Mechanisms of radiation leukaemogenesis, characterisation of haematopoietic stem cells and modulation of risk

A thesis submitted for the degree of Doctor of Philosophy, University of Oxford

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Abstract

Haematopoietic bone marrow cells are amongst the most sensitive to ionising radiation (IR), initially resulting in cell death or genotoxicity that may later lead to leukaemia development, most frequently Acute Myeloid Leukaemia (AML). The target cells for radiation-induced Acute Myeloid Leukaemia (rAML) are believed to lie in the haematopoietic stem and progenitor cell (HSPC) compartment. Using the inbred strain CBA/Ca as a murine model of rAML, progress has been made in understanding the underlying mechanisms, characterisation of target cell population and responses to IR. Complex regulatory systems maintain haematopoietic homeostasis which may act to modulate the risk of rAML. However, little is currently known about the role of metabolic factors and diet in these regulatory systems and modification of the risk of AML development.

This study characterises cellular proliferative and clonogenic potential as well as metabolic changes within murine HSPCs under oxidative stress and X-ray exposure. Ambient oxygen (normoxia; 20.8% O_2) levels were found to increase irradiated HSPC-stress, stimulating proliferative activity compared to low oxygen (3% O_2) levels. IR exposure has a negative influence on the proliferative capability of HSPCs in a dose-dependent manner (0–2 Gy) and this is more pronounced under a normoxic state. One Gy x-irradiated HSPCs cultured under normoxic conditions displayed a significant increase in oxygen consumption compared to those cultured under low O_2 conditions and to unirradiated HSPCs. Furthermore, mitochondrial analyses revealed a significant increase in mitochondrial DNA (mtDNA) content, mitochondrial mass, and membrane potential in a dose-dependent manner under normoxic conditions. Our results demonstrate that both IR and normoxia act as stressors for HSPCs, leading to significant metabolic deregulation and mitochondrial dysfunctionality which may affect long term risks such as leukaemia.

Diet can greatly impact health, while caloric restriction and fasting regimens possess conferring potential benefits for disease prevention and longevity. However, comprehensive investigations are required to elucidate the precise effects of distinct dietary protocols (e.g., caloric restriction, fasting, amino acid depletion), on haematopoietic malignancies including radiation-induced acute myeloid leukaemia. Our research highlights a remarkable reduction in the proliferative capacity of short-term primary HSCs when subjected to dietary valine depletion, displaying metabolically less active phenotype. Moreover, employing an in vivo fasting regimen using a CBA mouse model of rAML, we observe a significant reduction in mCherry loss, particularly in the initial stages following radiation exposure. This study provides a fundamental platform for further investigation into the intricate interplay between dietary modifications and the progression of radiation-induced acute myeloid leukaemia. Despite the current inherent limitations within the available dataset, the implementation of dietary interventions holds a great potential and may lower the risk of long-term complications in cancer patients.

Keywords Haematopoietic stem and progenitor cell, oxidative stress; ionising radiation; metabolism; diet, alternate fasting, radiation leukaemogenesis; mitochondrial dysfunction; hypoxia; reactive oxygen species; acute myeloid leukaemia.

Preface

Various parts of chapter 1 and chapter 3 were previously published by myself, Dr Rosemary Finnon, Dr Lourdes Cruz-Garcia, Dr Mark A. Hill, Dr Tatsuhiko Imaoka, Dr Anna A. Friedl and Dr Christophe Badie [Karabulutoglu, M.; Finnon, R.; Imaoka, T.; Friedl, A.A.; Badie, C. Influence of Diet and Metabolism on Haematopoietic Stem Cells and Leukaemia Development Following Ionising Radiation Exposure. Int. J. Radiat. Biol. 2019, 95, 452–479] and [Karabulutoglu, M.; Finnon, R.; Cruz-Garcia, L.; Hill, M.A.; Badie, C. Oxidative Stress and X-ray Exposure Levels-Dependent Survival and Metabolic Changes in Murine HSPCs. Antioxidants 2022, 11, 11. https://doi.org/10.3390/ antiox11010011]. I was responsible for analysing and writing the manuscripts with excellent guidance and editing by other authors contributions.

All animal practices completed in this doctoral project followed by the UK Animals (Scientific Procedures) Act, 1986, Amendment Regulations 2012, and experimental procedures were evaluated and accepted by the local Ethics Committee and the Home Office.

List of Abbreviations

Aa	amino acid
ADF	alternate day fasting
ADI-PEG	20 pegylated arginine deiminase
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
AML1-ETO	leukemogenic t (8;21) fusion protein
AMPK	AMP-activated protein kinase
APL	acute promyelocytic leukaemia
Asn	asparagine
ASS1	argininosuccinate synthase 1
ATRA	all-trans-retinoic acid
BM	bone marrow
Bp	base pair

BP-CML	blast phase- chronic myeloid leukaemia
CC3	cleaved caspase 3
CCCP	carbonyl-cyanide 3-chlorophenylhydrazone
CFC	colony forming cell
CFU	colony forming unit
Ch	chromosome
CHO	carbohydrate
CLP	common lymphoid progenitor
CLP	common lymphoid progenitor
CML	chronic myeloid leukaemia
CMP	common myeloid progenitor
CO_2	carbon dioxide
CP-CML	chronic phase CML
CR	caloric restriction
CRR	clinically relevant radioresistant cell lines
CSC	cancer stem cell
DAMPs	damage associated molecular patterns
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3A
Dsbs	DNA double-stranded breaks
ECAR	extracellular acidification rate
ECM	extracellular matrix
ETC	electron transport chain
ETS	erythroblast transformation specific
FACs	fluorescent activated cell sorting
FAO	fatty acid metabolism
FAT	low fat
FBS	foetal bovine serum
FCCP	trifluoromethoxy carbonylcyanide phenylhydrazone
FITC	fluorescein isothiocyanate
FLT3	fms related receptor tyrosine kinase 3
FMD/FMN	fasting mimicking diets
FOXO	forkhead box transcription factors
FSC/SSC	forward scatter/side scatter
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic DNA
GFP	green fluorescent protein
Gln	glutamine
GMP	granulocyte/macrophage progenitor
Gy	gray
h	hours
H_2O_2	hydrogen peroxide
HDACs	histone deacetylases
HepG2	human hepatoma cells
HIBCH	3-hydroxyisobutyryl-Co-A
HIF-1alpha	hypoxia-inducible factor 1-alpha
HSC	haematopoietic stem cell
HSPC	haematopoietic stem and progenitor cell
IF	intermittent fasting

IGF	insulin growth factor
IL	interleukin
IMDM	iscove's modified dulbecco's media
IR	ionising radiation
IRES	internal ribosome entry
Kb	kilobase
LCD	low carbohydrate diet
LEPR	leptin receptor
LET	linear energy transfer
Lin	lineage
LKB1	serine/threonine kinase or liver kinase 1
LSC	leukaemic stem cell
LSK	Lin-Sca1+cKit+
LT-HSC	long-term haematopoietic stem cells
mCh	mCherry
MDR	minimal deleted region
MELODI	multidisciplinary European low dose initiative
MEP	megakaryocyte/erythroid progenitor
min	minutes
ml	millilitre
mM	millimolar
MP	myeloid progenitors
MPP	multi-potent progenitors
mPTP	mitochondrial permeability transition pore
MRC	mitochondrial chain genes
MSR	MitoSox^{TM} Red
MSv	millisievers
mTATP6	mitochondrially encoded ATP synthase membrane subunit 6
mtDNA	mitochondrial DNA
MtMP	mitochondrial membrane potential
mTOR	mammalian target of rapamycin
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NEAA	non-essential amino acid
Neo	neomycin cassette
nM	nanomolar
NOX	nadph oxidase
NPMI	nucleophosmin
NRF2	nuclear factor erythroid 2-related factor 2
NSG	mouse NOD scid gamma mouse
O_2	oxygen
OCR	oxygen consumption rate
OLIGO	oligonucleotide
OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PF	prolonged fasting
PGC-1	peroxisome proliferator-activated receptor gamma coactivator1
PML-RARA	promyelocytic leukaemia/retinoic acid receptor alpha
PRDMI	pr domain zinc finger protein 1
Prot	protein

qRT-PCR	quantitative real time polymerase chain reaction
rAML	radiation-induced AML
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature
SC	stem cell
SEM	standard error of the mean
Sirt1	sirtuins 1
SSpan	stemspan
ST- HSC	short-term HSC
STAT	signal transducer and activators of transcription
tAML	therapy-related AML
TME	tumour microenvironment
TNBC	triple-negative breast cancer
Tp53	tumour protein p53 or transformation-related protein 53
TRE/TRF	time restricted eating/time restricted fasting
TVB	tail vein bleeding
URE	upstream regulatory element
VLCD	very low-calorie diet
WBC	white blood cell
ZFN	zinc finger nuclease
µg	microgram
µm	micrometre

Acknowledgements

I wish to extend my sincere gratitude to my supervisors, Dr. Christophe Badie and Dr. Mark A. Hill, for graciously hosting me within their research groups over the past four years and for their unwavering guidance and support throughout this academic journey. My profound appreciation extends to my valued colleagues at the UK Health Security Agency, particularly Dr. Rosemary Finnon, whose invaluable advice, unwavering support, and technical expertise have been pivotal, rendering this project feasible. Special recognition is also due to Paul Finnon and Dr. Lourdes Graces, whose contributions significantly enhanced my scientific and experimental skills. In addition, I would like to express my gratitude to Dr. Christiana Kartsonaki for her substantial contribution to the in vivo data analysis of this project as well as her generous sharing of expertise that has enabled me to improve my data analysis skills.

Furthermore, I express gratitude to the Radiation Effects Department at Harwell for providing me with a UKHSA-funded studentship, enabling this research.

The year 2022 posed significant challenges for me, marked by the loss of three exceptional individuals. Their absence created considerable difficulties in maintaining my focus. Despite these challenging circumstances, I am elated to have successfully completed this thesis. As I bring my remarks to a conclusion, I wish to dedicate this thesis to the memory of my grandfather, Mustafa Kemal Gorgulu. Undoubtedly, he would have been profoundly proud of my accomplishments.

CHAPTER 1

Introduction

1.Introduction

1.1 Radiation Carcinogenesis: Overview

"Cancer" is a multifaceted disorder characterised by accumulation of DNA mutations and altered gene expression, resulting in uncontrolled growth of the mutated cell. With around 14 million new cancer incidents occurring every year and approximately 8 million cancer-related deaths, it is one of the most serious health issues globally (Klaunig, 2020). Yet, the molecular events and associated mechanisms by which a normal cell becomes malignant are not fully understood.

Before delving into the effects of IR on cancer development, it is essential to comprehend the process of carcinogenesis. While various cancer types possess distinct molecular biologies, they also share fundamental principles of tumour formation (reviewed in (Bertram, 2000; Hanahan & Weinberg, 2011). Over the past 50 years, there has been a refinement in understanding the sequence of events involved in carcinogenesis, identifying both variations and commonalities amongst rodent and human models (Balmain & C.Harris, 2000; Hahn & Weinberg, 2002). The majority of adult epithelial tumours show an exponential rise in cancer incidence with increased age (Cairns, 1981), implying that the tumour progression proceeds over a series of sequential stages. Epidemiological and experimental investigations on murine skin carcinogenesis (along with other organ systems) have conceptualized carcinogenesis into four distinct steps tumour initiation, promotion, malignant conversion, and progression (Barrett & Wiseman, 1987; Fearon & Vogelstein, 1990; Loeb & Harris, 2008; Trosko, 2001). Underlying most multi-step models of carcinogenesis lies the involvement of genetic and epigenetic changes in a multitude of independent genes. These alterations enable cells to grow uninhibitedly by disrupting the equilibrium between proliferative and apoptotic signalling.

Furthermore, the control of tumour progression is closely intertwined to the activity of cytotoxic and adaptive immune cells. As neoplastic tissue transforms into detectable tumours, cancer cells utilise strategies resembling peripheral immune tolerance, thereby evading tumoricidal attacks of the immune system. The concept of tumour-associated inflammation, initially proposed by Virchow in 1893, has significantly shaped our understanding of cancer progression (Balkwill & Mantovani, 2001). Presently, it is broadly acknowledged that chronic inflammation serves as a critical hallmark of cancer, accounting for approximately 25% of cancer cases (Beaugerie et al., 2013; Coussens & Werb, 2002; Hussain et al., 2000). An example illustrating the impact of inflammation on tumour treatment is the successful use of a vaccine containing an attenuated strain of Mycobacterium bovis to induce acute inflammation in the bladder effectively treating squamous cancer (Askeland et al., 2012). Hence, infiltration of immune cells and subsequent inflammation can both initiate and, in some cases, eliminate tumour cells thereby impeding tumour growth (Shalapour & Karin, 2015).

While the precise relationship between inflammation and tumour development is not fully understood, it is evident that the tumour microenvironment undergoes various changes that activate immune-inflammatory responses. Gonzales et al., provided a comprehensive overview of immune cells and their crucial roles in either promoting or impeding tumour development (Gonzalez et al., 2018). Advancement of innovative technologies in molecular analysis, including microRNAs, gene expression profiling, and pathway analysis, shows carcinogenesis to be more complex than merely clonal progression of a cell. Existing multi-step models of carcinogenesis comprise of at least 80 cancer gene alterations or mutations, where a dozen of these genes are classified as drivers of the cancer growth. Additionally, the majority of human cancers involve exposure to non-genetic factors including environmental carcinogens such as diet, lifestyle, infectious agents, and occupation (Anand et al., 2008; Boffetta & Nyberg, 2003;

Lutz & Fekete, 1996; Parsa, 2012). These factors can initiate and/or advance the development of malignant tumours, changing the expression and activity of genes which are critical to maintain cell growth, differentiation, cell cycle control, DNA repair, apoptosis, among others (Higginson, 1993; Sabo-Attwood et al., n.d.). Other carcinogens which can trigger malignant transformation are a broad group of endogenous factors comprising hormones, age, genetic and hereditary risk factors (Ames, 1989; Lutz & Fekete, 1996). Carcinogens can be classified into physical, chemical, and biological carcinogens and their different mechanisms and carcinogenesis processes are displayed in Figure 1.1 below (S. Das et al., 2020)

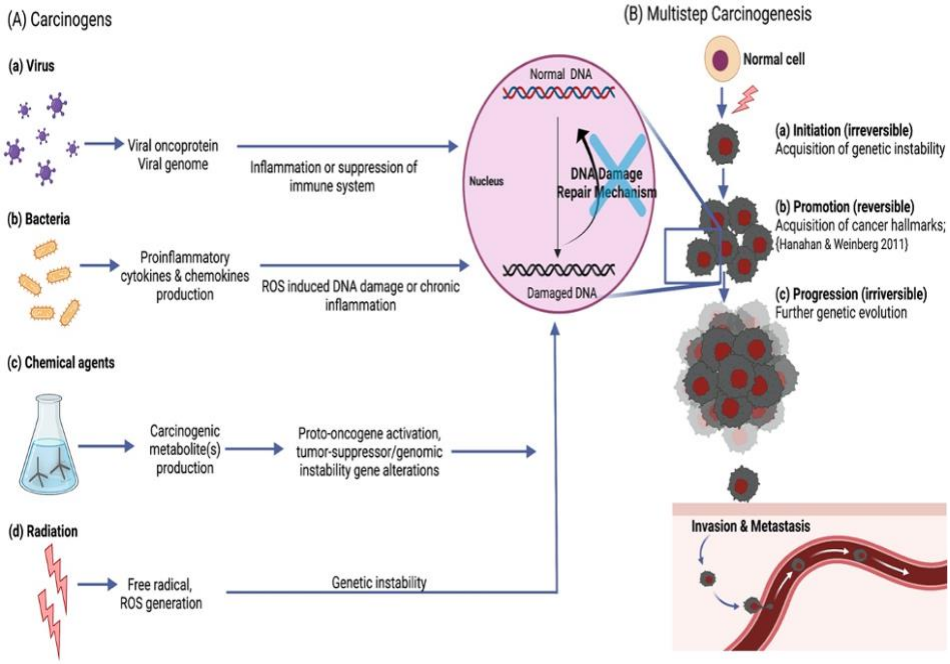


Figure 1. 1 Multistage carcinogenesis.

Exposure to carcinogens results in activation of proto-oncogenes and inactivation of tumour suppressor genes, resulting from a covalent damage to DNA. Multistage cancer occurs by the accumulation of mutations, regardless of their order of occurrence (Fearon & Vogelstein, 1990). (I) initiation stage, being irreversible, was ascribed as the result of DNA damage leading to mutagenesis; (II) promotion, being reversible, was believed to be caused by epigenetic mechanisms; (III) malignant conversion, is the transformation of a preneoplastic cell into one that expresses the malignant phenotype. This process requires further genetic changes. (IV) progression, being irreversible, was believed to be caused by genetic instability and altered gene expression. Tumour progression comprises the expression of the malignant phenotype and the tendency of malignant cells to acquire more aggressive characteristics over time. Metastasis may involve the ability of tumour cells to secrete proteases that allow invasion beyond the immediate primary tumour location. During progression, neoplasms show progressively increased invasiveness, developing the ability to metastasize e.g., via secretion of proteases from tumour cells which allow invasion beyond primary tumour (S. K. Das et al., 2017).

1.1.2 *Ionising radiation-related cancer risk*

Ionising Radiation (IR) is widely recognised as a comprehensive carcinogen, capable of inducing multiple types of genotoxic and cytotoxic damage to DNA and other macromolecules as it transverses through the cell. IR can also be termed as 'universal' carcinogen, as it has the potential to trigger cancer development across diverse tissues, irrespective of age or species. All cells in the body can be susceptible to the detrimental impacts of IR, and the extent of damage corresponds to specific physical parameters, which subsequently determine the dose received by individual cells or tissues. While fundamental aspects of radiation-induced cancer and cellular transformation are firmly established, ongoing research is delving deeper into the intricate effects of radiation on cellular and molecular mechanisms. Consequently, IR has emerged as a critical tool for advancing our insight into carcinogenesis, enabling the examination of molecular and cellular alterations to DNA damage.

The physiological effects of ionising radiation can be classified as either direct or indirect (Dertinger & Jung, 1970). Direct effects refer to immediate damage, caused by energy deposition in the macromolecules (e.g., within cellular nuclei such as single or double strand breaks in DNA). Conversely, indirect effects signify the interaction between IR and other molecules such as water which involve in generating free radical and reactive oxygen species (ROS). These damage events are resolved by cell death or DNA repair pathways, which may then result in tumour-initiating gene mutations and chromosomal aberrations. These can further lead to genomic instability leading to tumour progression/promotion and ultimately to cancer development (Dertinger & Jung, 1970; Huang et al., 2003).

Large-scale tumour induction studies were carried out to define numerous features of radiation carcinogenesis and were supported by epidemiological analysis from human populations who are exposed to IR through variety of sources ranging from naturally occurring environmental

sources e.g., cosmic radiation, (Sigurdson & Ron, 2004), radon gas (Darby et al., 2005; Krewski et al., 2005), man-made sources comprising medical exposures and accidental radiation exposures e.g., nuclear power plants (Morley, 2004). According to the most recent and comprehensive review (UK Health Protection Agency-Radiation Protection Division: HPA-RPD), most of the dose received by the general population comes from naturally occurring (84%) (Hughes et al., 2005).

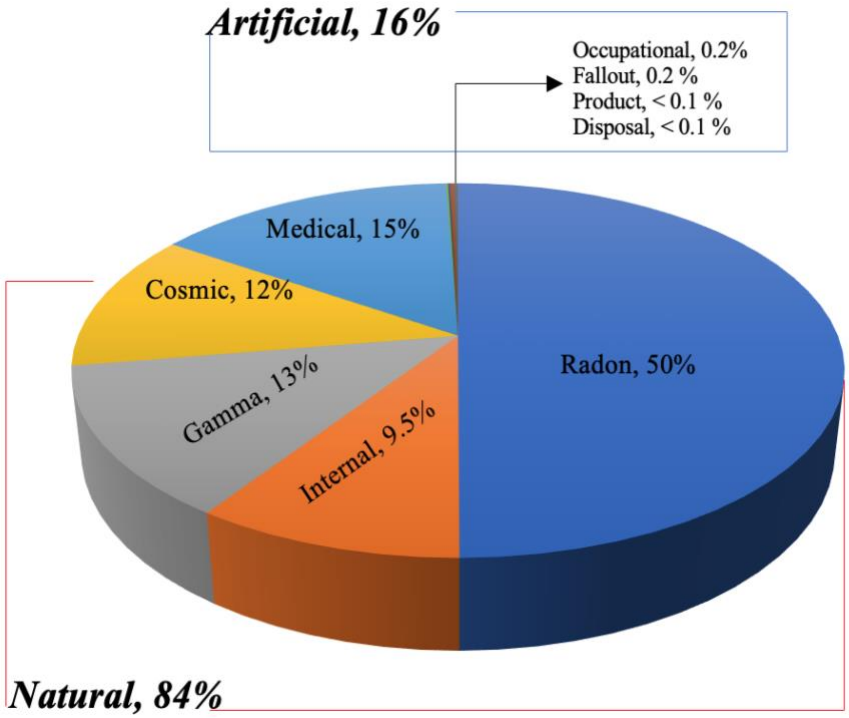


Figure 1. 2 Summary of the relative contributions from numerous artificial and natural sources of ionising radiation to the average annual dose received by the UK population.

Categories are as follows; Cosmic: radiation exposure from extra-terrestrial resources at ground level, as well as from average air travel. Gamma: terrestrial gamma radiation from ground levels and buildings. Internal: intakes of radionuclides occurring in drinks and food stuff (radon exclusive). Radon: from indoor radon gas concentrations resulting from natural radon emitted from the ground. Medical: radiology, nuclear medicine & patient exposure. Occupational: exposure at the workplace, comprising medical, airline workers, and miners. Consumer Products: e.g., smoke alarms, vaseline glass, uranium glazed ceramics. Disposals: discharges of radioactive waste. Fallout: consequence of nuclear weapons testing and nuclear accidents such as the Chernobyl incident. Source: HPA 2005 review (Hughes et al., 2005).

Substantial data supporting the association between IR exposure and leukaemia incidence have only begun to emerge through Life Span Studies. These studies involved cohorts of Japanese atomic bomb survivors as well as patients receiving high doses of therapeutic radiation for cervical cancers, tinea capitis, and ankylosing spondylitis (Hall & Giaccia, 2012; Hall & Giaccia AJ., 2006; Little et al., 1999; Preston et al., 1994; Wakeford et al., 2009; Weiss et al., 1994). Over the past two decades, data from the Chernobyl disaster have also provided a more comprehensive dataset on age dependence, doses, and latencies, offering insights into excess risk estimates of leukaemia in adults and children (Ivanov et al., 1997; Ivanov, Gorski, et al., 2003; Ivanov, Gorskiĭ, et al., 2003; Noshchenko et al., 2010). Among the adult population, acute and chronic myeloid leukaemias (AML and CML) are the predominant types of radiation-induced leukaemias observed with the highest frequency (Little et al., 1999; Little, Wakeford, Tawn, et al., 2009; Preston et al., 1994, 2004; TOMONAGA, 1962; Weiss et al., 1995). Furthermore, younger children, especially those exposed between the ages of 5 and 9, exhibit a greater susceptibility to acute lymphoblastic leukaemia (ALL), while older children have a higher probability of developing AML. The highest risk of leukaemia development occurs within the first decade following exposure, after which it gradually decreases over time but never returns to the baseline risk (Little et al., 1999; Little, Wakeford, & Kendall, 2009; Noshchenko et al., 2010; Preston et al., 1994). Several studies have also reported sex-specific variations in leukaemia type and associated risk (Noshchenko et al., 2010; Preston et al., 1994, 2004; Weiss et al., 1995).

Similarly, an increased risk of leukaemia with evidence of dose response was documented in patients who underwent radiotherapy for urological system (Radivoyevitch et al., 2016; Travis, 2000; Wright et al., 2010) and breast cancer (Kaplan et al., 2013; G.-P. Yu et al., 2006; Zeidan et al., 2017). Whilst there is a well-established link between acute radiation exposure and leukaemia incidence when administering high-to-moderate doses (Kendall et al., 2013;

Spycher et al., 2015; Nikkilä et al., 2016), this link is less clear when low doses are applied over time and cumulative over long periods. Yet a recent study conducted by Little, and colleagues were able to quantify the excess risk for leukaemia and other myeloid malignancies following low-dose exposure to IR in childhood, using data from nine historical cohort studies. Accordingly, more than 2-fold increased risk was detected for cumulative exposure to less than 100 millisievers (mSv) (Little et al., 2018).

Another study focusing on low-dose medical radiation exposure, reported that children who underwent computed tomography (CT) scans, resulting in cumulative doses of approximately 50 mGy, had nearly tripled the risk of developing leukaemia later in life (Pearce et al., 2012). Another population-based cohort study comprising more than 12 million South Korean adolescents, revealed that individuals who exposed to low-dose ionising radiation show an increased cancer incidence particularly, mouth and pharynx, breast, thyroid, lymphoid, haematopoietic malignancies as well as myelodysplasia (Hong et al., 2019). Recently, positive association between low dose or protracted external radiation exposure and cancer has been reported from National Registry for Radiation Workers (NRRW) study, where the risks are coherent with those seen in the atomic bomb survivor studies (Gillies et al., 2019). While radiation exposure at moderate to high levels has been well-characterised, the health effects of exposure at low levels and chronic levels are still a topic of debate (National Research Council U.S, 2006). Nevertheless, it has been widely recognised that epidemiological studies have limitations for statistical reasons for estimation of radiation risks at low doses (<100mGy) and very low doses (<10mGy). This principle is highlighted as a primary objective of the Multidisciplinary European Low Dose Initiative (MELODI) program, emphasising the importance of studying and elucidating the intricate mechanisms associated with low-dose radiation exposure. Besides, the National Registry for Radiation Workers (NRRW), known as a critical component of the latest international radiation worker collaboration (Haylock RGE

et al.) and the International Nuclear Workers Studies (INWORKS) provide more precise quantitative estimates of the risk of chronic, low-level exposure to IR, hence improved understanding of the association between dose effect and mortality due to solid and haematological cancers (e.g. leukaemia, excluding chronic lymphocytic leukaemia: CLL), as well as circulatory diseases (Gillies et al., 2019; Gillies & Haylock, 2022; Hamra et al., 2016; Hunter & Haylock, 2022; Laurier et al., 2017; Wakeford, 2021).

1.2. Radiation leukaemogenesis

After the discovery of X-rays by Roentgen in 1895, IR became one of the most widely recognised and extensively studied carcinogens. Acute myeloid leukaemia (AML) is the predominant radiation-associated leukaemia, accounting for approximately 80% of excess leukaemia cases (Folley et al., 1952; Hsu et al., 2013; Ozasa et al., 2012; Preston et al., 2003; Richardson et al., 2009). Whilst a clear link between radiation and leukaemia development is acknowledged, 85 years since Nobel Prize winner Marie Curie died from radiation-induced leukaemia, the underlying mechanisms remain unknown. For instance, the identity and nature of the target cell of origin for AML within the bone marrow is currently unknown, and increased understanding of the effect of radiation on potential target cell populations and their radiation response, radiosensitivity and factors that mitigate this is required.

1.3 Radiation-induced Acute Myeloid Leukaemia (rAML)

Leukaemias are a group of neoplasms that develop within the haematopoietic (blood-forming) system and are characterised by the accumulation of excess abnormal/dysfunctional blood cells. They can affect any of the lineages of blood cells, lymphoid or myeloid, and these can also be either acute or chronic depending on rapidity of presentation and the

number/immaturity of the abnormal cells produced (Chennamadhavuni A, Lyengar V and Shimanovsky A., 2022).

AML is an aggressive neoplasm affecting the myeloid cell lineage *i.e.*, granulocytes (neutrophils, basophils, and eosinophils) and monocytes/macrophages. In myelopoiesis, differentiation is blocked, resulting in the failure of mature myeloid cells, followed by an excessive proliferation of immature and functionally abnormal myeloid cells (known as leukaemic or myeloblasts), which invade bone marrow, blood, and spleen, destroying their structure and function, eventually resulting in haematopoietic system failure (Döhner et al., 2015).

Multiple AML subtypes exist, classified by clinical features, immunophenotyping, karyotype, cell morphology and molecular genetics which define disease entities of clinical significance and continue to be refined as new discoveries are made (most recent revision WHO classification of haematological malignancies, (Arber et al., 2016). Although treatment options for patients with AML have improved over the last few decades (H. Liu, 2021), the prognosis for these patients remains poor. Many patients with AML who have achieved initial remission are likely to relapse, and many older patients are unable to receive the most intensive treatments available (Bertoli et al., 2017; Döhner et al., 2017). We must therefore continue to understand the mechanisms of this disease to identify more targets for treatment and to accelerate the development of more effective and less toxic treatments.

1.4 Murine modelling of radiation-induced leukaemia

Radiation-induced leukaemogenesis is a complex, multi-step process difficult to study in humans due to lack of suitable samples, and the inability to study pre-leukaemic events (Finch, 2001; Gruszka et al., 2017; Rivina et al., 2014; Roussel et al., 2020). Several groups have

demonstrated the significance of mouse models of rAML in assessing the relative biological effectiveness of radiation and investigating the underlying molecular and chromosomal mechanisms (Alexander et al., 1995; Azumi & Sachs, 1977; Hayata et al., 1983; Rithidech et al., 1993; Silver et al., 1999). Given that approximately 99% of mouse genes share homology with the human genome and considering the power of murine genetic interventions, these models remain invaluable in advancing our understanding of mammalian biology and pathology from embryonic development to metabolic disorders, immunology, and cancer (Guenet, 2005).

The CBA inbred strain is considered as the primary model for rAML as it has a low spontaneous background rate ensuring that the tumours studied are directly radiation-induced, and a consistent induction rate of 15-20% AMLs following 3 Gy whole body dose of X-rays (Major & Mole, 1978). Alongside the CBA, three additional mouse strains including, the RF, SJL/J, and C3H/He strains were defined as relatively susceptible to rAML (Rivina & Schiestl, 2012).

1.5 Cytogenic Alterations and Sfpi-1 point mutation in murine rAML

The first experimental murine rAML cases were first described in early 1930s (Krebs et al., 1930) yet further specifications were not clear until 1977. Over the past 3 decades, it has been identified that one copy of chromosome 2 (ch2) has suffered from a deletion in most murine radiation-induced myeloid leukaemia (Alexander et al., 1992; Azumi & Sachs, 1977; Haran-Ghera, 1989; Hayata et al., 1983; Resnitzky et al., 1992). Chr2 deletions occur in almost 90% of rAML cases regardless of the radiation quality or strain and at approximately 2-fold greater frequency when compared with similar lesions ch1 & ch3 (Bouffler et al., 1996; Hirouchi et al., 2008; Rithidech et al., 1993; Steffen et al., 2013). Whereas chromosomal aberrations in

ch1,2, & 3 in C57BL/6 and AKR non-susceptible mouse strains were statistically indistinguishable (Darakhshan et al., 2006).

Deletion of ch2 homologue is detectable twenty-four hours post-irradiation in bone marrow cells from a single acute to 3 Gy whole body X-ray exposure (Bouffler et al., 1997; Peng et al., 2009). Prevalence of ch2 deleted-clones increases with time, being detectable in 25% and >50% of mice by nine-to-twelve months after exposure respectively. Despite this, rAML presentation rate is only 15-20%, with long latency (around 14 months), suggesting that whilst ch2 deleted clones appear to have a growth advantage, this is not sufficient for full AML presentation (See Figure 1.3 below). Alongside the consistent partial ch2 deletion, secondary chromosomal anomalies were affirmed in more than 30% of murine rAML incidents including (i) loss/gain of ch6 (Bouffler et al., 1996; Rithidech et al., 1993) (ii) loss/gain of the Y chromosome (Bouffler et al., 1996; Rithidech et al., 1993) and (iii) allelic loss on ch4 (Lyr2/TLSR5) (Cleary et al., 2001).

Sfpi-1 was identified within the minimal deleted region (MDR) on ch2, encoding the haematopoietic regulatory transcription factor PU.1, which is a member of the Ets transcription factor family and acts as a powerful inducer for commitment and maturation of myeloid and lymphoid lineages (McKercher et al., 1996; Scott et al., 1994; Silver et al., 1999). Sfpi1 exerts a direct role over the orchestration of haematopoietic stem cells (HSCs) machinery. It achieves this through the inhibition of factors that propel cell cycle advancement while concurrently facilitating the activation of cell cycle inhibitors. Consequently, any perturbation in Sfpi1 autoregulatory mechanisms triggers a disruption in the precise equilibrium governing cell cycle control. This perturbation, in turn, catalyses a cascade of events denoted by unexpected cellular proliferation, the emergence of point mutations, and the subsequent progression towards HSC exhaustion or leukaemia manifestation underscores the complexity of the pathogenic

mechanisms involved (Staber et al., 2013; Steffen et al., 2013). Intriguingly, the second Sfpi1/PU.1 allele in myeloid leukaemia carries a point mutation, impairing its ability to bind DNA (Cook et al., 2004; Suraweera et al., 2005). This point mutation affects the arginine residue at position 235 of the protein (R235), part of the ETS-binding domain resulting in amino acid substitutions of cysteine (R235C, 62%), histidine (R235H, 9%), serine (R235S, 8%) or leucine (R235L, 2%) (Figure 1.3 below) (Cook et al., 2004; Suraweera et al., 2005). Sequencing analysis of exon 5 in a large murine rAML panel (>100 cases) showed no mutation outside of the codon 235, Genik et al. reported that R235 is not a major rate limiting step in murine rAML cases (Genik et al., 2014). Hence, in the majority of the rAMLs, Sfpi-1/Pu.1 is biallelically mutated, and so according to Knudsons two-hit hypothesis, likely acts as a tumour suppressor gene in murine rAML (Knudson, 1993).

A recent review conducted by O'Brien et al. discovered new pathways following analysis of 123 mouse radiation-induced AML (rAML) samples for the presence of mutations which were previously identified in human AML. Accordingly, they have disclosed three gene mutations previously *Sfpi1* R235 (68%), *Flt3*-ITD (4%) and *Kras* G12 (3%), of which G12R was previously unreported. An important finding was that Sfpi1 gene expression was significantly lower in rAML samples without an Sfpi1 R235 mutation and was associated specifically with upregulation of mir-1983 and mir-582-5p. Besides, dysregulation of Sfpi1/PU.1 plays a vital role in AML development in both mouse and human cases, revealing a common pathway of myeloid disruption. A detailed discussion of the discovery of new pathways in murine rAML can be found in this paper (O'Brien et al., 2020).

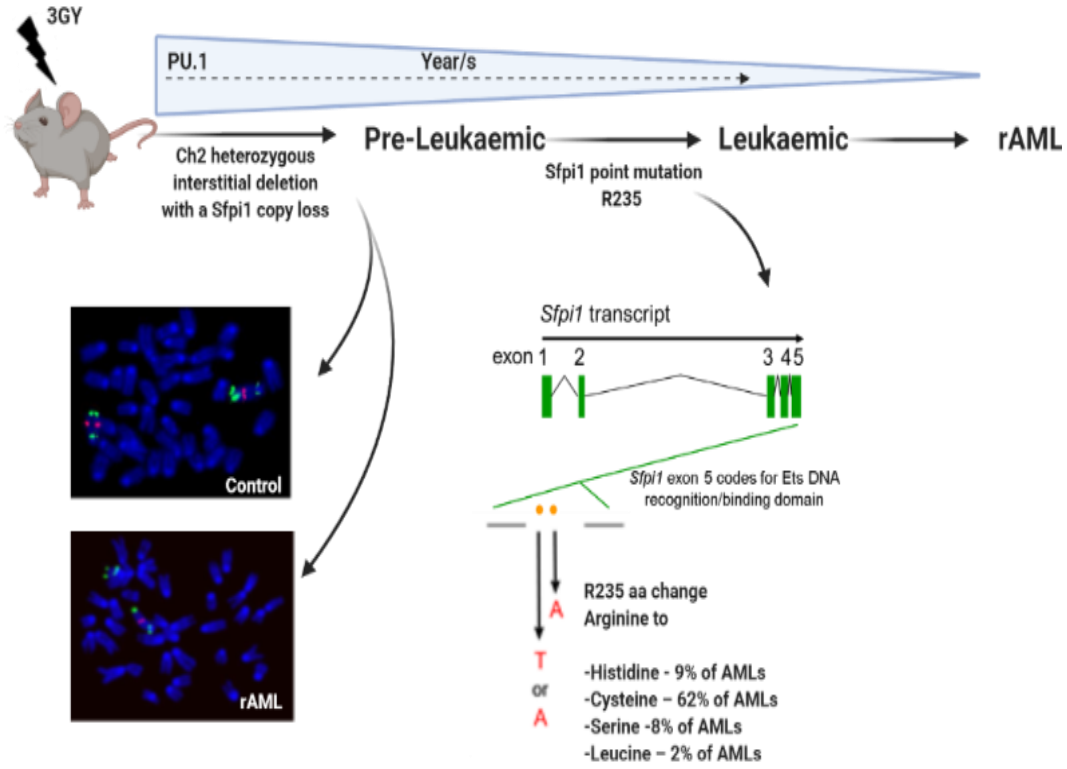


Figure 1. 3 Key cytogenic and molecular events in mouse radiation-induced leukaemogenesis in CBA rAML.

