Investigation of the Stem Cell Compartment in Patients with Chronic Cytopenias and Low-Risk MDS

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Dedicated to all patients who are struggling, especially those who participated in this research study,

AND

To all those who supported me throughout this academic journey including my husband, my family and my supervisors.

Abstract

Myelodysplastic syndromes (MDS) are defined by the WHO as a group of clonal haematopoietic stem cell (HSC) disorders which are characterised by cytopenias, ineffective haematopoiesis, dysplasia, the presence of blasts and an increased risk of developing Acute Myeloid Leukaemia (AML) (WHO,2022) Patients with persistent cytopenias are relatively frequent encounters in routine Haematology screening and the clinical management of these patients can be challenging due to unpredictable clinical course. This study aims to investigate the stem cell compartment of patients with persistent, unexplained cytopenias, and provide insights into the clinical behaviour of these conditions, aiding in better clinical management.

In this study, the stem cell compartment of 53 patients was investigated in two separate cohorts: Cohort A (n=30) included patients with persistent cytopenias (potential pre-MDS conditions, or low-risk MDS) and Cohort B (n=23) patients with high-risk MDS and AML, as a control group. A one-tube flow cytometric assay was used for the detection of leukaemic stem cells (LSCs) using a combination of 13 different monoclonal antibodies, to identify immunophenotypic aberrancies. Molecular studies by next-generation sequencing (NGS) were carried out using a targeted myeloid NGS panel to detect any molecular aberrations. Immunophenotypic findings were then correlated with the molecular findings to confirm or otherwise the clonal nature of cytopenias.

LSCs were found in 60% of patients from Cohort A and 91% of patients from Cohort B with the most common LSC markers being CD45RA and Combi markers. LSCs were detected at higher percentages in Cohort B. Various molecular aberrations which are commonly associated with MDS and AML were also detected in both Cohorts. There was high agreement between Immunophenotyping and Molecular results in Cohort A (56.6%) and Cohort B (91.3%). Cohort A was further sub-classified into low-risk MDS (50%), ICUS (33%), CCUS (7%) and 'Other' (10%) based on cytopenias, dysplasia and the presence of molecular aberrations. The presence of LSCs in 80% of LR-MDS patients, is an important adjunct finding that may prompt clinicians to monitor these patients more closely, with early therapeutic interventions in certain cases.

In conclusion, patients with persistent cytopenias together with the presence of LSCs and molecular aberrations might have an increased risk of leukaemic progression and should be monitored more closely. The LSC assay provides valuable information on the stem cell compartment, better guiding clinicians on the course of action for patients with persistent cytopenias. Detection of LSCs is also important in view of the development of therapeutic targets such as immunotherapy targeted towards aberrant markers including CD33, CD123, TIM-3 and CLL-1 leading to more specific and personalised treatments (Hansen *et al.,* 2022). Molecular analysis is also very important for patient stratification, prognosis and targeted therapy. The strong concordance between immunophenotyping and molecular results shows the importance of using a holistic approach when investigating patients with persistent cytopenia and suspected MDS.

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List of Abbreviations

ADCs	Antibody-Drug Conjugates
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
AML	Acute Myeloid Leukaemia
AML-MR	Acute Myeloid Leukaemia - Myelodysplasia Related
AML-NOS	AML not otherwise specified
ASXL1	Additional sex combs like 1
BM	Bone Marrow
CAR-T	Chimeric Antigen Receptor Therapy
CBC	Complete Blood Count
CBP	CREB-Binding Protein
CCUS	Clonal Cytopenias of Undetermined Significance
CD	Clusters of Differentiation
CHIP	Clonal Haematopoiesis of Indeterminate Potential
Clec12a	C-type Lectin Domain Family 12 Member a
CLL-1	C-type Lectin-Like Molecule-1
CoA	Co-Activator
CoR	Co-Repressor

CST	Cytometer Setup and Tracking
DNA	Deoxyribonucleic Acid
DNMT3A	DNA-methyltransferase 3 alpha
ELN	European LeukaemiaNet
ELN iMDS FLOW WG	European LeukaemiaNet international MDS Flow Working Group
EZH2	Enhancer of Zeste 2 Polycomb Repressive Complex 2
FISH	Fluorescence In Situ Hybridization
FLT3	Fms-like Tyrosine Kinase 3
FREC	Faculty Research Ethics Committee
FSC	Forward Scatter
GLI	Glioma-associated Oncogene homolog
GO	Gemtuzumab Ozogamicin
HA	Hyaluronic Acid
HAS	Human Serum Albumin
HMA	Hypomethylating Agents
HR-MDS	High-Risk Myelodysplastic Syndrome
HSC	Haematopoietic Stem Cell
H_0	Null Hypothesis
H_1	Alternate hypothesis

ICC	International Consensus Classification
ICUS	Idiopathic Cytopenias of Unknown Significance
IDUS	Idiopathic Dysplasia of Unknown Significance
IL-1	Interleukin-1
IL-3RA	Interleukin-3 Receptor
IPSS	International Prognostic Scoring System
IPSS-M	Molecular International Prognostic Scoring System
IPSS-R	Revised International Prognostic Scoring System
IKK	IκB Kinase
ITD	Internal Tandem Duplications
JAK/STAT	Janus Kinase-Signal Transducer and Activator of Transcription
JAK2	Janus Kinase 2
LCO	Leukaemia Cell of Origin
LIC	Leukaemia Initiating Cells
LR-MDS	Low-risk Myelodysplastic Syndrome
LSC	Leukaemic Stem Cell
MCL-1	Myeloid Cell Leukaemia 1
MDH	Mater Dei Hospital
MDS	Myelodysplastic Syndromes

MDS-bi *TP53*	MDS with biallelic *TP53* inactivation
MDS-EB	Myelodysplastic Syndrome with Excess Blasts
MDS-f	Myelodysplastic Syndrome with Fibrosis
MDS-h	Myelodysplastic Syndrome, hypoplastic
MDS-IB	Myelodysplastic Syndrome Increased Blasts
MDS-LB	Myelodysplastic Syndrome Low Blasts
MDS-MLD	Myelodysplastic Syndrome with Multilineage Dysplasia
MDS-RS	Myelodysplastic Syndrome with Ring Sideroblasts
MDS-SLD	Myelodysplastic Syndrome with Single Lineage Dysplasia
MFI	Mean Fluorescence Intensity
MGUS	Monoclonal Gammopathy of Undetermined Significance
MRD	Measurable Residual Disease
NAD	Nicotinamide Adenine Dinucleotide
NCAM1	Neural Cell Adhesion Molecule
NECD	NOTCH extracellular domain
NF-κB	Nuclear factor-kappa B
NGS	Next-Generation Sequencing
NHANES III	The Third National Health and Nutrition Examination Survey
NICD	NOTCH intracellular domain

NK	Natural Killer
NOS	Not Otherwise Specified
NPM1	Nucleophosmin 1
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMT	Photomultiplier Tube
PPV	Positive Predictive Value
Pre-LSC	Pre-Leukaemic Stem Cell
PTCH1	Patched-1
QC	Quality Control
RBC	Red Blood Cells
RCC	Refractory Cytopenia of Childhood
RNA	Ribonucleic Acid
RUNX1	Runt-related Transcription Factor 1
sAML	Secondary Acute Myeloid Leukaemia
SAMOC	Sir Anthony Mamo Oncology Centre
SBS	Sequencing by Synthesis
SF3B1	Splicing factor 3b, subunit 1

SMO	Smoothened
snRNP	Small Nuclear Ribonucleoproteins
SRSF2	Serine/arginine-rich splicing factor 2
SSC	Side Scatter
SUFU	Suppressor of Fused
TCF/LEF	T-cell Factor/Lymphoid Enhancer Factor
TET2	Tet Methylcytosine Dioxygenase 2 Human Gene
TIM-3	T cell Immunoglobulin and Mucin Protein 3
TKD	Tyrosine Kinase Domain
TNFα	Tumour Necrosis Factor α
TP53	Tumor protein p53
UMC	University Medical Center
VAF	Variant Allele Frequency
VUS	Variants of Unknown Significance
WBC	White Blood Cells
WHO	World Health Organization

Chapter 1

Introduction

1.1 Background and Statement of the Problem

Myelodysplastic Syndromes (MDS) are defined by the World Health Organization (WHO) as a group of clonal haematopoietic stem cell disorders characterized by cytopenias, ineffective haematopoiesis, dysplasia, the presence of blasts and molecular aberrations (WHO, 2018). Patients with MDS have an increased risk of developing leukaemia, specifically Acute Myeloid Leukaemia (AML). This is because leukemogenesis involves a multi-step process in which there is an evolution from low-risk MDS to high-risk MDS which can then finally transform into AML (Pandolfi, Barreyo and Steidl, 2013). Sometimes patients present with cytopenias or dysplasia but they do not fulfil all the necessary diagnostic criteria for MDS and this creates some challenges in their clinical management. These patients might be suffering from potential pre-MDS conditions. In July 2016, an international consensus group met in Vienna, Austria to address this challenge in MDS where patients present with cytopenias or dysplasia but they do not fulfil the diagnostic criteria for MDS. Several potential pre-MDS conditions have been described including idiopathic cytopenias of unknown significance (ICUS), clonal cytopenias of undetermined significance (CCUS), idiopathic dysplasia of unknown significance (IDUS) and clonal haematopoiesis of indeterminate potential (CHIP). These potential pre-MDS conditions may progress to MDS and AML over time (Valent, 2018).

Patients with persistent cytopenias and low-risk MDS are relatively frequent encounters in routine Haematology clinics and the clinical management of these can be challenging as it is very difficult to predict their course. Flow cytometry has been a useful tool in diagnosis of AML and to some extent high-risk MDS. However, the use of flow cytometry in CCUS/ICUS and low-risk MDS patients is not well defined. In this study,

flow cytometric techniques will be used for patients with CCUS/ICUS and low-risk MDS (Cohort A) by looking into the immunophenotypic properties of the stem cell compartment with the possibility of finding leukaemic stem cells which are the first steps in the process of leukemogenesis. A leukaemic stem cell tube which was designed in Amsterdam in 2015 and is specific for the identification and characterisation of the stem cell compartment will be used in this study. In this study, bone marrow samples of patients with persistent cytopenias will be collected (Cohort A – patient group). Another cohort group of patients will be recruited including patients with high-risk MDS and newly diagnosed AML patients and this will constitute the positive control group (Cohort B). Molecular studies by next-generation sequencing (NGS) will then be used to check for the presence of any mutations which are commonly mutated in MDS and AML. A targeted NGS panel including 40 genes will be used. Flow cytometry findings will then be correlated with molecular findings. Investigation of the stem cell compartment may shed light in the clinical behaviour of these conditions and thus aid in better clinical management.

1.2 Aims and Objectives

- To investigate the stem cell compartment of patients with persistent cytopenias (Cohort A) and newly diagnosed AML and high-risk MDS patients (Cohort B) by flow cytometry using the leukaemic stem cell tube on the BD FACS Canto II flow cytometer.
- To investigate the presence of any molecular abnormalities in the bone marrow samples of both cohort groups by NGS using Ion GeneStudio S5 NGS system by Thermo Fisher Scientific.

- To correlate flow cytometry findings with molecular findings.

Hypothesis

We postulate that patients with persistent cytopenias and potential pre-MDS conditions can have stem cells which have been transformed to leukaemic stem cells making them more prone to leukaemic transformation. Genetic mutations which are commonly found in MDS/AML patients might be present in patients with persistent cytopenias. Leukaemic stem cells and molecular aberrations should also be identified in Cohort B, the positive control group.

Chapter 2

Literature Review

2.1 Normal Haematopoiesis and the Haematopoietic Stem Cell

2.1.1 Haematopoiesis

Normal haematopoiesis involves the production of all cellular elements of the blood which serve oxygen delivery needs (erythrocytes), coagulation (platelets), and immune host defences (leucocytes) (Ghiaur and Jones, 2018). Haematopoiesis starts with the $CD34^{+}CD38^{-}$ haematopoietic stem cell (HSC) which differentiates into the $CD34^{+}CD38^{+}$ common myeloid progenitor or a $CD34^{+}CD38^{+}$ common lymphoid progenitor as can be seen in Figure 1 below. The progenitor cells continue to proliferate and differentiate into mature blood cells (Attar, 2014).

Adult haematopoiesis occurs in the bone marrow, spleen, thymus and lymph nodes. Each tissue is important for the growth and differentiation of haematopoietic cell lineages. Another important haematopoietic tissue is blood itself, where mature blood cells travel to all parts of the body to carry out their function (Hoffbrand, Mehta, Higgs and Keeling, 2016).

The haematopoietic activity is regulated by the bone marrow microenvironment to meet the physiological needs of individuals. The two major components of the microenvironment are the cellular elements (haematopoietic stem cells and progenitor cells) and non-cellular elements (growth factors, transcription factors and extracellular matrix). Growth factors promote survival, proliferation and differentiation of HSCs and progenitors. Transcription factors are also very important in the regulation of haematopoiesis as they determine differentiation of lineage-specific cells (Ghiaur and Jones, 2018).

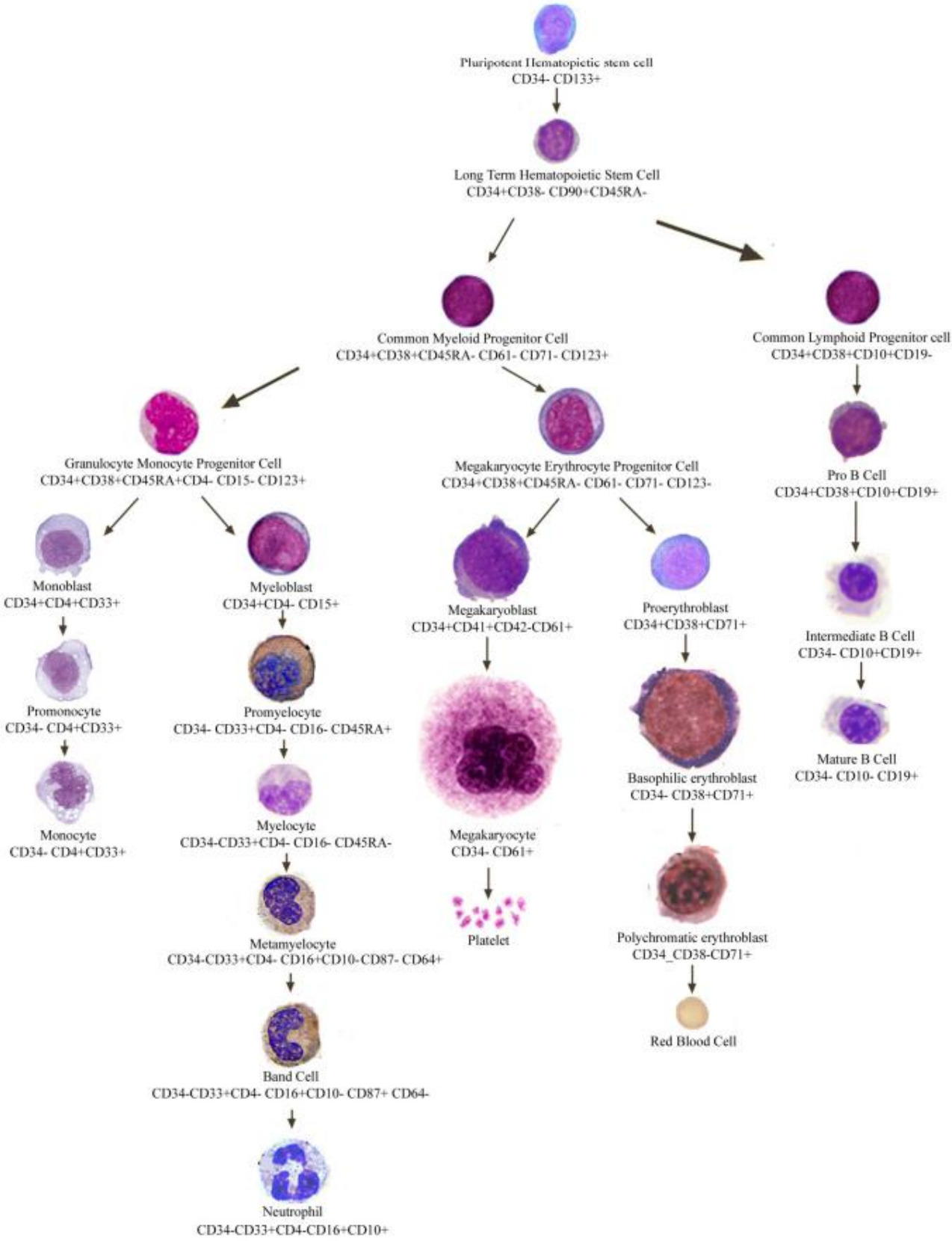


Figure 1: The Haematopoietic System and Cell Surface Markers. The Haematopoietic system is a hierarchical system which starts with the pluripotent stem cell which differentiates and proliferates to form the mature blood cells. Reproduced from Attar, A., 2014. Changes in the Cell Surface Markers During Normal Hematopoiesis: A Guide to Cell Isolation. Global Journal of Hematology and Blood Transfusion, 1(1), pp.20-28.

2.1.2 Haematopoietic Stem Cells

Haematopoietic stem cells (HSC) are defined by their high proliferative potential, their ability to self-renew and their capacity to give rise to all haematopoietic lineages. HSCs produce immature progenitor cells which progressively and gradually become restricted in lineage differentiation through a series of proliferation and differentiation events. The restricted progenitors then produce the differentiated functional blood cells. Unlike HSCs, the mature blood cells are restricted to one specific lineage and are not proliferative (Hoffbrand, Mehta, Higgs and Keeling, 2016).

When haematopoietic stem cells divide into two, only one of the resulting daughter cells starts to proliferate and differentiate into mature cells. The other daughter cell remains undifferentiated, and this phenomenon keeps the stem cell pool constant (Attar, 2014). Research on HSCs can further our understanding of their normal behaviour and their role in haematopoietic disease and leukaemia and thus helping us provide new, improved, patient-specific stem cell therapies.

2.2 Myelodysplastic Syndromes

2.2.1 Definition of Myelodysplastic Syndromes

The World Health Organization (WHO) defines Myelodysplastic Syndromes (MDS) as a group of clonal haematopoietic stem cell disorders which are characterised by cytopenia(s), ineffective haematopoiesis and dysplasia in one or more of the major myeloid lineages. Patients with MDS have an increased risk of developing Acute Myeloid Leukaemia (AML) (Swerdlow, 2008).

2.2.2 Aetiology and Epidemiology of MDS

MDS is a clonal myeloid stem cell disorder which may occur *de novo* or secondary to other factors such as exposure to chemotherapy (treatment-related MDS), radiation and environmental toxins such as benzene. The aetiology of *de novo* MDS has not been fully understood yet, but it is assumed to occur from an oncogenic process resulting in one or more somatic mutations. Advances in gene sequencing have identified several genes which are commonly altered in MDS (80-90%) and these include *SF3B1*, *DNMT3A*, *TET2*, *RUNX1*, *SRSF2*, *TP53, ASXL1*, *U2AF1*, and *EZH2*. These driver mutations correlate with different clinical features including the blast percentages, cytogenetics, the severity of cytopenias and prognosis of patients (Dotson JL and Lebowicz, 2021).

The incidence of MDS increases with age with the median age at presentation being 70 years. MDS is commoner in males and Caucasians except for MDS with isolated del(5q) which is found to be commoner in women. Rare cases have been reported in children (Aster and Stone, 2021).

2.2.3 Investigation of Patients with Suspected MDS

MDS patients may be clinically asymptomatic for many years. These patients may have incidental findings of cytopenias when doing a routine blood test. Other patients show signs and symptoms including fatigue, bleeding and frequent infections as a result of bone marrow failure which may be gradual and progresses with time.

2.2.3.1 Complete Blood Count (CBC) and Peripheral Blood Smear

The first step in the investigation of patients with suspected MDS includes a complete blood count (CBC) with a peripheral blood smear. When one or more cytopenias are

present, MDS may be suspected. The most common manifestation in MDS is anaemia which is typically normocytic or macrocytic. It is very important to rule out other causes of anaemia through additional laboratory testing including B12 and folate levels, ferritin levels, checking for haemolysis with lactate dehydrogenase, haptoglobins and Direct Antiglobulin test, immunofixation and serum protein electrophoresis to rule out multiple myeloma. Neutropenia and thrombocytopenia may also be present with anaemia, or else occur at a later stage in the disease. On examination of the peripheral blood smear, neutrophils may be hyposegmented (pseudo-Pelger-Huet anomaly) and hypogranular and platelets may be larger in size and lacking granules (figure 2). Myeloblasts are rarely seen in the peripheral blood smear of patients with MDS and if they are seen, they should raise suspicion for acute myeloid leukaemia (Dotson JL and Lebowicz, 2021).

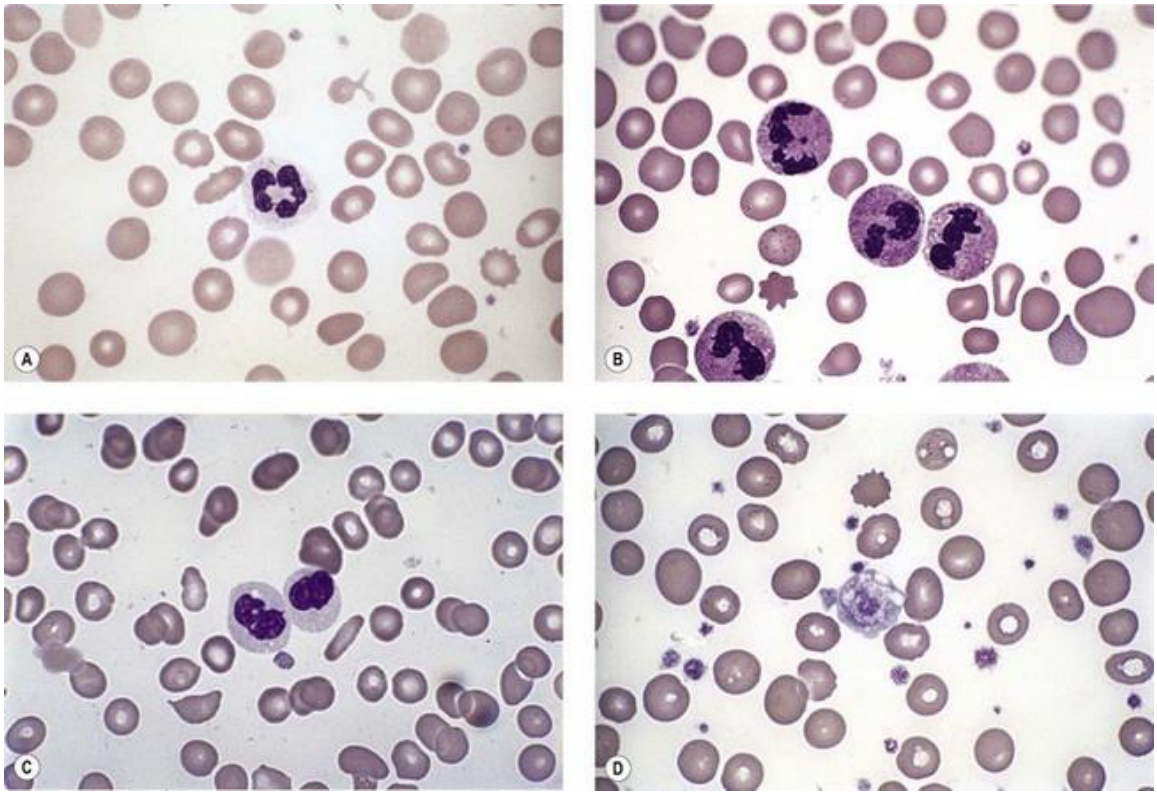
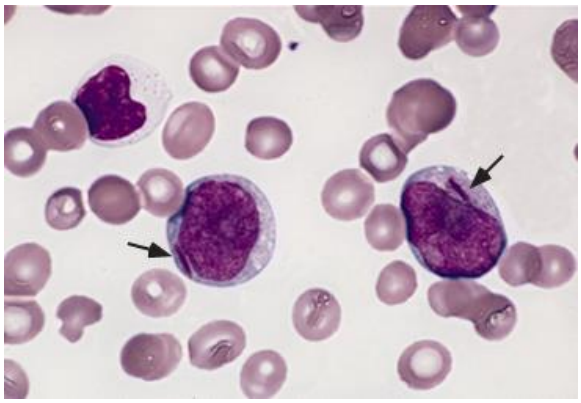


Figure 2: Blood film from MDS patient showing (A) hypogranular neutrophil, (B) dysplastic neutrophils, (C) pseudo-Pelger-Huet cells and (D) giant platelet. Reproduced from: Wilkins, B. and Porwit, A., 2022. *Myelodysplastic syndromes*. [online] Clinical Gate. Available at: <https://clinicalgate.com/myelodysplastic-syndromes-5/> [Accessed 29 April 2022].

Further investigations for diagnosis of MDS must include a bone marrow aspirate and trephine biopsy, flow cytometry for immunophenotyping, cytogenetic evaluation by karyotype and fluorescence in situ hybridization (FISH), together with genetic testing to look for somatic mutations including *TET2*, *DNMT3A*, *SF3B1*, *SRSF2*, *U2AF1*, *ASXL1*, *RUNX1*, *TP53* and *EZH2* (Dotson JL and Lebowicz, 2021).

2.2.3.2 Bone Marrow Smear and Immunophenotyping

Bone marrow smears are stained using Giemsa and iron stain. For MDS diagnosis, granulocytic cells must have more than 10% dysplasia. Moreover, quantification of the number of blasts present in the bone marrow is also required. Myeloblasts are cells with high nuclear to cytoplasm ratio (N:C ratio), have visible nucleoli, fine nuclear chromatin and might have granules and should account for less than 20% of nucleated cells in the bone marrow (figure 3a). A higher percentage of blasts would be considered as acute myeloid leukaemia. The iron stain is carried out to evaluate the presence of ring sideroblasts; a ring of iron containing mitochondria around the nucleus of erythroid progenitors (figure 3b).



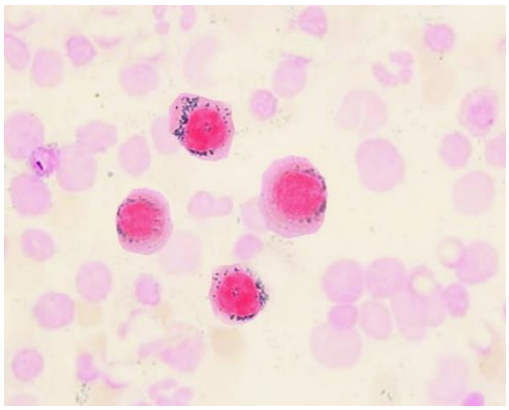


Figure 3a (Left) – Myeloblasts with auer rods and Figure 3b (Right) – ring sideroblasts. Figure 3 reproduced from: Aster, J. and Stone, R., 2021. *Clinical manifestations and diagnosis of myelodysplastic syndromes (MDS)*. [online] Uptodate.com. Available at: <https://www.uptodate.com/contents/clinical-manifestations-and-diagnosis-of-myelodysplastic-syndromes-mds/contributors> [Accessed 29 April 2022]. Figure 4 reproduced from: Sticco KL, Yarrarapu SNS, Al Obaidi NM. Refractory Anemia With Ring Sideroblasts. [Updated 2021 Aug 13]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. [Figure, Ringed sideroblasts. Image courtesy S Bhimji MD] Available from: https://www.ncbi.nlm.nih.gov/books/NBK537073/figure/article-17540.image.f2/

Immunophenotyping by flow cytometry is important to quantify the percentage of blasts present in the bone marrow. The Haematologist then correlates the bone marrow smear findings with immunophenotyping (Dotson JL and Lebowicz, 2021). Flow cytometry is used to identify any populations which are abnormally increased or decreased. Moreover, it is also used to detect any aberrant expressions of mature or immature lineage markers. The WHO recommends the presence of more than 3 immunophenotypic abnormalities for an MDS diagnosis to be made (Bento *et al.,* 2017).

In 2008, a European LeukaemiaNet (ELN) working group started in Amsterdam in order to standardise the use of flow cytometry in MDS. Representatives from 23 countries participated in the workshops to propose guidelines for the standardisation of Flow in MDS. Several MDS panels have been designed across the world. Therefore, guidelines are required for standardisation of MDS diagnosis. Table 1 below shows the recommended minimal requirements to assess dysplasia by flow cytometry. It includes the different bone marrow subsets (immature myeloid and monocytic progenitors, maturing neutrophils, monocytes, progenitor B cells and the erythroid compartment), the recommended analyses and aberrant patterns. It includes some of the most important cluster of differentiation (CD) markers which should be used in MDS panels to assess dysplasia and immunophenotypic patterns (Westers *et al*., 2012).

Bone Marrow Subset	Recommended analyses	Aberrancy
Immature myeloid and mononcytic progenitors	Percentage of cells in nucleated fraction Expression of CD45 Expression of CD34 Expression of CD117 Expression of HLA-DR Expression of CD13 and CD33 Asynchronous expression of CD11b, CD15 Expression of CD5, CD7, CD19 and CD56.	Increased percentage Lack of/decreased/increased Lack of/decreased/increased Homogeneous under/overexpression Lack of/increased expression Lack of/decreased/increased Presence of mature markers Presence of lineage infidelity markers
Maturing neutrophils	Percentage of cells as ratio to lymphocytes SSC as ratio vs SSC of lymphocytes Relationship of CD13 and CD11b Relationship of CD13 and CD16 Relationship of CD15 and CD10	Decreased Decreased Altered patterns Altered patterns Altered patterns; ex. Lack of CD10 on mature neutrophils
Monocytes	Percentage of cells Distribution of maturation stages Relationship of HLA-DR and CD11b Relationship of CD36 and CD14 Expression of CD13 and CD33 Expression of CD56	Decreased/increased Shift towards immature Altered patterns Altered patterns (Homogeneous) under/overexpression Presence of lineage infidelity marker
Progenitor B cells	Enumeration as fraction of total CD34+based on CD45/CD34/SSC in combination with CD10 and CD19	Decreased or absent
Erythroid compartment	Percentage of nucleated erythroid cells Relationship CD71 and CD235a Expression of CD71 Expression of CD36 Percentage of CD117-positive precursors	Increased Altered patterns Decreased Decreased Increased

Table 1: Recommended minimal requirements by ELN to assess dysplasia by flow cytometry. Adapted from: Westers, T.M. *et al.* (2012) 'Standardization of flow cytometry in myelodysplastic syndromes: A report from an international consortium and the European LeukemiaNet Working Group', *Leukemia*, 26(7), pp. 1730–1741. doi:10.1038/leu.2012.30.

2.2.3.3 Cytogenetics

Cytogenetic analysis by FISH is important when investigating patients with suspected MDS as identification of chromosomal abnormalities can influence both treatment and prognosis of patients. The commonest alterations include del(5q) which is listed as one of the WHO MDS classification list, monosomy 7 or del(7q), trisomy 8 and del(20q) (Dotson JL and Lebowicz, 2021).

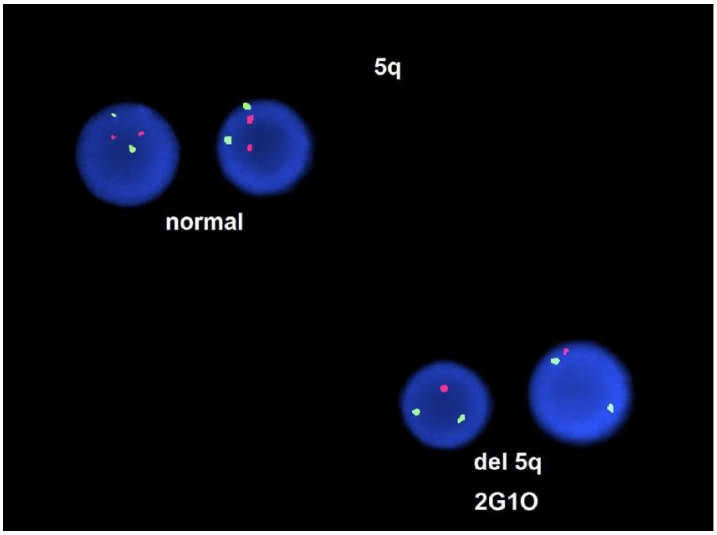


Figure 4: FISH showing chromosome 5q deletion. Reproduced from: Parylo, S., Vennepurredy, A. and Terjanian, T., 2017. Rapidly Progressing Myelodysplastic Syndrome Initially Presenting as Acute Leukemia. *Cureus*,.

2.2.3.4 Molecular Testing

Several studies have been published over the past years describing genetic mutations in myeloid neoplasms. Targeted sequencing by Next Generation Sequencing (NGS) could detect mutations which are present in 80-90% of MDS patients. The most commonly mutated genes include *SF3B1*, *SRSF2*, *ASXL1*, *TET2*, *DNMT3A*, *RUNX1*, *U2AF1*, *TP53* and *EZH2* (Hong and He, 2017).

2.2.4 Criteria for Diagnosis of MDS

For a patient to be diagnosed with MDS the following criteria are required:

- One or more peripheral blood cytopenias (anaemia- Hb <10g/dL, neutropenia- absolute neutrophil count $<1.8x10^9$/L and/or thrombocytopenia- platelets $<100x10^9$/L) which cannot be explained by other causes.
- Blasts which make up less than 20% of the nucleated cells in the peripheral blood and bone marrow.
- Evidence of dysplasia which is more than 10% in red cell precursors, granulocytes or megakaryocytes (Dotson JL and Lebowicz, 2021).

2.2.5 Classification of MDS (WHO,2016)

In 2016, the WHO revised the 2008 classification of MDS. The new classification introduced refinements in cytopenia and morphologic changes and the importance of genetic information for the diagnosis and classification of MDS. The WHO classification is again based on cytopenias, dysplasia and the presence of blasts. Cytopenias are still defined as haemoglobin <10g/dL, platelets $<100x10^9$/L and absolute neutrophil count $<1.8x10^9$/L. The new classification has removed the terms 'refractory anaemia' and 'refractory cytopenias' and replaced them with 'myelodysplastic syndrome' because MDS prognosis depends on the degree and not the lineage of cytopenia. This means that the diagnosis of MDS must be determined first and then classification is done. The new terms used for adult MDS are MDS followed by single lineage dysplasia (MDS-SLD) versus multilineage dysplasia (MDS-MLD), ring sideroblasts (MDS-RS), excess blasts

(MDS-EB, MDS-EB-1, MDS-EB-2) or the del(5q) cytogenetic abnormality. In childhood MDS, refractory cytopenia of childhood (RCC) remains (Hong and He, 2017).

Dysplasia is still defined as 10% dysplastic cells in myeloid lineages and common dysplastic features include: megaloblastoid erythroid maturation, erythroid precursor with nuclear abnormalities, or ring sideroblasts, neutrophil hypogranulation or hypolobulation and small megakaryocytes. Blast percentage is carried out by a marrow aspirate differential count and by flow cytometry and includes myeloblasts, monoblasts, promonocytes, erythroblasts and megakaryoblasts (Hong and He, 2017).

Type	**Dysplastic lineages**	**Cytopenias**	**Ring sideroblasts in erythroid elements of BM**	**Blasts**	**Cytogenetics**
MDS-SLD	1	1 or 2	RS <15% (or <5% with *SF3B1* mutation)	PB <1% BM <5% No Auer rods	Any, unless fulfils criteria for isolated del(5q)
MDS-MLD	2 or 3	1-3	RS <15% (or <5% with *SF3B1* mutation)	PB <1% BM <5% No Auer rods	Any, unless fulfils criteria for isolated del(5q)
MDS-RS MDS-RS-SLD	1	1 or 2	RS ≥15% (or ≥5% with *SF3B1* mutation)	PB <1% BM <5% No Auer rods	Any, unless fulfils criteria for isolated del(5q)
MDS-RS-MLD	2 or 3	1-3	RS ≥15% (or ≥5% with *SF3B1* mutation)	PB <1% BM <5% No Auer rods	Any, unless fulfils criteria for isolated del(5q)
MDS with isolated del(5q)	1-3	1-2	None or any	PB <1% BM <5% No Auer rods	del(5q) alone or with 1 additional abnormality except -7 or del(7q)
MDS-EB MDS-EB-1	0-3	1-3	None or any	PB 2-4% or BM 5-9%, No Auer rods	Any
MDS-EB-2	0-3	1-3	None or any	PB 5-19% or BM 10-19% or Auer rods	Any
MDS-U with 1% PB blast	1-3	1-3	None or any	PB = 1%, BM <5%, Auer rods	Any
With SLD and pancytopenia	1	3	None or any	PB <1% BM <5% No Auer rods	Any

Defining cytogenetic abnormality	0	1-3	<15%	PB <1% BM <5% No Auer rods	MDS defining abnormality
RCC	1-3	1-3	None	PB <2% BM <5% No Auer rods	Any

Table 2: Classification of Myelodysplastic Syndromes (WHO, 2016) (Hong and He, 2017).

2.2.6. Acute Myeloid Leukaemia

Acute Myeloid Leukaemia (AML) is characterised by the clonal expansion of immature blast cells in the bone marrow and peripheral blood resulting in further bone marrow failure and ineffective haematopoiesis. AML is the commonest leukaemia among the adult population (80% of cases) and is mainly secondary to Myelodysplastic Syndromes. Other risk factors include several congenital disorders like Down Syndrome; environmental exposures like tobacco smoke and radiation; and previous exposure to chemotherapy (Vakiti and Mewawalla, 2022).

AML is a heterogeneous disease caused by several mutations of genes involved in haematopoiesis. These genetic alterations in the haematopoietic stem cell cause ineffective haematopoiesis and bone marrow failure. Some mutations associated with AML include *NPM1*, *FLT3*, *RUNX1* and *TP53* and these are also associated with MDS (Vakiti and Mewawalla, 2022).

Patients with suspected AML experience several symptoms including recurrent infections, anaemia, excessive bleeding, bruising easily and bone pain. Like patients with suspected MDS, a CBC together with a blood film are required for diagnosis of AML. Further tests include bone marrow aspirate for morphology, flow cytometry, cytogenetics and molecular studies (Vakiti and Mewawalla, 2022).

In 2016, the WHO classified AML into AML with recurrent genetic abnormalities and AML not otherwise specified. AML with recurrent genetic abnormalities includes:

- AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
- AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
- APL with t(15;17)(q22;q12); *PML-RARA*
- AML with t(9;11)(p21.3;q23.3); *MLLT3-KMT2A*
- AML with t(6;9)(p23;q24); *DEK-NUP214*
- AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2*, *MECOM*
- AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1
- AML with mutated *NPM1*
- AML with biallelic mutations *CEBPA*

AML not otherwise specified (NOS) includes:

- AML with minimal differentiation
- AML without maturation
- AML with maturation
- Acute myelomonocytic leukemia
- Acute monoblastic/monocytic leukemia
- Pure erythroid leukemia
- Acute megakaryoblastic leukemia

- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis

2.3 Leukemogenesis

During healthy haematopoiesis (Figure 5), the haematopoietic stem cell (HSC) gives rise to committed progenitors which differentiate into the mature blood cells. Leukemogenesis involves a multistep process in which there is an evolution from low-risk to high-risk MDS which can finally transform to secondary acute myeloid leukaemia (sAML). Several studies have shown that MDS and sAML arise from transformed immature haematopoietic cells following multiple genetic and epigenetic changes in HSC and committed progenitors. The compromised HSC gives rise to a leukaemia cell of origin (LCO), which after a series of transforming events gives rise to a pre-leukaemic stem cell (pre-LSC) clone (Figure 5), a feature which is likely shared with MDS. The pre-LSC acquires further aberrations and then fully transforms into the leukaemic stem cells (LSC) (also known as leukaemia initiating cells – LIC) which are capable to give rise to leukaemia. The aberrations which are represented by lightning bolt symbols in the figure below, include mutations or deregulation of transcription factors, metabolic factors, epigenetic factors and signal transduction and cell cycle regulation proteins. The LSC/LICs are capable of self-renewal and contain genetic and epigenetic changes which lead to blocked haematopoietic differentiation and accumulation of leukaemic blasts (Pandolfi, Barreyro and Steidl, 2013).

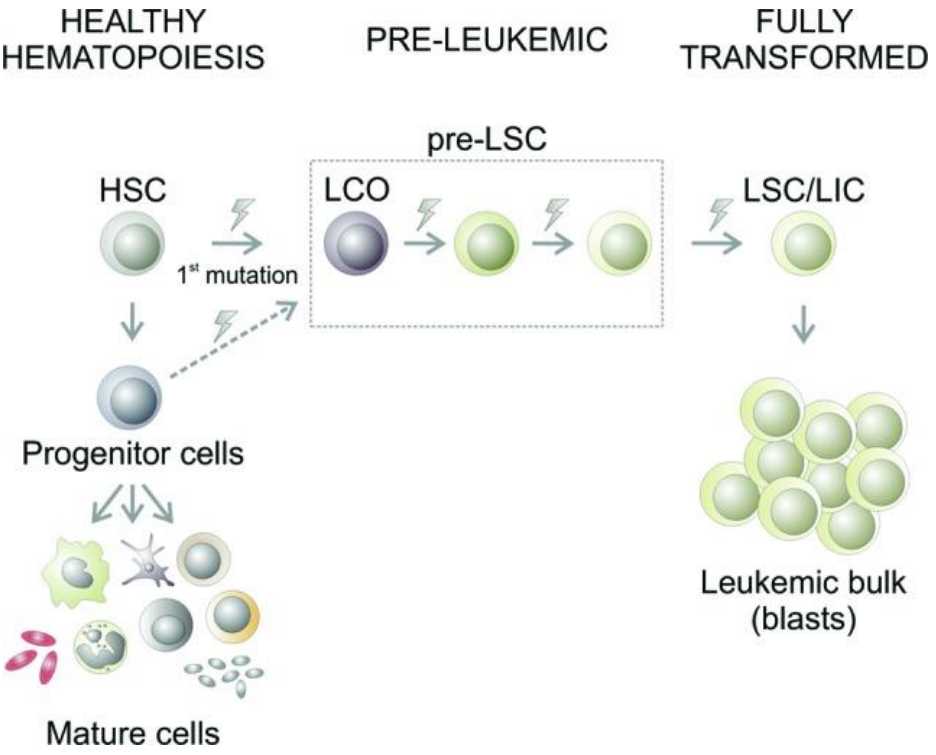


Figure 5: Multistep transformation of leukaemic stem cells. Reproduced from: Pandolfi, A., Barreyro, L. and Steidl, U., 2013. Concise Review: Preleukemic Stem Cells: Molecular Biology and Clinical Implications of the Precursors to Leukemia Stem Cells. *Stem Cells Translational Medicine*, 2(2), pp.143-150.

MDS and sAML share mutations in many of the same genes with functions including DNA methylation, RNA splicing and cell signalling, and this confirms that they are a disease continuum. Disease progression in MDS is associated with clonal evolution which is defined by the emergence of a subclone with a unique set of mutations (Menssen and Walter, 2020).

Early evidence of leukemogenesis and pre-leukaemia has been shown in murine models studies. These studies included the reduction in expression of transcription factors and overexpression of fusion oncogenes and resulted in distinct pre-leukaemic phases which are characterised by blocked differentiation and the accumulation of immature myeloid cells in the bone marrow and blood. AML development occurred in

mice after 2-6 months and this indicated the existence of a pre-leukaemic phase which includes stem cells and molecular alterations (Mitchell and Steidl, 2019).

Recent studies have focused on identifying the initial molecular changes contributing to leukemogenesis by conventional sequencing and NGS. The identification and investigation of specific LSC and pre-LSC populations is important for understanding the genesis of leukaemia and for developing strategies by which these cells can be eradicated. Pre-LSC provide a silent reservoir for the re-formation of LSC and hence relapse of leukaemia. Therefore, the therapeutic elimination of both LSC and pre-LSC is essential to achieve lasting cures (Pandolfi, Barreyro and Steidl, 2013), (Mitchell and Steidl, 2019).

2.4 Pre-MDS Conditions

Several studies during the past few years have shown that many mutations identified in patients with MDS were also detected in some elderly individuals who were apparently healthy, and this creates some challenges in this complex field. Some of these individuals may also have mild cytopenia(s) with no morphologic or cytogenetic abnormalities and thus do not fulfil the diagnostic criteria for MDS (Figure 6). However, numerous studies have described these conditions as pre-MDS states that can potentially transform to overt MDS and secondary AML (Valent, 2018).

In July 2016, an international consensus group met in Vienna to discuss and address this challenge in MDS. Several potential pre-MDS conditions have been described, including idiopathic cytopenias of unknown significance (ICUS), clonal cytopenias of undetermined significance (CCUS), idiopathic dysplasia of unknown significance (IDUS)

and clonal haematopoiesis of indeterminate potential (CHIP). These potential pre-MDS conditions may persist without clinical manifestations, may progress to MDS over time or could progress to other haematopoietic neoplasms (Valent, 2018).

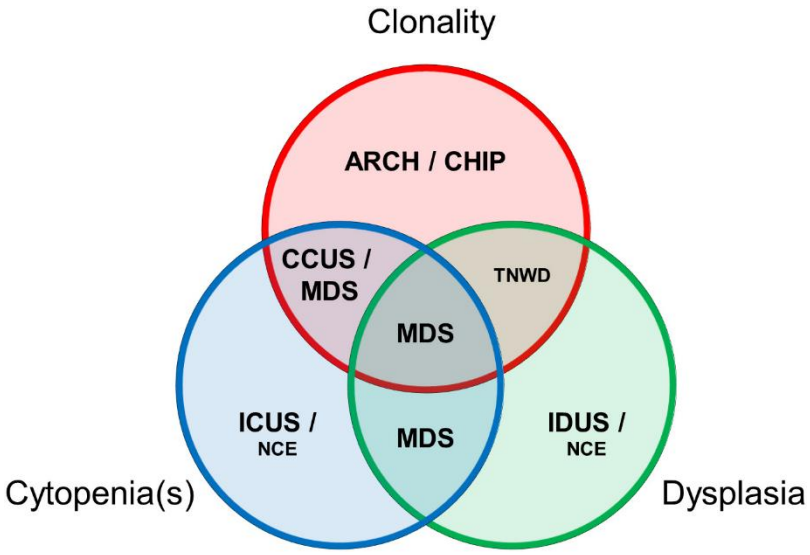


Figure 6: Relationship between clonality, dysplasia and cytopenia(s) in MDS and pre-MDS conditions. Reproduced from: Caponetti, G. and Bagg, A., 2020. Mutations in myelodysplastic syndromes: Core abnormalities and CHIPping away at the edges. *International Journal of Laboratory Hematology*, 42(6), pp.671-684.

2.4.1 Idiopathic Cytopenias of Unknown Significance (ICUS)

One of the challenges faced in Haematology when evaluating patients is unexplained persistent cytopenias. Sometimes, despite careful assessment to rule out other common causes for blood cytopenias such as nutritional deficiency, haematologic neoplasia, myelosuppressive medication or an auto-immune disorder, these cytopenias remain unexplained. Although many patients with unexplained persistent cytopenias have stable blood counts for years, some patients may suffer clinical consequences and may have progression to haematologic neoplasms such as MDS and sAML (Steensma, 2019).

The term ICUS can be used for patients with one or more unexplained persistent cytopenias (≥4 months) who do not meet the diagnostic criteria for MDS or another haematologic neoplasm. Patients with ICUS have no MDS-related mutations, no or only mild dysplasia (<10%) and blast cells (<5%). Patients with ICUS can be further subdivided as can be seen in table 3 below:

Variant of ICUS	Diagnostic feature
ICUS-A (anaemia)	ICUS criteria fulfilled (MDS excluded); persistent anaemia for at least 4 months, normal platelet and neutrophil counts.
ICUS-N (neutropenia)	ICUS criteria fulfilled (MDS excluded); persistent neutropenia for at least 4 months, normal haemoglobin and platelet counts.
ICUS-T (thrombocytopenia)	ICUS criteria fulfilled (MDS excluded); persistent thrombocytopenia for at least 4 months, normal haemoglobin and neutrophil counts.
ICUS-PAN (bi/pancytopenia)	ICUS criteria fulfilled (MDS excluded); persistent bi- or pan-cytopenia for at least 4 months.

Table 3: Variants of ICUS (Valent, 2018).

The prognosis of the different variants of ICUS remains uncertain. However, patients with ICUS-PAN may have a higher risk of transformation into a haematopoietic neoplasm than the other variants. Further testing including FISH, flow cytometric analysis and molecular analysis must be carried out on patients with persistent cytopenias with MDS-related features to confirm or exclude BM failure and the presence of a clonal population. When molecular aberrations are found in patients with ICUS, the diagnosis must be changed to CCUS or MDS if the diagnostic criteria for MDS are met (Valent, 2018).

A thorough diagnostic work-up is required to formulate a diagnosis of ICUS and repeated tests should be carried out to reach a conclusive diagnosis. The proposed

definition for ICUS has some limitations. One of the limitations is that an adequate period of observation is required and even persistent cytopenias of shorter duration must be considered carefully if a diagnosis has not been made yet. Another limitation is that unlike other haematologic conditions such as monoclonal gammopathy of undetermined significance (MGUS), a diagnosis of ICUS does not require the evidence of a clonal disorder. Limited studies on patients with ICUS were reported in literature. Larger prospective studies are required to investigate the prevalence of ICUS and the potential to transform into MDS or other haematologic neoplasm (Malcovati and Cazzola, 2015).

2.4.2 Clonal Cytopenias of Undetermined Significance (CCUS)

In patients with CCUS, cytopenia and clonal abnormalities can be detected, but no dysplasia is observed and MDS criteria are not fulfilled (Valent, 2018). The clonal abnormalities found in patients with CCUS are somatic mutations of myeloid malignancy-associated genes. Some genes involved include those of RNA splicing (*SF3B1, SRSF2, U2AF1, ZRSR2*), DNA methylation (*TET2, DNMT3A, IDH1/2*), transcription regulation (*RUNX1*), DNA repair (*TP53*), histone modification (*ASXL1, EZH2*), signal transduction (*CBL, NRAS, KRAS*) and cohesin complex (*STAG2*) (Malcovati and Cazzola, 2015).

The definition of CCUS implies that several investigations must be carried out to exclude MDS and to diagnose CCUS including a thorough BM investigation. In some patients it is difficult to distinguish between MDS and CCUS. Patients with CCUS have a high probability of progression to myeloid neoplasms upon follow-up.

2.4.3 Idiopathic Dysplasia of Unknown Significance (IDUS)

Patients with IDUS have no peripheral cytopenias and no MDS-related mutation is found. Dysplasia is found in ≥10% of neutrophilic, erythroid and/or megakaryocytes. However, MDS criteria are not fulfilled.

Several patients may be referred for investigation because of peripheral blood abnormalities including band neutrophils, hypogranulated neutrophils Pseudo-Pelger forms, and unexplained macrocytosis. Bone marrow examinations of these patients may show signs of dysplasia in one or more lineages. In the absence of any cytogenetic or molecular abnormalities and absence of any detectable cytopenia, the term IDUS can be used.

Several reactive conditions and other pathologies can also cause mild or marked dysplasia with or without cytopenias. In many of these conditions a non-haematopoietic disease or deficiency such as copper or vitamin B12 deficiency may be found during follow-up. Other conditions which may cause dysplasia include chronic conditions such as autoimmune processes and chronic treatments such as chemotherapy. It is very important to exclude clonal conditions (BM neoplasms and CHIP) and non-clonal conditions before a diagnosis of IDUS can be established. Sometimes, the aetiology of the dysplasia is only detected after follow-up and re-investigation of the patient. When persistent cytopenias and other criteria related to MDS are found, the diagnosis changes to MDS (Valent, 2018).

2.4.4 Clonal Haematopoiesis of Indeterminate Potential (CHIP)

The diagnosis of CHIP is based on the presence of at least one or more somatic mutations which are found in MDS; the absence of persistent cytopenia; and the exclusion of MDS or other haematopoietic neoplasms. Investigation of the bone marrow is always required to establish a diagnosis of CHIP. The variant allele frequency (VAF) of the aberrations detected must be at least 2% to count as CHIP-defining mutations. VAF is defined as 'the percentage of sequence reads observed matching a specific DNA variant divided by the overall coverage at that locus of a gene' (Valent,2018).

CHIP may be the earliest detectable phase of a haematopoietic myeloid neoplasm. Relevant somatic mutations may be acquired early by preleukaemic stem cells during leukemogenesis. CHIP is a common age-related condition characterised by the clonal expansion of HSC bearing mutations in certain genes, mainly *DNMT3A*, *TET2* and *ASXL1*, which are mutations commonly associated with MDS (Jaiswal and Libby, 2019) (Bejar, 2017). CHIP mutations increase with age (figure 7), and they may also be found in elderly individuals who are apparently healthy. Because of this, CHIP is sometimes also termed as age-related clonal haematopoiesis (Malcovati and Cazzola, 2015). Recent studies have shown that CHIP mutations may also predispose atherosclerosis and related cardiovascular events (Valent, 2018).

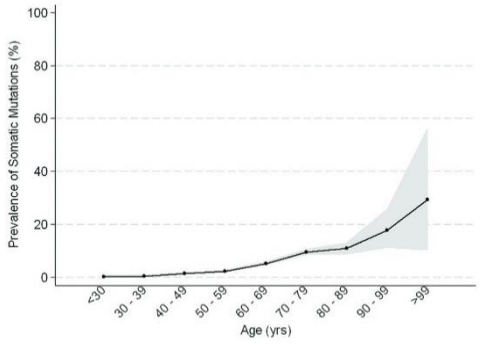


Figure 7: Prevalence of Somatic Mutations according to age (Malcovati and Cazzola, 2015)

A study by Malcovati and Cazzola in 2015, compared the frequency of the recurrent somatic mutations in MDS and CHIP in different studies. *TET2* mutations were more commonly found in MDS whilst *DNMT3A* mutations were more commonly found in CHIP. *ASXL1* mutations were found with the same frequency in both MDS and CHIP. Other common mutations in MDS and CHIP include *SF3B1*, *SRSF2*, *RUNX1* and *TP53* amongst other listed in Figure 8.

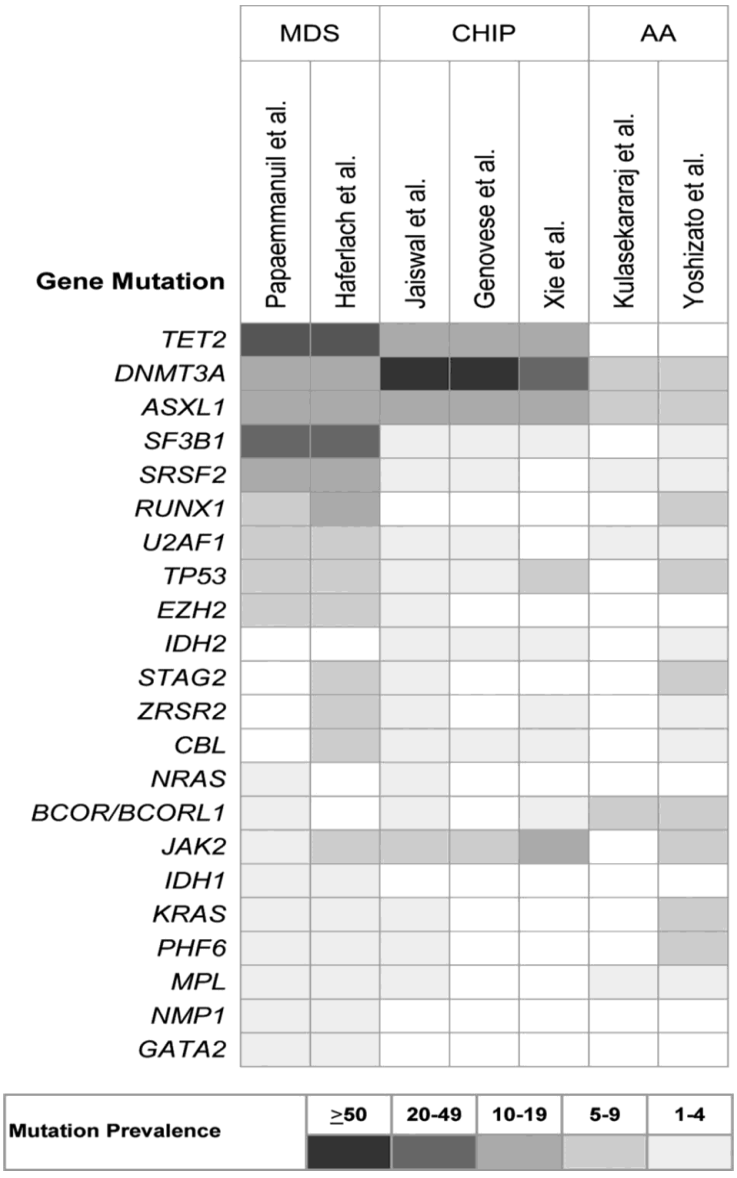


Figure 8: Frequency of recurrent somatic mutations in MDS, clonal haematopoiesis of indeterminate potential (CHIP) and aplastic anaemia (AA) (Malcovati and Cazzola, 2015).

Table 4 below summarizes the typical features and criteria of pre-MDS conditions, low-risk and high-risk MDS.

	Pre-MDS Conditions and MDS					
Feature	**ICUS**	**IDUS**	**CHIP**	**CCUS**	**LR-MDS**	**HR-MDS**
Dysplasia *	-	+	-	-	+	+
Cytopenia(s) **	+	-	-	+	+	+
BM Blasts	<5%	<5%	<5%	<5%	<5%	<20%
Flow Abnormalities	+/-	+/-	+/-	+/-	++	+++
Molecular aberration/s	-	-	+	+	++	+++

Table 4: Pre-MDS and MDS conditions: typical features and criteria as proposed by Valent et al. in 2018.

* at least 10% of all cells in a given lineage (erythroid, neutrophil, or megakaryocyte) are dysplastic.
**persistent cytopenias for at least 4 months.

2.4.5 Incidence and prevalence of unexplained persistent cytopenias

The global incidence and prevalence of unexplained cytopenias is not known with any confidence. The Third National Health and Nutrition Examination Survey (NHANES III) in North America indicated that anaemia as defined by the WHO is common among the elderly. Anaemia was found to be present in 48-63% of patients living in nursing homes, around 20% in males and females aged above 65 years who live independently and in around 20% of males and females aged above 85 years. According to this survey, one third of the cases of anaemia were caused by nutritional deficiencies. Another third of the cases were caused by renal insufficiency. The cause for the other one third of cases remains unexplained (Steensma, 2019).

The incidence of neutropenia is difficult to estimate as there is a difference in the reference ranges of the absolute neutrophil count according to different ethnicities.

Data from the NHANES III survey showed that a neutrophil count less than $1x10^9$/L was uncommon and found in less than 0.6% of adults.

Thrombocytopenia is very common in the general population. Most unexplained thrombocytopenia are most probably immune mediated. However, the lack of specific and sensitive tests to distinguish immune-mediated thrombocytopenia from other causes is a limiting factor in haematology practice (Steensma, 2019).

2.5 Immunophenotypic properties of normal haematopoietic stem cells and leukaemic stem cells

2.5.1 Principles of Flow Cytometry

Multiparameter flow cytometry enables the rapid measurement of chemical and physical characteristics of cells as they pass through beams of laser light in a focused fluid stream. Flow cytometry is most often used for immunophenotyping in which antibodies are used to identify cells based on the types of markers or antigens found on cell surfaces. These markers are usually functional membrane proteins involved in cell metabolism, adhesion and cell communication. Although cell markers are useful in the identification of a specific cell population, they are often expressed on one or more cell type. However, methods for immunophenotyping cells with two or more antibodies at the same time have been developed. Several antibodies can be used together but using different fluorochromes and a given cell population can be identified and quantified (O'Donnell, Ernst and Hingorani, 2013).

A flow cytometer is made up of five components which are the light source (laser), the flow chamber, the optical system, detectors and the computer (Figure 9).

