ELUCIDATING THE ROLE OF MESENCHYMAL STEM CELLS, IMMUNE AND TELOMERE BIOLOGY IN REGENERATION AND DIFFERENTIATION OF HAEMATOPOIETIC STEM CELLS

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SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES ANDTECHNOLOGY, THIRUVANANTHAPURAM

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A THESIS SUBMITTED BY

ARUNA BARADE

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IN PARTIAL FULFILMENT OF THE

REQUIREMENTSFOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2024

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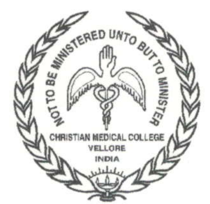

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LIST OF ABBREVIATIONS

AA	Aplastic anaemia
AGM	Aorta-gonad mesonephros
ALT	Alternative lengthening of telomeres
AML	Acute myeloid leukaemia
ATG	Anti-thymocyte globulin
BM	Bone marrow
BMA	Bone marrow adipocytes
BMF	Bone marrow failure
CAB	Cajal body
CAMT	Congenital amegakaryocytic thrombocytopenia
CAR cells	CXCL12-abundant reticular cells
CFU-C	Colony-forming units-culture
CLPs	Common lymphoid progenitors
cMAT	Constitutive marrow adipose tissue
CMPs	Common myeloid progenitors
CS	Carnegie stages
CSA	Cyclosporine A
CXCL	C-X-C motif chemokine ligand
Cy	Cyclophosphamide
DBA	Diamond-Blackfan anaemia
DC	Dyskeratosis congenita
DKC1	Dyskerin
DSBs	DNA double-stranded breaks
ECs	Endothelial cells
FACS	Fluorescence-activated cell sorting
FA	Fanconi anaemia
FGF	Fibroblast growth factor
Flu	Fludarabine

GAR1	H/ACA ribonucleoprotein complex subunit 1
GFs	Growth factors
GMPs	Granulocyte-macrophage progenitors
GVHD	Graft-versus-host disease
HLA	Human leukocyte antigen
HR	Hazard ratio
HSCs	Haematopoietic stem cells
HSCT	Haematopoietic stem cell transplantation
HSPC	Haematopoietic stem and progenitor cell
IAHCs	Intra-aortic hematopoietic clusters
IBMFS	Inherited Bone Marrow Failure Syndromes
IFN-γ	Interferon - gamma
IPSS	International Prognostic Scoring System
IST	Immunosuppressive therapy
LT-HSCs	Long-term HSCs
MDS	Myelodysplastic syndrome
MEPs	Megakaryocyte-erythrocyte progenitors
MPPs	Multipotent progenitors
MSCs	Mesenchymal stromal cells
MSD	Matched sibling donor
MUD	Matched unrelated donors
NHEJ	Non-homologous end-joining
NHP2	Non-histone protein 2
NK cells	Natural killer cells
NOP10	Nucleolar protein 10
NSAA	Non-Severe Aplastic Anaemia
PBSC	Peripheral-blood stem cells
PCR	Polymerase Chain Reaction
PNA	Peptide nucleic acid
POT1	Protection of telomeres protein 1

Q-FISH	Quantitative Fluorescence in Situ Hybridization
qPCR	Quantitative Polymerase Chain Reaction
RAP1	Repressor and activator protein 1
rMAT	Regulated marrow adipose tissue
rTL	Relative telomere length
SAA	Severe Aplastic anaemia
SCF	Stem cell factor
SCN	Severe congenital neutropenia
SDS	Shwachman–Diamond syndrome
STELA	Single telomere length analysis
ST-HSCs	Short-term HSCs
TCAB1	telomerase cajal body protein 1
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TFs	Transcription factors
TGF-β	transforming growth factor - Beta
Th1	T helper 1 cells
Th17	T helper 17 cells
TIN2	TRF1-interacting nuclear protein 2
TL	Telomere length
TNF-α	Tumour necrosis factor - alpha
TPP1	TIN2-interacting protein
Tregs	Regulatory T cells
TRF	Telomeric repeat-binding factor
TRF	Terminal Restriction Fragment
VCAM1	Vascular cell adhesion protein 1
VSAA	Very-Severe Aplastic Anaemia
vWf	von Willebrand factor

ELUCIDATING THE ROLE OF MESENCHYMAL STEM CELLS, IMMUNE AND TELOMERE BIOLOGY IN REGENERATION AND DIFFERENTIATION OF HAEMATOPOIETIC STEM CELLS

SYNOPSIS

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Background

The indefinite self-renewal and potential to differentiate into other types of cells represent stem cells as frontiers of regenerative medicine. Haematopoietic stem cells (HSCs) are well-characterized tissue-specific stem cells and are in routine use clinically. Stem cell transplantation banks on the unique potential of stem cells to regenerate the entire haematopoietic system. The destruction of HSCs by various causes, such as chemicals, cytotoxic drugs, radiation, viral infections, and immune disorders, leads to bone marrow failure (BMF). One such scenario is observed in aplastic anemia (AA), where immunologic mechanisms are believed to be the prime cause, along with other causative factors, namely telomere biology and constitutional gene defects, leading to the loss of HSCs. Certain antigens are thought to stimulate autoreactive T-cells to proliferate and attack host HSCs. Such immune responses induce the production of inflammatory cytokines, which can terminate the growth of HSCs; and eventually truncate cell cycling and cause cell death by apoptosis of HSCs. Aplastic anemia results in peripheral pancytopenia and marrow hypoplasia, and its incidence is believed to be 2-3 times higher in Asia. Haematopoietic stem cell transplantation (HSCT) or immunosuppressive therapy using a combination of anti-thymocyte globulin (ATG) and cyclosporine+/-Eltrombopag are the mainstays of treatment of AA. HSCT is associated with cure rates of over 80-90% in patients below the age of 20 years and over 70% in patients between 20-40 years of age.

Haematopoietic stem cells reside in specialized microenvironments (niches) in the bone marrow. The stem cell niche is thought to provide signals that support fundamental HSC properties, including self-renewal capacity and long-term multilineage repopulating ability. Mesenchymal stromal cells (MSCs) possess the capacity to differentiate into specific cell types, abundant production of soluble growth factors and cytokines, and their immunomodulating properties. Several studies have examined how the functional and immunosuppressive property

of MSCs in patients with AA differs from bone marrow MSCs in healthy individuals. Despite a certain degree of variability, various studies showed a decreased proliferation potential in AA-MSCs and their differentiation capacities, for instance, an increased tendency to differentiate towards adipocytes and a decreased propensity towards the osteogenic lineage. Furthermore, AA-MSCs showed a reduced ability for immunomodulation and hematopoietic support compared to healthy controls. Incongruously, few recent data have shown that the proliferative, functional, and immunomodulatory properties of MSCs are comparable to normal controls. The discrepancy in the data could be due to the heterogeneity of the study populations.

The proliferative potential of HSCs reduces with age and leads to the shortening of telomeres. Telomeres are DNA-protein structures consisting of tandem TTAGGG repeats that protect the ends of linear chromosomes from degradation. Human telomeres range from 5-15kb and are remarkably heterogeneous among individuals and vary with age, organ, and proliferative history of each cell. Telomere erosion results in critically short telomeres, leading to cellular senescence, including apoptosis, and genomic instability. In stem cells and germline cells, telomere erosion is counteracted by a ribonucleoprotein enzyme, telomerase encoded by the TERT (telomerase reverse transcriptase) and TERC (telomerase RNA component), along with additional factors such as DKC1, NOP10, NHP2 and GAR1. Telomerase is essential in preventing replication-dependent loss of telomere and cellular senescence in highly proliferative cells. Studies have demonstrated that the telomere length (TL) of leukocytes in patients with AA is shorter than in age-matched healthy controls. In addition, lower telomere length has been shown to be associated with frequent relapses, clonal evolution to MDS, and poor survival in patients undergoing treatment with ATG. Donor and patient telomere length also influence outcomes in patients undergoing unrelated stem cell transplantation for aplastic anaemia. There is limited data in studying the telomere length in patients with AA in the Indian population. The association of telomere length in patients undergoing matched sibling donor

transplants for aplastic anaemia has not been reported. Therefore, we aimed to investigate the role of mesenchymal stromal cells, and molecular and immune mechanisms that support HSCs regeneration in aplastic anaemia.

Objectives

1. To study the mechanism of mesenchymal stem cells, which provide a microenvironment for HSCs, in the condition of HSCs loss.
2. To investigate the role of telomere biology in the maintenance of HSCs self-renewal property.
3. To understand the immune mechanism in aplastic anemia that leads to the loss of HSCs regeneration.

Materials and Methods

Study participants

Patients diagnosed with aplastic anaemia (AA) at Christian Medical College, Vellore, India, between 2001 and 2022 were enrolled in the study. The Institutional Review Board and the institution's Ethics Committee approved this study. A total of 650 patients with AA were included, whose DNA samples were available. Clinical data of all these patients were collected from individual medical records and institutional databases. Patients with AA were categorized into very severe (VSAA), severe (SAA), and non-severe aplastic anaemia (NSAA) using the standard Comitta criteria. Eight hundred transplant donors, excluding donors of beta-thalassemia and aplastic anaemia, were taken as healthy controls (HC). A total of 281 AA patients underwent matched related sibling donor (MSD) transplants during 2001-2019, of which 194 DNA samples of patients and donors were available, with a final analysis of 163 patients. Transplant outcomes, such as graft failure, time to neutrophil recovery, acute and chronic graft incidence versus host disease, and overall survival, were recorded. A cohort of 72 AA patients treated with ATG was included to understand the immune and molecular response

to the treatment. Bone marrow samples were collected during the diagnostic procedure from AA patients, and lymphoma patients with no abnormal cells were considered normal marrow for MSCs culture.