The two-hit model of rAML comprises an interstitial deletion with the loss of Sfpi1 gene on one copy of chromosome 2 which is detectable as early as 24 hours' post-irradiation in a dose dependent manner, creating intermediate cells with a growth advantage, which are not fully malignant. Afterward, intermediate cells acquire a secondary mutational hit (point mutation in the remaining Sfpi/Pu.1 copy), leading in the formation of malignant cells which undergo further clonal expansion, developing into cancers. Adapted from (Dekkers et al., 2011; Verbiest et al., 2015).

In contrast to the two-hit model, which supports that Sfpi1 mutations are only detected in rAML with partially deleted ch2 homologues (Hirouchi et al., 2008; Steffen et al., 2013) . Our group has reported a rAML with a single point mutation in Sfpi1 (R235H), exclusive of ch2 deletions (Brown et al., 2015). The type of R235 mutation (C, H or S) differentially alters mRNA levels, protein abundance and possibly phosphorylation status, with some leading to complete lack of protein function. Even though point mutations are likely to accumulate through spontaneous processes which relies on DNA replication and perhaps 5-methyl cytosine deamination (Ban & Kai, 2009; Peng et al., 2009), it remains unknown at what time these point mutations arise after being exposed to radiation.

In humans, there is little available data on the involvement of PU.1 in the development of therapy-related acute myeloid leukaemia (tAML) (Suraweera et al., 2005). However, numerous genetic and epigenetic variations have been associated with the downregulation of PU.1 in human primary AML (reviewed by (Verbiest et al., 2015)). Accordingly, these pre-leukaemic cells harbour some, but not all of the mutations detected in leukaemic cells (Corces-Zimmerman & Majeti, 2014). Various pre-leukaemic and leukaemic mutations cause PU.1 downregulation in human primary AML, including the top three most commonly mutated genes FLT3, DNMT3A and NPM1 (Jawad et al., 2006; Ley & Cancer Genome Atlas Research Network, 2013). Consistent with the murine models, analysis from the mutation spectrum of 24 human primary AML cases verified that C to T amino acid transitions, as the most common single nucleotide mutation seen in several AML-related genes (Welch et al., 2012). Additionally, TP53 deletion, FLT3-ITD mutation (Finnon et al., 2012; Pedersen-Bjergaard et al., 2008) and the recurring PML-RARA [t (15;17)] and AML1-ETO [t (8;21)] translocations have been proven to downregulate PU.1 expression in human AML cases (Vangala et al., 2003; Walter et al., 2005). Even though the molecular targets vary between species, a common Sfpi1/PU.1 inactivation pathway occurs in both murine rAML and human primary AML cases.

Multiple epigenetic or genetic mutations (Ley & Cancer Genome Atlas Research Network, 2013) are seen in de novo acute myeloid leukaemia but mouse rAML has not been fully examined for these mutations. Recently O'Brien et al., revealed Kras mutation and PU.1 promoter methylation as novel pathways in murine radiation induced-AML (O'Brien et al., 2020). According to their findings, the major pathway consists of ch2 deletion and R235 with no other alterations, with a small minority (3%) also having Kras mutations. Minority have a

ch2 deletion without R235 (Figure 1.4 below). These show a significant rise in *Sfpi1* DNA methylation with a reduced *Sfpi1* transcriptional expression. Furthermore, a small number of cases do not carry ch2 deletion or R235 but do have a prominent rise in Sfpi1 DNA methylation with decreased Sfpi1 transcriptional expression. One of these cases also has a *Kras* G12 mutation. Overall, this work provides new insight into the pathways leading to rAML development, involving genetic, as well as epigenetic changes and, for the first time, specific gene DNA methylation.

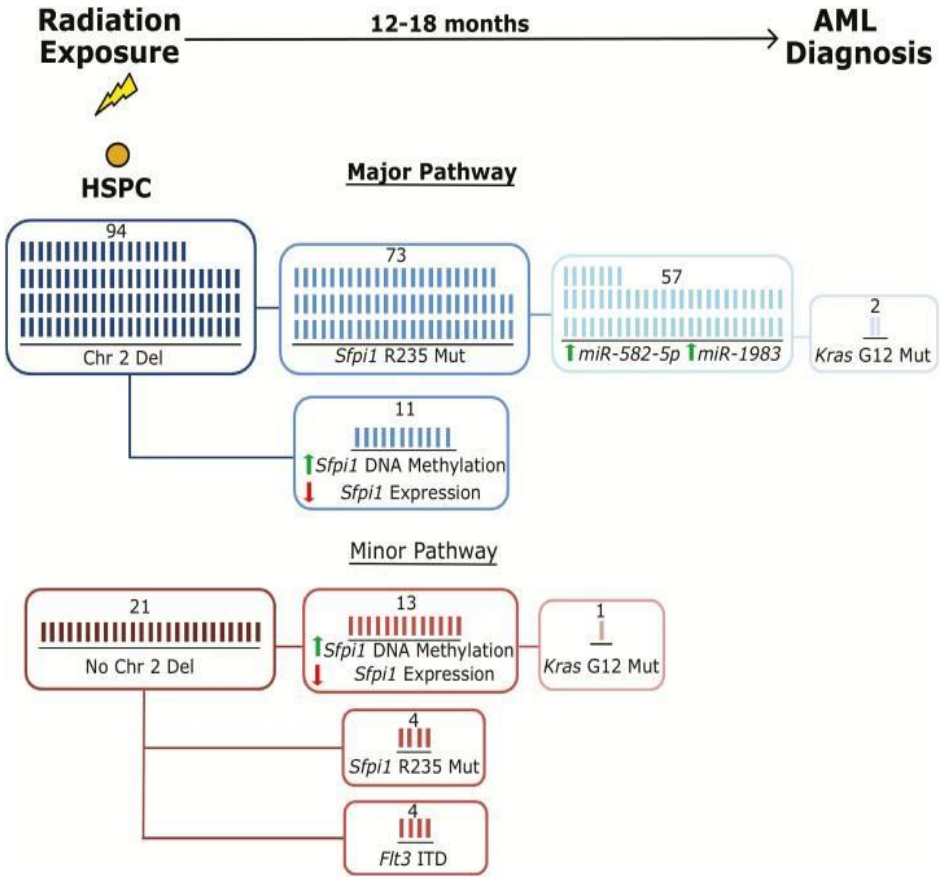


Figure 1. 4 Genetic and epigenetic pathways involved in rAML.

Following IR exposure, the development of rAML appears to be driven by at least two pathways. Ch2 deletion along with Sfpi1 R235 mutation constitutes the major pathway. Minor pathway involves increased Sfpi1 DNA methylation along with reduced Sfpi1 transcriptional expression as well as Flt-2-ITD, Sfpi1 R235 or Kras G12 mutations. Each vertical bar & numbers on top vertical bars (e.g., 94, 21, 13) represent individual mice with total number of mice affected from specified genetic/epigenetic pathway. Arrows in green indicate an increase in expression, while arrows in red indicate a decrease in expression. Figure is taken from (O'Brien et al., 2020). Copyright permission can be found via the link. The figure has not been modified).

1.6 Haematopoietic stem cells and maintenance of blood system homeostasis

Haematopoietic stem cells (HSCs) have been the most extensively studied stem cell model regarding their differentiation pathway ever since they were discovered in the 1960s (Till & McCulloch, 1961). HSCs are the only cell types which possess two essential properties: multi-potency and self-renewal and are defined functionally by the capability of mediating a long-term repopulation of all blood cell lineages in lethally irradiated recipient mice (Spangrude et al., 1988; Till & McCulloch, 1961). According to the classical tree-like model of haematopoiesis, HSCs are positioned atop of a hierarchy of increasingly committed progenitors which are divided into at least three multipotent populations, $CD34^-$ long-term (LT)-HSCs and $CD34^+$ short-term (ST)-HSCs and Multi-Potent Progenitors (MPPs, a cell population which have no detectable self-renewal ability) (Akashi et al., 2000; Kondo et al., 1997; Manz et al., 2002; Morrison et al., 1997; Yang et al., 2005).

Even though this conventional model has been broadly valuable for understanding the differentiation process of HSCs, it oversimplifies the complexity of haematopoietic stem and progenitor cells (HSPCs) and is based only on the specific cell surface markers and transplantation assays, using bulk harvested cells. According to these bulk cell analyses, each HSPC possesses the same phenotype and identical function. Yet, with the advances in single cell technology and mouse genetics, there is more evidence supporting the heterogeneity within HSCs and progenitor cell populations, particularly in the elucidation of megakaryopoiesis (H. Cheng et al., 2020).

According to the findings from Trump and Passegué labs, MPP populations have further divided into four different subpopulations (MPP1, 2, 3, & 4), regarding to their

immunophenotype, lineage bias, cell cycle status, bone marrow abundance as well as resistance to drug treatment (Pietras et al., 2015; Wilson et al., 2008). It has been revealed that MPP1 is more equivalent with the previously defined LT- or ST- HSC, whereas MPP2/3/4 do not hold self-renewal potential and only reveal short-term myeloid reconstruction capability. Although HSCs can give rise to all three-lineage biased MPPs autonomously, none of the MPP cells are capable of generating other MPPs *in vivo*. Further transplantation assays revealed that HSCs initially generate myeloid-biased MPPs (1/2), which is followed by the lymphoid-primed MPP4 subpopulation to rebuild the lymphoid compartment. So far, the model in Figure 1.5, signifies the most supreme model for haematopoietic differentiation, yet with further advancements in single cell technology, more subtypes of stem and/or progenitor cell populations may well be revealed.

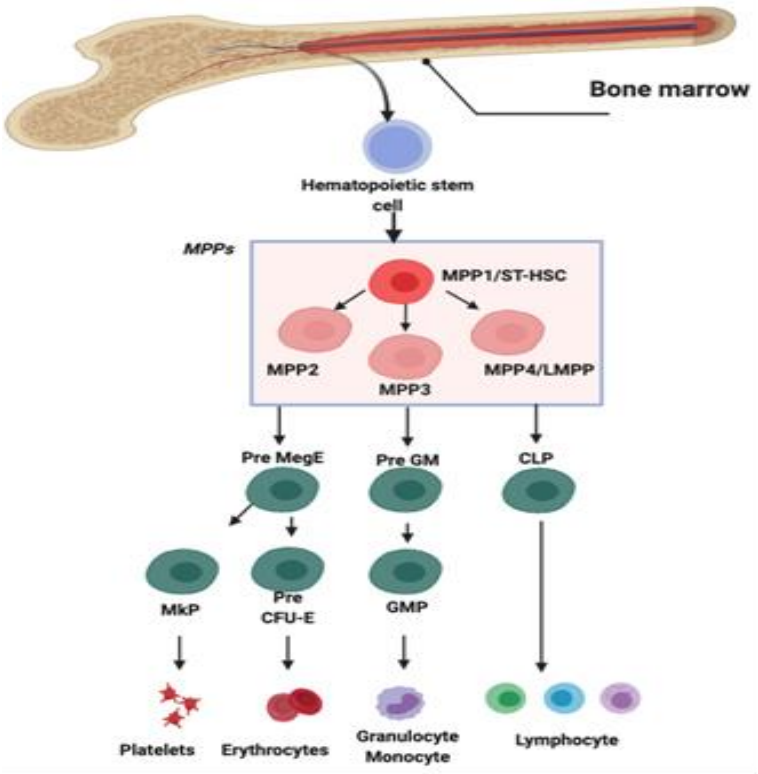


Figure 1. 5 Up-to-dated reconciled model for haematopoietic stem cell (HSC) differentiation.

In this model, HSCs primarily differentiate into MPP1/ST-HSC, then give rise to MPP2,3 and 4 (LMPP). MPP2 can give rise to pre MegE, and subsequently, Pre MegE gives rise to either platelets through or produce erythrocytes. MPP3 generally produces granulocytes & monocytes, whereas MPP4/LMPP mainly contribute to lymphocyte lineages. The figure is taken from (H. Cheng et al.,

2020), and has been modified. Permission details can be found from the link, which permits unrestricted use, disruption, and reproduction.

Haematopoiesis relies on the fine regulation of HSC biology in response to continuously changing internal and external cues from the niche microenvironment (Crane et al., 2017; Morrison & Scadden, 2014). Most HSCs exist in a quiescent, non-motile state *in vivo* which acts as a 'protective state' to reduce stress triggered by DNA replication, cellular respiration, and exogenous sources (Orford & Scadden, 2008; Semenza, 2012; Suda et al., 2011), Figure 1.6. The dynamic balance between quiescence, self-renewal and differentiation is crucial for preserving a functional blood system throughout the life of an organism (Ito & Suda, 2014). The process is regulated by multiple complex systems, involving transcription factors, growth factors, auto-regulatory feedback mechanisms and interaction with the local microenvironment. Failure to maintain haematopoietic homeostasis can lead to the development of haematopoietic disorders such as leukaemia (Zon, 2008), as well as manifestation of ageing (López-Otín et al., 2013)

HSCs possess a distinct metabolic profile, which is essential for maintaining their functions, quiescence, and long-term self-renewal (Suda et al., 2011; Zhang & Sadek, 2014). Metabolic equilibrium is critical to endorse HSC maintenance, restraining the generation of reactive oxygen species (ROS), yet reliance on this leaves HSCs vulnerable to changes in redox status. Many dietary modifications can lead to alterations in stem cell function, by directly influencing the availability of nutrients or regulating hormone level, growth factors controlling tissue homeostasis and tumour initiation, different signalling factors including insulin and insulin-growth factor, and epigenetic patterns (Mihaylova et al., 2014; Rafalski et al., 2012). As such it represents an area with potential for identifying factors and developing novel treatments capable of mitigating long-term potential deleterious effects following IR exposure.

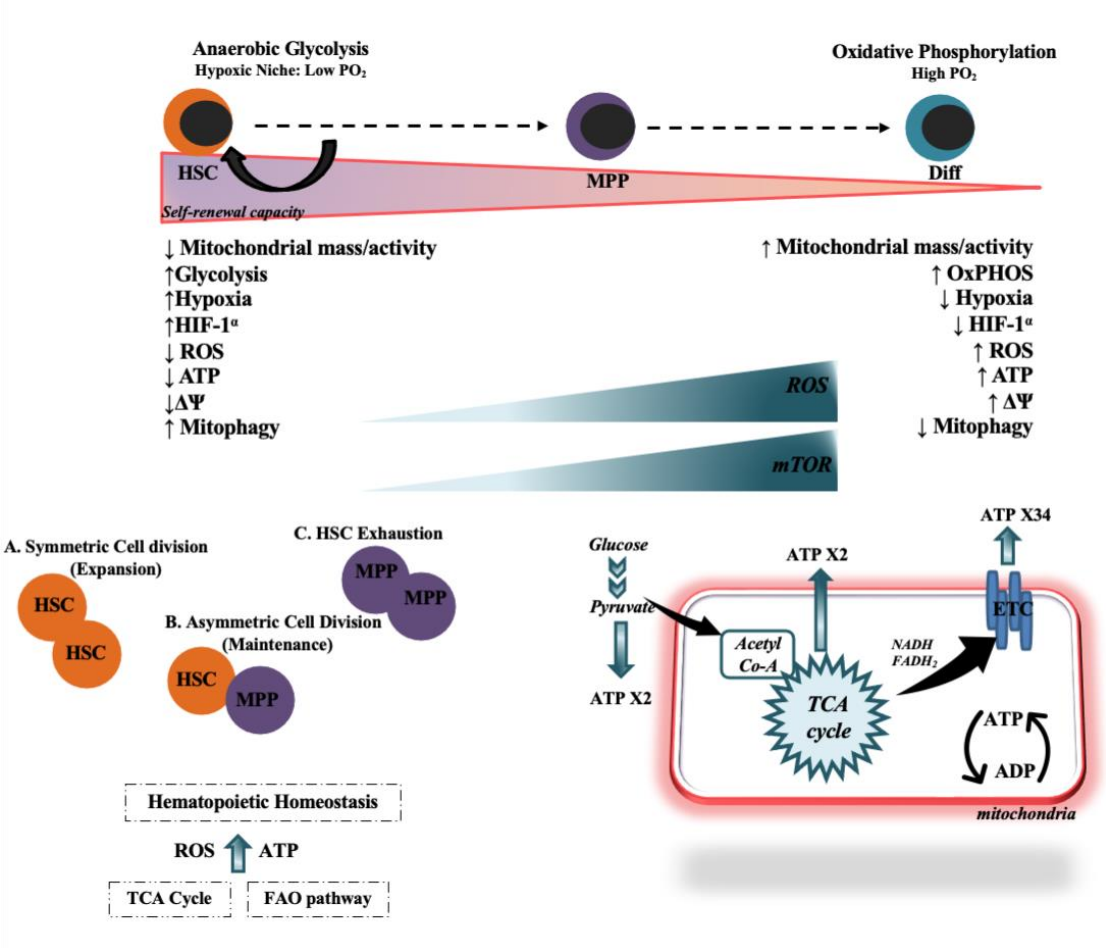


Figure 1. 6 Metabolic regulation of mammalian haematopoietic stem cell fate.

(**A, B)** HSCs display high glycolytic activity and low metabolic status with low ATP, ROS, and membrane potential (ΔΨM) that retain the quiescence of adult HSCs during steady state or stress conditions in contrast to their more differentiated progenitors which display high metabolic status and use oxidative phosphorylation (OXPHOS). Additionally, stabilised HIF-1α is indispensable to support stemness and self-renewal potential. (**B, C)** Stimulation of metabolic pathways such as tricarboxylic acid (TCA) cycle and fatty acid oxidation (FAO) pathways produce high levels of ROS and ATP, thus triggering asymmetric cell division and leading HSCs to adopt more committed and differentiated cell fates. A blockage of these pathways, on the other hand, causes symmetric division, which either results in the expansion of HSCs or their differentiated progenies. Adapted from (Kohli & Passegué, 2014; Kumar & Geiger, 2017).

1.7 Impact of radiation exposure on HSCs, homeostasis, and leukaemia development

HSPCs are among the most sensitive to the adverse effects of IR, including cell death or genotoxic responses, potentially resulting in radiation-induced acute myeloid leukaemia (Verbiest et al., 2015). Target cells for rAML are believed to lie in the HSPC compartment.

Studies using genetically modified mice have led to better understanding of underlying mechanisms and characterisation of target cells (Verbiest et al., 2018) as well as their responses to IR. Previous results from our group demonstrated that interstitial ch2 deletions exist in the HSC-enriched Lin-Sca1+cKit+ subpopulation but are not detected in the more differentiated lineage-negative cells (Olme et al., 2013). Recently, our group used a F1 CBA Sfpi1/PU.1$^{Gfp/mCh}$ reporter mouse to detect expanding clones of del2-positive leukocytes in peripheral blood (Verbiest et al., 2018).

The complex regulatory processes regulating haematopoietic homeostasis could influence rAML development (O'Brien et al., 2019). Yet, little is known about the role of metabolic and dietary factors in this (Karabulutoglu et al., 2019). Various lifestyle and environmental factors play a role in the initiation, progression, and promotion of cancer (Longo & Fontana, 2010), strongly suggesting that inadequate physical activity, excessive adiposity, and unhealthy diets were supporting factors in the pathogenesis and prognosis of various cancers including leukaemia (Lichtman, 2010; de Pergola and Silvestris, 2013). The link between metabolism and cancer development on a cellular level has long been recognised (Karabulutoglu et al., 2019). Diet and energy metabolism influence the regulation of tissue stem cells and homeostasis to change tissue composition and growth, stating an interconnection between diet, stem cell function and cancer/leukaemia development.

Research is revealing an intricate network of dietary and metabolic factors which interact to form a metabolic regulation system maintaining haematopoietic homeostasis and modulating stem cell radiosensitivity and rAML risk (O'Brien et al., 2019, 2020). The effect of dietary alterations such as calorie restriction, fasting, depletion of specific nutrients (e.g., individual amino acids, vitamins) and other metabolic changes on HSC regulatory systems and leukaemia

risk requires further investigation and represents a significant research area of interest for both the Radiation Protection and Public Health fields.

1.7.1 Caloric Restriction, HSC homeostasis, and leukaemia incidences

Dietary restriction, particularly caloric restriction (CR), is characterised by a reduction in overall caloric intake for a duration of more than 12 hours, without resulting in malnutrition. The primary objective of CR is to establish a sustained energy deficit, which can elicit various physiological and metabolic alterations. CR has been identified as a significant modulator of carcinogenesis in experimental studies and has demonstrated the ability to enhance longevity, promote a healthy lifespan, and decrease cancer incidence across a range of species, including primates (Colman et al., 2009; Fontana et al., 2010; Holloszy & Fontana, 2007; Kenyon, 2010; Mattison et al., 2012; Mihaylova et al., 2014; Mohrin et al., 2010; Signer & Morrison, 2013; Speakman & Mitchell, 2011; Weindruch et al., 1986; Weindruch & Walford, 1982). Haematopoietic stem cells (HSCs) rely critically on specific metabolic programs which prevent aerobic metabolism for sustaining their self-renewal capacity and quiescent state. Nevertheless, less is known about the impact of caloric restriction on the radiation carcinogenesis/leukaemogenesis (C.-W. Cheng et al., 2014; Ito et al., 2012; Takubo et al., 2013; W.-M. Yu et al., 2013).

According to a previous study, CR can reduce radiation-induced neoplasm by 36%, however their outcomes were only limited to lymphatic neoplasms, not for rAML (Gross, 1988; Gross & Dreyfuss, 1984, 1986, 1990). Subsequent studies carried out by Yoshida et al. revealed the effect of CR on reducing the occurrence of rAML, following single whole-body radiation exposure in C3H/He mice models (K. Yoshida et al., 1997). The CR groups displayed a delay in disease onset with an extended lifespan, following IR exposure. Furthermore, reduction in

frequency was more prominent when CR started prior to irradiation rather than later. Taken together, these results strongly suggest that CR could be used to mitigate the effect of IR exposure, playing a critical role throughout initiation and promotion phases of radiation-induced leukaemogenesis to diminish the risk (K. Yoshida et al., 1997, 2006). Despite the beneficial effects of CR on the incidence and progression of cancers, the mechanisms are still poorly understood.

Investigations into possible modes of action have shown that CR alters DNA methylation, oncogene expression levels, activity from free radical formation, cell cycle activity as well as modulates apoptosis, by eliminating preneoplastic cells (Feuers et al., 1993; Grasl-Kraupp et al., 1994; Hass et al., 1993; Hursting et al., 1994; James & Muskhelishvili, 1994; Lok et al., 1988; Longo & Fontana, 2010; Nakamura et al., 1989). In the case of mouse rAML, it has been indicated that HSCs, including the cycling fraction, displayed significant reduction in number (K. Yoshida et al., 1997, 2006). It is implied that all these mechanisms stimulate metabolic adaptation to CR, possibly by altering specific nutrient-responsive pathways (Figure 1.7), anabolic hormone production; ROS production as well as modifying antioxidant systems to attenuate oxidative stress and free radical-induced DNA damage (Kalant et al., 1988; Kemnitz et al., 1994; Kenyon, 2010; Merry & Holehan, 1981; Sohal et al., 1994; Sohal & Weindruch, 1996; Sonntag et al., 1999; Youngman et al., 1992). Besides, more recent studies signified the importance of mechanistic target of rapamycin complex 1 (mTORC1) signalling and CR. Thus, by reducing mTORC1 signalling in Paneth cells, CR improved the SC functionality and preserved intestinal SCs from DNA damage, however, further investigations are required to determine whether similar pro-regenerative effects can also be observed in other organs (Yilmaz et al., 2012; Yousefi et al., 2018).

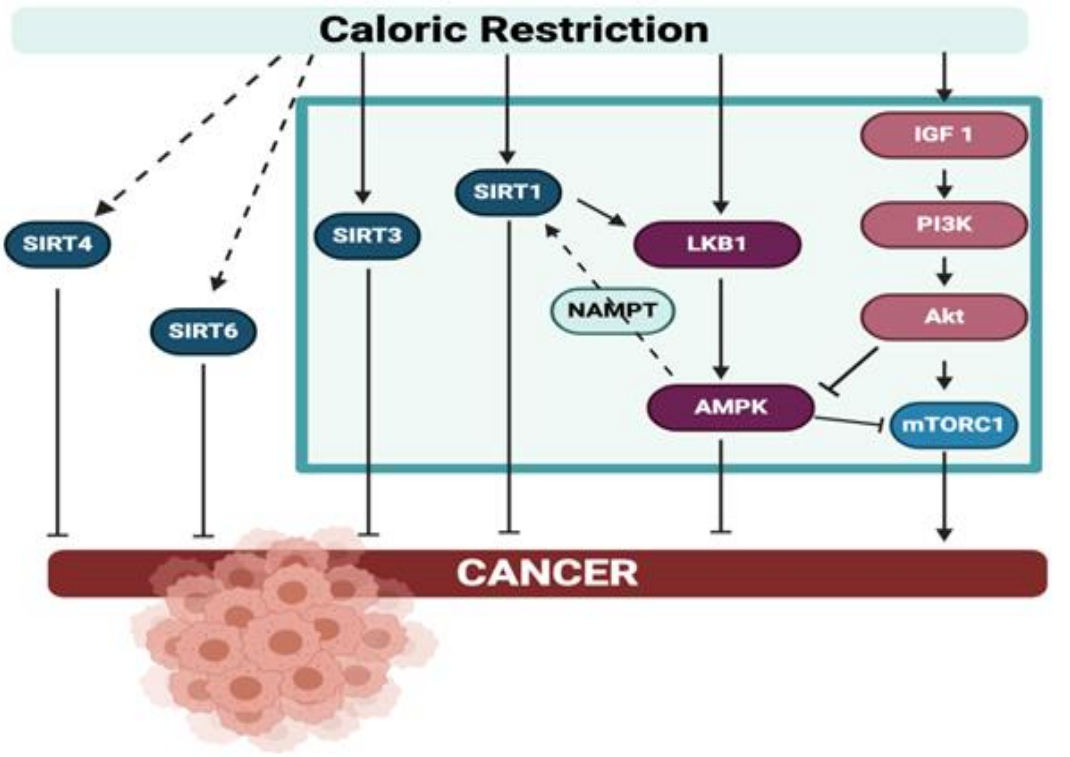


Figure 1. 7 Molecular pathways induced by caloric restriction to inhibit cancer.

Caloric restriction (CR) can impede IGF-1/AKT, mTORC1 and stimulate AMPK, SIRT1 & SIRT3 pathways, proposing that CR uses longevity pathways to restrain cancer (Dreyfuss, 1984; Dunn et al., 1997; Kalaany and Sabatini, 2009; Gao et al., 2012; Price et al., 2012; Curry et al., 2013; Faubert et al., 2013; Johnson, Rabinovitch and Kaeberlein, 2013b). However, the regulation of SIRT4 and SIRT6 by CR remains undefined (Csibi et al., 2013). Although there are a lot of controversies about the anticancer role of SIRT 1,2,4 & 6, (Chang & Guarente, 2014; D. Chen et al., 2008; W. Chen et al., 2013; Csibi et al., 2013; Jeong et al., 2013), the crosstalk between SIRTs and AMPH pathways are undoubtedly confirmed. AMPK can induce activation of SIRT1 via NAMPT and SIRT1 can activate AMPK via LKB1 regulation (Fulco et al., 2008). Abbreviations: IGF-1, insulin-like growth factor 1; PI3K, phosphoinositide 3-kinase; mTORC1, mammalian target of rapamycin complex 1; NAMPT, nicotinamide phosphoribosyltransferase; SIRT, sirtuin; LKB1, liver kinase B1. The figure is taken from (Meynet & Ricci, 2014) and slight additions are made to the original image. The copyright permission to use JC1 content can be found from the link with licence number 559571361527.

The study undertaken by Tang et al. provides the first experimental evidence that long-term CR alters lymphocyte differentiation, resulting in immune system dysfunction in the haematopoietic system (Tang et al., 2016). Despite the long-term CR conditions, both HSC repopulation capacity and quiescence showed significant improvements from youth to midlife. Overall, the findings of this study showed that both positive and negative effects on HSC

function could be attributed to subtle stress signalling mediators (such as IL-6 and 7) and growth factors (IGF-1) (Ertl et al., 2008; Tang et al., 2016).

Although the beneficial effects of CR on whole-body metabolism, including enhanced insulin sensitivity and glucose regulation, have been extensively documented for decades, recent investigations have unveiled a noteworthy discovery. At the cellular level, CR impacts molecular pathways similar to the ones affected by current biological substances used to target cancer metabolism (e.g., CR on triple-negative breast cancer (TNBC) (Saleh et al., 2013). Notably, at the molecular level, tumours subjected to both IR and CR showed decreased proliferation and increased programmed cell death (apoptosis). Their cDNA array analysis indicated that the IGF-1 pathway plays a significant role this physiological role, and various components of the IGF-1R pathway, including IRS, PIK3ca, mTOR, and IGF-1R were found to be being downregulated (Saleh et al., 2013). It's worth noting that while CR hasn't been formally tested as a cancer treatment in clinical trials, it has been effectively utilised in trials for other diseases with careful dietary guidance and behavioural modifications (Brandhorst & Longo, 2016; Klement & Pazienza, 2019; Murata et al., 2015; Safdie et al., 2012a). Therefore, applying CR could serve as a complementary approach to cancer treatment, offering the unique advantage of simultaneously influencing multiple molecular targets. This approach might also help mitigate the potential adverse effects associated with using multiple targeted agents.

1.7.2 Fasting, HSC homeostasis, and leukaemia incidence

While a wealth of literature has been devoted to the mechanisms and impacts of CR, there is substantial concern about how CR might intensify malnutrition in cancer patients, resulting in a loss of lean body mass, a reduction in steroid hormone production, and an impairment of immunity (S. K. Das et al., 2017; Dirks & Leeuwenburgh, 2006; Fontana, 2007; Fontana & Partridge, 2015; Holloszy & Fontana, 2007). It can therefore be argued that fasting or fasting-

mimicking diets (FMDs) offer more promising and discrete regenerative, metabolic, and protective profiles and are, therefore, conceivably more efficient than CR. Similar to CR, intermittent fasting (IF) acts as a carcinogenic modifier which involve in the treatment of various solid tumours and has been disclosed to stimulate anti-cancer effects of chemotherapy (Bordone & Guarente, 2005; Fontana & Partridge, 2015; Hursting et al., 2003; Kalaany & Sabatini, 2009; C. Lee et al., 2012; Longo & Mattson, 2014; Mihaylova et al., 2014).

Intermittent fasting is characterised by alternating cycles of fasting and feeding periods (Keenan et al., 2022). Numerous intermittent fasting approaches exist, such as the 16/8 method, involving a 16-hour fasting period followed by an 8-hour eating window daily. Another approach is alternate-day fasting, which includes fasting every other day or restricting food intake on 1 or 2 non-consecutive days per week. The latter is commonly referred to as the 6:1 or 5:2 diets respectively. During the feasting days, there is no restriction in the food intake and individuals are allowed to eat normally or ad libitum., Figure 1.8 below (Bruce-Keller et al., 1999; Anson et al., 2003; Harvie et al., 2011; Rothschild et al., 2014; Fung, 2016). Clifton et al. provided a comprehensive summary of various fasting regimens in both rodents and humans, with a particular focus on the biological adaptations that could potentially lower cancer incidence and improve overall health outcomes (Clifton et al., 2021). Despite the differences between caloric restriction, intermittent fasting, and time-restricted feeding, these approaches exhibit commonalities in their potential implications for cancer prevention and treatment. They entail energy restriction, triggering metabolic adaptations, influencing hormone profiles, exerting anti-inflammatory effects, enhancing cellular stress resistance, and possibly augmenting treatment efficacy. Further research is required to comprehensively understand their effectiveness, optimal protocols, and potential risks in cancer prevention and treatment.

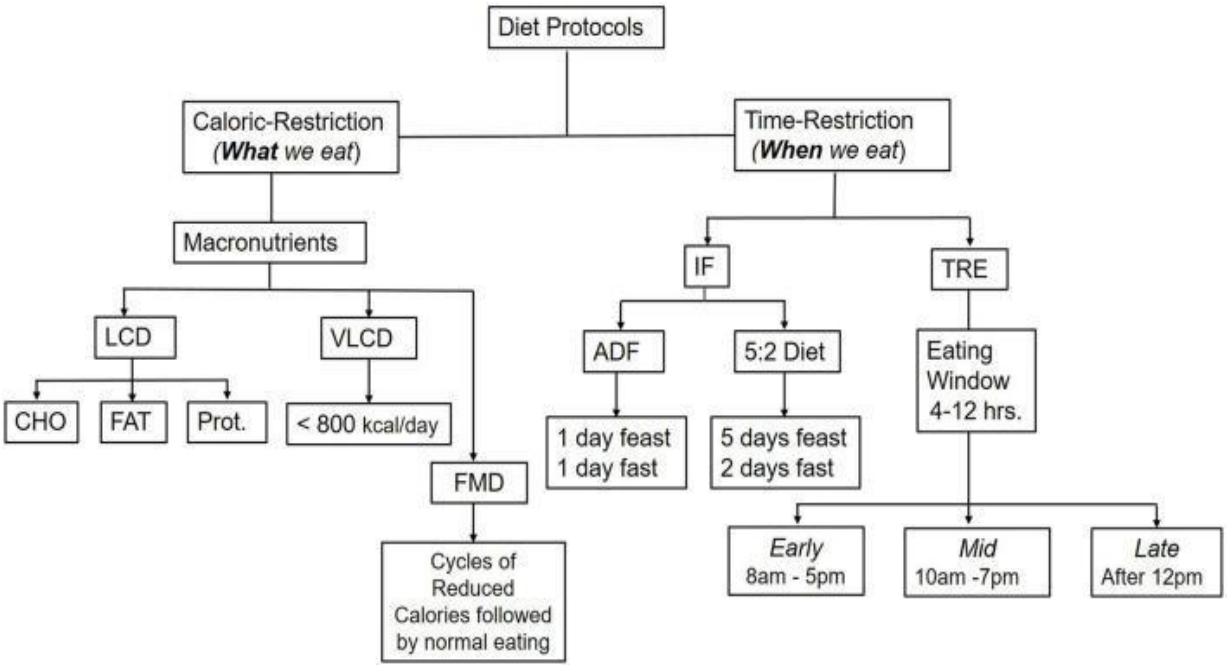


Figure 1. 8 Schematic representation of common dietary protocols.

Low-calorie diet (LCD) may include low carbohydrate (CHO), low fat (FAT), or, in certain cases, low protein (Prot.). Medical supervision is necessary when implementing a very low-calorie diet (VLCD) that provides only 800 kcal/day. Time-restricted diets involve specific eating windows, such as intermittent fasting (IF), time-restricted eating (TRE), and fasting-mimicking diets (FMN). TRE can be practised in different time frames. The figure is taken from (Soliman, 2022) and has not been modified. The article is an open-access article distributed under the terms of CC BY, the use of figure is fully permitted.

There has been extensive research conducted on intermittent fasting in preclinical mouse models of cancer, with mixed but promising results. For instance, a study from DBA mice found that IF did not prevent mammary cancer or slow tumour growth spontaneously (Tannenbaum & Silverstone, 1950). Nevertheless, xenograft mouse models of breast cancer, melanoma, and neuroblastoma showed the same efficacy of 48-hour fasts at reducing tumour growth as two cycles of chemotherapy (C. Lee et al., 2012). Furthermore, the IF regimen combining two consecutive 24-hour fasting periods demonstrated at least a trend (hazard ratio 0.59-0.65; $P>0.05$) toward delayed tumour growth and improved survival despite no differences in body weight in a small study of a xenograft LAPC-4 human prostate cancer model (Buschemeyer et al., 2010). Detailed information on intermittent fasting in mouse

models of cancer is presented in Table 2 of the review paper by Clifton et al (Clifton et al., 2021).