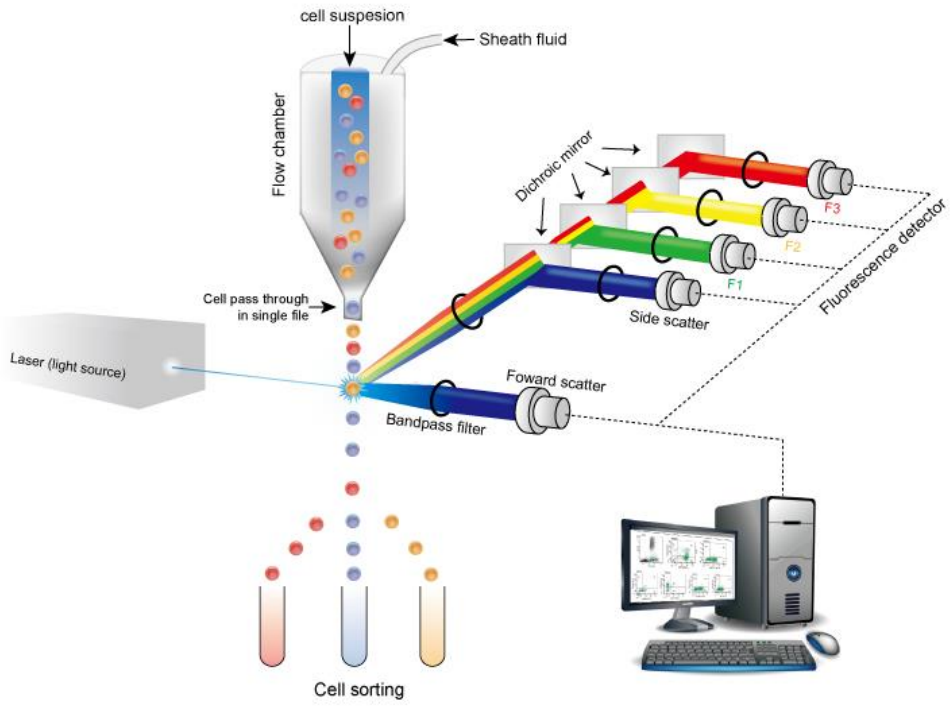


Figure 9: Diagram of a Flow Cytometer. Reproduced from: https://www.creative-diagnostics.com/flow-cytometry-guide.htm

The cell suspension is placed on the flow cytometer and the flow chamber moves the sample into a narrow channel. The cells pass through the laser beam in a single file and cells are scattered according to their size, shape and internal complexity. The flow cytometer detects forward scatter, which is proportional to the cell size, and side scatter, which is proportional to the granularity of the cell. Immunophenotyping is then carried out by using fluorescent-labelled antibodies directed against surface markers. Intracellular analysis is also carried out with antibodies detecting multiple intracellular targets including proteins and nucleic acids. The fluorochromes are excited by a laser with the corresponding excitation wavelength. Light is emitted from the fluorochrome and is splitted into defined wavelengths and directed along a path by a set of filters and mirrors within the flow cytometer. The fluorescent light is filtered and each sensor detects fluorescence only at a specific wavelength (Flow Cytometry Guide - Creative Diagnostics, 2022).

Immunophenotyping by flow cytometry is a very sensitive method to diagnose MDS. According to several studies, flow cytometry showed 60%-98% sensitivity in identifying MDS and a specificity of 93%-100%. Available evidence also shows that immunophenotyping by flow cytometry is also highly sensitive in cases with mild dysplasia by morphology (Malcovati and Cazzola, 2015).

2.5.2 Haematopoietic stem cells vs. Leukaemic stem cells

Hematopoietic stem cells and leukaemic stem cells are similar to each other because they are both capable of self-renewal. They are both capable of remaining undifferentiated and give rise to a copy of themselves and also to a progenitor cell which is more differentiated, and which is able to continue to differentiate. On the other hand, HSC and LSC differ in many characteristics including cell surface protein expression and activation of intracellular signalling pathways. In LSCs, dysregulation of some signalling pathways which are important for regulation of self-renewal, survival proliferation and differentiation occurs. Some signaling pathways which are dysregulated in LSCs include JAK/STAT, Nuclear factor-kappa B (NF-κB), Wnt/β-catenin, Hedgehog and Notch. All of these lead to the oncogenic potential of LSCs and leukemogenesis. Some of these dysregulated pathways may also lead to drug resistance and this results in increased relapse risk in some patients after treatment (Hansen *et al*., 2022).

2.5.2.1 JAK/STAT Pathway

The Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway is important for the regulation of proliferation, self-renewal and survival of haematopoietic stem cells. JAK 1 and JAK 2 are intracellular non-receptor tyrosine kinases and they become activated when extracellular cytokines bind to them. This

binding results in phosphorylation and activation of the STAT proteins. STAT proteins consist of transcription factors which regulate proliferation, differentiation and apoptosis of cells. In leukaemic stem cells, STAT3 and STAT5 are activated continuously, and this results in uncontrolled proliferation of blast cells and chemotherapy resistance. The JAK/STAT pathway is represented in figure 10 below (Hansen *et al*., 2022).

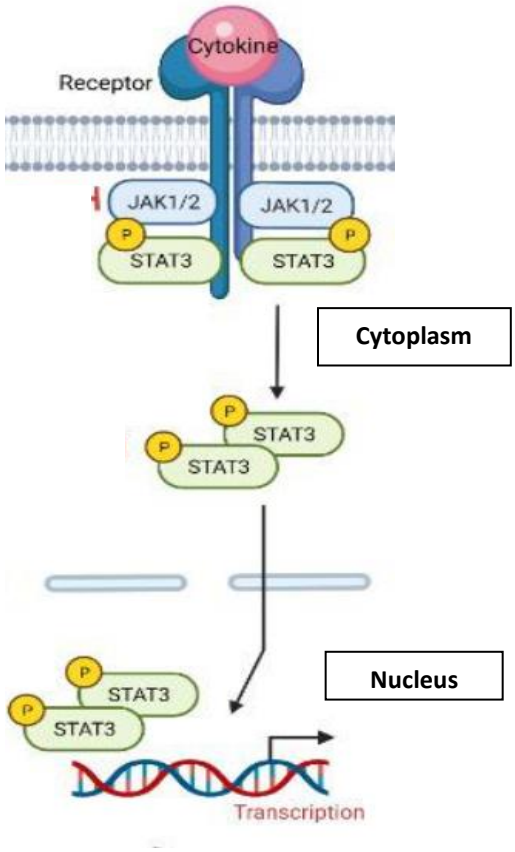


Figure 10: JAK/STAT Pathway. Adapted from: Hansen *et al*., 2022

2.5.2.2 Nuclear factor-kappa B (NF-κB) Pathway

The nuclear factor-kappa B (NF-κB) is a transcription factor which regulates cell proliferation, survival, inflammation and stress responses. Extracellular signals such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα) (proinflammatory cytokines) are

released when there is tissue injury or infection. These cytokines bind to cell receptors and activate IκB kinase (IKK), leading to phosphorylation of IκB. This phosphorylation releases NF-κB which is a pro-inflammatory transcription factor. NF-κB was found to be continuously activated in leukaemic stem cell and this leads to disease progression and drug resistance. The nuclear factor-kappa B (NF-κB) pathway is represented in figure 11 below (Hansen *et al*., 2022).

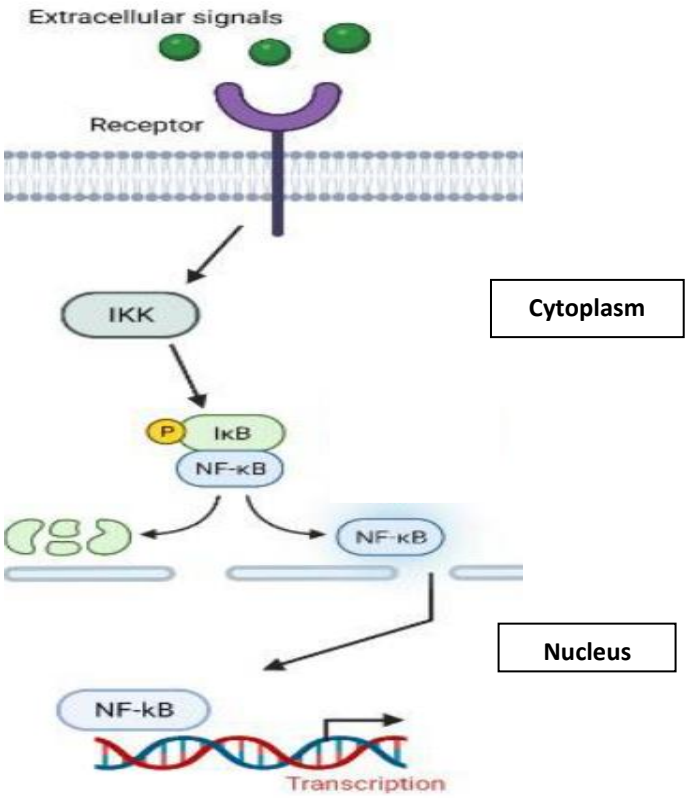


Figure 11: The NF-κB pathway. Adapted from: Hansen *et al*., 2022

2.5.2.3 Wnt/β-catenin signalling Pathway

The Wnt/β-catenin signalling pathway plays a key role in tissue homeostasis and regulates cell proliferation, differentiation, stem cell renewal and survival. B-catenin is a transcriptional coactivator and is very important in this pathway. Wnt ligand binds to its receptor Frizzled and the β-catenin degradation is blocked. This results in the

accumulation of β-catenin which enter the nucleus. In the nucleus, β-catenin binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and activates the coactivators such as the CREB-binding protein (CBP). This results in transcription of target genes responsible for self-renewal and proliferation. This is represented in figure 12 below. Several studies showed that leukaemic stem cells overexpress β-catenin (Hansen *et al*., 2022).

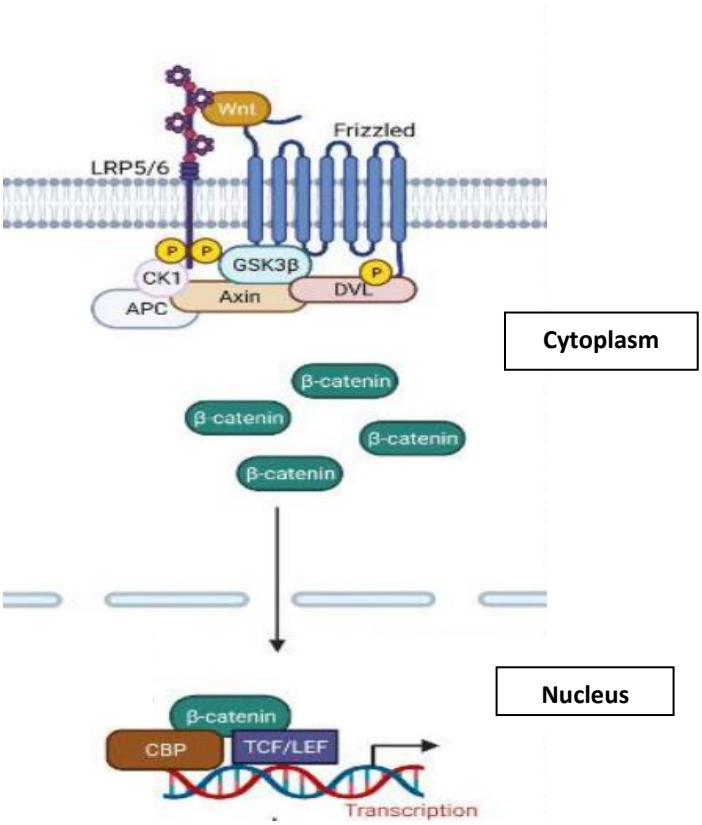


Figure 12: Wnt/β-catenin signalling Pathway. Adapted from: Hansen *et al*., 2022

2.5.2.4 The Hedgehog Pathway

The Hedgehog signalling pathway plays an important role in stem cell maintenance and dysregulation of this pathway leads to expansion of leukaemic stem cells. Hh ligands (Sonic Hh, Indian Hh and Desert Hh) bind to Patched-1 (PTCH1) 12-transmembrane receptor protein found on the cell surface. When inactive, PTCH1 inhibits Smoothened

(SMO) activity. When the Hh ligands bind to PTCH1 complex, the latter is degraded, SMO is activated and translocates to the primary cilium. The accumulation of SMO at the primary cilium results in activation of the glioma-associated oncogene homolog (GLI) family (transcription factors). These transcription factors then translocate to the nucleus and the expression of Hh target genes is restrained (figure 13B). When the Hh pathway is not activated, the GLI transcription factors are kept in an inactive state by a protein complex including suppressor of fused (SUFU) (figure 13A) (Jamieson *et al.,* 2020).

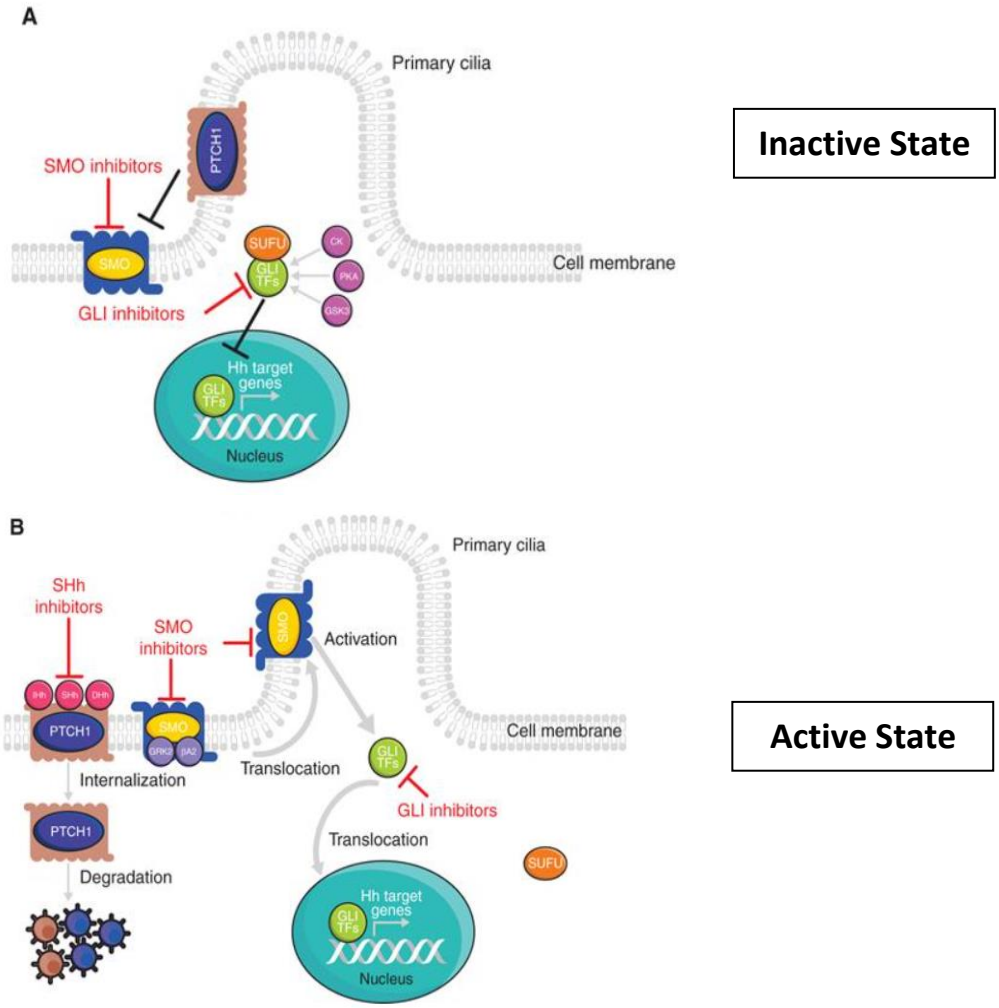


Figure 13: The Hedgehog Signalling Pathway. Adapted from: Jamieson *et al.,* 2020

2.5.2.5 The Notch Pathway

The Notch pathway is another signalling pathway which can be dysregulated in leukaemic stem cells leading to disease progression and leukemogenesis. Delta and Jagged ligands interact with NOTCH and this binding results in the activation of this signalling pathway. The metalloproteinase, ADAM, binds to the NOTCH extracellular domain (NECD), and NOTCH intracellular domain (NICD) is released by γ-secretase. NICD then translocates into the nucleus and replaces co-repressor proteins (CoR). The target genes are then promoted with the help of co-activator proteins (CoA) and CSL (BFI/RBPJ-κ) (figure 14) (Láinez-González *et al.*, 2022).

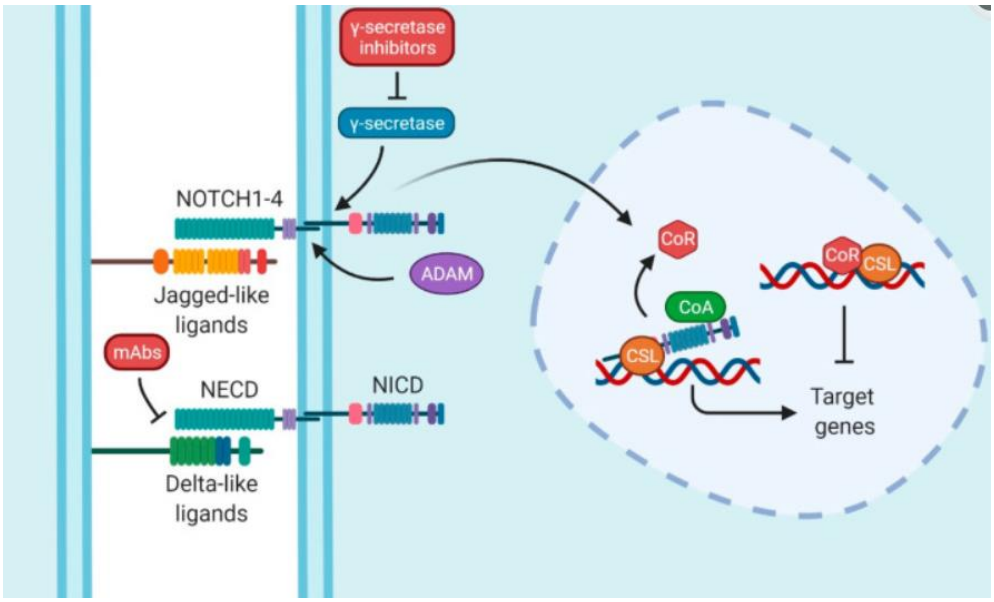


Figure 14: The Notch Signalling Pathway. Adapted from Láinez-González *et al.*, 2022.

2.5.2.6 Immunophenotypic differences of haematopoietic stem cells and leukaemic stem cells

Leukaemic stem cells (LSCs) are known to be the first step in leukemogenesis and lead to the development of MDS and sAML. LSCs are also responsible for relapse after successful treatment of patients with AML. Relapses occur in a considerable number

(20-70%) of patients. Detection of LSCs is therefore very important both at the time of diagnosis and follow-up to monitor relapse risk. Different cellular compartments may contain LSCs. These include: $CD34^{+}CD38^{+}$, $CD34^{+}CD38^{-}$ and $CD34^{-}$. However, $CD34^{+}CD38^{-}$ stem cells seem to be the most resistant to therapy and least immunogenic (Zeijlemaker *et al.,* 2015).

Current detection methods of leukaemic stem cells are complex, time-consuming and require a large volume of bone marrow sample since a multiple-tube approach is typically used. A group of scientists from Amsterdam University Medical Centre (UMC), designed a single 8-colour detection tube in 2015, including the common markers CD45, CD34 and CD38, and specific markers CD45RA, CD123, CD33, CD44 and a marker cocktail (CLL-1/ TIM-3/ CD7/ CD11b/ CD22/ CD56) in one fluorescence channel as shown in table 5 below.

FITC	PE	PerCP-cy5.5	PE-CY7	APC	APC-H7	BV421	HV500c
CD45RA	CLec12a/CLL-1	CD123	CD34	CD38	CD44	CD33	CD45
	TIM-3						
	CD7						
	CD11b						
	CD22						
	CD56						

Table 5: Leukaemic Stem Cell tube as proposed by Zeijlemaker *et al.* in 2015.

The immunophenotypic differences of HSC and LSC are illustrated in table 6 below. Both HSC and LSC are CD34+CD38-. Leukaemic stem cells have a higher FSC and SSC because they are larger in size than normal HSC. The highlighted markers are considered to be the most relevant leukaemic stem cell markers as these are usually absent on the stem cells of healthy individuals. However, the other three LSC markers (CD44, CD33

and CD123) are also considered relevant leukaemic stem cell markers as these are usually mildly expressed on normal HSC and brightly expressed on LSCs (Zeijlemaker *et al.*, 2015).

Normal Haematopoietic Stem Cell	Leukaemic Stem Cell
CD34+	CD34+
CD38-	CD38-
FSC^{low}	FSC^{high}
SSC^{low}	SSC^{high}
CD44+	CD44++
CD33+/-	CD33++
CD123+/-	CD123+++
Clec12a-	Clec12a+
TIM-3-	TIM-3+
CD7-	CD7+
CD11b-	CD11b+
CD22-	CD22+
CD56-	CD56+
CD45RA-	CD45RA+

Table 6: Normal Haematopoietic Stem Cells vs Leukaemic Stem Cells (Zeijlemaker *et al.,* 2015).

CD34 is a transmembrane phosphoglycoprotein and is regarded as a marker of haematopoietic stem cells and haematopoietic progenitor cells. Studies in haematopoietic cells suggest that the role of CD34 is in cytoadhesion and regulation of cell differentiation and proliferation (Sidney *et al.,* 2014).

CD38 is a multi-functional transmembrane glycoprotein. It is an ecto-enzyme that hydrolyses nicotinamide adenine dinucleotide (NAD), a co-enzyme used in metabolism (Hartman *et al.*, 2010). The expression of CD38 fluctuates markedly during

differentiation of haematopoiesis. CD38 is usually found to be present on bone marrow progenitor cells (common myeloid progenitor cells and common lymphoid progenitor cells). Most $CD34^{+}$ haematopoietic stem cells lack CD38 expression (Campana, Suzuki, Todisco and Kitanaka, 2000).

CD45 is a common leucocyte antigen. It is a transmembrane protein tyrosine phosphatase, and it is located on most haematopoietic cells (Ngo, Patel, Isaacson and Naresh, 2006). CD45RA is an isoform of CD45 and is a specific marker for leukaemic stem cells. It is absent on normal HSCs. In a study by Kersten et al., in 2016, it was found that CD45RA contributes to accurate detection of leukaemic stem cells, and it is recommended to include CD45RA in stem cell panels (Kersten *et al.,* 2016).

CD44 is a trans-membrane glycoprotein. It has many functions including cell division, adhesion, migration and signaling. CD44 is activated by binding to hyaluronic acid (HA). This binding regulates stem cell homing on the bone marrow. CD44 is usually weakly expressed on normal HSCs. CD44 can also be present on leukaemic stem cells. High expression of CD44 is an early marker of neoplastic stem cell proliferation (Basakran, 2015). CD44 variants are overexpressed in AML and that is associated with shorter survival rate and hence a poor prognosis (Thapa & Wilson, 2016).

CD33 is a marker associated with the myeloid lineage and belongs to the sialic acid-binding immunoglobulin (Ig)- like lectin (Siglec) family. Normal HSCs, show weak expression of CD33. LSC on the other hand, highly express CD33. CD33 is also highly expressed in acute myeloid leukaemia (Suwannasom *et al.,* 2019).

CD123 is also known as the alpha-chain of the interleukin-3 receptor (IL-3RA) and is a cytokine receptor. It is overexpressed in various haematological malignancies. CD123 expression on haematopoietic stem cell is usually absent or very weak, whilst on LSC it is highly expressed and this makes it a relevant leukaemic stem cell marker (Testa, Pelosi and Frankel, 2014). A study by Yue *et al.,* in 2010, investigated the expression of CD123 on the stem cells of patients having myelodysplastic syndrome. This study found that 48% of patients showed overexpression of CD123. This study also showed that CD123 expression in patients with high-risk MDS was similar to that of patients with AML (Yue *et al.,* 2010). Another study by Stevens *et al.,* in 2018, also showed that expression of CD123 is increased in patients with MDS. This study also showed that the stem cells of individuals having MDS exhibited higher levels of protein synthesis and significant changes in cellular energy metabolism (Stevens *et al.,* 2018).

TIM-3 (T cell immunoglobulin and mucin protein 3) is a membrane-bound glycoprotein and it has a function in the biological responses in the immune cells of humans. It also has an important role in immune responses in AML. TIM-3 is not expressed on normal haematopoietic stem cells. However, it can be expressed on leukaemic stem cells making it a leukaemic stem cell marker. It can be used in immunotherapy (such as anti-TIM3 monoclonal antibody Sym023) to treat AML/MDS, and this makes TIM-3 a potential therapeutic marker of LSC in AML (Wang *et al.,* 2021).

CD7 is a T-cell differentiation marker which identifies multiple CD8 T-cell effector subsets. CD7 is also found to be expressed by leukaemic cells whilst absent on normal HSCs (Tien and Wang, 1998).

CD11b is a leucocyte-specific receptor. It is a marker for monocyte/macrophages, granulocytes and natural killer cells. CD11b regulates the adhesion of leucocytes and migration to mediate the inflammatory response. CD11b is present on leukaemic stem cells and it is involved in the interaction of LSC with the microenvironment. CD11b is absent on normal HSCs (BAYSAL *et al.,* 2020).

CD22 is a B-lymphocyte specific cell surface glycoprotein. Its function is the downregulation of B-cell receptor mediated signalling (MoyrOn-QuirOz *et al.,* 2002). CD22 can also be expressed on $CD34^{+}CD38^{-}$ LSC. However, it is always negative on $CD34^{+}CD38^{-}$ HSC (Terwijn *et al.,* 2014).

CD56 is a neural cell adhesion molecule (also known as NCAM1) and is a marker of natural killer cells. CD56 can be highly expressed on LSC and is a marker which can be used to monitor minimal residual disease in AML. CD56 is never expressed on normal HSCs (Sasca *et al.,* 2019).

Clec12a (C-type lectin domain family 12 member a) has several functions including cell adhesion, cell signaling, roles in immune responses and inflammation. Clec12a is a leukaemic stem cell marker which is absent on normal HSCs. This makes it a potential treatment target (Bill *et al.,* 2018).

Validation analysis of the study by Zeijlemaker *et al*. in 2015, showed that the single tube approach was as sensitive as the multiple-tube approach with the additional benefit of requiring the least possible amount of bone marrow sample. The frequency of LSC population is low (0.2-625 cells per 10^6 mononuclear cells) and this makes the identification of LSC challenging. However, the single tube approach was found to

enable the identification of LSC both at time of diagnosis and follow-up. This method is also less expensive and more efficient than the current detection strategies of leukaemic stem cells (Zeijlemaker *et al.,* 2015).

A study by Li *et al.,* in 2022, compared the traditional multiparametric flow cytometry method and the LSC MRD assay proposed by Zeijlemaker *et al.,* in 2015. This study confirmed the high sensitivity of LSC based MRD method and it concluded that the LSC tube should be used routinely for AML MRD in order to predict relapse risk and prognosis. A limitation of the study was that the LSC tube could only be used on AML patients with a CD34 +ve leukaemia immunophenotype. Therefore, they suggested that another panel including CD117 should be designed for CD34 negative leukaemias (Li *et al.,* 2022).

2.6 Molecular aberrations in MDS and pre-MDS conditions

The first steps of leukemogenesis in MDS are expected to show one or few somatic mutations in driver genes that are frequently mutated in myeloid malignancies. Several studies have found common mutations which might be found in patients with MDS and potentially in pre-MDS conditions. As already mentioned, some common mutations include *DNMT3A*, *SF3B1*, *ASXL1*, *TET2*, *SRSF2* and *TP53* (Valent et al., 2017).

In the past years, numerous sequencing studies have been carried out and it was found that recurrent mutations are detectable in a majority of patients with MDS and that mutation profiling data can confirm diagnosis of MDS and also prognosis. Recent studies have focused on identifying the initial molecular changes contributing to leukemogenesis (Pandolfi, Barreyro and Steidl, 2013). This can be done by NGS.

2.6.1 Next generation sequencing (NGS)

NGS is a new DNA sequencing technology which has revolutionised research genomics. During NGS, millions of small fragments of DNA are sequenced in parallel. Bioinformatic analysis is then used to interpret the data and put these fragments together (Behjati and Tarpey, 2013). Patients with MDS may have multiple somatic mutations which can be identified by NGS, and the variance allele frequency (VAF) of the different mutations can change during leukemogenesis. The introduction of NGS has helped in the early diagnosis of MDS and pre-MDS states as it increased our understanding of the initial genetic changes associated with the development and progression of MDS. Detection of the molecular changes in MDS is also important for prognosis and targeted therapy. The identification of certain mutations such as *IDH1* and *IDH2* may become more important in the future for personalised treatments with *IDH1* and *IDH2* inhibitors for these 2 mutations. Moreover, molecular mutations have also become integrated in risk scores such as the IPSS-R which is a detailed prognostic scoring system (Bonadies and Bacher, 2019).

The initial step in next-generation sequencing is to extract DNA in order to isolate and purify the nucleic acid. After DNA extraction, library preparation, sequencing and data analysis must be carried out (NGS Workflow Steps | Illumina sequencing workflow, 2022).

Step 1: Library Preparation

Library preparation is an important step in NGS which prepares the DNA or RNA samples to be compatible with a sequencer. Libraries are prepared by fragmenting DNA

and adding specialised adapters to both ends as can be seen in Figure 15. These adapters contain complementary sequences which allow the DNA fragments to bind to the flow cell. The fragments can then be amplified and purified.

Step 2: Sequencing

After library preparation, libraries are loaded onto a flow cell and placed on the sequencer. During a process called cluster generation, the DNA fragments clusters are amplified resulting in millions of copies of single-stranded DNA. Then, chemically modified nucleotides bind to the DNA template strand by natural complementarity during a process called Sequencing by Synthesis (SBS). Each nucleotide contains a fluorescent tag and a reversible terminator which blocks incorporation of the next base. The fluorescent signal indicates which nucleotide has been added. The terminator is then cleaved so that the next base can bind. Following reading of the forward DNA strand, the reads are washed away and the process repeats for the reverse strand.

Step 3: Data Analysis

Data analysis is then carried out after sequencing. During a process called base calling, the instrument software identifies nucleotides and the predicted accuracy of those base calls (NGS Workflow Steps | Illumina sequencing workflow, 2022).

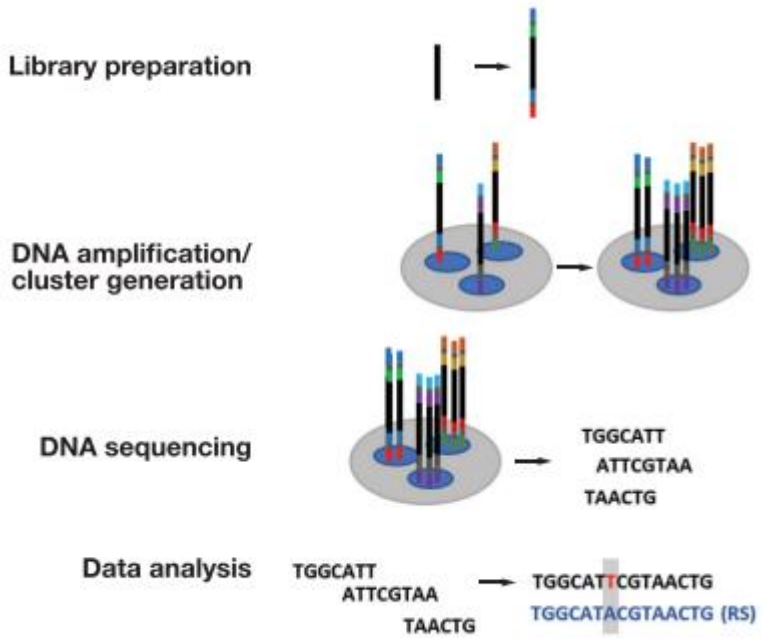


Figure 15: Main steps in Next-generation sequencing work-flow. Reproduced from Hanbazazh, M., Harada, S., Reddy, V., Mackinnon, A., Harbi, D. and Morlote, D., 2021. The Interpretation of Sequence Variants in Myeloid Neoplasms. *American Journal of Clinical Pathology*, 156(5), pp.728-748.

2.6.2 Common Mutations in MDS and AML

Several genes are commonly mutated in MDS. However, only 5 genes are known to be mutated in more than 10% of cases; *SF3B1*, *TET2*, *SRSF2*, *ASXL1* and *DNMT3A*. Other relatively common mutations include *RUNX1* and *TP53* (Caponetti and Bagg, 2020). These mutations are also commonly found in AML. Table 7 shows an overview of the type of mutations which are commonly mutated in MDS, AML and potentially also in pre-MDS conditions.

Gene Name	Gene Abb.	Chromosome Location	Type of Mutation
DNA-methyltransferase 3 alpha	*DNMT3A*	2p23	Most *DNMT3A* mutations occur at the methyltransferase domain and the most common is a **missense mutation** G>A at nucleotide position 2645 which leads to the substitution of an arginine with histidine residue at amino acid position 882 (*DNMT3A* R882H) (Walter *et al.,* 2011). Other mutations are nonsense and frameshift which are found scattered throughout the gene.
Tet methylcytosine dioxygenase 2 human gene	*TET2*	4q24	*TET2* mutations include **frameshift mutations** (43%), **nonsense** (47%) and **missense** (47%) (Patrick *et al.,* 2016).
Additional sex combs like 1	*ASXL1*	20q11	*ASXL1* mutations include **frameshift** and **nonsense mutations** (Gelsi-Boyer *et al.,* 2012).
Splicing factor 3b, subunit 1	*SF3B1*	2q33.1	*SF3B1* mutations are typically heterozygous, **point mutations** which are suspected to be deleterious with R625 and K700E described as major mutation hotspots (Mortera-Blanco *et al.,* 2017).
Serine/arginine-rich splicing factor 2	*SRSF2*	17q25.1	Most common mutations are the **missense mutations** including P95H, P95L, P95R and P95A. There is also the P95_R102del(c.284_307del), a 24-base pair **deletion** resulting in an 8-amino acid deletion starting from proline 95 (Wu *et al.,* 2012).
Tumor protein p53	*TP53*	17p13.1	The common **missense mutations** in the *TP53* gene disrupt the ability of p53 to bind to DNA and consequently to transactivate downstream genes. There is also the del(5q) syndrome **deletion** (Kato *et al.,* 2003).

Table 7: Type of mutations in genes detectable in patients with MDS and potentially in pre-MDS conditions.

A study by Liu e*t al.,* in 2021, investigated mutations in patients with MDS and also in patients who progressed to AML from MDS. A total of 214 mutations were found across 99 patients. The most common mutations were *U2AF1* (13.55%), *ASXL1* (10.28%), *TP53* (7.09%) and *RUNX1* (7.09%). Mutations were detected in 85% of patients of the MDS

group and in 100% of the patients from the MDS/AML group. The mutated genes were classified into six classes depending on the function of the gene. The six classes included epigenetic regulatory genes (*ASXL1, ASXL2, EZH2, DNMT3A, IDH1, IDH2* and *TET2*), transcription factor genes (*CEBPA, GATA2, ETV6, RUNX1* and *NPM1*), splicing factor genes (*SF3B1, SRSF2* and *U2AF1*), signal transduction genes (*CBL, FLT3, CSF3R, JAK2, JAK3, NRAS, KRAS* and *PTPN11*), tumour suppressor genes (*TP53, PHF6* and *WT1*) and other genes (*CALR* and *SETBP1*). These mutations are represented in figure 16 below. This study concluded that as MDS disease progresses, mutations increase especially in transcription factor and epigenetic regulatory genes (Liu *et al.,* 2021).

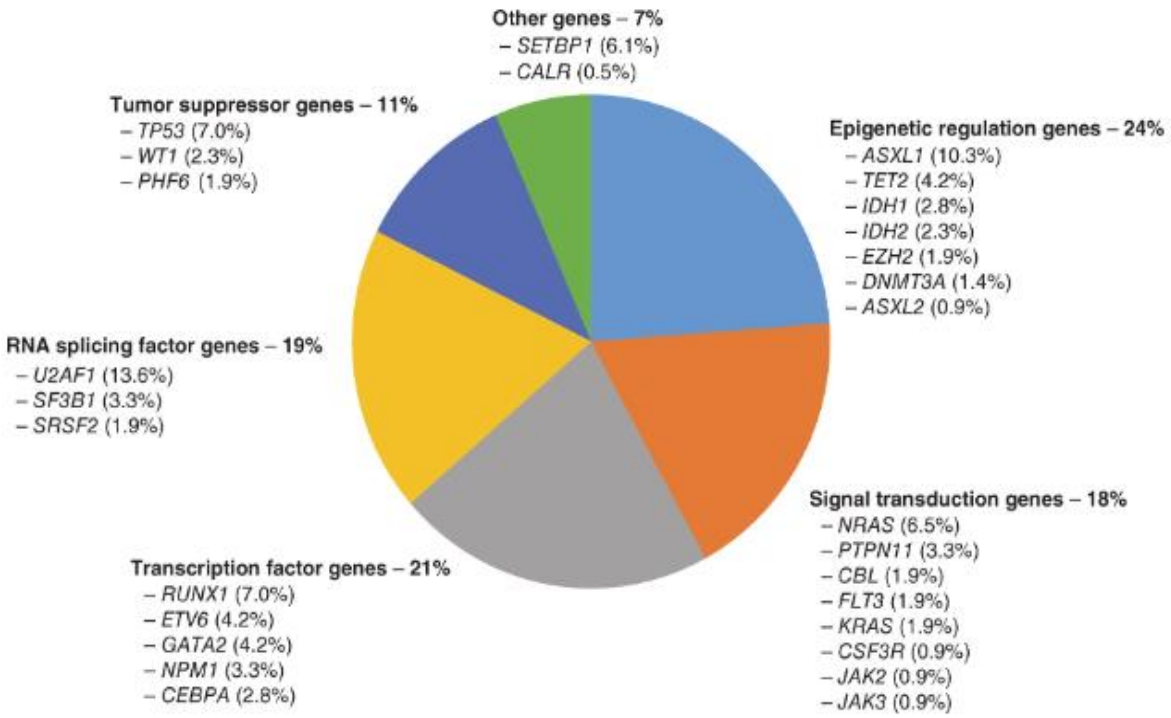


Figure 16: Common mutations in MDS and AML. Reproduced from: Liu *et al.,* 2021.

2.6.2.1 *SF3B1* gene mutations

The splicing factor 3b subunit 1 (*SF3B1*) gene forms part of the largest subunit (155kDa) of the splicing factor 3b protein complex and is located on the long arm of chromosome 2 (2q33.1). When the *SF3B1* complex interacts with the 12s unit and the splicing factor 3a, the interaction gives rise to U2 small nuclear ribonucleoproteins (snRNP) which are important for spliceosome assembly and mRNA splicing. Mutations in the *SF3B1* gene result in translation of new proteins with abnormal function. This is represented in figure 17 below (Cilloni *et al.,* 2022).

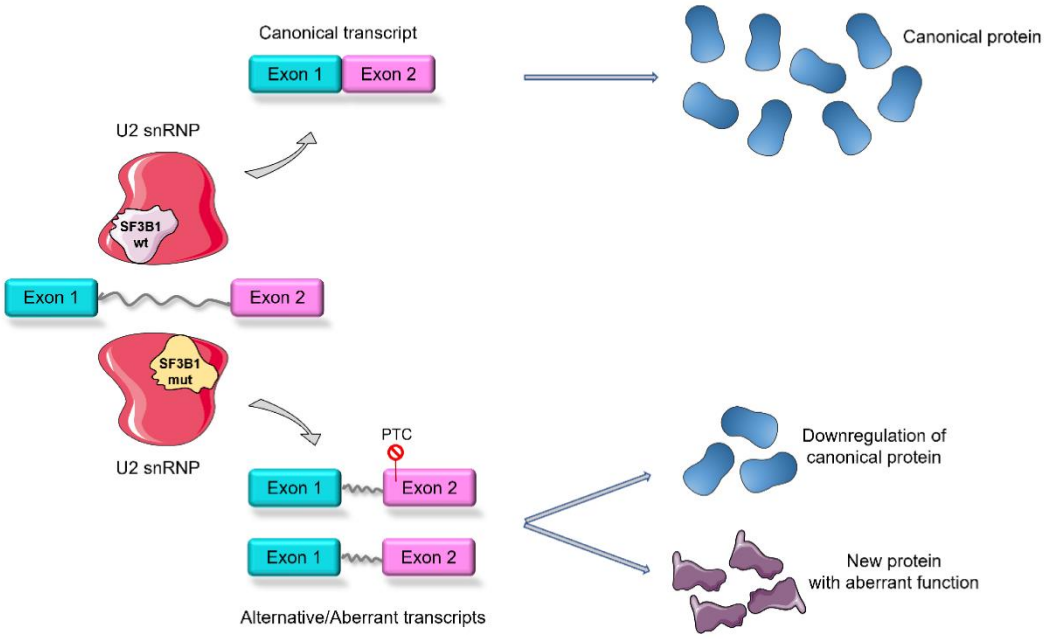


Figure 17: *SF3B1* gene in normal function and mutated. Reproduced from: Cilloni *et al.,* 2022.

SF3B1 mutations in MDS, result in aberrant splicing of some mitochondrial genes involved in iron metabolism including *PPOX, ABCB7* and *TMEM14C*. About 80% of MDS patients who have ring sideroblasts, were also found to have the *SF3B1* gene mutation (Malcovati *et al.,* 2020). Several studies have confirmed this in recent years, and the 5th WHO classification of MDS included this mutation as one of the disease entities (MDS-*SF3B1*). It is the only mutation that has been included in the WHO classification, 2016.

SF3B1 mutation is associated with low-risk of transformation to AML and also with a good prognosis (Cilloni *et al.,* 2022).

2.6.2.2 *SRSF2* gene mutations

Serine and arginine rich splicing factor 2 (*SRSF2*) is another spliceosomal gene which is commonly mutated in MDS. *SRSF2* mutations affect the RNA binding activity of *SRSF2* leading to *EZH2* (enhancer of zeste 2 polycomb repressive complex 2) mRNA degradation and also abnormal haematopoietic differentiation (figure 18) (Kim *et al.,* 2015).

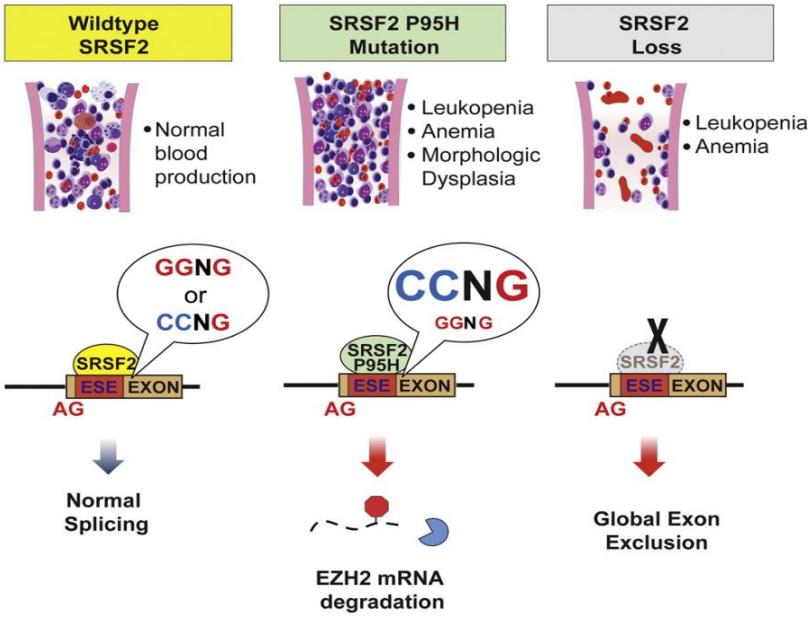


Figure 18: The *SRSF2* gene in normal function and mutated. Reproduced from: Kim *et al.,* 2015.

In a study by Wu *et al.,* in 2012, *SRSF2* gene mutations were found to be associated with older age and commoner in males. The prognostic role of *SRSF2* mutations is still controversial in MDS patients. *SRSF2* mutations were found to be commonly mutated with other genes including *RUNX1, ASXL1* and *IDH2. SRSF2* mutations were found to remain stable during disease progression (Wu *et al.,* 2012).

2.6.2.3 *ASXL1* gene mutations

Additional sex combs-like (*AXL1*) is an epigenetic regulator gene which is frequently mutated in myeloid malignancies and is associated with poor prognosis. *ASXL1* mutations occur in exons 11 and 12 and are mostly nonsense or frameshift mutations. Loss of function of *ASXL1* results in dysregulation of gene expression and impairment of histone H3 methylation. *ASXL1* mutations have been documented frequently also in CHIP and CCUS (Hanbazazh et al., 2021). *ASXL* recruits polycomb repressive complex 2 (PRC2) and causes repression of transcription by trimethylation of histone H3 on lysine 27 (H3K27me3). Genetic alterations and mutations in *ASXL1* result in decreased H3K27me3 levels and development of myeloid malignancies (Medina, Delma and Yang, 2022).

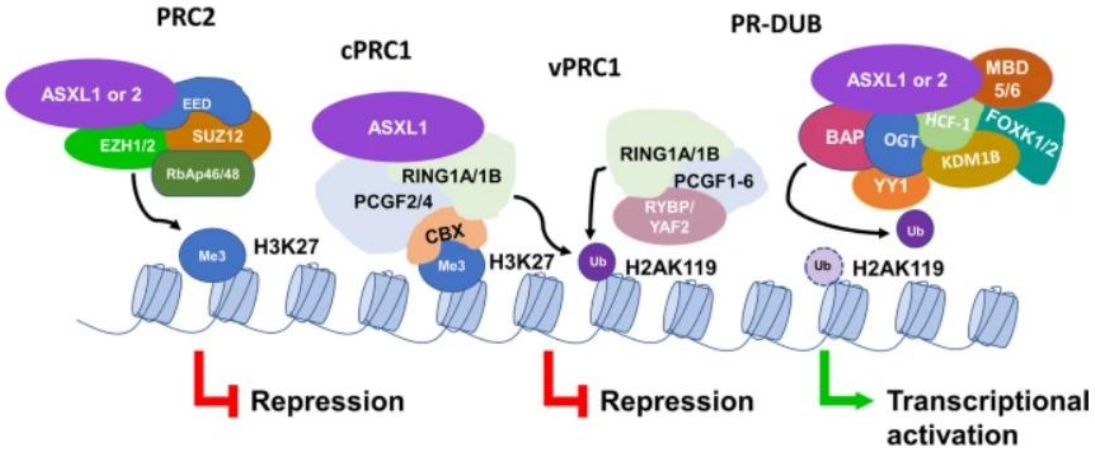


Figure 19: *ASXL1* gene function. Reproduced from: Medina, Delma and Yang, 2022.

2.6.2.4 *DNMT3A* gene mutations

DNA methyltransferase 3A (*DNMT3A*) gene has an important role in DNA methylation and mutations in this gene result in abnormal stem cell differentiation, self-renewal and also haematological malignancies (Yang, Rau and Goodell, 2015). *DNMT3A* mutations include nonsense, frameshift, missense and splice-site mutations and can be seen in

almost all myeloid neoplasms. *DNMT3A* mutations are also commonly mutated in CHIP and CCUS (Hanbazazh et al., 2021). Regulatory domains of *DNMT3A* allow interactions with histones and histone methyltransferase and this regulates gene expression (figure 20). The role of *DNMT3A* mutations as a prognostic marker is still not very clear. Several studies have found that *DNMT3A* mutations result in poor prognosis, whilst others found that *DNMT3A* status does not affect prognosis (Yang, Rau and Goodell, 2015).

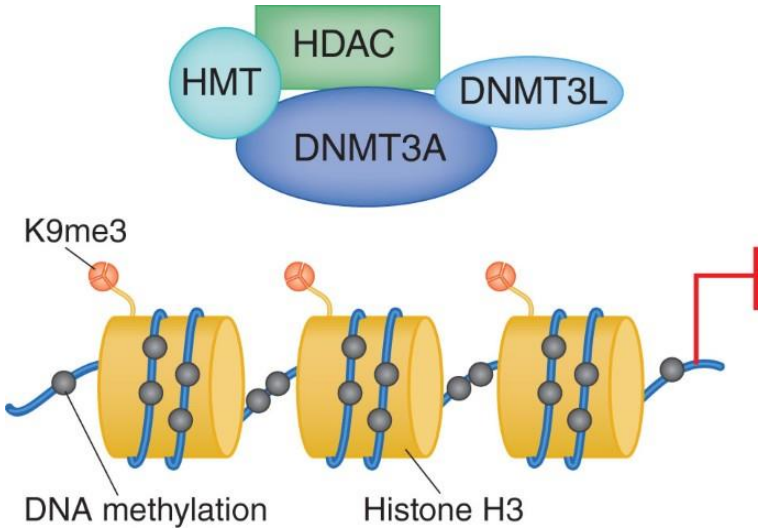


Figure 20: *DNMT3A* gene function. Reproduced from: Yang, Rau and Goodell, 2015.

2.6.2.5 *TET2 gene* mutations

Ten-eleven translocation 2 (*TET2)* gene is another gene which regulates DNA methylation. *TET2* hydroxylates 5-methylcytosine to 5-hydroxymethylcytosine which is then changed to unmodified cytosine by a number of mechanisms (figure 21). *TET2* gene has an important role in haematopoiesis as several *TET2* mutations were reported in a variety of haematological malignancies (Nakajima and Kunimoto, 2014). Mutations in *TET2* affect proliferation of HSCs after deregulation of gene expression which results from inhibition of *TET2*-mediated DNA methylation (Hanbazazh et al., 2021). *TET2* mutations lead to abnormal stem cell function and cellular differentiation.

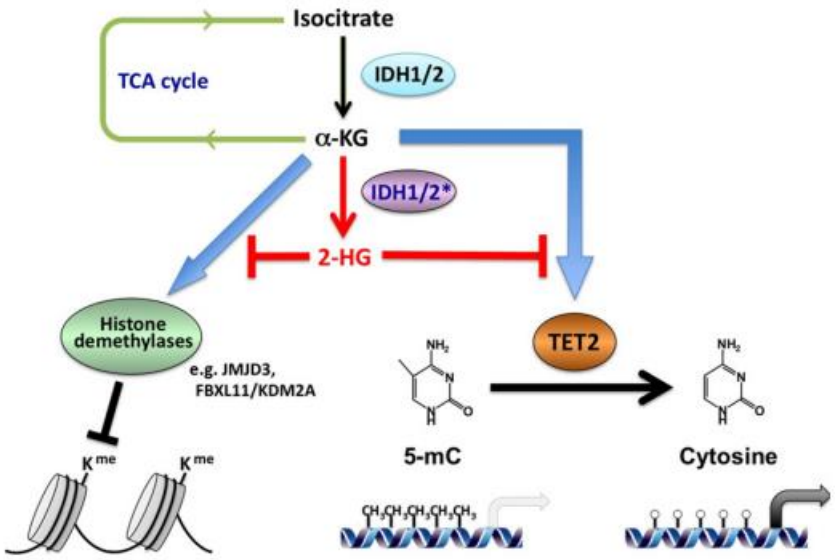


Figure 21: *TET2* gene. Reproduced from: Nakajima and Kunimoto, 2014.

2.6.2.6 *RUNX1* gene mutations

Runt-related transcription factor 1 (*RUNX1*) is a transcription factor gene and is involved in several functions including haematopoietic differentiation, cell cycle regulation, ribosome biogenesis and regulation of p53 and TGFβ pathways (figure 22) (Sood, Kamikubo and Liu, 2017).

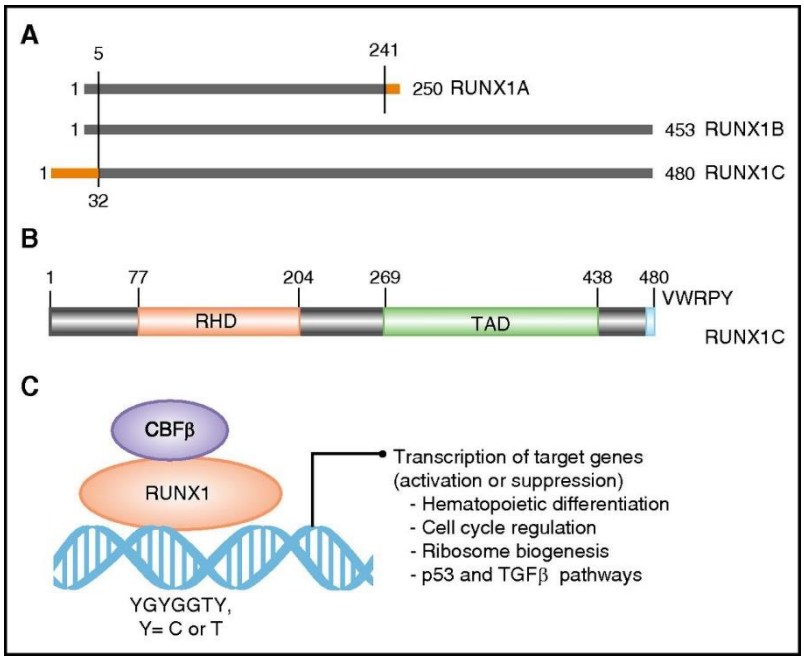


Figure 22: *RUNX1* gene structure and function. Reproduced from: Sood, Kamikubo and Liu, 2017.

RUNX1 mutations are found in 5-10% of AML cases and they tend to occur together with other mutations such as *ASXL1*, *SRSF2* and *SF3B1*. They are commoner in older age and in secondary AML which results from MDS. Most *RUNX1* mutations result in loss of function but they can also be missense mutations (Hanbazazh et al., 2021).

2.6.2.7 *TP53* gene mutations

Tumour protein p53 gene (*TP53*) is a tumour suppressor gene. *TP53* regulates the cell cycle, apoptosis and expression of stress response genes. P53 is activated by several stress factors such as DNA damage, hypoxia, nutrient deficiency, oncogenic signalling and oxidative stress. All these factors result in increased activity of p53 leading to cell cycle arrest, DNA repair or cell apoptosis (figure 23) (Moulder *et al.,* 2018). *TP53* mutations result in loss of function, loss of tumour suppressor function and increased DNA damage. *TP53* mutations are found to be commoner in AML and other cancers rather than MDS (Caponetti and Bagg, 2020).

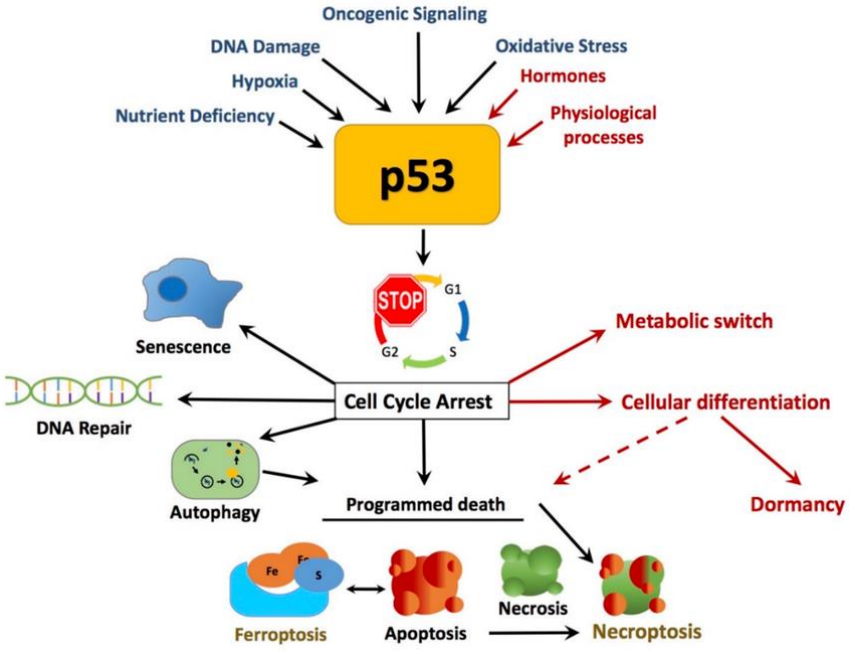


Figure 23: *Tp53* gene function. Reproduced from: Moulder *et al.,* 2018.

2.6.2.8 *FLT3* gene mutations

Fms-like tyrosine kinase 3 (*FLT3*) gene is also known as CD135 and is another gene which is known to contribute to leukemogenesis. The *FLT3* gene has a receptor tyrosine kinase which is important for the regulation of normal haematopoiesis. *FLT3* mutation is one of the commonest mutations in AML, being present in around one-third of AML patients. *FLT3* mutations result in abnormal cell proliferation, impaired differentiation, increased survival of leukaemic cells and an increased risk of relapse. There are 2 types of *FLT3* mutations – internal tandem duplications (ITD's) and point mutations within the tyrosine kinase domain (TKD) as can be seen in figure 24 below (Meryem Jalte *et al.,* 2023).

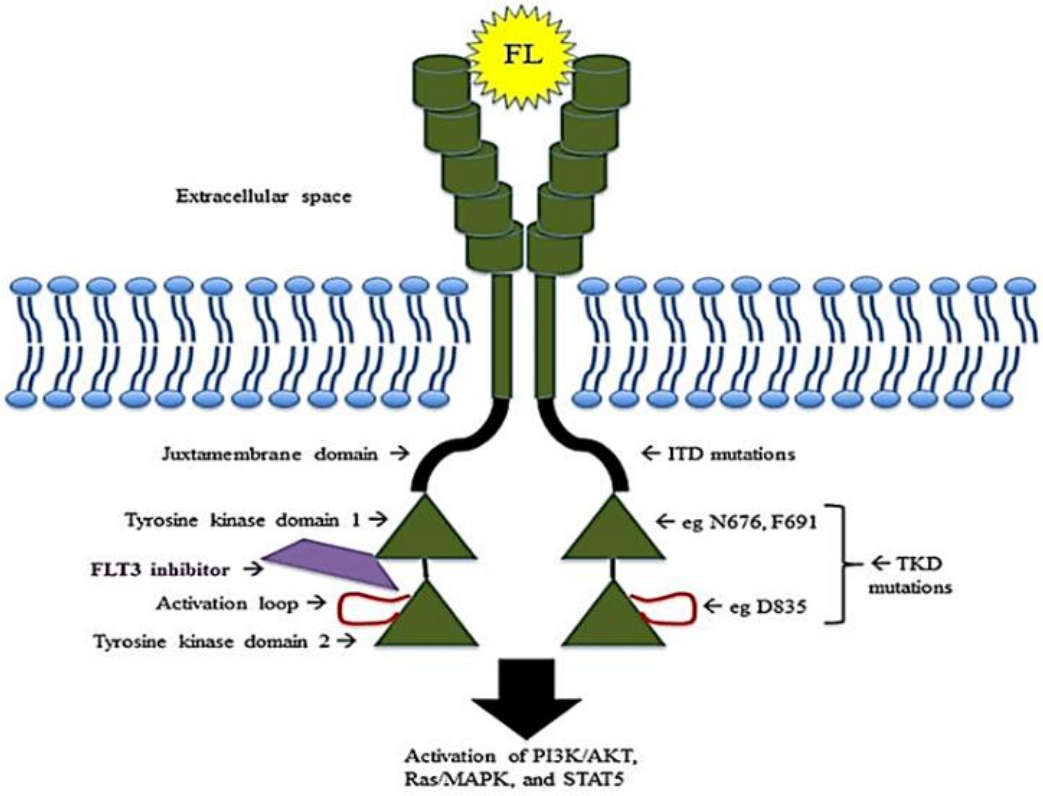


Figure 24: *FLT3* gene structure and mutations. Reproduced from: Meryem Jalte *et al.,* 2023.

2.6.2.9 *NPM1* gene mutations

Nucleophosmin 1 (*NPM1)* is one of the commonest mutated genes in AML. It is found in about 20-30% of AML cases. *NPM1* mutations are associated with a good prognosis (Heath *et al.,* 2017). *NPM1* proteins have multiple functions and are involved in several pathways including mRNA transport, apoptosis and chromatin remodelling. It is also a tumour suppressor gene like Tp53. In mutated *NPM1* in AML, myeloid differentiation is blocked, cell proliferation is promoted and DNA damage repair is impaired (Falini *et al.,* 2020).