Mesenchymal stromal cells culture

Bone marrow-mononuclear cells (MNCs) were separated by the Ficoll-gradient method and suspended in DMEM medium with 10% FBS and 1% pen/strep for 3-4 weeks. Upon reaching approximately 80% confluence, cells were passaged, and the cell adhering capacity of MSCs was observed using an inverted light microscope.

Differentiation of MSCs

To promote osteogenic and adipogenic differentiation, cells were incubated in the differentiation medium. On day 21, cells were stained for Alizarin Red and Oil red O stain for osteogenic and adaptogenic differentiation, respectively. The lineage-specific genes were analysed using qPCR.

Immunosuppression property of MSCs

To evaluate the immunosuppression potential of MSCs, their effect on mitogen-induced T-cell proliferation was determined. Carboxyfluorescein succinimidyl ester (CFSE) pre-labeled PB-MNCs were stimulated with the mitogen phytohemagglutinin (PHA) and co-cultured with MSCs for 96 hours. Flow cytometry determined the percent of T-cell proliferation by reducing CFSE intensity through cell divisions.

Telomere length measurement

Genomic DNA was extracted from the peripheral blood of patients and donors using QIAGEN Puregene gentra Kit and stored at 4^{0}C till analysis. The purity of the DNA samples was assessed using a nanodrop spectrophotometer by estimating the absorbance ratio at 260/280 nm, and samples' ratios within the range of 1.8-2 were considered pure. The relative

telomere length (rTL) was measured using quantitative real-time polymerase chain reaction (qPCR) from the extracted DNA, as described by Cawthon 2002.

Briefly, for the standard curve, two master mixes of PCR reagents were prepared, one with the TEL primer pair and another with S (36B4) primer pair. One reference DNA sample was diluted serially to produce five concentrations of DNA ranging from 10 to 0.625 ng/ml. PCR was performed using 10uL reaction volumes consisting of 5ul of SYBR Green PCR Master Mix (Takyon, Eurogentec), 0.5ul of lambda DNA, 2.25ul of sterile water, and 0.125ul of an assay-specific mix of primers. The ratio between telomeric DNA (T) and a single copy gene (S) is computed for each DNA sample. The relative T/S ratio is calculated about a reference genomic DNA sample. The relative T/S ratio was obtained using the formula $2^{-(\Delta Ct-\Delta Cs)} = 2^{-\Delta\Delta Ct}$. This T/S ratio is directly proportional to the average telomere length. All telomere and 36B4 reactions were performed in duplicates. The laboratory personnel conducting the telomere-length assay were blinded to patient characteristics and clinical outcomes before the statistical analysis.

Flow cytometry analysis

Peripheral blood (PB) whole blood cells were stained with the following antibodies to analyse T-cells subsets and NK cells: CD3-PerCP, CD4-FITC, CD8-PE, CD16-PE, CD56-APC, Human regulatory T-cell cocktail (BD Biosciences). The cells were acquired on Navios EX flow cytometer (Beckman Coulter), and data were analysed using Kaluza C software.

Immunoassay

Serum or plasma was obtained from peripheral blood. Analysis of 8 cytokines (IFN-G, TNF-A, IL-2, IL-4, IL-17A, IL-22, TGF-B, IL-10) was performed using simple plex assay from Protein simple according to manufacturer instructions.

Statistics

Statistical analysis was done using the IBM SPSS 24.0 Software.

Major findings

We demonstrated that MSCs from patients with AA are phenotypically like those of normal controls, in differentiation and immunosuppression properties that help in hematopoietic support. The telomere length in AA MSCs was comparable to that of normal MSCs. Telomere shortening plays a crucial role in biological aging. We established age-wise relative telomere length in large normal Indian population. The median relative telomere length in patients with aplastic anemia was significantly shorter than in healthy controls. About half of the AA patients' population had shorter telomere length than age-matched healthy controls. Telomere length was not associated with the severity of AA. We found that donor telomere length rather than recipient telomere length was significantly associated with transplant outcomes. Shorter donor telomere length was an independent risk factor for survival along with older age, presence of acute GVHD, and graft failure. The highest quartile of donor relative telomere length was associated with better overall survival, similar to observations seen in patients undergoing unrelated transplants by Gadalla *et al*., JAMA 2015. Telomere length was measured in CD34+ cells, and observed that rTL was comparable between AA and normal. The telomere length did not influence immunosuppression therapy (ATG) outcomes in patients with AA, consistent with the published data by Scheinberg *et al.,* JAMA, 2010. The analysis of immune cells in the cohort of AA patients treated with ATG showed that the percentage of $CD3^{+}$, $CD4^{+}$, $CD8^{+}$ T cells, and NK cells was not associated with response to ATG in AA patients. Similarly, the levels of pro- and anti-inflammatory cytokines did not differ in response to ATG in AA patients. The percentage of regulatory T cells was significantly higher in AA patients who responded to ATG.

Significance of the findings

This is the first Indian study to establish age-wise relative telomere length in a large normal population and patients with aplastic anemia. The healthy controls telomere length was categorized into percentiles age-wise; patients with rTL below the 50th percentile are considered shorter for diagnostics. This is the first study to report the effect of donor and recipient telomere lengths on transplant outcomes in patients with aplastic anemia undergoing matched sibling donor transplantation. The telomere length measurement in aplastic anemia MSCs and CD34+ cells was demonstrated for the first time.

1.0. INTRODUCTION

The marrow produces billions of blood cells daily; however, the function of bone marrow was not understood in ancient times and was experimentally defined in the late 19th century. Hippocrates, a renowned Greek physician (460 to 375 BC), formulated his conclusions through observation and reasoning rather than mysticism. He believed that bone tissue derives its nutrients from the marrow, a prominent view shared by Galen (130 to 200 AD). Aristotle (384 to 322 BC) held a different perspective on the matter; he believed that the marrow was a waste product of bones, referred to as "excrementum ossium". During the 18th century, anatomists were fascinated by the vascularity of the marrow and supported Hippocrates' belief that it was the vascular element of bone. Charles Robin, in 1872 observed that the marrow is formed after the bone during development, which further implies that it is unlikely to be a source of bone nutrition (Robin, 1875).

The existence of red blood cells was first documented in the 17th century, but it wasn't until the 19th century that scientists began investigating their origins. Cell theory in 1838 by Schleiden and Schwann identified the cell as the basic building block of life, enabling the idea of red blood cell production. Neumann and Bizzozero first observed bone marrow as the site of blood formation. In the late 1800s, there were significant advancements in morphologic methods, mainly due to Paul Ehrlich's innovations in aniline dyes and heat-fixed films for blood smears and bone marrow analysis. Through this discovery, an understanding of blood production was obtained and eventually confirmed the haematopoietic stem cell, as hypothesised by Neumann. The bone marrow microenvironment significantly influences the expansion of haematopoietic stem cells (HSCs) (Cooper, 2011).

Self-renewing haematopoietic stem cells maintain the blood system while residing in the bone marrow of adult mammals; being atop a hierarchy, they become progressively restricted to single lineages. The blood system serves as a model for comprehending the biology of tissue stem cells and their involvement in ageing, disease, and oncogenesis. Stem cells are necessary throughout life to replenish haematopoietic precursors committed to individual lineages due to the short lifespan of mature blood cells. The haematopoietic system is a prominent illustration of the effective application of regenerative medicine. Stem cell transplantation has revolutionized the treatment of various blood disorders and cancers, becoming a standard practice for over three decades (Orkin, 2000). The bone marrow environment comprises two main components, the cellular and acellular compartments. The cellular compartment consists of HSCs, mesenchymal stromal cells (MSCs), and other stromal cell types, while the acellular compartment comprises scaffold proteins called the extracellular matrix. During the coculture of HSCs and MSCs, direct contact between cells and cytokines secreted by MSCs is crucial for haematopoiesis (Atmar et al., 2022).

Telomeres are located at the end of chromosomes and comprise long $(TTAGGG)_n$ nucleotide repeats and an associated protein complex. Their primary function is to sustain chromosomal integrity by preventing end-to-end fusion of chromosomes. With each cell division, telomeres shorten, which makes their length a marker of replicative capacity, cell ageing, and senescence. The attrition of telomeres in human cells is a type of DNA damage with limited repair mechanisms (Gadalla & Savage, 2011). Maintaining proper telomeres in HSCs is crucial as blood cells require continuous replication throughout one's life. Telomeres undergo rapid shortening after birth and throughout an individual's life. Research conducted in the past has shown that the umbilical cord HSCs ($CD34^+CD38^-$) contain longer telomeres compared to the same cell

population in adult bone marrow or peripheral blood. Studies have shown that the shortening of telomeres in peripheral blood mononuclear cells is four times higher in the initial three years of life compared to adults (Hills et al., 2009). The high replication and growth of HSCs during the early years of life may cause significant shortening of telomeres, but the rate slows down as one ages. Evidence supporting the replicative senescence theory shows that haematopoietic progenitor cells ($CD34^{+}$) have longer telomeres than subsets of mature cells like naïve and memory $CD4^{+}$ and $CD8^{+}$ T-cells and granulocytes. Naïve $CD4^{+}$ and $CD8^{+}$ T-cells have longer telomeres than their corresponding memory or effector cells. This may be due to the higher replicative demands of naïve cells (Kimura et al., 2010). The connection between telomere biology and medicine was initially reported in bone marrow failure.