Another systematic review examined (Valayer et al., 2020) all experiments on humans, animals, and eukaryotic cells in PubMed, Cochrane Library, and specialised databases comparing the effects of irradiation post-caloric restriction or fasting to those of a non-nutritionally restricted control group on a broad range of outcomes ranging from molecular to clinical. There were 2,653 records found in the initial search, while 11 studies were included in the final analysis. The majority of studies investigated survival rates and/or cancer incidence. Pre-exposure caloric restriction had no benefit, except when combined with post-radiation caloric restriction. However, pre-exposure fasting may enhance resilience against ionising radiation.

Various studies have demonstrated that changes in circulating hormones and metabolites during fasting or FMDs have a valuable outcome against numerous cancer types by reducing the intracellular signalling cascades such as IGF1R–AKT–mTOR–S6K and cAMP–PKA signalling, increases autophagy, which helps normal cells resist stress and stimulate anticancer immunity (Anson et al., 2003; Brennan & Mantzoros, 2006; C.-W. Cheng et al., 2014; Di Biase et al., 2016; Ito et al., 2012; Jardé et al., 2011; C. Lee et al., 2010; Pietrocola et al., 2016; Pollak, 2012; Takubo et al., 2013; Wan et al., 2003). In addition, during fasting, ketone bodies can inhibit the activity of histone deacetylases (HDACs), decreasing tumour growth and stimulating differentiation via epigenetic mechanisms (Newman & Verdin, 2014).

In the study of haematological malignancies, intermittent fasting was shown to decrease the rate of development of both B-cell and T-cell acute lymphoblastic leukaemia, with fasting mice exhibiting 0.48%± 0.1% of leukaemic GFP^{+ve} cells in the peripheral blood (post-

transplantation) when compared with control mice (67.7% ± 8.4%) (Lu et al., 2017). As a result of their analysis, fasting increased LEPR expression and its downstream effector PRDM1, which drives ALL blast cells to differentiate by depleting leukaemic cells, but not in AML. Further research is required to clarify the effect of fasting on radiation-induced AML models based on their data. In another study, OF1 mice were found to have a 33% reduction in lymphoma frequency after four months of alternate day fasting (ADF) (Descamps et al., 2005). A similar finding was observed in the colon cancer study (P. Sun et al., 2017), where fasting mice consumed nearly twice the amount of food as control mice on their feast days, indicating that alternative mechanisms beyond weight change could contribute to the effects of fasting (Descamps et al., 2005).

Although rats can sustain longer fasts than mice, most studies conducted on these animals have been negative, with very few exceptions (Caderni et al., 2002; Hikita, 1997; Rocha et al., 2002; Siegel et al., 1988; Tessitore, 1998; Tomasi et al., 1999). In an early study (Siegel et al., 1988), rats fed alternate day fasting one week before injection with mammary ascites tumour cells had a longer survival rate than those fed ad libitum (ad-lib) (50% vs 12.5% survival at 10 days, respectively). In another study, male Wistar rats were used to test how intermittent fasting (IF) affects hepatocarcinogenesis after exposure to liver chemical carcinogenesis. After fasting 48 hours prior to carcinogenesis, rats were randomised into an ad-lib diet or were subjected to IF, with 48 h fasting every week for 48 weeks. Fasting prior to exposure did not affect the development of neoplastic lesions, however, long-term IF was associated with an anti-promotion effect on rat hepatocarcinogenesis (Rocha et al., 2002). Furthermore, there have been observations indicating that certain types of leukaemia can undergo lineage switching upon relapse, a phenomenon associated with the capacity to halt fasting and the ability to inhibit LEPR-induced differentiation (Dorantes-Acosta & Pelayo, 2012).

Studies of radioprotective effects of fasting and caloric restriction on humans are scarce. Following caloric restriction, an *in vitro* pilot study using human serum revealed an increased resistance to oxidative stress (Allard et al., 2008). Several other studies demonstrated the link between food intake and oxidative stress and provided potential explanations by interacting with energy sensing pathways (Fontana & Partridge, 2015; Martens & Seals, 2016). A number of proteins are implicated in the mediating the stress resistance and antioxidant response to fasting and caloric restriction, including Sirtuins (Cantó & Auwerx, 2009; Vassilopoulos et al., 2011), FOXO (Brunet et al., 2004), TOR (Johnson, Rabinovitch and Kaeberlein, 2013a), AMPK (Greer et al., 2007), or NRF2 (Martín-Montalvo et al., 2011). According to the studies so far, CR or fasting may mitigate the biological damage caused by indirect effects of IR by interacting positively with the cellular antioxidant system, as well as reducing the incidence of various diseases such as cancer (de Cabo & Mattson, 2019). However, deeper understanding of the underlying mechanisms and discrepancies observed in preclinical studies of intermittent fasting (IF) is crucial to develop modified diets that can serve as more effective therapeutic interventions for reducing disease burden in cancer and leukaemia patients. Similar to personalised medicine, achieving precision nutrition requires the targeted delivery of appropriate nutrients to specific patients at optimal times. To accomplish this, future research should focus on analysing how tumours utilise nutrients and investigating the biochemical processes involved in the digestion and absorption of macronutrients and micronutrients.

1.7.3 Amino Acids, HSC homeostasis and leukaemia incidence

Studies by Kornberg et al. demonstrated that rats fed a low-protein diet developed haematological disorders such as severe anaemia and granulocytopenia which could be successfully treated with purified amino acids (Kornberg A, 1946; Kornberg et al., 1946). The importance of amino acids in HSC homoeostasis has been reported in various studies (Girotra

et al., 2020; Ito & Suda, 2014; Z. Li et al., 2015; Suda et al., 2011). Valine is an essential amino acid for HSC proliferation and survival (Taya et al., 2016; Yamazaki & Nakauchi, 2015) and mice fed with valine-depleted diets experienced drastic reduction in HSC frequency within one week. Furthermore, dietary valine restriction cleared the mouse BM niche, facilitating donor HSC engraftment without the need for chemo-irradiative myeloablation (Taya et al., 2016).

Aas are not only key for protein synthesis, but also serve as intermediate metabolites. Besides, they are crucial for the interconnected metabolic pathways used by HSC, particularly under stress conditions, and can directly enter the TCA cycle, by generating acetyl-coA (Martínez-Reyes & Chandel, 2020). Thus, they can contribute to the haematopoietic stress response in the same way as glucose, FFA and lactate during the TCA cycle. Furthermore, Aas are involved in regulating a variety of signalling pathways that are important for determining the metabolic profiles of the HSCs. Branching chain Aas, for instance, were found to regulate MEIS 1 and p21 levels, which are essential for maintaining HSC quiescence and expansion (X. Liu et al., 2018). Additionally, Aas, particularly glutamine (Oburoglu et al., 2014) have been reported to play a role in regulating HSC differentiation.

Because of the multiple effects described, it is not possible at present to simply define the roles that amino acids play in HSC metabolism, and they are likely to be influenced by the context of the broader cellular and niche environment (Morrison & Scadden, 2014; Taya et al., 2016). There is an increasing body of evidence that malignant cells use amino acids for growth (Jones et al., 2018) and it is likely that AML hijacks mechanisms inherent to HSCs to achieve maximum growth potential. Thus, a better understanding of amino acid metabolism in AML may enhance our knowledge of their role both as a steady state and a response to stress.

Increasing metabolic demands can cause rapidly dividing cancer cells to genetically reprogram their nutritional requirements (Emadi, 2015). Aas, such as glutamine (Gln) are known to

promote cancer cell growth and survival (Kroemer & Pouyssegur, 2008). A study conducted by Esen et al. revealed that Gln is an exchange factor that facilitates the import of essential amino acids into cells, increasing protein translation through the mTORC1 signalling pathway, which in turn promotes cellular proliferation (Esen et al., 2016). Similarly, interrupting Gln metabolism impairs mitochondrial function by reducing intracellular ATP levels, oxygen consumption rate, and apoptosis, impeding AML cell survival (Jacque et al., 2015; Souba, 1993; Willems et al., 2013; Wise & Thompson, 2010; Yuneva et al., 2007). These findings highlighted Gln's importance in cell metabolism and provide a new research direction for future AML therapies. In addition, various groups have reported that arginine deprivation has influence against various cancer cell types including pancreatic, prostate, breast cancers as well as primary AML, by altering distinct signalling pathways (Bowles et al., 2008; Daylami et al., 2014; R. H. Kim et al., 2009a; Miraki-Moud et al., 2015; F. Qiu et al., 2015). Hence, valine or arginine depletion could possibly reduce the incidence of radiation-induced leukemogenesis, altering the frequency and maintenance of HSCs. Finally, while it has been suggested that diets deficient in certain Aas may be an effective means of reducing the effects of IR exposure, no substantiated evidence is currently available. For future applications, it is critical to consider numerous features (e.g., low toxicity, nonimmunogenic, fast-acting) while developing an Aa-depleting agent against cancer.

1.8 Metabolism, HSC, and leukaemia incidence

A growing body of evidence suggests that the significance of mitochondrial content and membrane potential in HSCs has been underestimated. The regulation of mitochondrial physiology has emerged as a pivotal intrinsic biological signal, influencing both healthy and transformed cell populations. Numerous comprehensive reviews underscore the centrality of metabolism as a governing factor in determining HSC fate (Ito et al., 2019; Karabulutoglu et

al., 2019; Morganti et al., 2022). Given this context, a more profound understanding of how distinct metabolic mechanisms are orchestrated to shape the fate of HSCs carries significant biological implications. Such insights will prove pivotal in the development of novel therapeutic strategies for haematological malignancies.

1.8.1 Influence of mitochondria in HSC and leukaemia incidence

Long-term HSCs residing in a hypoxic niche environment have a highly glycolytic nature with a low mitochondrial activity and are sensitive to oxidative stress (Ito et al., 2004; Ito & Suda, 2014; Maryanovich et al., 2012; Norddahl et al., 2011; Parmar et al., 2007; Simsek et al., 2010; Spencer et al., 2014; Suda et al., 2011; Vannini et al., 2016). Conversely, progenitor cells with a reduced capacity for self-renewal (e.g., short-term HSCs or MPP cells) produce energy, shifting their metabolic status from anaerobic glycolysis to oxidative phosphorylation (OXPHOS) (Figure 1.6 above) (Takubo et al., 2013; W.-M. Yu et al., 2013).

Mitochondria are biosynthetic hubs and have key roles in numerous fundamental processes including OXPHOS, ROS regulation, apoptosis, calcium signalling, TCA cycle (Ansó et al., 2017; Birsoy et al., 2015; Chandel, 2015) (Figure 1.9). Mitochondria are the main site for adenosine triphosphate (ATP) production through OXPHOS which creates the metabolic centre of the cell. During OXPHOS, ROS are produced as by-products of mitochondrial respiration (Bigarella et al., 2014a). Adaptation of cells to higher energy status largely depends on the mitochondrial complex, which is controlled by *de novo* synthesis, degradation as well as fission and fusion processes (H. Chen & Chan, 2005; Hock & Kralli, 2009; Xu et al., 2013). Regulatory pathways correlating HSC metabolism, energy demands, mitochondrial function, and HSC quiescence/self-renewal have been defined. For instance, tumour suppressor protein *Lkb1* functions upstream of AMP-activated protein kinase pathway and loss of *Lkb1* in mice displayed a significant reduction in mitochondrial membrane potential and showed alterations

in nucleotide and lipid metabolism, leading to loss of HSC quiescence (Gan et al., 2010; Nakada et al., 2010). Mitochondria act as a vital determinant of stem-cell fate, where its quality and quantity undertake numerous modifications throughout the self-renewal, proliferation, and differentiation phases (Joshi & Kundu, 2013; Parker et al., 2009). Therefore, disruption in any of the regulatory processes can result in mitochondrial damage or dysfunction, which impairs cellular function (Joshi & Kundu, 2013). Besides, subtle but detectable alterations in mitochondrial metabolism may distinguish normal blood and LSCs (Lagadinou et al., 2013). Mitochondria are not only main players in LSC survival and malignancy but also change the TME to keep the LSC alive. They regulate redox status, bioenergetics, nutritional dependence, and metabolic products according to the available substrates, as well as modifying the surrounding immune milieu of the tumour.

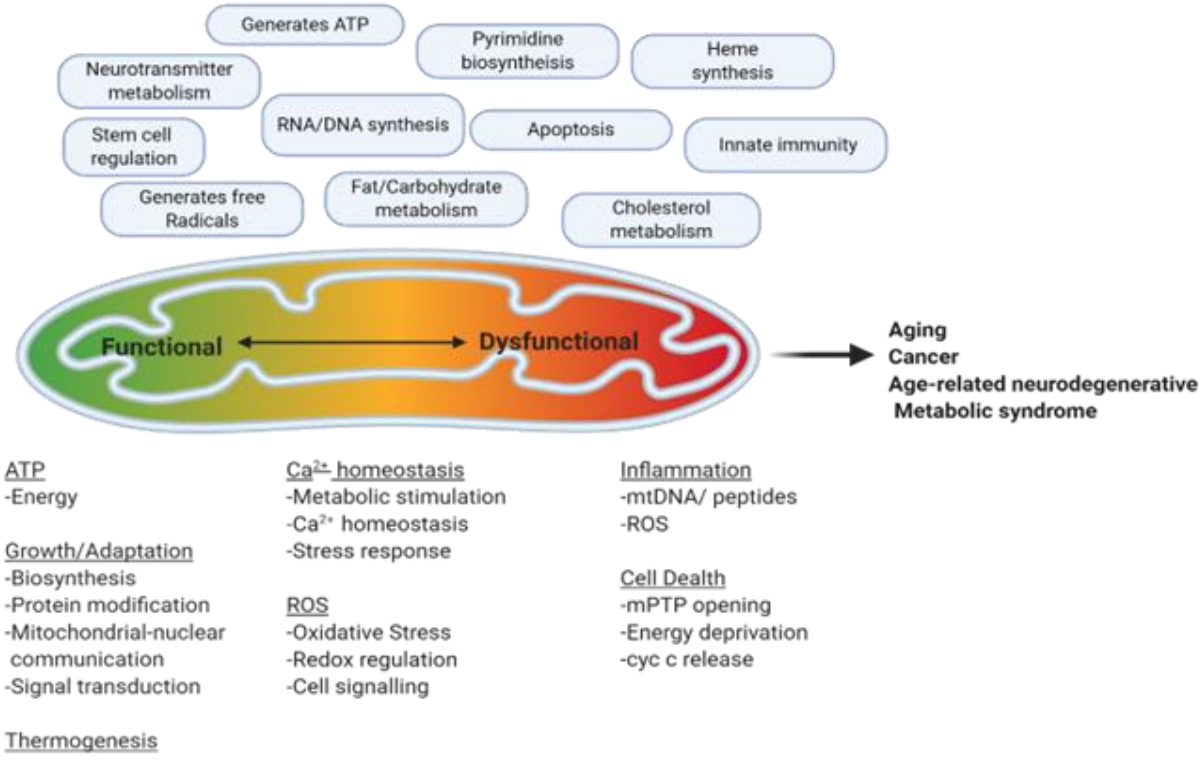


Figure 1. 9 An outline of mitochondrial function in health and disease.

Under normal circumstances, oxidative metabolism generates ATP as well as heat in various specialised cell types e.g., brown adipocytes. Intermediate metabolism in mitochondria leads to the generation of metabolites which are essential for protein modification, signal transduction and biosynthesis. OXPHOS results in ROS generation, which can be used as a molecular signal or can lead to cell damage and/or death. Calcium has a significant role in regulating mitochondrial metabolism but, under

pathological conditions excess calcium can trigger the opening of mitochondrial permeability transition pore (mPTP). Upon release of mitochondrial contents, such as *cytochrome c*, cells can undergo apoptosis, while the loss of membrane potential can result in ATP deprivation and necrosis. Likewise, the leak of damage-associated molecular patterns (DAMPs), including mitochondrial DNA, peptides, or excess ROS production, can lead to inflammation and further tissue damage. Consequently, a shift in mitochondria from a power source to a death engine is the predominant cause of mitochondrial related disorders, including neurodegenerative diseases, cardiomyopathies, metabolic syndrome, cancers, and obesity. The figure is taken from (Zhou & Tian, 2018) and slight additions are made to the original figure. The copyright permission to use JC1 content can be found from the link.

Mitochondria and particularly mitochondrial DNA (mtDNA) are highly sensitive to IR (Kam & Banati, 2013). IR exposure can lead to alterations in metabolic activity, stimulate ROS-generating oxidases and modulate antioxidant enzymes in response to oxidative stress. Due to the limited DNA repair capacity and the lack of protective histones in mtDNA, excessive ROS may impair OXPHOS, or alter expression of proteins critical for mitochondrial function, resulting in mitochondrial dysfunction and cellular damage (Azzam et al., 2012; Copeland et al., 2002; G. J. Kim, Chandrasekaran, et al., 2006). Radiation-induced mitochondrial-ROS altered gene expression levels, mitochondrial DNA copy number, apoptosis, induced mutations, genomic instability, leading to neoplastic transformation, Figure 1.10 (Antipova et al., 2011; Chaudhry & Omaruddin, 2011; Q. Chen et al., 2003; Cherbonnel-Lasserre & Dosanjh, 1997; Chiu et al., 2011; Cuisnier et al., 2003; Du et al., 2009; Limoli et al., 2003; Lomonaco et al., 2009; Malakhova et al., 2005). Furthermore, a study conducted by Shidara and colleagues, specified the carcinogenic potential of mtDNA, where a single point mutation in MTATP6 (component of ATP synthase), triggered apoptotic resistance, ultimately resulting in tumour formation (Shidara et al., 2005). It is important to note that a single gene mutation in mtDNA is insufficient to cause high frequency genomic instability and chronic oxidative stress. Thus, mitochondrial dysfunction, intertwined with other factors, can maintain oxidative stress, leading to chromosomal rearrangements which may contribute to haematological disorders.

In summary, mitochondrial biology and tumourigenesis interact on multiple levels: tumourigenesis may be initiated directly by mitochondria (such as through mutations in mtDNA) or through alteration of mitochondrial functions of metabolism and bioenergetics by oncogenic signalling pathways. Hence, understanding the mechanisms of cancer-initiating cell metabolism can aid in developing novel anticancer drugs that target these aspects.

1.8.2 Influence of Reactive Oxygen Species in HSC and leukaemia incidence

To maintain HSC function and homeostasis, it is necessary to strike a balance between self-renewal and differentiation. Reports from pluripotent and adult stem cells indicated that this balance is partly synchronised by ROS (Bigarella et al., 2014b). ROS are highly reactive and induce chain reactions between molecules, leading to acute oxidative stress (Ludin et al., 2014). ROS were initially considered to be harmful by-products of metabolism, but there is growing evidence for moderate levels of ROS influencing cell-fate signalling, stem cell function, migration, development, and in BM microenvironment (Finkel, 2003; Harris et al., 2013; Janssen-Heininger et al., 2008; Ludin et al., 2014). Numerous intrinsic factors (derived from stem cells) and extrinsic factors (derived from the microenvironment of the BM stroma and endothelial cells) play a role in maintaining ROS levels for the proper function of HSCs within BM reservoir (Ludin et al., 2014) For instance, under hypoxic environments, transcription factors HIF-1a, FOXO3, and ATM downregulate intracellular ROS and maintain HSC pool in a BID, p16- and AKT- dependent manner (Ito et al., 2004; Maryanovich et al., 2012; Miyamoto et al., 2007; Suda et al., 2011). Conversely, when subjected to stress conditions (e.g., IR exposure), increased NADPH oxidase activity and mitochondrial respiration result in augmented ROS production, concurrent with an attenuation in HIFα, triggering cell cycle entry and HSC proliferation (Kohli and Passegué, 2014; Ludin et al., 2014).

IR-induced ROS can increase transiently though; i) the direct hydrolysis of water; ii) increase in the expression levels of inflammatory cytokines (Iyer et al., 2000; Morgan et al., 2002) for iii) damaging the mtDNA or proteins (G. J. Kim, Chandrasekaran, et al., 2006). Upon activation, these pathways induce aberrantly high ROS levels, acting as toxic compounds, leading to aberrant cell proliferation (Oberley & Oberley, 1988; Weydert et al., 2006). Furthermore, multiple studies have demonstrated a dual role for ROS in the development of haematological malignancies, including AML, ALL, MDS, and CML (Battisti et al., 2008; Chung et al., 2014; Pawlowska & Blasiak, 2015; Testa et al., 2016; Udensi & Tchounwou, 2014). Accordingly, ROS can either i) stimulate cell death (e.g., apoptosis), which in turn provides a possible protective mechanism for cancer treatment (Khoshtabiat et al., 2016) or ii) provoke carcinogenesis through hindering apoptosis, prompting cell survival, proliferation (D. Cheng et al., 2015), extracellular matrix (ECM) independency, metastasis (Liou & Storz, 2010; Zhu et al., 2016), as well as drug resistance (D. S. Das et al., 2016; Ma et al., 2016). Understanding the biochemical and molecular events under oxidative stress environments in IR-induced cells and tissues may provide new strategies to defuse the adverse health effects of IR.

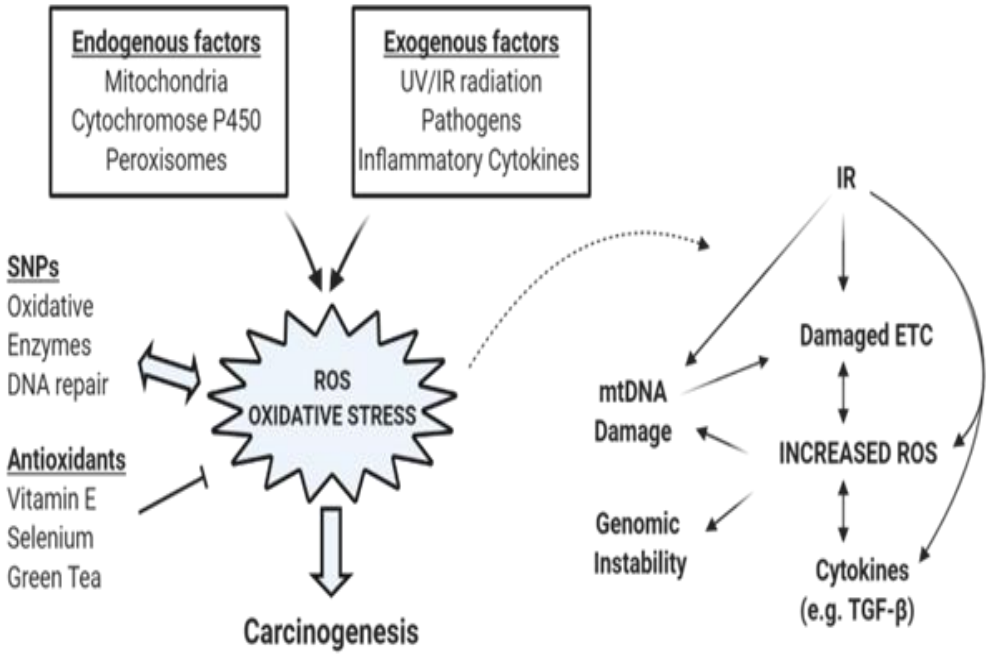


Figure 1. 10 The concept of oxidative stress effects in genomic instability.

ROS levels are dynamic and reversible where HSCs can change from ROS-high to ROS-low status, by switching their cell fate and function correspondingly. Fine-tuning of ROS levels is key in maintaining HSC function and normal haematopoietic homeostasis. IR can elevate ROS levels in a variety of ways; through the hydrolysis of water, increased levels of inflammatory cytokines, and the damage to mtDNA. Once activated, all these pathways can maintain elevated ROS levels, resulting in increased oxidative stress and genomic instability, which can culminate in HSC exhaustion or pre-leukaemic transformation. The figure incorporates elements from Kim et al. 2006, with various enhancements, and copyright permissions have been updated accordingly with licence number 5598880787204. Details can be found from the link (Karabulutoglu et al., 2019; G. J. Kim, Fiskum, et al., 2006).

1.9 Research Objectives

Radiation-induced Acute Myeloid Leukaemia (rAML) is the most common cancer seen in humans following IR exposure (Hsu et al., 2013) and bone marrow cells are amongst the most sensitive to the damaging effects of IR, comprising initially of cell death or genotoxicity, eventually leading to rAML. While the target cells for rAML are believed to reside within the Haematopoietic Stem and Progenitor Cell (HSPC) compartment, there is a notable dearth of knowledge concerning their characteristics and reactions to radiation exposure. Furthermore, intricate regulatory systems are in place to maintain haematopoietic equilibrium, potentially influencing the risk of rAML, particularly metabolic factors affected by diet (Karabulutoglu et

al., 2019). The emerging role of dietary and metabolic factors in regulating normal HSC homeostasis and their response to radiation exposure holds profound implications in radiation protection and public health.

The CBA mouse strain is regarded as the primary model for studying rAML (Verbiest et al., 2015). A novel genetically modified CBA mode, enabling the tracking of preleukaemic cells carrying ch2 deletions in vivo, has been recently developed (Verbiest et al., 2018). This model presents an opportunity to explore the impact altered diets e.g., caloric restriction, intermittent fasting, or amino acid depletion, on the sequence of molecular events occurring during radiation-induced leukaemic clonal evolution. This will, perhaps, lead to the development of protocols which can mitigate the risk in previously exposed populations.

This PhD thesis is underpinned by two primary aims:

1. Characterise the cellular aspects (proliferative/clonogenic abilities) and metabolic aspects (e.g., mitochondrial/glycolytic changes), using the Seahorse XFp analyser, within HSPCs following IR exposure.
2. Investigate the potential influence of dietary modifications, e.g., caloric restriction, amino-acid depletion, intermittent fasting on the progression of radiation leukaemogenesis in a genetically modified CBA model of rAML.

CHAPTER 2

Methodology

2. Methodology

2.1 Mouse models

Two genetically modified models with PU.1 expression reporter gene were used. CBA Sfpi1/Pu.1^{GFP} mice were generated as previously described (Olme et al., 2013) and mated to CBA Sfpi1/Pu.1^{Mcherry} (Verbiest et al., 2015) to generate F1 CBA Sfpi1$^{mCh/GFP}$ mice expressing mCherry from one chromosome 2 and GFP from the second copy of chromosome 2. Our group's initial aim was to track the allogeneic haematopoietic cells and to overcome the variable reporter expression within the haematopoietic cell population. GFP is under the control of the *Sfpi1* promoter and is expressed in myeloid lineages but variably in others (e.g., T-cells are GFP-negative). Hence, we chose a promoter which is constitutively active (rather than the Sfpi1 promoter, which has a variable expression depending upon the differentiation stage of the haematopoietic cell). mCherry is positioned in the minimal deleted region (MDR) near *Sfpi-1* under the control of the ubiquitous Rosa26 promoter, resulting in a constitutive expression of mCherry within all haematopoietic cells; from primitive HSCs to mature as well as terminally differentiated granulocytes and lymphocytes. The insertion site on chromosome 2 was selected at 92,823,765 – 92,823,766 bp (roughly 1.7 Mb away from Spfi1). By using Zinc Finger Nuclease methodology, transgenic one-cell embryos were produced at SAGE® Labs (Boyertown, USA), followed by rederivation at MRC Harwell (UK) and establishment of a breeding colony at our animal facilities. Due to its position in the MDR, it can act as an accurate reporter for *Sfpi-1* loss events without the variable reporter expression in the haematopoietic cells, as occurs with the GFP model (Figure 2.1).

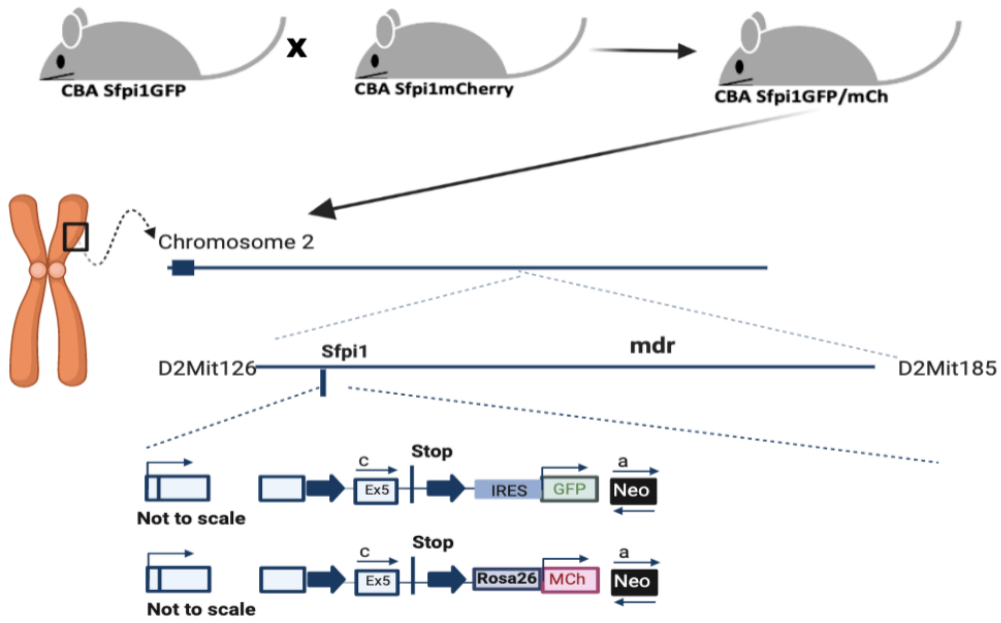


Figure 2. 1 Schematic representation of location of the GFP and mCherry genes on chromosome 2.

The Sfpi1 gene is positioned in the AML Minimal Deleted Region (MDR) between D2Mit126 and D2Mit185. The IRES-GFP cassette in the 3'untranslated region of the Sfpi1 gene is under control of Sfpi1 promoter, resulting in a variable GFP expression throughout the different haematopoietic subpopulations. Whereas mCherry construct is located under control of Rose26 promoter in the MDR region. Exons are denoted as boxes with the coding regions in grey and introns as a navy line, where exon 5 is particularly denoted in the figure. The direction of transcription is marked by arrows. Three primer binding sites are forward a, reverse b and forward c are all indicated. Stop: stop codon, IRES: internal ribosome entry site, Neo: neomycin cassette.

All animals were bred and handled according to the UK Animals (Scientific Procedures) Act, 1986, Amendment Regulations 2012. Animal protocols were reviewed and approved by the local Ethics Committee and the Home Office. Mice were provided with water and food ad libitum. Regular body weight analysis and body condition scoring proceeded (Figure 2.2 below) according to the method of Ullman-Culleré and Foltz (Ullman-Culleré & Foltz, 1999). Scoring was accomplished by keeping the mouse on a flat surface and holding the base of the tail with the thumb and index finger of one hand. The degree of flesh and fat cover was determined either by running the little finger of the same hand over the sacroiliac region or palpating the sacroiliac region with the fingers of the opposite hand.

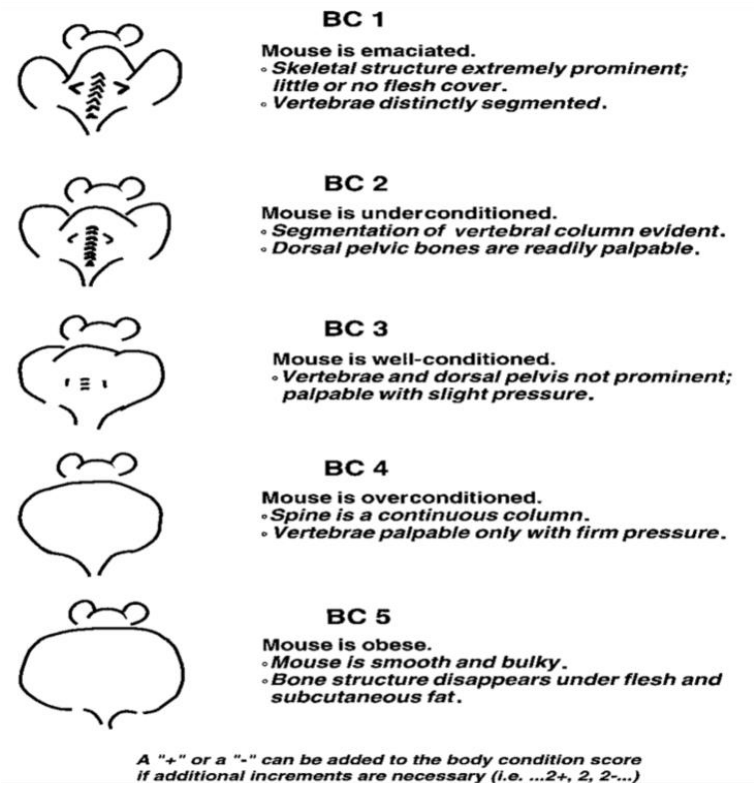


Figure 2. 2 Line drawings and description of body-conditioning (BC) scoring. The figure is taken from (Ullman-Culleré and Foltz, 1999) and no alterations have been made.

2.2 In Vivo X-Ray Exposure & rAML induction

In our experimental investigations, we employed distinct radiation dosages. For the induction of acute myeloid leukaemia (AML) in mice, we utilised a specific dose of 3 Gy, as it has been established as the optimal AML-induction dose in prior research (MAJOR & MOLE, 1978). Several assays were conducted using a dose of 100 mGy, which corresponds to the defined threshold for low dose radiation protection. Moreover, we incorporated intermediate dose levels (2 and 1 Gy) to construct dose-response curves in specific assays e.g., haematopoietic stem and progenitor cell (HSPC) proliferation assays.

Throughout the experimental protocols, mice were given single 3Gy whole-body X-irradiation at 10-13 weeks of age (54 males and 59 females). Irradiations were performed using an AGO

X-ray set (AGO, Reading, UK) running at 250 kV (constant potential) with a Cu/Al filter producing a beam of 1.2 mmCu HVL, with a high dose rate- 500 mGy/min (13 mA, source to shelf distance of 60 cm) or low dose rate 4.9 mGy/min (0.2 mA, source to shelf distance of 91 cm) and filtration remained the same under all circumstances. Radiation exposures were carried out at the Radiation, Chemical and Environmental Hazards Directorate (RCE, formerly CRCE), Chilton, Oxfordshire.

2.3 Dietary Restriction

The food regime used in this study involved alternate day (ADF) feeding/fasting [also known as every other day feeding], based on the method described in the paper published by Lu et al., 2017 "Fasting selectively blocks development of acute lymphoblastic leukaemia via leptin-receptor upregulation" (Lu et al., 2017). At around 8 weeks of age, mice were randomly assigned to Ad libitum (ad-lib) or ADF (alternate day fasting) groups. Mice were subjected to six x 1 day fasting/ 1 day feeding cycles from day 2 post-irradiation. Body weight analysis and conditioning scores were recorded three times weekly to ensure no significant weight loss occurred (no more than 20% starting body weight). Following this, mice are returned into ad libitum diet regimens and monitored weekly.

2.4 Tail vein Blood sampling and Flow Cytometry

Tail vein blood (TVB) samples examined bi-monthly by using flow cytometry post-irradiation to detect the expanding haematopoietic clone which has lost either GFP or mCherry fluorescence (also the ch2 homologue carrying the fluorescence construct).

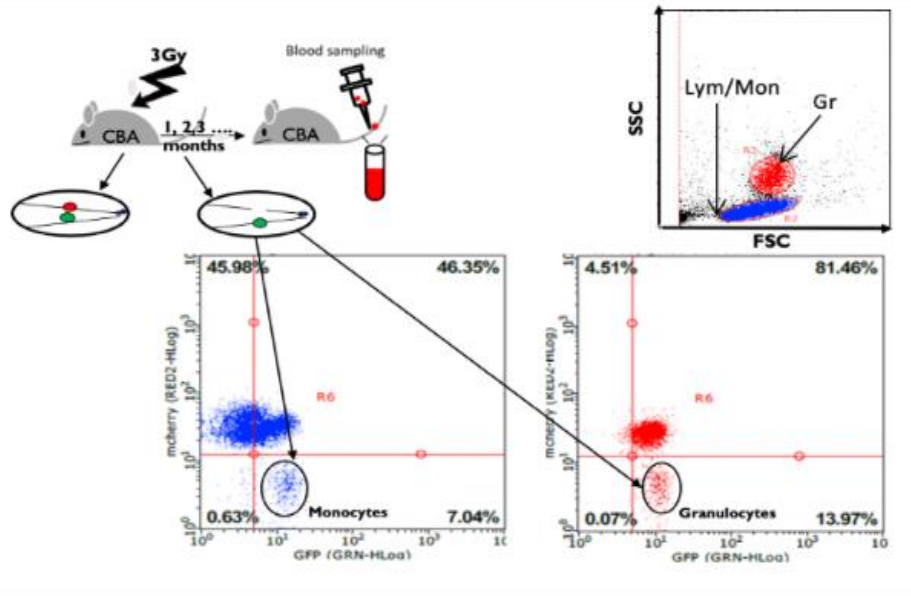


Figure 2. 3 Mouse model CBA Sfpi1$^{GFP/mCh}$ carries a different fluorescent marker on ch2 and allows tracking of peripheral mature blood cells following radiation exposure.

