- and post-treatment ranging from 0.17-0.35 mM and a maximum post-treatment concentration of 0.81 mM observed.

4 | DISCUSSION

A novel method for the detection of histone H4 acetylation by imaging flow cytometry has been developed and its applicability in cell lines (in vitro), white blood cells (ex vivo) and clinical samples obtained from patients receiving treatment with valproic acid investigated. The assay showed increases in acH4 positivity in both in vitro and ex vivo concentration-dependent experiments following exposure to valproic acid.

This is the first assay which describes the quantification of acH4-positive cells using an imaging flow cytometry approach, although previous studies have described methods to detect histone acetylation levels by conventional flow cytometry.[11-13] Unlike these previous approaches, imaging flow cytometry as utilised in our study can also provide valuable information on localisation of the antibody:antigen interaction in WBCs, allowing identification of nuclear expression of acH4. This function allows us to measure histone H4 acetylation by quantifying the number of nuclear acH4-positive cells, as compared to previously published techniques used to measure acH4, based on observing the expression of acH4 intensity.[11-13] Our results suggest that nuclear acH4 positivity is a markedly more sensitive approach to measuring relative changes in acH4 expression as compared to mean and median intensity, although absolute numbers were lower.

In our assay, acH4 expression in lymphoid cells was significantly higher than expression observed in myeloid cells, a finding that may support the use of PBMCs to measure acH4 expression in previously published clinical trials.[7,8] However, our results suggest that a larger dynamic range in response to valproic acid treatment, in terms of the magnitude of increase from baseline, was observed in myeloid as compared to lymphoid cells. To compare the expression of acH4 in WBC, therefore, the percentage of myeloid cells in whole blood should ideally be considered.

The clinical application of the current assay was tested using blood samples obtained pre- and post-treatment from

children being treated on the SIOP Ependymoma II clinical trial. In contrast to in vitro and ex vivo experiments, changes in acH4 positivity observed were modest. In this respect, it should be noted that plasma drug concentrations achieved in the study patients were markedly lower than concentrations used in ex vivo experiments (Figure 5), thus resulting in smaller increases in percentages of acH4-positive cells. In a previously published clinical trial, an approximate threefold increase in H4 acetylation was observed at a valproic acid plasma concentration of ≥1 mM,[8] markedly in excess of plasma concentrations observed in the patients in the current study. Another clinical trial reported no clear changes in acH4 expression ratios between post- and pre-valproic acid treatment at a mean valproic acid concentration of 0.65 mM.[9] Similarly, results from a third study suggested that patients with significant increases in histone acetylation (>4-fold) had valproic acid plasma levels in excess of 0.8 mM.[10] It is highly likely that the small increases in acH4 positivity observed in our limited number of patient samples analysed to date, reflect the low plasma concentrations of valproic acid observed in these patients at the doses administered at early time points in the current study. Further clinical samples collected from patients receiving increased valproic acid doses will be analysed as the trial progresses.

In conclusion, a novel assay for the detection of histone H4 acetylation in myeloid white blood cells by imaging flow cytometry has been developed and optimised. The method can be used for the measurement of acH4 as a pharmacodynamic biomarker for HDACIs in drug development and monitoring of drug activity in a clinical setting.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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