Bone marrow failure syndromes are a distinct group of benign haematological disorders that exhibit the absence of single or multi-lineage marrow and peripheral blood cytopenia. These syndromes can be categorised as constitutional or acquired based on their underlying pathophysiology. In inherited conditions, germline mutations appear in the haematopoietic stem and progenitor cell (HSPC), leading to a gradual decline in normal haematopoiesis. The genetic aetiology of inherited bone marrow failure syndrome (IBMFS) is attributed to germline mutations in crucial biological processes, such as telomere biology, DNA repair, and ribosomal biogenesis. Early and precise genetic diagnosis is crucial for managing developmental abnormalities and the increased possibility of cancer. In acquired syndromes, external cytotoxic sources can disrupt the stem cell pool/HSPC compartment or attack them by an autoimmune mechanism. The immune pathophysiology of bone marrow failure (BMF) is caused by abnormal T or B cells and innate immune responses. The primary evidence of this concept is haematological improvement after anti-complement or standard immunosuppressive therapies. Pro-inflammatory

cytokines significantly impact immune responses and inflammation during marrow failure, making them a crucial component of immune derangement (Giudice & Selleri, 2022).

Aplastic anaemia (AA) is a disease of historical significance. In 1885, a young Paul Ehrlich identified the first patient with "anaemia aplastique". Later, in 1904, Vaquez identified its origins, and Cabot and other pathologists described clinical features in the early 20th century. It is a nonmalignant condition that affects the bone marrow, causing it to become injured, markedly hypocellular, and ineffective. Aplastic anaemia is a type of bone marrow failure and one of the main causes is an autoimmune attack by cytotoxic T cells against HSPCs. This is sustained by type-I interferons, leading to early T helper 1 (Th1) cell response and later T helper 17 (Th17) and effector memory $CD8^+$ T cell responses in severe cases. Cytokines and chemokines are essential players in immune responses and can inhibit the growth of HSPCs, causing apoptosis, with interferon-γ and tumour necrosis factor α being examples (Giudice & Selleri, 2022). Recent studies suggest that besides the immune component, intrinsic defects of HSPCs and dysfunction of the BM microenvironment and molecular pathogenesis may contribute to the pathophysiology of AA.

In the past, it was believed that aplastic anaemia occurred due to a lack of haematopoietic stem cells caused by harmful effects on the stem cells. Efforts to cure AA through the transfusion from a genetically identical twin's bone marrow have proven ineffective in restoring haematopoiesis for most patients. Many of these patients underwent a successful retransplant after a preparative regimen involving high-dose cyclophosphamide, indicating that the pathophysiology of AA is more complex (Bayever et al., 1984). During the late 1960s, Mathé et al. were among the pioneers to propose that aplastic anaemia had an immune foundation. The authors conducted a bone marrow transplant on patients with AA by using partially mismatched donors and dispensed anti-lymphocyte globulin as an immunosuppressive conditioning regimen beforehand (Mathé G,

1970). The investigators observed that while some patients did not successfully engraft, they still experienced autologous restoration of haematopoiesis. The findings indicate that functional HSCs are present in individuals with AA; however, the immune system appears to be inhibiting their growth and differentiation. The positive outcome of immunosuppressive therapy provided the initial conclusive proof that aplastic anaemia is indeed an autoimmune disease. Laboratory experiments further acknowledged the autoimmune activity that plays a role in developing aplastic anaemia. In particular, the first experiment conducted on T lymphocytes from patients was observed to hinder the development of haematopoietic colonies in vitro (Nissen et al., 1980).

When it comes to treating severe AA (SAA) in newly diagnosed patients, the first choice is allogeneic haematopoietic stem cell transplantation (HSCT) from a human leukocyte antigen (HLA)-matched sibling donor (MSD). However, the transplantation procedure has been limited by certain complications, such as graft rejection and graft-versus-host disease (GVHD). Additionally, the availability of suitable donors also poses a challenge. Stem-cell transplantation replaces hematopoietic and immune system cells. The conditioning process involves using cyclophosphamide. It may not be strong enough to destroy the bone marrow; it still suppresses the immune system to prevent and eliminate any remaining host marrow through a graft-versus-marrow effect. The results are usually excellent if the transplant is performed quickly after diagnosis in young patients using a histocompatible sibling donor. Young children have a more than 90% long-term survival rate, while adolescents have more than 80%. Additionally, the short- and long-term complications rate is relatively low (Dufour et al., 2015). Historically, graft rejection has been a common issue in HSCT for aplastic anaemia. However, it is now reduced in patients who receive early transplantation and minimal transfusions. Nowadays, conditioning regimens that do not involve irradiation can achieve engraftment and avoid numerous long-term complications

associated with irradiation, including late cancers. Cyclophosphamide and fludarabine, with or without ATG, have high success rates in engraftment and survival in unresponsive patients who received heavy transfusions with transplant procedures involving mobilised peripheral blood stem cells (PBSC) (Young, 2018).

For patients who do not have MSD, which is the case for 70% of AA patients, the recommended first-line treatment is immunosuppressive therapy (IST) (Young et al., 2006). Studies have shown that both methods yield comparable survival rates exceeding 80%. For over three decades, the combination of anti-thymocyte globulin (ATG) and cyclosporine A (CSA) has been widely regarded as the standard immunosuppressive therapy for patients with AA. This treatment has shown a response rate of 50-60% and an overall survival rate of 60% after one year. Additionally, children have demonstrated higher rates of recovery and survival than older patients Marsh et al., 2009). When used with cyclosporine, ATG can result in haematologic responses in approximately two-thirds of patients. Regardless of treatment, most of patients do not regain normal blood counts. About one-third of patients experience a relapse or need to use cyclosporine for an extended period to sustain their progress. If a relapse occurs, it typically responds well to additional immunosuppression (Bacigalupo, 2017).

Patients can receive immunosuppressive therapy instead of a transplant, which is less difficult and accessible to everyone. However, since it does not replace the damaged marrow or immune system, there is a risk of experiencing long-term effects from the disease. Sometimes patients may experience relapses, which can be improved but require long-term use of cyclosporine. Still, a more severe concern is the possibility of clonal evolution, where myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML) may develop even after stable blood counts. In most cases, clonal evolution appears as a cytogenetic abnormality in

chromosome 7 and is observed in approximately 15% of aplastic anaemia patients after the initial immunosuppression. Chromosome 7 aneuploidy is linked to a poor prognosis and necessitates a transplant (Young, 2018). Specific genetic mutations have been linked to varying clinical outcomes, including their effect on response to IST, the likelihood of developing MDS or AML, and overall survival. The clinical significance of a mutation depends on various factors, such as the specific gene and mutation involved, the size and dynamics of the mutated cells, the hierarchy of mutations, and how the variant allelic fraction changes over time. The mutations act as markers for clonally derived populations in HSC mosaicism. They help monitor the clonal dynamics of the stem cell compartment and predict the progression of MDS/AML. (Yoshizato et al., 2015)

Recently, defective MSCs residing in the bone marrow microenvironment as a possible contributor to the pathophysiology of AA have been put forward. Numerous studies have shown varying and conflicting outcomes regarding the effect of MSCs on the expansion, proliferation, self-renewal, and differentiation of HSCs in vitro in aplastic anaemia. The evidence so far needs to be more sufficient in providing a clear understanding and requires a well-defined study population with a better investigation of cellular and molecular aspects of MSC biology in AA patients (Atmar et al., 2022).

The existence of innate biological defects in HSCs can affect the development of AA adversely. In a significant subset of AA patients, defective homeostasis of telomeres has been recognised to be an emerging category of disease. Specifically, short telomeres were found in the leukocytes of acquired AA patients, and this was followed by the identification of mutation in telomerase genes in individuals with dyskeratosis congenita. Sometimes, sporadic AA may be linked to a very short telomere length or changes in the telomerase complex genes, which could indicate telomeropathy. In cases where the mutation has low penetrance, it may not necessarily

predict the disease's severity, phenotype, or response to IST. While there is increasing research on telomere length measurement and analysis of variants in telomere-related genes in AA patients, there is a paucity of data from India.

1.1. Rationale of the study

The regulation of MSCs, immune mechanisms, and telomeres maintain HSC regeneration and differentiation. Bone marrow failure results in a severe reduction of hematopoietic stem cells, leading to a lack of bone marrow function. The inherited form includes genetic variants in DNA damage, telomere biology or ribosomal pathway, whereas in the acquired form the causative factors are heterogeneous. Our study utilized BMF as a model to gain insights into the factors that contribute to the loss of haematopoietic stem cells. Mesenchymal stem cells play a major role in supporting haematopoiesis. Studies on the role of MSCs in aplastic anaemia patients are conflicting. Telomere length maintenance aids in preserving self-renewal HSCs. Research has shown that telomere length in AA patients is shorter than in healthy controls. The pathophysiology of AA is widely accepted to be immune-mediated. We analysed factors influencing immunosuppressive treatment outcomes and mechanisms leading to reduced HSCs. Literature exists on individual components contributing to HSC maintenance in BMF separately, but there are no comprehensive studies. Therefore, the present study focused on comprehensively examining the role of mesenchymal stromal cells and molecular and immune aspects in understanding the pathophysiology of aplastic anaemia patients in India.