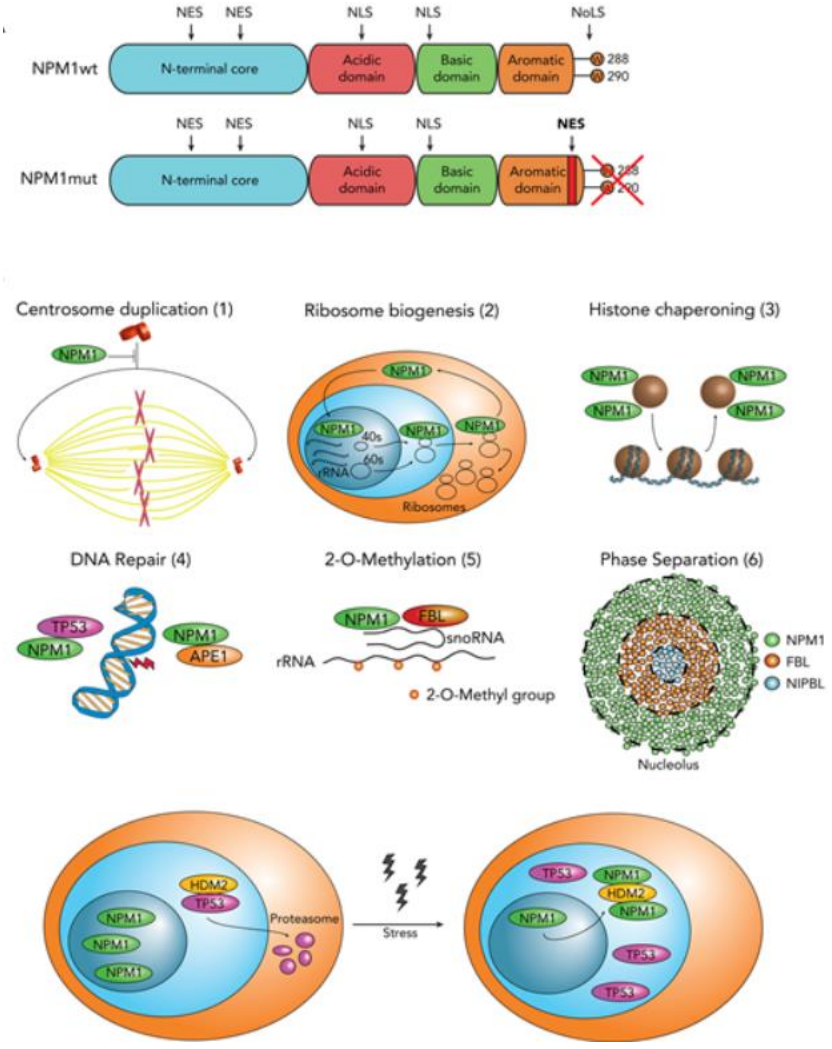


Figure 25: Different functions of NPM1. Reproduced from: Falini *et al.,* 2020.

2.6.3 Mutations in CHIP and CCUS

CHIP involves the presence of somatic mutations in individuals aged 70 years or more with an allele frequency of at least 2% when the criteria for a haematological malignancy are not fulfilled. Common mutations in CHIP include *DNMT3A*, *TET2* and *ASXL1*. In clinical practice, CHIP is not usually a significant problem since patients without any abnormality in blood counts are rarely investigated and sequenced. However, detecting individuals with CHIP may become more important in the future. This is because patients with CHIP have an increased risk of developing myeloid neoplasms and if these patients donate their stem cells, there might be poor haematopoietic engraftment in the recipients.

In CCUS, patients present with cytopenias and mutations but they do not fulfil the diagnostic criteria for MDS. Studies have shown that patients with CCUS can present differently; with different mutation patterns, different mutations in different genes, different number of mutations and different VAF of the mutations. Mutations in two or more genes carry a positive predictive value of 88% of developing a future myeloid neoplasm. Mutations with a VAF of 8.7% or above are associated with an 86% positive predictive value of future myeloid neoplasms. Certain mutations such as *RUNX1* and *JAK2* also increase the risk of development of myeloid neoplasms. Patients with 'high-risk' CCUS behave similarly to low-risk MDS patients without increased blasts in terms of overall survival and have a 10% per-year risk of progression (Hanbazazh et al., 2021).

Detection of somatic mutations in patients with potential pre-MDS conditions is important for their clinical management and prognosis. Detection of certain mutations can also help the clinicians to give personalised treatment and this will lead to better

survival of patients. For example, certain mutations in MDS show an increased response to hypomethylating agents such as those affecting *TET2* and *DNMT3A* and *TP53*. In this study, the presence of any somatic mutations in patients with potential pre-MDS conditions will be investigated by looking into the stem cell compartment to get a better understanding of these conditions (Caponetti and Bagg, 2020).

Chapter 3

Methodology

3.1 Ethical Approval and Permissions

Ethics approval was obtained from the Faculty Research Ethics Committee (FREC) prior to initiation of study – Reference number: MED-2022-00106 (Appendix A).

Permission from the Chairman of Pathology, Dr. Chris Barbara was granted to use the facilities within the Pathology Department, Mater Dei Hospital (MDH) as well as from Dr. David James Camilleri, Lead Consultant in Haematology, to use the Haematology Laboratories premises. Permission from the Chief Executive Officer, Ms. Celia Falzon was obtained to use MDH facilities together with permission from the Data Protection Officer, Mr. Simon Caruana, to be able to collect data from MDH. A Data Protection Clearance Declaration Form was also filled via email to obtain this permission (Appendix A).

Permission from Sir Anthony Mamo Oncology Centre (SAMOC) Chairman, Dr. Nick Refalo, and from Dr. David James Camilleri for recruitment of patients at SAMOC were obtained through the SAMOC Approval Data Search form. Permission was also granted from Ms. Donna Micallef, SAMOC HR and Administration Manager for recruitment of patients (Appendix A).

Permissions from the Haematology Consultants, Prof. Alexander Gatt, Dr. David James Camilleri, Dr. David Busuttil and from Dr. Mark Grech were obtained to recruit patients under their care at SAMOC. Permission from Dr. Erika Sultana, Higher Specialist Trainee in Haematology, was obtained to act as an intermediary in this research project and to approach and consent patients for this study (Appendix A).

A data management plan was developed as one of the requirements for University Research Ethics Committee approval (Appendix B).

3.2 Location of Research Study

This research study was carried out at the Haematology Laboratories (Flow Cytometry and Molecular Labs) within Mater Dei Hospital, Msida, Malta.

3.3 Patient Recruitment and Study Cohort Groups

Patients were recruited from SAMOC and MDH by the intermediary. Eligible patients were approached by Dr. Erika Sultana, and they were asked if they would like to participate in this research study. An information sheet together with a consent form (Appendix C) were given to each patient prior to their diagnostic bone marrow aspiration. The information sheet was read to each patient and any questions were answered. All the participants who gave their informed consent, were also given a short questionnaire asking their age, sex, smoking history, any medications they were taking, if they had any recent infections, history of malignancy/radiotherapy/chemotherapy, other clinical conditions, and their current symptoms (such as fatigue, shortness of breath, frequent infections, fever, bleeding and bruising easily) (Appendix C). The signed informed consent forms which included patient details were kept encrypted and password protected by the intermediary. Codes were given to each sample to replace patient details.

Bone marrow samples of patients with persistent cytopenias (anaemia, neutropenia, thrombocytopenia or pancytopenia) for more than 4 months were collected. This was the study patient group – Cohort A. Bone marrow samples of patients with newly

diagnosed Acute Myeloid Leukaemia or high-risk MDS were also collected, and this was the positive control group – Cohort B. A total of 55 patients were recruited for this study including 31 patients in Cohort A and 24 patients in Cohort B. However, two patients had to be excluded from the study (one from each cohort group) because one was diagnosed as having Hairy Cell Leukaemia and the other Blastic Plasmacytoid Dendritic Cell Neoplasm. Cohort A patients were patients with a potential pre-MDS condition (ICUS or CCUS) or LR-MDS. Patients with other potential pre-MDS conditions such as CHIP and IDUS had to be excluded from this study. This is because these patients have only clonality and/or dysplasia without cytopenias and these are difficult to find by routine diagnostic Haematological screening. Therefore, Haematology consultants will not typically request bone marrow sampling for these patients. Bone marrow samples were collected as part of the routine diagnostic work-up of patients by Haematology doctors. Only residual bone marrow samples after the requested diagnostic tests were carried out, were used for this study. Immunophenotypic investigation of the stem cell compartment by flow cytometry was carried out using the leukaemic stem cell tube, as detailed in Section 3.4.1. Immunophenotypic analyses were performed within 24 hours of sample collection, with the remaining sample frozen at -80°C for molecular testing.

A complete blood count (CBC) and a blood film were done from a peripheral blood sample which was also collected as part of the diagnostic work-up of these patients. The CBC and blood film were carried out at the Haematology Laboratory, using the Sysmex XN-1000 and Sysmex SP-50 analysers respectively (Sysmex Corporation, Kobe, Japan). The type of cytopenia and any dysplastic features were reported.

3.4 Immunophenotypic investigation of the stem cell compartment by Flow Cytometry

3.4.1 Local validation of the Leukaemic Stem Cell tube

The leukaemic stem cell tube is a single 8-colour tube which was developed by a group of scientists from Amsterdam University Medical Centre (UMC) in 2015. This tube includes leucocytes, blast and stem cell markers CD45, CD34 and CD38, respectively, and leukaemic stem cell markers including CD45RA, CD123, CD33, CD44 and other markers combined in one fluorescence channel (CLL-1/ TIM-3/ CD7/ CD11b/ CD22/ CD56) (Zeijlemaker *et al.,* 2015). This tube was used in this study to investigate the stem cell compartment of patients with persistent cytopenias and to check for the presence of leukaemic stem cells. The LSC tube was also used for patients with high-risk MDS or newly diagnosed AML patients, as positive controls.

Collaboration with the research team in Amsterdam led by Prof. Jacqueline Cloos, greatly aided in the optimisation of the technique for this research project. The optimisation phase spanned over a period of approximately five months and included adjustments in monoclonal antibody volumes, fine tuning of the instrument settings, sample processing and flow cytometry data analysis involving a very specific gating strategy. In order to ensure that the LSC technique was being performed correctly, the local research team was corresponding constantly with the expert group in Amsterdam, including sharing of FSCs files for trial runs. Training on the technique was consolidated by an onsite visit to the Amsterdam UMC, where the entire process could be observed and specific training on data analysis was provided. Once the optimisation phase was completed, patient recruitment and sample collection was initiated.

3.4.1.1 Volumes of LSC tube antibodies

A paper was published in 2018 including a comprehensive protocol on how to sample and process bone marrow samples for leukaemic stem cell detection. In the supplementary information section of this paper, the dilution of all the antibodies used in this tube were included in a table. This table (table 8) was used to calculate the volumes of the LSC tube antibodies to be used (Cloos et al., 2018).

Tube	FITC	dilution	PE	dilution	PerCP-CY5.5	dilution	PE-CY7	dilution	APC	dilution	APC-H7	dilution	BV421	dilution	HV500c	dilution
5	CD45RA	10	Clec12a	200	CD123	10	CD33	20	CD38	50	CD44	50	CD34	20	CD45	20
			TIM-3	20												
			CD7	20												
			CD11b	200												
			CD22	20												
			CD56	50												

Table 8: Standard antibodies and dilutions used in the Jove protocol. (Retrieved from: Cloos *et al.,* 2018).

In the first trial run, the following antibody volumes were used (table 9). These volumes were calculated using the dilutions in the Jove paper (Cloos et al., 2018).

FITC	Vol.	PE	Vol.	PerCP-CY5.5	Vol.	PE-CY7	Vol.	APC	Vol.	APC-H7	Vol.	BV421	Vol.	HV500c	Vol.
CD45RA (50µg/mL)	20µl	Clec12a (50µg/mL)	1µl	CD123 (25µg/mL)	20 µl	CD33 (25µg/mL)	10 µl	CD38 (25µg/mL)	10 µl	CD44 (400µg/mL)	10 µl	CD34 (100µg/mL)	10 µl	CD45 (100µg/mL)	10 µl
		TIM-3 (50 µg/mL)	10 µl												
		CD7(12.5µg/mL)	10 µl												
		CD11b (0.2µg/mL)	1 µl												
		CD22 (12.5µg/mL)	10 µl												
		CD56 (50µg/mL)	4 µl												

Table 9: Volumes of antibodies used in trial 1.

After the first trial, some changes in antibody volumes were suggested as displayed in Table 10 together with an additional 12µl of phosphate buffered saline (PBS) (0.05% azide-0.1% human serum albumin (HAS) to have a total volume of 120µl. These volumes gave better results in the trial runs and therefore, they were used throughout the study.

FITC	Vol.	PE	Vol.	PerCP-CY5.5	Vol.	PE-CY7	Vol.	APC	Vol.	APC-H7	Vol.	BV421	Vol.	HV500c	Vol.
CD45RA (50µg/mL)	20µl	Clec12a (50µg/mL)	1µl	CD123 (25µg/mL)	20 µl	CD33 (25µg/mL)	10 µl	CD38 (25µg/mL)	4 µl	CD44 (400µg/mL)	4 µl	CD34 (100µg/mL)	4 µl	CD45 (100µg/mL)	10 µl
		TIM-3 (50 µg/mL)	10 µl												
		CD7(12.5µg/mL)	10 µl												
		CD11b (0.2µg/mL)	1 µl												
		CD22 (12.5µg/mL)	10 µl												
		CD56 (50µg/mL)	4 µl												

Table 10: Final volumes of antibodies used in this study.

3.4.1.2 Instrument Settings

After optimising the antibody volumes to be used in the study, we encountered some issues with the instrument settings. At the Flow cytometry lab at MDH, the flow cytometer setup is based on EuroFlow's recommendations, as also recommended for the LSC analysis, by the protocols published by Jove et. al. However, sample preparation for the LSC involved the use of a specific lysing agent, which is the BD Pharm Lyse (BD Biosciences, New Jersey, USA). This lysing agent tends to preserve cells better for eventual fluorochrome labelled monoclonal antibody staining (figure 26), however it also affects the forward and side scatter properties of cells (figure 27). The conventional lysing reagent used within the Flow cytometry lab at MDH is FACS lysing solution (BD Biosciences, New Jersey, USA) which gives different forward and side scatter results and also impacts the instrument settings.

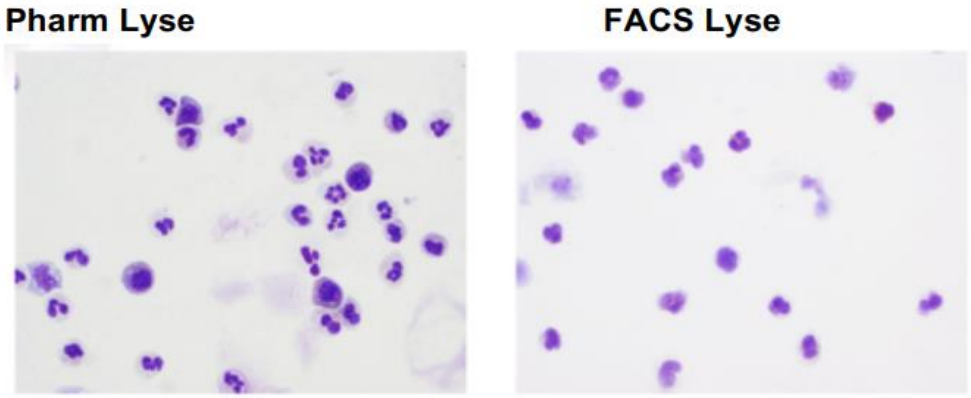


Figure 26: The FACS lysing agent (right) causing more cell damage. Retrieved from: O'Donahue & Johnson, 2016.

Therefore, Pharm lysing solution (BD Biosciences) had to be used both to setup the instrument for the leukaemic stem cell tube and also during sample preparation.

The scatter and staining properties of the cells change depending on the lysing agent being used. Figure 27 shows the different forward scatter and side scatter results when using both the PharmLyse and the FacsLyse (O'Donahue & Johnson, 2016). Using the FacsLyse resulted in a lower forward and side scatter and this affected the results of the previous trial runs.

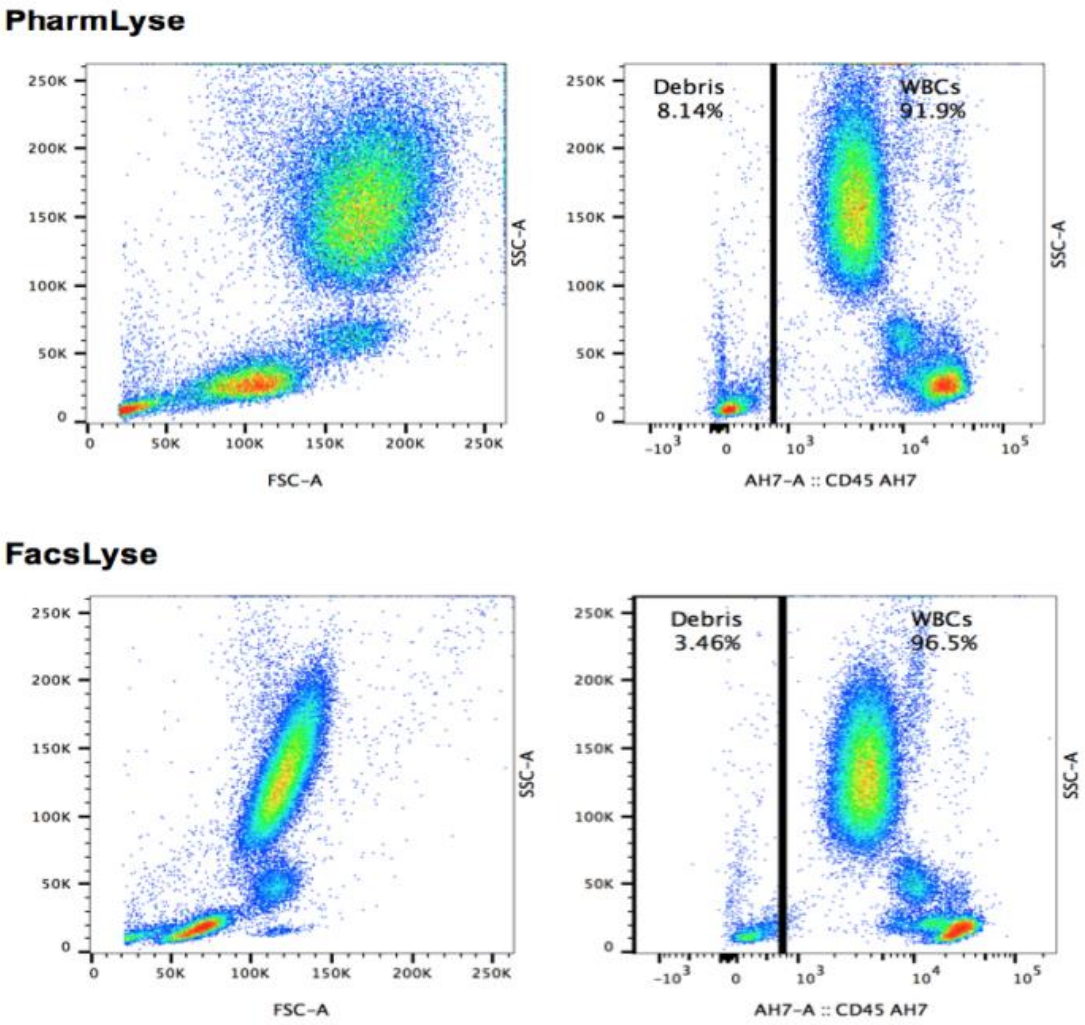


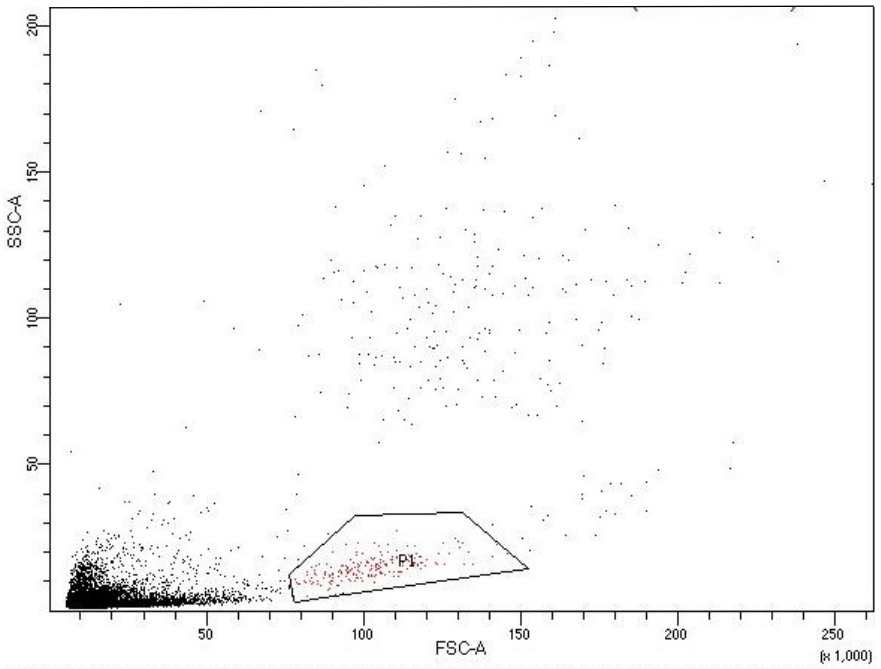
Figure 27: FSC and SSC changes when using the PharmLyse and FacsLyse. (Retrieved from: O'Donahue & Johnson, 2016).

The instrument settings to be used for the LSC tube were set-up once using the BD Pharm Lyse lysing agent, and each patient sample was analysed using these specific settings. The first step when setting up the instrument settings was to set up the photomultiplier tubes (PMT) voltages for target fluorescence channels and FSC and SSC parameters. After the BD FACS Canto II was connected, the Cytometer Setup and Tracking (CST) beads (BD biosciences, Catalog No:655051) were run to ensure that the lasers power and voltages were according to preset instrument values. A normal peripheral whole blood sample, specifically the lymphocyte population, was used to set up the forward and side scatter settings within the Facs Canto II. To the whole blood, 2mls of Pharm lysing solution (BD biosciences, Catalog No: 555899) were added, mixed gently and incubated for 10 minutes at room temperature. After incubation, the tube was centrifuged using a benchtop centrifuge (Eppendorf Centrifuge 5810) for 7 minutes at 800g at room temperature. The supernatant was then discarded using a Pasteur pipette. The cell pellet was vortexed and resuspended in 15mL phosphate buffered saline (PBS) (0.05% azide-0.1% human serum albumin (HAS) at room temperature) (Sigma Aldrich). The tube was centrifuged again for 7 minutes at 800g at room temperature. The supernatant was again discarded, and the cell pellet was vortexed and resuspended in 500µL PBS/0.05%azide-0.1%HSA. A new experiment was created on the FACSDiVa software. The new experiment included a FSC versus SSC dot plot for setting up the FSC and SSC parameters. The lysed blood was acquired and with specific gating on the lymphocyte population, the FSC and SSC voltages were adjusted to reach the following mean target values:

FSC: 100.000 (95.000-105.000)

SSC: 15.000 (13.000-17.000)

The data was then recorded, recording 10,000 events. After recording, the mean FSC and SSC target values for the gated lymphocytes were verified again. The data obtained is represented in figure 28 below. The recorded FSC was 101V and SSC was 15V.



Tube: Lysed/Washed Blood FSC/SCC

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	200	2.0	2.0

Experiment Name:	Daily Rainbow LS...	CYTOMETER CO...	2007-01-02T12:0...
Specimen Name:	Daily Rainbow Cal...	CST BEADS EXPI...	False
Tube Name:	Lysed/Washed Blo...	CST PERFORMA...	2023-01-18T08:5...
Record Date:	Feb 14, 2023 12:3...	CST REGULATOR...	CE-IVD Performan...
CYTOMETER CO...	3-laser, 8-color (4-...	GUID:	e7a1f55c-d695-43...

Population	#Events	%Parent	FSC-A Median	SSC-A Median
All Events	10,000	####	11,731	2,519
P1	200	2.0	100,944	14,512

Figure 28: Recorded FSC and SSC voltages when setting up instrument settings.

The 8-peak Rainbow calibration beads (Spherotech, Lake Forest, IL, USA, Euroflow RCP-30-5a) were then used to set up the fluorescence channels PMT voltages. A new experiment was created and was titled 7-peak Spherotech Beads. A worksheet "Target MFI" was created with all necessary dot plots (n=2; FSC versus SSC, FITC versus PE), histograms (n=8; one histogram for each fluorescence detector) and statistics showing

the reference peak values (MFI and CV) for each fluorescence channel. A freshly prepared solution containing 1 drop (0.05ml) of Spherotech 8-peak Rainbow beads was prepared in 1ml of deionised water, vortexed and run. The PMT settings of the FSC and SSC which were setup previously, were used to run the 8-peak Rainbow beads solution which were acquired at "Low" flow rate with a threshold of 5.000. The singlet beads – population P1 was gated from the FSC versus SSC dot plot. The 7th peak – population P2 was gated from the FITC versus PE dot plot.

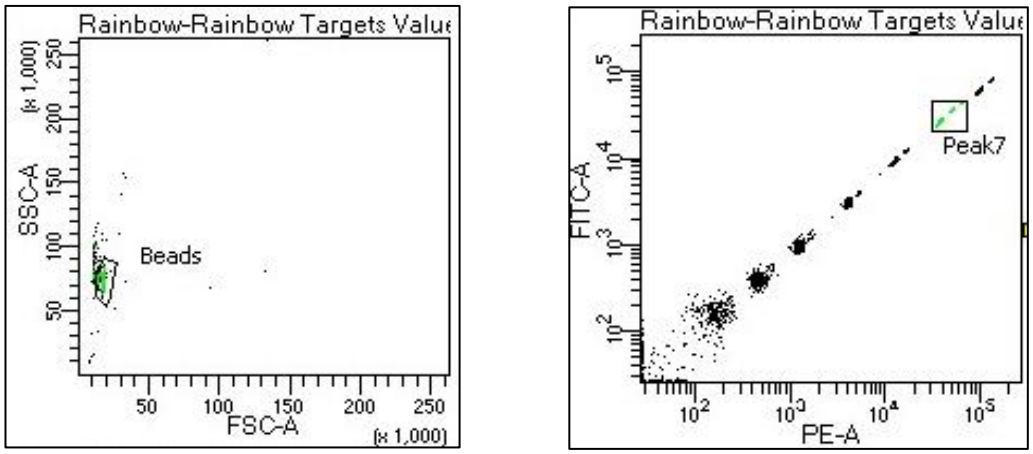


Figure 29: 7th Peak Population gated from FITC versus PE dot plot.

The target values for the fluorescence channels were provided with the specific bead lot. Using the 7-peaks Rainbow bead suspension the PMT voltages in all fluorescence channels were adjusted to reach the target MFI values according to the EuroFlow MFI target channels. About 2,000 events were recorded and the MFI for the 7th Peak beads was checked. Once the target MFI values for the 7th Peak were reached as can be seen in figure 30, the file was recorded for the final PMT values. The final PMT values were saved as LSCSetup and dated accordingly. This PMT setup was used for correcting the spill over for each fluorescence dye (Kalina *et al.,* 2012).

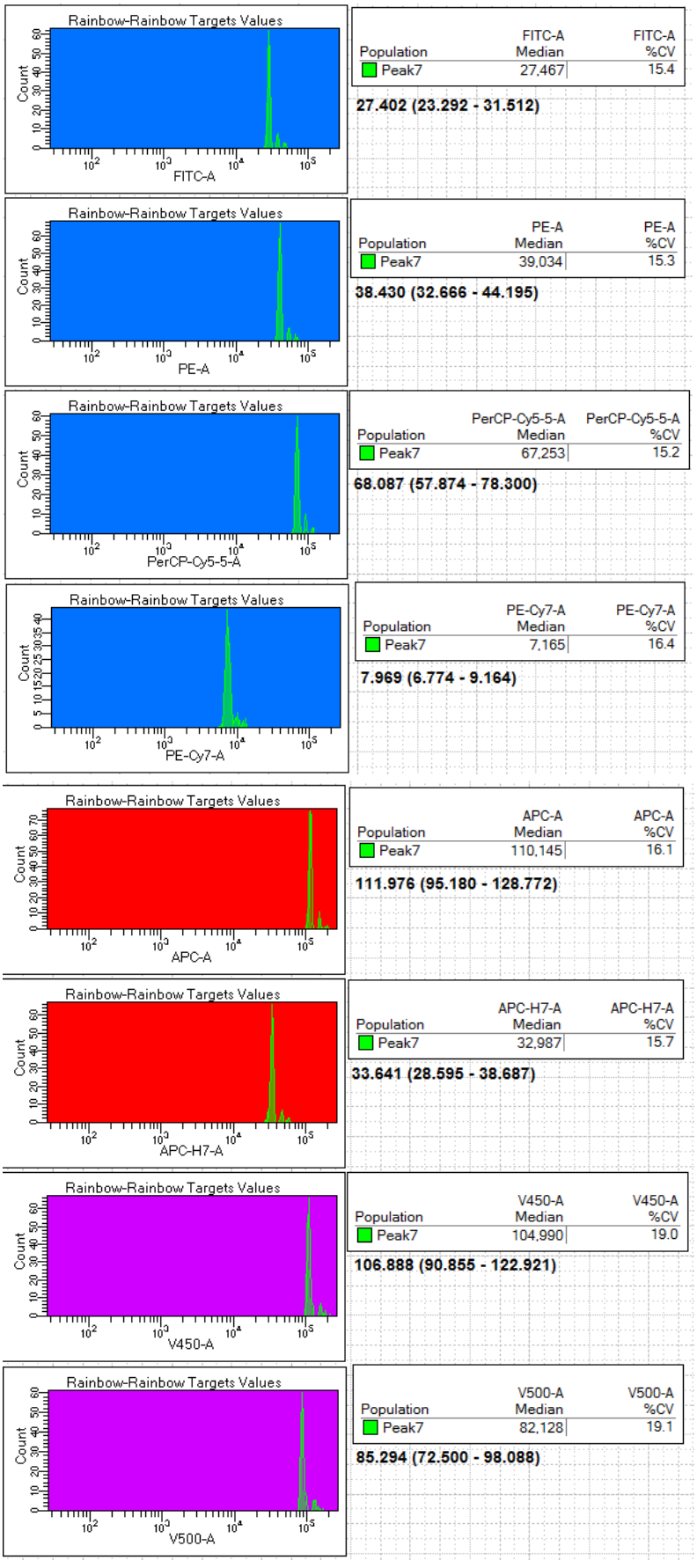


Figure 30: Setting up the PMT voltages of the fluorescence channels using the 8-peak Rainbow Calibration Beads.

For the fluorescence compensation settings, a tube for the unstained control and another for each fluorochrome conjugated antibody were labelled. In each tube, 100µL of PBS/0.05%azide-0.1%HSA were pipetted. The vial of Multicolor CompBeads (BD 644204) was vortexed thoroughly and a full drop (approximately 60µL) was added to each tube. The appropriate volume of fluorochrome-conjugated antibody sufficient for one test were pipetted to stain 10^6 cells into the corresponding tubes and were vortexed thoroughly. The tubes were incubated for 15 minutes in the dark at room temperature. After incubation, 4mL of PBS/0.05%azide-0.1%HSA were added to each tube. The tubes were then centrifuged at 300g for 10 minutes. The supernatant was then removed and the bead pellet was resuspended by adding 0.2mL of wash buffer to each tube. The BD FACSDiva software was then opened and an experiment was created. The compensation controls were created including label-specific tubes as needed. The following were then selected: 'Experiment', 'Compensation Setup' and 'Create Compensation Controls'. The unstained control tube was then loaded and the P1 gate was adjusted around the singlet bead population. The P1 gate was right-clicked and 'Apply to All Compensation Controls' was selected. A minimum of 10,000 events were recorded for all the single labeled fluorescence tubes. After verifying that the P2 gate encompasses the positive population on each fluorescence diagram, the compensation was calculated. Then the following were selected: 'Experiment', 'Compensation Setup' and 'Calculate Compensation'. A name was then entered (LSC Tube Settings) and saved. The tube settings were then used for every experiment i.e. for each patient sample (Zeijlemaker *et al.,* 2015).

3.4.2 LSC tube Analysis

3.4.2.1 Bone marrow sample processing

Bone marrow samples were processed within 24 hours upon arrival at the Flow cytometry lab. The bulk lysing technique was used on each BM sample. This allowed the reduction or elimination of the red cells from the sample, thus facilitating isolation of the leucocytes, precursors and stem cells during analysis. Although the bulk lysing technique is very important in the staining process of the LSC tube, it has some limitations including possible loss of cells, shifts in SSC and FSC and changes to antibody staining for cytometric analysis (O'Donahue & Johnson, 2016).

The bone marrow sample taken in an EDTA vacutainer, was inverted six times to ensure homogeneity. The concentration of the bone marrow cells (total white blood cell count) was determined pre-lysing using the Sysmex XN-1000 analyser. The red cells were lysed using Pharm lysing solution (BD Biosciences, Catalog No:555899), by mixing 2mLs of bone marrow sample with 50mL of lysing solution in a freestanding polypropylene tube. The tube was mixed gently by inversion and was incubated for 10 minutes at room temperature on a rotator. The tube was then centrifuged for 7 minutes at 700g at room temperature. The supernatant was discarded using a Pasteur pipette. The cell pellet was resuspended in PBS/0.05%azide-0.1%HSA at room temperature. The maximum volume of the tube was used. The tube was vortexed and centrifuged again for 7 minutes at 700g at room temperature. The supernatant was again discarded using a Pasteur pipette and the cell pellet was resuspended in PBS/0.05%azide-0.1%HSA. Another cell count was performed post-lyse. The minimum cell concentration required for this technique was $20x10^6$/mL and the maximum cell concentration was $60x10^6$/mL.

These cell counts allowed optimal antibody concentration staining, reducing non-specific bindings. If the cell concentration post bulk lysing was higher than $60x10^6$/mL, the cells were further diluted with PBS/0.05%azide-0.1%HSA.

Once optimal cell counts were obtained, staining of cells was carried out to determine the presence or otherwise of LSC and immunophenotype. Each antibody was pipetted in a pre-labelled polystyrene Facs tube. Then, 80µL from the lysed cell solution were added to the tube. The tube was gently vortexed and incubated for 15 minutes at room temperature in the dark. After incubation, PBS/0.05%azide/0.1%HSA was added and the tube was centrifuged for 5 minutes at 400g at room temperature. The supernatant was discarded using a Pasteur pipette and the cell pellet was vortexed and resuspended in 500µL PBS/0.05%azide/0.1%HSA. Acquisition of the sample was then carried out acquiring the maximum number of events possible. The guidelines provided by the experts at UMC suggested a minimum of 5,000,000 events per sample (Zeijlemaker *et al.,* 2015).

3.4.2.2 Gating Strategy

The LSC tube developed in Amsterdam UMC, contains the following markers:

- CD45: this marker was used for the identification of the different BM populations.
- CD34: this marker was used for the identification of the primitive populations and blasts.
- CD38: this marker was used for the identification of the stem cell compartment (CD34+CD38-).

- Aberrant markers (CD45RA, Clec12a, TIM-3, CD7, CD11b, CD22, CD56, CD33, CD123 and CD44): these markers were used for the characterisation of the stem cell compartment (normal HSCs vs LSCs).

The first step during sample analysis was gating the white blood cells (WBCs). During gating of WBCs, the low FSC cells (Figure 31- A) and low CD45 cells (Figure 31- B) were eliminated. Any debris or doublet cells (Figure 31- C) were also eliminated.

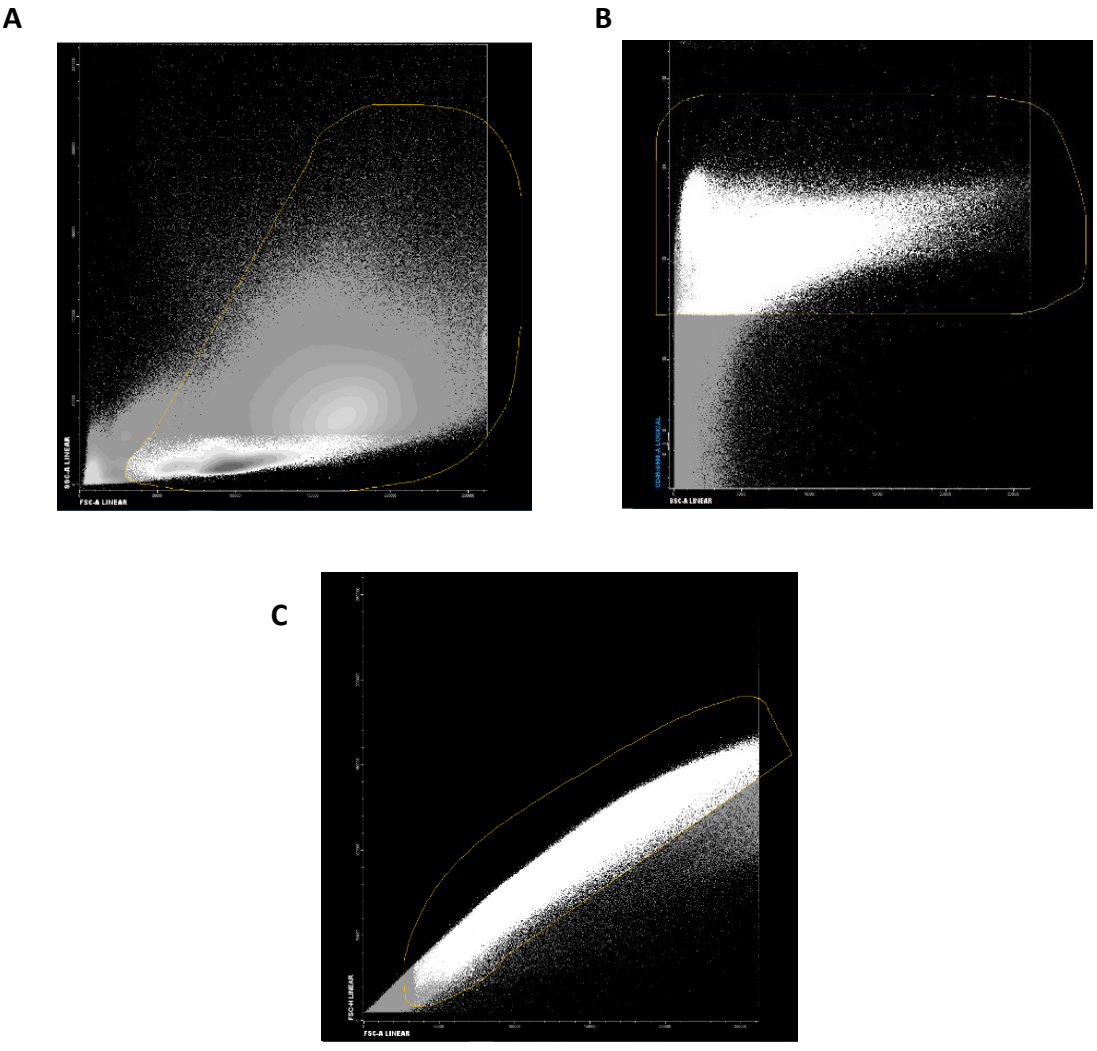


Figure 31 (A, B & C): Gating the WBC population.

After the WBC population was identified (blue population in figure 32), gating of the lymphocyte population (green population) was carried out by identifying the CD45 high/SSC low population, and by gating the FSC low/ SSC low population. Also, the

lymphocyte population was negative for CD33. The lymphocyte population was eventually used as a cut-off/ negative control population for the following markers: CD33, CD123, CD45RA, and CD44.

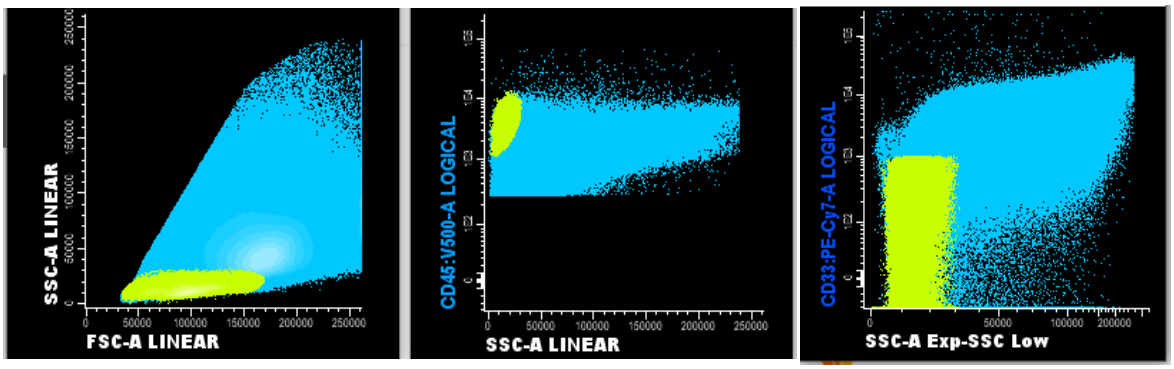


Figure 32: Gating the lymphocyte population (green population).

The blast cell population was then gated by selecting the CD45dim/ SSC low population. The blast cell population (dark blue population in figure 33) was then used to identify the CD34 positive compartment.

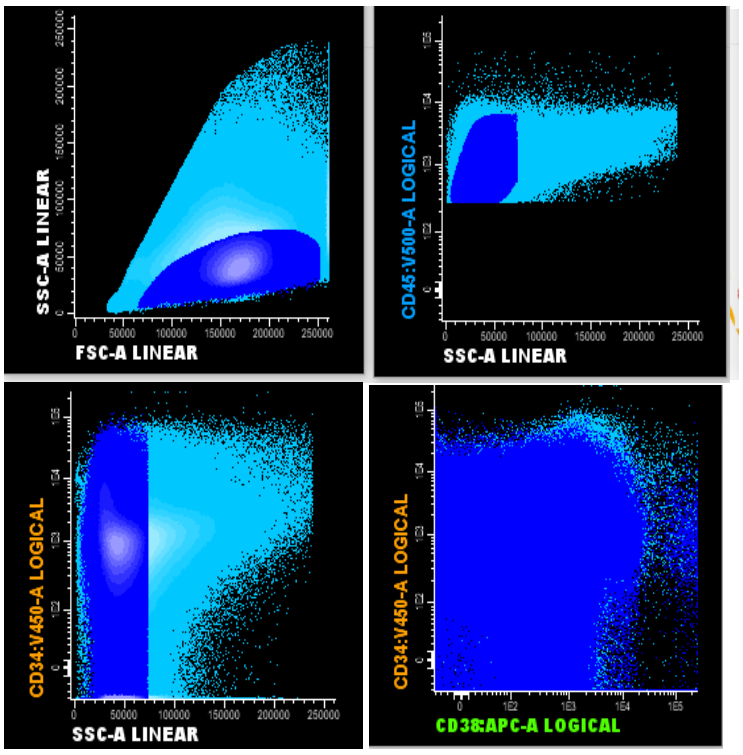


Figure 33: Gating the blast cell population (dark blue population).

The next step was to gate the CD34+ve blast cells (light blue population in figure 34). The CD34+ve population was gated from the blast cell population (dark blue population). The cut-off used on the CD34/SSC-A dot plot was approximately 10^4. However, cut-offs may have differed from sample to sample. If there was a homogeneous population at the 10^4 cut-off, it was never gated through, but it was lowered further down so to gate the whole population.

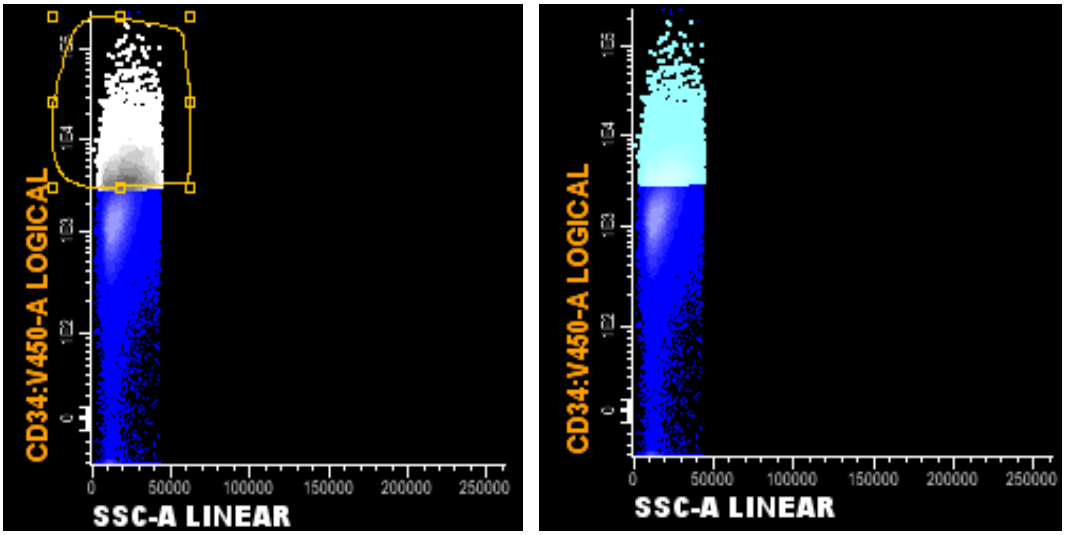


Figure 34: Gating the CD34+ve Blast Cells

The stem cell population was then identified (CD34+CD38-) from the CD34+ve compartment. To identify stem cells, a histogram (density/CD38) was used. The CD34+ve compartment was divided into 3: CD34+CD38+ (>10^3 on the histogram), CD34+CD38dim (between 10^2 and 10^3 on the histogram) and CD34+CD38- (<10^2 on the histogram).

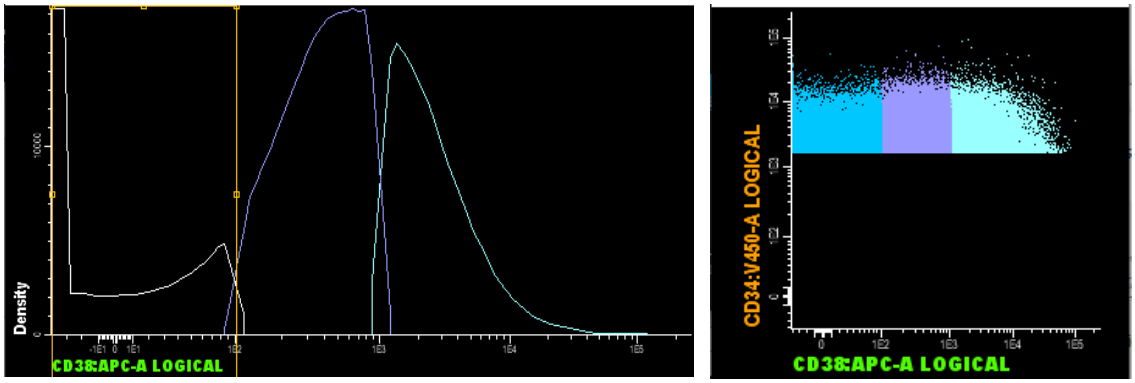


Figure 35: Histogram used to divide the CD34+ve compartment (Left) and CD34+ compartment divided into 3 (Right).

After the stem cell compartment was identified (dark blue population in figure 35), all the leukaemic stem cell markers were gated separately for each patient. The first marker which was gated was CD33, being weakly expressed on normal haematopoietic stem cells. However, it can be brightly expressed on leukaemic stem cells. The lymphocyte population was used as a cut-off population/negative control for the CD33 marker. Anything beyond the lymphocyte population was considered to be positive for CD33. In figure 36, normal haematopoietic stem cells are represented by the dark green population whilst leukaemic stem cells are represented by the red population. The dot plot on the left is showing how the lymphocyte population was used as a cut-off.

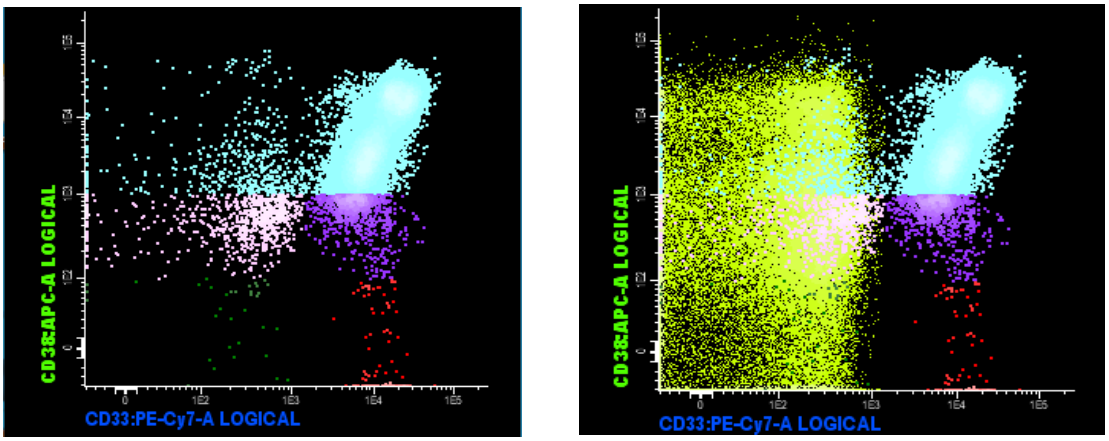


Figure 36: Normal HSCs (green) and LSCs (red) on the CD33 dot plot.

CD123 is also weakly expressed on normal haematopoietic stem cells but can be brightly expressed on leukaemic stem cells. The lymphocyte population was again used as a cut-off population/negative control for the CD123 marker. Anything beyond the lymphocyte population was considered to be positive for CD123. In figure 37, normal haematopoietic stem cells are represented by the dark green population whilst leukaemic stem cells are represented by the red population.

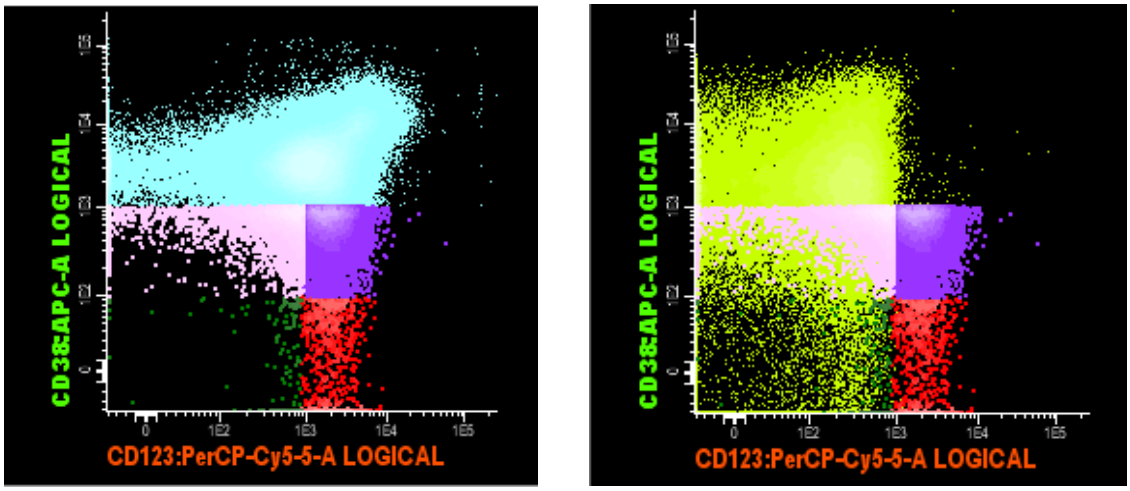


Figure 37: Normal HSCs (dark green) and LSCs (red) on the CD123 dot plot.

CD44 is also weakly expressed on normal haematopoietic stem cells but can be brightly expressed on leukaemic stem cells. The lymphocyte population once again used as a cut-off population/negative control for the CD44 marker. Anything beyond the lymphocyte population was considered to be positive for CD44. In figure 38, normal haematopoietic stem cells are represented by the dark green population whilst leukaemic stem cells are represented by the red population.

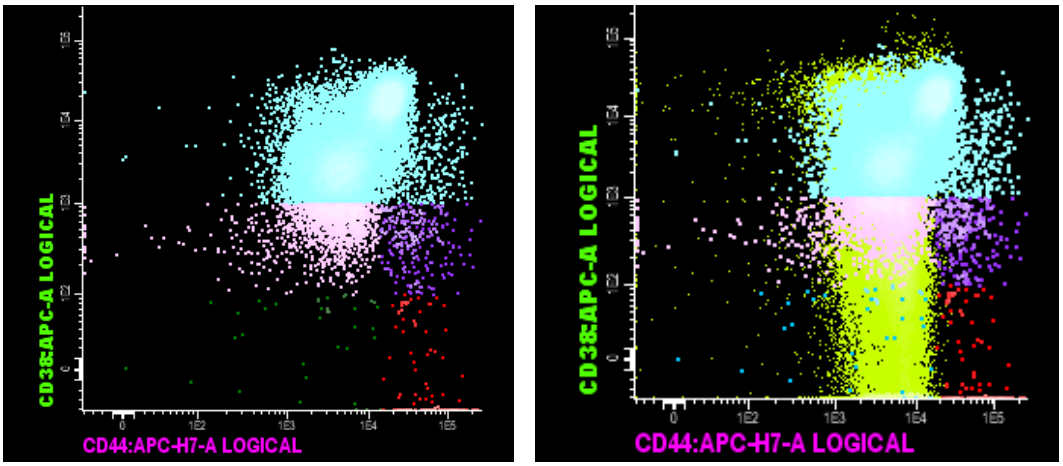


Figure 38: Normal HSCs (dark green) and LSCs (red) on the CD44 dot plot.

CD45RA is absent on normal haematopoietic stem cells and can be expressed on leukaemic stem cells. The lymphocyte population was again used as a cut-off population/negative control for the CD45RA marker. However, the expression of CD45RA on normal lymphocytes is different from the previous markers. As can be seen in figure 39 on the left, there are two lymphocyte populations in the CD45RA dot plot, the CD45RA positive B-lymphocytes and CD4+ve T-lymphocytes, and the CD45RA negative T-lymphocytes (Beckam Coulter, n.d.). In this case, anything beyond the first lymphocyte population (CD45RA-ve T-lymphocytes) was considered to be positive for CD45RA. In figure 39 (left), normal haematopoietic stem cells are represented by the dark green population whilst leukaemic stem cells are represented by the red population.

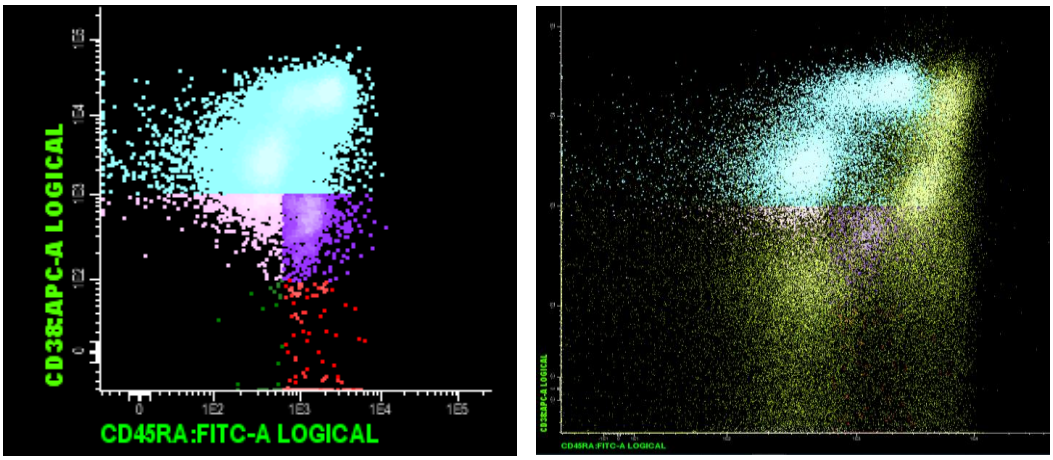


Figure 39: Normal HSCs (dark green) and LSCs (red) on the CD45RA dot plot.

The last leukaemic stem cell markers which were gated were the Combi markers i.e. Clec12a, TIM-3, CD7, CD11b, CD22 and CD56 all linked to PE fluorochrome, thus, all detected within the PE channel. All these antigens are absent on normal haematopoietic stem cells and can be expressed on leukaemic stem cells. When gating the Combi markers, the erythroid population was used as the negative/cut off population. The erythroid population was gated by selecting the population with low FSC, low SSC and by selecting the CD45-ve population (figure 40).

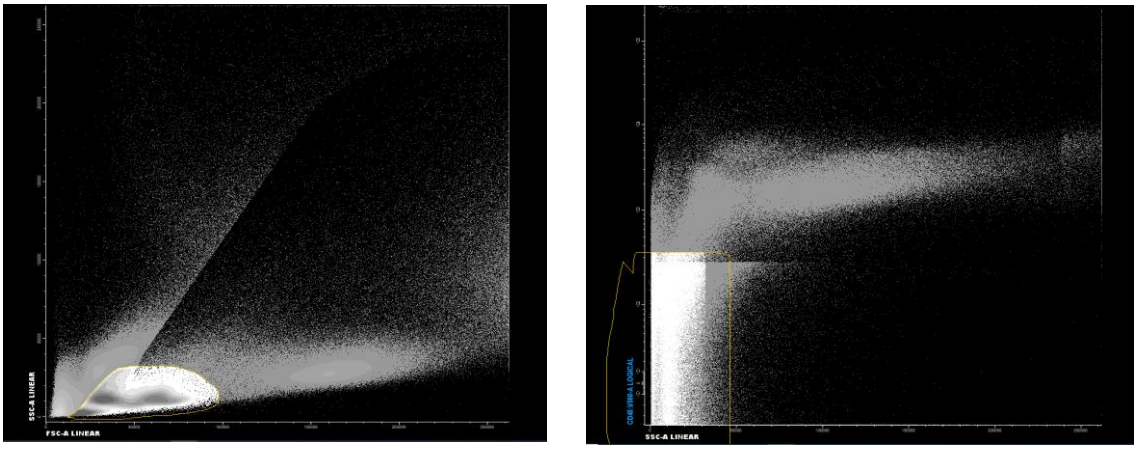


Figure 40: Gating the erythroid population.

Any stem cell population beyond the erythroid population was considered to be positive for the Combi markers. In figure 41 (left), normal haematopoietic stem cells are represented by the dark green population whilst leukaemic stem cells are represented by the red population.

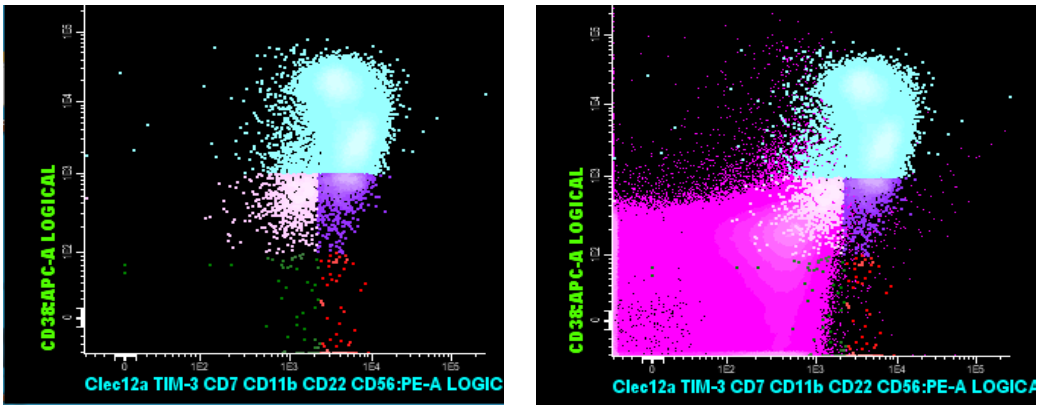


Figure 41: Normal HSCs (green) and LSCs (red) on the Combi markers dot plot.

After gating each leukaemic stem cell marker, a report was generated giving the leukaemic stem cell percentage of each marker. Some examples of the reports generated can be found in Appendix D.

Population	Events	Partial %	Visibility %
WBC	3330617	75.2678	100.0000
Lymfocyten	139073	4.1756	4.1756
Blasten	2397089	71.9713	71.9713
CD34+	138808	5.7907	4.1677
CD34+CD38-	64905	46.7588	1.9488
CD38- LAP neg	0	0.0000	NA
CD38- LAP pos	64903	99.9969	1.9487
CD34+CD38dim	55205	39.7708	1.6575
CD38dim LAP neg	0	0.0000	NA
CD38dim LAP pos	55205	100.0000	1.6575
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

Population	Median
Lymfocyten	
FSC-A	96100.7188
SSC-A	12323.7803
CD38- LAP neg	
FSC-A	NA
SSC-A	NA
ƒ MFI SSC LAP neg/lymfo's: NA	
CD38- LAP pos	
FSC-A	167313.6250
SSC-A	32965.3828
ƒ MFI FSC LAP pos/lymfo's: 1.7...	
ƒ MFI SSC LAP pos/lymfo's: 2.6...	