This model is of great importance as it provides that the loss of mCherry fluorescence in peripheral mature blood cells derived from haematopoietic stem cells could solely be due to interstitial deletion of ch2 and simultaneous loss of the Rosa26-mCherry construct.

Tail veins were punctured with an insulin syringe and blood collected using a heparinised capillary tube (Thermo Fisher Scientific UK) and transferred to a 0.2 ml Eppendorf tube on ice (Starlab, Milton Keynes, UK). Red blood cells were lysed using lysis buffer (20:1vol: vol; 20.75 g NH4Cl, 2.5 g $NaHCO_3$ and 0.093 g Na_2EDTA in 1,000 ml dH_2O) for 5min at room temperature (RT) then washed in 190 µl phosphate buffered saline (PBS). Samples were then spun down at 1200 x rpm for 5 minutes; pellets were resuspended in PBS and 1.5x 10^5 WBCs were transferred to a 5 ml FACS tube (BD Biosciences) for analysis by flow cytometry. Acquisition was accomplished by using a Guava® easyCyte Single Sample flow cytometer and examined using InCyte^{TM} software (Merck Millipore, Watford, UK). Cells were gated for size, shape, and granularity, using forward (FSC) and side (SSC) scatter parameters. Regions were drawn around major populations of cells on an FSC/SSC dot plot. Incidents with low FSC/SSC were anticipated to be dead cells or debris and exempt from analysis.

2.5 Tissue harvest & immunogenic negative selection of HSPCs

Mice were sacrificed with a rising concentration of CO_2 and tibias, femur, iliac crests, and spine dissected and cleaned of remaining muscle and connective tissue. To extract bone marrow (BM) cells the cleaned bones were crushed in a pestle and mortar in a small volume of Iscove's Modified Dulbecco's Media (IMDM) and a single cell suspension generated by disaggregating with an 18G needle and filtering through a 40 µm cell strainer (BD Biosciences, Wokingham, UK) into a centrifuge tube. A single cell bone marrow suspension was generated, disaggregating with a 18G needle and filtering through a 40 µm cell strainer (BD Biosciences). The cells were centrifuged for 5 min at 1200 rpm and the pellet resuspended in 1 mL IMDM ready for use.

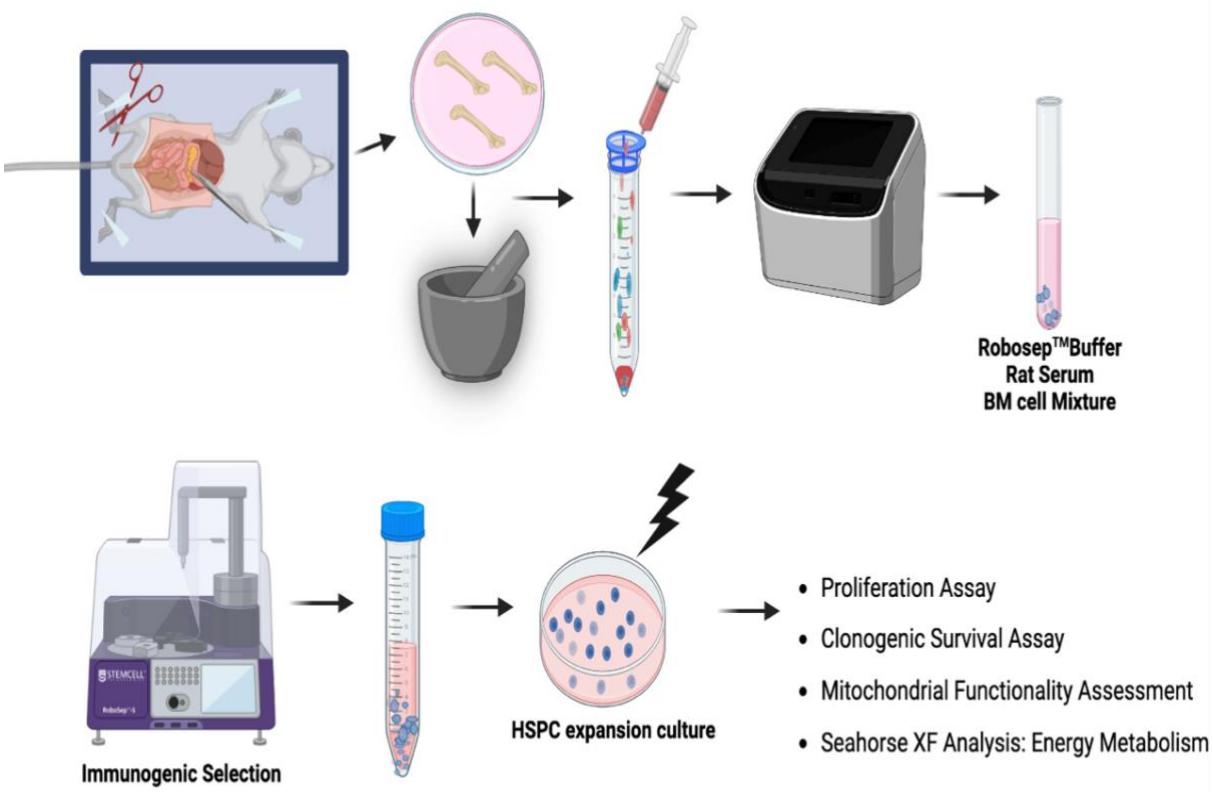


Figure 2. 4 Schematic representation of mice dissection and immunogenic selection protocol.

After the extraction of BM cells, the cell densities adjusted at a concentration of $1x10^8$ cells/mL. Immunogenic selection mixture was prepared (BM cells + Robosep™ Buffer+ Rat Serum), and fully automated mouse haematopoietic progenitor cell isolation protocol proceeded. Following immunogenic selection HSPCs seeded at desired cell densities, expanded either in 20.8% or 3% oxygen environments

and exposed to various radiation doses (0-3 Gy). After IR exposure, cells were extracted and various assays e.g., proliferation, survival, energy metabolism used to further investigate the effect of oxidative stress and X-ray exposure on murine HSPCs. For each assessment protocol, additional details are provided below.

The immunogenic negative selection kit targets non-progenitor cells for removal with biotinylated antibodies recognizing specific cell surface markers. The first step for the isolation of mouse haematopoietic stem and progenitor cells (HSPCs) from bone marrow consists of removing mature cells that express 'lineage' (Lin) antigens specific to terminally differentiated blood cells. Unwanted cells are labelled with biotinylated antibodies and magnetic particles and separated without columns using an RoboSep™ magnet and the desired cell population are simply separated in a new tube. Lineage antigens are absent or weakly expressed on HSPCs. Lineage antigens include CD3, CD11b, CD19, CD45R (B220), Ly6G/C (Gr-1), and TER119. In many mouse strains, HSPCs are positive for Sca1 (Ly-6A/E) and c-Kit (the receptor for SCF, also known as CD117) and referred to as LSK (Lin-Sca1+c-Kit+). For the lineage negative selection of HSPCs, BM cell numbers were determined using a Neubauer haemocytometer and cell densities were adjusted at a concentration of 1×10^8 cells/mL in RoboSep™ Buffer in a 14 mL polystyrene round-bottom tube (Thermo Fisher Scientific, Loughborough, UK) and 50 μL/mL of Normal Rat Serum added, and the tube placed in the RoboSep machine. Fully Automated Mouse haematopoietic progenitor cell isolation protocol #catalogno 19856 (Stem Cell Technologies, Cambridge, UK) was selected, the machine was loaded with antibody cocktail, RapidSpheres™, RoboSep™ buffer and RoboSep™ filter tips, following on-screen instructions. All reagents were obtained from Stem Cell Technologies, unless otherwise stated.

2.6 Cell Culture

All plastic materials for cell culture were procured from Falcon (BD Biosciences, UK), unless otherwise stated. All media were pre-warmed at 37ºC for at least 15 minutes before use. A Sorvall Legend RT benchtop Centrifuge (ThermoFisher Scientific UK) was used for pelleting cells during cell culture. Light microscopy was performed using a Nikon TS-100F (Nikon, UK) inverted phase contrast microscope with a DS5M digital camera attachment (Nikon) for documenting primary data.

2.6.1. Culture Conditions for HSPC Expansion

Following immunogenic negative selection, HSPCs were seeded at the required concentration in a specialised StemSpan (SSpan)^{TM} and a custom-made (amino acid-free) StemSpan^{TM} serum-free expansion medium supplemented with 50 ng/mL recombinant murine stem cell factor, 100 ng/mL recombinant human Flt3 ligand, 100 ng/mL recombinant human interleukin 11, 40 µg/mL low density lipoprotein (Sigma-Aldrich; Invitrogen, Alfa Aesar, Lancashire, UK), 100 U/mL penicillin, 100 µg/mL streptomycin (Thermo Fisher Scientific) and 50 mM 2-mercaptoethanol (Gibco, Life Technologies, Loughborough, UK) in 6-well plates.

For amino acid depletion, a stock of non-essential amino acid mix and aliquots of 16 essential amino acids (arginine·HCl·, arginine, cystine·2HCl, cysteine·HCl· H_2O, glutamine, histidine ·HCl·H2O, hydroxyl proline, isoleucine, leucine, lysine·HCl, methionine, phenylalanine, threonine, tryptophan, tyrosine ·2Na·2H_2O and valine, all L-isomers; Sigma-Aldrich, Gillingham, UK) were added individually to yield a variety of individual media deficient in single amino acids. Cells were subjected to incubation at 37ºC either in normal culture conditions or amino acid depleted conditions. The incubation was carried out in two different oxygen environments: ambient (20.8%) or low oxygen (3%). The low oxygen (hypoxic) environment was achieved by using Tri-Gas incubator, where CO_2 was supplied as usual while

nitrogen (N_2) was introduced to reduce the oxygen level. Cultures were established in triplicate wells, and each experiment was conducted in triplicate.

2.7 Irradiation of HSPCs

HSPCs were checked for attachment by using a phase contrast microscope prior to radiation exposure. The transport times between incubators and radiation sources were held to a minimum, in which cells were returned to an incubator within 5 minutes of radiation exposure. All exposures were executed at room temperature and under normoxia (20.8% O_2) as previously described for *in vivo* irradiations (section 2.2).

2.8 HSPC Proliferation Assay

HSPCs were plated at a concentration of 2×10^5 cells/mL in StemSpan medium into a 6-well tissue culture plate and incubated at 37ºC either in a 20.8% or 3% O_2 incubator for 2 h. Cells were then removed from the incubator and exposed to different radiation doses (0.1–2 Gy) prior to being incubated at 37ºC either in a 20.8% or a 3% O_2 incubator. Growth rates were examined, counting the number of HSPCs on days 2, 5, 7, 9, 13, 15 and the average number of cells were represented by generating a growth curve. One mouse per sample was used unless otherwise stated and all experiments were performed in triplicates.

2.9 Seahorse XFp Assay for Assessment of Energy Metabolism

The Seahorse system, also known as the Seahorse Extracellular Flux (XF) Analyzer, is a powerful tool used to measure cellular bioenergetics, including the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR). It provides valuable insights into cellular metabolism by monitoring real-time metabolic changes in living cells.

For the measurement of OCR, the Seahorse system uses fluorescent sensors to measure the dissolved oxygen concentration in the media. The sensors are embedded in the sensor cartridges, which contain microwells for cell culture (figure 2.5B, below). These sensors are calibrated to provide accurate and quantitative measurements of oxygen level. During the experiment, cells are plated in sensor cartridges, and media is equilibrated with a fixed concentration of oxygen. As cells start to consume oxygen through mitochondrial respiration, dissolved oxygen concentration within the media declines. Hence, by continuously analysing this fluctuation in oxygen concentration over time, the system calculates the OCR based on the rate of oxygen depletion. For the assessment of Extracellular Acidification Rate (ECAR), the Seahorse system measures ECAR as a proxy for glycolysis, which is associated with the production of lactate and protons (H+). The change in pH caused by the release of protons during glycolysis is monitored using pH-sensitive fluorophores embedded within the sensor cartridges. Following the OCR measurement, the Seahorse system introduces a chemical compound, such as glucose or a mitochondrial inhibitor, to stimulate glycolysis. As the cells metabolise glucose and produce lactate and protons, the pH-sensitive fluorophores detect the resulting change in pH. The Seahorse system measures this change in pH over time and calculates the ECAR based on the rate of extracellular acidification.

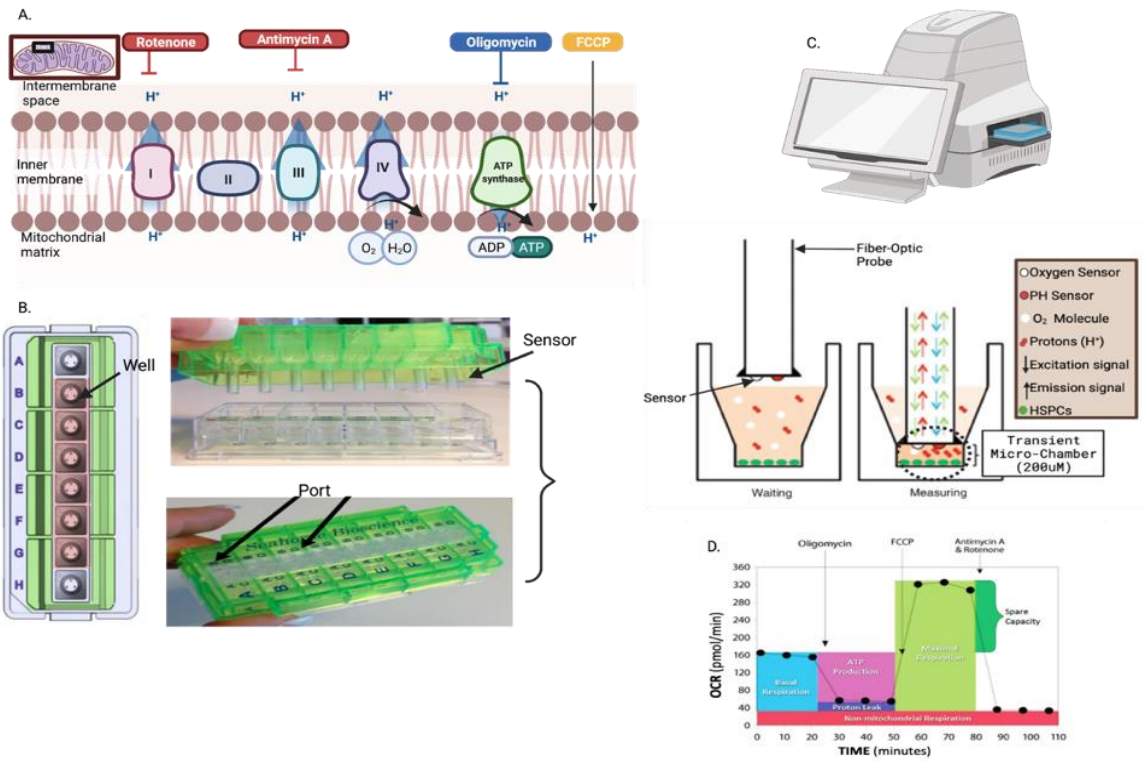


Figure 2. 5 Simplistic illustration of Seahorse XF technology workflow.

Illustrates the complexes of the electron transport chain (ETC) and target of action of all the compounds in the Seahorse XF Cell Mito Stress Test Kit. Oligomycin impedes ATP synthase (complex V), FCCP uncouples oxygen consumption from ATP production, antimycin A and rotenone hinder complexes 1,3. **(B)** Shows the micro sensors (green) which measure rates of OCR and ECAR and XFp miniplate (right), providing a power for pairwise comparison between two different sample groups/cell types. Injection ports provide up to 4 compounds, by leading to pathway perturbation, dose-response, agonist/antagonist response analysis for each sample. **(C)** Sensor probes gradually lower to form 'a transient microchamber', leading real-time measurement of alterations in O_2 and proton (H^+) concentration. **(D)** Displays key parameters of mitochondrial respiration. Sequential compound injections quantify basal respiration, ATP generation, proton leakage, non-mitochondrial respiration, maximal respiration, and spare respiratory capacity. The figure incorporates elements from Leonard et al., 2016, with various enhancements. The copyright agreement has been established with Springer Nature ("Springer Nature") under Licence number 5598890624668. The terms and conditions, as provided by Springer Nature and Copyright Clearance Centre, can be accessed through the following link (Pelgrom et al., 2016).

2.9.1. Real-time ATP Rate or Cell Energy Phenotype Analysis

Initially, the optimal seeding density was evaluated, using a range of cell numbers from 1×10^4, 1×10^5 or 2×10^5 cells/well. A 2×10^5 cells/well was selected as an optimal seeding density for a consistent confluent monolayer. The Agilent Seahorse XFp Analyzer was turned on overnight to warm up and the sensor cartridge was hydrated in Seahorse XF Calibrant (Agilent) at 37ºC in a humidified non-CO_2 incubator as per the manufacturer's guidelines (Agilent). Agilent

Seahorse cell culture miniplates were coated beforehand, using Cell-Tak solution (22.4 μg/mL). For each assay, 0.25 mL of Cell-Tak solution was made per plate, as per manufacturer's directions (Agilent Technologies, Didcot, UK) and 25 μL was applied to each well for 20 min at room temperature. Wells were washed twice using 200 μL distilled water and stored at 4ºC. HSPCs were harvested from the expansion cultures at day 4, 7 and 11 and plated into pre-warmed Cell-Tak coated miniplates in 180 μL XF base medium (DMEM non-buffered pH 7.4) supplemented with 200 mM glutamine, 1 M glucose and 100 mM pyruvate. For adherence, cells were spun down at 200 ×g for 2 min and incubated in a humidified non-CO_2 incubator at 37ºC for 1 h before the assay.

For ATP-rate analysis, pre-warmed oligomycin (1.5 μM final), Rotenone and Antimycin A (0.5 μM final) were diluted in the assay medium and loaded into ports A or B of the hydrated sensor cartridge. For Cell Energy Phenotype Assay, a stressor mixture was prepared with diluted oligomycin (1.0 μM final) and FCCP (1.0 μM final) and loaded into port A, respectively. After calibration of the sensor cartridge, the Seahorse XF cell culture miniplate was inserted (Seahorse Bioscience, Billerica, MA, USA), and the assay continued either using Real-time ATP Rate or Cell Energy Phenotype protocols. Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were taken over time under basal conditions and after the addition of mitochondrial inhibitors. With the simultaneous injection of these stressor reagents two events proceeded: Oligomycin impeded mitochondrial ATP production and, consequently, there was a compensatory surge in glycolytic rate. FCCP depolarized the mitochondrial membrane which triggered the OCR to preserve the mitochondrial membrane potential. For data analysis, the XFp report generator programme was used automatically to calculate test parameters from Wave software. In all experiments three replicate wells were

used. Respiration and acidification rates were presented as the mean ± SEM. All consumables were purchased from Seahorse Bioscience Inc. (North Billerica, MA, USA) (Agilent).

2.10 Colony forming cell (CFC) assays: Cell survival assay

Following immunogenic negative selection, cells were washed in 50 mL centrifuge tubes with room temperature IMD medium by centrifuging at 300 xg for 8 minutes. Supernatant was removed gently, and cells are resuspended in 10mL IMDM with gentle pipetting to generate single cell suspension. Cultures were set up by adding appropriate volume of HSPCs (6,000 cells total) to 4 ml Methocult^{TM} (Stem Cell Technologies) before transferring to 6-well plates, using a 2.5 ml syringe and 18G blunt ended needle (1.1 ml/well, in triplicate). Sterile water was added into empty wells to maintain the humidity necessary for colony development. Cultures were incubated at 37ºC and 5% CO_2 either in 3% and 20.8% O_2 environment for 16 hours before radiation exposure (IR exposures undertaken at 20.8% oxygen environment). Following exposure**,** cultures were incubated either in 3% or 20.8% O_2 for 12 days without disturbing the dishes during the incubation period to avoid shifting of the colonies. At Day 12, colonies were counted (>50 cells) by using EVOS XL core imaging system and average colony count was calculated as the mean colony count of three wells.

2.11 Mitochondrial Functionality Assessment

Following lineage depletion, HSPCs were expanded in suspension cultures at different oxygen states and X-irradiation exposures, as described in Section 2.5.

2.11.1 Mitochondrial mass

The MitoTracker family of dyes, initially developed in the mid-1990s, belongs to the class of cell-permeant dyes that selectively target mitochondria. Specifically, MitoTracker Red and

Orange dyes carry a positive charge, suggesting that mitochondrial membrane potential (ΔΨm) will affect their uptake by mitochondria. These MitoTracker probes undergo passive diffusion through the plasma membrane and accumulate in active mitochondria in a potential-dependent manner.

HSPCs were harvested from expansion cultures at day 7, resuspended in Advanced RPMI 1640 ($1x10^6$ cells/mL) and stained with 100 nM MitoTracker® Deep Red FM (Thermo Fisher Scientific) dissolved in DMSO at 37ºC/ 5% CO_2 for 30 min as per manufacturer's guidelines. Cells were then washed in pre-warmed dPBS, resuspended in Advanced RPMI, and stored on ice until being examined using a Guava® easyCyte Single Sample Flow cytometer, using 532nm excitation with 575 emission filters and analysed using InCyte™ software (Merck Millipore, Watford, UK).

2.11.2 Mitochondrial membrane potential

HSPCs were harvested from expansion cultures at day 7, resuspended at $1x10^6$ cells/mL in RoboSep™ Buffer and incubated with 2 µM MitoProbe™ JC-1 cationic dye (Thermo Fisher Scientific; dissolved in DMSO) at 37ºC/5% CO_2 for 30 min as per manufacturer's guidelines. For control sample, 50 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Thermo Fisher Scientific) was added simultaneously. Cells were washed, resuspended in RoboSep™ buffer, and stored on ice until analysis. Samples were analysed on a Guava® easyCyte Single Sample flow cytometer, using 488 nm excitation with 530/590 nm emission filters. JC-1 is a lipophilic, cationic dye that can selectively enter mitochondria and reversibly change colour from green to red as the membrane potential increases. In healthy cells with high mitochondrial (ΔΨm), JC-1 instinctively generates complexes known as J-aggregates (oligomers) with red fluorescence. Whereas, in unhealthy or apoptotic cells with low (ΔΨm), JC-1 maintains its monomeric form with a green fluorescence.

2.11.3 Mitochondrial superoxide

MitoSox™ Red is a novel fluorogenic dye for the detection of highly selective superoxide within mitochondria of live cells. MitoSox™ Red (MSR) reagent is a live-cell permeant which rapidly and selectively targets the mitochondria. Once in mitochondria, the MSR reagent is oxidised by superoxide and exhibits bright red fluorescence, respectively.

HSPCs were harvested from expansion cultures at day 7, then resuspended at $1x10^6$ cells/mL in RoboSep™ Buffer and incubated with 5 µM MitoSox™ Red (Thermo Fisher Scientific; dissolved in DMSO) at 37ºC/5% CO_2 for 30 min as per manufacturer's instructions. After incubations, cells were washed, resuspended in 0.5 mL RoboSep™ Buffer, and stored on ice until examined by using a Guava® easyCyte Single Sample flow cytometer, employing 532 nm excitation with 575 emission filters and analysed using InCyteTM software (Merck Millipore, Watford, UK).

2.11.4 Mitochondrial DNA Copy Number Assay

The ratio of mitochondrial DNA (mtDNA) to genomic DNA (gDNA) in HSPCs was measured using Quantitative Real-time Polymerase chain reaction (qRT-PCR) to quantitatively assess the ratio of genes on the mitochondrial and nuclear genome. To amplify the mitochondrial gene; Mus musculus mitochondrion, complete genome (mtND1: NCBI Reference number NC_005089.1) and the genomic gene; Mus musculus glyceraldehyde-3- phosphate dehydrogenase (GAPDH: NCBI Reference number NM_001289726.1), primers for both genes were designed using the online Primer3Plus software, obtained from Integrated DNA technologies and primer sequences can be found in Table 1. Total DNA (mtDNA and gDNA) was extracted from cells, using Dneasy Blood and Tissue Kit: Spin- Column Protocol (Qiagen, Manchester, UK) by following the manufacturer's guidelines.

Table 1 Genomic and mitochondrial primer sequences.

Primer	Sequence
mtND1 Forward	5'-CCCATTCGCGTTATTCTT-3'
mtND1 Reverse	5'-AAGTTGATCGTAACGGAAGC-3'
GAPDH Forward	5'-CAAGGAGTAAGAAACCCTGGACC-3'
GAPDH Reverse	5'-CGAGTTGGGATAGGGCCTCT-3'

Quantitative Real-time Polymerase chain reaction (qRT-PCR) was performed using a Rotor-Gene Q (QIAGEN, Hilden, Germany) with PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD, USA). The samples were run in triplicates in 10 µl reactions with 1 µl of the DNA sample (1 ng/µl) together with primer sets for mtND1 and GAPDH (Table 1). Reactions were performed with the following cycling conditions: 2 min at 95°C, then 40 cycles of 10s at 95°C (denaturation), 60s at 60°C (annealing/elongation). Fluorescence data acquired during the extension phase were normalised to housekeeping gene GAPDH by the delta-delta method (Livak & Schmittgen, 2001).

2.12 Hydrogen peroxide measurement

2.12.1 Sample Collection

HSPCs are harvested from expansion cultures at day 7 and centrifuged at 1200 xg for 5 minutes. 300 µl of supernatant (free of debris/cells) from each condition (3 wells/condition) was transferred into 0.5 ml Eppendorf tubes and stored at -20^0C until analysis. Quantitative measurement of hydrogen peroxide was ensued by using a hydrogen peroxide microsensor probe (World Precision Instruments, UK).

2.12.2 Polarisation, Calibration and Testing of Microsensor Probe

The hydrogen peroxide concentration was measured in samples using an amperometric Hydrogen Peroxide Microsensor electrode with a 100 mm tip diameter connected to a Free

Radical Analyser system (WPI, UK) set in the 10 nA range. Data was recorded and analysed using LabTrax 2 software. The microsensor electrode was polarised overnight to a baseline current (around 1000 pA) by plunging the electrode into a 40 ml PBS solution with constant magnetic stirring. The following day, a stock of 200 mM H_2O_2 was freshly made, and 4 to 64 ml was added every 45 sec to the PBS solution to produce a concentration standard ranging from 20 nM to 320 nM for calibration of the electrode. Following this, 100 µl of media from each condition was then sequentially added every 45 sec into the same PBS solution. This whole process was repeated every 15 samples (including the standard) into a new 40 ml PBS solution. All reagents were acquired from Sigma-Aldrich, unless otherwise specified.

2.13 Statistical Analysis

GraphPad Prism 7.04 was used for statistical evaluation. An assessment of normality was evaluated by applying the D'Agostino & Pearson analysis. Comparison between two groups tested, using Student's t-test, and between more than two groups, analysis of variance (ANOVA) as well as multiple comparison tests were applied. Additionally, software R was used for the analysis of our *in vivo* data (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/). All data was expressed as mean values ± Standard Error of the Mean (±SEM) unless otherwise stated. Asterisk * signifies the significance.

CHAPTER 3

Results

3. Results & Discussion

3.1 Effect of Ionising Radiation and Oxidative Stress on the Proliferative & Clonogenic Capability of HSPCs

Stem cells possess the unique ability to regulate their self-renewal and differentiation in response to various intrinsic and extrinsic factors which influence their fate. Notably, oxygen (O_2) plays a crucial role in determining the phenotype of stem cells. Existing body of evidence highlights the limitations of replicating physiologically relevant oxygen tension and gradients in culture environments which can further impair SC behaviour in vitro and after transplantation. Conventional incubation practices expose SCs to non-physiological oxygen tension (typically ambient oxygen levels; 21%), further exacerbating these limitations. Hence, investigating the intricate mechanisms and signalling pathways that become activated in response to low oxygen tensions, resembling the hypoxic conditions found in native microenvironments characterised by hypoxia, presents a valuable avenue for comprehending the fundamental role of oxygen in preserving stem cells' pluripotency and regulating their differentiation processes (Nombela-Arrieta & Silberstein, 2014; Wielockx et al., 2019). Maintenance of HSC in their favoured quiescent state is susceptible to changes in the redox state within the hypoxic bone marrow niche environment (Eliasson & Jönsson, 2010). Hypoxia is known to exert a protective environment for HSC quiescence and self-renewal, stimulating a metabolic shift towards anaerobic glycolysis to protect cells from oxidative stress. Numerous in vitro studies utilising hypoxic cultures with oxygen concentrations of 1-3% have revealed enhanced production of erythroid, megakaryocytic, and granulocytic-monocytic progenitors (Bradley et al., 1978; Katahira & Mizoguchi, 1987; Koller et al., 1992; LaIuppa et al., 1998), as well as improved expansion and engraftment of HSCs (Cipolleschi et al., 1993; Danet et al.,

2003; Ivanovic et al., 2000). In this chapter, we assess the effect of IR exposure, which is known to produce ROS, on the growth potential of HSPCs under a low oxygen environment (3% Oxygen).

3.1.1 Growth curve analysis of HSPC Expansion Cultures under stress conditions

Bone marrow cells were harvested from CBA^{Gfp} mice and negatively selected for HSPCs by lineage depletion as described in the Materials and Methods section 2.5. Following this, HSPCs were seeded at a concentration of $2x10^5$ cells/ml in StemSpan culture medium and incubated in 3% O_2 incubator for 2 hours prior to X-ray exposure and returned to 3% environment post exposure. IR exposed-HSPCs were counted every 3–4 days up to Day 22 then the data was plotted on a growth curve.

The analysis of the growth curve (Figure 3.1 below) reveals a statistically significant reduction in growth rates post-irradiation under low oxygen conditions, compared to non irradiated HSPCs with the effect increasing in a dose-dependent manner (*p* values; 0.1 Gy < 0.001; 0.5 Gy <0.001; 1.0 Gy < 0.0001; 2.0 Gy < 0.0001). This is particularly marked after exposure to the highest dose of 2 Gy. This effect on the growth rate is affected by radiation-induced cytotoxicity, which results in a drop in cell number at day 2 post-irradiation. The remaining survivor-cells continued to divide after the exposure, and all cultures reached a stationary phase (plateau) with a similar number of cells, regardless of dose and/or growth rate, due to limited substrate availability.

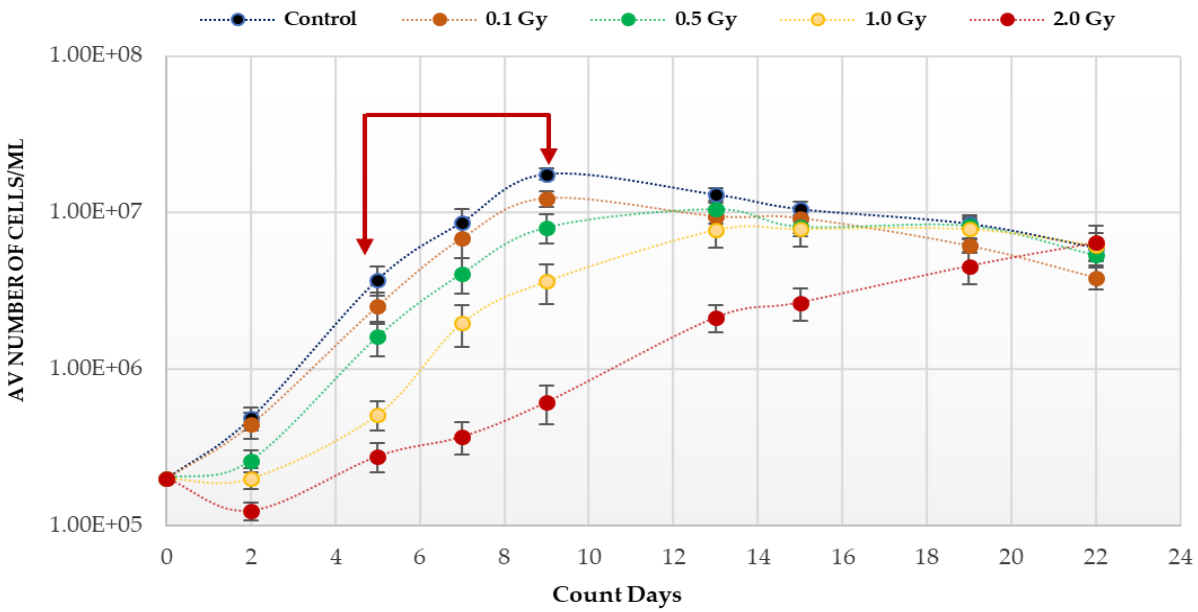


Figure 3. 1 Proliferative capability of HSPCs following IR exposure under low oxygen environments (3% Oxygen).

HSPCs (2×10^5 cells/well, x-irradiated (0–2 Gy) were incubated in 3% O_2 and regular cell counts carried out up to day 22 (N = 3/dose, 3 wells/dose/experiment. Error bars: ±SEM). Two-way ANOVA and Dunnett's multiple comparison test compared IR- and non-IR exposed HSPCs. The statistical significance was established profoundly within the exponential phase (red arrows) (0.1 Gy $p< 0.001$; 0.5 Gy $p< 0.001$; 1.0 Gy $p< 0.0001$; 2.0 Gy $p< 0.0001$). Inter-dose comparisons showed significant differences in all scenarios (student t-test $p< 0.05$, $p< 0.005$). Red arrows indicate exponential growth phase (days 5–9). Time and dose showed statistical significance.

Normoxia (20.8% O_2) alters the redox state by favouring aerobic OXPHOS over anaerobic glycolysis. To determine the impact of this on HSPCs, we compared the growth potential of irradiated HSPCs cultured in low oxygen or normoxic environments (Figure 3.1). A significant increase in proliferative activity during exponential growth phase (between days 5-9, signified by the red arrow in Figure 3.1 above) is observed among IR- and non-IR-exposed HSPC cultures under normoxic conditions, compared to cells cultured in low oxygen environments (Figure 3.2a, day seven cell counts representative of the midpoint of exponential growth). Under both normoxic and low oxygen conditions, growth rates are reduced in a dose-dependent manner, but the effect is more pronounced in normoxia ($p<0.0001$ Figure 3.2b), with a higher fractional change in day 7 cell counts.

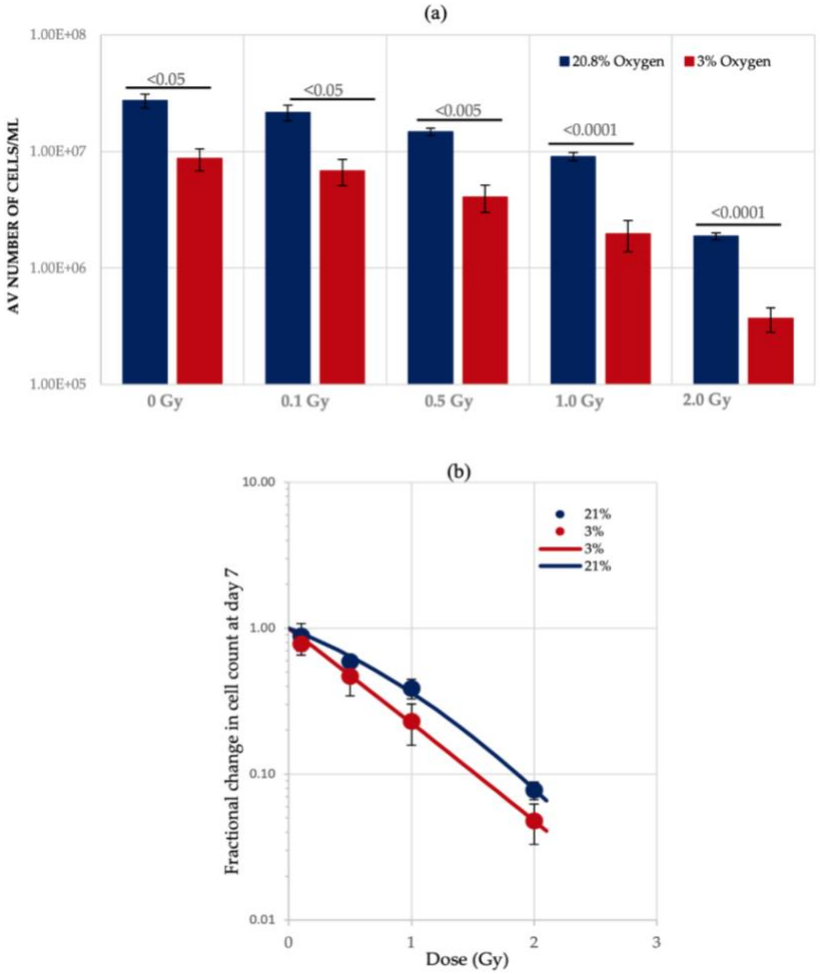


Figure 3. 2 (a, b) Effect oxygen stress and IR exposure on the proliferative capability of HSPCs at day7.