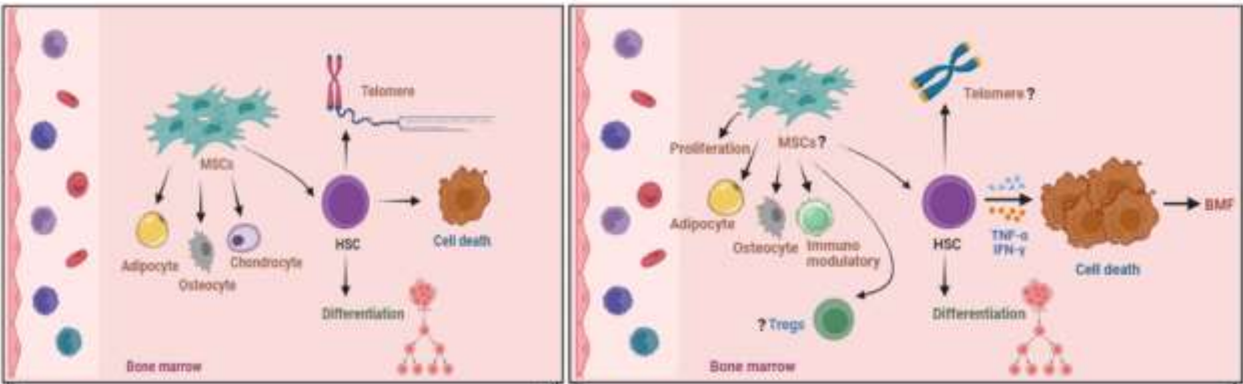


Figure 1.1.: Factors influencing HSC regeneration and differentiation leading to bone marrow failure

In normal homeostasis, MSCs in the BM microenvironment play a role in supporting HSC. Telomere maintenance is essential for protecting self-renewal capacity while the immune mechanism maintains a balanced HSC differentiation. Abnormality in the function of MSCs, telomere and immune aspect may lead to loss of HSC, causing BMF.

1.2. Aim of the study

The present study aims to investigate the comprehensive role of molecular and immune mechanisms and MSCs in supporting HSC regeneration.

1.3. Objectives of the study

1. To study the mechanism of mesenchymal stem cells, which provide a microenvironment for HSCs, in the condition of HSCs loss.
2. To investigate the role of telomere biology in the maintenance of HSCs self-renewal property.
3. To understand the immune mechanism in aplastic anaemia that leads to the loss of HSC regeneration.

1.4. A brief overview of chapters

1.4.1. Literature Review

Following the introduction, rationale and objectives of the study, the next significant chapter is the literature review, which briefly introduces haematopoiesis and the bone marrow environment. The role of mesenchymal stromal cells and bone marrow fat is described concisely. A detailed overview of telomeres, their structure and function, and telomere length measurement is explained. Further, the disease model of this study, the pathophysiology, diagnosis criteria, and treatment options are elaborated in this section.

1.4.2. Materials and Methods

A detailed description of study participants, recruitment, and inclusion criteria was provided in this section. The various techniques and methods used for conducting the experiments and analysing the data were outlined. Beginning with the haematological, biochemical, and molecular analyses, which include establishing telomere length measurement by qPCR method. Furthermore, target gene panel and whole exome sequencing using the next-generation sequencing were described. The cell-culture techniques, immunophenotyping by flow cytometry and finally, statistical analysis were summarised in this section.

1.4.3. Results

The study describes the properties of mesenchymal stromal cells derived from the bone marrow of the participants, including their morphology, immunophenotype, proliferative capacity, and differentiation potential. In addition, the immunosuppressive capabilities and telomere length assessment of MSCs have been reported. The telomere length measurement and its significance in the Indian population and bone marrow failure syndromes were discussed. The effect of telomere

length in aplastic anaemia patients undergoing matched sibling donor transplantation has been reported. NGS results have identified germline variants in AA patients. The immune and molecular aspects in patients with AA treated with immunosuppressive therapy are investigated.

1.4.4. Discussion

This section provides a detailed description of the significant results obtained in the study and their significance. The findings from this study have been analysed in conjunction with existing scientific data, and their clinical implications have been discussed.

1.4.5. Summary and Conclusion

This chapter has condensed and easily accessible information on the study's important discoveries and insightful observations. Additionally, suggestions for future work have been included. The bibliography consist of all the references cited in the text.

2.0. REVIEW OF LITERATURE

2.1. Haematopoiesis

The haematopoietic system is highly organised to preserve functional integrity and accommodate lifelong organismal demand. The haematopoietic system is highly plastic as an organ. It sustains diverse biological processes by generating myeloid and lymphoid cells, leading to the innate and adaptive immune systems to defend the organisms against a host of attacks. The erythrocytes transport oxygen all through the body to oxygenate all tissues and produce the platelets to cease bleeding and symphonise tissue repair. Altogether, these functions are essential for the organism's survival during its life span, and haematopoiesis has advanced substantial levels of regulation and protection to maintain its functional integrity and meet blood production demands (Wilson et al., 2008).

Haematopoiesis is orchestrated to regenerate blood cells continuously by maintaining homeostasis at a steady state and augmenting the output to sufficiently harmonise for acute blood loss in physical trauma, infection, and metabolic or toxic stress. This adaptive response requires the haematopoietic system to switch from its steady state of slow production to an activated state. Nevertheless, haematopoiesis involves adaptive regulation in progenitors, and this fundamentally starts at the level of haematopoietic stem cells (HSCs) (Foudi et al., 2009).

Blood is among the superlative regenerative tissues, where approximately one trillion (10^{12}) cells arise daily in adult human bone marrow (BM). Earlier anatomists, while examining the BM noted significant differences in cellular morphology in various blood lineages and differentiation stages. Russian biologist Maximow speculated that haematopoiesis is a cellular hierarchy procured from a common precursor, a haematopoietic stem cell (Maximow, 1909). The

noxious aftermath of radiation during the atomic era determined that the issue was caused by BM failure, which implies evidence for the existence of HSCs. The exposed recipients were said to be rescued following the injection of spleen or marrow cells from unirradiated donors (Lorenz, 1951). The study of haematopoiesis moved from observational to functional, and the regenerative potential of HSCs was evaluated using in vivo repopulation assays, thus establishing the multipotential nature of HSCs (Till, 1961; Becker et al., 1963). After this discovery, researchers were thrilled and developed clonal in vitro assays. These assays, combined with cell surface antibodies and flow sorting, have provided a detailed understanding of the blood system as a developmental hierarchy. At the top of this hierarchy are the multipotent HSCs, while the differentiated cells are located at the bottom. Most of the understanding of haematopoiesis comes from the mouse, as HSCs can only be identified and measured through functional repopulation assays, which creates a barrier to studying human HSCs (Doulatov et al., 2012). However, with the advent of xenotransplantation, potent in vitro clonal assays, and sorting strategies, considerable progress has been made toward defining the human blood hierarchy.

2.2. Developmental regulation of Haematopoiesis

In vertebrates, the production of stem cells is accomplished at definite embryonic cells, and the site changes during development (Galloway & Zon, 2003). In mammals, the location of haematopoiesis constitutes the yolk sac, an area encompassing the dorsal aorta described as the aorta-gonad mesonephros (AGM) region, the fetal liver, and lastly, the bone marrow (**Figure 2.2**). The elementary production of blood in the mammalian yolk sac is referred to as primitive. The primary function of primitive haematopoiesis is the production of red blood cells that assist tissue oxygenation as the embryo encounters active growth. The primitive haematopoietic system is fugitive and swiftly replaced by adult haematopoiesis, termed “definitive.” Of late, a

supplementary site, the placenta, has been perceived to engage from the AGM to the fetal liver period. Therefore, there is a difference in the properties of HSCs at each site, indicating distinct niches that contribute towards HSC expansion and differentiation and its intrinsic characteristics at each stage. HSCs in the fetal liver are active, while adult bone marrow HSCs are usually quiescent (Orkin & Zon, 2008).