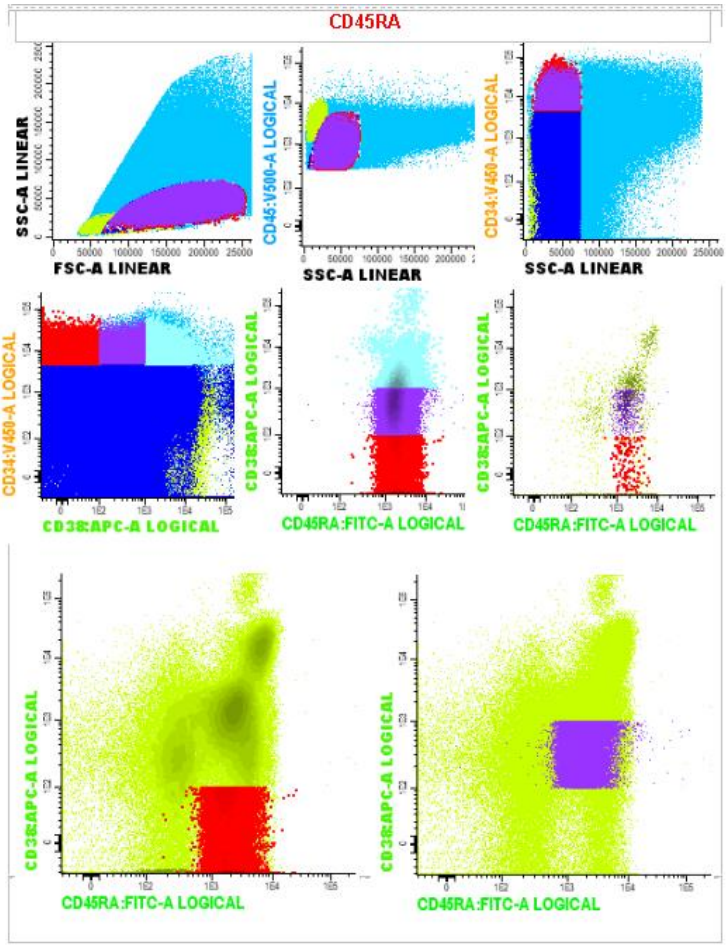


Figure 42: An example of a report generated after gating.

3.5 Molecular analysis of BM samples by NGS

Molecular analysis was carried out at the Molecular Haematology Laboratory, Mater Dei Hospital and it was carried out as part of the diagnostic work-up of patients. Molecular analysis was carried out using Ion Torrent™ Oncomine™ Myeloid targeted NGS panel. The targeted panel shown in table 11 included all relevant DNA mutations and fusion transcripts which are associated with myeloid disorders. The targeted panel included 40 DNA target genes, 29 driver genes and a broad fusion panel. The genes of interest for this study were the hotspot genes (23) and the full genes (17) which are represented in the first two columns in table 11 (Thermo Fisher, 2023).

Hotspot genes (23)		Full genes (17)		Fusion driver genes (29)			Expression genes (5)	Expression control genes (5)
ABL1	*KRAS*	*ASXL1*	*PRPF8*	*ABL1*	*HMGA2*	*NUP214*	*BAALC*	*EIF2B1*
BRAF	*MPL*	*BCOR*	*RB1*	*ALK*	*JAK2*	*PDGFRA*	*MECOM*	*FBXW2*
CBL	*MYD88*	*CALR*	*RUNX1*	*BCL2*	*KMT2A*	*PDGFRB*	*MYC*	*PSMB2*
CSF3R	*NPM1*	*CEBPA*	*SH2B3*	*BRAF*	*(MLL)*	*RARA*	*SMC1A*	*PUM1*
DNMT3A	*NRAS*	*ETV6*	*STAG2*	*CCND1*	*MECOM*	*RBM15*	*WT1*	*TRIM27*
FLT3	*PTPN11*	*EZH2*	*TET2*	*CREBBP*	*MET*	*RUNX1*		
GATA2	*SETBP1*	*IKZF1*	*TP53*	*EGFR*	*MLLT10*	*TCF3*		
HRAS	*SF3B1*	*NF1*	*ZRSR2*	*ETV6*	*MLLT3*	*TFE3*		
IDH1	*SRSF2*	*PHF6*		*FGFR1*	*MYBL1*			
IDH2	*U2AF1*			*FGFR2*	*MYH11*			
JAK2	*WT1*			*FUS*	*NTRK3*			
KIT								

Table 11: Ion Torrent™ Oncomine™ Myeloid targeted NGS panel (Thermo Fisher, 2023).

The steps involved in NGS included DNA extraction using Thermo Kingfisher Flex™, library preparation and templating using Ion Chef™, sequencing using Ion GeneStudioS5 system and data analysis using Ion Reporter™ software.

3.5.1 DNA extraction using Thermo Kingfisher Flex™

Bone marrow samples were stored at -80°C. Samples were removed from the freezer and thawed until they reached room temperature. The first step of NGS was DNA

extraction/purification. DNA extraction was performed using MagMAX™ DNA Multi-Sample Ultra 2.0 kit on the instrument Thermo KingFisher Flex™ (Thermo Fisher Scientific). The first step was to prepare the Elution and Wash Plates using deep well plates. These were prepared using reagents from the MagMAX™ DNA Multi-Sample Ultra 2.0 kit except for Wash 2 Solution. Wash 2 solution was freshly prepared by adding 20ml deionised water to 80ml absolute ethanol (80% ethanol). The required reagents were added as follows:

PLATE	REAGENT	VOLUME
Wash 1 Solution Plate	Wash 1 Solution	500 µL
Wash 2 Solution Plate1	Wash 2 Solution	500 µL
Wash 2 Solution Plate 2	Wash 2 Solution	500 µL
Elution Plate	Elution Solution	100 µL
Tip Comb	Place 96 Deep Well Tip Comb in a standard plate	

Table 12: Preparation of Elution and Wash Plates Reagent Volumes.

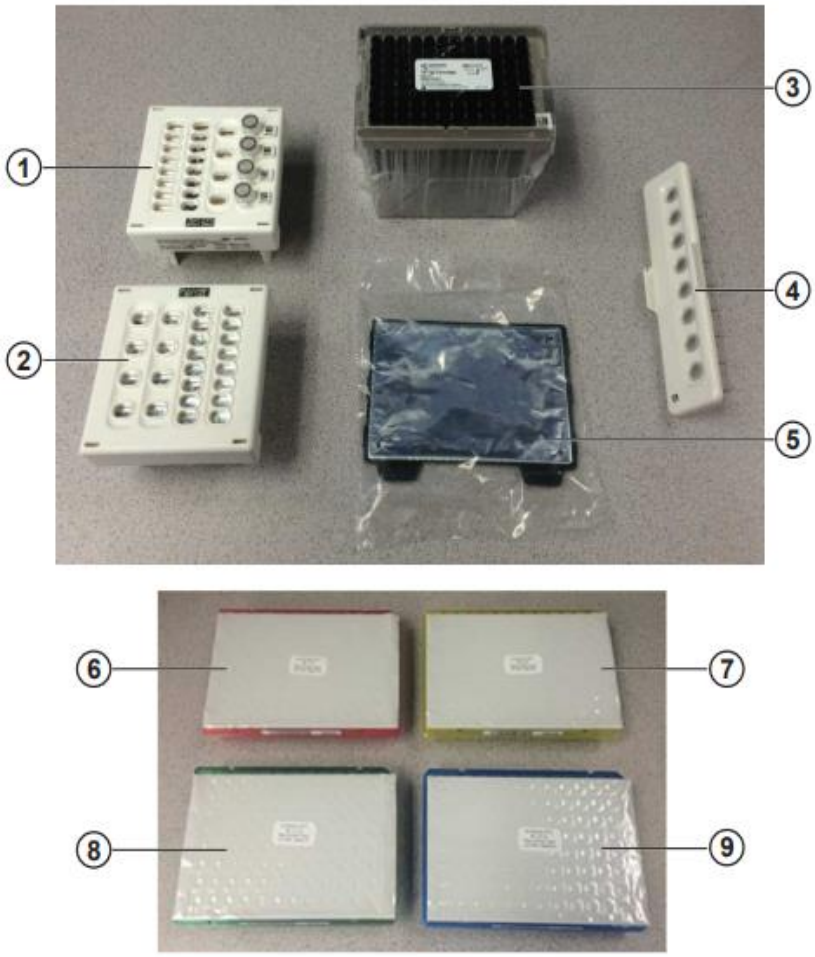
The next step was to prepare the sample plate, by pipetting 20µL Enhancer Solution, 200µL bone marrow sample and 20µL of Proteinase K in the mentioned order, in a deep well plate. After preparing the sample plate, the KingFisher Flex™ instrument was switched on and the user protocols tab was selected from the display. The program **MagMAX_Ultra2_400µL_Flex** was then selected. The previously prepared processing plates were then loaded in their respective positions as instructed by the instrument and the run was initiated. After 20 minutes, DNA Binding Bead Mix, consisting of 200µL Binding Solution and 20µL DNA Binding Beads, was added. This allows DNA purification, by separating the nucleic acid from proteins and cell remnants present in solution. The sample plate was removed from the instrument and the DNA Binding Bead Mix was

added to each sample. The plate was then reloaded onto the KingFisher Flex™ to complete the purification process. At the end of the run, the plate was immediately removed from the instrument. The elute for each sample was transferred into properly labelled, sterile tubes. The DNA extract material was quantified using Qubit4 fluorometer. The DNA extract had to be more than 10ng/µL, thus ensuring adequate nuclear material for further sequencing.

3.5.2 Library Preparation using Ion Chef™

After DNA extraction, library preparation was carried out. This was an automated process during which nucleic acids were isolated, fragmented, end-repaired and covalently linked to adapters using ligation or tagmentation methods. Each sample was tagged with a specific barcode by a process called multiplexing so that it could be analysed independently at later stages. Multiplexing increased the efficiency of NGS methodology by maximizing the number of samples processed in each sequencing run (Thermo Fisher Scientific, 2024).

Library preparation was performed using the Ion AmpliSeq™ Kit for Chef DL8 (DNA to library, 8 samples/run) using the Ion Chef™ instrument. The Ion AmpliSeq™ Kit contained the following consumables which are listed in figure 43 below and Ion AmpliSeq™ DNA primer pools which were stored at -20°C. In figure 43, there are 4 IonCode PCR plates (6,7,8 and 9) with different colours. Only one IonCode PCR plate was used in each run.



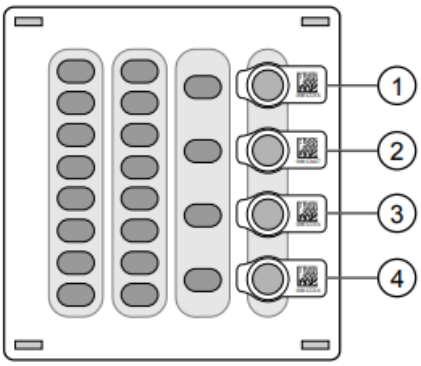
Ion AmpliSeq™ Kit for Chef DL8 consumables

① Ion AmpliSeq™ Chef Reagents DL8 cartridge
② Ion AmpliSeq™ Chef Solutions DL8 cartridge
③ Ion AmpliSeq™ Tip Cartridge L8
④ Enrichment Cartridge
⑤ PCR Frame Seal

⑥ IonCode™ 0101–0108 in 96 Well PCR Plate (red)
⑦ IonCode™ 0109–0116 in 96 Well PCR Plate (yellow)
⑧ IonCode™ 0117–0124 in 96 Well PCR Plate (green)
⑨ IonCode™ 0125–0132 in 96 Well PCR Plate (blue)

Figure 43: Ion AmpliSeq™ Kit consumables. Caption retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013432_Ion_AmpliSeq_Library_Prep_on_Ion_Chef_UG.pdf (Accessed: 06 March 2024).

After measuring DNA concentration using the Qubit4 fluorometer (mentioned in section 3.5.1), dilution of DNA using nuclease-free water was done to standardise the DNA concentrations and to have an end DNA concentration of 20ng. The next step was to set up the instrument and reagents. The Ion AmpliSeq™ Chef reagents DL8 cartridge (figure 43 -1) and the Ion AmpliSeq™ DNA primer pools were left to thaw at room temperature for about 20 minutes. It was ensured that the Ion Chef™ instrument and the Torrent Server were connected. The following step was to create a 'Sample Set', which enables tracking of samples on the Torrent Server from library and template preparation through chip loading, sequencing and data analysis. The Sample Set was created by logging in to the Torrent Server using the username and password used at the Haematology Molecular Laboratory. All the run details were inputted including sample codes, sample PCR plate position and also the Myeloid library preparation protocol. After inputting all the required details, the 'Sample Set' was saved. The next step was to set-up the Ion Chef™ system. All the 4 tubes in positions A, B, C and D (figure 44) in the Ion AmpliSeq™ Chef reagents DL8 cartridge were uncapped.



① Position A (150 µL Primer Pool 1 at 2X concentration)
② Position B (150 µL Primer Pool 1 or 2 at 2X concentration)
③ Position C (Empty tube)
④ Position D (Output tube)

Figure 44: Ion AmpliSeq™ Chef reagents DL8 cartridge. Retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013432_Ion_AmpliSeq_Library_Prep_on_Ion_Chef_UG.pdf (Accessed: 06 March 2024).

Primer panels were added to the Ion AmpliSeq™ Chef Reagents DL8 cartridge. The primer pool tubes were mixed by vortexing and centrifugation. The caps were removed, then the tubes in Positions A (Tube 1 of 2) and B (Tube 2 of 2) of the Ion AmpliSeq™ Chef Reagents DL8 cartridge were replaced with Chef-ready panel tubes. The tube in Position D contained 700µL of combined barcoded libraries at completion of the run.

The next step was to load the Ion Chef™ instrument which was loaded as seen in figure 45.

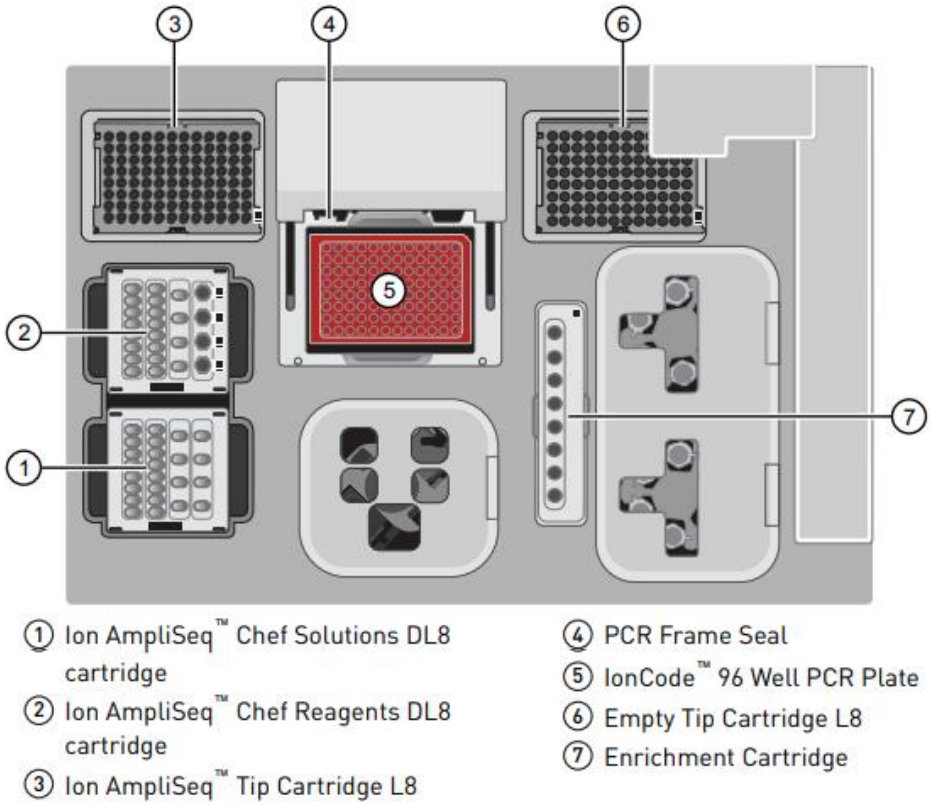


Figure 45: Loading the Ion Chef™ instrument. Retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0013432_Ion_AmpliSeq_Library_Prep_on_Ion_Chef_UG.pdf (Accessed: 06 March 2024).

In the IonCode™ plate (figure 45 – 5), 15µl of each sample were pipetted in positions A1 till H1. Care was taken that no cross over between samples occurred, in order to prevent cross-contamination between samples. After all the reagents and consumables were loaded, the instrument door was closed and the Ion Chef™ instrument was

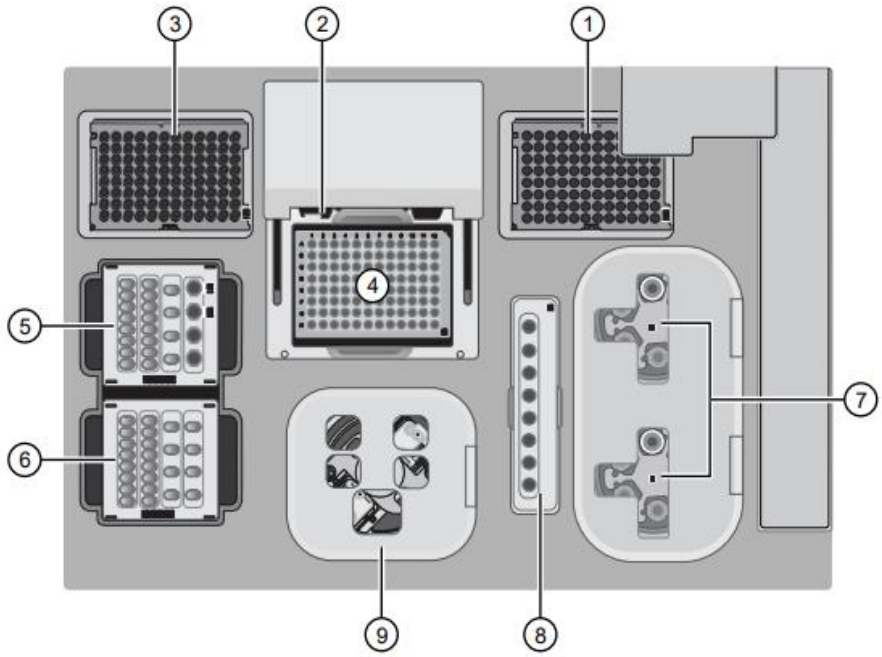
switched on. From the touchscreen, 'Set up run' was selected followed by 'Library Preparation'. The required checks were carried out and the run was started. Library preparation took about 8 hours to complete. The tube containing the combined library at approximately 100pM concentration was removed from Position D of the reagent's cartridge. All the used consumables were removed from the instrument. Cleaning of the instrument was then carried out by selecting the clean cycle on the instrument.

3.5.3 Templating using Ion Chef™

The next step was templating in preparation for sequencing. During templating, the library was settled in a semi-conductor microchip so that it could be further amplified. Templating was carried out using the Ion 530™ kit on the Ion Chef™ instrument. The Ion 530™ kit contained reagents and consumables for both templating and sequencing. The reagents and consumables used for templating included a chip adapter, enrichment cartridge, tip cartridge, PCR plate, PCR plate frame, frame seal, recovery station disposable lid and recovery tubes. The Ion 530™ Chip kit was also required for templating.

The first step of templating was to create a Planned Run. This was done by logging in the Torrent Server using the username and password used at the Haematology Molecular Lab. All the required data was inputted including kit data, library data and samples data. The planned run was then saved. The following step was to dilute the library, which was previously prepared to a concentration of 100pM, using nuclease-free water. The diluted library was then added to the Ion Chef™ library sample tube by pipetting 25µL of the diluted library in the tube. The library sample tube was then capped and stored on ice until it was ready to be loaded on the Ion Chef™ instrument.

After library dilution, the consumables from the Ion 530™ kit were prepared. The Ion 530™ Chef reagents cartridge was allowed to warm to room temperature for 45 minutes before used. The Ion Chef™ instrument was then loaded as can be seen in figure 46.



① Empty tip rack (move from new Tip Cartridge position)
② Frame Seal v2
③ New Tip Cartridge
④ PCR Plate and PCR Plate Frame
⑤ Ion 540™ Chef Reagents cartridge
⑥ Ion S5™ Chef Solutions cartridge
⑦ Recovery Tubes and Recovery Station Disposable Lid v2
⑧ Enrichment Cartridge v2
⑨ Chip Adapter/Chip assemblies

Figure 46: Loading the Ion Chef™ instrument for templating. Retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0010851_Ion_540_Kit_Chef_UG.pdf. (Accessed: 13th March, 2024).

First, the pipette tip racks and PCR plates were loaded, followed by loading of reagents and solution cartridges. The library sample tube was then uncapped and loaded onto positions A and B on the Reagents cartridge as seen in figure 47.

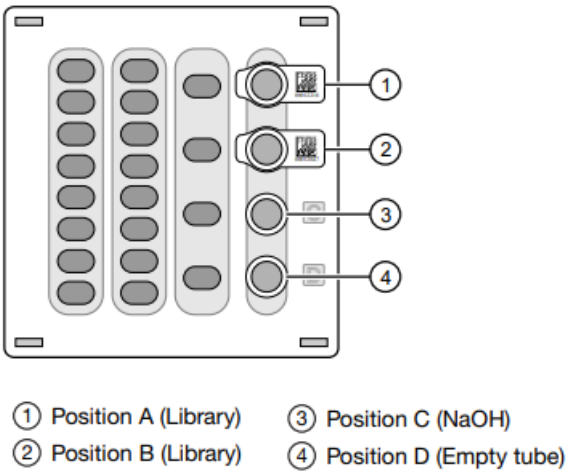


Figure 47: Loading the Library Sample Tube. Retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0010851_Ion_540_Kit_Chef_UG.pdf. (Accessed: 13th March, 2024).

The recovery tubes and enrichment cartridge were then loaded by inserting the Ion Chip™ in the Chip-loading centrifuge. It was first loaded on the Chip bucket and adapter as seen in figure 48, and then on the centrifuge.

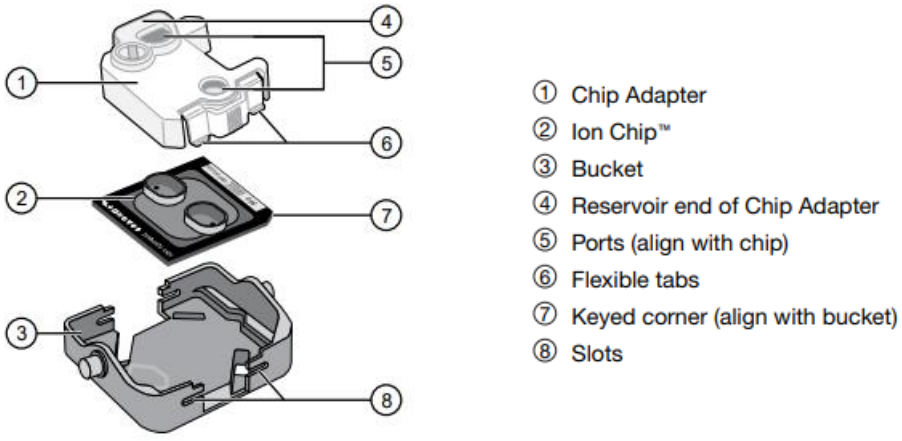


Figure 48: Loading the Ion Chip™ on the bucket and adapter. Retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0010851_Ion_540_Kit_Chef_UG.pdf. (Accessed: 13th March, 2024).

After confirming that the consumables were correctly installed, the Ion Chef™ run was started and took about 18 hours to complete. The Ion Chip™ was then unloaded for sequencing and cleaning of the Ion Chef™ instrument was then carried out.

3.5.4 Sequencing using Ion GeneStudio S5 System

After templating, the Ion Chip™ was then loaded onto the Ion S5™ sequencer and a sequencing run was initiated. During sequencing, the sequence of each amplicon in the library was read and data was transmitted for computational analysis. Figure 49 shows the Ion GeneStudio S5 system.

① Touchscreen
② Power button
③ Ion S5™ Sequencing Reagents cartridge
④ Chip clamp
⑤ Ion S5™ Wash Solution bottle. Waste reservoir located behind the Ion S5™ Wash Solution bottle (shown on the right).
⑥ Ion S5™ Cleaning Solution bottle
⑦ Waste reservoir

Figure 49: Ion GeneStudio S5 system. Retrieved from: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0010851_Ion_540_Kit_Chef_UG.pdf. (Accessed: 27th March, 2024).

The Ion S5™ Sequencing reagents cartridge was unboxed 45 minutes prior use, allowing it to reach room temperature. The wash solution bottle was unboxed and mixed thoroughly by inverting it 5 times within its bag which was vacuum-sealed. The sequencer was initialised by touching the touchscreen main menu and tapping **Initialize**. The door, chip and reagent cartridge clamps were unlocked. The wash solution bottle was removed when prompted in order to be able to remove and empty the waste reservoir. The empty waste reservoir was then reinstalled. The previously used Ion

S5™sequencing reagent cartridge was replaced with a new cartridge. The wash solution bottle was thoroughly mixed again and installed, as was the chip. Once all the consumables and chip were properly installed, the instrument was ready for the sequencing run. The previously prepared 'Planned Run' was selected in the **Run Selection** tab and the run was started. The sequencing run took about 5 hours followed by another 12 hours data uploading.

3.5.4.1 Principle of Ion Torrent Sequencing

Sequencing of DNA was done using a semi-conductor chip. Chemically encoded information (A, C, G or T) was translated into digital information (0 or 1) or base calls on the semiconductor chip. A DNA sample was first cut into millions of fragments. Each DNA fragment then attached to a bead and was copied to cover the whole bead. Millions of beads were covered with millions of different DNA fragments. The beads then deposited into a well on the semiconductor chip, which was then flooded with nucleotides by the Ion Torrent™ next-generation sequencer to start base calling. A hydrogen ion was then released whenever a nucleotide was incorporated into a single strand of DNA. This resulted in a change in the pH of the solution of the well due to the charge released from the hydrogen ion. The ion sensors beneath the well then measured the change in pH and converted it into voltage. The voltage was then recorded, indicating that the base was called and chemical information was converted to digital information. The process was repeated every 15 seconds with a different nucleotide and occurred in millions of wells at the same time, hence why it is called massively parallel sequencing (Thermo Fisher, 2023).

3.5.5 Data Analysis using Ion Reporter™ Software

The Ion Reporter™ software was then used to analyse the data. The first step when analysing data was to check the quality control (QC) checkpoints. If any of the QC points failed, the run would not be valid so data interpretation was not carried out. QC checkpoints included checking the colour of the Ion Chip™ (red: passed, yellow/green/blue: low quality DNA), the total number of reads (15-20M), polyclonality (<45%), low quality reads (<20%), mean raw accuracy (>99%). Other quality checkpoints included checking every sample quality i.e. checking uniformity (>97%), mean read base pair length (>200) and total reads per sample (>1.4M). After ensuring that all QC criteria were up to standard, data analysis was carried out. Any gene variants detected in the sequences were matched with databases of known relevant biomarkers. The Ion Reporter™ software supported the analysis of data from the Ion GeneStudio™ S5 series instrument. Detection of variants and limit of detection were dependent on the percentage of disease progression, DNA input concentration and DNA quality. A minimum depth variance allele frequency (VAF) of 5% was required for variant calling. The sensitivity for oncology hotspot control variants included in the assay was >99%, with a positive predictive value (PPV) of 100%. During data analysis, any mutations detected were reported including the VAF.

3.6 Statistical Analysis

The data collected was analysed using IBM SPSS Statistics, version 29 (IBM Corporation, USA). Statistical analysis was carried out with the help of Prof. Liberato Camilleri, Professor of Statistics and Operations and Research at the University of Malta. Some descriptive statistics including graphical representations were also generated

using Microsoft Excel. The sample size of the research study was a total of 53 BM samples collected through a year period starting January 2023 to January 2024. The most appropriate statistical analysis were non-parametric tests.

A level of significance of 0.05 was used for hypothesis testing. When a probability value (p-value) exceeded the 0.05 level of significance, the null hypothesis (H_0) was accepted. When the p-value was lower than 0.05, the alternative hypothesis (H_1) was accepted. The following statistical tests were carried out:

- Mann Whitney test to compare the percentage leukaemic stem cell results obtained from Cohort A and Cohort B.
- Mann Whitney test to compare the number of mutations which were detected in Cohort A and Cohort B.
- Chi-squared test to compare the different mutations detected in Cohort A and Cohort B.
- Kappa test to investigate agreement between immunophenotypic aberrations, that is LSC detected by Flow Cytometry and Molecular aberrations identified by NGS.
- Difference of two proportions test to compare cytopenia prevalence, that is if low erythrocytes, neutropenia or thrombocytopenia.

Chapter 4

Results

4.1 Patient Demographics

4.1.1. Gender frequency in Cohort A and Cohort B

In this study, the male gender was found to be more common than the female gender in both Cohort A and Cohort B. This is represented in the bar chart in figure 50 below. In Cohort A, 22 out of 30 patients (73%) were males, whilst only 8 patients (27%) were females. In Cohort B, 16 out of 23 patients (70%) were males and only 7 patients (30%) were females.

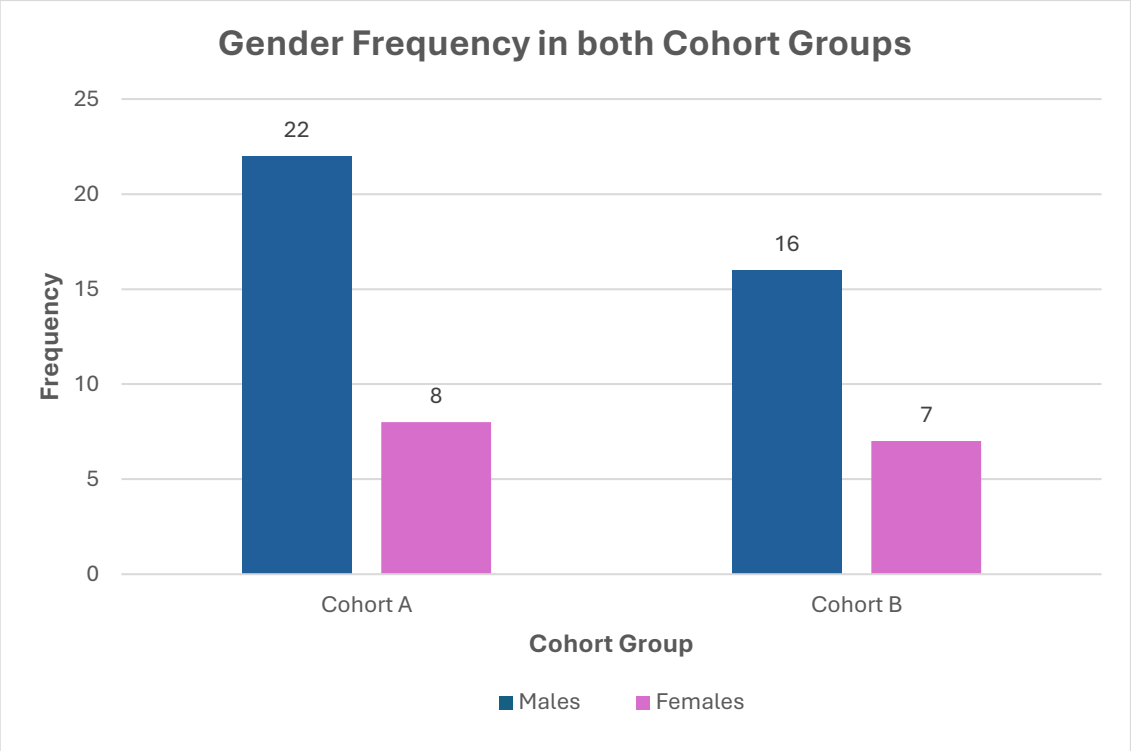


Figure 50: Gender frequency in Cohort A and Cohort B.

4.1.2 Patient demographics of Cohort A

All patient demographics of Cohort A are tabulated in table 13. The mean age of patients was 69 years (range 25-88 years). The majority of patients were non-smokers (87%). More than half of the patients were taking medications (57%) and only 13% of

patients had recent infections. Most of the patients (87%) did not have any history of chemotherapy or radiotherapy, although 13% of patients had received such therapies for other cancers. Some patients (33%) had other underlying medical conditions including diabetes (3 patients), hypertension (4 patients), thyroid problems (1 patient), heart problems (2 patients) and others (4 patients). The majority of patients (67%) did not have any other medical conditions whilst being investigated for MDS.

Variable	Result / Mean ±SD
Age (Years)	69 ± 14
Gender	
Male	22 (73%)
Female	8 (27%)
Smokers	
Yes	4 (13%)
No	26 (87%)
Medications	
Yes	17 (57%)
No	13 (43%
Recent Infections	
Yes	4 (13%)
No	26 (87%)
History of Chemotherapy/Radiotherapy	
Yes	4 (13%)
No	26 (87%)
Other Medical Conditions	
Diabetes	3
Hypertension	4
Thyroid Problems	1
Heart Problems	2
Others	4
No	20

Table 13: Patient demographics of Cohort A

4.1.3 Patient Demographics of Cohort B

All patient demographics of Cohort B are tabulated in table 14. The mean age of patients was 69 years (range 42-85 years). Most patients were non-smokers (83%). More than half of the patients were taking medications (65%) and only 13% of patients had recent infections. Most of the patients (87%) did not have any history of chemotherapy or radiotherapy, but 13% of patients had. Some patients (26%) had other medical conditions including diabetes (2 patients), hypertension (2 patients), thyroid problems (2 patients) and others (1 patient). Most patients (74%) did not have any other medical conditions.

Variable	Result / Mean ±SD
Age (Years)	69 ± 12
Gender	
Male	16 (70%)
Female	7 (30%)
Smokers	
Yes	4 (17%)
No	19 (83%)
Medications	
Yes	15 (65%)
No	8 (35%)
Recent Infections	
Yes	3 (13%)
No	20 (87%)
History of Chemotherapy/Radiotherapy	
Yes	3 (13%)
No	20 (87%)
Other Medical Conditions	
Diabetes	2
Hypertension	2
Thyroid Problems	2
Others	1
No	17

Table 14: Patient demographics of Cohort B

4.1.4 Cytopenia Categories in Cohort A

Persistent cytopenia was the main criterion for patient recruitment in Cohort A. Table 15 shows the type of cytopenia in each patient and was obtained from the CBC results.

Patient Number	Type of Cytopenia
A1	↓ RBCs
A2	↓ Neutrophils
A3	Pancytopenia
A4	↓ RBCs
A5	↓ RBCs
A6	Pancytopenia
A7	Pancytopenia
A8	↓ Neutrophils
A9	Pancytopenia
A10	↓ RBCs
A11	Pancytopenia
A12	↓ RBCs
A13	↓ RBCs
A14	↓ RBCs, low platelets
A15	↓ Neutrophils
A16	↓ Platelets
A17	↓ RBCs, low platelets
A18	↓ RBCs
A19	↓ RBCs, low platelets
A20	↓ RBCs, low platelets
A21	↓ Neutrophils
A22	↓ RBCs
A23	Pancytopenia
A24	↓ RBCs
A25	↓ RBCs, low platelets
A26	↓ RBCs
A27	↓ RBCs
A28	Pancytopenia
A29	Pancytopenia
A30	Pancytopenia

Table 15: Type of Cytopenia in Cohort A Patients from CBC Results.

Figure 51 shows the different cytopenia categories and their frequency. Decreased red blood cells (RBCs) was the most common cytopenia and was present in 24 out of 30 patients (80%). Thrombocytopenia was the second most common cytopenia and it was present in half of the patients (50%). The least common cytopenia was neutropenia and was present in 12 out of 30 patients (40%).

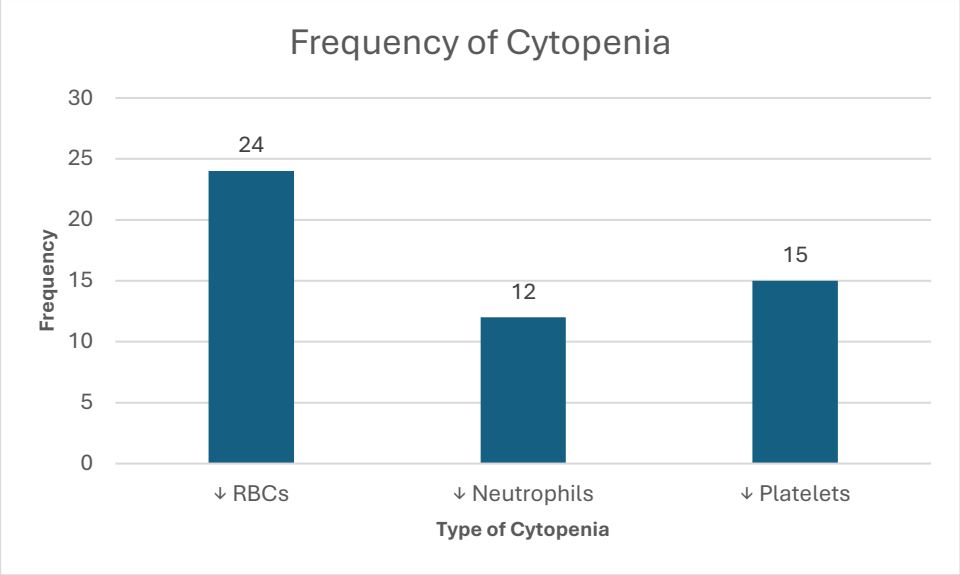


Figure 51: Different cytopenia categories and their frequency.

A difference of two proportions test was carried out to compare cytopenia prevalence between the three different cytopenia categories. The low RBCs category was significantly more prevalent than the other categories.

Difference of Two Proportions Test

		Frequency	Percentage
Cytopenia	Low RBCs	25	83.3%
	Low Neutrophils	13	43.3%
	Low Platelets	15	50.0%

Table 16: Difference of Two Proportions Test.

The percentage difference between Low RBC's and Low Platelets was 33.3% and was found to be statistically significant (z=3.215, p=0.001). Similarly, the percentage

difference between Low RBC's and Low Neutrophils was 40.0% and was also found to be statistically significant (z=2.739, p=0.006). Alternatively, the percentage difference between Low Neutrophils and Low Platelets was 6.7% and was not found to be statistically significant (z=0.518, p=0.603).

4.1.5 Symptoms of Patients in Cohort A and Cohort B

Figure 52 is showing the symptoms experienced by patients from both Cohort A and Cohort B. The most common symptom was fatigue and it was observed in 16 patients from Cohort A and in 17 patients from Cohort B. The second most common symptom was shortness of breath followed by bleeding and bruising, fever and frequent infections. Since these clinical findings are commonly experienced by patients with acute leukaemia, all patients of Cohort B had one or more of these symptoms.

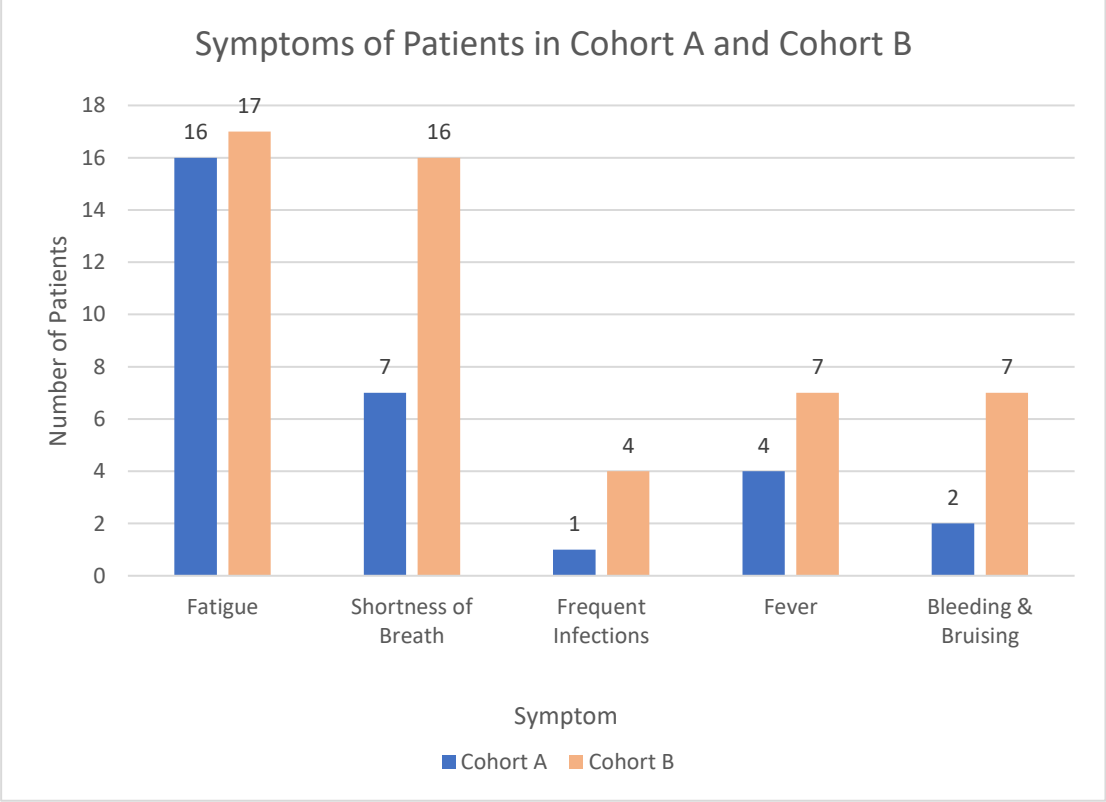


Figure 52: Patient symptoms in Cohort A and Cohort B.

4.2 Leukaemic Stem Cells Results

4.2.1 Leukaemic stem cell results in Cohort A

The expression of each leukaemic stem cell marker was analysed separately for all patients, and the leukaemic stem cell percentages for each antigen was determined. The raw data can be found in Appendix D. Leukaemic stem cells were detected in 60% of patients from Cohort A. The bar charts below represent the percentage for each individual LSC marker for all patients in Cohort A.

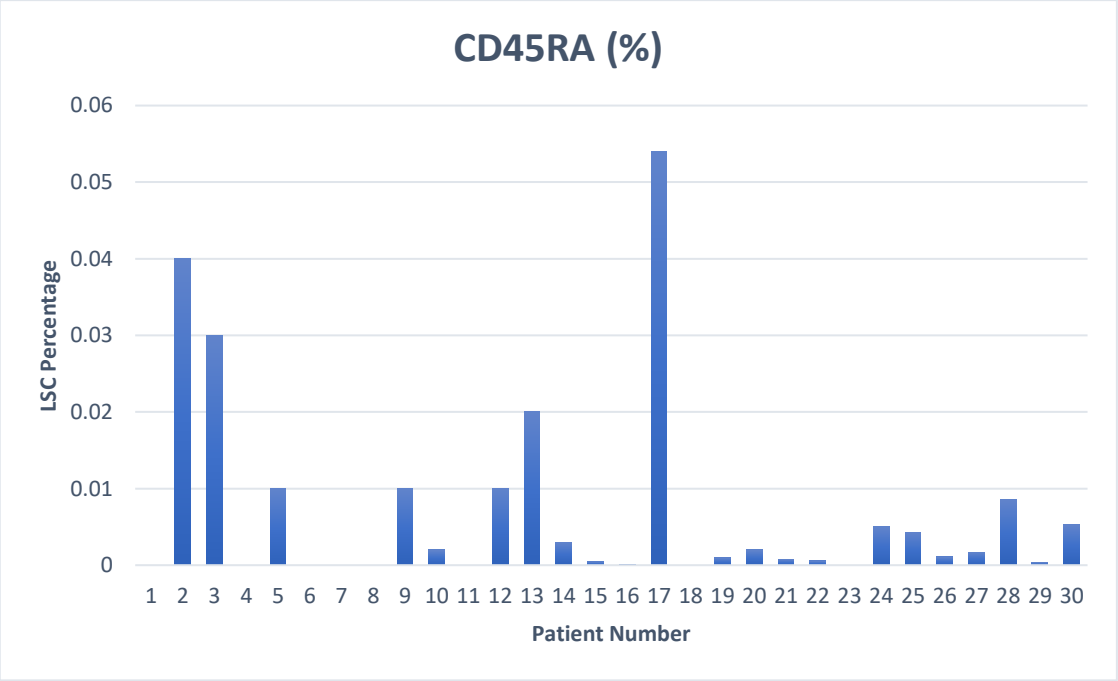


Figure 53: CD45RA expression in all patients of Cohort A.

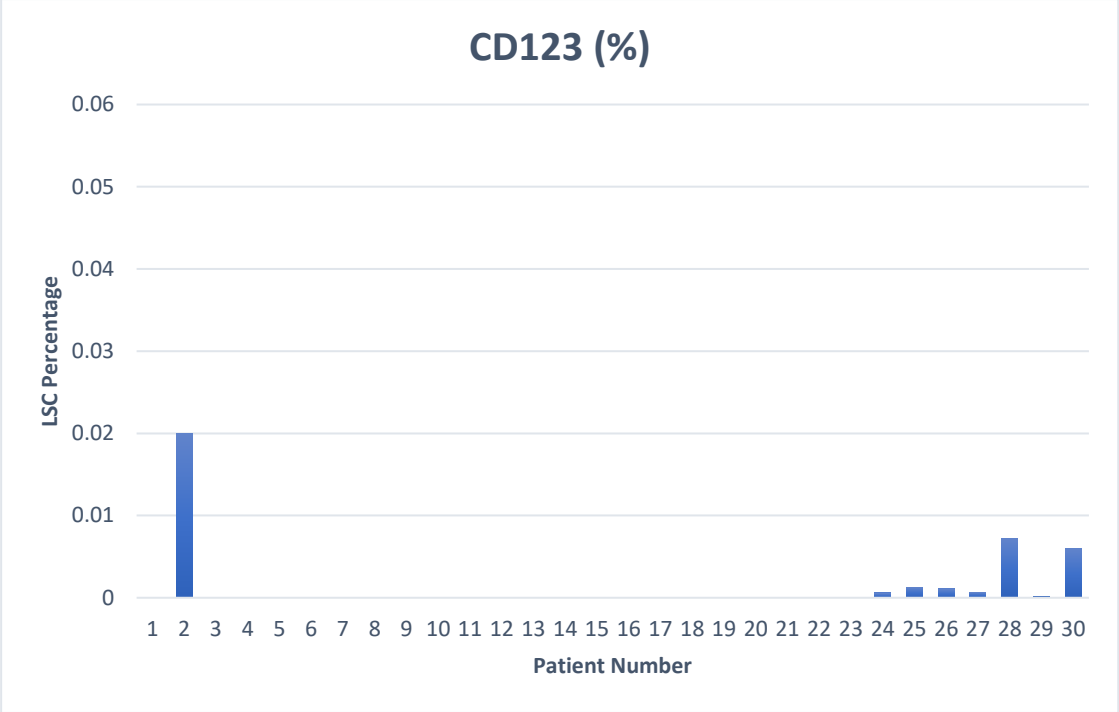


Figure 54: CD123 expression in all patients of Cohort A.

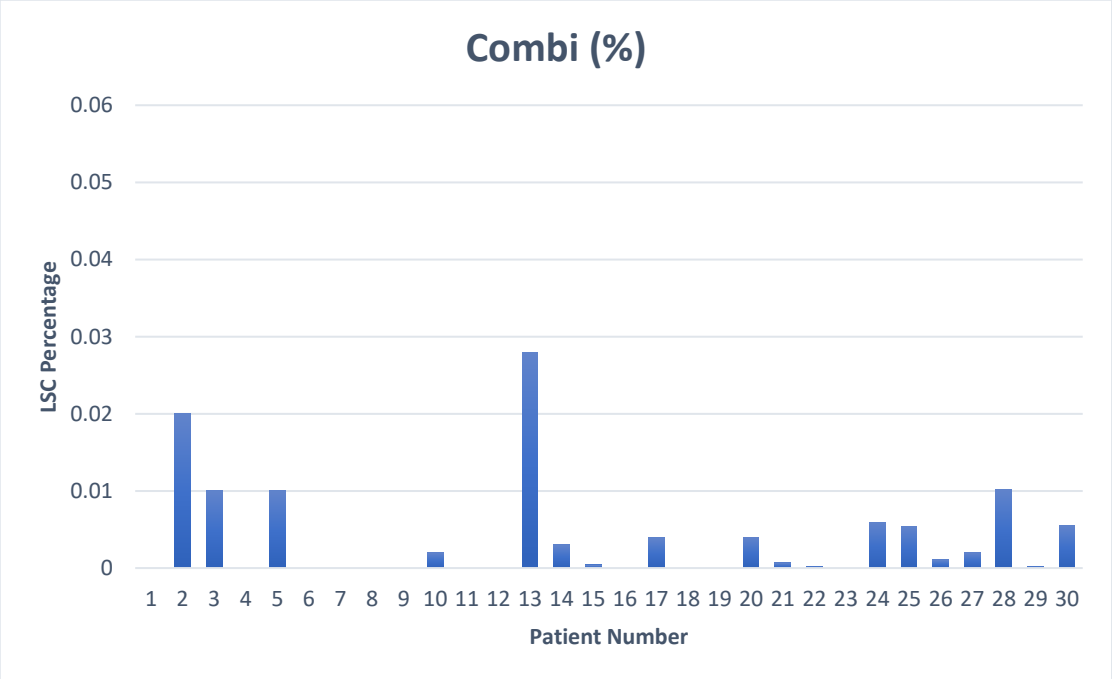


Figure 55: Expression of CD markers in the Combi channel (CLec12a, TIM-3, CD7, CD11b, CD22 and CD56) in all patients of Cohort A.

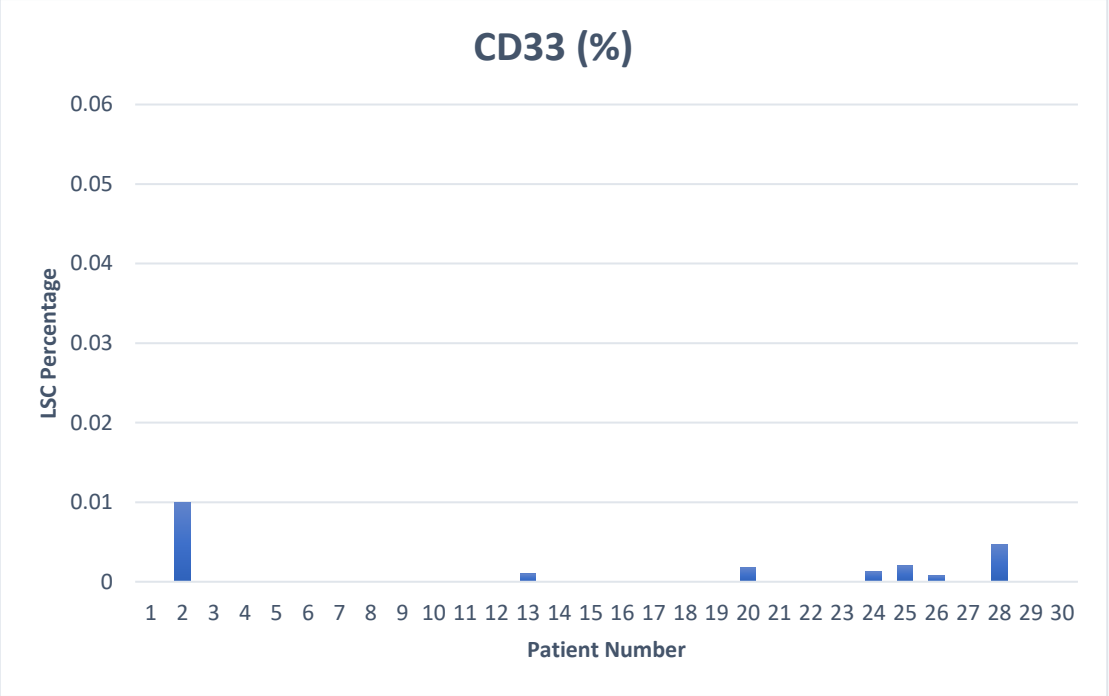


Figure 56: CD33 expression in all patients of Cohort A.

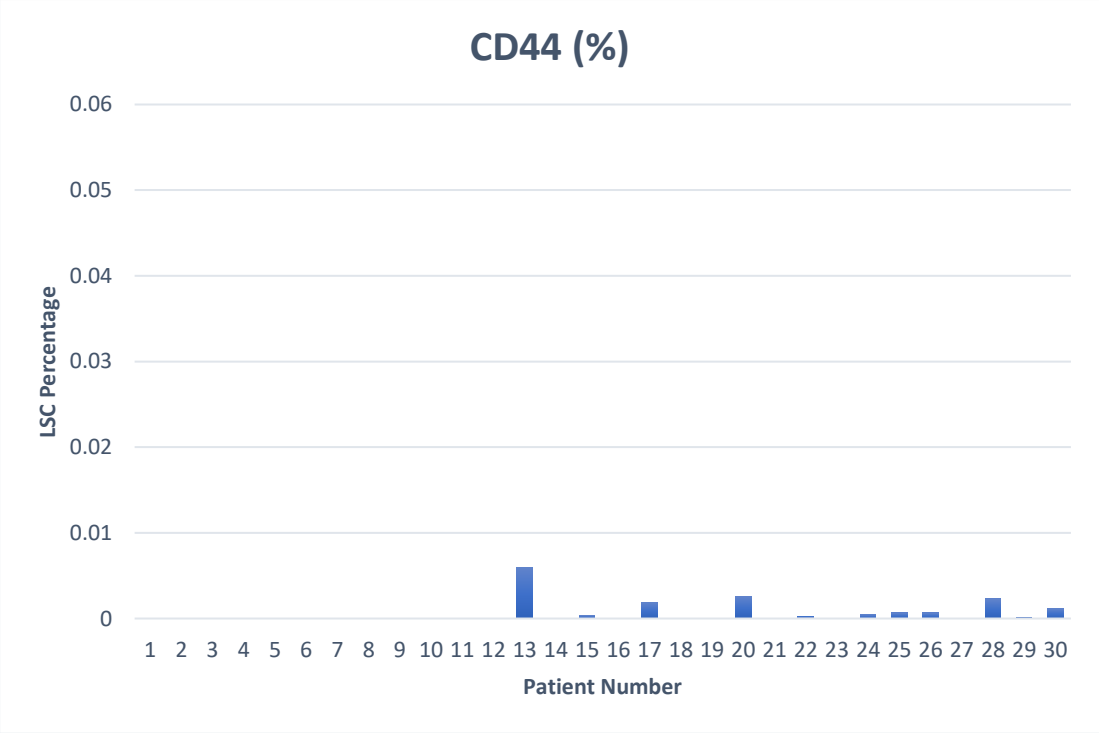


Figure 57: CD44 expression in all patients of Cohort A.

4.2.2 Leukaemic stem cell results in Cohort B

Leukaemic stem cells were found to be present at higher percentages in patients from Cohort B. This was expected as Cohort B (the positive control group) was composed of patients presenting with Acute Myeloid Leukaemia (21 patients) and HR-MDS (2 patients). Out of the 21 AML patients, 7 had a CD34 negative myeloid leukaemia with presence of LSCs. Overall, leukaemic stem cells were detected in 91% of patients from Cohort B. The bar charts below represent the percentage for each individual LSC marker for all patients in Cohort B.

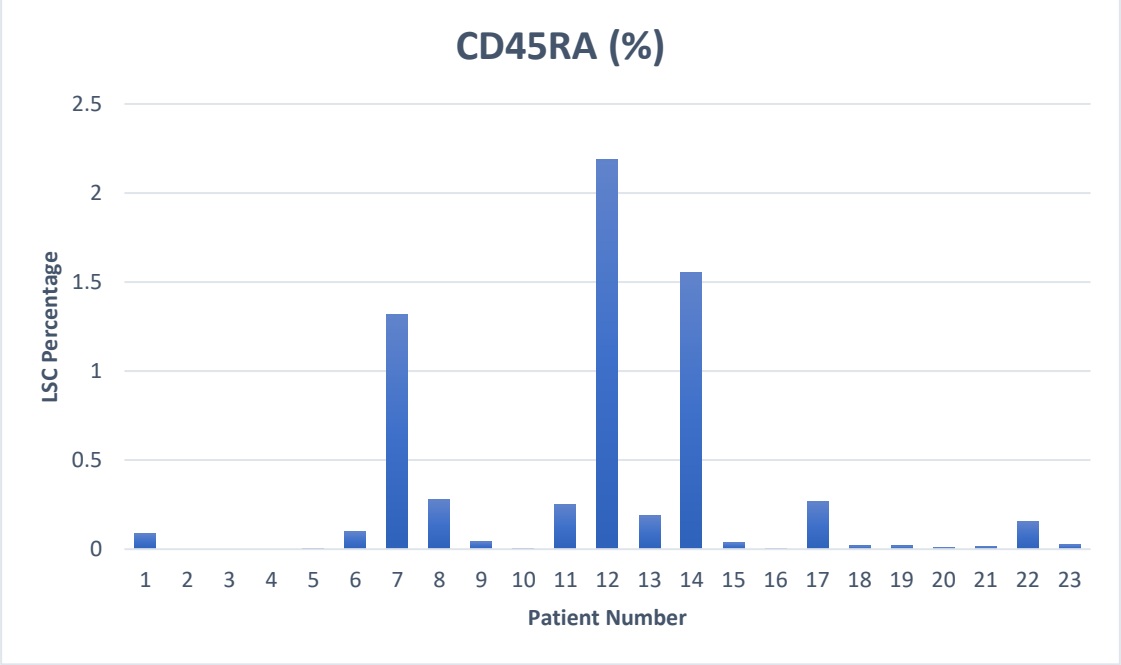


Figure 58: CD45RA expression in all patients of Cohort B.

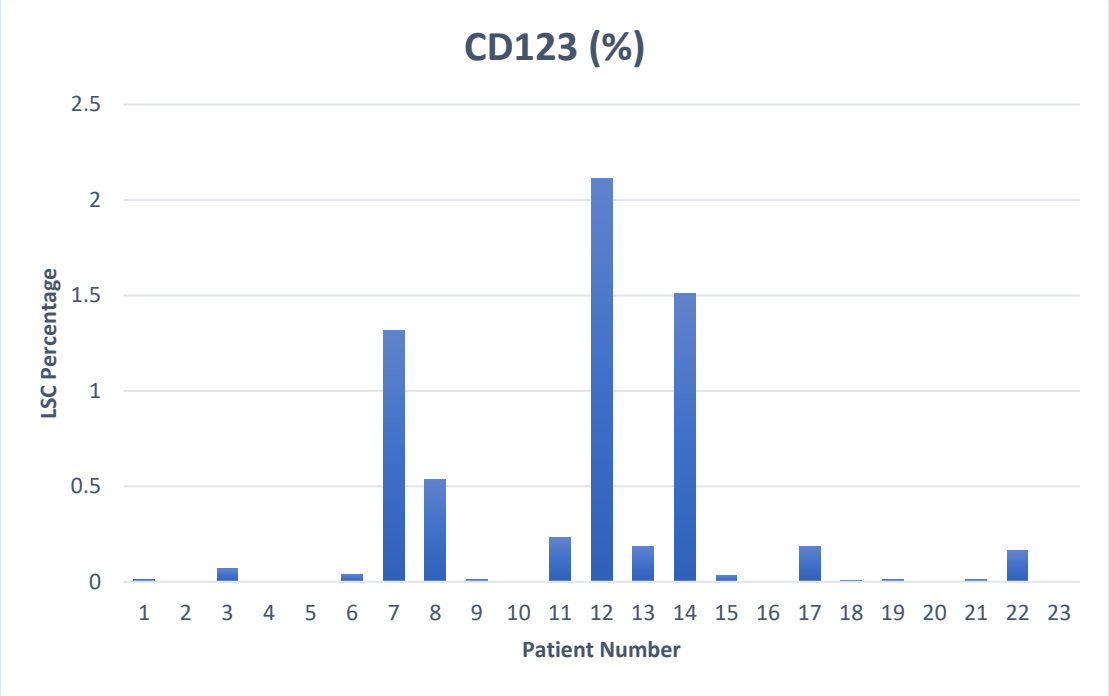


Figure 59: CD123 expression in all patients of Cohort B.