HSPCs (2×10^5 cells/well) were irradiated (0-2Gy) under normoxic environment and subsequently incubated 3% and 20.8% oxygen incubators and regular cell counts were carried out up to day 14 (N= 3; 3 wells/dose/experiment; error bars represent ±SEM). **(a)** compares the growth potential of HSPCs under stress conditions involving differing oxygen levels and IR exposure. **(b)** displays the fractional change in HSPC count at day 7 under stress conditions (blue 21%; red 3% oxygen conditions). ANOVA and multiple comparison tests were applied. Time and O_2 level showed statistical significance in each dose with p= 0.0001 or p< 0.0001. The subject was not significant. Plating efficiency in both normoxic and low O_2 cultures was ~75–80%.

3.1.2 Mouse Colony forming assay to assess HSPC survival under stress conditions.

The effect of IR exposure and differing oxygen environments are also evaluated by using colony forming (CFC) assay. As described previously in section 3.1, BM was harvested from 10 weeks old CBA^{Gfp} mice, followed by immunogenic selection HSPCs. Colony forming cell (CFC) assays were set-up in triplicate at concentration of $2x10^3$ cells/well in 2 ml Methocult™ semi-solid media. Cultures were incubated at 37 ^oC/5% CO_2 in 3% and 20.8% O_2 environments

for 16 h prior to radiation exposure (0-2 Gy). Following IR exposure**,** cultures were incubated, and colonies counted (>50 cells/colony) at day 12, by using EVOS XL core imaging system. The average plating efficiency [PE= (colonies counted/colonies inoculated) x100], for HSPCs was approximately 38%. According to the results presented in Figure 3.3, dose-dependent reduction in the CFC formation was detected, when compared with unirradiated colonies. IR exposure had a more prominent impact on colonies exposed to 1 and 2 Gy, which experienced 1.7- and 2.5-fold reduction in average colony numbers, respectively.

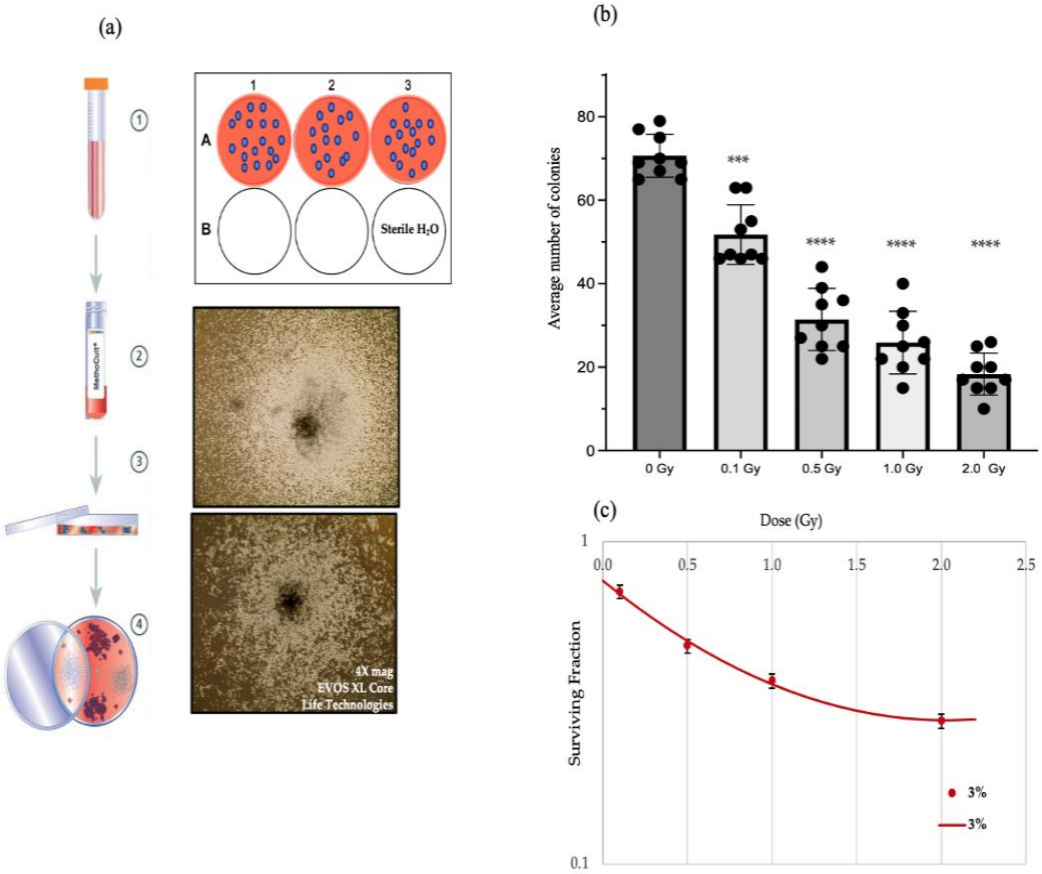


Figure 3. 3 Methylcellulose colony forming assay under low oxygen environment.

(a) Representative fluorescent images of colony formation in MethoCult™ media; x40 magnification. **(b)** Represents the radiation effect on clonogenic survival of HSPCs in low oxygen environments. **(c)** A dose-dependent reduction in colony formation was observed following radiation exposure. Colonies from triplicated cultures were counted. Values are expressed as the mean ± standard error of the mean. One-way ANOVA with Dunnett's multiple comparison test was applied to determine the statistical significance comparing the average colony number of each group with the control. *Signifies the significance difference, P values are presented on the graph for each radiation dose; (***<0.005, ****p<0.0001)

Our results in Figure 3.4 (below) represents the influence of differing oxygen levels, where the normoxic environment showed a significant reduction in CFC formation when compared with colonies treated with a low oxygen environment. Colonies showed dose-dependent reduction in both oxygen environments when compared with unirradiated cultures. A low oxygen environment generated significantly higher numbers of colonies than a normoxia environment, possibly as a result of hypoxic state promoting stemness and quiescence (Simon & Keith, 2008).

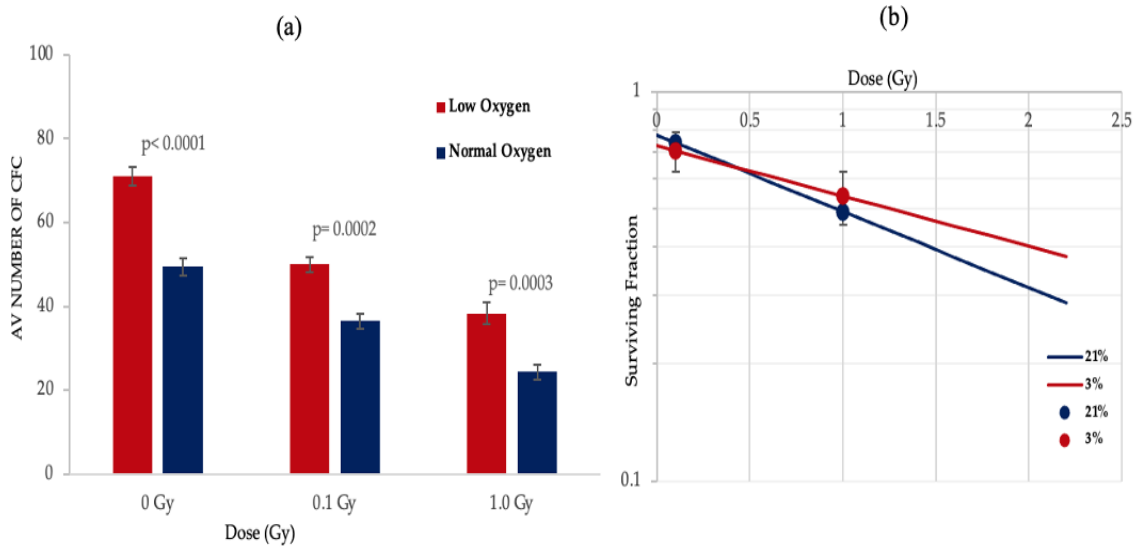


Figure 3. 4 Methylcellulose colony forming assay comparison between different oxygen states.

(a) Represents the effect of differing oxygen levels on the clonogenic survival of HSPCs. **(b)** A low oxygen environment promotes the growth of significantly higher colonies. Dose-dependent reduction in colony formation was observed following radiation exposure both in low oxygen and normoxic environments. Colonies from triplicated cultures were counted. One-way ANOVA with Dunnett's multiple comparison test was applied to determine the statistical significance comparing the average colony number in differing oxygen levels and p values are represented on the graph. Values are expressed as the mean ± standard error of the mean.

3.1.3 Discussion

A growing body of evidence demonstrates that oxygen availability and metabolism affect haematopoietic homeostasis and cellular responses to environmental oxidative stressors, such as ionising radiation. Multiple internal and external factors influence oxygen metabolism within the bone marrow, such as diet and exposure to oxidative stressors, potentially impacting

haematopoietic homeostasis and HSPC radio-sensitivity, thereby elevating the risk of developing rAML. This chapter investigated both the effects of oxygen levels (ambient normoxia 20.8% vs. low O_2 3%) and IR exposure on murine HSPC growth and clonogenic potential. Previous *in vitro* studies used varying oxygen levels (1–7%) to assess the impact of hypoxic culture on numerous stem cell micro-environments (Atkuri et al., 2007; G. Chen et al., 2011; Chow et al., 2001; Elabd et al., 2018; Ito & Suda, 2014; Ivanovic et al., 2000; Koller MR et al., 1992; Mohyeldin et al., 2010; Tsai et al., 2011; Wierenga et al., 2014; Y. Yoshida et al., 2009). Accordingly, hypoxic culture preserved the redox environment (Atkuri et al., 2007), enhanced cellular fitness, differentiation potential, short-term proliferation capability, long-term expansion efficacy, stemness as well as inhibited senescence.

Our growth curve analyses of HSPCs showed that normoxia increases proliferative capacity in unirradiated HSPC, but also enhances IR-induced reduction in proliferative capacity in irradiated HSPC. For instance, increasing oxygen levels favours HSPC proliferation and differentiation over HSC quiescence as well as increase HSPC radiosensitivity compared to HSPC cultured in low O_2 states. Haematopoietic reconstitution following radiation exposure requires the release of the surviving HSCs from their quiescent state into the G1 phase of the cell cycle. Nearly 60% of the surviving HSCs actively cycle for more than 10 months after radiation exposure and the number of cell divisions per surviving HSC specified to be ten times higher than unexposed mice (Ban & Kai, 2009). Therefore, our data are consistent with an initial decline in the growth rate due to cell death followed by proliferation to replace the cells, meaning that all the samples had the same number of cells by the end of the short-term culture period.

Earlier studies on haematopoietic colony formation under hypoxic conditions have been undertaken with varying outcomes (J. Chen et al., 2016; Cipolleschi et al., 1993; C. D. Dunn

et al., 1980; Ivanovic et al., 2000; Lord & Murphy, 1973; T. P. McDonald et al., 1979; Murphy & Lord, 1973; Peschle et al., 1977). Study conducted by Ivanović et al., in one instance indicated that growing human cord blood $CD34^+$ cells under 3% O_2 augmented CFC formation and did not change the $CD34^+$ cell phenotypic profiles or proliferation, implying that low ambient oxygen supports stem cell renewal and hence prevents exhaustion within tissue culture (Ivanovic et al., 2004). Likewise, 4% O_2 promoted primitive and definitive neural stem cell colony formation, by inhibiting distinctive cell death pathways (Clarke and van der Kooy, 2009). Although the observations may differ, our results indicate that 3% O_2 environment enhances more colonies, which may possibly because this state favours stemness and stem cell quiescence, whereas normoxia (20.8% O_2) favours stem cell proliferation.

In addition to nuclear DNA damage, IR stimulates a state of oxidative stress (OS) in cells via direct hydrolysis of water molecules to produce ROS, increasing expression of inflammatory cytokines and damaging mtDNA. Activation of these pathways can result in a transient rise in ROS levels, eventually leading to HSC exhaustion or pre-leukaemic transformation (Azzam et al., 2003; Hei et al., 2010; K. Hei et al., 2011; Laiakis et al., 2007). According to our data presented in Figure 3.2, ambient oxygen (normoxia) levels were found to increase irradiated HSPC-stress, stimulating proliferative activity when compared with low oxygen levels. Normoxia increases HSPC radiosensitivity, stimulating both IR-induced ROS production and genotoxicity but also increases cell division. Due to their open DNA structure and increased DNA content, proliferating HSPCs are more radiosensitive, with increased vulnerability to IR-induced oxidative damage under normoxia. Our results are in line with the well-established finding that ionising radiation damages a variety of cells to a greater extend in the presence of oxygen when compared to anaerobic environments (Laser, 1954). The presence of low oxygen,

on the other hand, provides a radio-protective environment for HSCs, preserving the quiescent state and hence, diminishing both cell division and ROS production.

3.2 Effect of Ionising Radiation and Oxidative Stress on Mitochondrial Metabolism, DNA content and Function

3.2.1 Influence of Radiation Exposure and Oxidative stress on HSPC Energy Metabolism

HSPC homeostasis is heavily dependent on mitochondrial function and health, and it is increasingly apparent that this dependence plays an important role in AML. The growth potential of HSPCs is redox state dependent, and in the previous section it was shown to be affected by both normoxia and IR exposure. To increase our understanding on the mechanisms which trigger these effects, we further investigated the alterations in energy metabolism of HSPCs. Researchers have employed a range of techniques including mass spectrometry, glucose & lactate measurements, oxygen & carbon dioxide measurements and nuclear magnetic resonance Spectroscopy. While these techniques offer valuable information about cellular metabolism, the Seahorse system, with its real-time measurement capabilities, offers a distinct advantage over other methods for assessing cellular metabolism. It allows continuous monitoring of metabolic changes, providing immediate insights into the dynamic responses of cells to experimental manipulations. In contrast to methodologies such as mass spectrometry and NMR spectroscopy, which necessitate sample extraction, the Seahorse system directly assesses metabolic parameters within intact cells, preserving their physiological state and mitigating potential artifacts. Additionally, its high throughput capabilities enable simultaneous analysis of multiple samples, ensuring efficient data acquisition and better reproducibility of results across experimental conditions.

ATP real-time assays synchronously measure ATP production in mitochondrial and glycolytic pathways and so can be used to identify the favoured metabolic pathways favoured by cells under different conditions. We applied this assay to IR- and non-IR exposed HSPC in a low oxygen environment (Figure 3.4 a–d). To start with, HSPCs were harvested from expansion cultures (at day 7) and plated ($2x10^5$ cells/ml) into Cell-Tak coated miniplates in a XF base medium supplemented as per manufacturer's instructions. Cells were spun down and incubated in a humidified non-CO_2 incubator at 37^{o}C, 1h prior to the assay for complete cellular adherence (Section 2.9). Oligomycin (ATP synthase inhibitor), rotenone and antimycin A (electron transport inhibitors) were diluted and loaded to specified ports before the calibration of the sensor cartridge (Figure 2.5). These compounds allow changes in various aspects of mitochondrial function to be measured under different conditions (Table 2 below).

Compound	ETC target	Effect on OCR
Oligomycin (Omy)	ATP synthase (complex V)	Decrease
FCCP	Inner mitochondrial membrane	Increase
Retenone/antimycin A	Complex 1 & 3 (respectively)	Decrease

Table 2. Summary of target and effect for the mitochondrial respiration modulators.

(Abbreviations: oligomycin (Omy); carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), electron transport chain (ETC), adenosine triphosphate (ATP) synthase, oxygen consumption rate (OCR))

For instance, the reduction in oxygen consumption rate upon injection of ATP synthase inhibitor (oligomycin) specifies the portion of basal respiration that was used to stimulate ATP production. Hence this will display ATP produced by mitochondria which contributes to meeting the energetic demands of the cells. Upon completion of the calibration stage, Seahorse XF cell culture miniplate was inserted and assay continued, using Real-time ATP rate protocol as described in section 2.9. Oxygen consumption rate (OCR) and extracellular acidification

rate (ECAR) were measured over time under basal conditions followed by the sequential addition of oligomycin, rotenone & antimycin A, see table 2 above for the summary of effects generated by these stressor compounds. Use of these mitochondrial inhibitors allowed for the estimation of the contribution of individual parameters for basal respiration, maximal respiration, non-mitochondrial respiration, and ATP production.

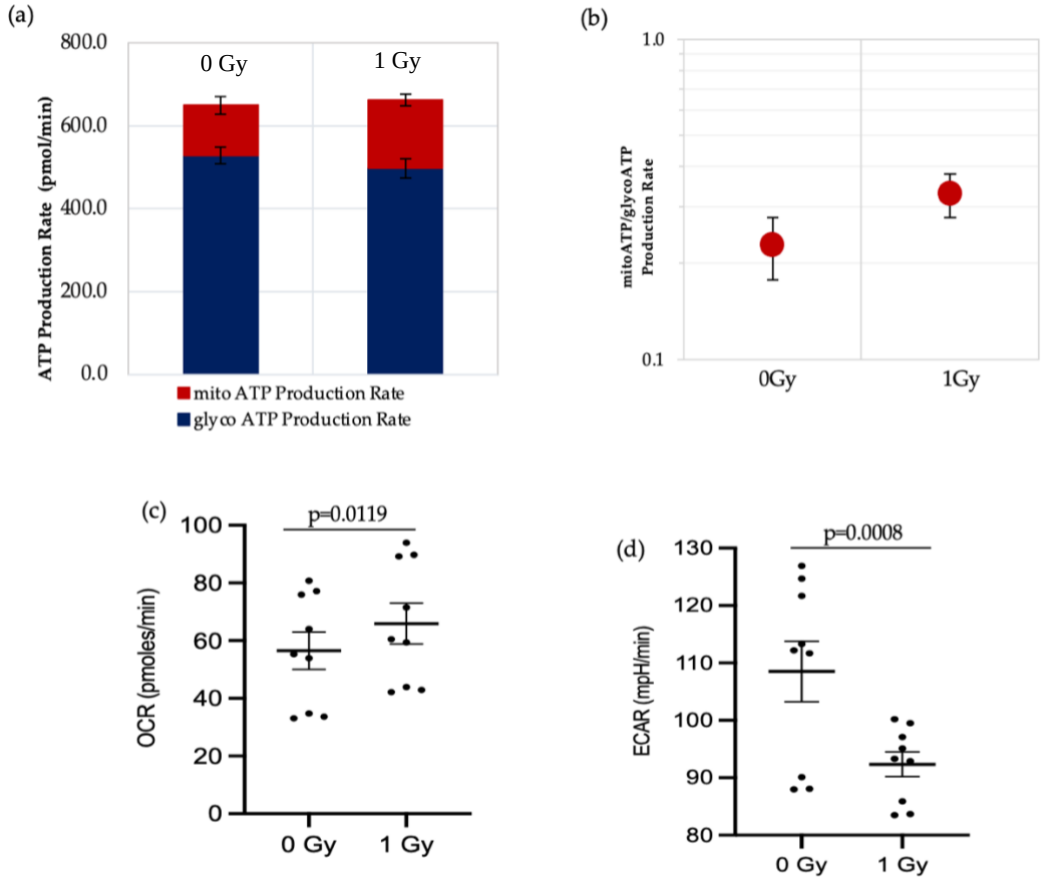


Figure 3. 5 Seahorse XF real-time ATP rate analysis of HSPCs under a low oxygen environment.

(a) Metabolic flux analysis shows increased OXPHOS following radiation exposure. **(b)** XF ATP rate index calculated from the data in panel a. **(c)** OCR: oxygen consumption rate **(d)** ECAR: extracellular acidification rate. Student t-test was used, and horizontal lines indicate statistical comparison made. Three independent experiments proceeded (N=3; 3 wells/dose/experiment; error bars represent ±SEM).

The data shows that IR-exposed HSPCs (Figure 3.5) switch to a more aerobic phenotype, as evidenced with an increase in mitochondrial-ATP production rate (OXPHOS), whilst non-IR exposed cells favour an anaerobic glycolytic pathway (Figure 3.5a glycoATP production

comparison between 0 Gy vs 1 Gy, p= 0.006). Within the BM niche, HSCs rely on anaerobic glycolysis which helps maintain the quiescence and stemness (Simsek et al., 2010; Suda et al., 2011; Takubo et al., 2013). It is evident that 1Gy-exposed HSPCs demonstrate increasing use of non-glycolytic metabolism, which can result in a switch from quiescence to a proliferation phase with a loss of stemness.

To provide a more comprehensive picture of changing energy metabolism in HSPC as a result of differing conditions, XF cellular energy phenotype tests were conducted. To determine the alterations in metabolic potential of IR- and non-IR exposed HSPC under normoxic or low O_2 environments when stress conditions are applied using a stressor compound as part of the assay (methodological stressor compounds, see section 2.10).

Metabolic potential is the cell's ability to meet an energy demand stimulated by a stressor and is indicated by the difference between baseline and stressed OCR/ECR. The cellular energy phenotype test uses oligomycin (ATP-synthase inhibitor, which causes an increase in glycolysis) and FCCP (mitochondrial-uncoupling agent, increases oxygen consumption rates) as methodological stressor compounds. Metabolic potential of non-IR exposed HSPCs was first examined under baseline and stress environments using different oxygen states (low vs normal oxygen states presented in Figure 3.6 below). Accordingly, both under normoxic and low oxygen surroundings HSPC populations revealed significant increase in metabolic response with respect to baseline when methodological stressor compounds were applied (Figure 3.6a–c). Conversely, in normoxia this is seen as increased mitochondrial oxygen consumption signifying an aerobic metabolic pathway, while low oxygen favours a glycolytic pathway, as seen in the XF real-time ATP rate assay described previously.

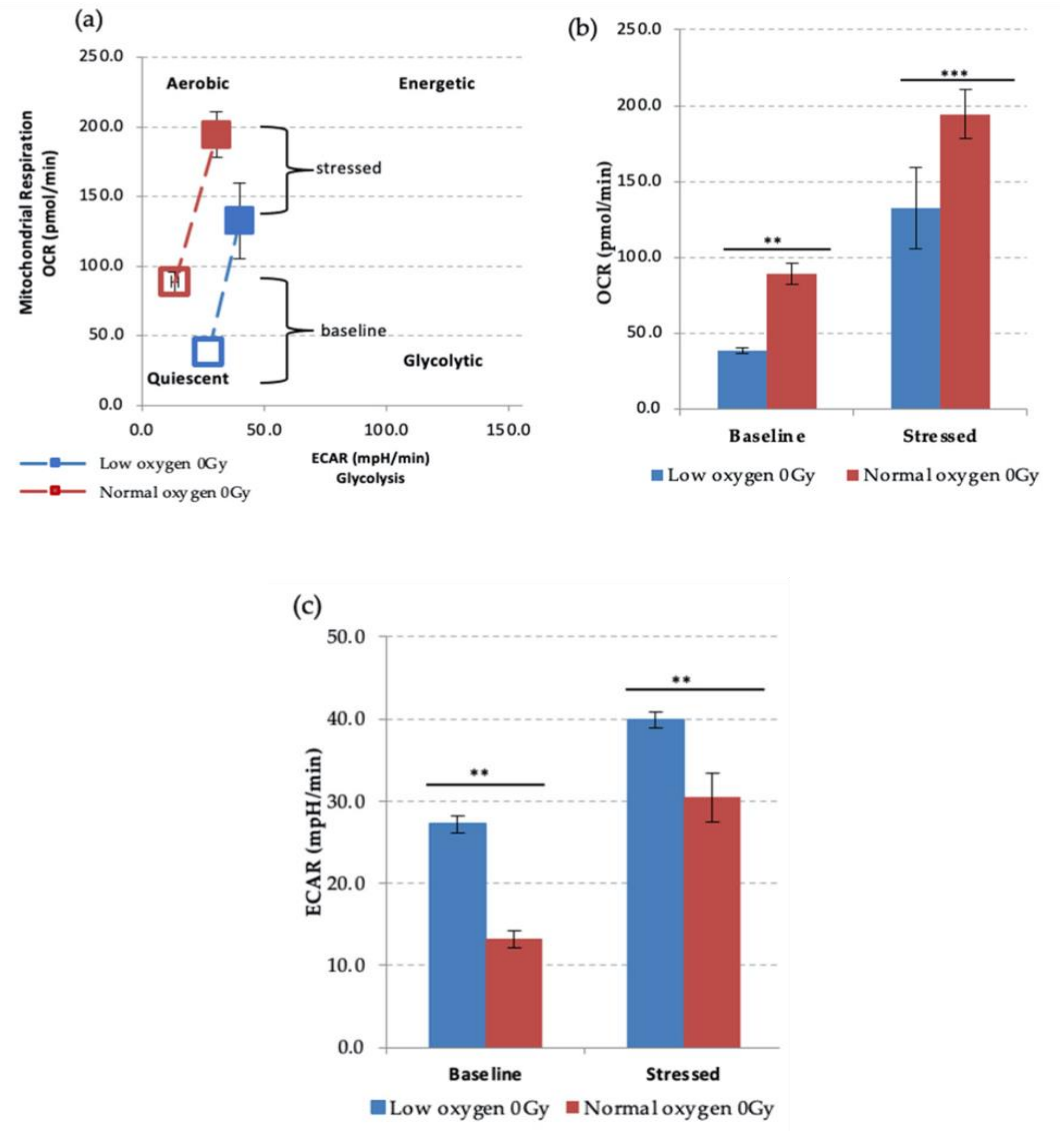
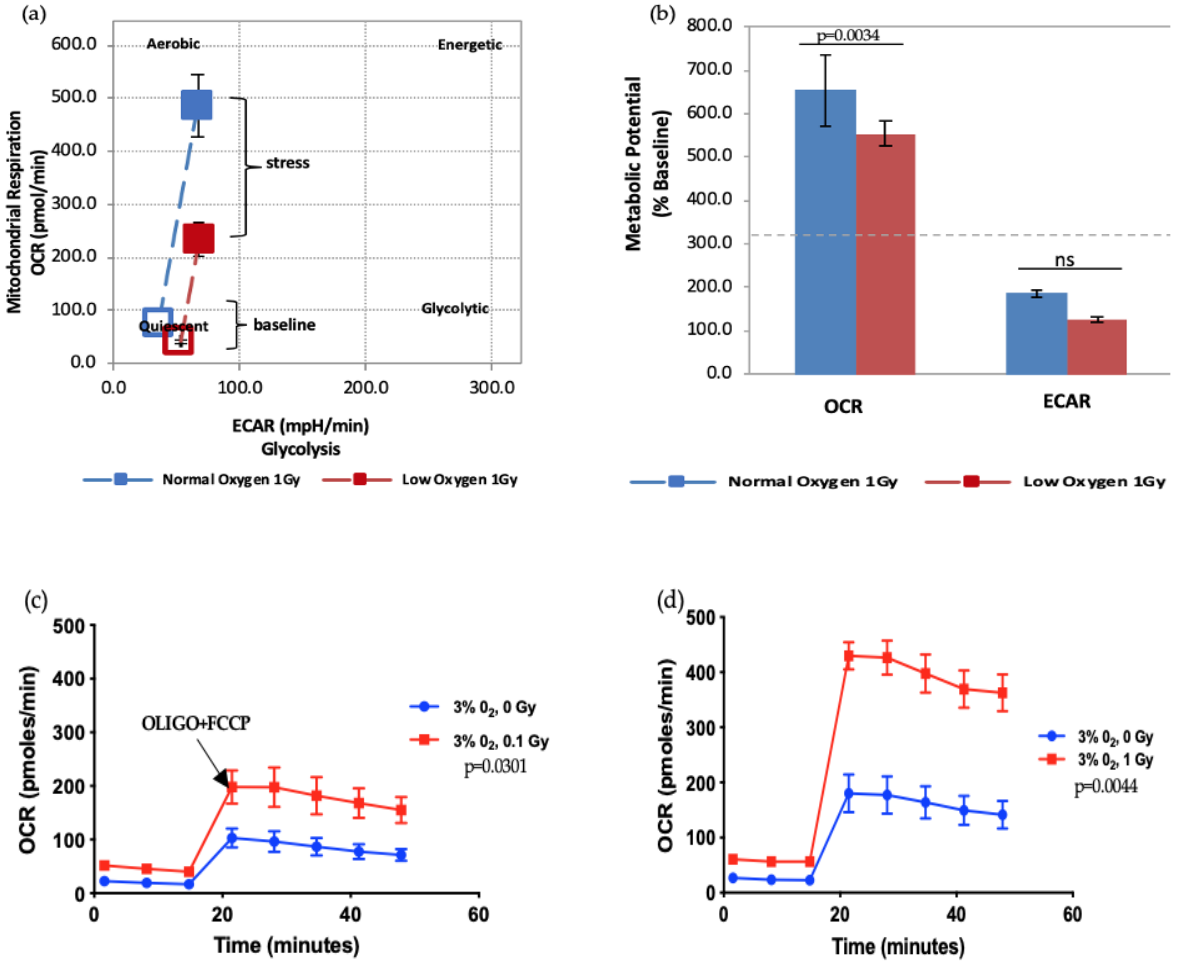


Figure 3. 6 Seahorse XF cell energy phenotype analysis of non-IR exposed HSPC population

HSPCs were harvested from day 7 expansion cultures and plated ($2x10^5$ cells/ml) into Cell-Tak coated miniplates as described previously. Once cell adherence was fully accomplished, stressor mixture was prepared and loaded into ports. Upon calibration of sensor cartridge, Seahorse XF miniplate was inserted and cell energy phenotype assay was followed as per manufacturer's instructions. **(a)** presents the phenotype map of non-IR exposed HSPC under basal and stress conditions. **(b)** shows the basal and stressed OCR **(c)** represents ECAR measurements in HSPC under normoxic (red column) and low O_2 (blue column) environments. Student t-test was applied to compare OCR (low oxygen vs. normal oxygen) and ECAR (low oxygen vs. normal oxygen). Asterisk * denotes the significance difference; ** $p<0.05$, *** $p<0.005$ (N=3, error bars represent ±SEM).

We further assessed the effect of radiation doses and oxygen status on the metabolic potential of HSPCs (Figure 3.7 below). Following 1Gy exposure, HSPCs which were kept in normoxic environment show higher mitochondrial respiration under baseline conditions when compared

with 1Gy exposed HSPCs incubated under a low oxygen environment. Likewise, when methodological stressors (OLIGO+FCCP) were added (black arrow marks the time-point when stressors added, in Figure 3.7c), cells exhibited more pronounced OCR levels when compared with 1Gy-exposed HSPCs in low oxygen (Figure 3.7a). In contrast, ECAR levels did not reveal any difference between normoxic and low oxygen conditions (Figure 3.7b).



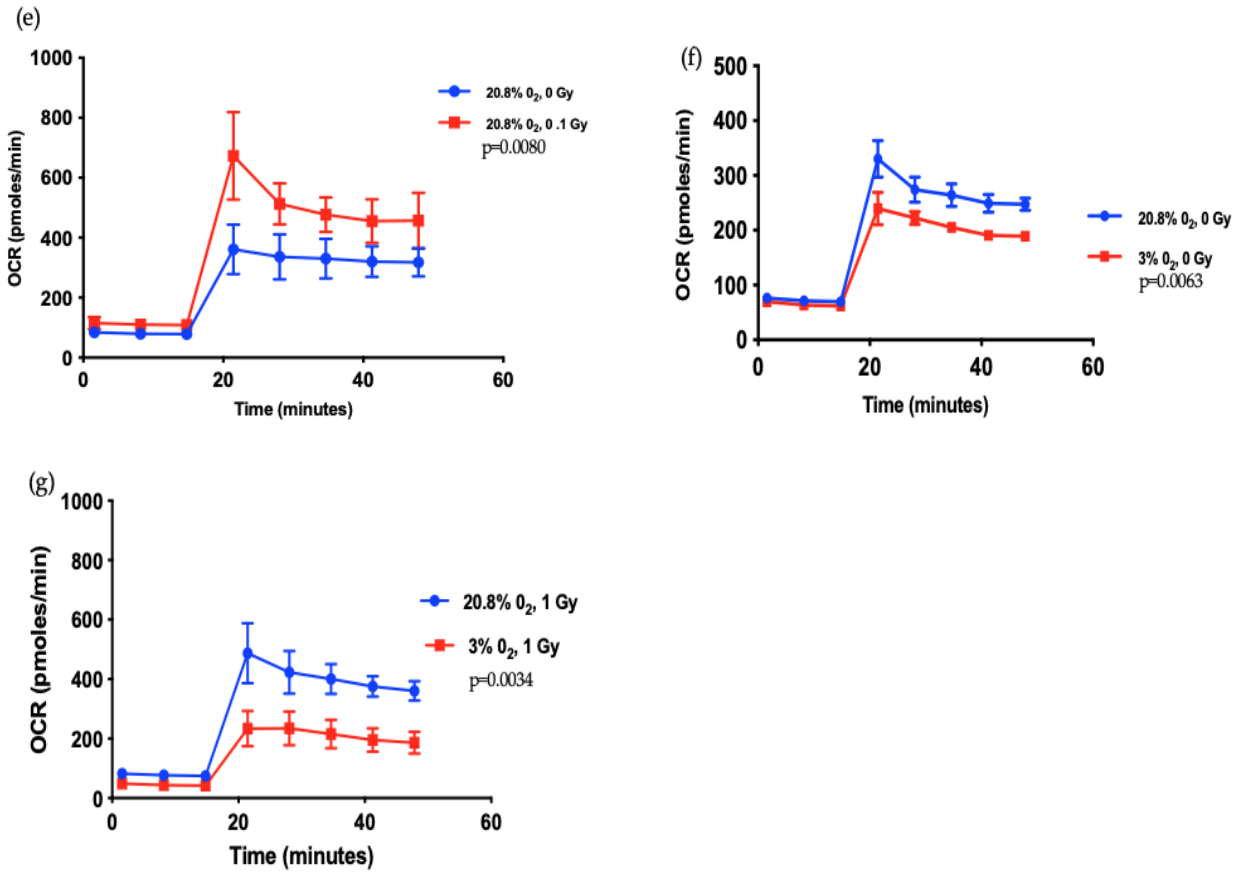


Figure 3. 7 Seahorse XF cell energy phenotype test of HSPCs under stress conditions.

HSPCs were harvested from day 7 expansion cultures and plated ($2x10^5$ cells/ml) into Cell-Tak coated miniplates as explained earlier. Stressor mixture was prepared and loaded into ports. Following calibration stage of sensor cartridge, Seahorse XF miniplate was inserted to the instrument and phenotype assay was proceeded as per manufacturer's guidelines. **(a, b)** The phenotype map of 1Gy-exposed HSPCs under normoxic and low O_2 states. **(c–g)** Effect of different oxygen levels and radiation exposure on the metabolic potential of HSPCs. Student t-test applied for statistical analysis; p values are indicated separately for each figure (N=3, error bars represent ±SEM).