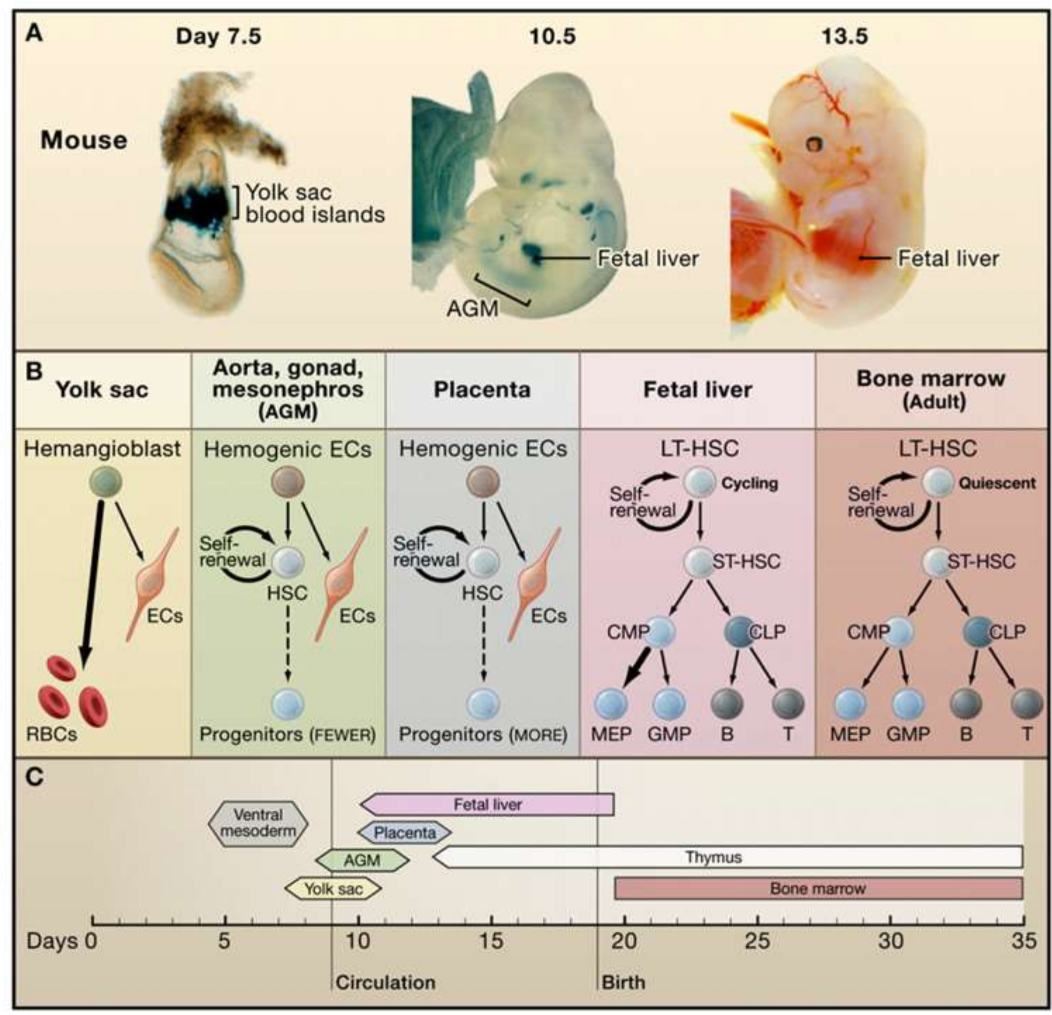


Figure 2.2. Developmental Regulation of Haematopoiesis in the Mouse
(Orkin & Zon, 2008)

(A) Haematopoiesis begins in the blood islands of the yolk sac and progresses to the aorta-gonad mesonephros (AGM) region, placenta, and fetal liver. The visualisation of yolk sac blood islands is achieved through LacZ staining of transgenic embryos expressing GATA-1-driven LacZ. In

Runx1-LacZ knockin mice, AGM and FL are also stained by LacZ. (B) Each location has a preference for producing specific blood lineages through haematopoiesis. (C) Haematopoiesis locations can shift during specific stages of development.

The subsequent site of haematopoietic potential after an embryo is the AGM region. About a century ago, haematopoietic cells were primarily identified in the aorta of the developing pig. Morphological examination of chick-quail chimaera disclosed that a sheet of lateral mesoderm migrates, caresses the endoderm, and then appears as a single aorta tube. Consequently, clusters of haematopoietic cells emerge in the ventral wall. In mice, adult HSCs capable of long-term reconstitution of irradiated hosts are found in the AGM region (Müller et al., 1994). HSC activity is visible on embryonic day 10.5, while by day 11, engrafting activity is present. In the embryo of the mouse, haematopoietic activity was noticed alternatively, where haematopoietic and endothelial cells are colocalized (Inman & Downs, 2007). Placental HSCs might arise by either de novo generation or colonisation upon circulation (Ottersbach & Dzierzak, 2005). The specific contribution of each of the above sites to the final pool of adult HSCs is largely unknown. Sequential definitive haematopoiesis involves colonising the fetal liver, thymus, spleen, and bone marrow.

2.2.1. The haematopoietic development in humans

In humans, haematopoiesis originates in the yolk sac and progresses into the liver momentarily, eventually establishing definitive haematopoiesis in the bone marrow and thymus. The presence of HSCs closer to endothelial cells is confirmed by the experiments performed with human embryos that support observations in the haemangioblast (a common precursor for endothelial and haematopoietic cells). The first human HSCs originated in the AGM has been

acknowledged by the transplantation of HSCs from human embryos into immune-deficient mice (Tavian et al., 2010; Ivanovs et al., 2011).

In the mouse, developmental stages can be illustrated by morphological changes occurring in less than a day. However, human embryo development takes a significantly longer time. Human development is categorized into stages, known as Carnegie stages (CS), which are determined by external morphological characteristics and cover several days each (O'Rahilly and Muller, 1987). Staging the human HSCs development according to the Carnegie classification is more precise than gestational age (Ivanovs et al., 2013). Regardless of the differences in developmental timescales, subsequent parallels between mouse and human haematopoietic development have been noted (**Figure 2.2.1**).

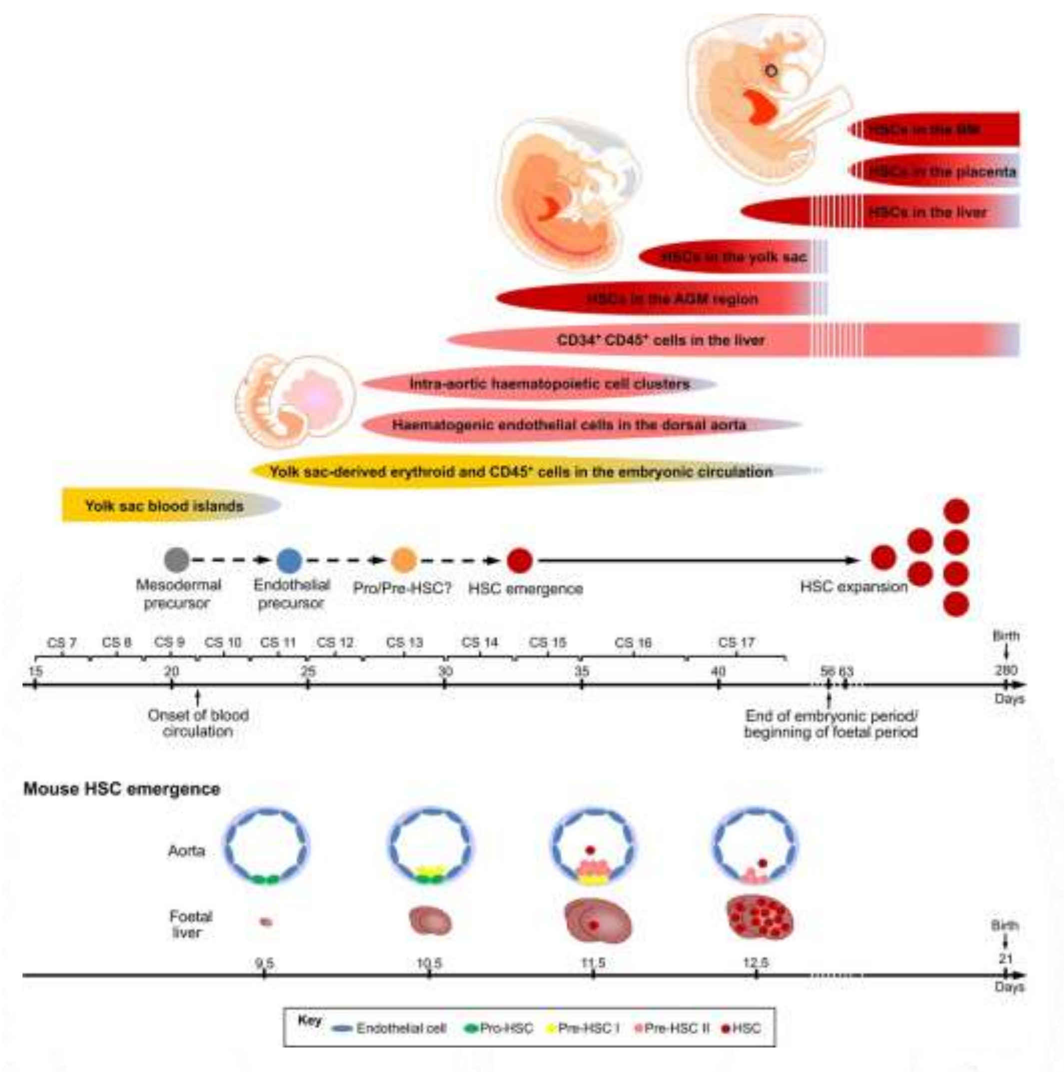


Figure 2.2.1.: Chronology of human haematopoietic development

(Ivanovs et al., 2017)

During CS 7 and 8 (equivalent to 16-18.5 days), the initial haematopoietic cells, including primitive erythroid cells and monocytes/macrophages, are generated in the yolk sac by the human body. At CS 10 (day 21), when cardiac contractions and blood circulation begin, haematopoietic cells derived from the yolk sac are spread throughout the developing embryo. Clusters of intra-aortic haematopoietic cells expressing $CD34^{+}CD45^{+}$ markers appear in the vitelline artery and dorsal aorta at CS 13 (day 27) but disappear by CS 16 (day 35-38). Stem cells (SCs) in the AGM region remain present from CS 14 (day 30) until CS 17 (day 42), which is the latest stage tested. The activity of haematopoietic stem cells (HSCs) in the AGM region occurs before it occurs in the yolk sac (CS 16; 35-38 days), liver (CS 17; 39-42 days), and placenta (around 63 days). The haematopoietic lineage originates from a mesodermal precursor (grey) and advances through the haematogenic endothelium intermediate phase before expanding. At the bottom of the figure is the gradual development of mouse HSCs from immature haematopoietic precursors, including pro-HSC, pre-HSC I, and pre-HSC II, for comparison purposes. It is unclear if human haematopoietic

development has similar precursors to those found in other species due to the unknown status of human pro/pre-HSC. Red, bona fide HSCs; light red, haematopoietic lineages which may or may not be related to HSC development; yellow, yolk sac haematopoietic differentiation. The fading of coloured bubbles to blue represents the extinction of the process. White striped lines represent a change in time scale (omission of several days for the mouse or weeks for the human).