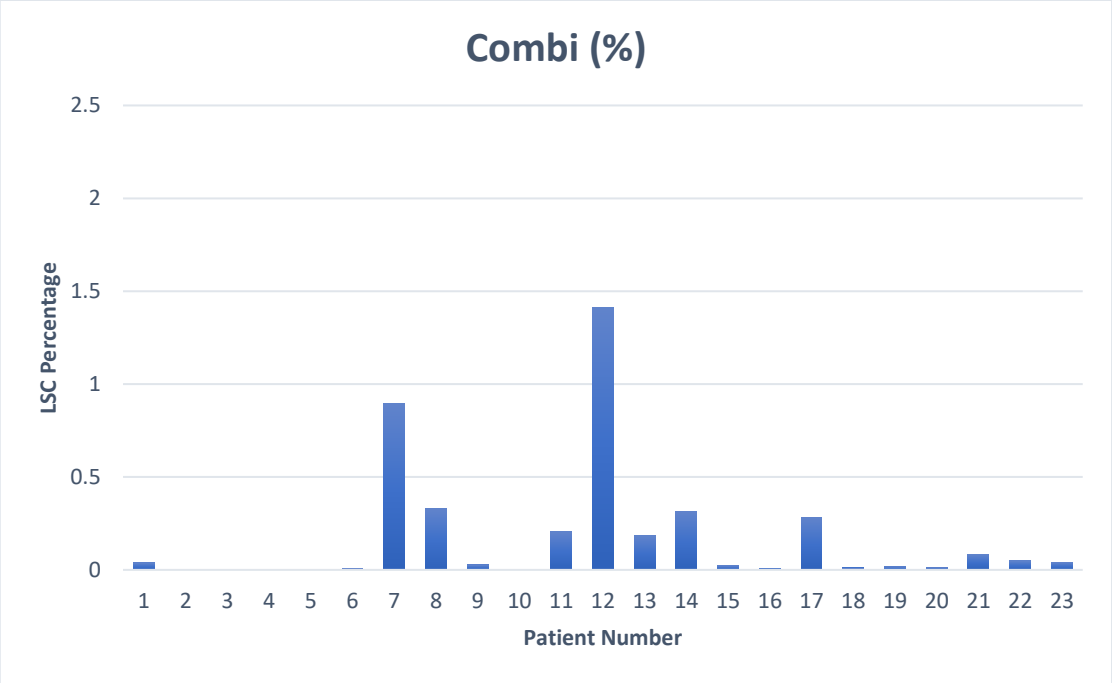


Figure 60: Expression of CD markers in the Combi channel (CLec12a, TIM-3, CD7, CD11b, CD22 and CD56) in all patients of Cohort B.

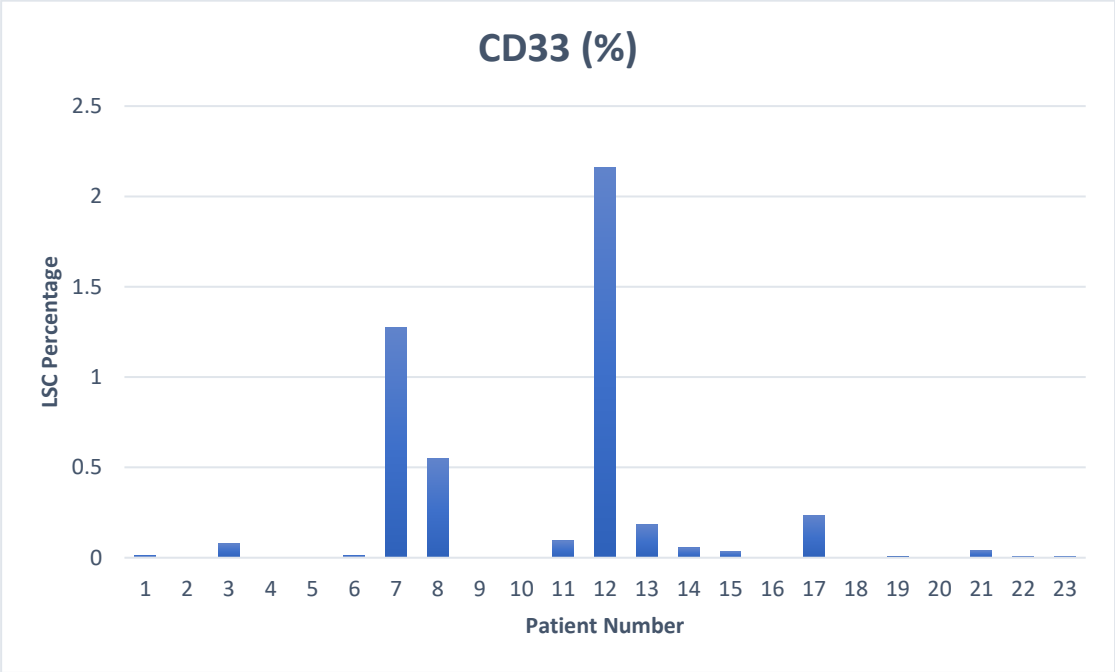


Figure 61: CD33 expression in all patients of Cohort B.

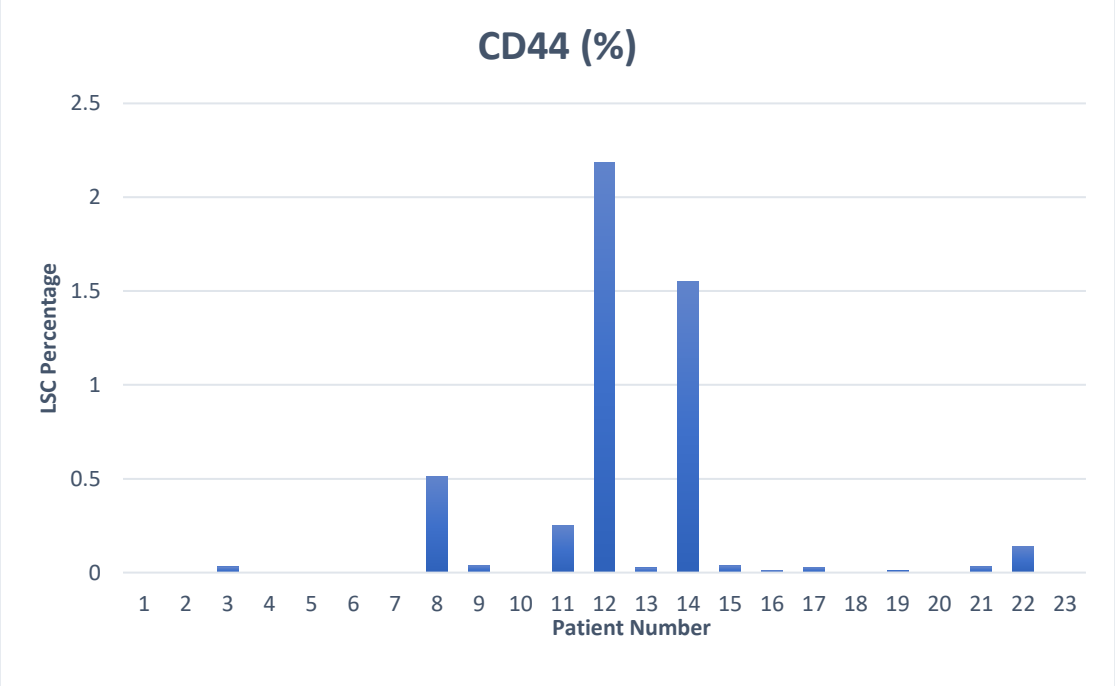


Figure 62: CD44 expression in all patients of Cohort B.

4.2.3 Comparing LSC Results in Cohort A and Cohort B

4.2.3.1 Box and Whisker Plots

Box and whisker plots were generated for Cohort A and Cohort B separately. The box and whisker plots are confirming the finding that leukaemic stem cells were found at higher percentages in Cohort B. In Cohort A, the most common leukaemic stem cell markers identified were CD45RA and markers in the Combi channel. These markers are considered to be the most relevant markers to differentiate between HSC and LSC and are usually absent on the stem cell compartment of healthy individuals. The other antigens CD123, CD33 and CD44 were found at very low percentages in Cohort A.

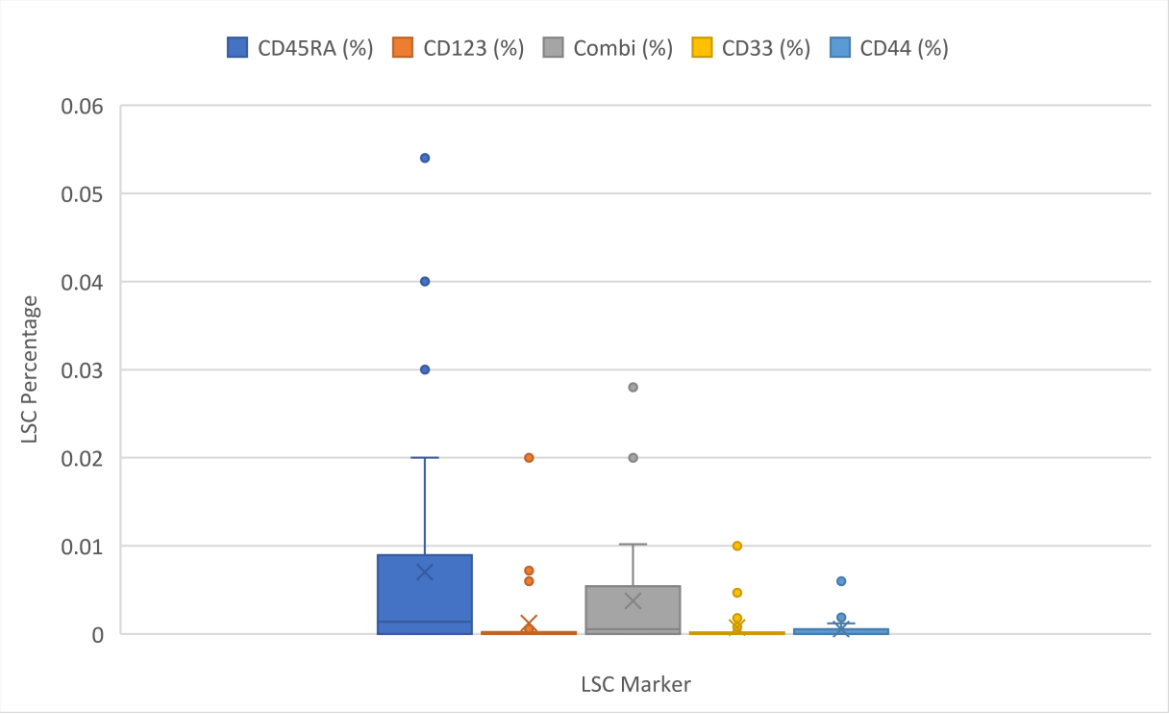


Figure 63: Box and Whisker Plot for Cohort A.

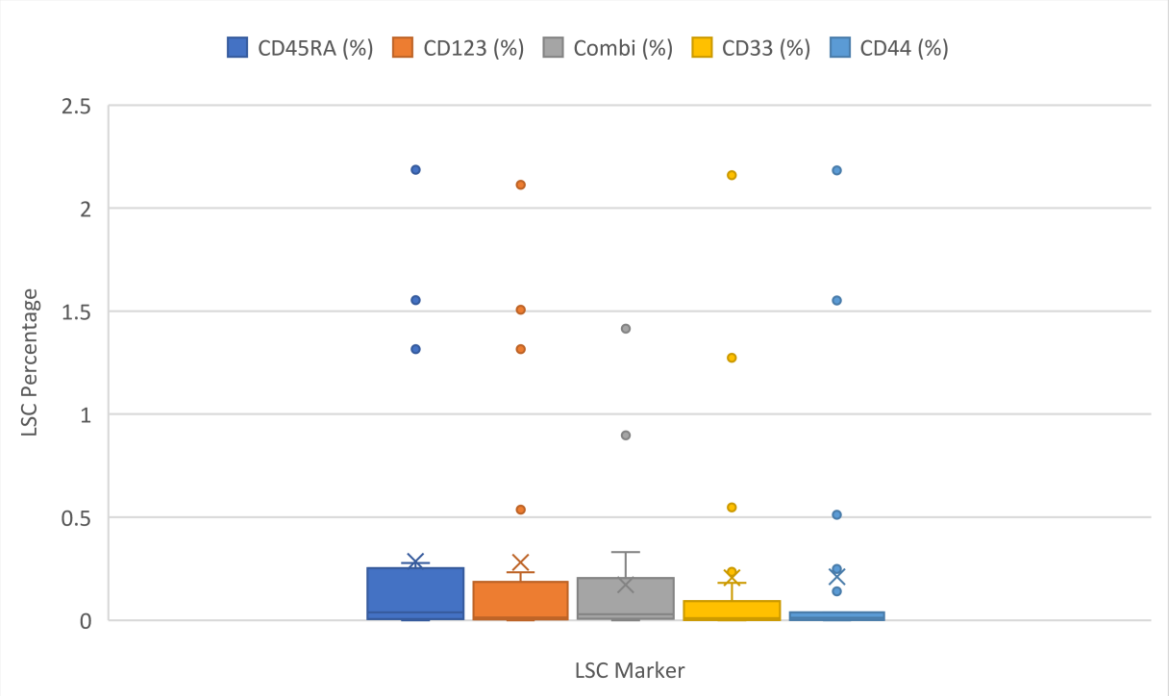


Figure 64: Box and Whisker plot for Cohort B.

4.2.3.2 Bar Graph comparing percentage of patients with LSCs in Cohort A and Cohort B.

The bar graph below is illustrating the percentage of patients expressing each leukaemic stem cell marker for both cohort groups. In Cohort A, the leukaemic stem cell markers with the higher percentages were CD45RA and markers in the Combi channel. The other markers CD33, CD123 and CD44 were found at lower percentages. In Cohort B, the positive control group, all leukaemic stem cell markers were detected in almost all patients. Such expected finding, provides assurance that the methodology for the identification of LSC, has been correctly validated.

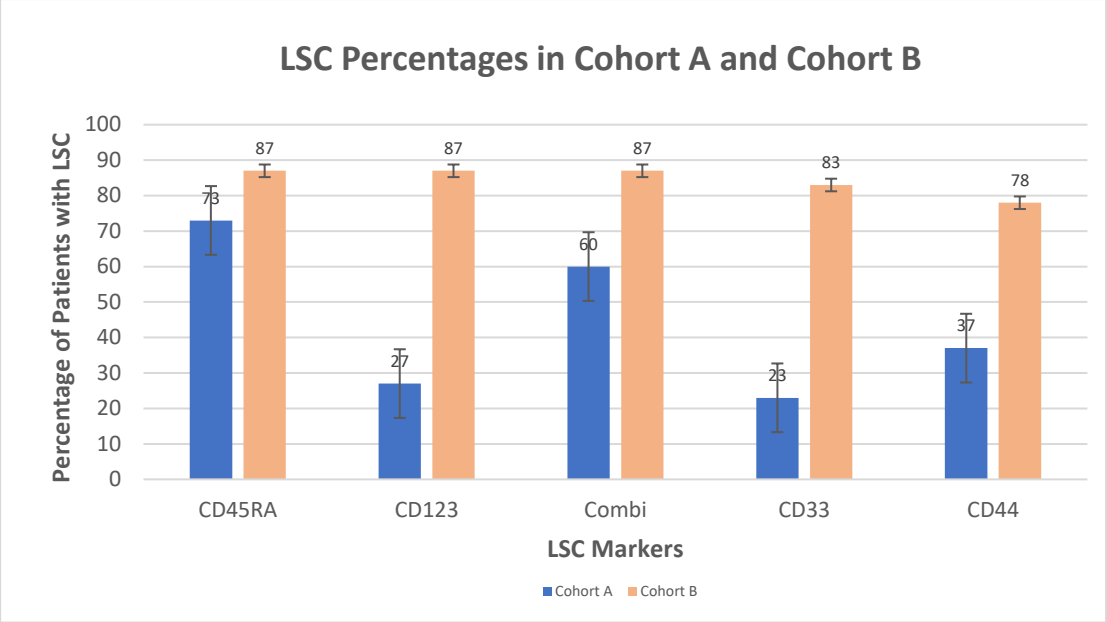


Figure 65: LSC percentages is Cohort A and Cohort B.

4.2.3.3 Comparison between leukaemic stem cell results in Cohort A and Cohort B.

The Mann Whitney test is a non-parametric test used to compare sample means. It was used to compare the average percentages of the leukaemic stem cell markers in Cohort A and Cohort B. For each leukaemic stem cell marker, the average percentage of Cohort B was significantly higher than the percentage of Cohort A since the p-value was <0.001 which is smaller than the 0.05 level of significance. All leukaemic stem cell markers were found at higher percentages in Cohort B than in Cohort A.

Mann Whitney Test

	Cohort	N	Mean	Std. Deviation	P-value
CD45RA	A	30	0.0070	0.0129	<0.001
	B	23	0.2859	0.5775	
CD123	A	30	0.0012	0.0039	<0.001
	B	23	0.2805	0.5683	
Combi	A	30	0.0038	0.0065	<0.001
	B	23	0.1725	0.3372	
CD33	A	30	0.0007	0.0020	<0.001
	B	23	0.2066	0.5098	
CD44	A	30	0.0006	0.0013	<0.001
	B	23	0.2111	0.5434	

Table 17: Mann Whitney Test comparing LSC results of Cohort A and Cohort B.

4.2.3.4 Heat Maps comparing leukaemic stem cell results in Cohort A and Cohort B.

Heat maps were generated for leukaemic stem cell results in Cohort A (left) and Cohort B (right). The colours of the heat maps are indicating the percentage of the leukaemic stem cell markers with the green colour being 0% and the red colour representing the largest percentage in each cohort group. Most of the patients of Cohort B had high percentages for all leukaemic stem cell markers. On the other hand, in Cohort A, not all patients had expression of all the leukaemic stem cell markers identified within the stem cell compartment. The mean percentage of LSCs in Cohort A was 0.0027% (standard deviation of 0.00711) and in Cohort B was 0.2313% (standard deviation of 0.50771).

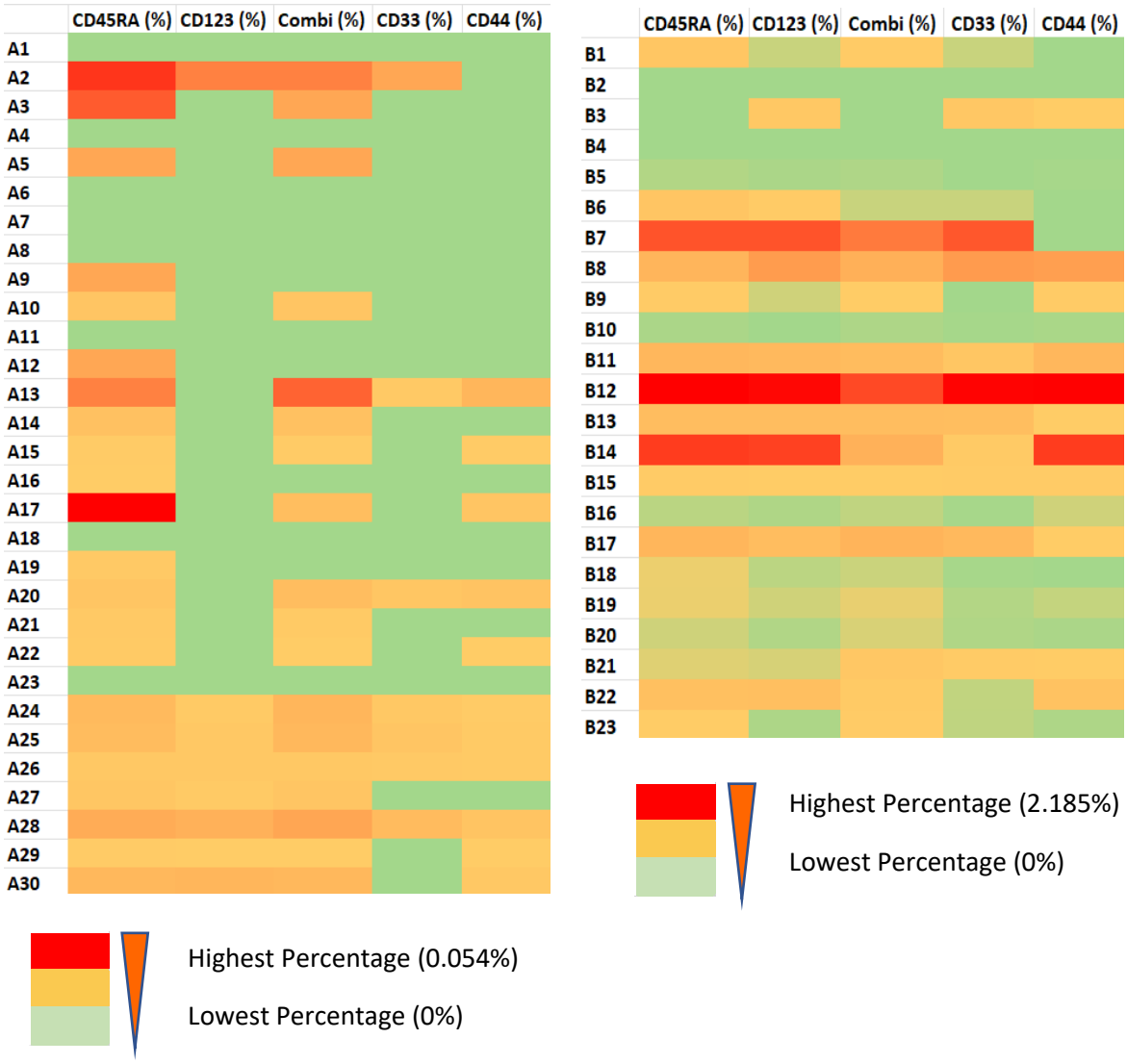


Figure 66: Heat maps representing LSC result in Cohort A and Cohort B.

4.3 Molecular Results

The data generated by NGS was analysed using the Ion Reporter™ software. The results gathered included mutated myeloid genes together with their variance allele frequency (VAF). Statistical analysis was then carried out using this data.

4.3.1 Chi Square Test comparing different mutations in Cohort A and Cohort B.

The Chi Square test was used to investigate the association between the mutations identified and the cohort group, that is Cohort A and Cohort B. The association was found to be statistically significant since the p-value (<0.001) was smaller than the 0.05 level of significance. This implies that some mutations were found to be more prevalent in Cohort A, while other mutation were more prevalent in Cohort B. Mutations which were more prevalent in Cohort A include *ASXL1*, *RUNX1*, *U2AF1* and *EZH2*. Mutations which were more prevalent in Cohort B include *FLT3*, *NPM1, IDH1, IDH2* and *TP53*. Some mutations were found to be commonly mutated in both cohort groups such as *TET2*, *NRAS*, *BCOR* and *STAG2*. The least common mutations found were *SETBP1*, *MPL*, *PHF6*, *KIT*, *JAK2*, *SH2B3* and *PTPN11*. The Chi Square test results are represented in table 18 below.

Chi Square Test

			Cohort		
			A	B	Total
Mutation	DNMT3A	Count	1	2	3
		Percentage	2.0%	3.1%	2.6%
	TET2	Count	7	7	14
		Percentage	13.7%	10.8%	12.1%
	BRAF	Count	1	0	1
		Percentage	2.0%	0.0%	0.9%
	SF3B1	Count	3	0	3
		Percentage	5.9%	0.0%	2.6%
	ASXL1	Count	6	3	9
		Percentage	11.8%	4.6%	7.8%
	SRSF2	Count	6	6	12
		Percentage	11.8%	9.2%	10.3%
	RUNX1	Count	6	1	7
		Percentage	11.8%	1.5%	6.0%
	U2AF1	Count	4	2	6

	Percentage	7.8%	3.1%	5.2%
SETBP1	Count	1	0	1
	Percentage	2.0%	0.0%	0.9%
ZRSR2	Count	2	0	2
	Percentage	3.9%	0.0%	1.7%
MPL	Count	1	0	1
	Percentage	2.0%	0.0%	0.9%
EZH2	Count	3	0	3
	Percentage	5.9%	0.0%	2.6%
NRAS	Count	3	4	7
	Percentage	5.9%	6.2%	6.0%
PHF6	Count	1	0	1
	Percentage	2.0%	0.0%	0.9%
KIT	Count	1	0	1
	Percentage	2.0%	0.0%	0.9%
JAK2	Count	1	0	1
	Percentage	2.0%	0.0%	0.9%
NFI	Count	1	2	3
	Percentage	2.0%	3.1%	2.6%
BCOR	Count	1	1	2
	Percentage	2.0%	1.5%	1.7%
STAG2	Count	2	1	3
	Percentage	3.9%	1.5%	2.6%
KRAS	Count	0	2	2
	Percentage	0.0%	3.1%	1.7%
FLT3	Count	0	6	6
	Percentage	0.0%	9.2%	5.2%
NPM1	Count	0	7	7
	Percentage	0.0%	10.8%	6.0%
TP53	Count	0	9	9
	Percentage	0.0%	13.8%	7.8%
SH2B3	Count	0	1	1
	Percentage	0.0%	1.5%	0.9%
CBL	Count	0	1	1
	Percentage	0.0%	1.5%	0.9%
IDH2	Count	0	6	6
	Percentage	0.0%	9.2%	5.2%
IDH1	Count	0	3	3
	Percentage	0.0%	4.6%	2.6%
PTPN11	Count	0	1	1

	Percentage	0.0%	1.5%	0.9%
Total	Count	51	65	116
	Percentage	100.0%	100.0%	100.0%

$X^2(27) = 55.5$, $p < 0.001$

Table 18: Chi Square test comparing different mutations in Cohort A and Cohort B.

4.3.2 *Pie Charts representing mutation prevalence in Cohort A and Cohort B.*

Pie charts were generated to represent mutation prevalence in Cohort A and Cohort B, separately. The most common mutation detected in Cohort A was *TET2* (14%). Other common mutations include *ASXL1 (12%)*, *SRSF2* (12%) and *RUNX1 (12%)*. Mutations detected at lower frequency include *U2AF1* (8%), *SF3B1* (6%), *EZH2* (6%) and *NRAS* (6%).

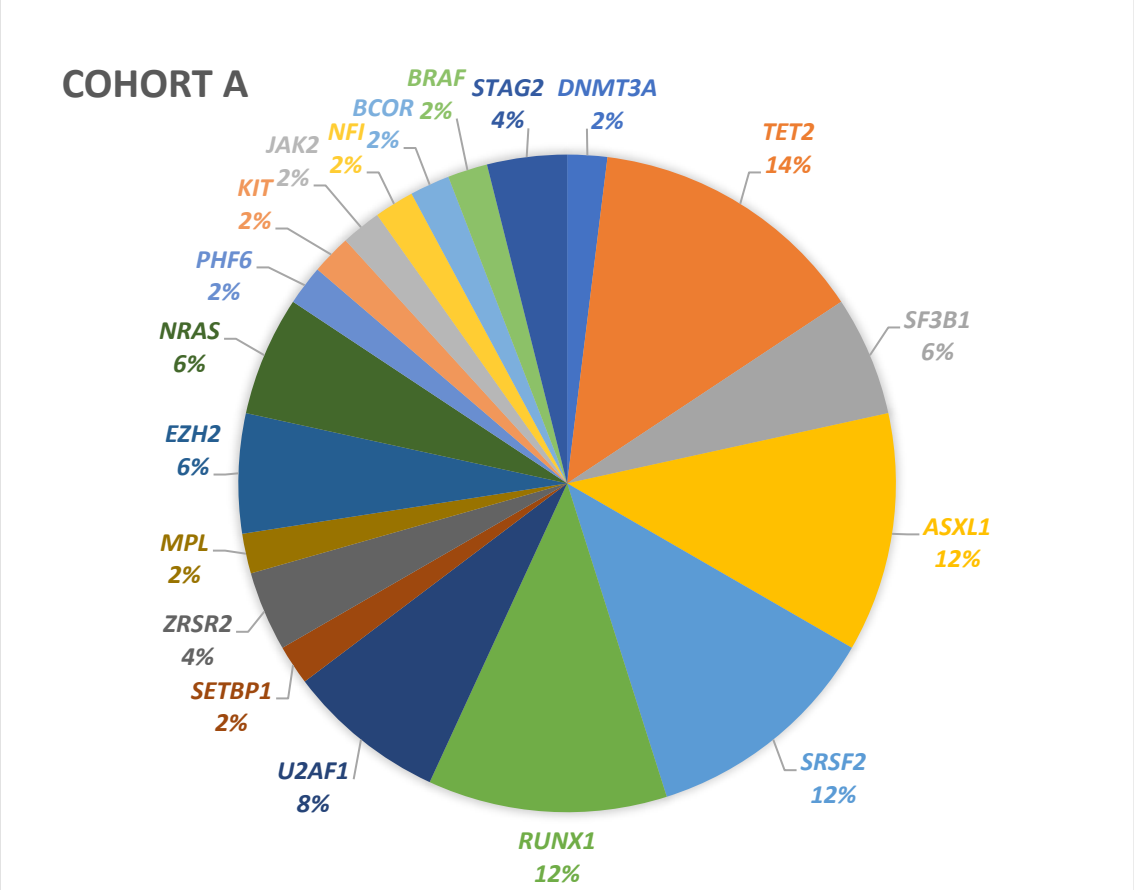


Figure 67: Pie Chart representing mutations detected in Cohort A.

In Cohort B, the most common mutation detected was *TP53* (14%). Other common mutations include *TET2* (11%), *NPM1* (11%), *FLT3* (9%), *SRSF2* (9%) and *IDH2* (9%). Mutations found at lower frequencies include *NRAS* (6%), *IDH1* (5%) and *ASXL1* (5%).

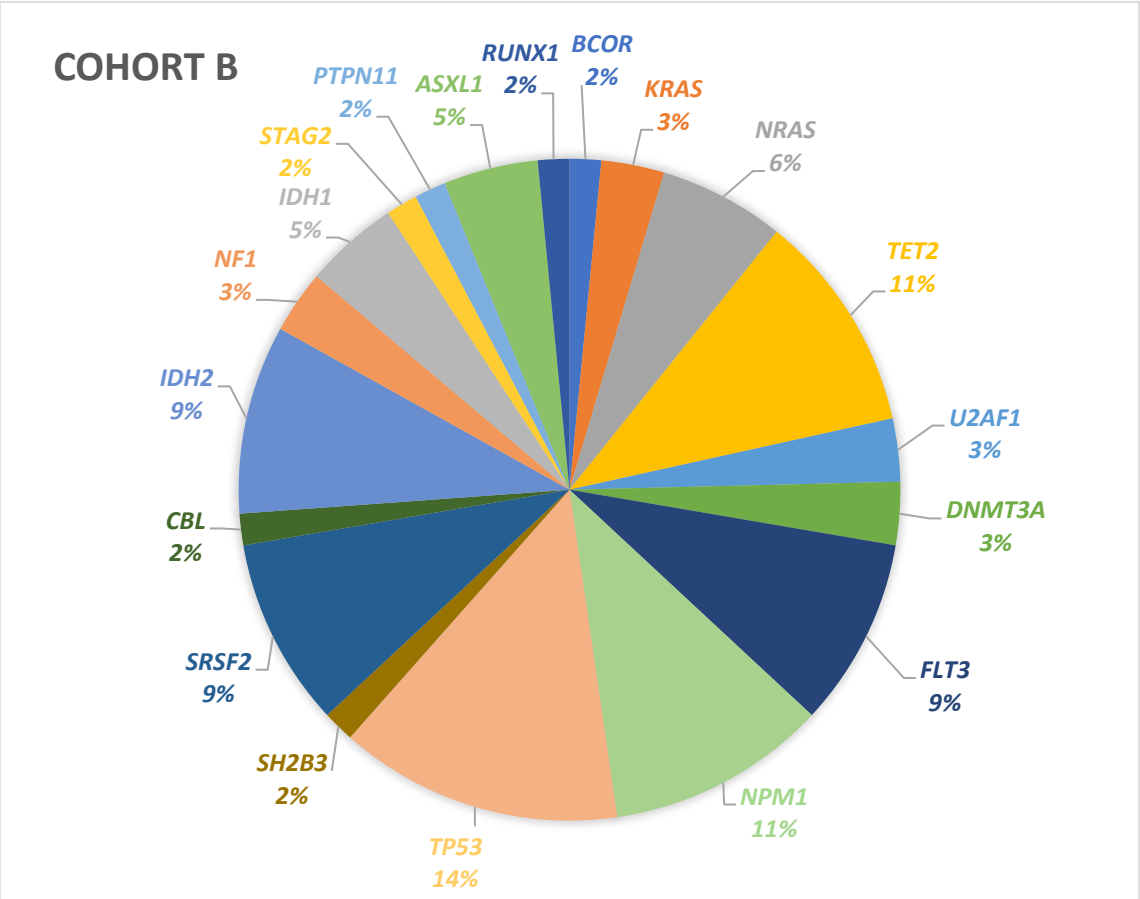


Figure 68: Pie Chart representing mutations detected in Cohort B.

4.3.3 Comparing the number of mutations in Cohort A and Cohort B.

The Mann Whitney Test was used to compare the average number of mutations between Cohort A and Cohort B. The average number of mutations of Cohort B (2.826) exceeds the average number of mutations of Cohort A (1.700) by 1.126 mutations (p-value 0.023). This difference is statistically significant because the p-value (0.023) is smaller than the 0.05 level of significance, thus showing more mutations were detected in Cohort B than in Cohort A.

Mann Whitney Test

	Cohort	Sample Size	Mean	Std. Deviation	P-value
Number of mutations	A	30	1.700	1.725	0.023
	B	23	2.826	1.642	

Table 19: Mann Whitney Test comparing the number of mutations in Cohort A and Cohort B

4.3.4 Heat Maps representing VAFs in Cohort A and Cohort B.

The Heat Maps generated are representing the variance allele frequencies (VAFs) results for Cohort A and Cohort B, respectively. The colours of the Heat Maps are indicating the percentage of the VAF with the green colour being 0% and the red colour representing the largest percentage in each cohort group. The Heat Maps clearly show that mutations detected in Cohort B had higher VAFs than mutations detected in Cohort A. Moreover, in Cohort B, the mutation *TP53* was the most commonly mutated and had the highest VAFs.

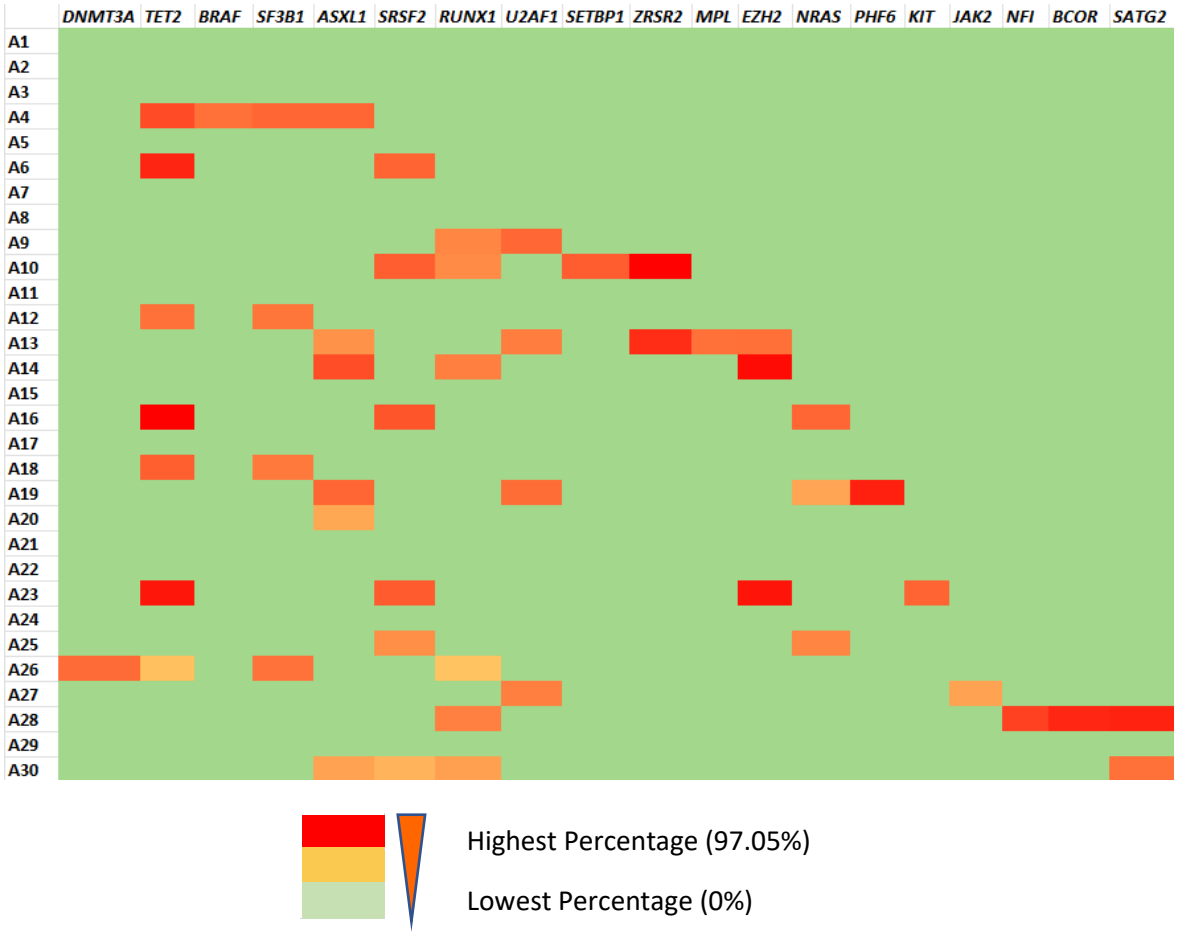


Figure 69: Heat Map representing VAFs of mutations detected in Cohort A.

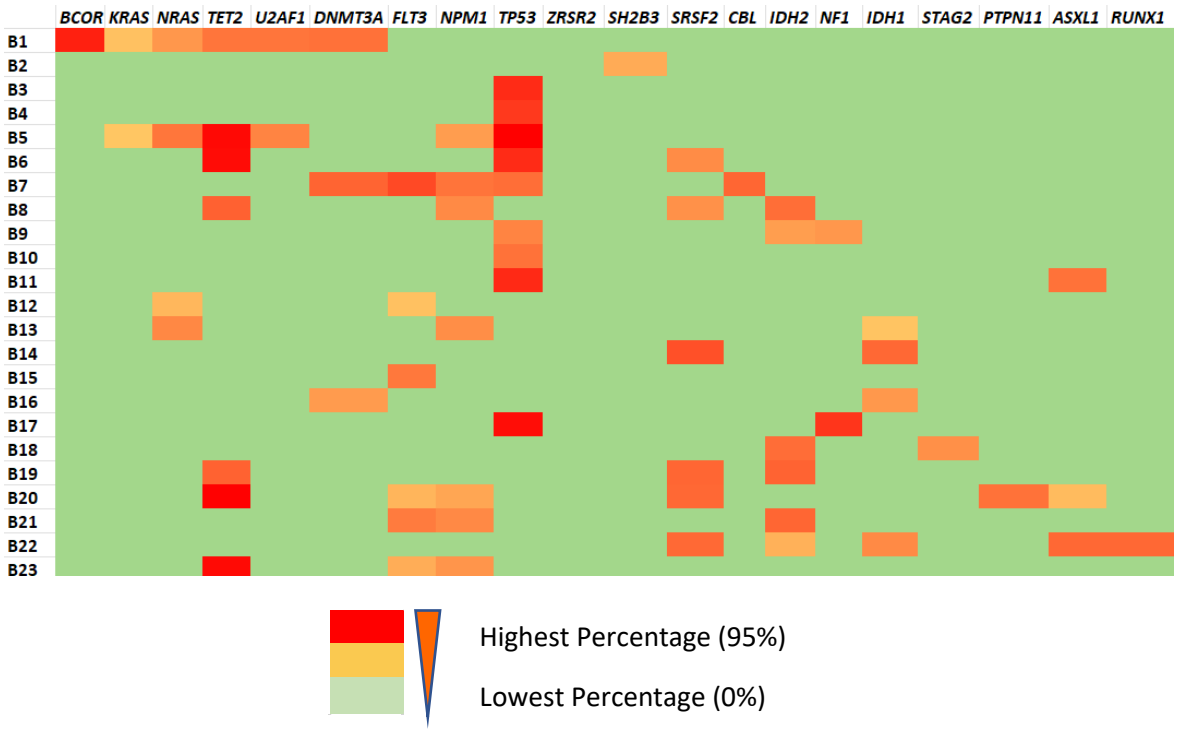


Figure 70: Heat Map representing VAFs of mutations detected in Cohort B.

4.4 Classification of Cohort A

4.4.1 Characteristics of MDS

One of the sample collection criteria for Cohort A subject recruitment was persistent cytopenia, which is one of the main features of MDS. All patients from Cohort A had cytopenias or pancytopenia. The other two characteristics of MDS are clonality and dysplasia. Clonality was found in 17 out of 30 patients (57%) from Cohort A. Dysplasia was found in 18 out of 30 patients (60%) from Cohort A. This is represented in figure 71 below.

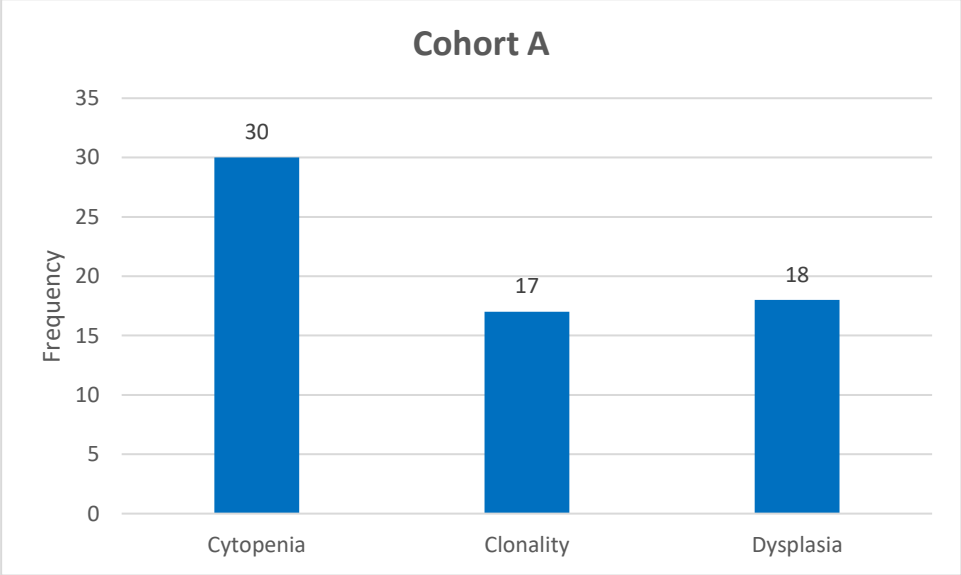


Figure 71: Characteristics of MDS in Cohort A.

4.4.2 Potential Pre-MDS conditions and low-risk MDS

Cohort A was further classified into some of the potential pre-MDS conditions and low-risk MDS depending on the characteristics of MDS which were described previously. Patients having all the characteristics for MDS i.e. cytopenia, dysplasia and clonality,

were classified as having low-risk MDS. Patients having both cytopenias and clonality without any dysplastic features were classified as having clonal cytopenia of undetermined significance (CCUS). Patient having only cytopenias without any other features of MDS (i.e. no dysplasia and no clonality), were classified as having idiopathic cytopenia of unknown significance (ICUS). The other group of patients had cytopenias and dysplasia but with no clonality. These group of patients were categorised as 'Others' as there is no 'pre-MDS' terminology for this group of patients, as yet. This is represented in table 20 below.

Patient Number	Cytopenia	Clonality	Dysplasia	Classification	LSC
A1	✓			ICUS	
A2	✓		✓	? Other	✓
A3	✓			ICUS	✓
A4	✓	✓	✓	LR-MDS	
A5	✓		✓	? Other	✓
A6	✓	✓	✓	LR-MDS	
A7	✓		✓	? Other	
A8	✓			ICUS	
A9	✓	✓	✓	LR-MDS	✓
A10	✓	✓	✓	LR-MDS	✓
A11	✓			ICUS	
A12	✓	✓	✓	LR-MDS	✓
A13	✓	✓	✓	LR-MDS	✓
A14	✓	✓	✓	LR-MDS	✓
A15	✓			ICUS	✓
A16	✓	✓	✓	LR-MDS	✓
A17	✓			ICUS	✓
A18	✓	✓		CCUS	
A19	✓	✓	✓	LR-MDS	✓
A20	✓	✓		CCUS	✓
A21	✓			ICUS	✓
A22	✓			ICUS	✓
A23	✓	✓	✓	LR-MDS	
A24	✓			ICUS	✓

A25	✓	✓	✓	LR-MDS	✓
A26	✓	✓	✓	LR-MDS	✓
A27	✓	✓	✓	LR-MDS	✓
A28	✓	✓	✓	LR-MDS	✓
A29	✓			ICUS	✓
A30	✓	✓	✓	LR-MDS	✓

Table 20: Classification of Cohort A. Classification of Cohort A patients was based on the presence of cytopenia/s, dysplasia and clonality. The last column also shows the presence or absence of LSC's. This was done to see how many patients from the different sub-classifications of Cohort A had LSCs.

Cohort A included 50% of patients with low-risk MDS, 33% of patients with ICUS, 7% of patients with CCUS and 10% of patients which were classified as 'Others' (figure 72).

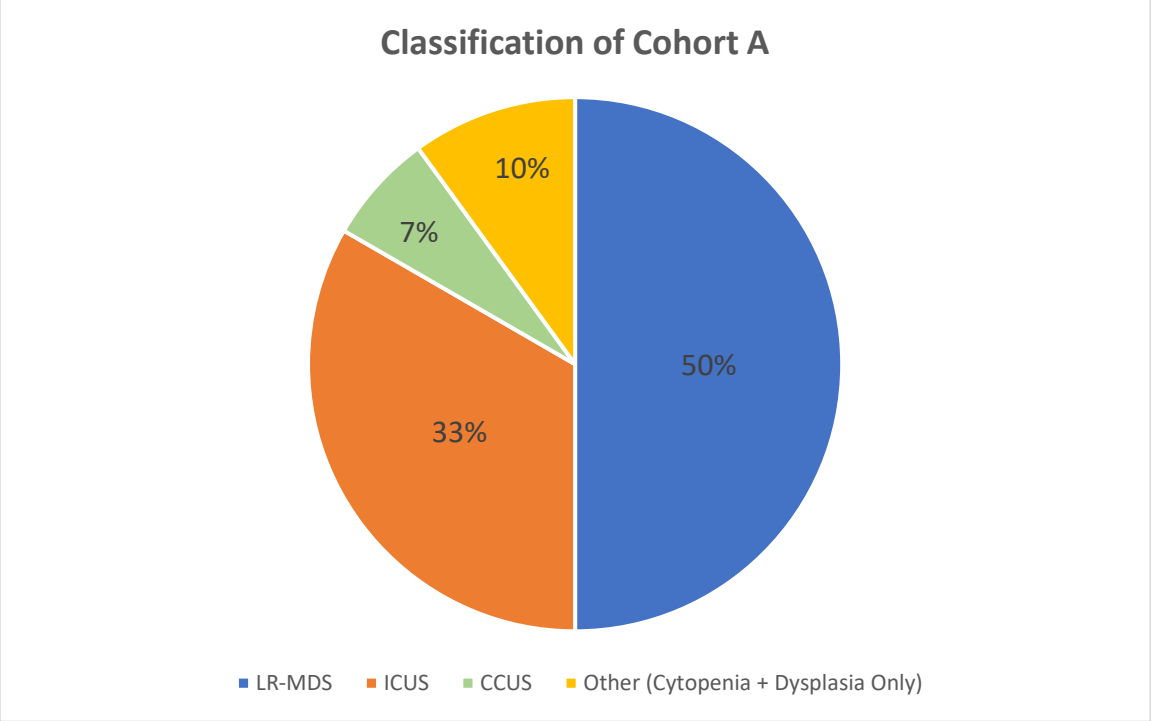
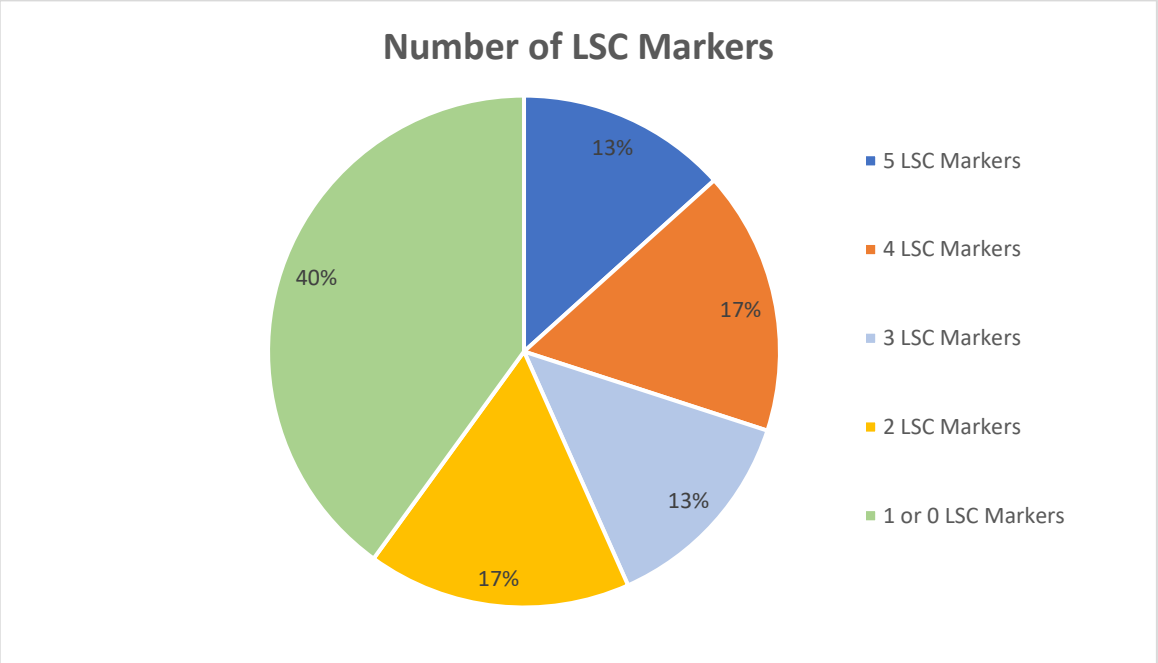


Figure 72: Classification of Cohort A.

4.4.3 Leukaemic stem cells in Cohort A

The pie chart below is showing that 40% of patients from Cohort A had 1 or 0 LSC markers. From these 40% of patients, 4 patients had 1 LSC marker and the other 8 patients had none. The other 60% of patients had 2 or more LSC markers.



	No. of patients	Percentage
All 5 LSC Markers present	4	13%
4 LSC Markers present	5	17%
3 LSC Markers present	4	13%
2 LSC Markers present	5	17%
1 or 0 LSC Markers present	12	40%

Figure 73: Number of LSC markers detected in Cohort A.

Cohort A was further subdivided into 4 different sub-groups: LR-MDS, ICUS, CCUS and Others. The stacked bar chart below is showing the number of patients with LSCs (blue) and without LSCs (orange) in each sub-group. In the LR-MDS sub-group, 12 out 15 patients (80%) had LSCs, thus showing that most patients from the LR-MDS sub-group had all the criteria for MDS (cytopenias, dysplasia, clonality) and also the presence of LSCs. In the ICUS sub-group, 7 out of 10 patients (70%) had LSCs. In the CCUS sub-group 1 out of 2 patients (50%) had LSCs, whilst in the 'Others' sub-groups 1 out of 3 patients (33%) had LSCs.

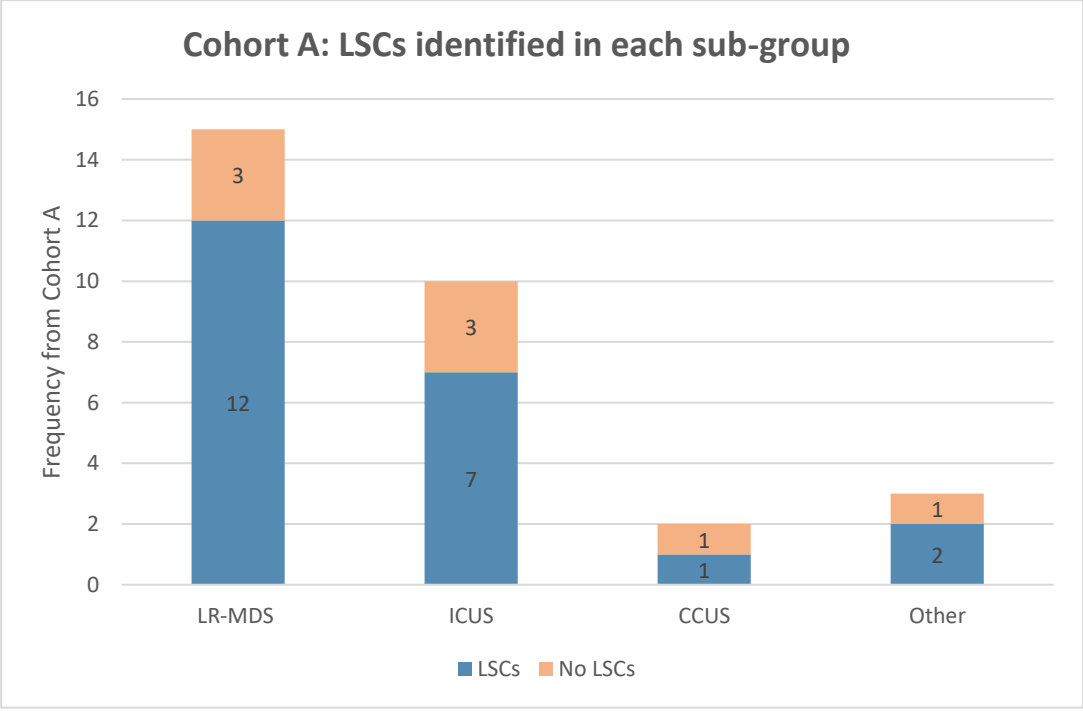


Figure 74: LSCs in each Cohort A Sub-group Classification.

4.5 Mutations in Cohort A Sub-groups and Cohort B

Figure 75 is showing the number of mutations in Cohort A sub-groups and Cohort B. As the disease progresses, the number of mutations increase.

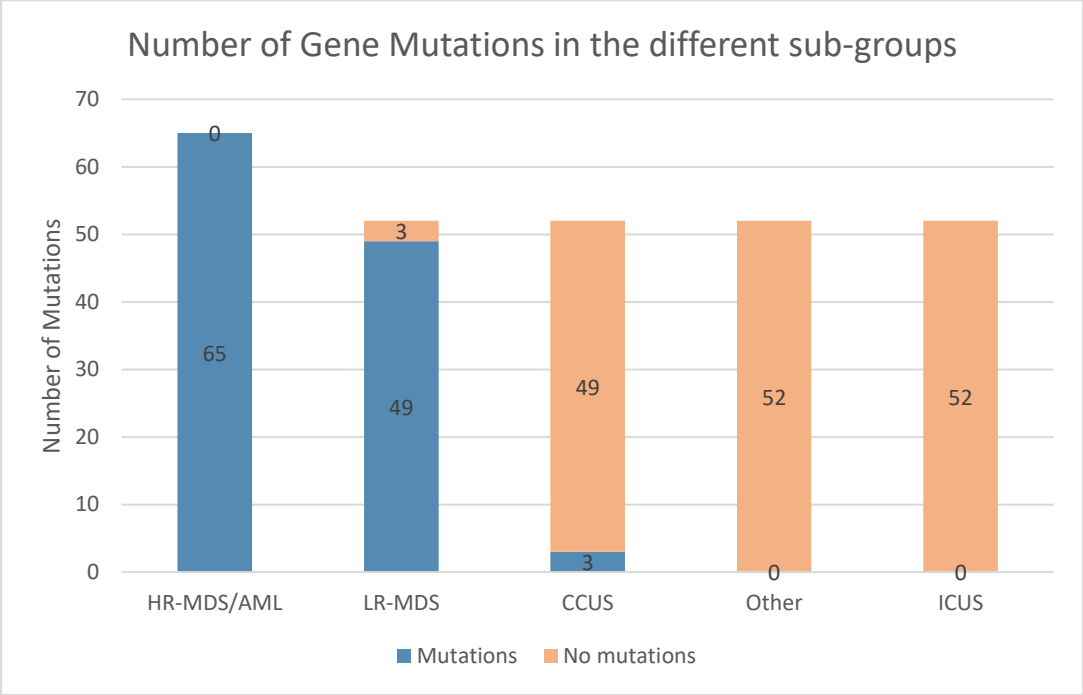


Figure 75: Number of gene mutations in the different sub-groups

4.6 Comparison of Immunophenotyping and Molecular Results

The Kappa Test was used to investigate any agreement between immunophenotyping results and Molecular results. In Cohort A, 'Presence' was identified in 43.3% of the cases by both Flow Cytometry and NGS. 'Absence' was identified in 13.3% of the cases by both Flow Cytometry and NGS. This means that there was 56.6% agreement between Flow Cytometry and Molecular results. However, in 30%

of the cases ‘Presence’ was identified by Flow Cytometry and ‘Absence’ by NGS. In 13.3% of cases ‘Presence’ was identified by NGS and ‘Absence’ by Flow Cytometry.

Kappa Test (Cohort A)

			NGS		
			Present	Absent	Total
Flow Cytometry	Present	Count	13	9	22
		Percentage	43.3%	30.0%	73.3%
	Absent	Count	4	4	8
		Percentage	13.3%	13.3%	26.7%
Total		Count	17	13	30
		Percentage	56.7%	43.3%	100.0%

Kappa = 0.076.

Table 21: Kappa Test to compare Immunophenotyping and Molecular Results in Cohort A.

In Cohort B, there was high agreement between Immunophenotyping and Molecular results. In fact, ‘Presence’ was identified in 91.3% of the cases by both Flow Cytometry and NGS. In 8.7% of cases ‘Presence’ was identified by NGS and ‘Absence’ by Flow Cytometry.

Kappa Test (Cohort B)

			NGS		
			Present	Absent	Total
Flow Cytometry	Present	Count	21	0	21
		Percentage	91.3%	0.0%	91.3%
	Absent	Count	2	0	2
		Percentage	8.7%	0.0%	8.7%
Total		Count	23	0	23
		Percentage	100.0%	0.0%	100.0%

Table 22: Kappa Test to compare Immunophenotyping and Molecular Results in Cohort B.

Chapter 5

Discussion

Patients with persistent cytopenias and low-risk MDS are relatively common encounters in routine Haematology screening and the clinical management of these patients can be challenging as it is very difficult to predict their course. In this study, investigation of the stem cell compartment was carried out to shed light on the clinical behaviour of these conditions and to aid in better clinical management. Investigation of the stem compartment was performed using both immunophenotyping by Flow Cytometry and molecular techniques by NGS, two very important techniques when investigating patients with suspected MDS.

In this study, the male gender was found to be more prevalent in both Cohort groups. Several studies have shown that both MDS and AML are commoner in males, except for MDS with isolated del(5q) which is more common in females (Aster and Stone, 2021) (Sekeres & Taylor, 2022) (Jackson *et al.,* 1999). A study by Tinsley-Vance *et al*. in 2023, included 4580 patients with MDS, out of which 66% were males and 34% were females (Tinsley-Vance *et al.,* 2023). These are similar findings to this study were 73% of patients from Cohort A and 70% from Cohort B were males. The incidence of MDS and AML increases with age and most cases occur after the age of 65 (Aster and Stone, 2021). The mean age in this study was 69 years in both cohort groups. The most common type of cytopenia in this study was anaemia which was present in 83.3% of patients from Cohort A, followed by thrombocytopenia (50%) and neutropenia (43.3%). This corresponds to current literature, since anaemia is also the most common type of cytopenia associated with MDS and is found in about 80% to 85% of patients (Samiev *et al.,* 2014. This was also reflected in the symptoms experienced by patients. The most common symptom was fatigue followed by shortness of breath and these are both

related to anaemia. Other symptoms included fever, frequent infections and easy bleeding and bruising related to neutropenia and thrombocytopenia, respectively.

5.1 Leukaemic Stem Cells

Leukaemic stem cells are known to be the first step in leukemogenesis and lead to the development of MDS and AML. A leukaemic stem cell tube was developed by a group of scientists from Amsterdam University Medical Centre (UMC) to identify LSCs both at diagnosis of AML and after treatment to assess risk of relapse (Zeijlemaker *et al.,* 2015). In this study, the LSC tube was used to identify and characterise the stem cell compartment ($CD34^{+}CD38^{-}$) of patients with persistent cytopenias to assess the risk of transformation to MDS and eventually AML. We postulate that patients with persistent cytopenias having a compromised stem cell compartment have an increased risk of leukaemic progression. The LSC tube was also used to assess the stem cell compartment of patients with high-risk MDS or newly diagnosed AML patients, as positive controls.