The findings from this assay demonstrate that IR exposure elicits a dose-dependent increaser in cellular metabolic response, as shown in Figure 3.7c-g. Under normoxic conditions, both IR and oxidative stress induce a notable elevation in mitochondrial-dependent ATP production, facilitating the fulfilment of energy demands for HSPCs in the presence of applied methodological stressors. Conversely, ECAR did not reveal a significant impact on ATP production (data not presented). Yet, we acknowledged several limitations, including inability to measure metabolic changes under all conditions simultaneously leading to day-to-day

variations in our results. To address this issue in our future studies, a 96-well format pro analyser will be used to conduct the experiments to minimise day-to-day variations and enhance the experimental accuracy respectively. In conclusion, IR exposure and normoxia act as physiological oxidative stressors on HSPC, resulting in increased mitochondrial ATP production, suggesting they may induce mitochondrial dysfunction.

3.2.2 Effect of Radiation Exposure and Oxidative Stress on Mitochondrial Mass, DNA content and Function

Amongst the various roles attributed to mitochondria, one of the utmost significances is facilitating oxidative phosphorylation for cellular energy production (Fernández-Silva et al., 2003). Encoded by the mtDNA, components of the electron transport chain (ETC) are critical for this process. Consequently, any ROS-induced mutations can lead to OXPHOS impairment, potentially increasing ROS production, thereby triggering further cellular and mitochondrial damage (Copeland et al., 2002; G. J. Kim, Chandrasekaran, et al., 2006; G. J. Kim, Fiskum, et al., 2006). Disruptions in mitochondrial function and consequent onset of oxidative stress have been implicated in numerous diseases, including, neurodegenerative, endocrine, and cardiovascular diseases as well as many cancer types including haematological malignancies (El-Hattab & Scaglia, 2016). Hence, a thorough understanding of the role of mitochondria in maintaining health and contributing to pathological conditions is of paramount importance (Bui & Schreiber, 2007; Erez & DeBerardinis, 2015; Kroemer et al., 2015; Porporato et al., 2018).

Our findings have revealed that both IR and normoxia function as metabolic stressors in HSPCs, prompting increased utilisation of aerobic mitochondrial-mediated respiration. Hence, this escalation further contributes to augmented ROS production, by increasing the risk of mitochondrial dysfunction. In pursuit of a deeper understanding, we extended our examination to explore the impact of IR exposure under varying oxygen conditions on markers of

mitochondrial function, with a particular focus on mitochondrial mass, DNA content superoxide production, and mitochondrial membrane potential (Figure 3.8a–d).

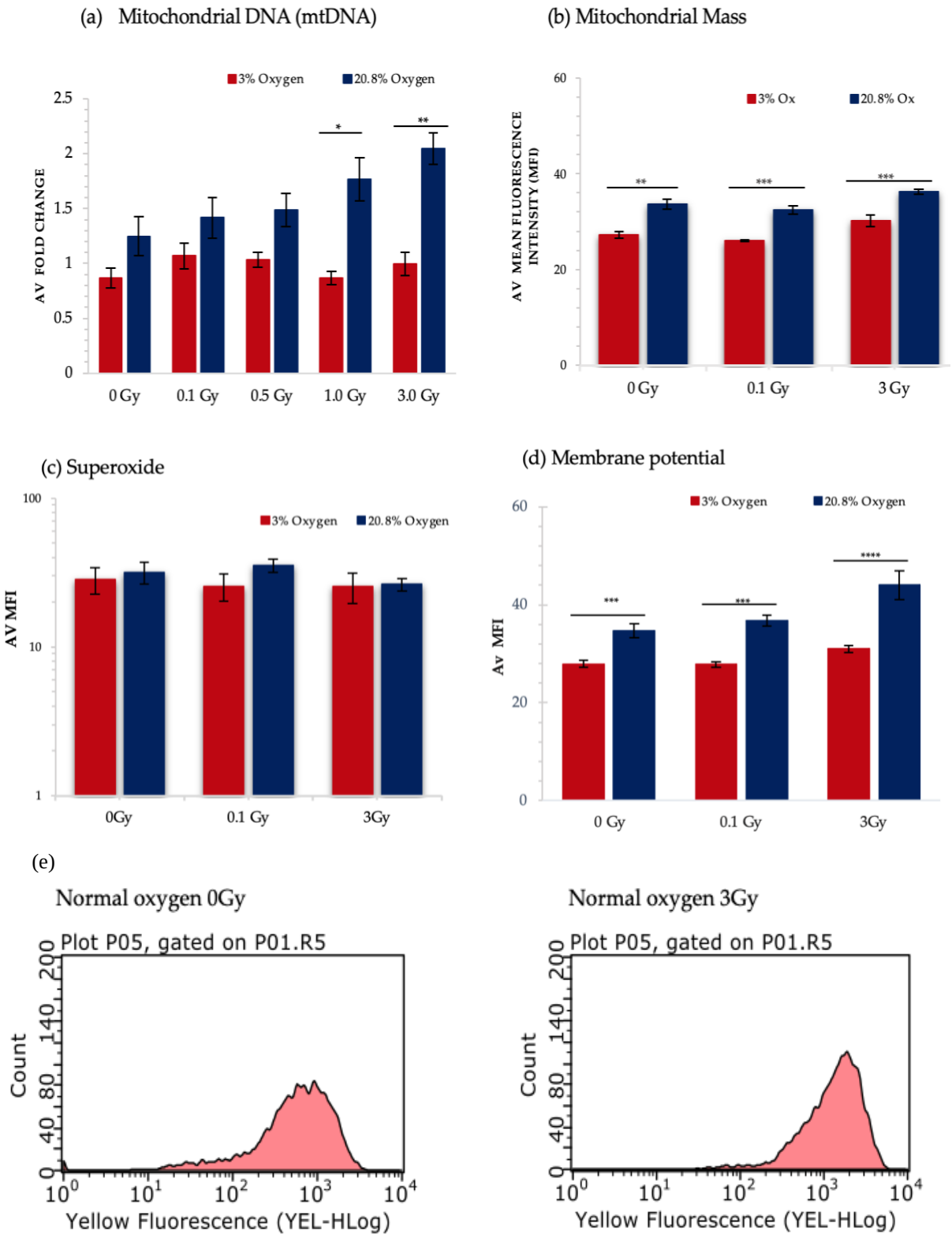


Figure 3. 8 Represents analysis from different mitochondrial functionality assays.

HSPCs for each assay were extracted from expansion cultures at day 7 as described previously. Differing oxygen levels and radiation doses (0-3 Gy) were compared to evaluate the influence on mitochondrial functionality. **(a)** Mitochondrial DNA (N=3); **(b)** Mitochondrial Mass (N=3), **(c)** Mitochondrial Superoxide(N=4), **(d)** Mitochondrial Membrane Potential (N=4). **(e)** Flow cytometric profiles of JC-1 displaying mean fluorescence intensity of 0 Gy- and 3 Gy-exposed HSPCs under normoxia. One-way ANOVA and multiple comparison tests are performed. Horizontal lines indicate statistical comparison made. Error bars represent ±SEM.

Figure 3.8a, reveals a significant rise in mtDNA content following IR exposure under normoxia in a dose dependent manner, whereas IR exposure did not reveal any alteration in mtDNA content under low oxygen. Contrastingly, IR exposure did not produce any significant alteration in mitochondrial mass irrespective for dose, however normoxia triggered a remarkable increase in mitochondrial mass when compared with a low oxygen state (Figure 3.8b). Mitochondrial ETC produces an electrochemical gradient which is involved in ATP synthesis (Mitchell, 1961) and creates mitochondrial membrane potential (MtMP), which acts as a key parameter for evaluating mitochondrial function and is used as an indicator of cell health (L. B. Chen, 1988). Correspondingly, our results demonstrate a substantial rise in MtMP under normoxic backgrounds for all three doses. Besides, significant change in MtMP was detected between 0 Gy vs. 3 Gy and 0.1 Gy vs. 3 Gy-exposed HSPC population both in normoxic and low oxygen conditions (Figure 3.8d).

The MitoSox assay results revealed no significant change in superoxide levels under different oxygen states and IR exposure (Figure 3.8c). Perhaps this is due to the transient nature of superoxide, meaning there were none detectable after 7 days in culture. Additionally, MitoSox test can only detect superoxide and does not identify other ROS or reactive nitrogen species (RNS). To resolve this issue, we decided to quantify the amount of hydrogen peroxide produced (H_2O_2) which is derived from superoxide. Our results presented in Figure 3.9 show a significant increase in H_2O_2 levels under a normoxic background when compared with a low

oxygen environment in all three doses (*p* values are specified on the chart below). We have found a significant increase in H_2O_2 yield between doses of radiation, most predominantly between 0 Gy and 3 Gy-exposed HSPCs under normoxic conditions ($p<0.0001$). Interestingly, no significant difference was observed between doses administered under low oxygen conditions.

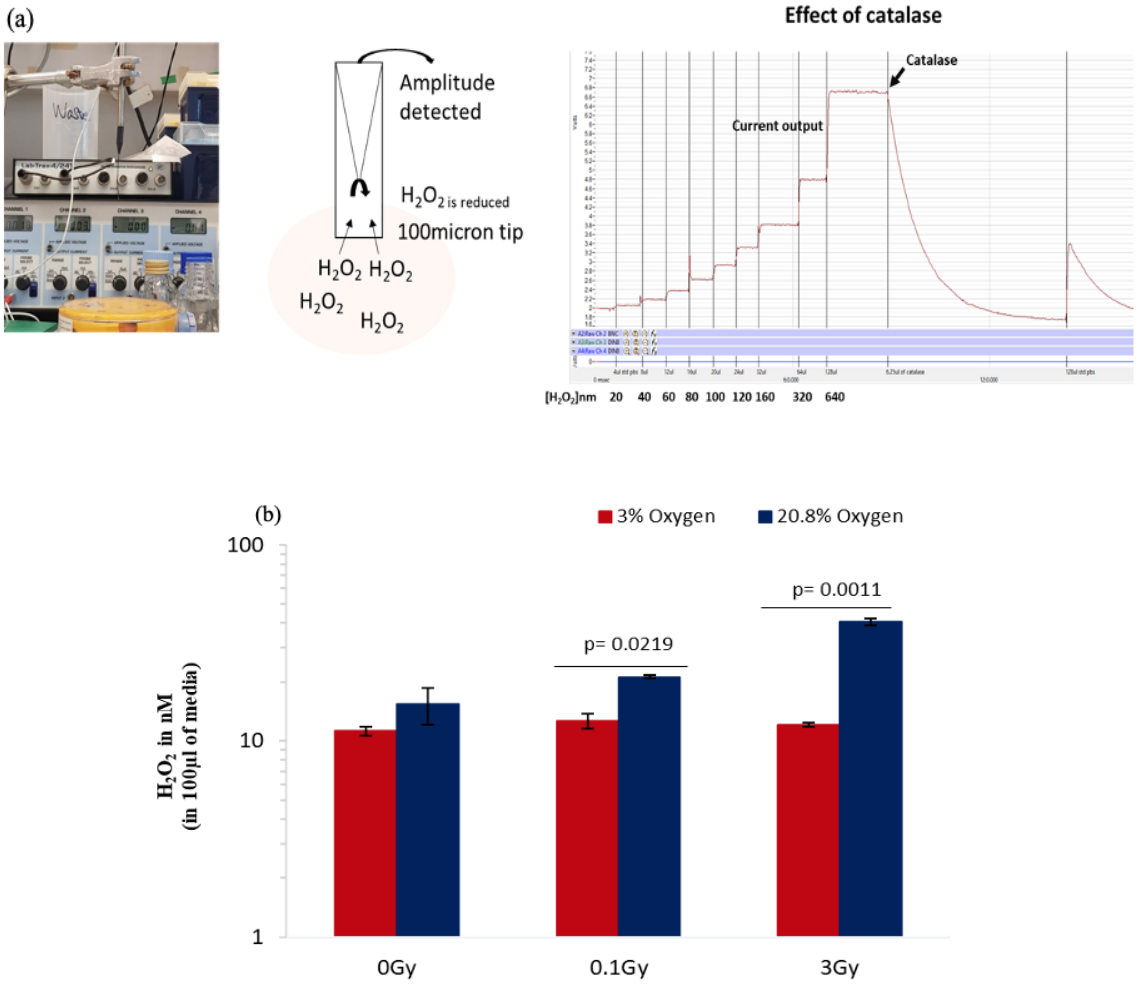


Figure 3. 9 (a) Simplistic illustration of direct quantitative measurement of hydrogen peroxide in biological samples (b) effect of oxygen stress and IR exposure on hydrogen peroxide levels of HSPC population extracted from day 7 cultures.

HSPCs for each assay are extracted from expansion cultures and H_2O_2 concentration measured in each sample, using an amperometric microsensor electrode. Differing oxygen levels and radiation doses (0, 0.1, 3 Gy) were compared. Two-Way ANOVA and multiple comparison tests were applied. Horizontal lines indicate statistical comparison made (N=3; Error bars ±SEM).

3.2.3 Discussion

Our results demonstrate that IR exposure leads to an increase in mitochondrial-ATP production (OXPHOS). Moreover, normoxia caused HSPCs to attain more aerobic phenotype, while HSPCs under low oxygen conditions favoured anaerobic glycolysis, which is known to be critical for stemness and quiescence (Ito et al., 2004; Norddahl et al., 2011; Parmar et al., 2007; Simsek et al., 2010; Suda et al., 2011; Vannini et al., 2016). The effects of IR exposure were reported by several groups to enhance NADPH-oxidase activity and mitochondrial-ROS production with reduced HIF-1a levels, which result in HSC proliferation and cell cycle entry (Kohli & Passegué, 2014; Ludin et al., 2014). One of the fundamental problems in cancer research is the identification of cells within the tumour microenvironment which maintains the growth of the neoplastic clone. The evidence for the existence of cancer stem cells (CSCs) first originated from the study of human AML due to the accessibility of quantitative stem cell assays for leukaemic stem cells (LSCs). It was shown in these studies that only a limited number of cells within the leukaemic clone had the ability to initiate AML growth after transplantation into NOD/SCID mice, establishing the hierarchy of AML. Clonal tracking studies have revealed that there are several classes of LSCs within the LSC compartment, each of which can be distinguished based on their ability to self-renew (Verbiest et al., 2018). These findings have important implications for understanding leukaemogenic processes and designing more effective therapies to eradicate AML by eradicating the LSCs (Dick, 2005).

CSCs exhibit a unique metabolic phenotype and several studies have shown that the metabolic characteristics of CSCs are highly heterogeneous (Emmink et al., 2013; Hammoudi et al., 2011). Unlike non-CSCs, CSCs display either glycolytic or OXPHOS-dependent metabolic phenotype depending on the niches where they are located. CSCs from a variety of tumour types (including acute myeloid leukaemia, glioblastoma, melanoma, and pancreatic cancer) are

highly reliant on OXPHOS and possess low glycolytic reserves (Adams & Strasser, 2008; Janiszewska et al., 2012; Lagadinou et al., 2013; Roesch et al., 2013). LSCs depend heavily on the "OXPHOS" process, and this characteristic may be used to target LSCs. Nonetheless, the reason why LSCs prefer OXPHOS over glycolysis is not fully understood, and the topic represents a critical avenue for future investigations. One possibility is OXPHOS is a highly efficient way for energy generation, hence it might be crucial for sustaining LSC energy requirements and survival (Mattes et al., 2019). Besides, LSCs exploit various other metabolic events such as protein/RNA synthesis, Aa, and FAO as a vital source for energy generation under metabolic stress conditions (Mattes et al., 2019). Numerous groups revealed that Aa and FAO metabolisms are altered in AML and significantly decrease the survival of LSCs when inhibited, and therefore directly affect the maintenance of OXPHOS in LSCs (Ito & Ito, 2018; Karigane & Takubo, 2017). Besides, it has also been reported that CSCs show increased utilisation of extracellular metabolites such as lactate, glutamic acid, glutamine, alanine, and ketone bodies to adapt to OXPHOS metabolism, which can possibly create a selective advantage for CSCs in specific tumour microenvironments (Panuzzo et al., 2020; Sancho et al., 2016).

Our analysis of several parameters of HSPC mitochondrial function and activity showed that both IR-exposure and normoxia caused a substantial increase in mtDNA, mitochondrial mass, membrane potential and hydrogen peroxide levels. A study conducted by Qiu et al. (J. Qiu et al., 2021), revealed that human $CD34^+$ HSPCs, $CD38^-$ HSPCs ($CD34^+CD38^-$), $CD90^+$ HSCs and $CD49f^+$ HSCs (Notta et al., 2011) revealed reduced mitochondrial activity (e.g., membrane potential; (Perry et al., 2011) under steady state conditions, indicating the significance of low mitochondrial activity for HSC maintenance. In contrast, AML cells show increased mitochondrial mass and mtDNA content, signifying that their mitochondrial activity has been

upregulated to compensate for their energy demands under stress environments (Panina et al., 2019). Mutations in mtDNA present in most tumour types, including lymphomas and leukaemias and affected mtDNA regions include genes which encode respiratory complexes, transcription, or replication factors (Copeland et al., 2002). As a result, these mutations can potentially impair mitochondrial function, increasing ROS levels thereby triggering chromosomal abnormalities (K. K. Singh, 2004).

Recent proteomic-based comparison study of AML LSCs with HSCs and AML blasts indicated that numerous components of ETC complexes I and V were consistently more abundant in LSCs (Raffel et al., 2020). Intriguingly, studies have found that OXPHOS has also been associated with transformed mesenchymal stem cells (MSCs) (Funes et al., 2007), breast cancer (K. Lee et al., 2017), and brain tumours (Molina et al., 2018), highlighting the significance of this metabolic pathway for cancer stem cells in a variety of malignancies (Jones et al., 2021). LSCs can additionally harness alternative metabolic pathways such as amino acid and fatty acid metabolism to serve as an essential source of energy during periods of metabolic stress (Mattes et al., 2019). In line with this, researchers have documented that inhibition of AA and FAO metabolism significantly diminishes LSC viability, exerting a direct impact on the maintenance of OXPHOS (Ito & Ito, 2018; Karigane & Takubo, 2017).

ROS, particularly (H_2O_2), plays a vital role in promoting proliferation and survival of tumour cells through the activation of redox signalling cascades (Lennicke et al., 2015). Depending on their intracellular concentration and localization, H_2O_2 exerts either anti- or pro-apoptotic activities (Holmström & Finkel, 2014; Lennicke et al., 2015; Schieber & Chandel, 2014).
It is well known that IR instantaneously induces the generation of water radiolysis products which contain ROS including superoxide, hydrogen peroxides, and hydroxyl radicals. In the presence of oxygen, H_2O_2 is converted into hydroxyl radicals by a Fenton-type reaction and

these radicals can lead to further cellular lesions which are similar to ones induced by IR (Birben et al., 2012; Prise et al., 1989). Hence, hydroxyl radicals stimulate chromosomal breaks and the formation of various micronuclei within dividing cells. IR induces DNA double strand breaks (dsbs), which are known as the most deleterious lesions yet, H_2O_2 does not generate DNA dsbs. Authors reported an increase in γH2AX-positive cells in the parental cells following IR exposure, but not in CRR (Clinically Relevant Radioresistant) cell lines (R. J. McDonald et al., 1993). From these results Kuwahara et al., postulated that CRR cells can efficiently repair not only DNA dsbs but also DNA single-strand breaks. Together, these results revealed that CRR cells are resistant to H_2O_2, but it is undetermined whether H_2O_2-resistant CRR cells are also resistant to X-rays (Kuwahara et al., 2020). Hence, cancer therapies should be carried out with awareness of the presence of these resistant cells, and as the next step, it is highly crucial to inspect the appearance rate of these cells instantly and take countermeasures.

Several novel therapeutic strategies have emerged to modify the redox state of tumour cells. These include methods such as hyperactivation of antioxidant enzymes to lower intracellular ROS levels or selective inhibition of cellular ROS sources e.g., NOX (Hole et al., 2013; S. J. Kim et al., 2019). However, the intricate mechanisms governing the interplay between the tumour progression process and redox signalling compounds remain substantially unexplored. Therefore, further investigations are vital to unravel the complexities of these signalling networks and to formulate innovative, targeted approaches for cancer treatment.

3.3 Dietary Restriction, Metabolic Reprogramming in HSCs homeostasis and cancer

The impact of diet and metabolism on various model organisms has been extensively explored, demonstrating their capacity to alter the functionality of stem cells (SCs). This alteration can encompass nutrient availability, hormone regulation, growth factors that govern tissue equilibrium, initiation of tumours, signalling molecules (e.g., IGF-1), and even epigenetic patterns (Colman et al., 2009; Fontana et al., 2010; Kenyon, 2010; Mihaylova et al., 2014; Rafalski et al., 2012; Speakman & Mitchell, 2011).Metabolic reprogramming stands as a hallmark of malignancy, with Haematopoietic Stem Cells (HSCs) employing specific metabolic strategies to sustain their self-renewal potential and maintain a preferred quiescent state (Karabulutoglu et al., 2019).

Within this intricate framework, a complex metabolic regulatory system upholds haematopoietic equilibrium. It does so by integrating dietary and metabolic factors that potentially modulate the radiosensitivity of stem cells and the risk of radiation-induced Acute Myeloid Leukaemia (rAML) (O'Brien et al., 2020). Upon delving further, the impact of dietary interventions such as caloric restriction, fasting, and the targeted depletion of specific nutrients (including individual amino acids and vitamins) on the governing mechanisms of HSCs and the risk of leukaemia require further exploration. This field of study holds significant promise, imparting insights into factors that influence leukaemogenesis and paving the way towards innovative therapeutic approaches that aim to alleviate the enduring risks associated with long-term risks of leukaemia development following IR exposure.

3.3.1 Effect of Amino Acid Depletion on HSPCs Growth Rates and Metabolism

The critical significance of amino acid metabolism in cancer is underscored by its pivotal role in supporting diverse aspects of cancer cell behaviour. In the context of cancer, metabolic reprogramming is a hallmark, facilitating the rapid proliferation and survival of malignant cells. Amino acids, as integral constituents of proteins and pivotal intermediates within metabolic pathways, assume a central position in these cancer-specific metabolic adaptations.

In line with literature, we decided to examine the effect of valine-depletion on the proliferative capability of short-term primary HSPCs under low oxygen states, to determine how this affected their ability to proliferate. HSPCs were cultured in StemSpan™ (SSpan™; a special medium used for *in vitro* culture and expansion of HSPCs isolated from human, mouse and other species when combined with growth factors and supplements, accordingly) and a custom-made SSpan (+/- Aas) for the evaluation of the changes in their proliferation rates. HSPCs were counted every two/three day as described previously, and the data displayed in Figure 3.9a represents the analysis of day 7 counts (as defined earlier, it is an integral part of the exponential growth phase). Accordingly, custom-made valine-depleted SSpan medium drastically reduced the proliferative ability of HSPCs when compared with cells cultured in normal SSpan media ($p<0.0001$) or custom-made SSpan medium with all Aa-added *(*$p<0.0012$).

Following this, the metabolic potential of HSPCs (extracted from day 7 expansion cultures) in media with and without valine under low oxygen was analysed, using Seahorse XF cell energy phenotype assay. HSPCs from valine-depleted cultures exposed to low O_2 levels revealed significantly lower OCR levels under both baseline and stressed conditions (Figure 3.10b), demonstrating a reduction in the mitochondrial-mediated respiration favoured by normally proliferating HSPC populations. Our findings correlate with previously published work in

normoxic conditions demonstrating a reduction in both HSPC number and proliferation capability, as well as a reduction in their ability to switch to OXPHOS respiration, a crucial function in maintaining HSPC proliferative state.

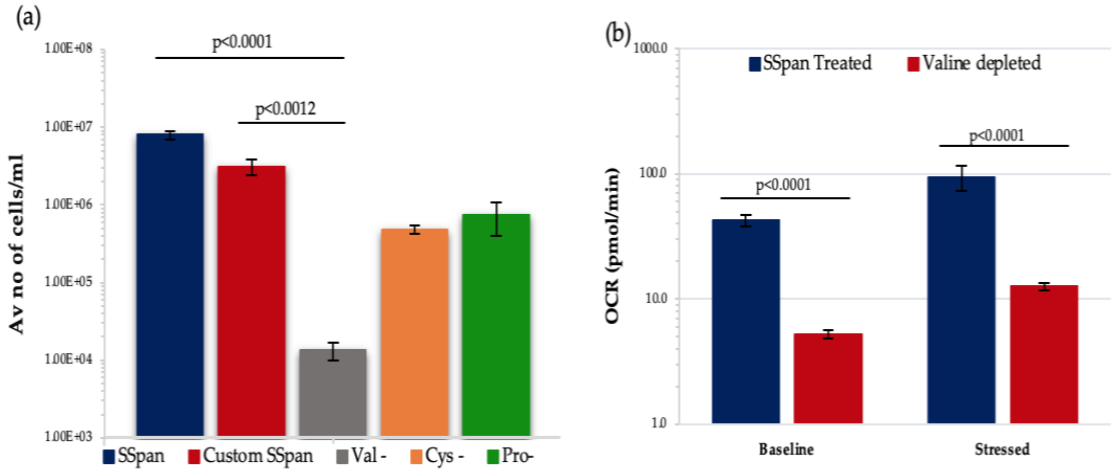


Figure 3. 10 Effect of amino acid depletion on proliferative capacity and metabolism of HSPCs under low oxygen environment, 3% O_2.

(a) Following lineage depletion, HSPCs (2×10^5 cells/well) were treated with custom-made SSpan medium (+/- Aas) and incubated in a 3% Oxygen environment. Under individual Aa-depleted conditions, cells were counted every two to three days, and the data presented on the graph are from the counts collected on day 7 (N=3; 3 wells/condition/experiment. Error bars ±SEM). **(b)** The effect of valine-depleted media on the metabolic potential of HSPCs was assessed under baseline and stress conditions with the addition of mitochondrial inhibitors: oligomycin and FCCP. HSPCs were extracted from day 7 cultures for this assessment. (N=3, 3 wells/condition/experiment. Error bars ±SEM). Anova and multiple comparison tests are applied. Horizontal lines indicate the statistical comparison made.

3.3.2 Discussion

Across numerous cancers, Aas are harnessed as alternative energy sources and a vital substrate to fulfil escalated demands. Notably, in the context of leukaemias, Aa metabolism undergoes a significant shift away from energy-dependent pathways intracellular Aa resynthesis to favouring Aa import from extracellular sources. This altered cellular metabolome plays a pivotal role in nurturing tumour growth and conferring resistance to chemotherapy (Eales et

al., 2016; Emadi, 2015) and thus targeting the increased reliance on Aas in LSCs could unveil novel therapeutic avenues.

LSCs have been suggested to possess a greater level of amino acid metabolism than leukaemic blasts, most of which is employed to generate TCA intermediates (Jones et al., 2018b). Studies revealed that various cancer types such as colorectal and lymphoma highly rely on exogenous serine uptake and dietary restriction of serine hindered the growth of these tumours (Maddocks et al., 2017; Tajan et al., 2021). Furthermore, dietary methionine restriction improved the treatment outcome of H3K27M mutant glioma rodent tumour models and colorectal soft tissue sarcoma (X. Gao et al., 2019; Golbourn et al., 2022). Translatability of such dietary restrictions to humans, attaining comparable outcomes as in mice has also been demonstrated (Epner et al., 2002; X. Gao et al., 2019).

Our investigations have revealed a significant decrease in HSPS proliferative capacity caused by a shortage of valine. Additionally, our analysis using the Seahorse XFp platform has demonstrated that HSPCs cultivated in valine-deficient media exhibit reduced metabolic activity compared to those grown in standard StemSpan media. This difference was observed under both normal conditions and when the cells were subjected to stress conditions (Figure 3.9b). The implications of valine on HSPCs suggest that valine depletion could potentially impact the incidence of rAML, by altering the frequency and persistence of the target cell population. Various studies have previously reported the impact of valine on the survival and apoptosis of CSCs in diverse cancer types, such as hepatocellular carcinoma, colorectal prostate cancer, and brain tumours. For instance, Michishita et al. observed elevated valine levels in canine mammary CSCs (Michishita et al., 2019), while a reduction in valine prompted apoptosis of CD13+ CSCs in hepatocellular carcinoma through unknown mechanisms (L. Sun

et al., 2020). The enzyme 3-Hydroxyisobutyryl-CoA hydrolase (HIBCH), pivotal in valine metabolism, has been linked to the initiation and progression of colorectal cancer. Elevated HIBCH levels led to increased tumour cell proliferation and resistance to bevacizumab while diminishing cancer cell autophagy, thus influencing cancer development and response to treatment (Kalita-de Croft et al., 2019; Shan et al., 2019).

Likewise, deprivation of arginine appears to influence numerous types of cancer cells, including prostate, breast, and pancreatic cancer, as well as primary AML cells by altering distinct signalling pathways (Bowles et al., 2008; Daylami et al., 2014; R. H. Kim et al., 2009b; Miraki-Moud et al., 2015; F. Qiu et al., 2015). Enzymatic degradation of Aas is used in induction therapy for acute lymphoblastic leukaemia (ALL) to deprive malignant cells. Study conducted by Miraki-Moud et al. revealed that most AML cells are lacking a critical enzyme essential for arginine synthesis, argininosuccinate synthase-1 (ASS1). Hence, ASS1-deficient AML cells are highly reliant on importing arginine extracellularly. They also analysed the effect of plasma arginine deprivation, using pegylated arginine deiminase (ADI-PEG 20) against primary AMLs in a xenograft model and *in vitro*. Accordingly, ADI-PEG resistant AMLs revealed higher expression of ASS1 than sensitive AMLs, suggesting that resistant AMLs survive by producing arginine though this metabolic pathway. Furthermore, AMLs treated with combined cytarabine chemotherapy and ADI-PEG 20 demonstrated greater responses than either treatment in 6 of 6 AMLs tested *in vivo* (Miraki-Moud et al., 2015).

Besides Aas such as Glutamine (Gln), stimulate the import of essential Aas, controlling protein translation via the mTORC1 signalling pathways, which results in cellular proliferation (Esen et al., 2016). Most tumours are metabolically flexible and can use glucose if deprived of Gln to replenish TCA cycle. Authors reported that inhibition of one metabolic pathway resulted in upregulation of another. Thus, combination therapy by using low doses of available with well-

studied drugs which deplete asparagine (Asn) and Gln, prevented their repletion, triggering cancer cell death (Esen et al., 2016). Another study conducted by Kovacević (Kovačević, 1971) revealed the importance of Gln metabolism in mitochondrial function as well as its contribution to the metabolic machinery of neoplastic cells. Disruption of Gln metabolism equally impaired mitochondrial function leading to reduced intracellular ATP and OCR levels as well as increased apoptosis, which further hindered AML cell survival (Jacque et al., 2015; Souba, 1993; Willems et al., 2013; Wise & Thompson, 2010; Yuneva et al., 2007).

In a recent study involving primary leukaemic stem and progenitor cells, a comprehensive screening was conducted by deliberately omitting certain amino acids. This investigation discovered several amino acid dependencies, with methionine emerging as the most pronounced among them (Cunningham et al., 2022). According to their analysis, dietary methionine starvation impacted the proteome, metabolome, and epigenome, perturbing AML progression *in vivo*. This *in vivo* analysis of methionine starvation was not only well-tolerated by mice, but significantly affected both cell lines as well as patient-derived AML progression.

Together all these results suggest that targeting altered tumour cell metabolism via specific amino acid starvation could possibly provide an attractive opportunity for patients with AML. Current research is centred around the investigation of amino acid (Aa) dependencies. Within the Aa metabolic pathway, there exist several susceptible nodes that offer selective targeting opportunities, including Aa transporters, transaminases, and synthases, which play pivotal roles in modulating biosynthesis (M. K. L. Fung & Chan, 2017; Maggi & Scotti, 2019; Pathria & Ronai, 2021; Tabe et al., 2019). Another cohort of researchers has shed light on the significance of targeting non-essential amino acids (NEAAs) like glutamate, aspartate, arginine, and cysteine. Their investigation in AML indicated that simultaneously targeting multiple NEAAs might yield superior antileukaemic efficacy compared to a single-agent therapeutic approach.

This approach could potentially enhance treatment outcomes while mitigating the risk of potential toxicities (Bhingarkar et al., 2021).

It is evident that SCs derived from different pathological types possess different metabolic patterns of Aas, which may be related to their microenvironment and genetic background (Wei et al., 2021). Therefore, based on the metabolic characteristics of individual CSCs, more antitumour therapies can be developed to combat specific CSC types. Hence, enhancing our comprehension of the interplay between CSC self-renewal, and other traits, as well as Aa metabolism holds promise for identifying novel targets in forthcoming cancer treatments. This knowledge could potentially offer avenues to mitigate the risk of rAML following IR exposure.

3.4 Radiation exposure and effect of Alternate fasting in murine radiation leukaemogenesis

The primary objective of this chapter was to systematically examine the potential impact of dietary interventions, such as alternate fasting, on the metabolic dynamics of haematopoietic stem and progenitor cells (HSPCs) and the maintenance of haematopoietic equilibrium in an in vivo context. Furthermore, the aim was to critically evaluate the conceivable capacity of such dietary modifications to attenuate the influence of radiation exposure on the susceptible pre-leukaemic cell population. This investigation aimed to effectively mitigate the potential hazards of aberrant cellular proliferation and oxidative stress, thereby culminating in the potential mitigation of the underlying risk for leukaemia development.

3.4.1. CBA$^{mCh/GFP}$ reporter mouse model & mCherry expression across HSCs and downstream haematopoietic lineages

The CBA Sfpi1^{Gfp} mouse model, where Gfp accurately reports Sfpi-1 expression levels, has previously been used by our group to detect live peripheral blood cells with Sfpi1/Gfp copy

loss i.e., ch2 deletion, derived from HSPC with radiation-induced ch2 deletion in the bone marrow, using flow cytometry analysis for reduced GFP expression (Olme et al., 2013). However, Sfpi1 has a variable expression pattern within haematopoietic cell population, e.g., HSCs, CLPs, and subset of CMPs are Sfpi1^{high} whereas, B-cells are Sfpi1^{low}, and T-cells are Sfpi1^{negative}. To overcome this confounding factor for ch2 deletion detection, our group developed a genetically engineered mouse model where a construct carrying the fluorescent protein gene, mCherry, is positioned within the ch2 MDR adjacent to Sfpi1. Unlike Gfp, mCherry is constitutively expressed from the Rosa26 promoter, meaning that mCherry fluorescence is detected at a consistent level in all haematopoietic cell lineages and throughout lifespan (Methodology section; 2.1, Figure 2.1 & Section 2.4, Figure 2.3). This was validated by a previous group member, Dr Tom Verbiest (Figure 3.11) and published in 2018 in Leukaemia (Verbiest et al., 2018), showing that positioning mCherry adjacent to Sfpi1 leads to del2-induced mCherry copy loss, which is detectable by FACS in mCherry heterozygote mice.

Crossing CBA$^{Gfp/Gfp}$ and CBA$^{MCherry/MCherry}$ mice results in the generation of F1 CBA$^{Gfp/MCherry}$ mice. Clonal expansion of pre-leukaemic HSPC carrying Ch2 deletion (del2) can be identified in live animals' post-irradiation. This expansion is evident through the presence of mature leukocytes in the peripheral blood with loss of either MCherry or Gfp indicating the presence of del2 (i.e., these mature leukocytes are derived from the pre-leukaemic HSPC). We can use this assay to investigate the potential effects of an alternate fasting diet on the induction of pre-leukaemic HSPCs, consequently influencing the risk of developing rAML. By monitoring increased or decreased presence of del2– carrying peripheral blood leukocytes over lifespan of the mice, we can gain further insight into the impact of diet on leukaemogenesis.