The initial wave of human haematopoiesis begins in the yolk sac, a structure that develops differently in humans and mice. The yolk sac in mice surrounds the embryo's body, whereas it develops in front of the embryo in a balloon-like form in humans. The significant haematopoietic output is represented mainly by primitive nucleated erythrocytes from the yolk sac at CS 7-8 (16-18.5 days post-conception, dpc), with the occasional presence of primitive macrophages and megakaryocytes (Fukuda, 1974; Luckett, 1978). The first primitive erythroblasts arise inside the cardiac cavity by CS 10 (21-22 dpc), marking the onset of blood circulation, followed by the appearance of the first $CD45^+$ ($PTPRC^+$) cells, as in the mouse (Tavian et al., 1999; Ghiaur et al., 2008). The intra-aortic haematopoietic clusters (IAHCs) emerging at CS 13 (27 dpc) are revealed by spatiotemporal analysis at the floor of the embryonic dorsal aorta and disappear by CS 17 (39-42 dpc). The endothelial lining of the human dorsal aorta upregulates CD34, which later marks adult HSCs from CS 9 (19 dpc) (Tavian et al., 2001; Oberlin et al., 2002). During embryonic development, $CD34^+CD45^+$ cells first appear in the pre-umbilical region of the dorsal aorta by CS 13 (27 dpc). Their numbers continue to increase, reaching several hundred by CS 15 (33 dpc).

The liver rudiment develops as a diverticulum from the embryonic gut floor at early CS 10 (21 dpc). The liver rudiment encompasses primitive yolk sac-derived erythrocytes and $CD45^+$ cells from late CS 10 (22 dpc), plausibly of monocytic/macrophage lineage. From CS 13 (27-29 dpc), the liver is seeded by an increase in numbers of $CD34^+CD45^+$ cells (Tavian et al., 1999), which probably represent yolk sac-derived cells like mouse erythro-myeloid progenitors (McGrath et al.,

2015). The liver is an essential niche for HSC expansion and differentiation until birth. The human placenta contains high numbers of $CD34^{+}$ cells, specifically immature $CD34^{+}CD45^{lo}$ cells that lack CD38 starting from weeks 5-6 of development. They comprise high colony-forming units-culture (CFU-C) numbers and $CD34^{+}CD45^{lo}$ cells committed to erythroid and myeloid differentiation containing fewer CFU-Cs (Barcena et al., 2009). At this juncture, the placenta is also a spot for substantial erythroid maturation, and primitive erythrocytes are enhanced at placental villi that express embryonic ζ-globin in association with macrophages, which facilitate their enucleation (Van Handel et al., 2010). Despite $CD34^{+}$ cells appearing in the human placenta as early as week 5 of gestation, true HSCs can be detected only after week 9 of pregnancy or later, which was established by xenotransplantation into immunocompromised mice (Robin et al., 2009; Muench et al., 2017)

The advancement of the definitive bone marrow niche is firmly associated with the influx of cartilaginous bone by blood vessels and bone ossification. This vascular invasion accelerates the seeding of bone marrow with haematopoietic progenitors and HSCs. The formation of bone marrow formation indiscriminately terminates the human embryonic period (CS 23; 56 dpc). The initial $CD34^{-}CD45^{+}$ haematopoietic cells occupy the cartilaginous bone, including mostly $CD68^{+}$ monocytes/macrophages possibly participating in chondrolysis, thereby following the colonisation by $CD34^{+}CD45^{+}$ progenitors and HSCs (O'Rahilly and Muller, 1987; Charbord et al., 1996). The onset of HSC activity in the human bone marrow remains unknown.

2.3. Haematopoietic stem cells

Haematopoietic stem cells are responsible for the production and regeneration of all blood cell types during the entire life of an organism. HSCs are a fundamental part of the entire adult blood system. Haematopoietic stem cells have two definitive characteristics: they can self-renew

by divide and generate new HSC daughter cells to preserve lifelong haematopoiesis. HSCs are also multipotent, as they have the capability to differentiate into adult haematopoietic cell lineages (Eaves, 2015). The ability of HSC progenitors to differentiate was discovered through years of experimentation by performing *in vitro* colony assays and transplantation of isolated cells into myeloablated mice. The progeny of HSCs undergoes lineage commitment to produce mature blood cells by differentiation, which is consecutively lost till they become restricted to forming one type of blood cell. The molecular mechanisms, cellular relationships, and estimate of lineage commitment are essential for regulating blood production in physiological homeostasis and disease (Seita & Weissman, 2010).

2.3.1. Identification of self-renewing HSCs

Describing and isolating the functional HSCs is crucial but progressive improvements over decades make it possible for mouse and human HSCs to be purified at high accuracy. During embryonic development, HSCs have been isolated from several anatomical regions, and functional HSCs were isolated from several adult tissues. Most studies were engaged in purifying and characterising HSCs within the adult mouse bone marrow microenvironment (Pinho & Frenette, 2019). Human research has centred on bone marrow HSCs, whereas HSCs derived from umbilical cord blood have also been studied. HSCs are mostly isolated with multicolour fluorescence-activated cell sorting (FACS). Since the mid-1980s, it is used to enrich HSCs on the cell-surface expression of specific proteins identified using antibody labelling and fluorescent reporter molecules (Spangrude et al., 1988). Diverse mouse HSC markers have been recognised, and most of the FACS-based HSC purification strategies use the $CD150^{+}$ $CD48^{-}$ $CD34^{lo/neg}$ $CD117^{+}$ $SCA1^{+}$ lineage marker-negative (LIN^{-}) bone marrow population to isolate adult mouse HSCs with long-term self-renewal capacity (Kiel et al., 2005; Yamamoto et al., 2013). Distinctly, human HSCs are

defined as $CD49f^{+} CD90^{+} CD45RA^{-} CD34^{+} CD38^{-} Lin^{-}$ cells. Nevertheless, no immunophenotype can identify functional HSCs with 100% purity in either mice or humans (Notta et al., 2011; Wilson et al., 2015). The advent of single-cell analyses of mouse and human HSCs (in vivo and ex vivo) has emphasised heterogeneity within these phenotypic populations (Notta et al., 2016; Wilkinson et al., 2019; Yamamoto et al., 2013). Additionally, the cell surface markers used to isolate HSCs are often no longer precise following the ex vivo culture of HSCs. For instance, in ex vivo culture CD49f and CD38 are not reliable markers for human HSCs, and EPCR and ITGAM3 have been found to be more reliable markers than CD49f and CD38 (Fares et al., 2017; Tomellini et al., 2019).

During cell division, HSC self-renewal occurs at the cellular level. Conceptually, HSCs undergoes three types of cell division event. One, symmetric self-renewal develops into two HSCs; two, symmetric differentiation generates two haematopoietic progenitor cells (HPCs); and three, asymmetric self-renewal produces one HSC and one HPC. The absence of defined prospective markers for functional HSCs and self-renewal cell divisions is challenging to track directly in real time. The self-renewal of multipotent HSCs is determined retrospectively and indirectly by functional assays (Wilkinson et al., 2020).

2.3.2. Identification of multipotent HSCs

HSCs must balance two opposing cell-fate decisions, self-renewal, and differentiation, to maintain the stem cell compartment and produce all the needed downstream progenitors and mature blood cells. Under normal circumstances, HSCs typically exhibit a low cycle rate and remain in a state of dormancy known as quiescence (Wilson et al., 2008; Foudi et al., 2009). HSC quiescence suffix as a precautionary mechanism by restricting replicative stress, which slows the functional HSCs with age (Flach et al., 2014). HSCs become activated rapidly upon receiving the

response to inflammatory signals that coordinate regeneration. This leads to the proliferation and expansion of the required progenitor compartments to replenish the blood system and induce the necessary effector cells. The hyperactivation of regenerative signalling prioritises differentiation over self-renewal, which leads to the loss of the immature stem and progenitor cell compartment (Sato et al., 2009).

The differentiation roadmap of HSCs was investigated half a century ago. The classical model of haematopoiesis is HSCs residing at the top of a hierarchy; where HSCs possess the self-renewal capacity and can sequentially produce all blood lineage cells. Nevertheless, over the past few years, the developmental scheme of HSCs is challenged by the advent of single-cell technologies. The hierarchy model of HSCs illustrates the relationship between an HSC and its progenies and their stepwise differentiation process from the immunophenotype data (Akashi et al., 2000; Morrison et al., 1997; Manz et al., 2002).