The LSC tube included the following LSC markers: CD45RA, CD33, CD44 and other six markers combined in one fluorescence channel, the Combi markers, namely CLL-1, TIM-3, CD7, CD11b, CD22 and CD56. In Cohort A, LSCs were identified in 60% of patients. The most common leukaemic stem cell markers found were CD45RA and Combi markers. These markers are considered to be the most relevant leukaemic stem cell markers as they are usually absent on the stem cell compartment of healthy individuals (Zeijlemaker *et al.,* 2015) (van Spronsen *et al*., 2023). The other CD markers CD123, CD33 and CD44 were found at very low percentages in Cohort A. In Cohort B, LSCs were identified in 91% of patients. The percentages of all leukaemic stem cell markers were found to be consistently high, in all patient cohort. The mean percentage of each

leukaemic stem cell marker for Cohort B, the positive control group, was significantly higher than that of Cohort A (p-value= <0.001), as expected. In addition, all leukaemic stem cell markers were detected in almost all patients from Cohort B which were mainly newly diagnosed leukaemia patients. This expected finding, provides confidence that the methodology for the identification of LSCs, has been correctly validated and implemented within this research study. It also demonstrates the evolution of the malignant clone both molecularly with the acquirement of more mutations and the appearance of more aberrant antigens. The heat maps generated clearly depicted that leukaemic stem cells were detected at higher percentages in Cohort B. The mean percentage of LSCs in Cohort A and Cohort B was 0.0027% and 0.2313% respectively. The higher percentages in Cohort B further confirms the scope of the LSC tube which was developed to detect LSCs both at diagnosis and follow-up of AML patients (Zeijlemaker *et al.,* 2015) (Hansen *et al.,* 2022). Hanekamp *et al.,* in 2017, also highlighted the importance of LSC identification and stated that LSC measurement is a valuable asset for clinical decisions in AML patient management. The convenient one-tube assay can be implemented easily as part of the diagnostic work-up of these patients. The LSC tube can also be used to identify leukaemic stem cells in patients who present with a CD34 negative AML. In fact, in our study, 7 patients from Cohort B had a CD34 negative AML with the presence of LSCs. This finding is particularly important since these patients usually have a better prognosis (Hanekamp *et al.,* 2017). Controversially to this, some literature reported challenges in LSC identification for CD34-ve AMLs, especially when it comes to MRD analysis. Therefore, Liu *et al.*, highlighted the need for a panel including CD117 for the CD117+CD34- AMLs (Liu *et al.,* 2022). However, within our study, LSCs were identified in all the CD34-ve AML Cohort, although the CD34+ve

compartment made up a minor proportion of the total bone marrow nucleated cells. This might be because patients were only investigated at diagnosis and not at an MRD stage.

Another recently published study by Das *et al.*, showed the importance of characterising LSCs in AML patients using a single tube 10-colour panel by multiparametric flow cytometry. Leukaemic stem cells were identified in 88.2% of the bone marrow samples analysed from AML patients. This is close to our findings, where LSCs were identified in 91% of patients from Cohort B. The expression of the LSC markers was significantly higher on LSCs than HSCs and hence, Das e*t al.,* concluded that the single 10-colour tube is an easy and reproducible tool for the identification and characterisation of normal HSCs and LSCs (Das *et al.,* 2024).

Cohort A was further sub-classified into some of the potential pre-MDS conditions and low-risk MDS depending on the characteristics of MDS i.e. cytopenia, dysplasia and clonality. Half of the patients from Cohort A were classified as low-risk MDS (50%). The other half of patients included ICUS (33%), CCUS (7%) and 'Other' (10%). In the LR-MDS sub-group, 12 out 15 patients had LSCs, thus showing that most patients from the LR-MDS sub-group had all the criteria for MDS together with the presence of LSCs. This further shows that the leukaemic stem cell tube can be used as an additional tool when investigating patients with suspected MDS as it can give more information about the stem cell compartment. The presence of leukaemic stem cells in LR-MDS patients is an important adjunct finding that may prompt clinicians to monitor these patients more closely or even initiate treatment at earlier timepoints as opposed to patients who do not have LSCs. The presence of LSCs in these patients poses an increased risk to

transformation to AML. Closer monitoring and early treatment may prevent the actual transformation from LR-MDS to HR-MDS/AML. Leukaemic stem cells were also identified in the other sub-groups of Cohort A (ICUS, CCUS and 'Other') and this further highlights the importance of the LSC tube in the diagnostic work up of suspected MDS. However, a larger sample size of these sub-groups would have given a better insight on the relevance of this tube in these categories (ICUS, CCUS and 'Other'). Since these categories include patients who are asymptomatic with incidental findings of pancytopenia, it is more clinically challenging to identify and investigate. Patients at this point are probably at an early stage of the leukemogenesis process. This explains why the ICUS, CCUS and 'Other' cohort was limited in number.

Several criteria must be used when interpreting the LSC tube results. The Amsterdam group highlighted three important scoring systems during the onsite training visit (personal communication). The first one being which LSC marker is most relevant to distinguish between normal HSC and LSC; with CD45RA being the most relevant LSC marker, followed by markers in the Combi channel (i.e. CLec12a, TIM-3, CD7, CD11b, CD22 and CD56), CD123, CD33 and CD44. CD45RA and the markers in the Combi channel are considered to be the most relevant LSC markers because they are totally absent on normal HSCs. Therefore, their presence indicates a definite abnormal stem cell compartment. The second criterion is that in order to classify the stem cell compartment as LSCs, a minimum of two LSC markers should be expressed on the stem cells. If only one LSC marker is identified, it is not enough evidence to classify the stem cell compartment as LSCs. The third criterion was that the LSC marker which gives the clearest positivity on the Flow cytometry dot plots generated (as seen in figures 37-40 in the methodology section) should be the chosen marker to report the LSC percentage.

In this study, 60% of patients from Cohort A had two or more LSC markers, in fact, these were the LSC positive results. In the other 40% of patients, 4 patients had only 1 LSC marker and 8 patients had none, and hence were counted as negative.

So far, studies have focused on the significance of the LSC tube in AML patients, highlighting its significance in Measurable Residual Disease (MRD) rather than MDS or pre-MDS conditions, which was one of the aims of this study. Therefore, the literature on the use of the LSC tube in MDS is so far limited. However, a study which has been recently published by van Spronsen *et al*., in 2023, mentioned that the presence of leukaemic stem cells in MDS patients can predict leukaemic progression. This study was carried out in Amsterdam UMC, in the same centre where the leukaemic stem cell tube was developed. Van Spronsen *et al*., hypothesized that aberrant expression of immunophenotypic markers on the stem cell compartment of MDS patients can predict leukaemic transformation. Bone marrows from 68 MDS patients and 53 normal controls were analysed using the same LSC tube used in our study. Leukaemic stem cells were identified in one-third of the MDS bone marrow samples. In our study, LSCs were identified in 60% of patients with persistent cytopenias. Similar to our findings, the most frequently expressed LSC markers were CD45RA and markers within the Combi channel. No relationship was however observed between the risk stratification (IPSS-R) of MDS and leukaemic stem cells. Molecular analysis by NGS was also carried out to assess for differences in mutations between normal HSCs and LSCs using amplicons covering regions in 27 genes which are commonly associated with MDS, on a small group of patients (n=10). Although mutations of tumour suppressor genes and DNA methylation genes were detected when LSCs were present, no molecular conclusions could be drawn from the study by van Spronsen *et al*., due to the small sample size (10 patients). In our

study, molecular testing was carried out on all samples from patients with persistent cytopenias. On the other hand, in the study by van Spronsen *et al*., MDS patients were followed-up for 2 years post initial LSC identification. The group reported that the presence of LSCs predicted leukaemia progression with a sensitivity of 83% and a specificity of 71%. Van Spronsen *et al*., concluded that the presence of LSCs in MDS patients can highly predict progression from MDS to AML. Patients who were classified as IPSS-R low-risk but had LSCs, eventually transformed to MDS with excess blasts or AML. Other patients with IPSS-R high-risk but without LSCs had a stable disease course. Another conclusion drawn from the study by van Spronsen *et al*., was that in the future, the presence of LSCs should be applied for risk assessment in order to and guide clinicians in the management of patients with MDS. Aberrant immunophenotypic findings should also be targets for therapy (van Spronsen et al., 2023), as explained in detail further down below.

Role of Leukaemic Stem Cell Markers in Leukemogenesis

Leukaemic stem cells are known to be part of the leukemogenesis process leading to MDS/AML. Expression of each LSC marker has a unique role in conferring the LSC immunophenotype to stem cells. Antigens expressed by LSCs are immunogenic and can be identified by immune cells and also by major histocompatibility complex molecules resulting in interaction with T-cells. As a result, innate immune responses to leukaemia were reported in patients (Tasian, Bornhäuser and Rutella, 2018). In leukaemic blasts, increased expression of CD123 has been associated with more cell cycle activity, resistance to apoptosis, activation of IL-3 and increased signal transducer and activator of transcription 5 (STAT5) (Hansel *et al.,* 2022). Increased expression of CD44 in LSCs

lead to increased cell survival signaling, cell proliferation, homing and engraftment (Jin *et al.,* 2006). Another LSC marker responsible for increased cell survival in TIM-3. TIM-3 is also responsible for galactin-9-mediated signal transduction resulting in expression of myeloid cell leukaemia 1 (MCL-1) protein. Clec12a, another LSC marker, is known for potential negative regulation of the immune system (Han *et al.,* 2004). CD56 expression on LSCs is responsible for the activation of MAP-kinase signaling pathway. This activation lead to proliferation and survival of leukaemic cells, together with increased resistance to chemotherapy (Sykes, 2019). CD11b expression on LSCs is known to suppress the immune system and can also result in hypercoagulation and coagulopathy (Baysal *et al.,* 2020). CD33 marker is responsible for cell growth, cell adhesion and cell signaling. Increased expression of CD33 on LSC's leads to increased cell growth (Walter *et al.,* 2012).

Immunophenotype-based therapeutic targeting of LSCs

Detection of leukaemic stem cells is also important as these can be therapeutic targets. Although most chemotherapeutic agents are able to eliminate most leukaemic blast cells in AML, most patients still relapse because of residual leukaemic stem cells which are resistant to chemotherapy. Therefore, elimination of leukaemic stem cells is essential to prevent relapse (Mitchell and Steidl, 2019). Although elimination of LSCs can be quite challenging, several clinical trials during the past years, have been investigating different therapies to target LSCs. Therapeutic targeting of LSCs can also be beneficial in patients with suspected MDS in order to prevent transformation to AML (Mitchell and Steidl, 2019). This could result in better patient management and reduced overall healthcare costs.

Haematopoietic stem cells and leukaemic stem cells differ in their expression of surface markers and activation of intracellular signalling pathways (Hansen *et al.,* 2022). Many clinical trials have focused on targeting both signalling pathways (using drugs such as Idarubicin and Parthenolide (Jordan, 2007) and surface markers of LSCs. Identification of the cell surface markers on LSCs can lead to more specific and personalised treatments. This highlights the importance of knowing the LSC immunophenotype of individual patients and hence the use of the LSC tube to characterise the stem cell compartment. Although chemotherapy and radiotherapy have been effective treatment options over the past years, they are known to cause severe side effects as they also target healthy cells. Therefore, more targeted treatment options are needed. Different therapeutic targeting strategies against surface proteins are available and are still being developed. One approach is the use of antibody-drug conjugates (ADCs) where monoclonal antibodies are attached via a chemical linker to a cytotoxic drug/toxin. The antibody part than attaches to leukaemic cells that express leukaemic associated proteins. The leukaemic cells uptake the ADC leading to apoptosis. This treatment strategy is directed against leukaemic cells with survival of healthy cells as opposed to traditional chemotherapy (Barbosa and Deshpande, 2023). Other forms of immunotherapy include unconjugated antibodies and multivalent recombinant antibodies (bi- or tri- specific antibodies). Unconjugated antibodies can facilitate Natural Killer (NK) cell antibody-dependent cell-mediated cytotoxicity (ADCC). In ADCC, antibodies bind to antigens on the surface of target cells such as leukaemic stem cell antigens. The NK cell receptors on CD16 recognise the antibodies bound to the cell and trigger degranulation and lyses the target cell resulting in cell death. In multivalent recombinant antibodies approaches, antibodies can engage either NK cells or T-cells to

target cancer cells. Another targeting strategy is the use of chimeric antigen receptor T-cells (CAR-Ts) directed against LSCs. In CAR T-cell therapy, the patient's T cells are genetically modified to express a synthetic receptor which binds to a tumour antigen or other antigens. The CAR T-cells are then infused back into the patient and they attack the tumour cells (Feins *et al.,* 2019) (Williams *et al.,* 2019) (Barbosa and Deshpande, 2023).

Some LSC targeted therapies mentioned in several studies include immunotherapy targeted against CD33, CD123, TIM-3 and CLL-1 (Hanekamp *et al.,* 2017) (Mitchell and Steidl, 2019) (Arnone *et al.,* 2020) (Hansen *et al.,* 2022). The most studied immunotherapies in AML so far include therapies targeted against CD33 as it is expressed by the majority of myeloid blasts. Gemtuzumab ozogamicin (GO) has been used as an anti-CD33 immunotherapy. However, since CD33 is also expressed by healthy HSCs, several toxicities including hepatotoxicity and cardiotoxicity have been reported with this treatment (Mitchell and Steidl, 2019) (Hansen *et al.,* 2022). For this reason, GO was initially withdrawn from clinical use after the first studies. GO has recently obtained re-approval for use in specific patients (Arnone *et al.,* 2020). Anti-CD123 therapies have also been reported to show anti-leukaemic effects. Monoclonal antibodies targeting CD123 were found to be effective by inhibiting IL-3 signaling and cell killing by innate immune effector cells. Although anti-CD123 seemed to be promising, several clinical trials were terminated because of risks to patients. Toxicities were observed since CD123 is also expressed by healthy HSCs. Further clinical trials using CD123 CAR T-cell therapy have been initiated (Arnone *et al.,* 2020). Another two interesting LSC targets are TIM-3 and CLL-1, which are both absent on the stem cells of healthy individuals. Clinical trials with agents targeting these surface markers show promising results

(Arnone e*t al.,* 2020). A study published by Akashi in 2015, used a xenogeneic transplantation system to target TIM-3 by an anti-TIM-3 cytotoxic antibody. Elimination of LSCs was successfully accomplished without effecting healthy haematopoietic stem cells. Akashi concluded that TIM-3 can be an ideal therapeutic target (Akashi, 2015). These findings were also demonstrated by another study by Kikushige and Miyamoto in 2013 who also found that TIM-3 is a promising therapeutic target to eliminate leukaemic stem cells (Kikushige and Miyamoto, 2013). Several studies have confirmed that CLL-1 could also be an ideal target for LSCs. CLL-1 targeted therapies include antibody-based therapies and CLL-1 CAR-T cell therapy. However, more research is needed to fully understand the function of CLL-1, considering the high cost and time needed to generate individualised CLL-1 CAR-Ts (Ma *et al.,* 2019).

Although LSC targeted therapies seem to be promising treatments, one must take into account several considerations before applying anti-LSC therapies. An important consideration is that healthy HSCs must not be targeted by treatment since both HSCs and LSCs share some common features including expression of some surface markers. LSC targeted therapies can harm healthy stem cells resulting in severe and harmful side effects. Therefore, more research is required to investigate how healthy stem cells are affected by LSC targeted therapy (Hansen *et al.,* 2022). The ideal LSC therapy would be one which is highly selective, that is, targets antigens which are significantly expressed by LSC and not present on healthy stem cells. (Hanekamp *et al.,* 2017).

5.2 Molecular Results by NGS

Myelodysplastic syndromes are a group of heterogeneous myeloid disorders resulting from a number of molecular aberrations that occur within the haematopoietic

stem cell compartment (Bănescu, Tripon and Muntean, 2023). Leukemogenesis involves a multi-step process in which there is evolution from low-risk to high-risk MDS which can then eventually transform to AML (Pandolfi, Barreyro and Steidl, 2013). A next-generation sequencing myeloid targeted panel was used in this study to investigate any mutations present in patients with persistent cytopenias and in the positive control group (newly diagnosed AML and HR-MDS patients). The use of a targeted NGS panel instead of whole genome sequencing to investigate patients with suspected MDS and AML has a lot of advantages. Firstly, a targeted approach is important so to focus on the genes which are more relevant to the disease investigated. By using a targeted approach, sequence coverage of challenging genomic regions can be obtained. Other cost benefits include less data storage, less informatics, shorter sequencing and workflow times and higher depth coverage of rare variants with a low allelic frequency. Moreover, more samples can be processed together in a single sequencing run and the data produced can be managed more easily (Thermo Fisher, 2023).

We compared mutations detected in both Cohort A and Cohort B. Mutations in *ASXL1*, *RUNX1*, *U2AF1* and *EZH2* genes were found to be more prevalent in Cohort A, whilst other mutations such as *FLT3*, *NPM1*, *IDH1*, *IDH2* and *TP53* were more prevalent in Cohort B (p= <0.001). Other mutations in *TET2, NRAS, BCOR* and *STAG2* genes were commonly mutated in both cohort groups. The least common mutations found in this study were *SETBP1, MPL, PHF6, KIT, JAK2, SH2B3* and *PTPN11*. The most common mutation detected in Cohort A was *TET2* (14%) followed by *ASXL1 (12%)*, *SRSF2* (12%) and *RUNX1 (12%)*. Other mutations which were detected at lower percentages and include *U2AF1* (8%), *SF3B1* (6%), *EZH2* (6%) and *NRAS* (6%). In literature, the most

common mutations found in MDS include *SF3B1, TET2, ASXL1, SRSF2, DNMT3A, RUNX1, U2AF1, ZRSR2, STAG2, TP53, EZH2, CBL, JAK2, BCOR, IDH2, NRAS* and *NF1* genes in a descending order (Christina-Nefeli Kontandreopoulou *et al.,* 2022). This is similar to our findings expect for *TP53* and *IDH2* which were found to be more prevalent in Cohort B. A study by Maurya *et al.,* in 2022 investigated 152 MDS patients using NGS and cytogenetics, out of which 111 patients had mutations. The most common mutations found were *SF3B1* (25.2%), *SRSF2* (19%), *U2AF1* (14.4%), *ASXL1* (9.9%), *RUNX1* (9.9%), *TET2* (9%), *TP53* (9%), *ATM* (6.3%), *NRAS* (5.4%) and *JAK2/3* (5.4%). Similarly to our findings, except for *TP53* which was found to be more prevalent in Cohort B, and *ATM* which was not included in the myeloid panel used in our study. Maurya *et al*., found that mutations in *TP53, JAK2/3, KRAS, NRAS* and *ASXL1* were associated with a poor prognosis and concluded that genetic mutations should be included in the prognostic stratification of patients with MDS (Maurya *et al*., 2022).

In Cohort B, the positive control group, the most common mutation detected was *TP53* (14%). This is a tumour suppressor gene and is commonly detected in leukaemias and other tumours (Olivier, Hollstein and Hainaut, 2009). Other common mutations found in Cohort B include *TET2* (11%), *NPM1* (11%), *FLT3* (9%), *SRSF2* (9%) and *IDH2* (9%). Other mutations present at lower percentages included *NRAS* (6%), *IDH1* (5%) and *ASXL1* (5%). Cohort B consisted mainly of newly diagnosed AML patients. AML is a heterogeneous haematopoietic stem cell disorder characterised by the presence of molecular aberrations (DiNardo and Cortes, 2016). In a study by Li *et al.,* in 2023, 197 AML patients were investigated. The most common mutations detected were *DNMT3A* (13%), *FLT3* (11%), *RUNX1* (9%), *NPM1* (9%), *TTN* (8%), *MUC16* (8%), *TP53* (8%), *IDH2* (8%), *KIT* (6%) and *NRAS* (6%). Although the sample size of this study was larger, the

mutations reported were also commonly found in our control group, except for *RUNX1,* which was only mutated in 2% of our patients. *KIT* mutation was detected in Cohort A but not in Cohort B. *TTN* and *MUC16* genes were not included in the myeloid gene panel used in our study (Li *et al.,* 2023). Some mutations are commonly shared between MDS and AML and this confirms that AML secondary to MDS or AML with MDS-related changes, are a result of this disease continuum (Vakiti and Mewawalla, 2022).

We also compared the number of mutations detected in each cohort group. The number of mutations was significantly higher in Cohort B (p= 0.023). This was expected because as leukemogenesis progresses, the number of mutations is expected to increase.

The advanced research in MDS and AML by NGS allowed the discovery of several molecular aberrations which can result in leukaemic transformation. Identification of genetic mutations is important for patient stratification, better patient management and more efficient therapeutic approaches such as targeted therapy (Bănescu, Tripon and Muntean, 2023). Somatic mutations in MDS and AML include mutations in splicing factor genes (*SF3B1, SRSF2* and *U2AF1*), epigenetic regulatory genes (*ASXL1, ASXL2, DNMT3A, EZH2, IDH1, IDH2* and *TET2*), transcription factor genes (*CEBPA, GATA2, ETV6, RUNX1* and *NPM1*), signal transduction genes (*CBL, FLT3, CSF3R, JAK2, JAK3, NRAS, KRAS* and *PTPN11*), tumour suppressor genes (*TP53, PHF6* and *WT1*) and other genes (*CALR* and *SETBP1*) (Liu *et al.,* 2021). A large study by Shukron *et al.,* in 2012, included 1019 patients with MDS and AML. This study found that mutations in spliceosome genes and epigenetic modifiers contribute to MDS pathogenesis, occur early in leukemogenesis, and are commonly observed in both MDS and AML. Mutations involved in signal

transduction genes are secondary events. It was also observed that mutations in epigenetic regulatory genes and cell signalling are acquired at later stages during MDS progression to AML (Shukron *et al.,* 2012).

Splicing Factor Genes Mutations

Mutations in splicing factor genes are the most commonly mutated genes in MDS. The spliceosome gene is a protein complex consisting of five small nuclear RNAs and 150 proteins which are necessary for the splicing reaction. The protein complex removes the non-coding sequences (introns) from precursor mRNA resulting in the formation of mature mRNA (Christina-Nefeli Kontandreopoulou *et al.,* 2022). Spliceosome gene mutations suggest AML that has progressed from MDS even if the patient has no history of MDS. In MDS, the most common spliceosome mutations include *SRSF2, SF3B1, ZRSR2* and *U2AF1*. These mutations were all commonly found in our study in Cohort A. *SF3B1* encodes the splicing factor 3b subunit 1 and is the most common spliceosome mutation in MDS. *SF3B1* is associated with MDS with ring sideroblasts (MDS-RS) and most patients with mutated *SF3B1* have a favourable prognosis. *SF3B1* mutation was included in the fifth edition of WHO classification of myeloid malignancies – MDS-*SF3B1*. Mutations in *SRSF2* are the second most common mutation in splicing factor genes, are associated with a poor prognosis and an increased risk of transformation to AML in MDS. Other less common splicing factor gene mutations include *U2AF1* and *ZRSR2*. *U2AF1* mutation is commonly associated with MDS with multilineage dysplasia and excess blasts, a poor prognosis and an increased risk of leukaemic transformation. *ZRSR2* mutations are more common in MDS with no ring sideroblasts and are also associated with a poor prognosis

and increased risk of transformation to AML. *ZRSR2* mutations are frequently found together with *TET2* mutations (Bănescu, Tripon and Muntean, 2023).

Epigenetic Regulatory Genes Mutations

Mutations in genes that are important for epigenetic regulation can affect post-translational DNA methylation and histone modification and are the second most common mutations in MDS. These mutations were found to silence tumour suppressor genes leading to transformation from MDS to AML (Christina-Nefeli Kontandreopoulou *et al.,* 2022). Epigenetic regulatory gene mutations include *DNMT3A*, *TET2*, *ASXL1*, *IDH1*, *IDH2* and *EZH2* and these were all commonly found in our study. *ASXL1* mutations are associated with a poor prognosis and an increased risk of transformation to AML. Tefferi *et al.,* in 2017 observed that *ASXL1* mutations commonly occur with other mutations. This was confirmed by Liu *et al* in 2021, who observed that *ASXL1* mutations are commonly mutated with *ETV6*, *RUNX1* and *SRSF2* mutations, as observed in our study. *IDH1* and *IDH2* genes are important for the generation of cellular energy by converting isocitrate to 2-ketoglutarate. *IDH2* mutations are more prevalent and are known to pose a higher risk of transformation as opposed to *IDH1*. Both mutations are associated with excess blasts and multilineage dysplasia and are treatment targets. *IDH1* mutations are commonly found together with *ASXL1*, *DNMT3A* and *SRSF2* mutations (Bănescu, Tripon and Muntean, 2023). *TET2* starts the DNA methylation process and hydroxylates methylated cytosines. *TET2* mutations include deletions, missense, nonsense and frameshift mutations and are commonly associated with older age and normal cytogenetic analysis. The prognostic impact of *TET2* mutations is not fully understood yet. However, *TET2* mutations have a higher rate of leukaemic transformation

(Christina-Nefeli Kontandreopoulou *et al.,* 2022). In our study, more than one *TET2* mutation was observed in the same patients and it was commonly mutated across both cohort groups. *DNMT3A* is another DNA methylation gene and is known to have a poor prognosis. *DNMT3A* and *TET2* mutations were found to appear in the early stages of MDS and have a higher risk of progression to AML. *EZH2* mutations are associated with oncogenesis and poor prognosis (Bănescu, Tripon and Muntean, 2023).

Transcription Factor Genes Mutations

Transcription factor genes mutations are known to result in AML transformation from MDS and include *RUNX1*, *GATA2*, *NPM1* and *CEBPA*. Interestingly, in our study, *RUNX1* was found more commonly in Cohort A. This might mean that patients with mutated *RUNX1* have a higher risk of transformation to AML and these patients should be monitored more closely. The other mutations *GATA2* and *CEBPA* were not found in our study. Patients with *RUNX1* mutations usually have a bad prognosis. *RUNX1* and *SRSF2* genes are commonly mutated together. *NPM1* was commonly mutated in Cohort B as expected because *NPM1* is more common in AML than in MDS. *NPM1* also has a poor clinical outcome (Bănescu, Tripon and Muntean, 2023).

Signal Transduction Genes Mutations

Signal transduction genes mutations include *NRAS*, *KRAS*, *JAK2*, *JAK3*, *PTPN11*, *CBL* and *FLT3*. *NRAS* and *KRAS* are involved in leukaemia pathogenesis and the presence of these mutations in MDS can be an indication of leukaemia progression. Hence, these patients have a poor prognosis. *PTPN11* mutations are uncommon in MDS. In fact, no *PTPN11* mutations were detected in Cohort A. The prognosis of patients with *PTPN11* mutation is still conflicting. Other less frequent mutations in MDS but commoner in AML

include *CBL* and *FLT3* and these mutations were not found in Cohort A. *CBL* is responsible for tyrosine kinase signalling and the degradation of certain important proteins such as *FLT3* and *STAT5* in myeloid malignancies. *CBL* mutations were found to be associated with MDS types which are more aggressive. *FLT3* mutations are one of the most common mutations in AML. This is close to our findings were *FLT3* mutations were detected in 9% of patients from Cohort B. In literature, most *FLT3* mutations which were detected in MDS patients were observed during leukaemic transformation and hence *FLT3* mutations should be considered as a disease progression marker. Patients with *FLT3* mutations are known to have shorter overall survival rates. Detection of *FLT3* mutations is important for earlier detection of leukaemic transformation, earlier treatment and better overall patient management (Bănescu, Tripon and Muntean, 2023). Many clinical trials have also focused on targeting *FLT3*. *FLT3* targeted therapy include small-molecule *FLT3* inhibitors such as midostaurin and gilteritimib (Tecik and Adan, 2022). The deletion of the long arm of chromosome 5 or loss of 5q results in *JAK2* mutation and this is also associated with leukaemia transformation. However, the prognostic impact of *JAK2* mutations still remain unclear (Bănescu, Tripon and Muntean, 2023).

Tumour Suppressor Genes Mutations

The *TP53* gene is a tumour suppressor gene and is located on chromosome 17. It is responsible for DNA repair, cell cycle arrest and cell apoptosis to protect cells during stress and damage. *TP53* gene mutation was the most common mutation detected in Cohort B which consisted mainly of leukaemia patients. *TP53* mutations can also be detected in other tumours, lymphoid leukaemias and also in MDS although in this study

no *TP53* mutations were detected in Cohort A. *TP53* mutations in MDS can imply a higher risk of transformation and poor prognosis. Therefore, the *TP53* status is crucial for diagnosis and therapy decisions (Bănescu, Tripon and Muntean, 2023).

The mutation variance allele frequency (VAF) is 'the percentage of sequence reads observed matching a specific DNA variant divided by the overall coverage at that locus' (Strom, 2016). From the Heat Maps generated in this study, it was observed that mutations detected in Cohort B had higher VAFs than mutations detected in Cohort A. Moreover, in Cohort B, the mutation *TP53* was the most commonly mutated and had the highest VAFs followed by *TET2*. The clinical outcome of the somatic mutations in myeloid malignancies is commonly studied. However, little is known on any associations between VAF and prognosis. A study by Wan-Hsuan Lee *et al.,* in 2022, investigated the VAF of myeloid mutations in 698 MDS patients. The results were correlated with their prognosis. Lee *et al.,* observed that high VAF of *DNMT3A*, *TET2, ASXL1, SRSF2, ZRSR2* and *TP53* were significantly associated with poor survival. Moreover, they concluded that the VAF is very important for risk stratification in patients with MDS and should be implemented in new scoring systems (Wan-Hsuan Lee *et al.,* 2022). This conclusion was further confirmed by Taegeun Lee *et al*., in 2023 who evaluated the prognostic impact of VAF in patients with acute myeloid leukaemia, myelodysplasia related (AML-MR). Taegeun Lee *et al.,* found that the survival rate of patients with MDS-related gene mutations with a VAF ≥10% was significantly lower than those patients with a VAF of 2-10%. Taegeun Lee *et al.,* also confirmed that the VAF has a prognostic impact and should be a predictor of survival rate (Taegeun Lee *et al.,* 2023).

Genetic variants can be classified as pathogenic, likely pathogenic, variants of unknown significance (VUS), likely benign and benign. In the clinical setting, variant classification is important as this information can influence treatment and care decisions. Pathogenic variants are considered to be the cause of the disease. Likely pathogenic variants are considered to be the probable cause of the disease. These variants must be interpreted with caution as there is still some degree of uncertainty. In VUS, there is not much evidence about these variants yet. But they might increase the risk of developing the disease. In benign and likely benign variants, the variants are not likely to be the cause of the disease (Susswein *et al.,* 2016). In this study, the mutations reported were all pathogenic, likely pathogenic or VUS. Benign or likely benign mutations were never reported as these are variants which are not causing the disease (MDS/AML).

Another factor which can affect disease progression in MDS is the number of mutations detected in each patient. In our study, 43% of patients from Cohort A had no mutations. The rest of patients had 1 mutation (3%), 2 mutations (20%), 3 mutations (10%), 4 mutations (20%) and 5 mutations (3%). Patients from Cohort B had 1 mutation (26%), 2 mutations (22%), 3 mutations (26%), 4 mutations (4%), 5 mutations (13%) and 6 mutations (9%). None of the patients had zero mutations. Cook, Karp and Lai in 2021, demonstrated that as the number of mutations increase, the survival rate of patients decreased (Cook, Karp and Lai, 2021).

Characterisation of the genomic profile of patients with myeloid malignancies has become a very valuable tool for accurate diagnosis, prognostication and treatment decisions. The best therapeutic approach in patients with the heterogeneous

Myelodysplastic Syndrome is to provide personalised treatment depending on their age, disease characteristics and any mutations present (Bănescu, Tripon and Muntean, 2023). Although there are various therapeutic approaches for MDS patients, allogeneic haematopoietic stem cell transplantation remains the only curative option. However, it is only available for a few patients. This is because of high risks associated with transplantation such as mortality especially in the elderly. To select which patients are eligible for transplantation, several factors must be considered including patient factors and disease factors such as genetic markers and stage of disease (Saber and Horowitz, 2016). Most MDS patients receive non intensive treatments such as iron chelation, growth factors, lenalidomide and hypomethylating agents. The aim of these treatments is to improve the quality of life of patients and cytopenias. Moreover, most treatments given are aimed to delay or prevent disease progression (Platzbecker, 2019). During the past years, major advances have been made when it comes to understanding the very heterogeneous MDS. However, more research is still required to provide more targeted therapy. When it comes to treatment, the two major sub-categories of MDS are low-risk MDS and high-risk MDS. The main aims of treatment in patients with low-risk MDS are to improve cytopenias, reduce transfusions, improve the quality of life, delay disease progression, improve survival and cure. The priorities in patients with high-risk MDS are to delay disease progression, improve survival and cure the disease (Bănescu, Tripon and Muntean, 2023) (Platzbecker, 2019). Current standard care in low-risk MDS patients include watchful observation, targeting anaemia and del(5q) with recombinant erythropoietin, using iron chelation to prevent iron overload from transfusions, lenalidomide to improve anaemia and immunosuppressive agents. Hypomethylating agents (HMA) such as azacitidine or decitabine are also a common treatment used in

MDS, especially in high-risk MDS. HMA improve the quality of life and increase survival and can be used in patients who are not eligible for chemotherapy. Intensive chemotherapy including an anthracycline-cytarabine combination or cytarabine in high-doses is the current standard care in high-risk MDS patients (Platzbecker, 2019). During recent years, novel therapies have been developed which are specific to the mutational status of the patient. This highlights the importance of using NGS to identify the mutational profile of patients so to be able to provide personalised treatment. This can also be beneficial in patients with persistent cytopenias and potential pre-MDS conditions who can be quite challenging to manage. Examples of targeted treatments include *IDH* inhibitors such as ivosiderib and enasidenib for patients with *IDH1* and *IDH2* gene mutations, lenalidomide for patients with del(5q), luspatercept for patients with *SF3B1* mutations and allogeneic stem cell transplantation in patients with mutated *NPM1* (Bănescu, Tripon and Muntean, 2023). Other targeted therapies are directed towards *TP53* mutation which is associated with a poor prognosis even after allogeneic stem cell transplantation. Recent studies have discovered immunotherapeutic targets directed against *TP53*. Some promising agents include magrolimab, flotetuzumab, sabatolimab and eprenetapot (Daver *et al.,* 2022).

5.3 Comparison of Immunophenotyping and Molecular Results

Immunophenotyping and Molecular results were compared to investigate any agreement between Immunophenotyping and Molecular results in both cohort groups and also to assess the relevance of the LSC tube when investigating patients with persistent cytopenias. In Cohort A, there was 56.6% agreement between Immunophenotyping and Molecular results, and disagreement in 43.3% of results. The

disagreement of results in Cohort A could have been a result of several reasons. The presence of LSCs and cytopenias in these patients can be a result of earlier stages of other haematological disorders. LSCs can be detected in other conditions such as lymphoid neoplasms and not only myeloid (which were investigated in this study). Another possibility of these findings might be that the NGS targeted panel used included 40 genes which are commonly mutated in myeloid malignancies. However, these patients might have had other mutations present which were not included in this panel.

In Cohort B, the positive control group, there was high agreement between Immunophenotyping and Molecular results. In fact, 'Presence' was identified in 91.3% of the cases by both Flow Cytometry and NGS. In 8.7% of cases 'Presence' was identified by NGS and 'Absence' by Flow. This can be due to one of the limitations of the LSC tube that was designed for the CD34+CD38- stem cells. No leukaemic stem cells were identified in some samples with CD34+CD38+ve AML, since the acquisition of CD38 indicates a well differentiated and committed AML. Therefore, although the LSC tube can be applied to most AML cases, there can be few cases in which the LSC tube will not provide any further information. As already mentioned before, the LSC tube is also found to be challenging in the CD34-ve AMLs especially when it comes to MRD analysis (Liu *et al.,* 2022). However, within our study, LSCs were identified in all the CD34-ve AML Cohort, although the CD34+ve compartment made up a minor proportion of the total bone marrow nucleated cells. This might be because patients were only investigated at diagnosis and not at an MRD stage. The high agreement between Immunophenotyping and Molecular results especially in Cohort B, confirms the relevance of the leukaemic stem cell tube when investigating patients with HR-MDS/AML. The leukaemic stem cell

tube provides additional information to the clinician on the stem cell compartment and might aid in the clinical management of these patients. The lower percentage of agreement between Immunophenotyping and Molecular results in Cohort A, shows that further studies are required to confirm the relevance of the leukaemic stem cell tube in patients with persistent cytopenias. So far, the only study available in literature which compared the presence of leukaemic stem cells and the genomic profile of patients was the one published by van Spronsen *et al.,* in 2023. In the study by Spronsen and colleagues, no relationship was found between the presence of LSCs and mutations. However, molecular testing was carried out on a small cohort of 10 patients and therefore, no solid conclusions could be drawn from this study when comparing the LSC findings to Molecular results (van Spronsen *et al.,* 2023). Up to the date of submission, no published data could be traced, to compare our findings. The studies available till now use different immunophenotypic techniques, such as different Flow Cytometry panels, which are used for the routine investigation of suspected MDS. One of the aims of our study was to investigate the relevance of the leukaemic stem cell tube in patients with persistent cytopenia, and to check whether it can be used as an additional tool when investigating these patients.

A study by Gao *et al.,* in 2022, compared the morphologic, immunophenotypic and molecular findings in patients with clonal cytopenias of undetermined significance (CCUS), MDS and AML with MDS-related changes. For immunophenotyping, an eight colour, 5-tube panel was used for AML patients and a 4-tube panel was used for MDS patients. Flow cytometric investigations showed accumulation of abnormalities when comparing CCUS, low-grade MDS, high-grade MDS and AML with MDS-related changes. Moreover, morphologic abnormalities also increased as the disease progressed. Gao *et*

al., also compared molecular and flow abnormalities. Immunophenotypic abnormalities in CCUS patients who also had high-risk mutations, were similar to those patients with low-grade MDS. This shows that CCUS can be an immediate precursor to low-grade MDS. CCUS patients had fewer mutations in splicing factor and epigenetic regulatory genes *TET2*, *DNMT3A* and *ASXL1* when compared to MDS patients. Similar to our study, patients with high-grade MDS and AML had more mutations in transcription factor genes such as *RUNX1*, *GATA2* and *CEBPA* and tumour suppressor genes such as *TP53*, showing that these mutations play a significant role in transformation from MDS to AML. A limitation of this study by Gao *et al.,* was that most CCUS patients were not followed up. This study concluded that the stepwise acquisition of mutations in genes coding for splicing factors, transcription factors and tumour suppressors was responsible for transformation from CCUS to MDS and eventually AML. Accumulation of mutations was also accompanied by additional morphological dysplasia and immunophenotypic abnormalities as the disease progresses (Gao *et al.,* 2022). In our study, only 2 patients were classified as having CCUS. The mutations detected in these patients were *SF3B1*, *TET2* and *ASXL1* which are all mutations in splicing factor and epigenetic regulatory genes. This further confirms that mutations in splicing factor and epigenetic regulatory genes occur very early in the evolution of MDS. Moreover, 1 out of the 2 CCUS patients investigated in our study also had leukaemic stem cells. Definite conclusions cannot be drawn from our study regarding CCUS patients due to the small sample size and lack of long term followup.

5.4 Updated WHO Classification of Myeloid Malignancies including some potential Pre-MDS conditions

In 2022, the 5th edition of the 'World Health Organization Classification of Haematolymphoid Tumours' was published including some important changes. Myeloid precursor lesions (CHIP and CCUS) were included in the classification of 'Myeloid proliferations and neoplasm'. This confirms the relevance of our study when investigating patients with potential pre-MDS conditions. These myeloid precursor lesions are now uniformly defined. CCUS was defined as having clonality and cytopenias and other conditions which do not meet the diagnostic criteria for MDS. The cytopenia definitions in the WHO Classification are the same for CCUS, MDS and MDS/MPN and include Hb <13g/dL in males and <12g/dL in females; neutrophils $<1.8x10^9$/L and platelets $<150x10^9$/L (Khoury *et al.,* 2022).

Myelodysplastic syndromes have been renamed as Myelodysplastic neoplasms, however, the abbreviation MDS was kept. The change in nomenclature highlights the fact that MDS is a neoplasia process and to harmonise this entity with Myeloproliferative neoplasia. Myelodysplastic neoplasm was divided into 3 major groups unlike the 2017 WHO MDS Classification which included 8 sub-groups. The WHO 2022 Classification of Myelodysplastic neoplasm includes: 'Myelodysplastic neoplasm post cytotoxic therapy', 'MDS with defining genetic abnormalities' and 'MDS defined by morphology'. The last two sub-groups were further subclassified as represented in table 23 below:

	Blasts	Cytogenetics	Mutations
MDS with defining genetic abnormalities			
MDS with low blasts & isolated 5q deletion	<5% BM & <2% in PB	5q deletion alone, or with 1 other abnormality other than monosomy 7 or 7q deletion	
MDS with low blasts and *SF3B1* mutation (MDS-*SF3B1*)	<5% BM & <2% in PB	Absence of 5q deletion, monosomy 7 or complex karyotype	*SF3B1*
MDS with biallelic *TP53* inactivation (MDS-bi*TP53*)	<20% BM and PB	Usually complex	Two or more *TP53* mutations, or 1 mutation with evidence of *TP53* copy number loss of cnLOH
MDS, morphologically defined			
MDS with low blasts (MDS-LB)	<5% BM & 2% PB		
MDS, hypoplastic (MDS-h)	<5% BM & 2% PB		
MDS with increased blasts (MDS-IB) • MDS-IB1 • MDS-IB2 • MDS with fibrosis (MDS-f)	 5-9% BM or 2-4% PB 10-19% BM or 5-19% PB or Auer rods 5-19% BM; 2-19% PB		

Table 23: WHO 2022 MDS Classification (Khoury *et al.,* 2022).

The WHO AML classification has also been updated, including more genetic abnormalities. The 2 main categories of AML are now 'AML with defining genetic abnormalities' and 'AML defined by differentiation'. The sub-classification of these 2 categories can be seen in table 24 below. There is also another category named 'Myeloid neoplasm post cytotoxic therapy'.

Acute myeloid leukaemia with defining genetic abnormalities
Acute promyelocytic leukaemia with *PML::RARA* fusion
Acute myeloid leukaemia with *RUNX1::RUNX1T1* fusion
Acute myeloid leukaemia with *CBFB::MYH11* fusion
Acute myeloid leukaemia with *DEK::NUP214* fusion
Acute myeloid leukaemia with *RBM15::MRTFA* fusion
Acute myeloid leukaemia with *BCR::ABL1* fusion
Acute myeloid leukaemia with *KMT2A* rearrangement
Acute myeloid leukaemia with *MECOM* rearrangement
Acute myeloid leukaemia with *NUP98* rearrangement
Acute myeloid leukaemia with *NPM1* mutation
Acute myeloid leukaemia with *CEBPA* mutation
Acute myeloid leukaemia, myelodysplasia-related (AML-MR)
Acute myeloid leukaemia with other defined genetic alterations
Acute myeloid leukaemia, defined by differentiation
Acute myeloid leukaemia with minimal differentiation
Acute myeloid leukaemia without maturation
Acute myeloid leukaemia with maturation
Acute basophilic leukaemia
Acute myelomonocytic leukaemia
Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryoblastic leukaemia

Table 24: WHO 2022 AML Classification (Khoury *et al.,* 2022).

The AML-MR (myelodysplasia-related) subclassification which was formerly termed AML-with MDS related changes, is now defined as 'a neoplasm with ≥20% blasts expressing a myeloid immunophenotype and harboring specific cytogenetic and molecular abnormalities associated with MDS, arising de novo or following a known history of MDS or MDS/MPN'. The new classification removed the morphological criteria of dysplasia, updated the cytogenetic criteria and introduced some mutations which are *SRSF2, SF3B1, U2AF1, ZRSR2, ASXL1, EZH2, BCOR* and *STAG2* (Khoury *et al.,* 2022). This further confirms the importance of an NGS myeloid panel when investigating patients with persistent cytopenias and suspected MDS equally as in AML patients. A major change in the WHO AML classification was that the >20% blasts cutoff was removed from most AML with defining genetic abnormalities except for AML with *BCR::ABL1*, AML with *CEBPA* mutation and AML-MR. The 20% blast cutoff still applies for AML defined by differentiation (Huber *et al.,* 2023).

In 2022, the International Consensus Classification (ICC) was also proposed by another expert panel in parallel to the WHO Classification. As opposed to the WHO 2022 classification, the ICC uses a blast cutoff of 10% for AML with defining genetic abnormalities. Cases with 10-19% blasts without defining genetic abnormalities were grouped into a new category which is AML/MDS. This new AML/MDS category acknowledges the disease continuum between MDS and AML and can also expand the treatment options for patients in this category. Another difference is the inclusion of AML with mutated *TP53* in the ICC and not in the WHO classification. The premalignant clonal cytopenias (CCUS) are now recognised in both ICC and WHO 2022 classifications. For low-risk MDS, the ICC has the following sub-classifications: 'MDS, NOS-SLD', 'MDS, NOS-MLD', 'MDS, NOS', 'MDS with mutated *SF3B1* (MDS-*SF3B1*)' and 'MDS-del(5q)'. As

mentioned earlier, the WHO classifies low-risk MDS as 'MDS-LB', 'MDS-*SF3B1*', 'MDS with low blasts and isolated 5q deletion' and 'MDS, hypoplastic'. For the high-risk MDS, ICC has the following sub-classifications: 'MDS with mutated TP53 (MDS-TP53)', 'MDS-EB' and 'MDS/AML mutated TP53, MR gene mutations or MR cytogenetic abnormalities' for patients with 10-19% blasts in peripheral blood or bone marrow. On the other hand, the WHO classification has the following sub-classifications: 'MDS with biallelic TP53 inactivation (MDS-bi TP53) (<20% blasts)', 'MDS-IB-1 (2-4%PB, 5-9%BM)', 'MDS-IB-2 (5-19%PB, 10-19%BM or auer rods)' and 'MDS with fibrosis (MDS-f)'. The AML with myelodysplasia-related changes has been changed to AML-myelodysplasia related in the WHO 2022 classification. As mentioned earlier this includes some genetic mutations (*ASXL1, BCOR, EZH2, SF3B1, SRSF2, STAG2, U2AF1* or *ZRSR2*), cytogenetic abnormalities and history of MDS or MDS/MPN. In ICC, AML with myelodysplasia related changes is classified in 4 sub-categories: 'AML with myelodysplasia- related gene mutations', 'AML with myelodysplasia-related cytogenetic abnormalities', 'AML with mutated *TP53*' and 'AML not otherwise specified (AML-NOS)'. The major differences from the WHO classification includes the addition of *RUNX1* mutation in the 'AML with myelodysplasia-related gene mutations' category, some cytogenetic changes and the inclusion of 'AML with mutated *TP53*'. The advancements in the pathological, immunophenotypic and molecular characterisation of myeloid neoplasms has led to refinement of the diagnostic criteria of myeloid malignancies. Although both ICC and WHO 2022 classifications can lead to more accurate diagnosis and risk stratification, the presence of two classifications lead to new challenges in clinical application. Ideally, these two new classifications must be integrated into one to facilitate clinical decisions and patient management (Wu *et al.,* 2023).

5.5 A Holistic Approach for Better Patient Management

The prognosis and treatment of MDS patients depends on a variety of factors and therefore, accurate diagnosis is crucial for the best management of these patients. A holistic approach must be used for the most accurate and reliable diagnosis of MDS. MDS diagnosis should be based on a CBC and morphology assessment of a peripheral blood, bone marrow morphology and histology, immunophenotyping by flow cytometry, molecular studies to check for mutations and cytogenetic analysis (Brunner *et al.,* 2022). Both the WHO and ICC classifications of myeloid malignancies use an integrated approach based on clinical, morphological, genetic, flow cytometric and molecular findings. The European Leukaemia Net international MDS Flow Working Group (ELN iMDS FLOW WG), published an article in 2021 discussing the importance of flow cytometry when investigating patients with persistent cytopenias and suspected MDS. Immunophenotypic aberrancies in precursor populations, erythroid, myeloid and monocytic populations can be detected by flow cytometry. In patients with CCUS and ICUS, flow cytometry can show dysplastic changes which cannot be detected by cytomorphology. This further confirms the importance of flow cytometry when investigating patients with suspected MDS. In addition, the absence of flow cytometric abnormalities can help with the exclusion of MDS. Flow cytometry is a recommended tool for the routine diagnostic investigations of MDS in the published European Leukaemia Net guidelines. An 8- or 10- colour flow cytometry screening tubes are suggested for the characterisation and evaluation of haematopoietic cells. Flow cytometry can also be instrumental in prognostication and in disease monitoring during treatment and therefore, repeated assessments by flow cytometry are strongly

recommended in ICUS, CCUS and MDS patients (van de Loosdrecht *et al.,* 2021). An additional flow cytometric test which can be used when investigating patients with persistent cytopenias is the leukaemic stem cell tube used in our study. The LSC tube was developed at the Cancer Center in Amsterdam UMC, a group which forms part of the European Leukaemia Net working group. So far, they focused on the use of the LSC tube in patients with AML to assess the risk of relapse after treatment and they are working to include the LSC tube in the prognostication system in AML through an ELN-David project which is part of the European LeukaemiaNet (ELN-David, 2022) (Heuser *et al.,* 2021). The LSC tube was also used in another study at Amsterdam UMC by van Spronsen *et al.* in 2023 in patients with MDS. This study concluded that the LSC tube in MDS can identify immunophenotypic aberrant stem cells and can be used as a biomarker for leukaemia progression (Spronsen *et al.,* 2023). However, more research is still needed to support this conclusion and the similar conclusion drawn from our study. We believe that the LSC tube can also be included in the diagnostic work-up of patients with suspected MDS in the future.

Another crucial diagnostic test when investigating patients with suspected MDS is next-generation sequencing to identify the mutational profile of individuals. NGS can also be very helpful when investigating patients with persistent cytopenias including ICUS and CCUS patients. A study by Marie Fredslund Breinholt in 2022, investigated the value of next-generation sequencing in routine diagnostics and management of patients with cytopenia and concluded that NGS analysis of bone marrow samples can highly contribute for an accurate diagnosis and management of these patients. They in fact included NGS analysis on bone marrow samples as part of their routine investigations for unexplained cytopenias (Marie Fredslund Breinholt *et al.,* 2022). Bone marrow

analysis is currently the gold standard when investigating patients with cytopenias. However, bone marrow sampling is an invasive procedure and can be quite painful to patients. Genomic analysis using peripheral blood samples instead of bone marrow samples would be a great advancement in the diagnostic work-up of MDS especially in the elderly individuals. A very recent study published by Huber *et al.,* in 2024 compared genomic analysis of 200 cytopenic patients from peripheral blood (PB) and bone marrow (BM) samples. The cohort group included 75 patients with MDS, 52 CCUS patients, 43 ICUS patients and 30 patients without a definite diagnosis. No mutations were detected in PB and BM samples of 70 out of the 200 participating patients. In 129 out of the 130 remaining patients, mutations were detected in both PB and BM samples. The number of mutations detected in PB and BM was the same in 118 patients. More mutations were detected in 9 BM samples and in 2 PB samples. Similar to our study, the most frequently mutated genes were *TET2* (15%), *DNMT3A* (10%) and *ASXL1* (9%). This study concluded that peripheral blood samples can be used as a first screening tool in patients with suspected MDS. The current guidelines recommend a BM examination when clonality is detected for a full diagnosis to be made and for prognostication. In CCUS and ICUS cases, analysis of NGS from peripheral blood can be used to monitor disease progression instead of the more invasive BM sampling. This will lead to better patient management (Huber *et al.,* 2024). Unfortunately, the same cannot be applied to the flow cytometric investigation of the stem cell compartment. Identification of stem cells by flow cytometry can be challenging even in bone marrow samples due to the small amount of stem cells. However, investigation of the stem cell compartment by flow cytometry using bone marrow samples can still be used as a secondary screening and monitoring tool when clonality is identified by NGS analysis of peripheral blood samples.

Another important element in the management of MDS patients is their prognosis which is based on disease severity and treatment eligibility. Prognostic factors include both patient related and disease related factors (Brunner *et al.,* 2022). Patient related factors include age at time of diagnosis, associated comorbidities and performance status. Disease related factors include genetic and cytogenetic results and clinical/haematological variables (percentage of marrow blasts, CBC, LDH, ferritin, albumin). The International Prognostic Scoring System (IPSS) was the first important prognostic scoring system for untreated MDS patients. Patients are classified into four risk categories by the IPSS scoring system; low, intermediate-1, intermediate-2 and high depending on the cytopenias, blast percentages and cytogenetic alterations. A limitation of this system was that it could not be applied to treated MDS patients. Therefore, a new IPSS score was proposed which was the IPSS-R. The revised IPSS-R takes into consideration haematologic parameters and emphasizes more on cytogenetic abnormalities. Patients are classified into five risk categories; very low, low, intermediate, high and very high risk (Brunner *et al.,* 2022) (Lucero, Al-Harbi and Yee, 2023). The most recent prognostic scoring system is the Molecular International Prognostic Scoring System (IPSS-M) which was described in 2022. Patients are classified into six risk categories: very low, low, moderate low, moderate high, high and very high risk. This IPSS-M takes into consideration the presence of somatic mutations and provide a personalised risk score (Bănescu, Tripon and Muntean, 2023) (Brunner *et al.,* 2022) (Lucero, Al-Harbi and Yee, 2023).

Using all the recommended tools (including both molecular and immunophenotyping techniques) when investigating patients with persistent cytopenias is the ideal approach for the diagnosis, patient management and personalised treatment. We can postulate

that the LSC result can serve as a prognostic marker to progression, but we cannot conclude on this since the sample size used in this study was small.

5.6 Limitations of the Study

The results of this study have shown the importance of the leukaemic stem cell tube and molecular testing when investigating patients with persistent cytopenias as they can guide clinicians better in the management of these challenging patients. However, a larger sample size would have provided more robust data for statistical analysis. A limitation of this study was time and funds due to high reagent costs. Moreover, bone marrow samples were collected and sample collection was carried out over a period of 12 months. Bone marrow sampling is an invasive procedure and samples were collected as part of the diagnostic work-up of patients. No sample was collected for research purposes only and this limited once again the sample size. It would have been ideal to have a larger sample size especially for the pre-MDS conditions ICUS and CCUS. No conclusions could be drawn from this study about ICUS and CCUS due to the very small sample size. The majority of patients from Cohort A were classified as low-risk MDS.

Long-term monitoring of patients with persistent cytopenias, particularly those with the presence of leukaemic stem cells and mutations, would have given a better insight into the prognostic value of these immunophenotypic and molecular aberrations. Due to time and financial restrictions, such monitoring could not be carried out through this project. However, an interesting future project would be the long-term monitoring of patients with persistent cytopenias to assess the risk of MDS/AML transformation in the leukemogenesis process and repeating the marrow to check for increasing LSCs.

Another limitation was that few patients had to be excluded from this study as they were vulnerable and could not be consented. Also, assessment of dysplasia was carried out by the actual Haematology Consultant taking care of the patient during the diagnostic work-up. Morphological assessment can be subjective, and it would have been ideal if a couple of specialists have reviewed the material independently and then came up with a unifying report.

The leukaemic stem cell tube was originally developed to monitor patients with AML after receiving treatment in order to identify residual leukaemic stem cells which are known to cause relapse (Zeijlemaker *et al.,* 2015) (Hansen *et al.,* 2022). In our study, the leukaemic stem cell tube was used to identify leukaemic stem cells in patients with persistent cytopenias before transformation to MDS-HR/AML. Leukaemic stem cells were identified in 60% of patients from Cohort A. However, although more than half of patients with persistent cytopenias had leukaemic stem cells, little is known about the use of the LSC tube in MDS patients and more research is required to support the conclusions drawn from our study. Identification of leukaemic stem cells can be challenging, especially in some MDS patients due to their low frequency. Therefore, a large number of cells (5,000,000 events) had to be acquired to be able to identify stem cells. The acquisition of 5,000,000 events also increases the sensitivity of the Flow Cytometric test. Another limitation of the LSC tube is that it can be challenging in some cases of AMLs, especially CD34-ve AMLs (Liu *et al.,* 2022). However, in our study, LSCs were identified in all the CD34-ve AML Cohort, although the CD34+ve compartment made up a minor proportion of the total bone marrow nucleated cells. This might be because patients were only investigated at diagnosis and not at an MRD stage. LSCs

were not detected in 2 patients who had a CD34+CD38+ leukaemia, since the acquisition of CD38 indicates a well differentiated and committed AML. Therefore, although the LSC tube can be applied to most AML cases, there can be few cases in which the LSC tube will not provide any further information. An ongoing study is currently being carried out at Amsterdam UMC to investigate the use of CD133 as a LSC marker in the CD34 negative AML cases. Takanashi *et al.,* in 2013 also identified CD133 as a CD34 negative stem cell marker in cord blood derived CD34-ve cells (Takahashi *et al.,* 2013).

Molecular analysis by NGS is another valuable tool when assessing patients with persistent cytopenias. A myeloid targeted NGS panel was used in this study. Although a targeted panel is much less expensive than whole-genome sequencing, the cost of targeted NGS is still expensive. In addition, the procedure is laborious and the turnaround time is of a minimum of four days. Another limitation of targeted NGS panels is that only data on targeted regions is obtained. Some relevant variants may be missed if they are not in the designed panel. However, considering this limitation, a targeted NGS myeloid panel is still the best option when investigating patients with suspected MDS and for diagnostic purposes. Whole genome sequencing generates a great amount of data which requires robust bioinformatic systems to aid in the diagnostic process. Unavailability of such systems and lack of expertise may result in more complicated diagnostic processes (ThermoFisher Scientific, 2023).

5.7 Future Recommendations

Although this study has shed some light on the usefulness of LSC quantification in patients with persistent cytopenias by investigating the stem cell compartment, the

time-frame of this study was not long enough to investigate the risk of leukaemic progression. Long-term monitoring of patients with persistent cytopenias, particularly those with the presence of leukaemic stem cells and mutations, would have given a better insight into the prognostic value of these immunophenotypic and molecular aberrations. Due to time and financial restrictions, such monitoring could not be carried out through this project. However, an interesting future project would be the long-term monitoring of patients with persistent cytopenias to assess the risk of MDS/AML transformation in the leukemogenesis process.

Other interesting future projects include the validation of the LSC tube for AML MRD purposes locally and genomic analysis using peripheral blood and bone marrow samples to further improve patient management. Another interesting project would be single cell analysis for mutations in LSCs. Originally, the plan was to do cell sorting to be able to do single cell analysis of the stem cell compartment. However, we were advised by the research team at Amsterdam UMC that it can be challenging to select the cut-off for the stem cell compartment because the CD34+CD38 expression pattern is like a smear. To be able to sort cells one needs a strict definition of positive vs negative. The way the stem cells are identified in the LSC tube is also including the heterogeneous expression of CD38 and this makes this methodology better for identification of leukaemic stem cells. It would have been ideal to do mutational analysis on the stem cell compartment.

Chapter 6

Conclusions

Conclusions

The results obtained from this study confirm that leukaemic stem cells are the first step in leukemogenesis and can lead to the development of MDS and AML. We believe that patients with leukemic stem cells might have an increased risk of leukemic progression and these patients should be monitored more closely. The high percentages of LSCs in the positive control group provide confidence that the methodology for the identification and characterisation of LSCs has been correctly validated. Moreover, the presence of LSCs in more than half of patients (60%) with persistent cytopenias show that the LSC tube can give additional information on the stem cell compartment to the clinicians leading to better patient management. However, more long-term research is required to further confirm this conclusion. The inclusion of the LSC count as part of the diagnostic work-up of patients with unexplained persistent cytopenias can lead to closer monitoring of patients and earlier treatment (including earlier stem cell transplantation for eligible patients) if LSCs are identified. Detection of LSCs is also important in view of the development of therapeutic targets towards aberrant markers identified using this tube. Identification of the cell surface markers on LSCs can lead to more specific and personalised treatments such as immunotherapy targeted against CD33, CD123, TIM-3 and CLL-1 (Hanekamp *et al.,* 2017) (Mitchell and Steidl, 2019) (Hansen *et al.,* 2022).

Molecular analysis by NGS is another valuable tool when assessing patients with persistent cytopenias. Identification of genetic mutations is important for patient stratification, prognosis, better patient management and more efficient therapeutic approaches such as targeted therapy. The presence of mutations in patients with persistent cytopenias but who do not fulfil the necessary diagnostic criteria for MDS,

classifies patients with CCUS; a terminology which has been recently added to the WHO and ICC 2022 classifications. The type of mutations, number of mutations and also the VAF are all factors which affect the prognosis and overall survival of patients.

The high agreement between immunophenotyping and molecular results shows the importance of using a holistic approach when investigating patients with suspected MDS. Moreover, the high agreement of results further confirm the benefits of adding the LSC count as part of the diagnostic work-up of patients with persistent cytopenias. The inclusion of myeloid precursor lesions (CHIP, ICUS and CCUS) in the recently updated WHO and ICC 2022 classifications, confirms the relevance of our novel research when investigating patients with potential pre-MDS conditions.

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Appendix A

Ethics Approval and Permissions

Ethics Approval

L-Università ta' Malta

Faculty of Medicine & Surgery
University of Malta
Msida MSD 2080, Malta

Tel: +356 2340 1879/1891/1167
umms@um.edu.mt

www.um.edu.mt/ms

Ref No: MED-2022-00106

19 September 2022

Ms Stephanie Vassallo,
78, Lwien, Flat 1,
Triq Hal-Dwin,
Haz-Zebbug,
ZBG3066.