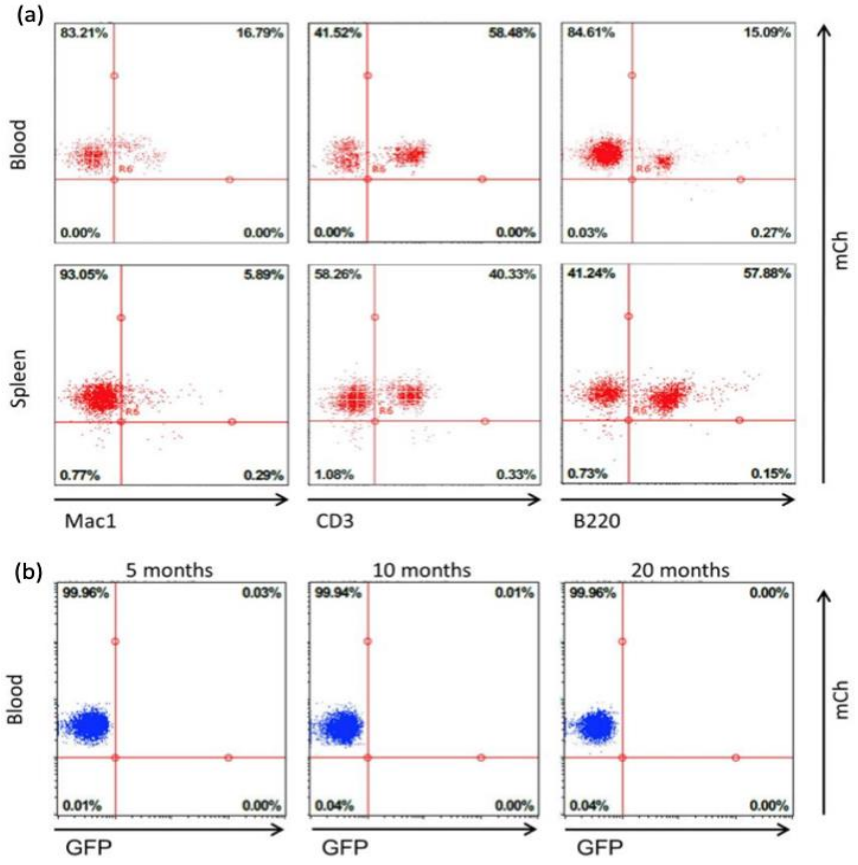


Figure 3. 11 Sfpi1 ^{mCh} mouse model characterisation.

(a) Blood and spleen were harvested from young mice and single cell WBC suspensions were labelled with Mac1, CD3, and B220 antibodies c, followed by flow cytometry analysis for cell surface marker and mCherry expression. Upper right-hand quadrant shows consistent mCherry expression in all of the major WBC lineages **(b)** Serial tail vein blood sampling was performed throughout the life span and mCherry expression was assessed, using flow cytometry. This shows no change in mCherry expression throughout lifespan. The figure showcases representative dot plots, and the content presented in this visual was generated by a former DPhil student, Dr. Tom Verbiest, from our research group. The figure is sourced from Dr. Verbiest's thesis with both his and Dr. Christophe Badie's permissions (Verbiest et al., 2015, 2018).

3.4.2. Clonal Expansion of chromosome 2 deleted WBCs following Radiation exposure

A cohort of hundred and thirteen F1 CBA Sfpi1$^{mCh/Gfp}$ mice was established and divided randomly into 4 groups (ad-lib fed (0 Gy and 3 Gy), alternate day fasting: ADF (0 Gy and 3 Gy)) and maintained for lifespan. At 10-13 weeks of age, one ad-lib and one ADF group (fifty-three Sfpi1$^{mCh/Gfp}$ mice in total: 25 male and 28 female) were exposed to a single acute 3 Gy whole body X-irradiation. 48 hours post-irradiation, the ADF groups were placed on their fasting regime, which involved x1 day fasting/1 day feeding of 6 cycles (Methodology, section

2.3). Mice were examined regularly via body weight analysis and BC scoring. Weight of ad-lib fed mice were recorded weekly and ADF mice daily for two weeks and then we continued with regular body scoring analysis for lifespan. In terms of weight data, fasted mice showed a similar pattern to that of ad-lib fed mice (see Figure 3.12 below), with no significant weight loss seen during the fasting period, and BC scored 3 and/or 4 accordingly (data not presented). Also, average body weight analysis of fasted male and female mice did not reveal any significant difference when compared to the ad-lib fed groups. Following the fasting period, the ADF mice continued to gain weight similar to the ad-lib fed groups. Interestingly, it seems likely that the mice in the ADF treatment groups consumed the similar quantity of food they would normally consume within 48 hours in the ad-lib condition. Hence, our records presented in Figure 3.12 demonstrate that AD fed-mice under these circumstances compensated for the loss of intake on fasting days which was previously reported by Anson et al. (Anson et al., 2003).

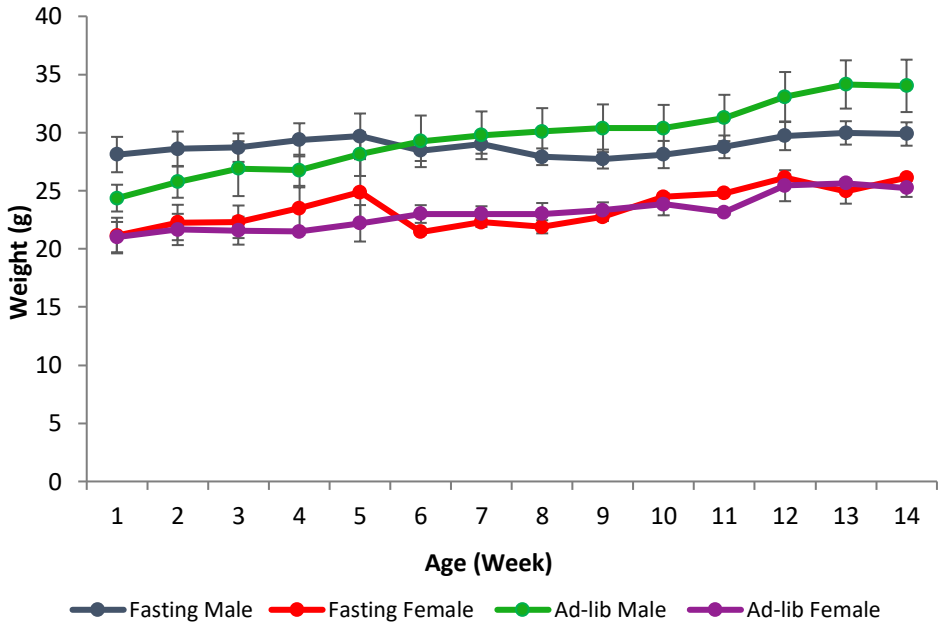


Figure 3. 12 Changes in body weight.

Represents the average body weight analysis of ad-lib (*n*= 46; male *n*= 26; female *n*= 22) and fasted male vs female mice (*n*= 55; male *n*= 22 & female *n*= 33; fasted-female mean BW ~ 21-26 g; fasted-male mean BW ~ 27-30 g). Ad-lib mice body weight was assessed weekly whereas, fasted mice examined daily for two weeks. No significance difference found between fasted and ad-lib groups (males & females analysed independently).

Tail vein bleeding (TVB) sample of each mouse was collected on a monthly basis following X-ray exposure *in vivo* and samples were analysed to track changes in both mCherry and GFP expression levels using flow cytometry (section 2.5). Blood samples were collected repeatedly until the mice revealed outward physical indications of rAML (such as poor grooming, decreased activity, pallor, hunched posture) or until they were euthanised due to poor conditions. Clonal expansion of mCherry negative (mCh-) leukocytes was detected as early as 3 months post-exposure in 9.4% (5/53) of irradiated animals. Figure 3.13a below shows the development over lifespan of clonal expansion of mCh negative WBCs in an individual mouse (101.2 female CBA Sfpi1 $^{mCh/Gfp}$ mouse), following IR exposure. Initially, at 2 months post-irradiation, no leukocytes showed loss of mCherry (Figure 3.13a). By around 15 months post-irradiation (Figure 3.13b), mCh loss can be seen in around 21% of lymphocytes (blue) and 11% myeloid cells (red) in the lower quadrants of the plots below. The percentage of irradiated mice showing mCh- clonal expansion in WBCs was 26% by 6th months post-irradiation, increasing to 38% over the 9th month's period. Finally, by the 15th month, the percentage showing mCh- clonal expansion reached 43.3% (23/53) and these results are in line with data previously reported by Verbiest et al, which was roughly 50% (Verbiest et al., 2018). The majority of the clonal expansion detected was of mixed lineage origin (e.g., lymphocytic, and granulocytic with minimal monocytic origin) and 11% (6/53) were potential leukaemic mice, showing hepatosplenomegaly, extremely elevated WBC counts (e.g., 89.2x10^6/ml and rapid mCh- clonal expansion >70%). Our study revealed a higher background rate of mCherry fluorescence loss which was approximately 3-4% in 0 Gy groups typically (compared to <1% in Verbiest's study), which might possibly arise due to loss of sensitivity of mCh detection within the Guava system.

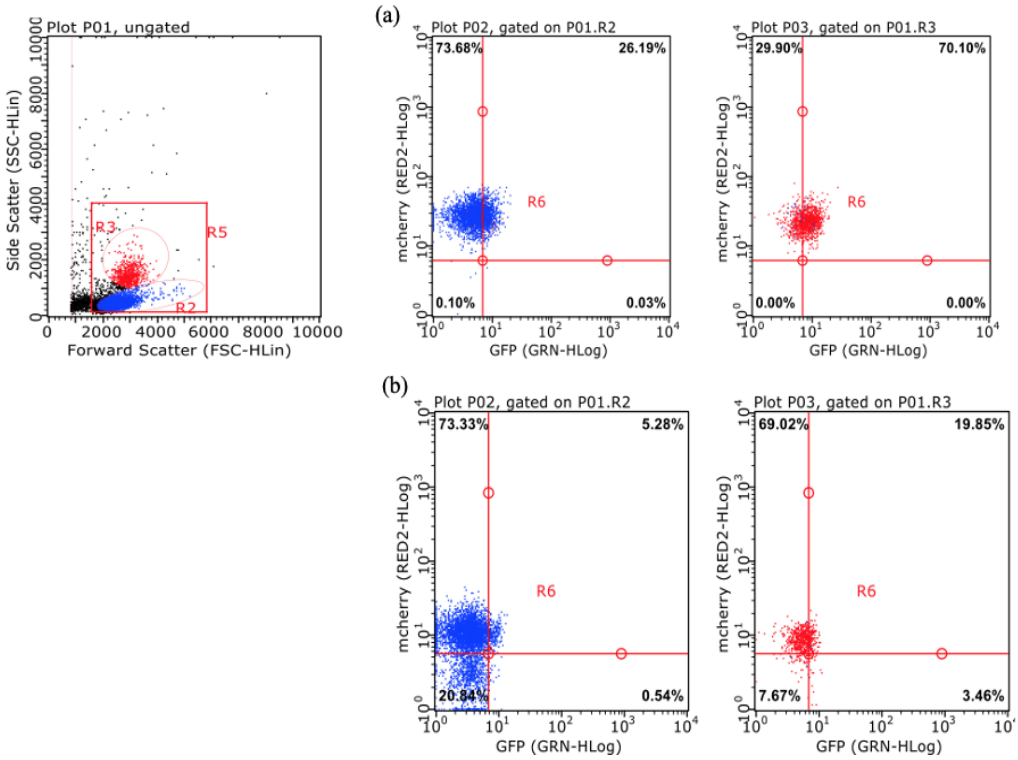


Figure 3. 13 CBA Sfpi1^{mCh} mice show mCh- clonal expansion following radiation exposure.

Following exposure, tail vein blood samples were examined using flow cytometry analysis for mCherry expression. Representative dot plots of 3 Gy irradiated female mice (upper panel **(a)** 3rd, lower panel **(b)** 6th month blood sampling analysis, respectively. The gates are as follows; forward scatter vs side scatter plots (size versus granularity) and the lower (blue) gate contains lymphocyte and monocyte cell populations. Lymphocytes do not express GFP and so they appear on the left side of PO2 plot, whereas monocytes which express GFP appear on the right side of the plot. The upper gate contains a granulocyte cell population (in red, higher side scatter), and this population contains some cells that express GFP (plot PO3). Due to pandemic restrictions, these experiments were carried out in collaboration with my colleagues, Dr Rosemary Finnon and Paul Finnon, who provided invaluable support and assistance.

According to our blood sample analysis, only 2/44 mice (approximately 4.5%) revealed clonal expansion within non-IR exposed groups, and this may arise as a result of clonal haematopoiesis, which is known to be closely associated with age (Papa et al., 2020). Following IR exposure (see figure 3.14 below) at month 3 male and female mice had similar percentages of mCh loss (25%, 28%). Strikingly, at 6th months the percentage of female mice with mCh- leukocytes was significantly higher when compared to male mice (45% and 33%, respectively). The percentage of mice with mCh- clonal expansion amplified 2.5-fold over the course of 18 months, respectively (female: 87% and male 67%; Figure 3.14 a, b). Even though

Sfpi1$^{mCh/GFP}$ mice with clonal expansion at months 9 and 15th had mixed lineage origin (myeloid and lymphoid), 38% of mice which presented with mCh- clonal expansion at 15 and 18th months following IR exposure had clonal expansion of myeloid only (female: 11/29 and male: 9/21 mice with mCherry-clonal expansion, respectively). Furthermore, at 18th month the percentage of mCherry-mixed myeloid-lymphoid leukocytes were significantly higher in female mice (female: 47% and male: 17% respectively) and 7% of female mice were diagnosed with leukaemia.

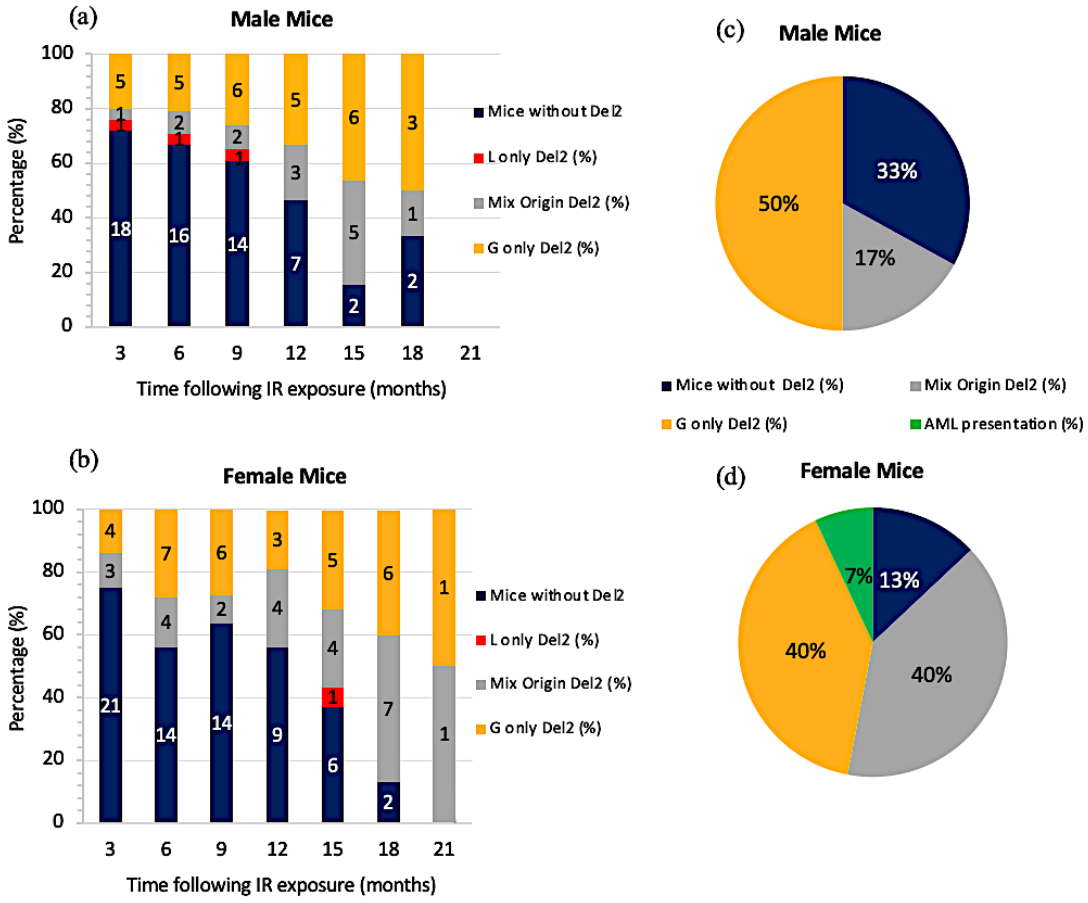


Figure 3. 14 mCherry- clonal expansion in the peripheral blood of an irradiated CBA Sfpi1$^{mCh/GFP}$ mice.

The blood of irradiated male and female mice (n = 25 and n = 28, respectively) was assessed monthly for the expression of mCherry and GFP. **(a,b)** Represents the percentage of male and female mice with mix origin both lymphoid and myeloid mCherry loss (grey), with only granuloid (orange) or lymphoid (red) mCherry loss and without mCherry loss (navy), detected in the blood, time following IR exposure. Numbers in the bar chart denote the actual numbers of mice alive at the specified time point. **(c,d)** Signifies the percentage of male (top panel) and female (bottom panel) mice at the time of death,

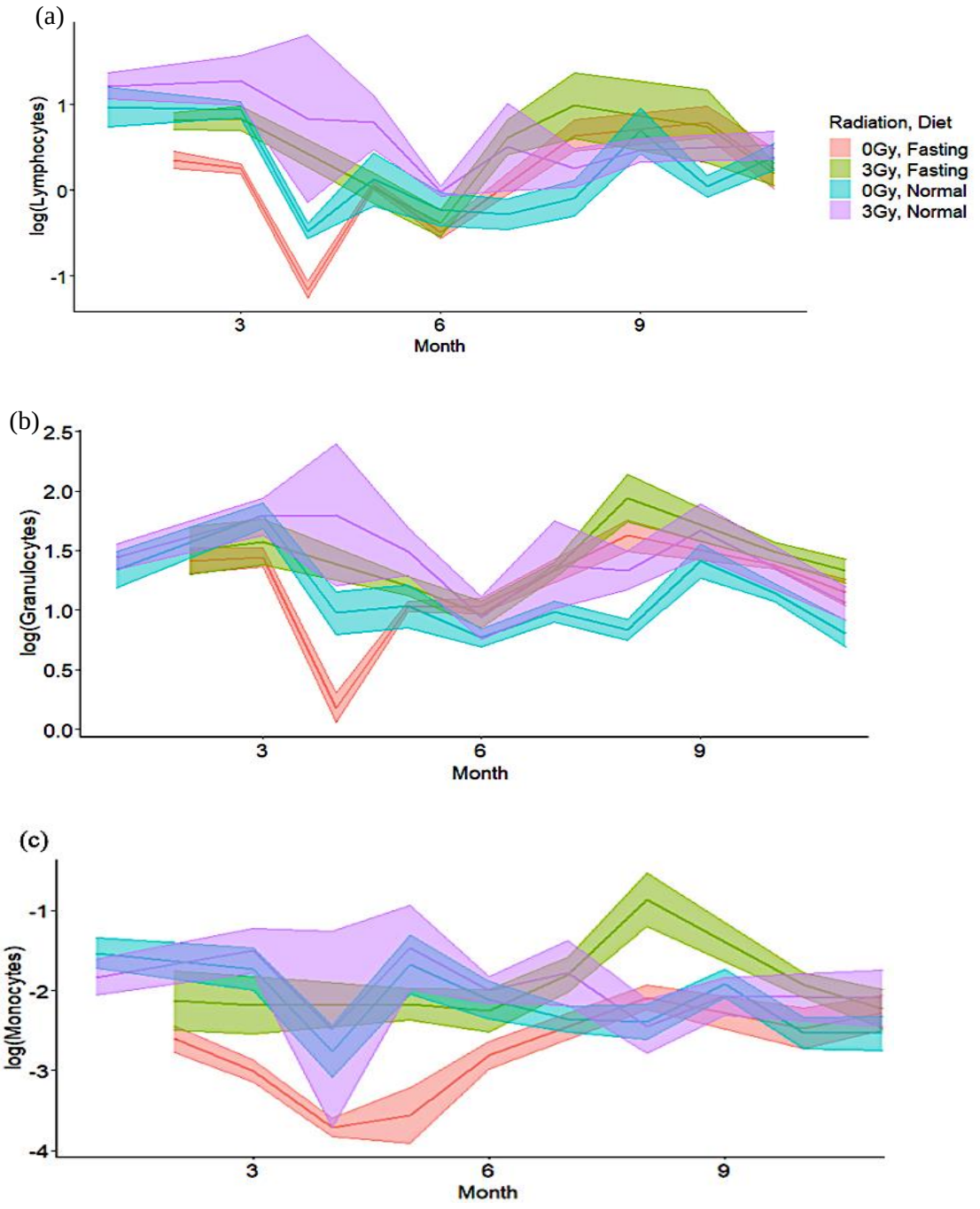
diagnosed without mCherry loss (navy), with both lymphoid and myeloid mCherry loss (grey), with granuloid (myeloid) mCherry loss (orange) or with leukaemia (green).

By using this well characterised CBA Sfpi1 $^{mCh/GFP}$ mice model, we wanted to examine whether alternate day fasting (ADF) could possibly reduce the incidence of rAML, altering the frequency and maintenance of rAML-target cells within the HSC compartment by tracking the changes on mCherry-clonal expansion in peripheral blood leukocytes derived from Ch.2 carrying HSPC. Following day 2 post-irradiation, mice were exposed to six x1 day fasting/1 day feeding cycles and body weight analysis and conditioning scores were followed consistently to ensure mice were not subjected to significant weight loss. After a 12 days fasting cycle, mice returned into ad-lib diet regimens and evaluated weekly. As presented in Figure 3.12 b above, we showed that fasted mice sustained a healthy body weight throughout their lifespan.

Data represented in Figure 3.15 below represents the average mCh^{-} loss within individual leukocyte cell population. According to our comparative analysis, there was no difference in mCh^{-} leukocytes between ad-lib 0 Gy and ADF 0 Gy groups. As presented in Figure 3.14 above, radiation exposure resulted in mCh-clonal expansion in CBA Sfpi1$^{mCh/Gfp}$ mice when compared with ad-lib 0 Gy. Likewise, Figure 3.15 below shows that IR exposure increases mean mCh- loss within leukocyte populations (ad-lib 0 Gy vs ad-lib 3 Gy), and this pattern was observed across all three cell populations. While IR exposure affected mean mCh- loss among groups, particularly between months 2-5 following exposure, the results did not reach statistical significance.

Next, we assessed the effect of fasting on mCh loss following radiation exposure. Accordingly, our results reveal that the mean mCh loss within 3 Gy fasting (green) groups display a decline

when compared with ad-lib 3 Gy (purple), within an early time frame (2-6 months). A significant reduction in mCh loss observed in lymphocyte cells at months 5 and 6 ($p < 0.047$; $p < 0.058$). However, strikingly, ADF 3 Gy data reveals an increase in mean mCh-loss among all cell populations, particularly at the 8th month, where both monocyte and granulocytes have experienced a statistical significance ($p < 0.0031$; $p < 0.029$).



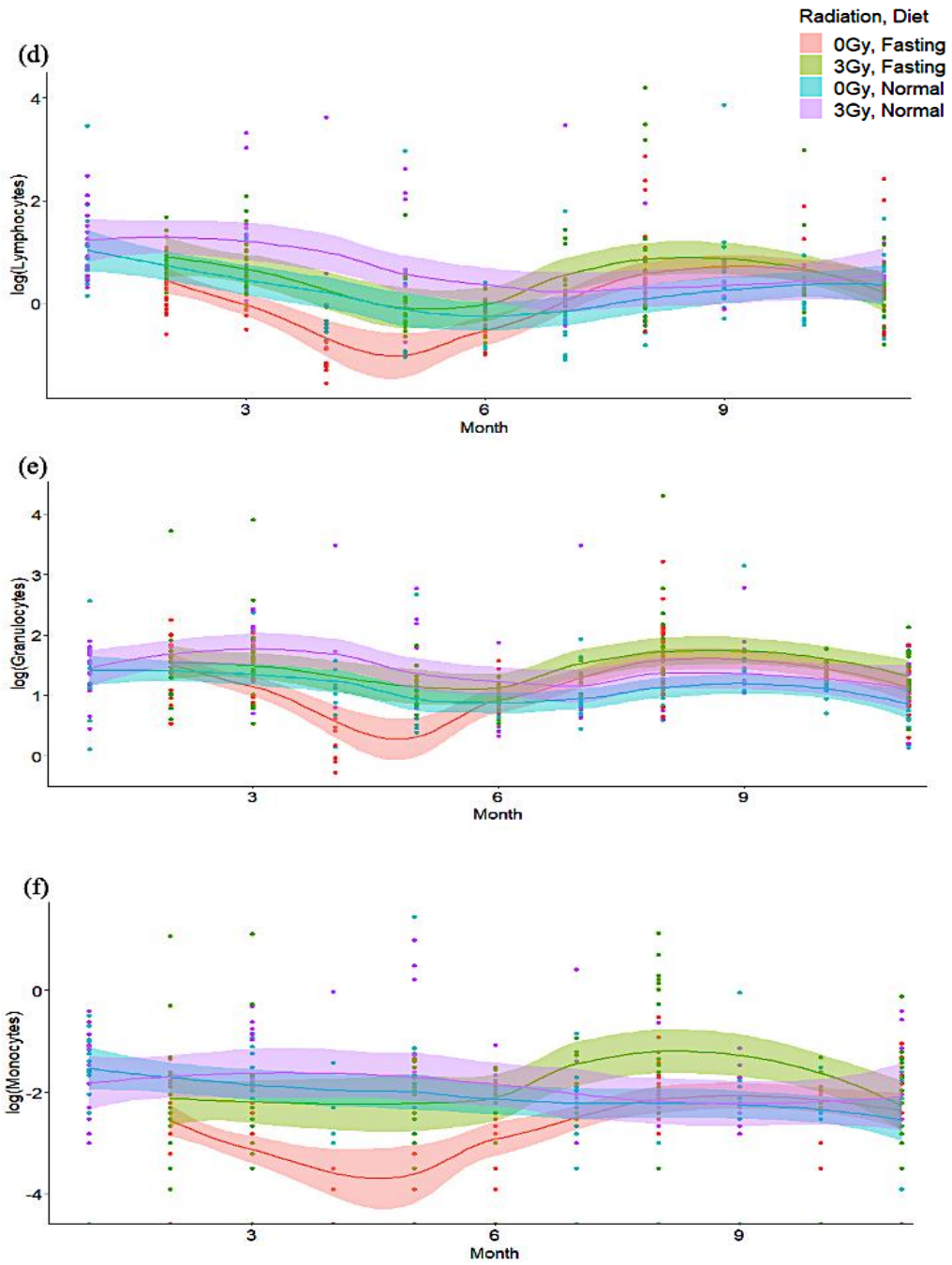
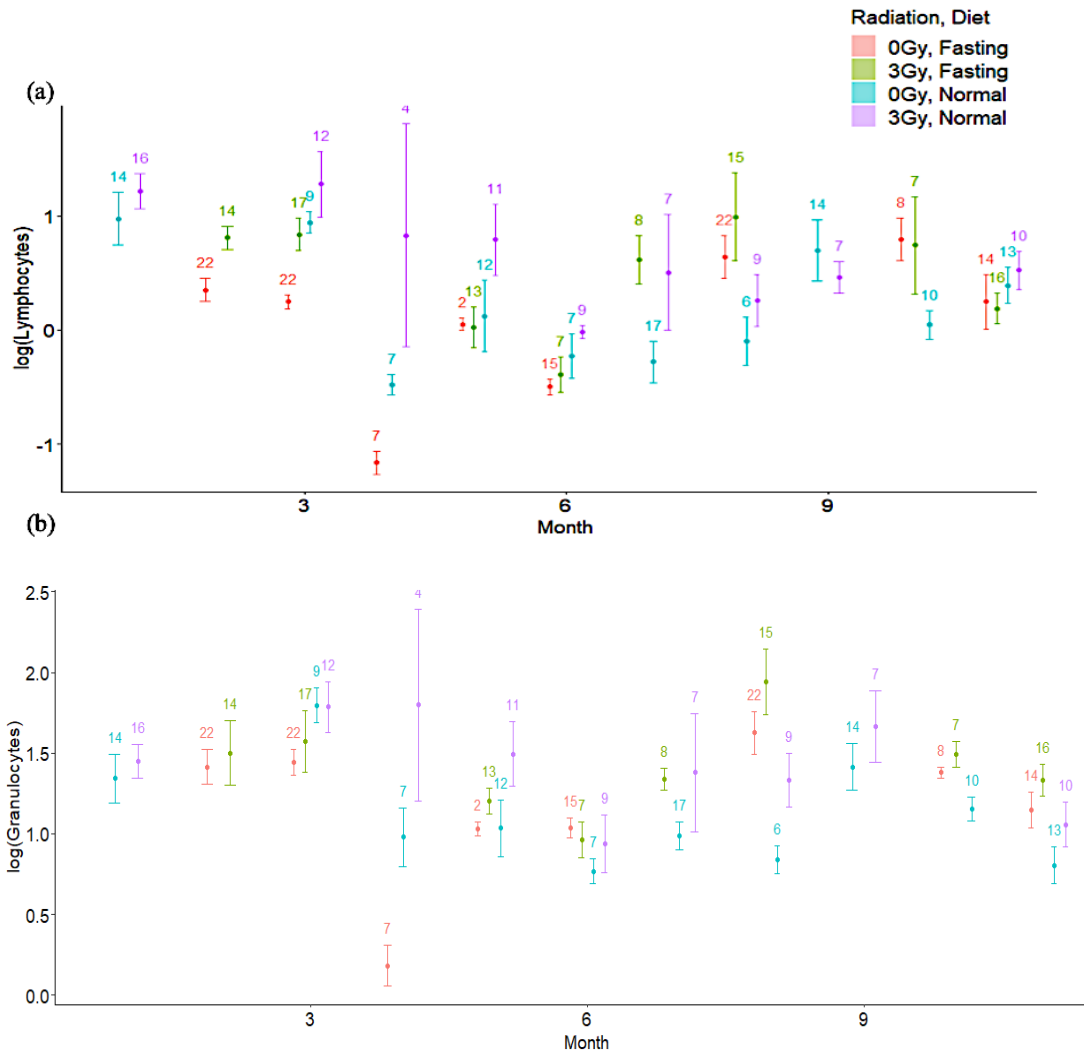


Figure 3. 15 Effect of radiation exposure and fasting regimens in CBA Sfpi1$^{mCh/GFP}$ mice.

Six cycles 1-day fasting/ 1-day feeding (for the duration of 12 days in total) was initiated at 2-day post-exposure. Tail vein blood samples were examined monthly using flow cytometry post-irradiation to detect whether fasting could induce alterations in the expanding haematopoietic clones: average mCherry loss in **(a)** lymphocytes, **(b)** granulocytes, **(c)** monocyte cell populations**. (d, e, f)** Local polynomial regression curves for mCh-loss within leukocytes. Each coloured dot signifies an individual mouse. Plots presented specify the mean values at each time point for each group e.g., radiation, diet. Colour codes: red (0 Gy fasting), green (3 Gy fasting), blue (0 Gy ad-lib), purple (3 Gy ad-lib).

Figure 3.16 (see below) presents the mean, standard error, as well as the number of mice used to collect blood samples every month for each experimental group. It can be seen from the plots that the number of mice used to conduct the analysis varies throughout the study, which has impacted the interpretation of our data. Inconsistencies between mice numbers have been attributed to several factors, primarily samples were failing to lyse properly, and occasionally very low blood volumes. All together, these findings of our study indicate that there are some possible dietary influences, particularly within the early stages following IR exposure. Nevertheless, the effect was not statistically significant and further investigation is required to reach a more reliable conclusion while minimising the technical issues associated.



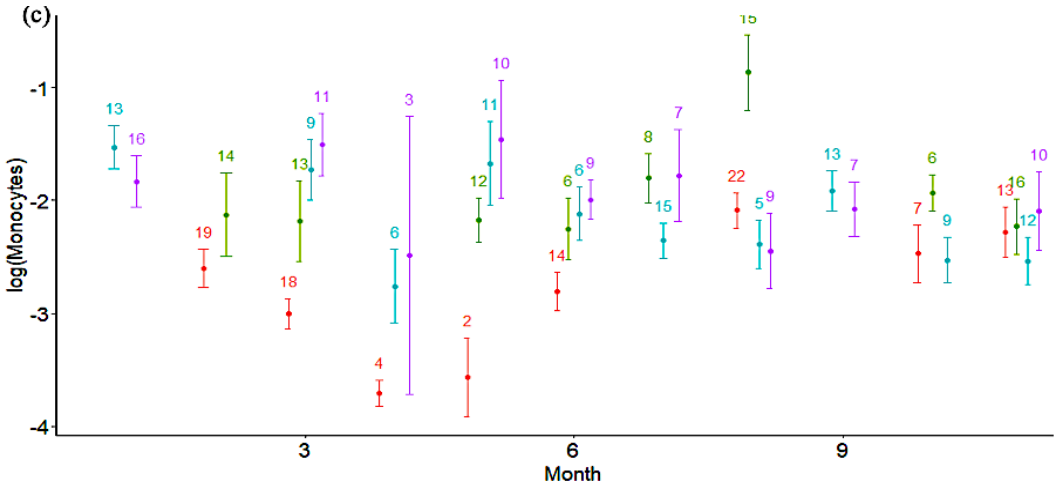


Figure 3. 16 Represents the SE bars and number of mice used at individual time points in (a) lymphocyte, (b) granulocyte, (c) monocyte cell populations from 1st to 11th months following IR exposure.

Points are indicator of means and numbers on top of the coloured error bars indicate how many mice were used for blood sampling every month. Colour codes: red (0 Gy fasting), green (3 Gy fasting), blue (0 Gy ad-lib), purple (3 Gy ad-lib).

Our preliminary study reveals that there is no statistically significant distinction in the presence of del2-carrying peripheral blood leukocytes. Additionally, no evident shifts were observed in in the number of pre-leukaemic HSPC induced within the bone marrow post-IR exposure between mice on ADF diet and those on ad-lib-fed diet. As a result, no differences were documented in the susceptibility to the development of acute myeloid leukaemia (rAML). It is critical to acknowledge that the timing of the ADF diet, initiated post-irradiation as described in the Lu et al., may have influenced the potential benefits on oxygen radical metabolism in HSPCs (Lu et al., 2017). Given additional time, the commencement of the diet before irradiation could hold potential advantages, as it would ensure the availability of potential benefits immediately upon alteration of oxygen metabolism. This may facilitate a more targeted investigation into the effects of the diet on HSPCs and their response to irradiation-induced changes in the microenvironment.

3.4.3 Discussion

In acute leukaemias, neoplastic progenitors over-proliferate and infiltrate normal haematopoietic tissues of the BM, spleen, and peripheral blood, eventually reducing the number of fully differentiated functionally normal white blood cells (leukopenia). There have been various therapeutic approaches which aim to force cancerous cells to resume lineage maturation as an alternative to cytotoxic chemotherapy. For instance, all-trans-retinoic acid (ATRA) has been used successfully for the treatment of acute promyelocytic leukaemia (APL), allowing the differentiation of APL leukaemic blast cells. However, there have only been a limited number of pharmacological agents that triggers the terminal differentiation of leukaemic cells (Nowak et al., 2009).

In a recent study, Lu et al. (Lu et al., 2017) investigated the potential therapeutic benefits of fasting; a dietary intervention which has been suggested to promote normal haematopoietic regeneration as a treatment for acute leukaemia (C.-W. Cheng et al., 2014). Accordingly in response to fasting, ALL cells revealed rapid proliferation, apoptosis, and differentiation rate. The group further carried out RNA sequencing and pathway analysis to gain better mechanistic insight into how fasting may possibly involve in the elimination of ALL cells. Based on their analysis, authors found a prominent signature of leptin-receptor (LEPR) signalling including a strong activation of PRDM1, a downstream target of the LEPR-mediated STAT pathway which involves in driving the terminal differentiation of lymphoid progenitors.

The fluorescence-tagged preleukaemic ALL and AML cells are transplanted into recipient mice and with the development of leukaemia in the mice, ALL cells expressed low levels of LEPR (Figure 3.17 below). Whereas fasting-induced gene-expression programme hinders leukaemia development at early stages, and further leads to differentiation and eventually depletion of the

leukaemic cells at later phases. In contrast AML cells express high levels of LEPR and are refractory to the effects of fasting. It has been indicated that in response to therapy, a subset of acute leukaemias can either shift lineages or attain mixed phenotypes (e.g., possessing both myeloid and lymphoid features) at relapse, possibly allowing those leukaemias to escape fasting- or LEPR-induced differentiation (Dorantes-Acosta & Pelayo, 2012). Thus, it will be necessary to circumvent such complications or mechanisms of escape with proposed therapeutic interventions. Despite these potential challenges, this study demonstrates that LEPR and PRDM1 signalling are important in leukaemic cell differentiation and that they might one day be exploited therapeutically to alleviate the disease burden of ALL patients.