2.3.2.1. The classical haematopoietic hierarchical model

According to the classical model, HSCs are classified into two subpopulations based on their CD34 expression $CD34^{-}$ long-term (LT-HSCs) and $CD34^{+}$ short-term (ST-HSCs). The LT-HSCs are a scarce, quiescent population in BM, having a reconstitution capacity of (> 3~4 months), while ST-HSCs have mostly < 1 month reconstitution ability. The differentiation begins with LT-HSCs into ST-HSCs, and subsequently to multipotent progenitors (MPPs), which do not have the visible self-renewal ability (Yang et al., 2005). The initial branching arises from MPPs to the common myeloid progenitors (CMPs, into myeloid, erythroid, and megakaryocytic lineage) and common lymphoid progenitors (CLPs, with only lymphoid potential). Further, CMPs bifurcate into bipotent granulocyte-macrophage (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs), and CLPs advance into T, B, NK, and dendritic cells, with GMPs differentiating into

granulocytes/monocytes whereas MEPs generate megakaryocytes/erythrocytes. Finally, a balanced hierarchy model of stepwise differentiation of HSCs to mature blood cells is formed, which includes crucial transcription factors (TFs) and cytokines (Zhu & Emerson, 2002; Robb, 2007; Zhang & Lodish, 2008; Seita & Weissman, 2010) (**Figure 2.3.2.1**)

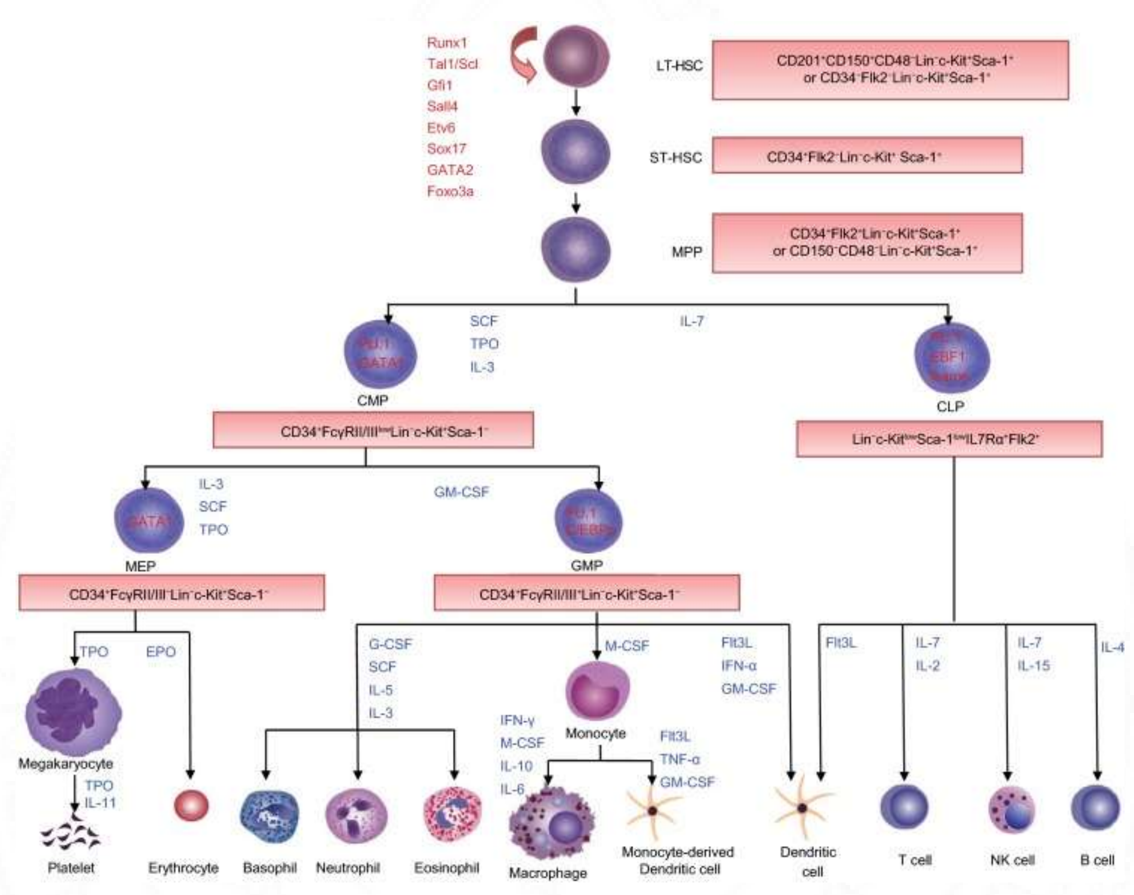


Figure 2.3.2.1.: The classical haematopoietic hierarchy model

(Cheng et al., 2020)

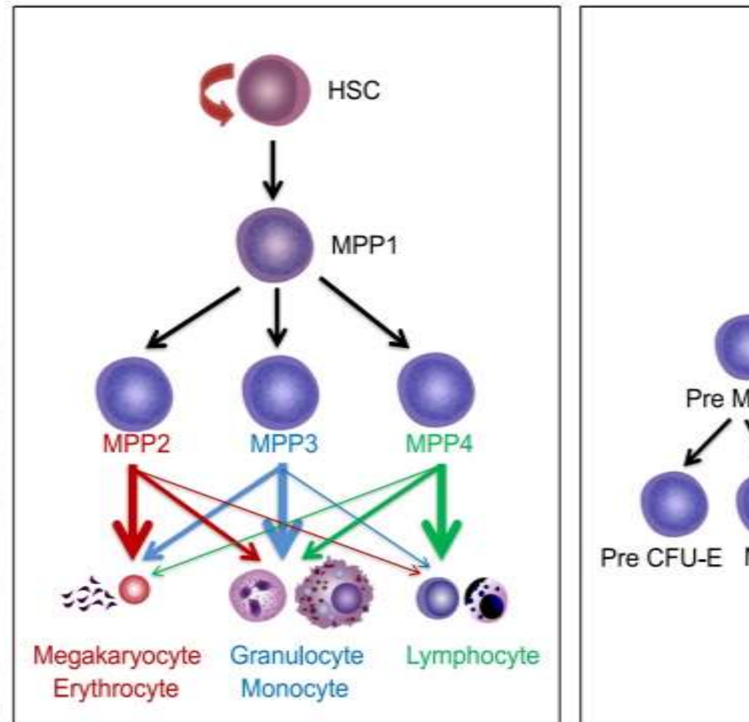
According to the classical model, LT-HSCs are at the highest point in the hierarchy. These LT-HSCs then progress into ST-HSCs, which eventually develop into MPPs but with a diminished capacity for self-renewal. When it comes to lineage commitment, there is a clear separation between the myeloid and lymphoid branches downstream of MPPs. The initial step is crucial, and CMPs can generate both MEPs and GMP. Lymphocytes and dendritic cells arise from CLPs, while megakaryocytes/platelets and erythrocytes are the result of MEP differentiation. GMPs, on the other hand, are responsible for producing granulocytes, macrophages, and dendritic cells. Haematopoietic differentiation is regulated by both external cytokines and internal transcription factors.

2.3.2.2. Advances in the haematopoietic hierarchy

Considering the reconstitution period, intermediate-term HSCs (IT-HSCs) in between LT-HSC and ST-HSC confining reconstitution up to 8 months after transplantation have been designated by specific labs (Benveniste et al., 2010; Yamamoto et al., 2013). The high-throughput sequencing tracked single HSCs and their heterogeneity. This assay demonstrated that HSCs do not contribute evenly to progenies, and two distinct HSC differentiation styles were found to co-exist in the same recipient mouse after irradiation. One of the patterns involves progenitor cell populations GMPs, MEPs, and CLPs, and the other group contains mature lymphoid blood cells (R. Lu et al., 2011). The self-renewing lineage-restricted progenitors prevail in phenotypically defined HSC, consisting of megakaryocyte-erythrocyte repopulating progenitors (MERPs), megakaryocyte repopulating progenitors (MkRPs), and common myeloid repopulating progenitors (CMRPs) observed by single-cell transplantation (Yamamoto et al., 2013). Thereby suggesting that oligo-, bi-, and unipotent cells co-exist in HSC populations. Moreover, SLAM family markers CD150 and CD229 can divide HSCs into distinct fractions based on differentiation reconstitution ability. $CD150^{hi}$ HSC showed more significant self-renewal potential with myeloid-biased differentiation than $CD150^{med}$ HSC (Morita et al., 2010) (**Figure 2.3.2.2**). Modern techniques and recent studies have exposed that the haematopoietic hierarchy is more intricate than prior known knowledge.

The MPP population was classified into four subtypes - MPP1, MPP2, MPP3 and MPP4 by the Passegue group (Pietras et al., 2015). This classification was based on their immuno-phenotype, cell cycle status, lineage bias, resistance to drug treatment and bone marrow abundance. MPP1 resembles IT-HSC or ST-HSC and can reconstitute multiple lineages for up to 4 months. MPP2/3/4 lacks self-renewal potential and only reconstitutes short-term myeloid cells

for less than a month. MPP2 and MPP3 produce low levels of T and B cells, while MPP4 generates low levels of myeloid cells in vivo. Compared to MPP3 and MPP4, MPP2 produces a higher level of platelets (Pietras et al., 2015). In the LKS- population, cells expressing CD41 and CD150 are classified as megakaryocyte progenitors (MkPs) and are responsible for megakaryocyte production. $CD41^{-}CD150^{-}$ $CD16/32^{+}$ cells are GMPs. In the $CD41^{-}CD150^{-}CD16/32^{-}$ population, there are four sub-populations: pre-MegEs, pre-GMs, Pre CFU-Es and CFU-Es. Single Pre-MegE cells can effectively produce megakaryocytic, erythroid, and mixed megakaryocyte/erythroid colonies. Pre-GM cells have a clonal lineage output similar to GMPs (Pronk et al., 2007).