With reference to your application submitted to the Faculty Research Ethics Committee in connection with your research entitled:

Investigation of the Stem cell compartment in patients with chronic cytopenias and low-risk MDS.

The Faculty Research Ethics Committee is granting ethical approval for the above-mentioned application.

Professor Anthony Serracino Inglott
Chair
Faculty Research Ethics Committee

cc Prof. Alexander Gatt

Permission from Chairman of Pathology

SPTAR MATER DEI

PATHOLOGY DEPARTMENT

tel: (00356) 2545-6317

Pathology and Laboratory Medicine Service
Approval for Assistance in Research

Title of Proposed Study: Investigation of the Stem cell compartment in patients with chronic cytopenias and low-risk MDS

Brief Description of Proposal: The ~~study~~ aim is to investigate the stem cell compartment of patients with chronic cytopenias and low-risk MDS. This study will investigate any immunophenotypic and molecular aberrancies within the stem cell compartment of these patients.

Number of Tests Required* 60

Estimated Start and End Dates March 2022 - January 2024

Pathology Departments/ Sections Involved:

- Haematology
- Clinical Chemistry
- Immunology
- Histopathology
- Cytology
- Virology
- Bacteriology
- Genetics
- Toxicology
- Mycology

Specification of Research Type:

- Pilot Study
- Clinical Trial
- Audit
- PhD Study
- Others: please specify Masters Degree Study (Part-time by Research)

Lists Tests Required:

- Immunophenotyping by Flow Cytometry
- Real-Time PCR at Molecular Diagnostics

Ethical Committee Approval Obtained: Yes / (No) → In the process / Not Applicable

Names of Researchers Involved in Research / Trial

Ms. Stephanie Magri (Principal Investigator), Prof. Alexander Galt (Supervisor), Ms. Patricia Bonnici (Co-supervisor) and Prof. Joseph Borg (Co-supervisor)

Magri

Signature of Lead Researcher / Applicant **

Contact Details

e-mail Stephanie.vassallo.1@gov.mt **pager number** ---

phone number 79935695 **Department** Pathology - Haematology

Who should be contacted in the case of Panic Values / Results if Applicable?

Principal Investigation ---- **Tick where appropriate**

On Call Physician ---

Other Consultant in charge of patient **Contact details:** alexander.gatt@um.edu.mt

Date of Application 17/3/2022

Declaration of Acceptance of Specimens *For laboratory Use Only*

Signature of Head of Department

Dr Christopher Barbara
Chairman
Signature of Chairman of Pathology Department
Hospital - Malta

Estimated Cost of Research (€12,500)
(€10,000 funded by MABS research fund, €2500 funded by University of Malta Research Funds)

For managerial Use only

Estimated Materials and Equipment Cost
€0

Estimated Human Resources Cost
€0

Estimated Total Cost €12,500.

** The applicant / researcher undersigned above acknowledges the necessity of the role of the Laboratory Service as an integral component of the above-mentioned Trial / Research / Study / Audit and binds himself / herself to lease with the relevant laboratory personnel to ensure smooth provision of service which includes appropriate notification of samples to be sent and appropriate identification of such specimens

Permission from MDH CEO

RE: Permission from Data Protection Officer for Research study: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS'.

CEO at Health-MDH

Mon 02/05/2022 21:32

To: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>;

Dear Ms Vassallo,

Kindly note that approval has been given by Ms Celia Falzon for you to conduct this study in line with applicable hospital protocols.
Regards

Carmen Farrugia
Personal Assistant To CEO

SPTAR MATER DEI

T +356 +356 25454102
E carmen.farrugia@gov.mt

Mater Dei Hospital, Triq id-Donaturi tad-Demm, l-Imsida, Malta MSD 2090 | Tel +356 2545 0000 |
https://deputyprimeminister.gov.mt/en/MDH/Pages/Home.aspx | https://www.facebook.com/materdeihospital/

From: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>
Sent: Saturday, 30 April 2022 10:32
To: CEO at Health-MDH <ceo.mdh@gov.mt>
Subject: Fw: Permission from Data Protection Officer for Research study: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS'.

Dear Ms. Celia Falzon,

I am Stephanie Magri (ID:221496M) working at the Haematology Lab. I am currently reading for a part-time Master's Degree within the Faculty of Medicine and Surgery, University of Malta. I will be carrying out a research project entitled: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS' under the supervision of Prof. Alexander Gatt, with Ms. Patricia Brincat and Prof. Joseph Borg as co-supervisors. The research project will initiate once UREC ethics approval is obtained and projections are that it should be completed by January 2024.

I am writing this email to ask for your approval as MDH CEO to carry out this research study. Kindly note that all the necessary permissions have been obtained including clearance from data protection, approval from Dr. Christopher Barbara and Dr. Nick Refalo (attached).

Thank you.

Permission from MDH Data Protection Officer

RE: Permission from Data Protection Officer for Research study: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS'.

Caruana Simon at Health-MDH on behalf of Data Protection at Health-MDH

Sat 30/04/2022 10:09

To: 'stephanie.vassallo.15@um.edu.mt' <stephanie.vassallo.15@um.edu.mt>;

Cc: Young Sharon at Health-MDH <sharon.young@gov.mt>; Data Protection Approval Form at Health-MDH <dpaform.mdh@gov.mt>; Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>;

Dear Ms Magri (Vassallo)

On the basis of the documentation you submitted, from the MDH data protection point of view you have been cleared to proceed with your study titled ***Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS*** provided that you obtain approval from MDH CEO (ceo.mdh@gov.mt - please provide the relevant documents including Dr Christopher Barbara's and Dr Nick Refalo's approval with this email).

- Your intermediary to approach potential participants on your behalf is *Dr Erica Sultana – Higher Specialist Trainee in Haematology, MDH*
- Your potential participants to reply your questionnaire and provide a small volume of the bone marrow sample taken by MDH as part of the diagnostic investigation and that will be utilised for genetic testing and flow cytometry for your Master's Degree project are *men and women over the age of 18 years who are receiving services at SAMOC, MDH*

All data stored must be anonymized and in no way should you retain any personal details you obtain from your research and these should be destroyed at the end of your study and /or if any of your participants decides to withdraw. Remember that participants reserve the right to be forgotten.

Anonymisation and Data minimisation

Participant consent forms must be separated from the answered questionnaires and any health data at source, meaning that there will be no correlation between one and the other that will indicate how participants replied and their health status.

ALL data presented to your supervisors / tutors or examiners or any other personnel from UOM or anyone else must be already anonymized; meaning that you must not divulge to anyone the identity of your participants and / or how they replied.

There shall not be any access to personal data for verification purposes by UOM personnel or anyone else since you didn't declare so with the consent documents.

Consent Criteria

This clearance does not allow access to medical records or MDH Health Information Systems.

All your participants must be reached and approached for invitation when physically at MDH grounds through Dr Erica Sultana and NOT via postal services, email, telephone or any other means. You cannot be handed any contact details of

potential participants, otherwise consent would be bypassed.

Potential participants must be approached through Dr Erica Sultana for invitation and not directly by you. You can approach only after the participants will consent.

The pseudonym list for the identity of our patients in respect of the bone marrow samples must only remain in your possession and of Ms Patricia Brincat since you declared so with your consent documents. Personal identifiable data such as signed consent forms and the said pseudonym list are not to be sent via email (not even relayed to yourself or to Ms Patricia Brincat), replicated and/or uploaded in any server, cloud storage, site or any other media since participants will not consent any service provider to store their personal identifiable data. All data that will be stored in an password protected spreadsheet must remain offline and permanently deleted at the end of your study.

Video, audio recordings and photography are not allowed for this research.

Genetic testing and flow cytometry shall only be carried out purposely for this study titled: ***Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS.***

Bone marrow samples cannot be processed or utilised for other projects neither in the present nor in the future.

All data obtained from the bone marrow samples must be utilised for your project titled ***Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS***.

Bone marrow samples must not leave the Haematology Lab, MDH; under no exception or circumstances.

It is your responsibility to ensure that bone marrow samples are processed and destroyed at the Haematology Lab, MDH after you extract the relevant data for your project titled ***Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS***.

It is your responsibility to coordinate the logistics of the samples to ensure their security in terms of access. Remember that genetic data is classified as personal data therefore, even if unlabelled, the samples contain personal data hence these must be accessed only by your good self and Ms Patricia Brincat.

Clarifications

This clearance does not cover ethical approval.

All documents presented to your participants must include UOM's logo.

This clearance does not allow verbal communications, meaning that verbal consent is not covered.

This clearance is valid for your report to be included with your dissertation only and not in medical journals or elsewhere since you are not obtaining approval from MDH legal office.

Your submitted documentation must remain unchanged.

What was declared during this clearance process is what you will abide to.

You must abide with all the articles of the GDPR (EU) 2016 / 679 throughout the data collection process and thereafter.

You are requested to submit a copy of your findings to this office at the end of your study.

Please communicate with Dr Erica Sultana and Ms Patricia Brincat to present this clearance email.

-

Data Protection Clearance Declaration Form

From: Vassallo Stephanie 1 at Health-MDH
Sent: 30 April 2022 09:13:40
To: Data Protection at Health-MDH
Cc: Young Sharon at Health-MDH; Gatt Alexander A at Health-MDH; Brincat Patricia at Health-MDH
Subject: Re: Permission from Data Protection Officer for Research study: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS'.

Dear Mr. Caruana,

Kindly find below the additional information requested:

How are subjects recruited? (also state their age)

> Patients will attend the Outpatient Haematology Clinic for a consultation. An intermediary will provide an Information Letter explaining the details about the study and a Consent Form which they will sign to give their consent if they are interested in participating in this study.

What do subjects do, or what is done to them, or what information is gathered?

> A small volume of their bone marrow sample will be used for this research study and will be required only once throughout the study. The bone marrow sample will be taken during the same procedure as that required for standard clinical care and patient will not be subjected to an extra procedure for this research.

How information is gathered, where it is stored and for how long?

> All the names and any other personal information used for this project will be completely anonymised and will never be published. The data will be retained by an intermediary person.

Who has access for data gathered?

All data will be stored in an excel sheet which is password protected and available only to the researchers involved (namely myself and Ms. Patricia Brincat) and discarded at the end of the study.

State the period of the study.

The study will start once Ethics approval is gained and it is projected that it will be finished by January, 2024.

State the data collection period.

Data collection will start once Ethics approval is gained and will finish when the amount of samples (40-60 samples) required for the study are collected.

Who will carry out this study? Please include any research assistants.

The study will be carried out by myself (Stephanie Magri) and Ms. Patricia Brincat (research assistant and co-supervisor).

How many times will observations, tests, etc., be conducted?

The tests required for this study will be carried out only once on each patient sample.

How long will participation take?

Participation will only be done once throughout the study.

Info letter and consent form should include UOM's logo: (updated)

Provide the questionnaire in English (kindly find attached)

Confirm that audio, video recordings or photography will not take place

I confirm that audio, video recordings or photography will not take place.

Confirm that info from the testing will be utilised only for your project and not for other side / future projects

I confirm that the info from testing will be utilised only for my research project and not for other future projects or side projects.

Provide us an endorsement from Ms Donna Micallef (an email would suffice) (attached)

Confirm that potential participants will be approached at MDH / SAMOC grounds

Potential participants will be approached at MDH SAMOC grounds.

We need a signed declaration from an intermediary stating that s/he will approach potential participants on your behalf (the documents that you provided are approvals to for recruitment and not a declaration that patients will be approached). Remember that researchers cannot approach patients before explicit written consent is obtained. (attached)

In the info letter please include more detail such as: (info letter updated with requested changes)

Thank you.

Kind regards,

Stephanie Magri

Permission from SAMOC Chairman

SIR ANTHONY MAMO ONCOLOGY CENTRE

FORM :	**Oncology Proposal/Approval Audit/ Research purposes**
Document Code: ONCO-GeFO-P/A-001. Ver.01	Reference SOP : ONCO-Ge-PD.AP--001.Ver.01

PROJECT TITLE: Investigation of the Stem cell compartment in patients with chronic cytopenias and low-risk MDS

Name & Surname (Researchers): Stephanie Magri

Email address: stephanie.vassallo.15@um.edu.mt

Tutor's name & Surname: Supervisor: Prof. Alex Gatt; Co-supervisors: Ms. Patricia Brincat and Prof. Joseph Borg

Proposal

Introduction:

Potential pre-MDS conditions include idiopathic cytopenias of unknown significance (ICUS) and clonal cytopenias of undetermined significance (CCUS). These conditions may be potential pre-phases of an MDS; a group of clonal haematopoietic stem cell disorders which are characterised by cytopenias, ineffective haematopoiesis and dysplasia in one or more of the major myeloid lineages. Patients with MDS have increased risk of development of Acute Myeloid Leukaemia (AML). Leukemogenesis involves a multistep process in which there is an evolution from low-risk to high-risk MDS which can finally transform into AML. Flow cytometry has been a useful tool in diagnosis of AML and to some extent high-risk MDS. However, the use of flow cytometry in CCUS/ICUS and low-risk MDS patients is so far limited.

In this study, flow cytometric techniques will be used for patients with CCUS/ICUS and low-risk MDS by looking into the immunophenotypic properties of the Stem cell compartment. Currently the Leukaemic Stem Cell compartment is being investigated as a prognostic and predictive marker for assessment of Minimal Residual Disease monitoring and relapse prediction in patients with AML. This study will investigate any immunophenotypic aberrancies within the Stem cell compartment in patients with CCUS/ICUS and low-risk MDS and correlate the immunophenotype with molecular findings.

Aim/s:

- This study aims to investigate the Stem Cell compartment of patients with chronic cytopenias (ICUS, CCUS and low-risk MDS), with the possibility of identifying Leukaemic Stem Cells.
- Molecular studies will be used to correlate the Flow cytometry findings in the patient cohort, with a control group composed of AML and high risk MDS patients.

Method (include the sample size)

This study will be carried out using bone marrow samples. Patient and Control cohorts will be divided as follows:

- **Cohort A** (CCUS/ICUS/low risk MDS): n=30 Unexplained persistent cytopenias (>4 months) with no or mild dysplasia (Patient group)
- **Cohort B** (high risk MDS/sAML): n=30 multilineage cytopenias, excess blasts >10% by morphology/flowcytometry. (Control group)

Immunophenotyping

Erythrocyte-lysed fresh bone marrow (BM) samples will be used to perform 8-color multiparameter flow cytometry using a FACS Canto II from BD. After immunophenotyping, gating strategy and marker selection will be carried out. BM samples will then be analysed using Infinicyt software (Cytognos).

Molecular Diagnostics

Genomic DNA will be extracted from the bone marrow aspirate samples used for Flow cytometry. Selected exons and flanking sequences of the myeloid genes listed in the table below will be amplified by **real-time polymerase chain reaction** (real-time PCR). Real-time PCR combines PCR amplification and detection into a single step. In real-time PCR, fluorescent dyes will be used to label PCR products using thermal cycling. Real-time PCR instruments will then measure the accumulation of fluorescent signal during the exponential phase of the reaction. Any mutations present on the genes of interest (*DNMT3A, SF3B1, TET2, SRSF2, ASXL1, TP53*) will then be detected.

Clinical Consultant Oncologist/s:

Professor Alexander Gatt

Dr. David James Camilleri

Dr. David Busuttil

Dr. Mark Grech

SIR ANTHONY MAMO ONCOLOGY CENTRE

FORM :	**Oncology Proposal/Approval Audit/ Research purposes**
Document Code: ONCO-GeFO-P/A-001. Ver.01	Reference SOP : ONCO-Ge-PD.AP--001.Ver.01

Heads of: Clinical Lead for Haematology Dr. David James Camilleri

Radiotherapy Department: *(if applicable)*

Radiography

Medical Physics

Nursing

Other SAMOC Departments/ Wards:

Clinical Chairperson (Haematology - Oncology):
Name and Surname (in block letters) and Signature:

Dr Nick Refalo
Chairman & Consultant Oncologist
Sir Anthony Mamo Oncology Centre
Reg No: 2662
22.10.21

Quality Assurance Manager:
Name and Surname (in block letters) and Signature:

An approval is granted to carry out the study/audit at any SAMOC Department. Patient information can be accessible only by complying with the following data protection principles, which are set out in the General Data Protection Regulation 2016. In summary these state that patient's data shall:

- ***Be obtained and processed fairly and lawfully and shall not be processed unless certain conditions are met. Therefore patient's information (including scans) should be made anonymous by an appointed radiotherapy staff (from the Head of section)***
- ***Be obtained for a specified and lawful purpose and shall not be processed in any manner incompatible with that purpose.***
- ***Be adequate, relevant and not excessive for those purposes (in the case of a study or audit).***
- ***Be accurate and kept up to date.***
- ***Not be kept longer than is necessary for that purpose***
- ***Be processed in accordance with the data subject's rights.***
- ***Be kept safe from unauthorised access, accidental loss or destruction.***
- ***Not be transferred to any third party unlawfully.***

Permission from SAMOC HR and Administration Manager

Re: Permission to recruit patients from SAMOC for research study

Vassallo Stephanie 1 at Health-MDH

Thu 28/04/2022 11:25

Sent Items

To: Micallef Donna at Health-SAMOC <donna.b.micallef@gov.mt>;

Thank you Ms. Micallef.

Get Outlook for Android

From: Micallef Donna at Health-SAMOC <donna.b.micallef@gov.mt>
Sent: Thursday, April 28, 2022 11:11:49 AM
To: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>
Cc: Data Protection at Health-MDH <datapro.mdh@gov.mt>
Subject: RE: Permission to recruit patients from SAMOC for research study

Dear Stephanie,

Approved from my end. Copying in Data protection officer for his direction accordingly.

Kind Regards,

Donna

Donna Micallef
HR & Administration Manager

T +356 +356 25452636
E donna.b.micallef@gov.mt

Sir Anthony Mamo Oncology Centre, Triq id-Donaturi tad-Demm, l-Imsida, Malta, MSD 2090 |
https://deputyprimeminister.gov.mt/en/MDH/Pages/Home.aspx | https://www.facebook.com/materdeihospital/

I am writing this email to ask for your approval to carry out this research study as advised by the Data Protection Officer. Kindly note that approval from Dr. Nick Refalo has already been obtained (document attached).

Thank you.

Kind regards,

Stephanie Magri

From: Cachia Vella Mary at Health-SAMOC
Sent: 25 April 2022 08:05
To: Vassallo Stephanie 1 at Health-MDH
Subject: RE: Permission to recruit patients from SAMOC for research study

Dear Ms Vassallo,

Good morning.

Please find attached signed research form by Dr Refalo.

Can you please let me know where to post the original copy for you?

Thank you.

Best wishes,

Maria

Mary Cachia Vella
Clerk
Secretary to Chairman
Oncology & Haematology Department

SIR ANTHONY MAMO ONCOLOGY CENTRE

T +356 25452478
E mary.cachia-vella@gov.mt

Permission from Haematology Consultants

RE: Permission to recruit patients under your care for Research Study

Gatt Alexander A at Health-MDH

Fri 18/03/2022 14:00

To: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>;

Cc: Brincat Patricia at Health-MDH <patricia.brincat@gov.mt>; Joseph Borg <joseph.j.borg@um.edu.mt>;

Approved
Alex

From: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>
Sent: Friday, 18 March 2022 13:56
To: Gatt Alexander A at Health-MDH <alexander.a.gatt@gov.mt>
Cc: Brincat Patricia at Health-MDH <patricia.brincat@gov.mt>; Joseph Borg <joseph.j.borg@um.edu.mt>
Subject: Permission to recruit patients under your care for Research Study

Dear Prof. Gatt,

I am writing this email to ask for your approval as a Consultant Haematologist to recruit eligible patients under your care for the study 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS' .

Thank you.

Best regards,

Stephanie Magri

Re: Permission to use Flow Cytometry Lab facilities for Research study and to recruit eligible patients under your care.

Vassallo Stephanie 1 at Health-MDH

Thu 14/04/2022 12:44

Sent Items

To: Camilleri David James at Health-MDH <david-james.camilleri@gov.mt>;

Thank you Dr. Camilleri.

Get Outlook for Android

From: Camilleri David James at Health-MDH <david-james.camilleri@gov.mt>
Sent: Thursday, April 14, 2022 12:25:41 PM
To: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>
Subject: RE: Permission to use Flow Cytometry Lab facilities for Research study and to recruit eligible patients under your care.

By all means Steph. Please proceed. Should you need me to sign documents, you know where to find me

TY
KW
DJ

David James Camilleri
Consultant

SPTAR MATER DEI

T +356 +356 25456349
M +356 99842719
E david-james.camilleri@gov.mt

Mater Dei Hospital, Triq Id-Donaturi Tad-Demm, Msida, Malta MSD 2090 | Tel +356 2545 0000 | https://careandcure.gov.mt/

From: Vassallo Stephanie 1 at Health-MDH
Sent: Thursday, 14 April 2022 11:06
To: Camilleri David James at Health-MDH <david-james.camilleri@gov.mt>
Subject: Re: Permission to use Flow Cytometry Lab facilities for Research study and to recruit eligible patients under your care.

Dear Dr. Camilleri,

Gentle reminder to the below email.

Thank you.

Kind regards,

Stephanie

From: Vassallo Stephanie 1 at Health-MDH
Sent: 18 March 2022 13:16:07
To: Camilleri David James at Health-MDH
Cc: Gatt Alexander A at Health-MDH; Brincat Patricia at Health-MDH; Joseph Borg
Subject: Permission to use Flow Cytometry Lab facilities for Research study and to recruit eligible patients under your care.

Dear Dr. Camilleri,

I am Stephanie Magri working at the Haematology Lab. I am currently reading for a part-time Master's Degree within the Faculty of Medicine and Surgery, University of Malta. I will be carrying out a research project entitled: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS' under the supervision of Prof. Alexander Gatt, with Ms. Patricia Brincat and Prof. Joseph Borg as co-supervisors. The research project will initiate once UREC ethics approval is obtained and projections are that it should be completed by January 2024.

In this research project, the stem cell compartment of patients with persistent cytopenias for more than four months (potential pre-MDS conditions) and low-risk MDS patients will be investigated by using flow cytometric techniques. The immunophenotyping properties of the stem cell compartment will then be correlated with molecular findings.

This study will be carried out using bone marrow samples which will be taken as part of the diagnostic process of the above-mentioned patients. Patient and Control cohorts will be divided as follows:

- Cohort A (CCUS/ICUS/low risk MDS): n=30 Unexplained persistent cytopenias (>4 months) with no or mild dysplasia (Patient group)
- Cohort B (high risk MDS/sAML): n=30 multilineage cytopenias, excess blasts >10% by morphology/flowcytometry. (Control group)

Genomic DNA will be extracted from the bone marrow aspirate samples used for Flow cytometry. Real-time PCR will be used to look for somatically mutated genes detectable in patients with MDS and potentially in pre-MDS conditions.

I am writing this email to ask for your approval as a Consultant Haematologist to recruit eligible patients under your care for this study, as well as for your permission as Lead in General Haematology to make use of the Flow Cytometry Lab facilities.

Your approval will be greatly appreciated. Should you require any further information about the study, please do not hesitate to contact me.

Thank you.
Best regards,
Stephanie Magri

RE: Permission to recruit patients under your care for Research Study

Busuttil David P at Health-MDH

Fri 18/03/2022 14:02

To: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>;

You have my approval.DB

From: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>
Sent: Friday, 18 March 2022 13:19
To: Busuttil David P at Health-MDH <david.p.busuttil@gov.mt>
Cc: Gatt Alexander A at Health-MDH <alexander.a.gatt@gov.mt>; Brincat Patricia at Health-MDH <patricia.brincat@gov.mt>; Joseph Borg <joseph.j.borg@um.edu.mt>
Subject: Permission to recruit patients under your care for Research Study

Dear Dr. Busuttil,

I am Stephanie Magri working at the Haematology Lab. I am currently reading for a part-time Master's Degree within the Faculty of Medicine and Surgery, University of Malta. I will be carrying out a research project entitled: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS' under the supervision of Prof. Alexander Gatt, with Ms. Patricia Brincat and Prof. Joseph Borg as co-supervisors. The research project will initiate once UREC ethics approval is obtained and projections are that it should be completed by January 2024.

In this research project, the stem cell compartment of patients with persistent cytopenias for more than four months (potential pre-MDS conditions) and low-risk MDS patients will be investigated by using flow cytometric techniques. The immunophenotyping properties of the stem cell compartment will then be correlated with molecular findings.

This study will be carried out using bone marrow samples which will be taken as part of the diagnostic process of the above-mentioned patients. Patient and Control cohorts will be divided as follows:

- Cohort A (CCUS/ICUS/low risk MDS): n=30 Unexplained persistent cytopenias (>4 months) with no or mild dysplasia (Patient group)

- Cohort B (high risk MDS/sAML): n=30 multilineage cytopenias, excess blasts >10% by morphology/flowcytometry. (Control group)

Genomic DNA will be extracted from the bone marrow aspirate samples used for Flow cytometry. Real-time PCR will be used to look for somatically mutated genes detectable in patients with MDS and potentially in pre-MDS conditions.

I am writing this email to ask for your approval as a Consultant Haematologist to recruit eligible patients under your care for this study.

Your approval will be greatly appreciated. Should you require any further information about the study, please do not hesitate to contact me.

RE: Permission to recruit patients under your care for Research Study

Grech Mark at Health-SAMOC

Sun 20/03/2022 15:23

To: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>;

Cc: Gatt Alexander A at Health-MDH <alexander.a.gatt@gov.mt>; Brincat Patricia at Health-MDH <patricia.brincat@gov.mt>; Joseph Borg <joseph.j.borg@um.edu.mt>;

Dear Stephanie,

Approved.

Regards,

Mark

From: Vassallo Stephanie 1 at Health-MDH <stephanie.vassallo.1@gov.mt>
Sent: Friday, 18 March 2022 13:18
To: Grech Mark at Health-SAMOC <mark.a.grech@gov.mt>
Cc: Gatt Alexander A at Health-MDH <alexander.a.gatt@gov.mt>; Brincat Patricia at Health-MDH <patricia.brincat@gov.mt>; Joseph Borg <joseph.j.borg@um.edu.mt>
Subject: Permission to recruit patients under your care for Research Study

Dear Dr. Grech,

I am Stephanie Magri working at the Haematology Lab. I am currently reading for a part-time Master's Degree within the Faculty of Medicine and Surgery, University of Malta. I will be carrying out a research project entitled: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS' under the supervision of Prof. Alexander Gatt, with Ms. Patricia Brincat and Prof. Joseph Borg as co-supervisors. The research project will initiate once UREC ethics approval is obtained and projections are that it should be completed by January 2024.

In this research project, the stem cell compartment of patients with persistent cytopenias for more than four months (potential pre-MDS conditions) and low-risk MDS patients will be investigated by using flow cytometric techniques. The immunophenotyping properties of the stem cell compartment will then be correlated with molecular findings.

This study will be carried out using bone marrow samples which will be taken as part of the diagnostic process of the above-mentioned patients. Patient and Control cohorts will be divided as follows:

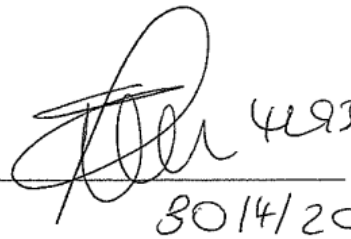
- Cohort A (CCUS/ICUS/low risk MDS): n=30 Unexplained persistent cytopenias (>4 months) with no or mild dysplasia (Patient group)

- Cohort B (high risk MDS/sAML): n=30 multilineage cytopenias, excess blasts >10% by morphology/flowcytometry. (Control group)

Genomic DNA will be extracted from the bone marrow aspirate samples used for Flow cytometry. Real-time PCR will be used to look for somatically mutated genes detectable in patients with MDS and potentially in pre-MDS conditions.

Permission from Intermediary

I am Dr. Erika Sultana (Higher Specialist Trainee in Haematology) and I hereby confirm that I will be an intermediary in the research study titled: 'Investigation of the Stem cell compartment in patients with chronic cytopenias and low-risk MDS'. I will approach potential participants on behalf of Ms. Stephanie Magri (researcher) and explain the Information Letter and Consent form and answer any basic queries that they might have. I will explain that participation in the research study is totally voluntarily and refusing to participate will not impact their care pathway at SAMOC MDH.

Signature of Intermediary: 

Date: 30/4/2022

Appendix B

Data Management Plan

Data Management Plan

Data Collection: Participants will be asked to participate in this research study through an intermediary, Dr. Erika Sultana (Higher Specialist Trainee in Haematology). Dr. Erika Sultana will provide an Information letter together with a consent form and questionnaire. I will not have any patient contact and samples will be pseudo-anonymised by Dr. Erika Sultana. Codes will be used to replace patient details. Patients will not be put under any pressure to participate and Dr. Sultana will explain that refusing to participate will not impact their care in any way. Moreover, participants may withdraw from the study at any time. Contact details will be provided so that participants can withdraw from the study.

Data Characteristics: During processing of bone marrow and peripheral blood samples collected with patient consent, data will be collected. The data which will be collected includes a Complete Blood Count (CBC) and peripheral blood film, immunophenotype of the stem cell compartment by flow cytometry and genetic data.

Storage and Security: Data will be stored and encrypted on a PC which is password protected and will be accessed only by myself (the main researcher) and Ms. Patricia Brincat (co-supervisor). The patient details will be kept by Dr. Erika Sultana and they will also be password protected and encrypted. Only Dr. Erika Sultana will have access to patient personal details.

Retention: The data collected during processing of samples will be stored for 10 years. The personal details of patients will also be kept by Dr. Erika Sultana for 10 years.

Access: The data collected during processing of samples will be encrypted and only myself and Ms. Patricia Brincat will have access to it. Personal details will be accessed only by Dr. Erika Sultana and codes will be provided to replace patient details.

Discarded: All data collected during this research project will be discarded after 10 years.

NB: The data collected will be used ONLY for Research purposes. The patients will be undergoing a Clinical investigation in parallel with the study and therefore, no data obtained will be used for the Clinical management of patients. Therefore, the Consultants do not need to know the codes used for this research. They will only inform Dr. Erika Sultana when one of their patients can be recruited for the study.

Appendix C

Information sheet, Consent Form and Questionnaire

(Maltese and English versions)

Information Letter for Patients

L-Università ta' Malta

Dear Patient,

I am Stephanie Magri (ID: 221496M), and I am currently reading for a part-time Master's Degree within the Faculty of Medicine and Surgery, University of Malta. I would like to invite you to participate in my research project entitled: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS' under the supervision of Prof. Alexander Gatt, with Ms. Patricia Brincat and Prof. Joseph Borg as co-supervisors.

Introduction

Myelodysplastic syndromes (MDS) are a group of disorders in which the bone marrow decreases the production of the blood cells or the production of blood cells is abnormal. In this study, we are going to investigate the cells which produce the different blood cells, these are the stem cells. The study will be performed on patients who have decreased production of blood cells that is persistent for more than 4 months also known as chronic cytopenias.

Benefit

Your participation in this research project will be of great benefit, both to you, and also to other patients with decreased production of blood cells from the bone marrow. This research project will give a better understanding of the health of the stem cells and will help for better management of patients with MDS or chronic cytopenias.

Procedure

To carry out this research project, we will be using a small volume of the bone marrow and peripheral blood sample which will be taken by the doctor as part of your diagnostic investigation. The testing which will be done include a technique called flow cytometry and genetic testing. The sample will be analysed, stored, and then discarded at the Haematology Lab at Mater Dei Hospital. All data collected for the research study will be stored in excel sheet which is password protected and available only to the researchers involved (namely myself and Ms. Patricia Brincat).

Risks

Kindly note that there are no risks associated with participation in this study. You will not be asked to take any extra samples for this research study. A small volume from the residual bone marrow and peripheral blood samples that will be taken as part of your Clinical investigation and that would otherwise be discarded will be used for this study.

Confidentiality

All the names and any other personal information used for this project will be completely anonymized and will never be published. All personal data collected for the study will be pseudonymized by using codes and these will be kept for 10 years in case needed for further studies. All personally identifiable data will be encrypted and stored safely and securely by the intermediary, Dr. Erika Sultana. There may be exceptional circumstances where the supervisors and examiner of this research study would also need access to personal data for verification purposes. All personal identifiable data will be discarded after 10 years, when no longer needed.

'Under the General Data Protection Regulation (GDPR) and national legislation that implements and further specifies the relevant provisions of said Regulation, you have the right to obtain access to, rectify, and where applicable ask for the data concerning them to be erased'.

Voluntary Participation

Your participation in this research project is entirely voluntarily. You may change your mind and withdraw from this study at any time, without giving a reason , even if you had agreed earlier. You can contact me on the email provided below and I will delete any data collected. Moreover, refusing to participate will not impact your care pathway at SAMOC MDH. Refusing to participate will involve no penalty or loss of benefits.

Contact Information

In case you have any questions you may want to ask, feel free to contact me, Stephanie Magri or my supervisor Prof. Alexander Gatt on the following email addresses and mobile number:

Stephanie Magri: stephanie.vassallo.15@um.edu.mt /79935695

Prof. Alexander Gatt: alexander.gatt@um.edu.mt

Signature of Prinicipal Investigator (Stephanie Magri) _____

Signature of Supervisor (Prof. Alexander Gatt) _____

Ittra bl-Informazzjoni għall-pazjent

Għażiż Pazjent,

Jiena, Stephanie Magri (ID:221496M), li bħalissa qed nagħmel Master's Degree (part-time) mal-Fakulta tal-Mediċina, fl-Universita' ta' Malta, qed nistiednek biex tipparteċipa fi studju ta' riċerka li jismu: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS'.

Introduzzjoni

'Myelodysplastic syndromes'-(MDS) huma grupp ta' mard fejn il-produzzjoni taċ-ċelloli tad-demm mill-mudullun tibda' tonqos jew ma ssirx b'mod tajjeb. F'dan l-istudju se nkunu qed ninvestigaw grupp ta ċelloli li jinstabu fil mudullun u jipproducu ic celloli differenti tad demm, jissejhu 'stem cells'. F'dan il progett ha ninvestigaw listem cells u naraw kemm huma b'sahhitom. Dan ha nagħmluh fuq pazjenti li ghandom nuqqas fic celloli tad demm ghal zmien ta aktar minn 4 xhur.

Benefiċċju

Il-parteċipazzjoni tiegħek f'dan l-istudju se jkun ta' benefiċċju kbir kemm għalik u kemm għal pazjenti oħra li għandhom il-kundizjoni tal-MDS jew nuqqas ta' produzzjoni ta' ċelloli tad-demm. Dan l-istudju se jagħtina iktar informazzjoni fuq dawn ċ-ċelloli rari tal-mudullunu se jgħin fir ricerka ta dan il-tip ta mard biex b'hekk pazjenti b' dawn il kundizjoni ikollhom trattament ahjar.

Proċedura

Din ir ricerka ser issir fuq volum zghir mill- kampjun tal mudullun u tad-demm li se jkun qed jittiehdulek mit-tabib tieghek bhala parti mill investigazzjonijiet li qed isirulek. It-testijiet li ha jkunu qed isiru jinkludu 'flow cytometry' u 'genetic testing'. Il-kampjun tieghek se jkun qed jinzamm, jigi analizzat u imbaghad jintrema gewwa l-Laboratorju tal-'Haematology', tal-isptar Mater Dei. L-informazzjoni migbura ghar-ricerka se tkun accessata biss mir-ricerkaturi prinicipali (jiena u Ms. Patricia Brincat) u se tkun sigura b'password.

Riskji

M'ghandek l-ebda riskju assocjat mall-partecipazzjoni tieghek f'dan l-istudju. Volum zghir mill-kampjuni tal-mudullun u tad-demm li ser jittiehdulek ghall-investigazzjoni medika tieghek ser jigu uzati ghall-istudju flok jintremew.

Kunfidenzjalita

L-ismijiet u d-dettalji personali użati għal dan il-proġett se jinżammu fuq bażi anonima u qatt mhu se jiġu ppublikati. Id-dettalji personali ser jigu anonimizzati permezz ta' uzu ta' kodici u dawn se jinzammu ghal ghaxar snin f'kaz li jkun hemm bzonn iktar studji. Id-dettalji personali ser jinzammu protetti ghand Dr. Erika Sultana. Jista' jkun hemm il-bzonn li s-'supervisors' u 'examiners' ta' dan l-istudju ikollhom bzonn access ghad-dettalji personali ghall-verifikazzjoni. Id-dettalji personali kollha ser jintremew wara ghaxar snin, meta ma jkunx hemm iktar bzonnhom.

'Taħt ir-Regolament Generali dwar il-Protezzjoni tad-Data (GDPR) u l-leġislazzjoni nazzjonali li timplimenta u tispeċifika aktar id-dispozizzjonijiet relevanti ta' limsemmi Regolament, għandek id-dritt li tikseb aċċess għal, tikkoreġi, u fejn applikabbli titlob li d-data li tikkonċerna lilek titħassar.'

Parteċipazzjoni Volontarja

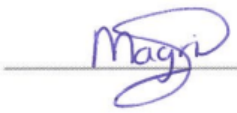
Il-parteċipazzjoni tiegħek f'dan l-istudju hija totalment volontarja. Inti tista' tibdel fehmtek u tirtira mill-istudju fi kwalunkwe ħin, minghajr ma taghti spjegazzjoni, anke jekk diġa' qbilt u ffirmajt il-formola tal-kunsens. Tista' tikkuntatjani fuq l-imejl provdut hawn taħt u jien inħassar l-informazzjoni tiegħek kollha. Għandek kull dritt li tirrifjuta u jekk tirrifjuta, it-trattament tiegħek gewwa l-Isptar tal-Onkologija, Mater Dei mhux se jigi affetwat u mintix se tigi penalizzat.

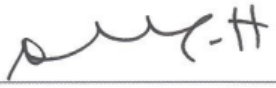
Kuntatt

Fil-każ li għandek xi mistoqsijiet, tista' tikkuntatja lili Stephanie Magri, jew lis-supervisor tiegħi, il-Professur Alexander Gatt, fuq dawn l-imejls u numru tal-mowbajl:

Stephanie Magri: stephanie.vassallo.15@um.edu.mt / 79935695

Il-Professur Alexander Gatt: alexander.gatt@um.edu.mt

Firma tar-riċerkatur (Stephanie Magri) 

Firma tas-supervisor (Il-Professur Alexander Gatt) 

Consent Form for Patient

L-Università ta' Malta

This consent form is for men and women who are attending a state general hospital and whom we are inviting to participate in the research project titled: 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS'. Kindly feel free to ask any questions prior to signing the consent form. You are also invited to participate in a questionnaire which will also be used for this study and will take about 5 minutes of your time to complete it. No risks are associated with participating in this research study.

I have read the information letter and any questions were clarified. I am aware that I can change my mind and withdraw from the study at any time without giving any reasons. I give my consent to voluntarily participate in the research project. I also give my consent to the researcher to use my bone marrow sample and blood sample for research purposes. I am aware that personal data will be anonymized and kept safely by Dr. Erika Sultana for 10 years and that the supervisors and examiners of this research study may request access to personal data.

Signature of patient: _____

Name of patient: _____

Date: _____

Statement by the person taking consent:

I have accurately read out all the information to the patient and I did, to the best of my ability, make sure that the patient understood that a small volume of the bone marrow sample collected will be used for the research project. Any questions that the patient asked, have been answered appropriately to the best of my ability. I confirm that the patient has not been forced or coerced into giving his/her consent and that the patient's consent has been given freely and voluntarily.

Signature of person taking consent: _____

Name of person taking consent: _____

Date: _____

Signature of Principal Investigator: _____

Contact details of Principal Investigator: stephanie.vassallo.15@um.edu.mt / 79935695

Signature of Supervisor: _____

Contact details of Supervisor: alexander.gatt@um.edu.mt

Formola tal-Kunsens għall-Pazjent

L-Università ta' Malta

Din il-formula tal-kunsens hija għall-irġiel u n-nisa li jattendu l-Isptar Ġenerali. Aħna qed nistednuhom biex jipparteċipaw fil-proġett ta' riċerka li jismu 'Investigation of the Stem Cell Compartment in patients with chronic cytopenias and low-risk MDS'. Jekk għandek xi mistoqsijiet li trid tagħmel qabel tiffirma, tista' ssaqsi lill-persuna li qed tieħu l-kunsens. Nixtiequ nistiednuk ukoll biex tippartecipa fi kwestjonarju li se jintuza wkoll ghal dan l-istudju u li jehodlok madwar 5 minuti biex tillestih. M'hemm l-ebda riskju assoccjat mall-partecipazzjoni tieghek f'dan l-istudju.

Jiena qrajt u fhimt l-ittra ta' informazzjoni u kull mistoqsija li kelli ġiet imwieġba. Naf li jekk nibdel id-deċiżjoni tiegħi nista' nieqaf xħin u meta rrid, minghajr ma naghti spjegazzjoni. Jien qed nagħti l-kunsens tiegħi li se nipparteċipa volontarjament f'dan il-proġett u biex il-kampjuni tieghi tal-mudullun u tad-demm jintuzaw ghal din ir-ricerka. Jiena naf li l-informazzjoni personali tieghi ser tkun qed tinzamm minn Dr. Erika Sultana ghal ghaxar snin u li jista' jkun li s-'supervisors' u l-'examiners' ta' dan l-istudju jitolbu access ghall-informazzjoni personali tieghi.

Firma tal-pazjent: _____

Isem tal-pazjent: _____

Data: _____

Dikjarazzjoni mill-persuna li qed tieħu l-kunsens:

Jiena qrajt l-informazzjoni kollha neċessarja lill-pazjent u kkonfermajt li l-pazjent fehem eżatt li volum żgħir mill-kampjun tal-mudullun li se jittieħedlu se jintuża għall-proġett. Kull mistoqsija li kellu l-pazjent, ġiet imwieġba kif suppost bil-kompetenzi kollha tiegħi. Nikkonferma li l-pazjent ma kienx sfurzat jew imġiegħel jagħti l-kunsens tiegħu u l-kunsens tal-pazjent kien mogħti volontarjament.

Isem tal-persuna li qed tieħu l-kunsens: _____

Firma tal-persuna li qed tieħu l-kunsens: _____

Data: _____

Firma tar-riċerkatur prinċipali: _____

Dettalji tar-riċerkatur prinċipali: stephanie.vassallo.15@um.edu.mt /79935695

Firma tas-supervisor: _____

Dettalji biex tikkuntatja lis-supervisor: alexander.gatt@um.edu.mt

Questionnaire

L-Università ta' Malta

1. Age (Years): _____

2. Gender (M/F/Other): _____

3. Smoker: Yes ☐
   No ☐

   If yes daily quanitity: _____

4. Are you on any medications? If so, kindly specify and for how long:

   _____

   _____

5. Did you have any recent infections? _____

   _____

6. History of malignancy/radiotherapy/chemotherapy: _____

   _____

7. Any other chronic clinical condition: _____

   _____

8. Kindly tick any symptom/s mentioned below that you might be experiencing:

   - Fatigue ☐
   - Shortness of breath ☐
   - Frequent infections ☐
   - Fever ☐
   - Bleeding and bruising easily ☐
   - Others: _____

Kwestjonarju

L-Università ta' Malta

1. Eta` (Snin): _____

2. Sess: _____

3. Tpejjep?: Iva ☐
   Le ☐

   Jekk iva, kemm tpejjep kuljum?: _____

4. Qed tieħu xi mediċina? Jekk iva, jekk jgħoġbok speċifika u għal kemm ilek tieħu l-mediċina?:

   _____

   _____

5. Kellek xi infezzjoni riċenti? _____

   _____

6. Kont tieħu xi kimoterapija jew radjoterapija fil-passat? _____

   _____

7. Tbati minn xi kundizzjoni medika oħra? _____

   _____

8. Jekk jgħoġbok immarka b'sinjal xi sintomi li qed tesperjenza:
   - Għejja ☐
   - Qtugħ ta' nifs ☐
   - Infezzjonijiet regolari ☐
   - Deni ☐
   - Titlef id-demm u titbenġel malajr ☐
   - Sintomi ohra: _____

Appendix D

Results

Leukaemic Stem Cell Results

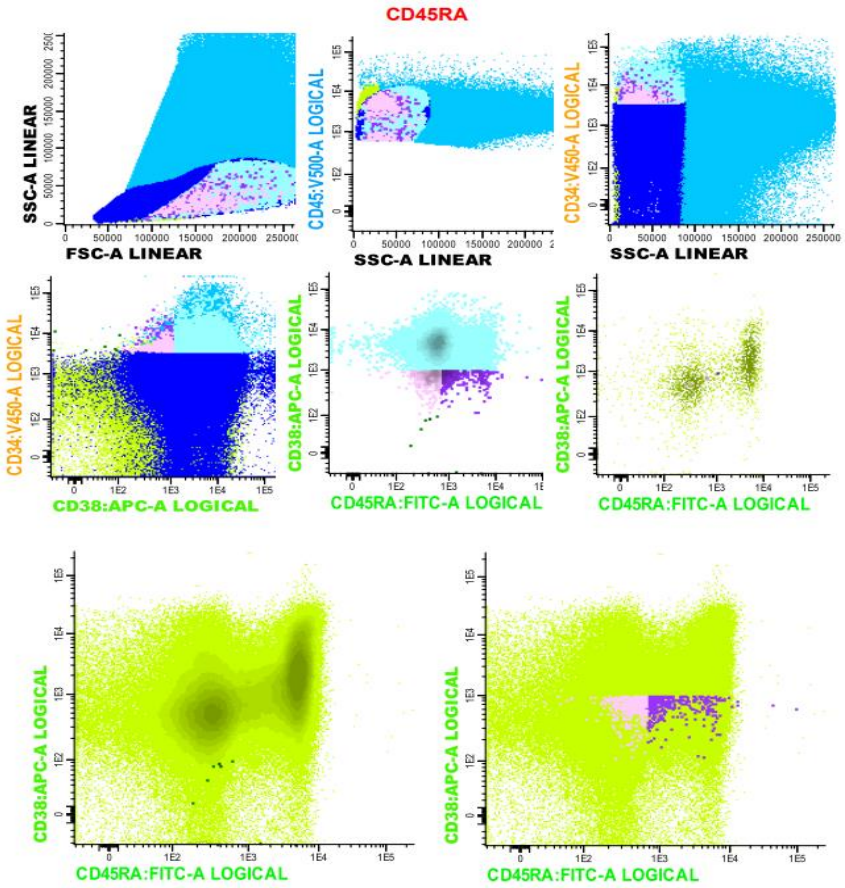
A report was generated for each LSC marker gated. Each patient had 5 reports with different LSC percentages for each marker. Below are 3 examples from 3 different patients: 1 without LSCs, 1 with LSCs at low percentages and 1 with LSCs at high percentages.

Patient 1: No LSC's present.

<u>CD45RA</u>

Population	Events	Partial %	Visibility %
WBC	1760056	35.2011	100.0000
Lymfocyten	238175	13.5322	13.5322
Blasten	424868	24.1395	24.1395
CD34+	28346	6.6717	1.6106
CD34+CD38-	9	0.0318	0.0005
CD38- LAP neg	9	100.0000	0.0005
CD38- LAP pos	0	0.0000	NA
CD34+CD38dim	766	2.7023	0.0436
CD38dim LAP neg	411	53.6554	0.0234
CD38dim LAP pos	355	46.3446	0.0202
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

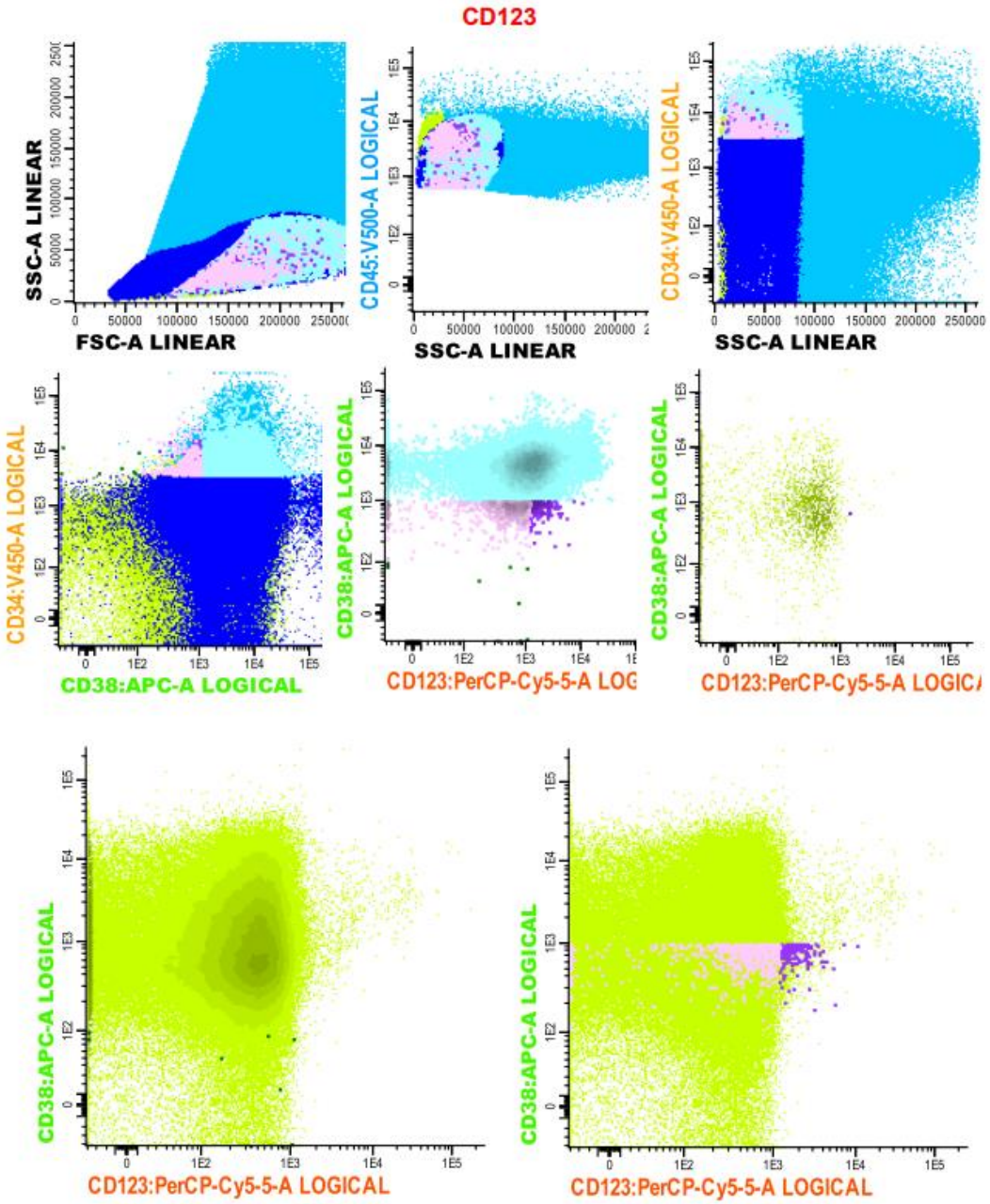
Population	Median
Lymfocyten	
FSC-A	110492.9141
SSC-A	13539.3604
CD38- LAP neg	
FSC-A	142779.0938
SSC-A	22603.1211
ƒ MFI FSC LAP neg/lymfo's: 1.2[illegible]	
ƒ MFI SSC LAP neg/lymfo's: 1.6[illegible]	
CD38- LAP pos	
FSC-A	NA
SSC-A	NA
ƒ MFI FSC LAP pos/lymfo's: NA	
ƒ MFI SSC LAP pos/lymfo's: NA	



CD123

Population	Events	Partial %	Visibility %
WBC	1760056	35.2011	100.0000
Lymfocyten	238175	13.5322	13.5322
Blasten	424868	24.1395	24.1394
CD34+	28346	6.6717	1.6105
CD34+CD38-	9	0.0318	0.0005
CD38- LAP neg	9	100.0000	0.0005
CD38- LAP pos	0	0.0000	NA
CD34+CD38dim	766	2.7023	0.0435
CD38dim LAP neg	602	78.5901	0.0342
CD38dim LAP pos	164	21.4099	0.0093
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

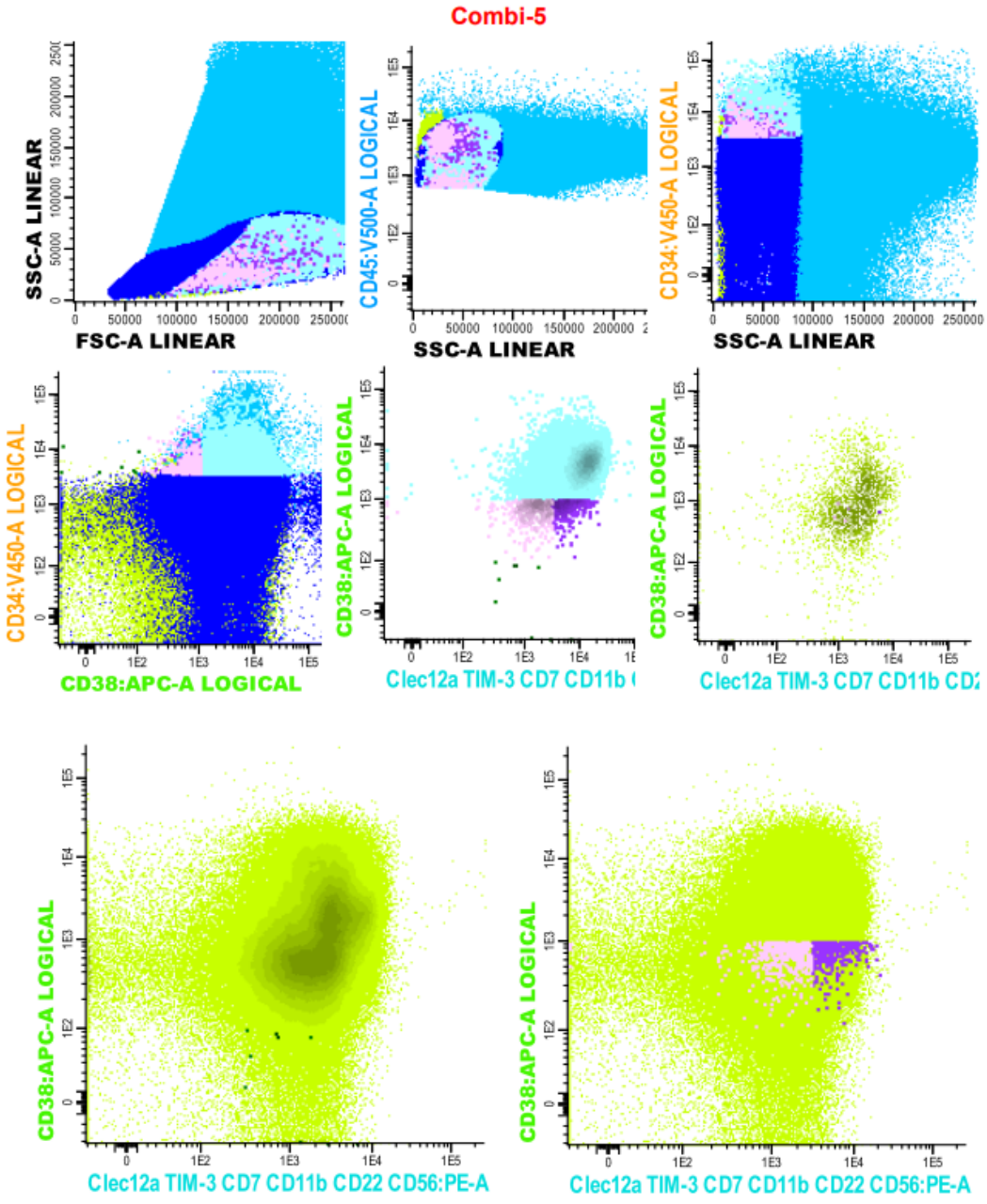
Population	Median
Lymfocyten	
FSC-A	110492.9141
SSC-A	13539.3604
CD38- LAP neg	
FSC-A	142779.0938
SSC-A	22603.1211
ƒ MFI FSC LAP neg/lymfo's: 1.2...	
ƒ MFI SSC LAP neg/lymfo's: 1.6...	
CD38- LAP pos	
FSC-A	NA
SSC-A	NA
ƒ MFI FSC LAP pos/lymfo's: NA	
ƒ MFI SSC LAP pos/lymfo's: NA	



Combi Markers

Population	Events	Partial %	Visibility %
WBC	1760056	35.2011	100.0000
Lymfocyten	238175	13.5322	13.5322
Blasten	424868	24.1395	24.1394
CD34+	28346	6.6717	1.6105
CD34+CD38-	9	0.0318	0.0005
CD38- LAP neg	9	100.0000	0.0005
CD38- LAP pos	0	0.0000	NA
CD34+CD38dim	766	2.7023	0.0435
CD38dim LAP neg	431	56.2663	0.0245
CD38dim LAP pos	335	43.7337	0.0190
pre	0	0.0000	NA
CD45/	2227998	44.5600	0.0000
beads	0	0.0000	NA

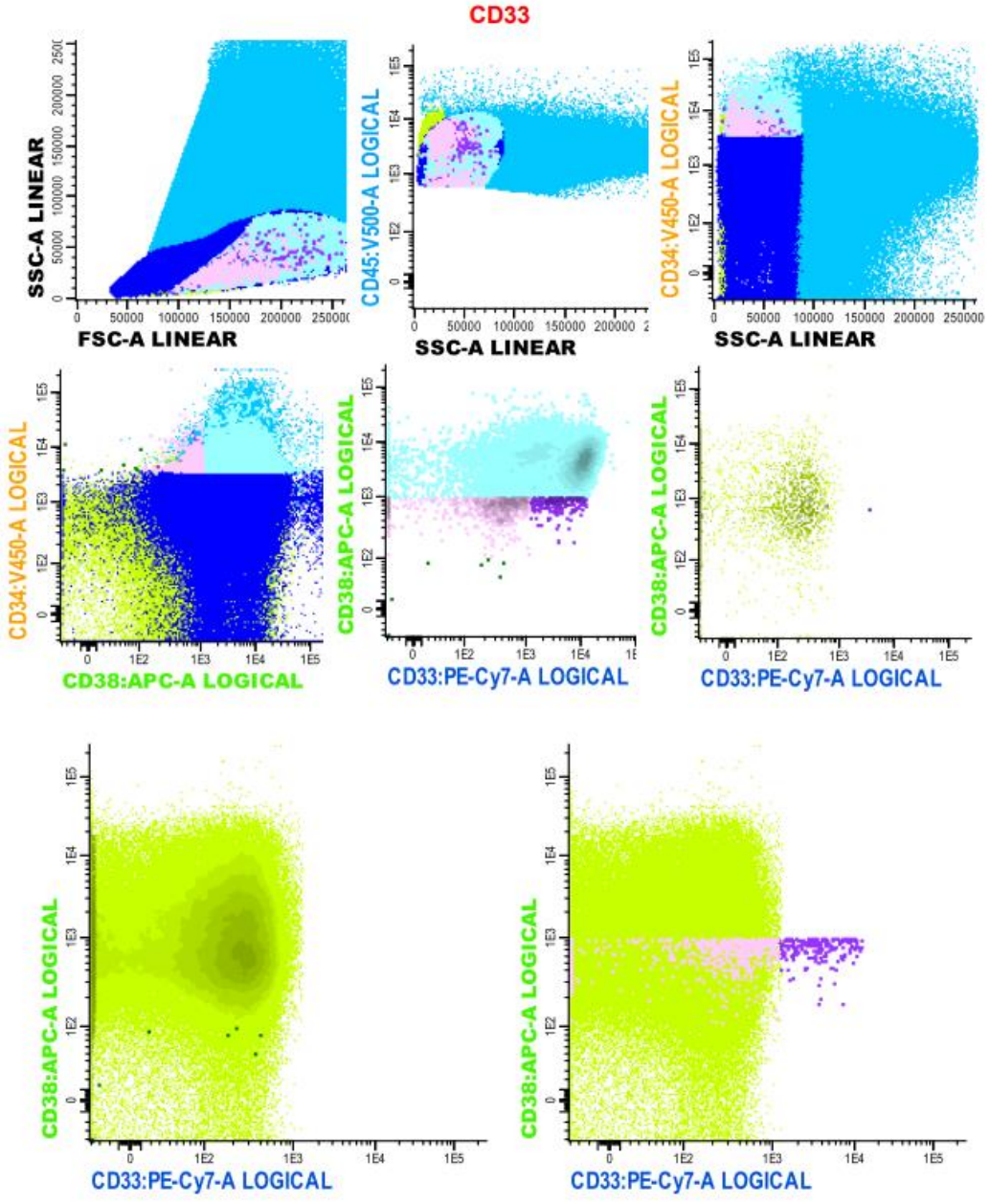
Population	Median
Lymfocyten	
FSC-A	110492.9141
SSC-A	13539.3604
CD38- LAP neg	
FSC-A	142779.0938
SSC-A	22603.1211
ƒ MFI FSC LAP neg/lymfo's: 1.2	
ƒ MFI SSC LAP neg/lymfo's: 1.6	
CD38- LAP pos	
FSC-A	NA
SSC-A	NA
ƒ MFI FSC LAP pos/lymfo's: NA	
ƒ MFI SSC LAP pos/lymfo's: NA	