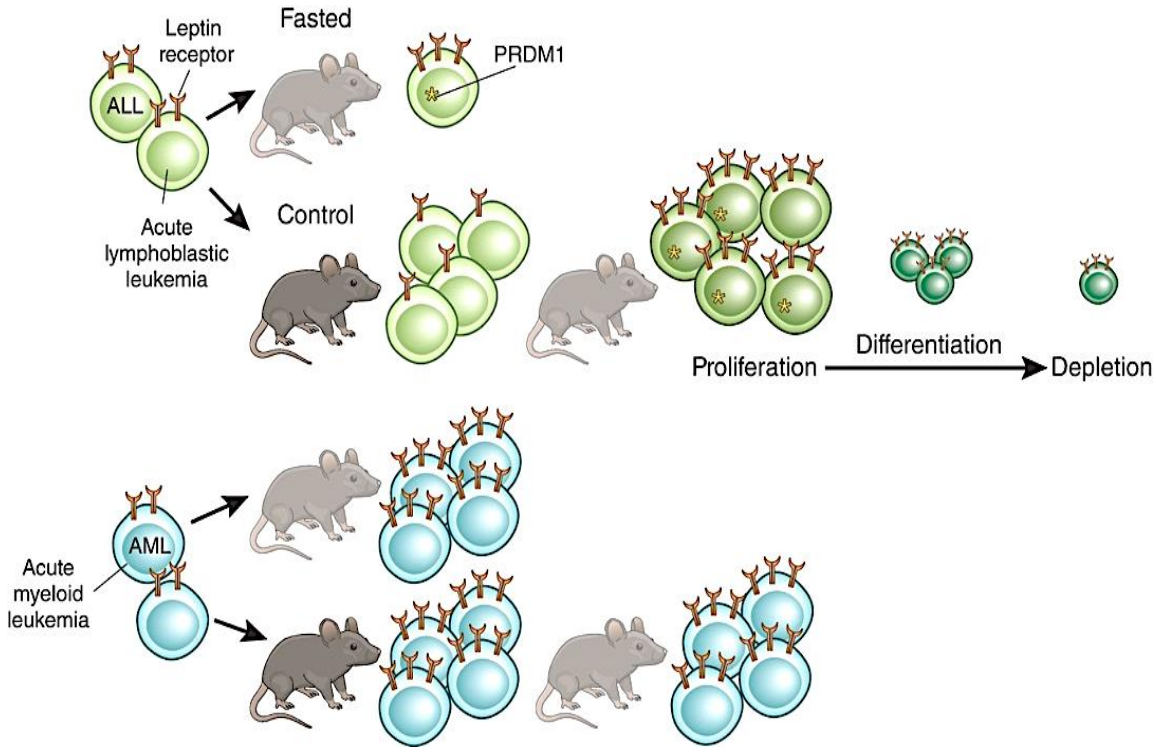


Figure 3. 17 Dietary fasting regulates LEPR-mediated leukaemia differentiation.

Figure is taken from a manuscript which is submitted to Nature Medicine by (C.-W. Cheng & Yilmaz, 2017) and no changes have been made. The copyright permission to use content can be found from the link with licence number 5602470065013.

Rapidly expanding body of research which studies the effects of caloric restriction and dietary fasting have revealed a multitude of benefits affecting a variety of physiological systems. Recently, Valayer and colleagues (Valayer et al., 2020) conducted a systemic review to investigate the effects of caloric restriction and dietary fasting on the physiological response to ionising radiation in humans and animals. A detailed review of the evidence for fasting and/or caloric restriction as an approach to radioprotection can be found in this specific report (Valayer et al., 2020). Although a number of physiological mechanisms have previously been proposed, there is now increasing evidence that fasting and caloric restriction can directly modulate and reduce cellular oxidative stress, thereby contributing to a longer lifespan (Sohal & Weindruch, 1996; Song et al., 2014).

A number of unique fasting sequences have been examined as an intervention prior to radiation exposure. Smith and colleagues have assessed the effect of fasting on the mean time of death of guinea pigs. In the study, animals were fasted 24 hours prior to exposure, which was followed by another fasting phase for 48 hours after the IR exposure. Accordingly, fasting was associated with an increase in mean survival time with an extremely large effect size (Smith et al., 1952). Another study conducted by (Kozubík & Pospísil, 1982) examined the survival rate of several strains of mice that had been exposed to different protocols of intermittent fasting. Each group revealed a higher survival rate, but the effects varied depending on the length of time of the fasting regimens. Survival benefits were seen more evidently when the fasting interventions were applied longer than a week. Moreover, shorter intervals of food access between fasting periods appeared to reinforce the survival effect although there was no indication of a dose-effect relationship (Cappelli et al., 1967; de la Cruz Bonilla et al., 2019). For instance, Bonilla et al., examined the effect of fasting on reducing intestinal radiotoxicity and enabling dose-escalated radiation therapy in pancreatic cancers. Interestingly, they have

reported no alternative in gamma-H2AX levels (a marker for monitoring DNA damage initiation and resolution), while the number of cleaved caspase-3 (CC3; marker of programmed cell death) positive cells were significantly higher in fasted animals 24 hrs after radiation exposure (de la Cruz Bonilla et al., 2019). Furthermore, the results of a study conducted by Li et al. (J. X. Li et al., 2010) indicate that short fasting periods in mice (e.g., 12, 48, 72 h with 8 mice per group) prior to radiation exposure led to an increase in survival rates of 0, 12.5 and 50%, respectively when compared to 0% survival in the control groups following 7.5 Gy γ-ray exposure. All together, these studies suggest that fasting (particularly continuous, intermittent, as well as fasting before, fasting before and after exposure) may possibly improve the survival rates and time to death following exposure, yet there are a lot of heterogeneity depending on the mouse strain, radiation type (e.g., γ-Rays, X-rays) and dose.

Various groups attempted to describe the cellular mechanisms responsible for the link between food intake and oxidative stress. Correspondingly, they have postulated that reduced oxidative stress coupled with increased antioxidant activity may account for the observed beneficial outcomes through energy sensing pathways such as FOXO (Brunet et al., 2004), mTOR (Johnson, Rabinovitch and Kaeberlein, 2013b), Sirtuins (Cantó & Auwerx, 2009; Guarente, 2013; Merksamer et al., 2013; X. Qiu et al., 2010; Traba et al., 2017; Vassilopoulos et al., 2011), AMPK (Greer et al., 2007) or NRF2 (Martín-Montalvo et al., 2011). Although human studies investigating fasting and CR's influence on radioprotective mechanisms are very limited, Allard and colleagues (Allard et al., 2008) investigated the effect of dietary regimens on health and longevity in humans. Accordingly, authors used serum collected from participants of two-pilot studies which assessed the effect of ADF, CR, as well as CR combined with aerobic exercise on several longevity and health markers. Human hepatoma cells (HepG2) cultured in serum collected at the end of the dieting period and compared to the HepG2 cells

cultured in serum collected at baseline (before the dietary phase). Accordingly, cells from ADF participants showed a 20% increase in Sirtuin1 (Sirt1) protein which is linked with reduced triglyceride levels. ADF serum stimulated a 9% reduction in proliferation and 25% heat resistance. Besides, cells cultured in serum from CR participants also revealed an increase in Sirtun1 protein expression by 17% as well as 30% increase in PGC-1 alpha mRNA levels.

Previous studies reported Sirt1 as a leading contender for proteins responsible for the longevity effect of CR in rodents (Safdie et al., 2012b; Traba et al., 2017). Sirt1 is an NAD-dependent deacetylase that activates and/or deactivates various proteins implicated in the regulation of growth, differentiation, oxidative damage, metabolism, as well as stress reactions (Lee et al., 1999; Luo et al., 2001; Brunet et al., 2004; Cohen et al., 2004; Higami et al., 2004; Yeung et al., 2004; Raffaghello et al., 2008; Pietrocola et al., 2016). Likewise, earlier studies claimed that the treatment of cells cultured in sera collected from rats and monkeys fed CR diets revealed comparable effects to those found by Allard et al (Allard et al., 2008; de Cabo et al., 2003). In summary, authors concluded that this first *in vitro* study resulted in increased stress-resistance as well as up-regulation of genes which are proposed to be markers for longevity. Although the data is inconclusive, there is some evidence suggesting that fasting and CR might play a protective role against radiation exposure in rodents. The radioprotective effect (i.e., lower cancer incidence and greater survival) with caloric restriction was only seen if implemented before and after irradiation whereas the benefits of fasting were seen regardless of timing. The potential application and mechanisms of radioprotection provided by dietary changes could have various applications in both terrestrial and space medicine. However, the transferability of our understanding from animal models to humans is still doubtful and given the scarcity of research within the field, all these observations are hypothesis generating. Therefore, forthcoming studies should prioritise exploring the impact of decreased caloric

consumption and fasting in humans, particularly inspecting the clinical consequences in patients receiving radiotherapy.

CHAPTER 4

General discussion and conclusions

4. General Discussion and Conclusions

4.1 General Discussion

As a next step from epidemiological investigations demonstrating an elevated leukaemia incidence following IR exposure, the target cells, and mechanisms responsible for radiation-induced leukaemia need to be further studied. To address this gap, our group developed a unique mouse model, wherein distinct fluorescent markers were introduced onto each ch2, located in the MDR that occur after IR exposure and has been identified as the initial leukaemogenesis trigger. In this tailored model, a significant proportion of symptomatic CBA Sfpi1$^{GFP/mCh}$ mice exhibited expanding clones of preleukaemic haematopoietic cells carrying a hemizygous interstitial ch2 after radiation exposure. Notably, upon isolating preleukaemic HSPCs that had been irradiated in their natural microenvironment, the study unveiled the presence of Sfpi1 point mutations within a rapidly expanding subset of these preleukaemic cells. Additionally, an unreported distinction in the preleukaemic cell and leukaemia phenotype based on gender was also monitored, suggesting a gender-related difference in the target cells susceptible to radiation-induced leukaemogenesis (Verbiest et al., 2018). In summary, our group provided ground-breaking insights into the chronological sequence of molecular events occurring during radiation-induced leukaemic clonal evolution, shedding light on previously charted aspects of this phenomenon.

Within the confines of our in vitro investigations, the effect of differing oxygen levels (ambient normoxia 20.8% vs. low oxygen 3%) and the impact of IR exposure upon the growth dynamics and metabolic phenotype of HSPC populations were evaluated. Previous *in vitro* studies used oxygen levels spanning from 1 to 7%) to assess the influence exerted by hypoxic culture settings on numerous stem cell micro-environments (Atkuri et al., 2007; G. Chen et al., 2011;

Chow et al., 2001; Elabd et al., 2018; Ivanovic et al., 2000; Koller MR et al., 1992; Tsai et al., 2011; Wierenga et al., 2014; Y. Yoshida et al., 2009). According to those findings, hypoxic culture has proven to preserve the redox environment (Atkuri et al., 2007), leading to improved cellular fitness, short-term proliferation capability; long-term expansion efficiency, differentiation potential, stemness and hindered senescence. The outcomes derived from our growth curve analysis of HSPCs elucidated a distinctive pattern where normoxia triggers proliferative potential of unirradiated HSPCs. Specifically, elevated oxygen levels emerge as a stimulant driving the dual processes of HSPC proliferation and differentiation, effectively tilting the balance away from the quiescent state of HSCs. This augmented oxygen environment stimulates increased radio-sensitivity within HSPCs as compared to their counterparts nurtured in low-oxygen conditions.

Following radiation exposure, haematopoietic reconstitution requires the release of the surviving HSCs from their quiescent state into the G1 stage of the cell cycle. It has been reported that around 60% of these surviving HSCs actively proliferate for more than 10 months following radiation exposure, with the number of cell divisions per surviving HSC being ten times higher compared to those unexposed mice (Ban & Kai, 2009). Our results are therefore consistent with an initial reduction in the growth rate due to cell death followed by proliferation to restore the cell pool so that all samples have a similar number of cells by the end of their short-term culture period. At the onset of the G1 phase of the cell cycle, DNA damage is typically repaired by error prone nonhomologous end-joining, which can promote the formation of de novo mutations (Mohrin et al., 2010). Besides, enhanced replicative stress can induce premature ageing and premature senescence of HSCs in a ROS-dependent manner (Shao et al., 2014), decreasing their DNA repair capacity and thereby making them more susceptible to spontaneous mutation.

This hypothesis gained additional support upon the identification of a common myeloid-progenitor-like leukaemic stem cell (Lin-Sca1-cKit+CD34+) in an experimental murine model of rAML. Authors have demonstrated that within a context of continuous cycling aimed at replenishing the CMP (common myeloid progenitor) population, HSCs bearing a deletion on chromosome 2 (ch2) exhibited further aberrations (Hirouchi et al., 2011; Verbiest et al., 2018). This phenomenon was accompanied by an escalation in the mutation rate, a trend often linked to advancing age. This enhanced mutation frequency potentially contributes to the accumulation of secondary point mutations, providing a rationale for the relatively prolonged interval between radiation exposure and the onset of rAML in murine models.

In past decades, the focus of cancer research centred around the inactivation of tumour-suppressor genes and the activation of oncogenes. While this paradigm has significantly enhanced our comprehension of cancer biology, there are additional factors that exert a substantial influence on carcinogenesis and must not be disregarded. Recent advancements in cancer research have prompted Hanahan and Weinberg to update their 2000 review on cancer hallmarks, reflecting the substantial progress made. One noteworthy advancement is the "reprogramming of energy metabolism" in cancer cells (Hanahan & Weinberg, 2011). Considering the significant role of energy metabolism in HSC homeostasis, the study of energy metabolism in leukaemic stem cells (LSCs) has become particularly relevant. Single cell RNA sequencing study conducted by Giustacchini et al, disclosed that within the same patient-derived CML sample, BCR-ABL^{+}LSCs overexpressed genes were found to be linked with OXPHOS when compared to non-malignant HSCs (Giustacchini et al., 2017). These results were in line with global mRNA microarray evaluations using HSCs and progenitor cells from CP-CML, BP-CML and non-leukaemic donors which were shown that CP-CML LSCs reveal an oxidative phenotype with highly expressed mitochondrial chain (MRC) genes (Flis et al., 2012).

Our findings presented in subsection 3.2.1 of our study unveil a notable augmentation in mitochondrial-derived ATP synthesis via OXPHOS following IR exposure. Intriguingly, our investigation further elucidates that the ambient oxygen milieu exerts a pivotal influence on HSPCs, stimulating a compelling transition towards an augmented aerobic phenotype. In contrast, under conditions of lower oxygen availability, HSPCs manifest a tendency for anaerobic glycolysis, a metabolic model widely recognised for its integral role in fostering stemness and quiescence within this cellular cohort (Ito et al., 2004; Ito & Suda, 2014; Norddahl et al., 2011; Parmar et al., 2007; Simsek et al., 2010; Suda et al., 2011; Vannini et al., 2016)**.** The current observations situate themselves within a broader context established by prior research endeavours. Specifically, existing studies have verified that exposure to IR triggers the generation of reactive oxygen species (ROS) within the mitochondrial compartment, associated with increased NADPH-oxidase activity. A notable consequence of this oxidative milieu is the reduction of hypoxia-inducible factor-1 alpha (HIF-1α) levels. The complex interplay of these molecular events collectively provokes cell cycle progression, which in turn facilitates the proliferation of HSCs.

Although OXPHOS stands as a predominant metabolic pathway used by LSCs, the rationale behind the preferential selection of OXPHOS over anaerobic glycolysis remains unclear. As leukaemic cells resort to mitochondrial respiration for energy acquisition, the mitochondrial respiratory chain (MRC) emerges as a pivotal source of intracellular ROS (Testa et al., 2016). Previous reports showed an increase in NOX activity among numerous leukaemic cell types including AML, chronic myeloid leukaemia (CML) as well as promyelocytic leukaemia, signifying that constitutive activation of NOX is an important source of intracellular ROS in LSCs (Dong et al., 2004; Hole et al., 2013; M. M. Singh et al., 2012). Furthermore, existing research has also shed light on the decrement in antioxidant defence mechanisms within distinct leukaemia subtypes (Battisti et al., 2008; Gaman et al., 2014; Rasool et al., 2015; Tahir et al.,

2017). This observation underscores the potential existence of a delicate equilibrium disruption between oxidative and antioxidant systems, thereby potentially explaining the escalated ROS levels characteristic of leukaemic cells.

Our comprehensive examination of mitochondrial function and dynamics unveiled substantial changes in diverse aspects of mitochondrial activity within HSPCs after exposure to ionising radiation (IR) and in a normoxic oxygen environment. This conclusion finds robust support in the data outlined in Section 3.2.2, which highlights notable rises in mitochondrial DNA (mtDNA) content, mitochondrial mass, and membrane potential within HSPCs. Further affirmation comes from reports indicating increased respiratory activity in AML cells, underscoring the pivotal significance of mitochondria not just in the domains of normal haematopoiesis but also in the context of leukaemogenesis (Jitschin et al., 2014; Panina et al., 2019; Sriskanthadevan et al., 2015; Vélez et al., 2013).

Exploring how metabolism orchestrates the behaviour of stem cells in their natural environment has shed light on a spectrum of metabolic patterns that span from normal equilibrium to cancerous states. This intricate landscape presents a fertile ground for investigating factors that might impact the initiation of leukaemia. Simultaneously, it offers a promising avenue for exploring novel therapeutic strategies aimed at diminishing the long-term susceptibility to leukaemia following IR exposure. Using the well-established CBA mouse model, we delved into the effects of dietary interventions and intermittent fasting on the step-by-step molecular changes that unfold during radiation-induced leukaemia. This work holds promise for devising novel approaches to reduce the risk of leukaemia occurrence in human populations whose bone marrow has been exposed to IR.

Multiple studies of alternate dietary restrictions on mouse models of leukaemia have been carried out previously. A group led by professor Valter Longo, at University of Southern

California Leonard Davis School has been working on a promising new approach with temporary fasting or fasting mimicking diets (FMD) to treat CLL. Their preliminary studies have already seen a 100% kill rate of CLL cells by using a combination of FMD and specific FDA-approved agents. Strikingly, fasting or FMD seems to protect normal cells from the toxic effect of these agents. This potentially occurs as a result of normal cells shutting off the biochemical pathways blocked by these drugs. Multiple cycles of fasting are shown to promote differential sensitization to stress in a variety of tumour types, which might be capable of replacing or augmenting certain chemotherapy drugs in the treatment of numerous cancers (C. Lee et al., 2012).

The data presented in section 3.4.3 indicate that our findings do not reveal a notable disparity in ch2-carrying peripheral blood leukocytes. The number of pre-leukaemic HSPCs induced after IR exposure revealed no evident shifts between ADF and ad-lib diets, resulting in no differences in AML susceptibility, which might be due to the timing of ADF initiation post-irradiation, as stated by Lu et al. (Lu et al., 2017). Although our study did not yield statistically significant results, it is crucial to recognise that the investigation into the impacts of intermittent fasting on disease remains an ongoing pursuit.

Given the scarcity of available research, it is presently impractical to draw a comprehensive conclusion regarding the widespread applicability of fasting in clinical practices. Yet, the possibilities for implementing these strategies in conjunction with existing and new treatment strategies remain interesting. For instance, as part of cancer immunotherapy, cancer-specific T cells are activated to enhance the killing effects on cancer cells (Pardoll, 2012). However, immunosuppression caused by chemotherapy can possibly diminish the efficiency of immunotherapy (Di Biase et al., 2016). Accordingly, it has been demonstrated that conventional fasting techniques can be beneficial in alleviating chemotherapy-induced

immunosuppression, treatment-related mortality rates and facilitating self-renewal of HSCs (C.-W. Cheng et al., 2014).

(Mercier et al., 2022) carried out an in-depth study that presented a thorough overview exploring both preclinical and clinical investigations that delve into the influence of dietary interventions on the toxicity and effectiveness of cancer treatments, particularly chemotherapy and radiotherapy (Mercier et al., 2022). In summary, there has been considerable interest in exploring the potential advantages of Caloric Restriction (CR) and Intermittent Fasting (IF) as dietary approaches for patients undergoing chemotherapy and/or radiotherapy. Although these dietary strategies have shown promise in animal studies, by reducing treatment side effects and enhancing chemotherapy drug effectiveness, there are several limitations associated with their implementation.

These limitations include the use of small participant groups, varying levels of adherence to prescribed diets among patients in certain cases, and significant drop-out rates from the studies. These factors collectively weaken these studies' statistical reliability. Importantly, the lack of substantial research conducted on human subjects from large and diverse populations highlights the need for caution. The inclusion of diverse types of cancer in some studies has also complicated the understanding of the effects of these dietary interventions.

In parallel, ongoing clinical trials investigate the effects of dietary interventions, some focusing on specific cancer types. However, it remains crucial to conduct substantial clinical trials involving much larger groups of patients before considering broader applications. Unlike pharmaceutical treatments, which can be expensive and involve medication schedules, dietary interventions provide a cost-effective approach that is accessible to patients regardless of their financial status. Moreover, these interventions empower patients to actively participate in their treatment journey, potentially influencing outcomes and promoting adherence.

While researchers anticipate more definitive trials to comprehensively evaluate the efficacy of these interventions, it is essential to determine optimal implementation strategies. The promising directions of dietary clinical trials envisions the formulation of dietary approaches capable of attenuating overall mortality risk by a substantial 5% to 10% across the general population, extending beyond just high-risk patients. To achieve these goals, larger study groups, longer follow-up periods, alignment with disease and mortality databases, and an effort to improve participant adherence are necessary (Mercier et al., 2022; Mirmiran et al., 2021; Mittelman, 2020; Vernieri et al., 2016). In summary, future dietary clinical trials should prioritise exploring dietary patterns, eating behaviours, social and economic factors that influence lifestyle changes and adherence to interventions. Notably, while the standards for evaluating study design, rationale, and statistical analysis of dietary interventions are similar to those for drug trials, innovative study approaches are essential to overcome common challenges and ensure high-quality dietary clinical trials.

4.2 Conclusions

This doctoral thesis has yielded a comprehensive exploration into the intricate interplay between ionising radiation, haematopoietic stem, and progenitor cells (HSPCs) derived from CBA/Ca rAML mouse model in short-term primary cell culture. Our investigations encompassed an in-depth examination of alterations in cellular oxygen metabolism, mitochondrial dynamics, proliferation kinetics, and the impact of alternate day fasting (ADF) within target cell population. The findings elucidate a multifaceted response to ionising radiation (IR), characterised primarily by an oxidative stress-induced cytotoxicity followed by a compensatory surge in proliferation of the surviving cells. This surge in cellular proliferation aligns with a concurrent elevation in mitochondrial activity, facilitating a shift towards oxidative phosphorylation (OXPHOS) respiration. Consequently, this metabolic transition

leads cells to adopt more aerobic phenotype, thereby fulfilling increased energy requisites within stress-induced environments.

While there is a growing awareness of the complex nature of haematopoiesis and the factors that influence it, such as genetic mutations and microenvironmental signals, the interplay between metabolic dynamics and dietary components in this context has not been extensively unravelled. By uncovering the intricate connections between metabolic processes, diet, and haematopoietic regulation, we may unveil strategies for preserving the integrity of the haematopoietic system and minimising the likelihood of rAML development. A striking finding from our recent *in vivo* analysis using CBA mouse models of rAML has showcased that ADF notably curtails early-stage mCherry loss primarily within leukocyte cell population following IR exposure. Nonetheless, no disparities were found in the rate of clonal expansion between the ad-lib and ADF fed groups.

Although the data remains incomplete, our preliminary study serves as the foundation for delving deeper into the impact of dietary modifications (such as calorie restriction, fasting, and the depletion of specific nutrients like individual amino acids) as well as other metabolic factors (e.g., oxidative stress). These factors are known to affect HSPC metabolism and haematopoietic homeostasis, which have the potential to act as modifiers of radiation exposure and rAML risk through various mechanisms, including the reduction of rAML target cell populations, curbing over proliferation risk, and mitigation of prolonged oxidative stress. These insights resonate not only within the academic sphere focused on radiation-induced leukaemogenesis, but also offer potential avenues for therapeutic intervention that utilise precise metabolic adjustments to target a spectrum of vulnerabilities associated with radiation.

CHAPTER 5

Future studies

5. Future Studies

The discoveries outlined in this thesis establish a platform for forthcoming inquiries into dietary interventions known to affect the metabolism of haematopoietic stem and progenitor cells (HSPCs). These alterations could pave the way for novel therapeutic approaches, focused on mitigating the risk of radiation-induced acute myeloid leukaemia. As a result, they are currently a focal point of extensive research and interest within the radiation protection field.

In upcoming studies, we intend to expand our *in vivo* fasting experiments incorporating additional treatment groups with different fasting protocols. Unfortunately, our preliminary study was limited in scope due to time constraints that were further exacerbated by the Covid pandemic. Various fasting strategies, such as continuous fasting, intermittent fasting, and fasting before and after exposure to IR, have been demonstrated in studies to potentially enhance survival rates and prolonged time to death post-exposure. Given this, a potential approach could involve initiating fasting regimens in mice prior to IR exposure, as the timing of fasting relative to IR exposure may affect any observed protective effects. Of course, in case of real-life exposure, diet-based treatments would only be viable after the exposure and ideally fasting protocols would be developed in this scenario.

Additionally, this project would aim to further examine the effect of valine depletion in clonal expansion of HSPCs and assess how this may change the rAML risk in CBA mouse model. Cancer cells require increased Aa uptake, and they undergo metabolic reprogramming to meet the energy demands associated with their proliferation. Our results showed that valine depletion significantly reduced the proliferative capacity of HSPCs, and metabolic analysis signified that cells grown in valine-depleted media are metabolically less active. Previously, Taya *et al* showed that valine is essential for HSC maintenance. Mice were either fed with a complete

diet or Val-depleted diet for 4 weeks before HSC harvest and transplantation into recipient mice. HSC derived from mice on the Val-depleted diet failed to produce any long-term reconstitution (Taya et al., 2016). Hence, we aim to incorporate additional groups which will be fed with Val diet only 4 weeks prior to IR exposure and another group which will take Val-depleted diet 4 weeks pre- and post-IR exposure. Groups will undergo monthly tail vein blood sampling throughout the lifespan, mCherry expression will be assessed using flow cytometry and survival rates of mice will be compared between different dietary regimens.

In conclusion, acute myeloid leukaemia is the most common form of acute leukaemia among adults. Based on the statistics reported in 2016, the 5-year overall survival rate of AML is approximately only 24%, however, the highest mortality rate of AML in the United States is among older patients (approximately 90% for ages >65), indicating the need for better treatment choices. Hence, because of the lower survival rate of this demographic and intolerance to aggressive chemotherapy, there are fewer curative possibilities offered to these older patients.

Current research is focusing on dietary restriction which has already been showing a positive therapeutic outcome both in animal models and humans, showing remarkable selective suppression in certain solid tumour types, when merged with chemotherapy (Bordone & Guarente, 2005; Fontana & Partridge, 2015; Hursting et al., 2003; Kalaany & Sabatini, 2009; Longo & Mattson, 2014; Mihaylova et al., 2014). Investigations have explored the potential mechanisms by which diet can enhance cancer outcomes such as metabolic, hormonal, immune/inflammatory effects, and presents the limited clinical research that has been published in this area. Despite the fact that there is little data to support the validity of diet interventions, they demonstrated their potential to reduce toxicity, improve chemotherapy

efficacy, and lower the risk of long-term complications in cancer patients. As such, it is of the utmost importance to improve our understanding and expand scientific knowledge of this critical yet complex adjunctive treatment for cancer (Mittelman, 2020).

CHAPTER 6

References

6. References

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CHAPTER 7

Appendix

7. Appendix

antioxidants

MDPI

Article

Oxidative Stress and X-ray Exposure Levels-Dependent Survival and Metabolic Changes in Murine HSPCs

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Abstract: Haematopoietic bone marrow cells are amongst the most sensitive to ionizing radiation (IR), initially resulting in cell death or genotoxicity that may later lead to leukaemia development, most frequently Acute Myeloid Leukaemia (AML). The target cells for radiation-induced Acute Myeloid Leukaemia (rAML) are believed to lie in the haematopoietic stem and progenitor cell (HSPC) compartment. Using the inbred strain CBA/Ca as a murine model of rAML, progress has been made in understanding the underlying mechanisms, characterisation of target cell population and responses to IR. Complex regulatory systems maintain haematopoietic homeostasis which may act to modulate the risk of rAML. However, little is currently known about the role of metabolic factors and diet in these regulatory systems and modification of the risk of AML development. This study characterises cellular proliferative and clonogenic potential as well as metabolic changes within murine HSPCs under oxidative stress and X-ray exposure. Ambient oxygen (normoxia; 20.8% O_2) levels were found to increase irradiated HSPC-stress, stimulating proliferative activity compared to low oxygen (3% O_2) levels. IR exposure has a negative influence on the proliferative capability of HSPCs in a dose-dependent manner (0–2 Gy) and this is more pronounced under a normoxic state. One Gy x-irradiated HSPCs cultured under normoxic conditions displayed a significant increase in oxygen consumption compared to those cultured under low O_2 conditions and to unirradiated HSPCs. Furthermore, mitochondrial analyses revealed a significant increase in mitochondrial DNA (mtDNA) content, mitochondrial mass and membrane potential in a dose-dependent manner under normoxic conditions. Our results demonstrate that both IR and normoxia act as stressors for HSPCs, leading to significant metabolic deregulation and mitochondrial dysfunctionality which may affect long term risks such as leukaemia.

Keywords: HSPCs; oxidative stress; ionising radiation; metabolism; radiation leukemogenesis; mitochondrial dysfunction; hypoxia; reactive oxygen species; acute myeloid leukaemia

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1. Introduction

Haematopoiesis relies on the fine regulation of bone marrow (BM) haematopoietic stem cell (HSC) biology in response to continuously changing internal and external cues from the niche microenvironment [1,2]. A functional link between stemness and reduced O_2 availability has been stated in multiple stem cell contexts and has been extensively studied in the haematopoietic stem and progenitor cell (HSPC) population [3,4]. The low O_2 environment of the BM endosteal stem cell niche supports the maintenance of long-term hematopoietic stem cells (LT-HSCs) in a protective quiescent state, whereas the actively differentiating/proliferating short-term HSCs (ST-HSCs) and progenitor cells are in the higher O_2 environment of the perivascular niches [3,5–9].

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REVIEW

Influence of diet and metabolism on hematopoietic stem cells and leukemia development following ionizing radiation exposure

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ABSTRACT

Purpose: The review aims to discuss the prominence of dietary and metabolic regulators in maintaining hematopoietic stem cell (HSC) function, long-term self-renewal, and differentiation.

Results: Most adult stem cells are preserved in a quiescent, nonmotile state in vivo which acts as a "protective state" for stem cells to reduce endogenous stress provoked by DNA replication and cellular respiration as well as exogenous environmental stress. The dynamic balance between quiescence, self-renewal and differentiation is critical for supporting a functional blood system throughout life of an organism. Stress-conditions, for example ionizing radiation exposure can trigger the blood forming HSCs to proliferate and migrate through extramedullary tissues to expand the number of HSCs and increase hematopoiesis. In addition, a wealth of investigation validated that deregulation of this balance plays a critical pathogenic role in various different hematopoietic diseases including the leukemia development.

Conclusion: The review summarizes the current knowledge on how alterations in dietary and metabolic factors could alter the risk of leukemia development following ionizing radiation exposure by inhibiting or even reversing the leukemic progression. Understanding the influence of diet, metabolism, and epigenetics on radiation-induced leukemogenesis may lead to the development of practical interventions to reduce the risk in exposed populations.

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HSC; leukemia; ionizing radiation; diet; metabolism

Introduction

Tissue development and homeostasis rely on fine regulation of tissue stem cell biology in response to continuously changing external and internal environments. Stem cells undergo two types of cell divisions: symmetric division generating a pair of stem cells contributing to stem cell pool expansion, and asymmetric division to produce a stem cell and a differentiated progeny for homeostasis (Morrison and Kimble 2006). Balance between these two types of division, as well as between quiescence and active cell proliferation, is controlled by various systemic and local cues in the organism (Mihaylova et al. 2014). As a result, failure to maintain the tissue stem cell system in homeostasis can lead to development of various diseases and manifestation of aging. Exhaustion of tissue stem cells is a hallmark of aging whereas cancer and leukemia are a consequence of deregulation of tissue stem cells (López-Otín et al. 2013).

Diet and physical activity are lifestyle factors that modulate energy metabolism and influence health and disease such as cancer. Dietary caloric restriction and exercise are known to delay aging and onset of various age-related morbidities whereas obesity is often associated with multiple comorbidities called metabolic syndrome and risk of cancer development (Rockenfeller and Madeo 2010). Epidemiological observation has inferred that a majority of human + malignancies is attributable to dietary and metabolic risk factors (Doll 1998), and prevalence of overweight, obesity and insufficient physical activity in developed countries are risk factors of cancers of many sites (Bianchini et al. 2002) among which are myelodysplastic syndrome and leukemia (Lichtman 2010; De Pergola and Silvestris 2013). Interestingly, diet and energy metabolism also influence the regulation of tissue stem cells and their homeostasis to alter tissue composition and growth, indicating interconnection between diet, stem cell function and cancer/leukemia development (Mihaylova et al. 2014).

Cancer is an epigenetic disease and this is also true for hematological malignancies (Langstein et al. 2017; Mansouri et al. 2018; Sun et al. 2018). Metabolism and diet affect epigenetic patterns (Etchegaray and Mostoslavsky 2016), suggesting that part of this interconnection is mediated by altered epigenetic marks.

In this review, we aimed to discuss the prominence of dietary and metabolic regulators in maintaining stem cell function, long-term self-renewal, and differentiation of the

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/irab.

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ORIGINAL ARTICLE

International expert group collaboration for developing an adverse outcome pathway for radiation induced leukemia

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ABSTRACT

Purpose: The concept of the adverse outcome pathway (AOP) has recently gained significant attention as to its potential for incorporation of mechanistic biological information into the assessment of adverse health outcomes following ionizing radiation (IR) exposure. This work is an account of the activities of an international expert group formed specifically to develop an AOP for IR-induced leukemia. Group discussions were held during dedicated sessions at the international AOP workshop jointly organized by the MELODI (Multidisciplinary European Low Dose Initiative) and the ALLIANCE (European Radioecology Alliance) associations to consolidate knowledge into a number of biological key events causally linked by key event relationships and connecting a molecular initiating event with the adverse outcome. Further knowledge review to generate a weight of evidence support for the Key Event Relationships (KERs) was undertaken using a systematic review approach.

Conclusions: An AOP for IR-induced acute myeloid leukemia was proposed and submitted for review to the OECD-curated AOP-wiki (aopwiki.org). The systematic review identified over 500 studies that link IR, as a stressor, to leukemia, as an adverse outcome. Knowledge gap identification, although requiring a substantial effort via systematic review of literature, appears to be one of the major added values of the AOP concept. Further work, both within this leukemia AOP working group and other similar working groups, is warranted and is anticipated to produce highly demanded products for the radiation protection research community.

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KEYWORDS
Leukemia; ionizing radiation; acute myeloid leukemia; AML; radiation-induced leukemia; adverse outcome pathway; key event; low-dose radiation; molecular initiating event; radiation protection; risk assessment

Introduction

Leukemia

Epidemiological studies consistently show that human populations exposed to ionizing radiation are at an increased risk of developing leukemia. In the late 1940s, an increase in the incidence of leukemia was the first late effect that was observed among the atomic bomb survivors in Hiroshima and Nagasaki (Folley et al. 1952). The Life Span Study of atomic bomb survivors is, to this day, an extremely important source of information for the system of radiation protection. The exposure regime for the atomic bomb survivors, however, is very different from exposure scenarios relevant for radiation protection: the atomic bomb survivors were exposed to acute, moderate-to-high doses of radiation, whereas for radiation protection purposes, protracted, low doses are more relevant. In this light, the study of exposed workers, such as the INWORKS study of French, American and British workers in the nuclear industry, are important complements to the life span study. The INWORKS study demonstrated an association between radiation dose and risk of leukemia for workers chronically exposed to low doses of radiation. The magnitude of the risk is similar to that for the acutely exposed A-bomb survivors of low doses (Hsu et al. 2013; Leuraud et al. 2015), and the results of the INWORKS study do not indicate the existence of a threshold dose below which no risk exists, strengthening the scientific basis for the current system of radiation protection. Uncertainties associated with the effects at low doses, however, remain large, and epidemiological studies would have to be of a formidable scale to reduce these further. A thorough understanding and description of the mechanisms involved in

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