CD33

Population	Events	Partial %	Visibility %
WBC	1760056	35.2011	100.0000
Lymfocyten	238175	13.5322	13.5322
Blasten	424868	24.1395	24.1395
CD34+	28346	6.6717	1.6106
CD34+CD38-	9	0.0318	0.0005
CD38- LAP neg	9	100.0000	0.0005
CD38- LAP pos	0	0.0000	NA
CD34+CD38dim	766	2.7023	0.0436
CD38dim LAP neg	573	74.8042	0.0326
CD38dim LAP pos	193	25.1958	0.0110
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

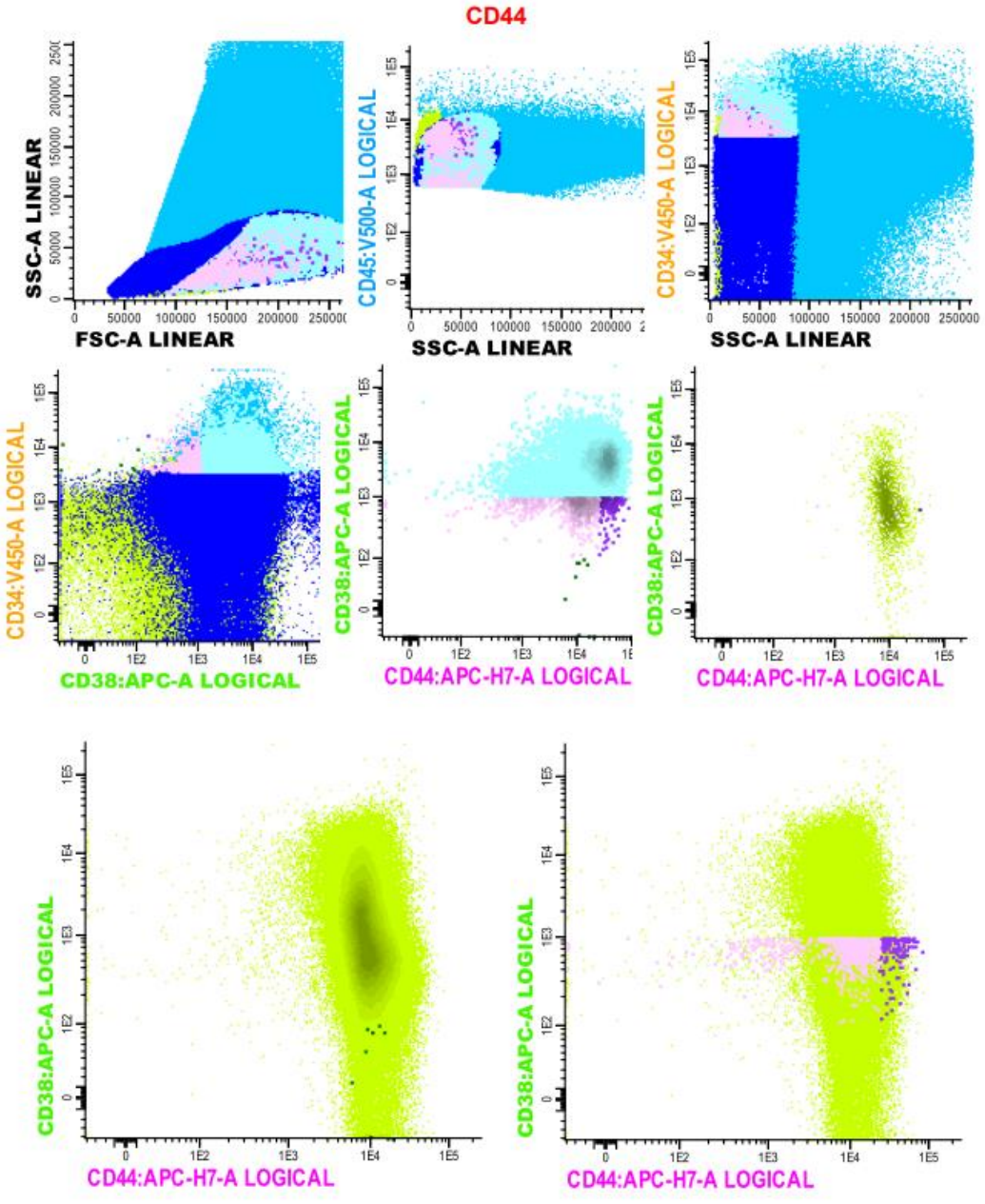
Population	Median
Lymfocyten	
FSC-A	110492.9141
SSC-A	13539.3604
CD38- LAP neg	
FSC-A	142779.0938
SSC-A	22603.1211
ƒ MFI FSC LAP neg/lymfo's: 1.2(	
ƒ MFI SSC LAP neg/lymfo's: 1.6(	
CD38- LAP pos	
FSC-A	NA
SSC-A	NA
ƒ MFI FSC LAP pos/lymfo's: NA	
ƒ MFI SSC LAP pos/lymfo's: NA	



CD44

Population	Events	Partial %	Visibility %
WBC	1760056	35.2011	100.0000
Lymfocyten	238175	13.5322	13.5322
Blasten	424868	24.1395	24.1394
CD34+	28346	6.6717	1.6105
CD34+CD38-	9	0.0318	0.0005
CD38- LAP neg	9	100.0000	0.0005
CD38- LAP pos	0	0.0000	NA
CD34+CD38dim	766	2.7023	0.0435
CD38dim LAP neg	630	82.2454	0.0358
CD38dim LAP pos	136	17.7546	0.0077
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

Population	Median
Lymfocyten	
FSC-A	110492.9141
SSC-A	13539.3604
CD38- LAP neg	
FSC-A	142779.0938
SSC-A	22603.1211
ƒ MFI FSC LAP neg/lymfo's: 1.2	
ƒ MFI SSC LAP neg/lymfo's: 1.6	
CD38- LAP pos	
FSC-A	NA
SSC-A	NA
ƒ MFI FSC LAP pos/lymfo's: NA	
ƒ MFI SSC LAP pos/lymfo's: NA	

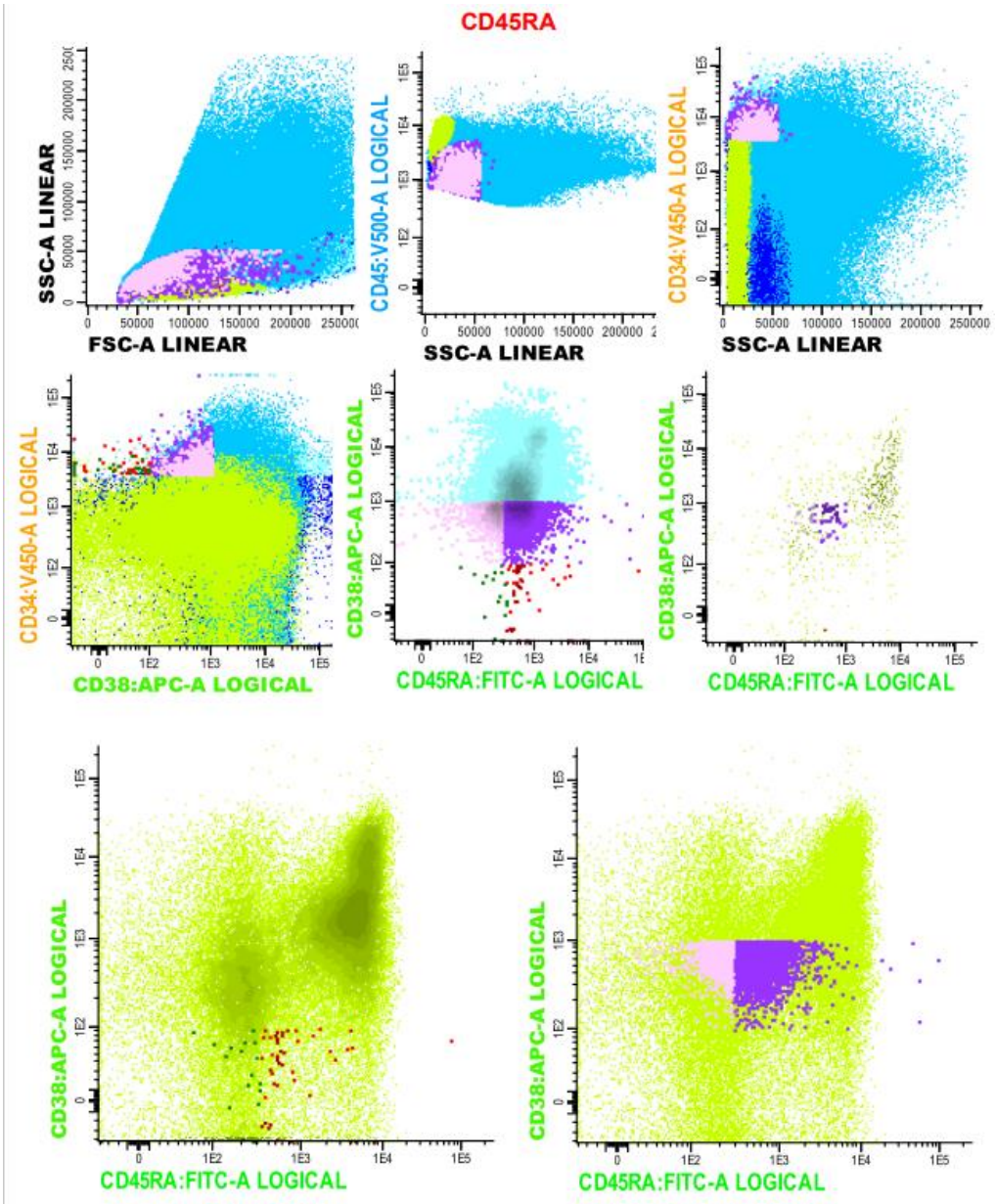


Patient 2: LSC's present at low percentages.

CD45RA

Population	Events	Partial %	Visibility %
WBC	1045538	20.9108	100.0000
Lymfocyten	68767	6.5772	6.5772
Blasten	478827	45.7972	45.7971
CD34+	21927	4.5793	2.0971
CD34+CD38-	65	0.2964	0.0062
CD38- LAP neg	20	30.7692	0.0019
CD38- LAP pos	45	69.2308	0.0043
CD34+CD38dim	6111	27.8697	0.5844
CD38dim LAP neg	1024	16.7567	0.0979
CD38dim LAP pos	5087	83.2433	0.4865
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

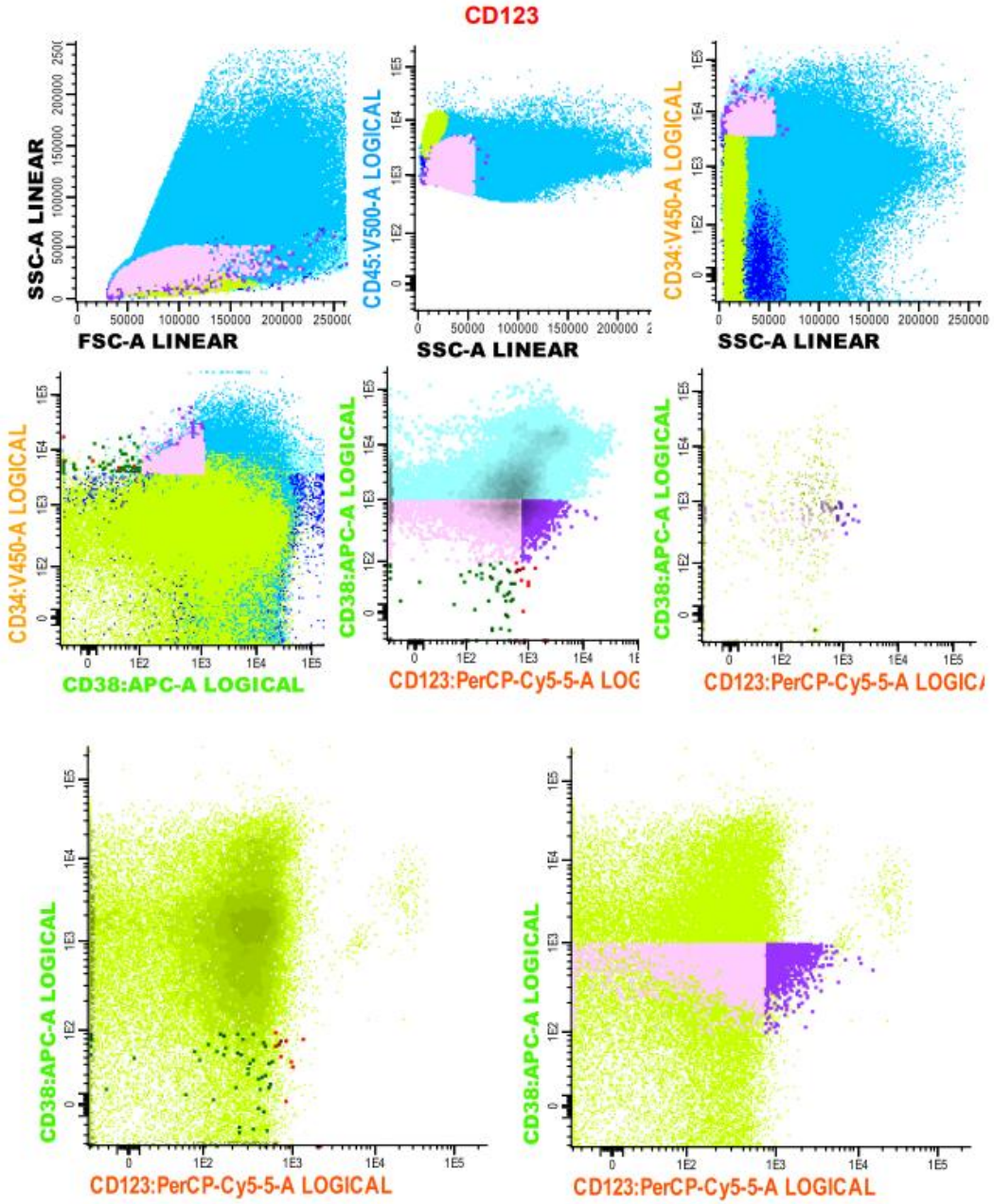
Population	Median
Lymfocyten	
FSC-A	103233.0781
SSC-A	11612.2305
CD38- LAP neg	
FSC-A	52551.3398
SSC-A	18072.7051
ƒ MFI FSC LAP neg/lymfo's: 0.5(	
ƒ MFI SSC LAP neg/lymfo's: 1.5(	
CD38- LAP pos	
FSC-A	72194.4375
SSC-A	23157.9609
ƒ MFI FSC LAP pos/lymfo's: 0.6(	
ƒ MFI SSC LAP pos/lymfo's: 1.9(	



CD123

Population	Events	Partial %	Visibility %
WBC	1045538	20.9108	100.0000
Lymfocyten	68767	6.5772	6.5772
Blasten	478827	45.7972	45.7972
CD34+	21927	4.5793	2.0972
CD34+CD38-	65	0.2964	0.0062
CD38- LAP neg	51	78.4615	0.0049
CD38- LAP pos	14	21.5385	0.0013
CD34+CD38dim	6111	27.8697	0.5845
CD38dim LAP neg	4796	78.4814	0.4587
CD38dim LAP pos	1315	21.5186	0.1258
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

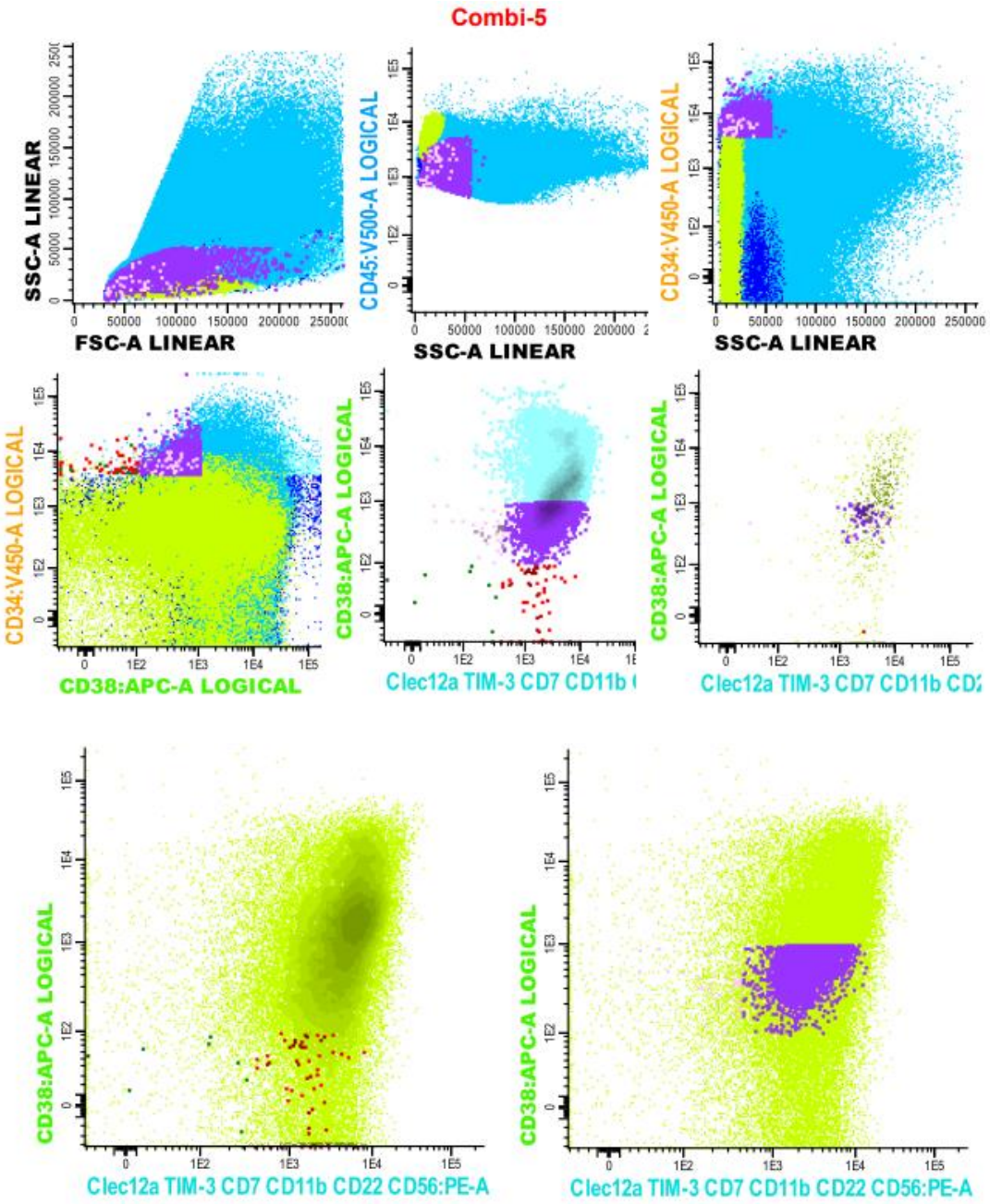
Population	Median
Lymfocyten	
FSC-A	103233.0781
SSC-A	11612.2305
CD38- LAP neg	
FSC-A	62051.8594
SSC-A	19500.4609
ƒ MFI FSC LAP neg/lymfo's: 0.6(...	
ƒ MFI SSC LAP neg/lymfo's: 1.6'...	
CD38- LAP pos	
FSC-A	75728.2344
SSC-A	27290.2715
ƒ MFI FSC LAP pos/lymfo's: 0.7...	
ƒ MFI SSC LAP pos/lymfo's: 2.3...	



Combi Markers

Population	Events	Partial %	Visibility %
WBC	1045538	20.9108	100.0000
Lymfocyten	68767	6.5772	6.5772
Blasten	478827	45.7972	45.7972
CD34+	21927	4.5793	2.0972
CD34+CD38-	65	0.2964	0.0063
CD38- LAP neg	9	13.8462	0.0009
CD38- LAP pos	56	86.1538	0.0054
CD34+CD38dim	6111	27.8697	0.5844
CD38dim LAP neg	37	0.6055	0.0035
CD38dim LAP pos	6074	99.3945	0.5809
pre	0	0.0000	NA
CD45/	2926806	58.5361	0.0000
beads	0	0.0000	NA

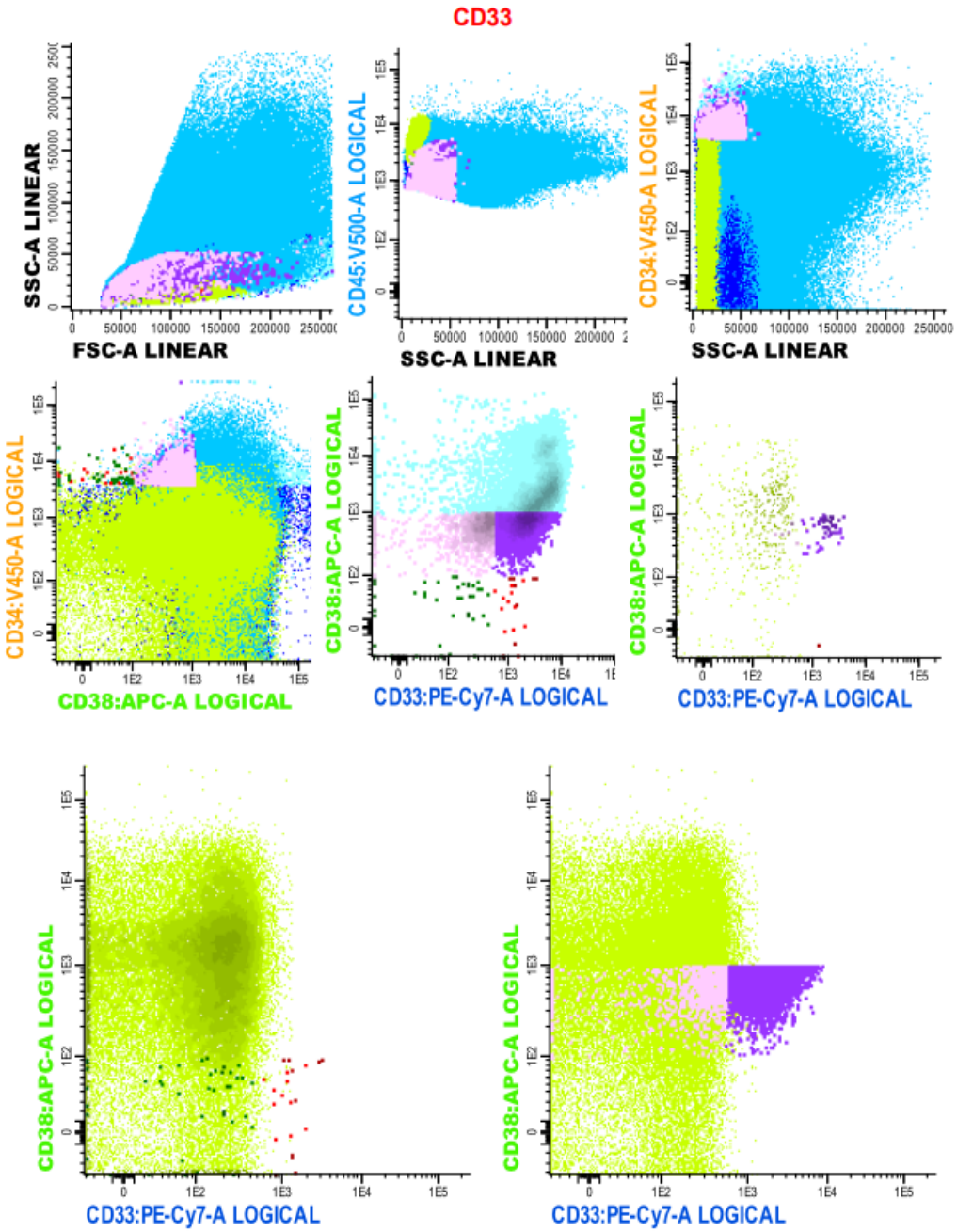
Population	Median
Lymfocyten	
FSC-A	103233.0781
SSC-A	11612.2305
CD38- LAP neg	
FSC-A	51172.9180
SSC-A	17151.6797
ƒ MFI FSC LAP neg/lymfo's: 0.4	
ƒ MFI SSC LAP neg/lymfo's: 1.4	
CD38- LAP pos	
FSC-A	68525.3438
SSC-A	23580.2363
ƒ MFI FSC LAP pos/lymfo's: 0.6	
ƒ MFI SSC LAP pos/lymfo's: 2.0	



CD33

Population	Events	Partial %	Visibility %
WBC	1045538	20.9108	100.0000
Lymfocyten	68767	6.5772	6.5772
Blasten	478827	45.7972	45.7972
CD34+	21927	4.5793	2.0972
CD34+CD38-	65	0.2964	0.0062
CD38- LAP neg	44	67.6923	0.0042
CD38- LAP pos	21	32.3077	0.0020
CD34+CD38dim	6111	27.8697	0.5845
CD38dim LAP neg	918	15.0221	0.0878
CD38dim LAP pos	5193	84.9779	0.4967
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

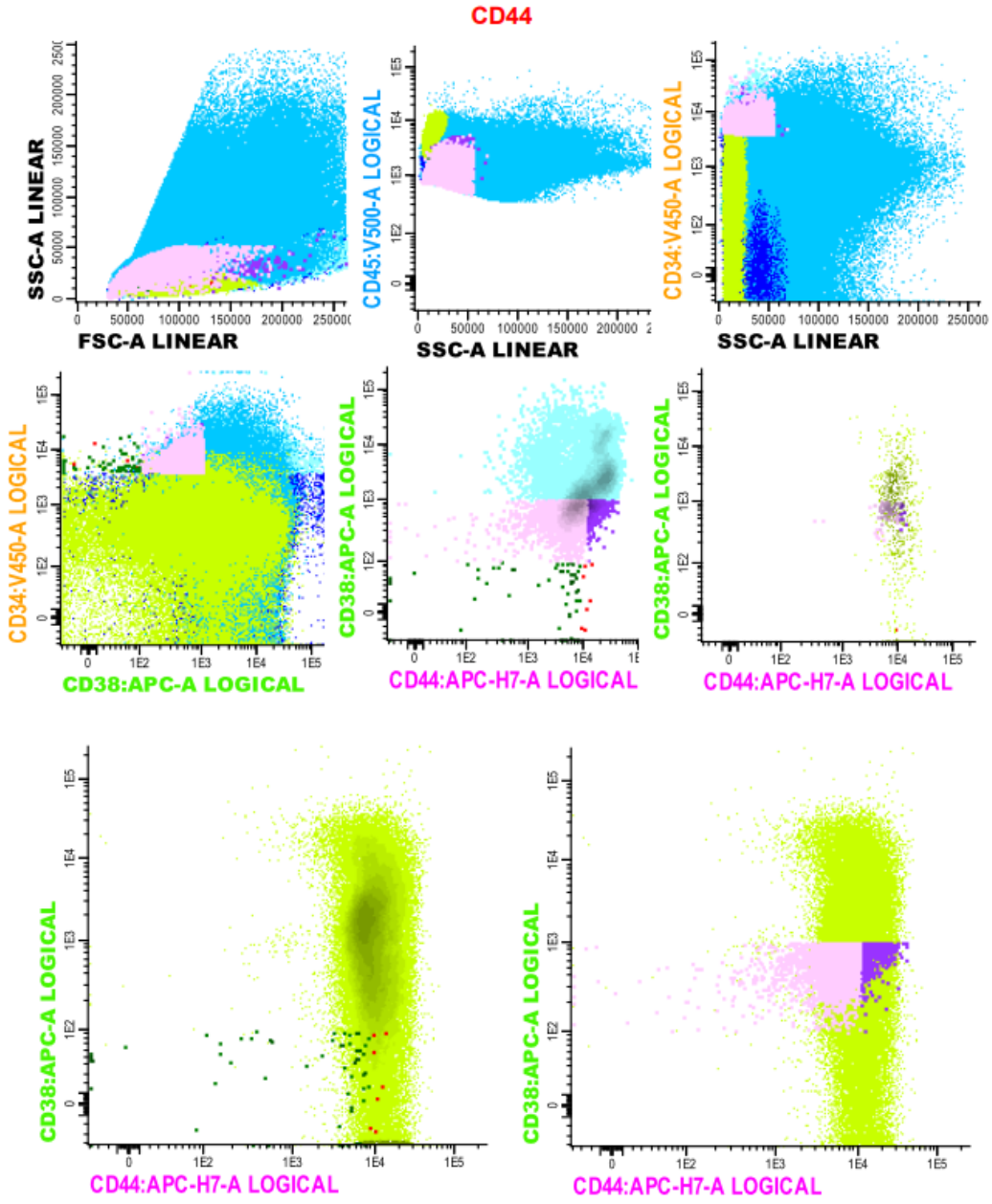
Population	Median
Lymfocyten	
FSC-A	103233.0781
SSC-A	11612.2305
CD38- LAP neg	
FSC-A	66099.7891
SSC-A	19128.7266
ƒ MFI FSC LAP neg/lymfo's: 0.6...	
ƒ MFI SSC LAP neg/lymfo's: 1.6...	
CD38- LAP pos	
FSC-A	72194.4375
SSC-A	24143.4902
ƒ MFI FSC LAP pos/lymfo's: 0.6...	
ƒ MFI SSC LAP pos/lymfo's: 2.0...	



CD44

Population	Events	Partial %	Visibility %
WBC	1045538	20.9108	100.0000
Lymfocyten	68767	6.5772	6.5772
Blasten	478827	45.7972	45.7971
CD34+	21927	4.5793	2.0971
CD34+CD38-	65	0.2964	0.0062
CD38- LAP neg	58	89.2308	0.0055
CD38- LAP pos	7	10.7692	0.0007
CD34+CD38dim	6111	27.8697	0.5844
CD38dim LAP neg	5132	83.9797	0.4908
CD38dim LAP pos	979	16.0203	0.0936
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

Population	Median
Lymfocyten	
FSC-A	103233.0781
SSC-A	11612.2305
CD38- LAP neg	
FSC-A	67151.8438
SSC-A	22517.5664
ƒ MFI FSC LAP neg/lymfo's: 0.6…	
ƒ MFI SSC LAP neg/lymfo's: 1.9…	
CD38- LAP pos	
FSC-A	71875.4609
SSC-A	16852.4297
ƒ MFI FSC LAP pos/lymfo's: 0.6…	
ƒ MFI SSC LAP pos/lymfo's: 1.4…	

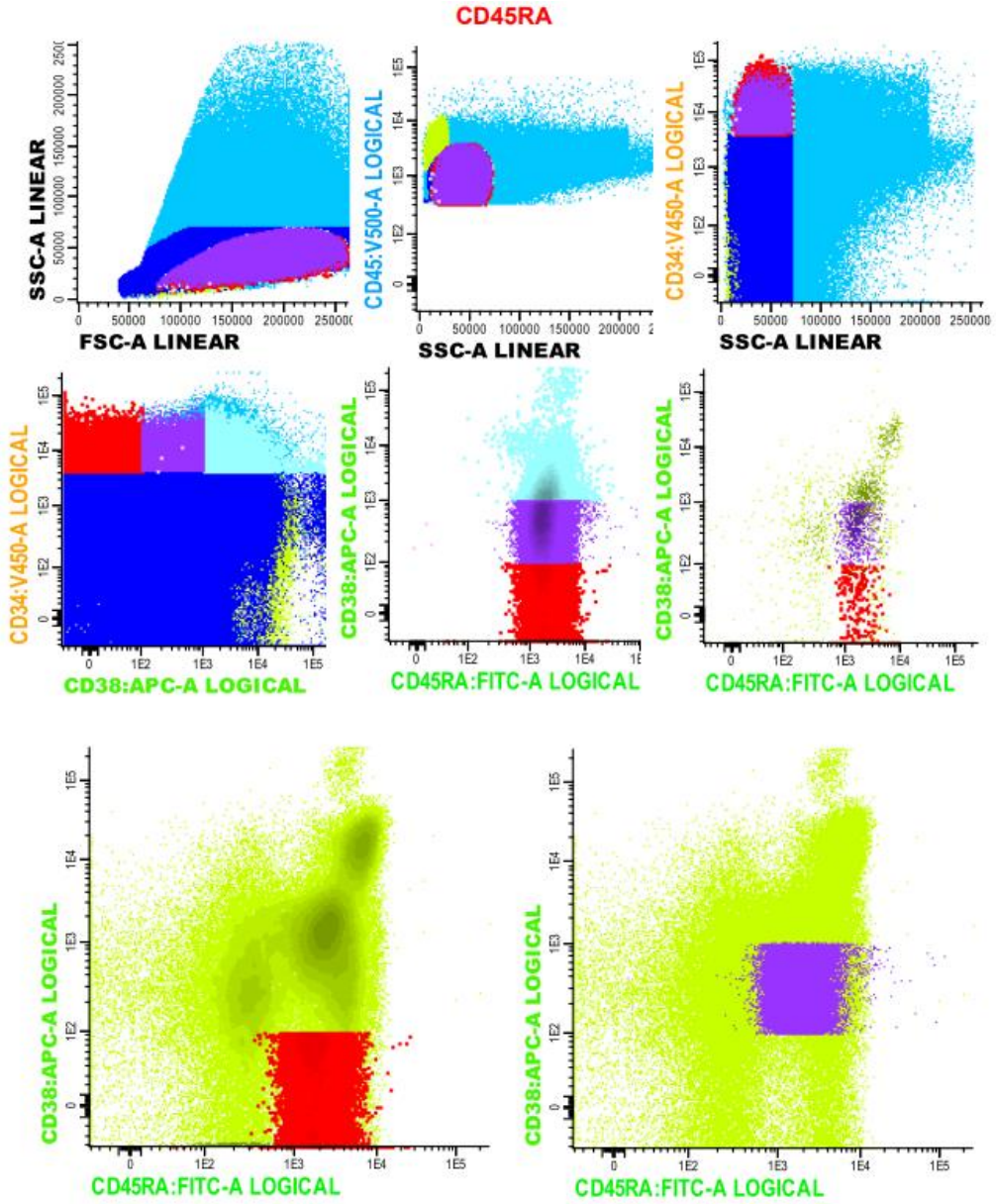


Patient 3: LSC's present at high percentages.

CD45RA

Population	Events	Partial %	Visibility %
WBC	3235476	73.1177	100.0000
Lymfocyten	136077	4.2058	4.2058
Blasten	2525630	78.0605	78.0605
CD34+	152079	6.0214	4.7003
CD34+CD38-	70725	46.5054	2.1859
CD38- LAP neg	0	0.0000	NA
CD38- LAP pos	70725	100.0000	2.1859
CD34+CD38dim	61251	40.2758	1.8931
CD38dim LAP neg	3	0.0049	0.0001
CD38dim LAP pos	61248	99.9951	1.8930
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

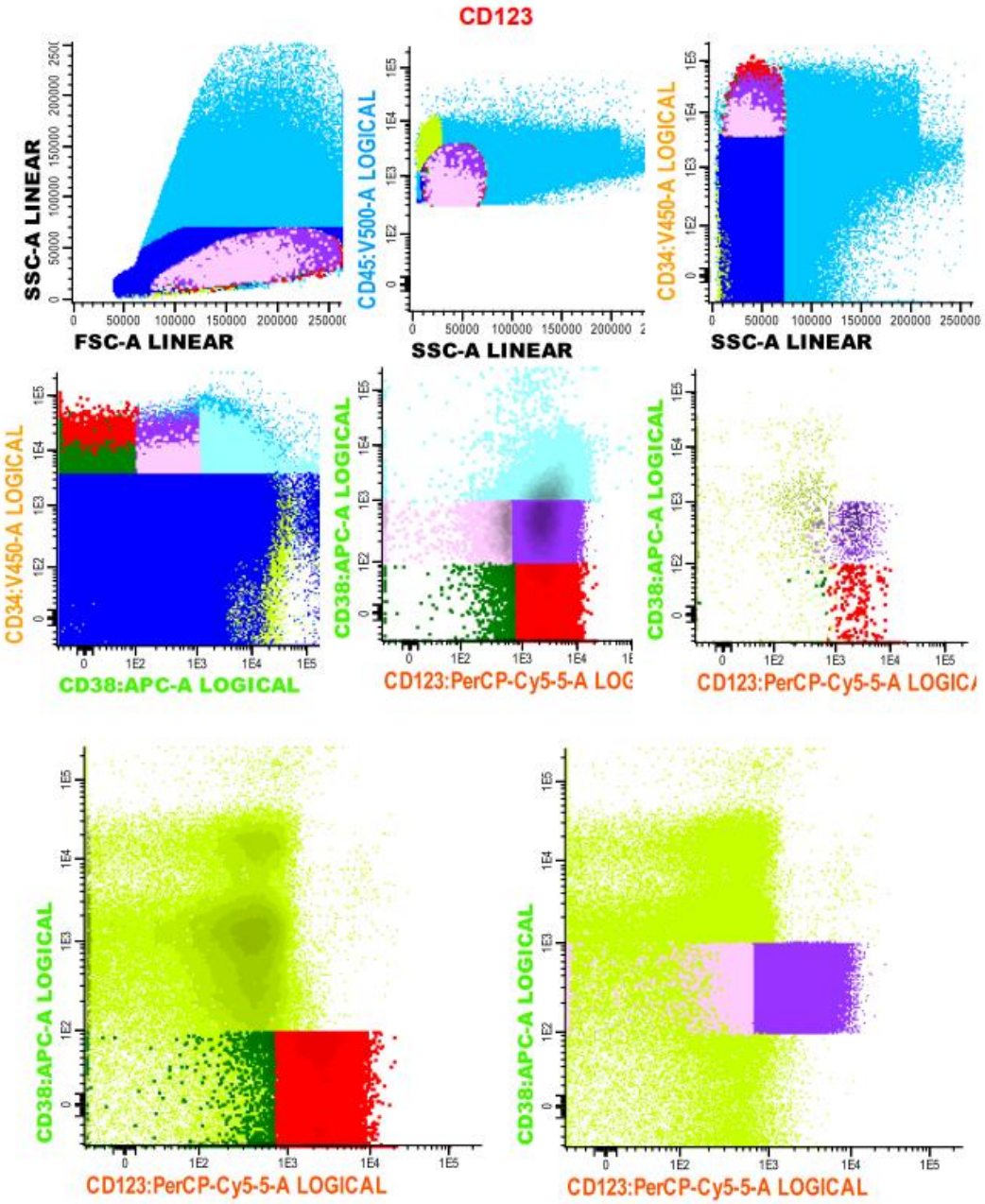
Population	Median
Lymfocyten	
FSC-A	96244.2188
SSC-A	12241.3203
CD38- LAP neg	
FSC-A	NA
SSC-A	NA
ƒ MFI FSC LAP neg/lymfo's: NA	
ƒ MFI SSC LAP neg/lymfo's: NA	
CD38- LAP pos	
FSC-A	167710.5000
SSC-A	32840.3594
ƒ MFI FSC LAP pos/lymfo's: 1.74...	
ƒ MFI SSC LAP pos/lymfo's: 2.68...	



CD123

Population	Events	Partial %	Visibility %
WBC	3235476	73.1177	100.0000
Lymfocyten	136077	4.2058	4.2058
Blasten	2525630	78.0605	78.0605
CD34+	152079	6.0214	4.7004
CD34+CD38-	70725	46.5054	2.1860
CD38- LAP neg	2370	3.3510	0.0733
CD38- LAP pos	68355	96.6490	2.1127
CD34+CD38dim	61251	40.2758	1.8931
CD38dim LAP neg	2334	3.8106	0.0721
CD38dim LAP pos	58917	96.1894	1.8210
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

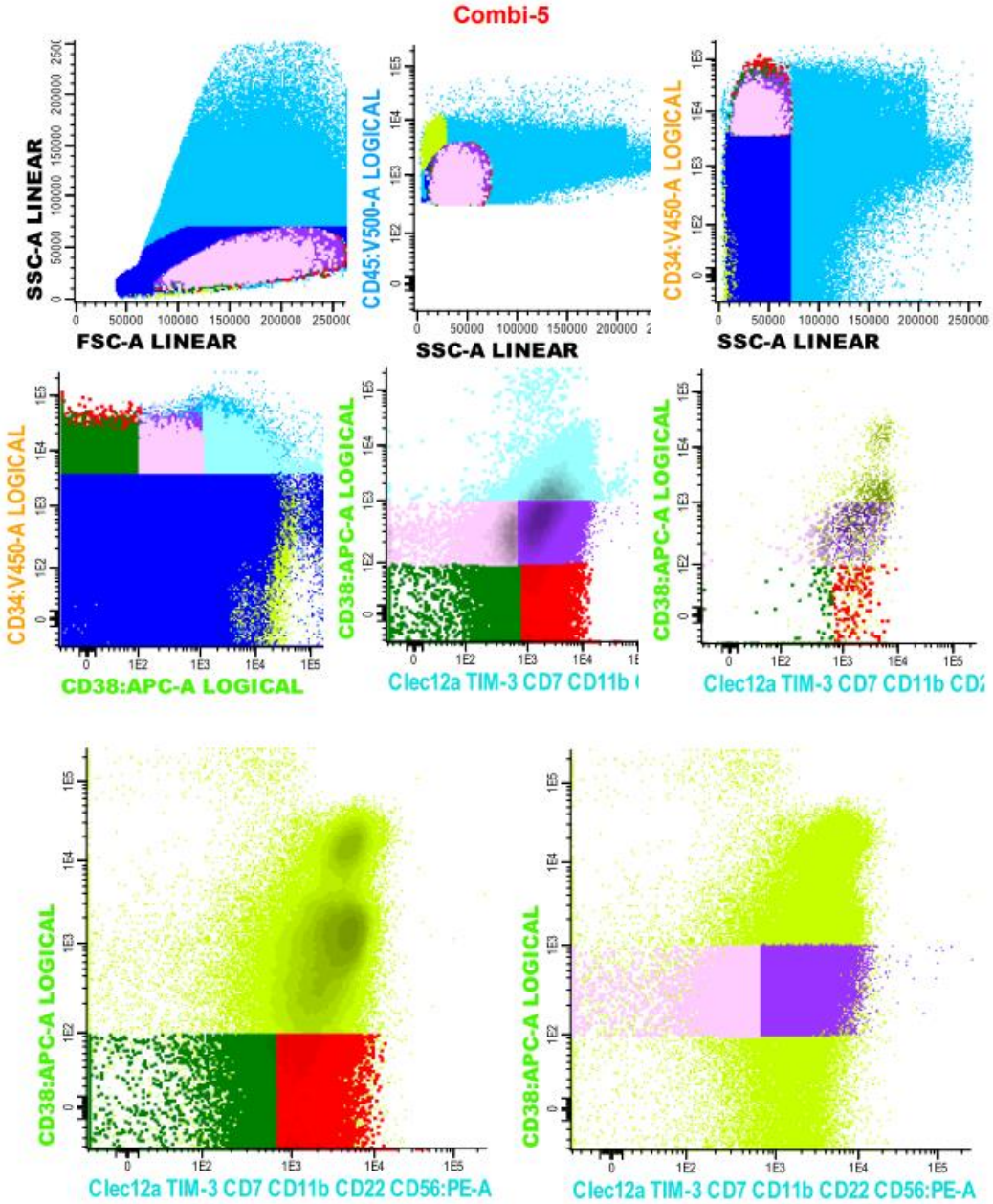
Population	Median
Lymfocyten	
FSC-A	96244.2188
SSC-A	12241.3203
CD38- LAP neg	
FSC-A	162877.4063
SSC-A	29835.2266
ƒ MFI FSC LAP neg/lymfo's: 1.6…	
ƒ MFI SSC LAP neg/lymfo's: 2.4…	
CD38- LAP pos	
FSC-A	167890.0781
SSC-A	32940.1094
ƒ MFI FSC LAP pos/lymfo's: 1.7…	
ƒ MFI SSC LAP pos/lymfo's: 2.6…	



Combi

Population	Events	Partial %	Visibility %
WBC	3235476	73.1176	100.0000
Lymfocyten	136077	4.2058	4.2058
Blasten	2525630	78.0605	78.0605
CD34+	152079	6.0214	4.7003
CD34+CD38-	70725	46.5054	2.1859
CD38- LAP neg	24943	35.2676	0.7709
CD38- LAP pos	45782	64.7324	1.4150
CD34+CD38dim	61251	40.2758	1.8931
CD38dim LAP neg	7937	12.9582	0.2453
CD38dim LAP pos	53314	87.0418	1.6478
pre	0	0.0000	NA
CD45/	553595	12.5106	0.0000
beads	0	0.0000	NA

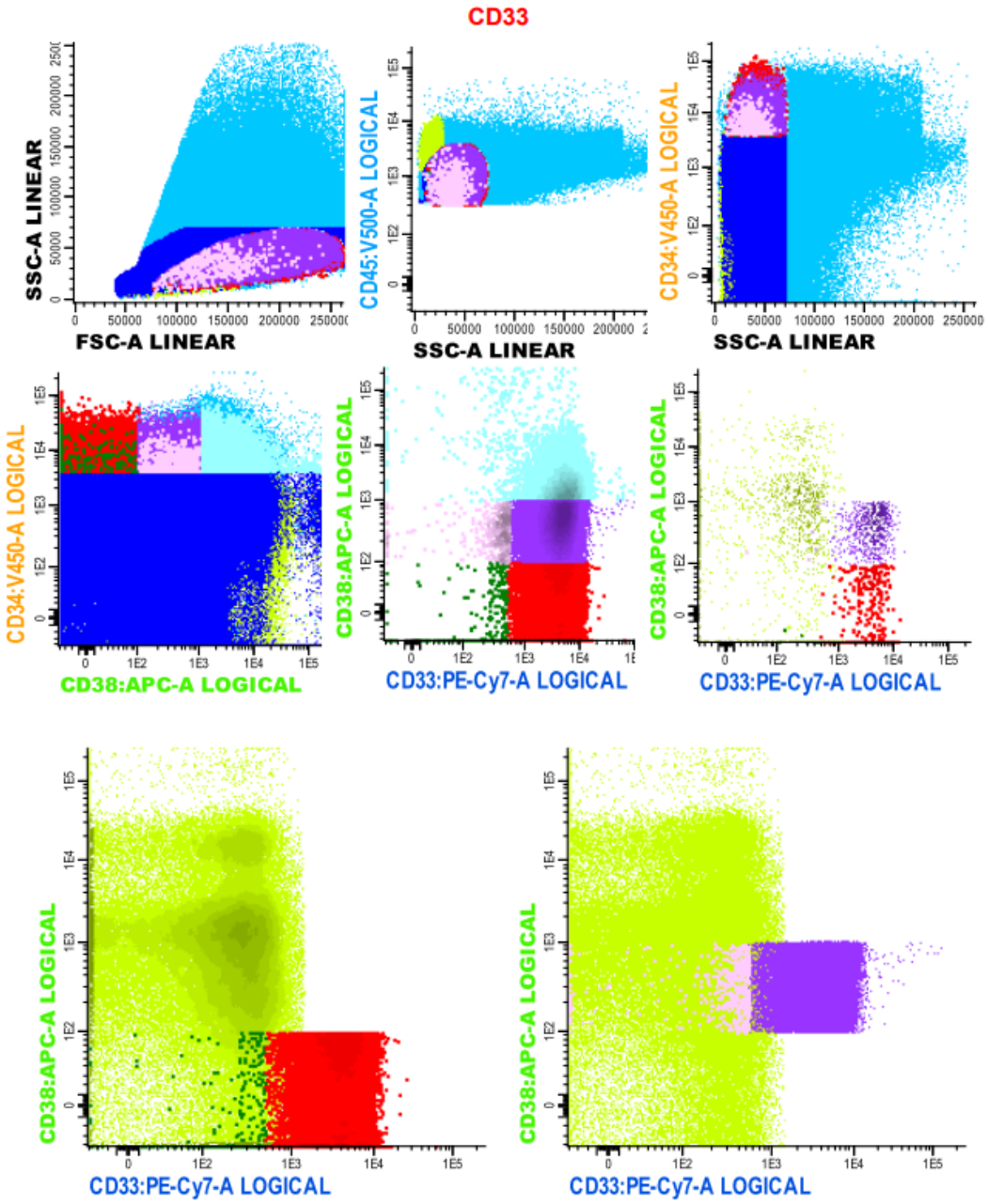
Population	Median
Lymfocyten	
FSC-A	96244.2188
SSC-A	12241.3203
CD38- LAP neg	
FSC-A	159942.6406
SSC-A	31532.9707
ƒ MFI FSC LAP neg/lymfo's: 1.6(	
ƒ MFI SSC LAP neg/lymfo's: 2.5	
CD38- LAP pos	
FSC-A	172157.3594
SSC-A	33635.0352
ƒ MFI FSC LAP pos/lymfo's: 1.7	
ƒ MFI SSC LAP pos/lymfo's: 2.7	



CD33

Population	Events	Partial %	Visibility %
WBC	3235476	73.1177	100.0000
Lymfocyten	136077	4.2058	4.2058
Blasten	2525630	78.0605	78.0605
CD34+	152079	6.0214	4.7003
CD34+CD38-	70725	46.5054	2.1859
CD38- LAP neg	821	1.1608	0.0254
CD38- LAP pos	69904	98.8392	2.1605
CD34+CD38dim	61251	40.2758	1.8931
CD38dim LAP neg	497	0.8114	0.0154
CD38dim LAP pos	60754	99.1886	1.8777
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

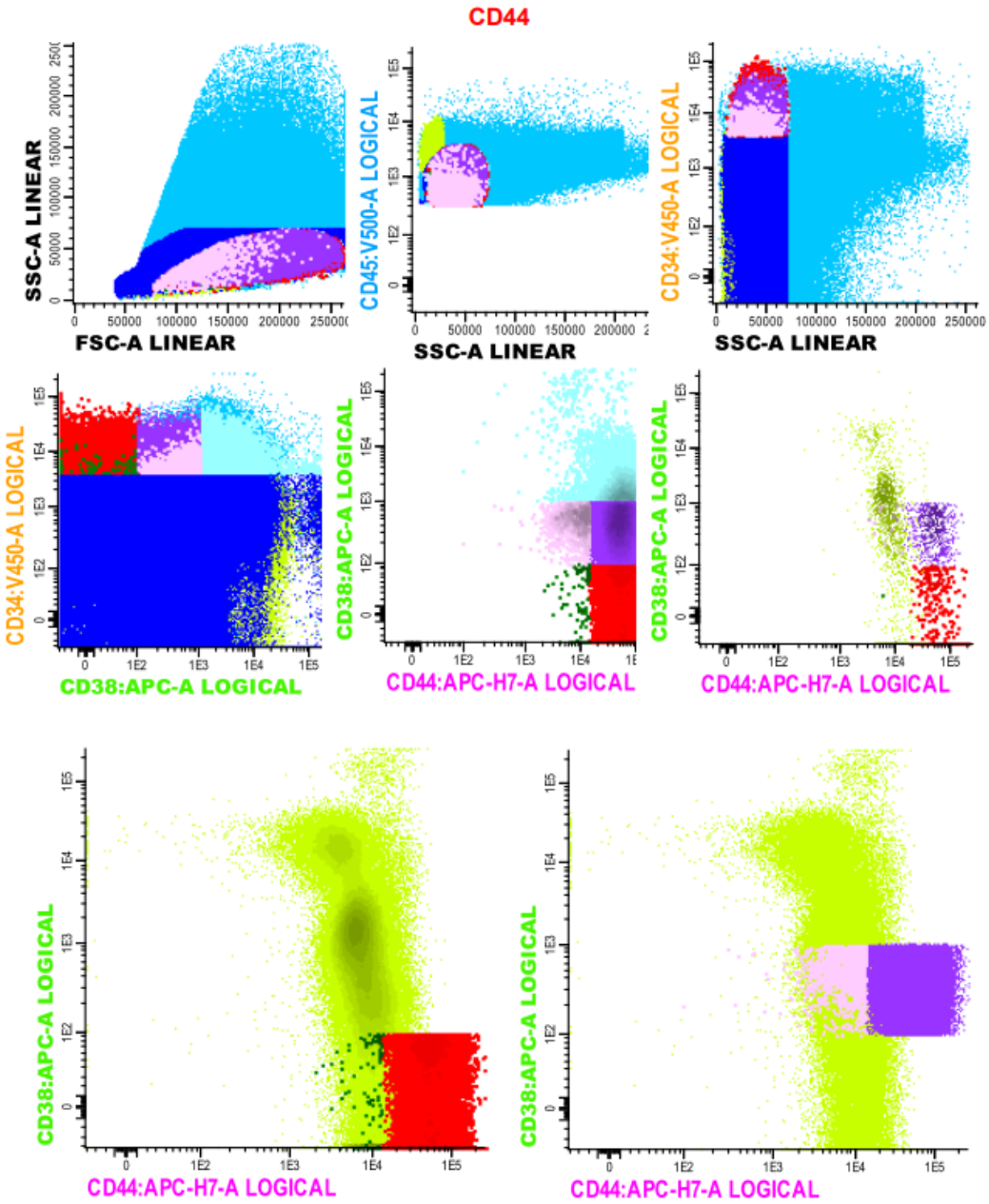
Population	Median
Lymfocyten	
FSC-A	96244.2188
SSC-A	12241.3203
CD38- LAP neg	
FSC-A	148321.5938
SSC-A	30918.5117
ƒ MFI FSC LAP neg/lymfo's: 1.5...	
ƒ MFI SSC LAP neg/lymfo's: 2.5...	
CD38- LAP pos	
FSC-A	167913.4375
SSC-A	32861.6406
ƒ MFI FSC LAP pos/lymfo's: 1.7...	
ƒ MFI SSC LAP pos/lymfo's: 2.6...	



CD44

Population	Events	Partial %	Visibility %
WBC	3235476	73.1177	100.0000
Lymfocyten	136077	4.2058	4.2058
Blasten	2525630	78.0605	78.0605
CD34+	152079	6.0214	4.7003
CD34+CD38-	70725	46.5054	2.1859
CD38- LAP neg	101	0.1428	0.0031
CD38- LAP pos	70624	99.8572	2.1828
CD34+CD38dim	61251	40.2758	1.8931
CD38dim LAP neg	1152	1.8808	0.0356
CD38dim LAP pos	60099	98.1192	1.8575
pre	0	0.0000	NA
CD45/	0	0.0000	NA
beads	0	0.0000	NA

Population	Median
Lymfocyten	
FSC-A	96244.2188
SSC-A	12241.3203
CD38- LAP neg	
FSC-A	121997.9609
SSC-A	27435.2402
ƒ MFI FSC LAP neg/lymfo's: 1.2(	
ƒ MFI SSC LAP neg/lymfo's: 2.2	
CD38- LAP pos	
FSC-A	167747.8125
SSC-A	32845.6797
ƒ MFI FSC LAP pos/lymfo's: 1.7	
ƒ MFI SSC LAP pos/lymfo's: 2.6(	



Leukaemic Stem Cell Results – Cohort A

	CD45RA (%)	CD123 (%)	Combi (%)	CD33 (%)	CD44 (%)
A1	0	0	0	0	0
A2	0.04	0.02	0.02	0.01	0
A3	0.03	0	0.01	0	0
A4	0	0	0	0	0
A5	0.01	0	0.01	0	0
A6	0	0	0	0	0
A7	0	0	0	0	0
A8	0	0	0	0	0
A9	0.01	0	0	0	0
A10	0.002	0	0.002	0	0
A11	0	0	0	0	0
A12	0.01	0	0	0	0
A13	0.02	0	0.028	0.001	0.006
A14	0.003	0	0.003	0	0
A15	0.0005	0	0.0004	0	0.0004
A16	0.0001	0	0	0	0
A17	0.054	0	0.0039	0	0.0019
A18	0	0	0	0	0
A19	0.001	0	0	0	0
A20	0.002	0	0.004	0.0018	0.0026
A21	0.0008	0	0.0007	0	0
A22	0.0006	0	0.0002	0	0.0002
A23	0	0	0	0	0
A24	0.005	0.0006	0.0059	0.0013	0.0005
A25	0.0043	0.0013	0.0054	0.002	0.0007
A26	0.0011	0.0011	0.0011	0.0008	0.0007
A27	0.0017	0.0007	0.002	0	0
A28	0.0086	0.0072	0.0102	0.0047	0.0023
A29	0.0004	0.0001	0.0002	0	0.0001
A30	**0.0053**	**0.006**	**0.0055**	**0**	**0.0012**

Leukaemic Stem Cell Results – Cohort B

	CD45RA (%)	CD123 (%)	Combi (%)	CD33 (%)	CD44 (%)
B1	0.09	0.01	0.04	0.01	0
B2	0	0	0	0	0
B3	0	0.07	0	0.08	0.03
B4	0	0	0	0	0
B5	0.0041	0.0024	0.0033	0	0.0012
B6	0.1	0.04	0.01	0.01	0
B7	1.315	1.316	0.897	1.274	0
B8	0.277	0.537	0.33	0.547	0.512
B9	0.044	0.0119	0.0283	0	0.0382
B10	0.0023	0	0.0031	0.001	0.0021
B11	0.253	0.233	0.204	0.093	0.249
B12	2.1859	2.1127	1.415	2.1605	2.1828
B13	0.1868	0.1858	0.1854	0.1817	0.0251
B14	1.5541	1.5076	0.3139	0.0551	1.5514
B15	0.0383	0.035	0.0248	0.0363	0.0363
B16	0.0064	0.0035	0.0079	0.0011	0.012
B17	0.2683	0.1855	0.2831	0.2352	0.0284
B18	0.0198	0.0065	0.0107	0.0012	0.0005
B19	0.0195	0.0121	0.0187	0.0045	0.0089
B20	0.0118	0.0039	0.0145	0.0037	0.0023
B21	0.0162	0.0138	0.0851	0.0419	0.0318
B22	0.1561	0.1629	0.051	0.0083	0.1407
B23	0.0268	0.0027	0.042	0.0077	0.003

Molecular (NGS) Results – Cohort A

	DNMT3A	TET2	BRAF	SF3B1	ASXL1	SRSF2	RUNX1	U2AF1	SETBP1	ZRSR2	MPL	EZH2	NRAS	PHF6	KIT	JAK2	NF1	BCOR	SATG2
A1																			
A2																			
A3																			
A4		61.37%	43.85%	49.12%	48.82%														
A5																			
A6		79.99%				49.85%													
A7																			
A8																			
A9							33.71%	48.05%											
A10						52.41%	31.23%		53.25%	97.05%									
A11																			
A12		43.60%		41.51%															
A13					27.75%			37.74%		76.88%	43.60%	44.36%							
A14					60.73%		37.16%					92.35%							
A15																			
A16		97.48%				57.02%							49.08%						
A17																			
A18		52.65%		39.76%															
A19					48.92%			45.65%					18.76%	82.45%					
A20					17.31%														
A21																			
A22																			
A23		87.41%				54.07%						88.29%			50.10%				
A24																			
A25						29.76%							34.05%						
A26	46.40%	5.95%		42.60%			4.60%												
A27								36.85%								20.17%			
A28							36.56%										66.53%	79.42%	81.24%
A29																			
A30					20.50%	12.03%	21.19%												43.50%

Molecular (NGS) Results – Cohort B

	BCOR	KRAS	NRAS	TET2	U2AF1	DNMT3A	FLT3 ITD	NPM1	TP53	ZRSR2	SH2B3	SRSF2	CBL	IDH2	NF1	IDH1	STAG2	PTPN11	ASXL1	RUNX1
B1	80.49%	5.15%	25.03%	40.86%	40.60%	42.72%														
B2											15.46%									
B3									75.09%											
B4									68.64%											
B5		3.00%	40.26%	91.17%	33.85%			22.30%	95.00%											
B6				89.64%					75.24%			30.13%								
B7						48.70%	0.61%	41.30%	43.97%				47.80%							
B8				50.15%				30.99%				27.73%		44.01%						
B9									33.65%					21.77%	25.05%					
B10									42.15%											
B11									76%										41.94%	
B12			10.05%				5.20%													
B13			32.07%					29.16%								4.11%				
B14												57.83%				46.81%				
B15							39.19%													
B16						23.20%										24.45%				
B17									89.20%						70.40%					
B18														44.53%			28.21%			
B19				49.77%								47.64%		48.97%						
B20				94.93%			11.15%	18.05%				46.74%						42.30%	8.19%	
B21							37.90%	31.40%						47.80%						
B22												46.16%		12.61%		31.13%			46.84%	46.61%
B23				90.55%			14.80%	25.83%												