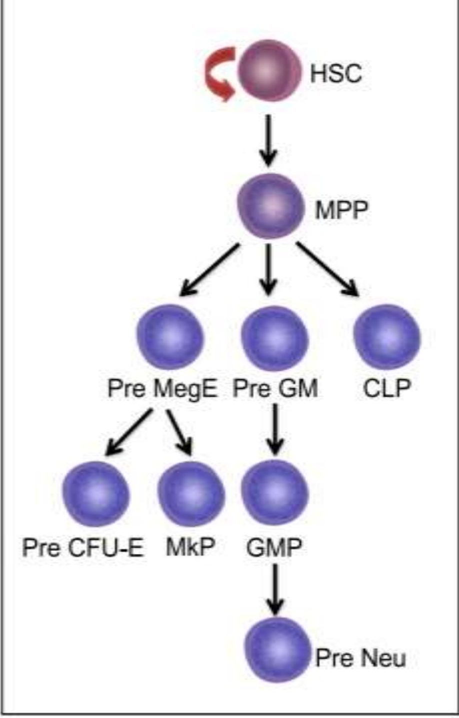


Figure 2.3.2.2.: Revised models of haematopoietic stem cell differentiation (Cheng et al., 2020)

(A) There are different subtypes of MPPs, namely MPP1-4. MPP1 has the ability to develop into all lineages. MPP2/3 tend to have a bias towards myeloid cells, while MPP4 leans towards lymphoid cells. Notably, MPP2 is known to have a preference for platelet production. (B) In this particular model, MPPs undergo differentiation into pre-MegE, Pre-GM, and CLP. Pre-MegE is located upstream of MkP and pre CFU-E. Pre-GM, on the other hand, gives rise to GMP and eventually produces newly identified neutrophil precursors (Pre Neu).

2.4. Bone marrow environment

2.4.1. Bone marrow Niche

The lifelong functional integrity of HSCs is preserved through strict control by both cell-intrinsic and extrinsic cues in the BM microenvironment, commonly referred to as the BM niche. HSC quiescence regulation is supervised by a hypoxic BM microenvironment, in which self-renewal promoting cytokines quiescence-enforcing are supported by endothelial and stromal niche cells (Schepers et al., 2015; Crane et al., 2017; Wei & Frenette, 2018). The most significant factors of HSC retention in the niche are the c-Kit receptor ligand stem cell factor (SCF) and c-x-c motif chemokine ligand 12 (CXCL12) (Ding et al., 2012). The colonisation of the BM niche during the development requires stromal-derived CXCL12. Its expression increases during DNA-damaging injury from chemotherapy or radiation to stimulate the transfer and repopulation of the BM niche by HSCs (Ara et al., 2003; Ponomaryov et al., 2000). The endothelial cells are sensitive and trigger HSCs to initiate emergency haematopoiesis and promote activation by secreting pro-inflammatory cytokines (Boettcher et al., 2014). Therefore, the HSC maintenance and function are supported by the niche cells and maintain HSC quiescence and self-renewal by synchronising regeneration in response to physiologic insult.

The components of the BM niche are distinctly heterogeneous. For several decades there has been tremendous interest in identifying definitive HSC niche components within the BM microenvironment, which includes soluble factors, cellular contributions, oxygen tension, extracellular matrix and physical factors. Regarding the physical landmarks within BM, besides preferential localisation of HSCs, BM niches are classified into two distinct niches: endosteal niche and perivascular niche (**Figure 2.4.1**).

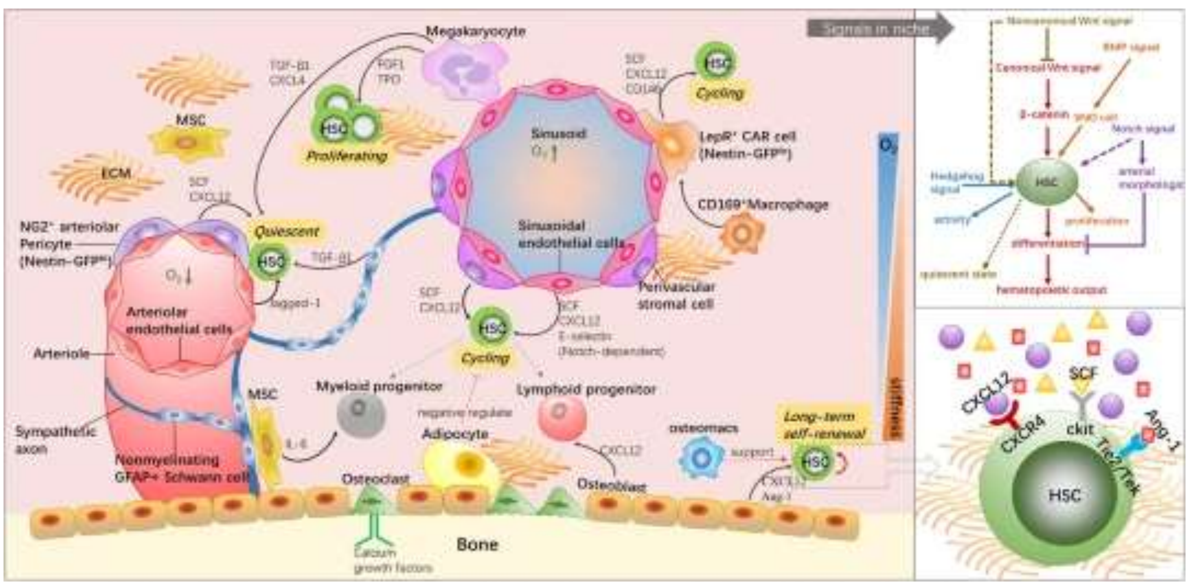


Figure 2.4.1.: The model of endosteal and perivascular niches in the bone marrow (Zhang et al., 2019)

HSC activities can be regulated directly or indirectly by various factors, including niche cells, cytokines, signals, ECM, and oxygen concentration gradient. The behaviour of HSCs varies across different sub-niches. The maintenance of LT-HSCs is aided by the endosteal niches, whereas the vascular niches activate the cell cycle and trigger cell proliferation and differentiation. Within the arterioles sub-niche, neural–glial antigen 2 ($NG2^+$) arteriolar pericytes play a crucial role in maintaining HSCs in a quiescent state near arterioles. The primary source of essential SCF and CXCL12 for HSC maintenance or mobilization comes from LepR-expressing perisinusoidal cells.

2.4.1.1. Endosteal niche

The intersection between the bone and BM, known as endosteum, is enclosed by layers of osteoprogenitors, bone-forming osteoblasts and bone-resorbing osteoclasts (Travlos, 2006). The journey of an endosteal niche was determined by earlier studies that preceded Schofield's niche concept decades ago (Gong, 1978) and further validated the activation of osteogenesis enriched HSCs in trabecular-rich bone areas (Calvi et al., 2003; J. Zhang et al., 2003). Consequently, immunofluorescence imaging reported endogenous HSCs located in direct contact with a few subsets of osteoblast-like cells, positive for Jagged-1, N-cadherin, and osteopontin (Calvi et al., 2003; Xie et al., 2008; Zhao et al., 2019). Contrarily, osteoclasts are redundant for HSC

mobilisation and act as negative regulators in the endosteal niche (Miyamoto et al., 2011). Therefore, HSCs populate in the endosteal region enhanced with osteoblasts and skeletal cells. Transplant models affirmed the actuality of the endosteal niche, which was used to trace HSCs homing and transfer to the micro-anatomy of the BM (Nilsson et al., 2001). The advent of imaging technology combined with transplant models, showed transplanted exogenous HSCs to be home to the endosteal bone surfaces in recipients (Lo Celso et al., 2008; Xie et al., 2008) and similar results were observed when human HSCs were transplanted into mice (Guezguez et al., 2013). The HSCs move towards the endosteal niches, which were mediated by local functional factors, such as angiopoietin-1 secreted from osteoblasts that bind to HSC receptor Tie2 for HSC retention to the niche (Arai et al., 2004)

2.4.1.2. Perivascular niche

The intricate vascular network inside the BM, which includes several perivascular stromal cells supporting haematopoiesis, leads to the postulation that HSCs reside near the vascular region. The SLAM family of receptors was found to purify HSCs and was present adjacent to the sinusoid endothelium than to the bone surface (Kiel et al., 2005b). A comprehensive quantitative analysis of HSC organisation within BM was performed using an imaging cytometry platform that observed most HSCs were exclusively beside sinusoids, which supports the concept of a perivascular niche (Nombela-Arrieta et al., 2013). Distinct sets of markers, such as Homeobox protein Hox-B5 (Hoxb5), α-catulin, and myelodysplasia syndrome 1 (MDS1), have been identified to indicate a high-level purity of HSCs. These markers prove that HSCs are located perivascularly in adult BM, mainly near sinusoidal blood vessels (Acar et al., 2015; Chen et al., 2016; Christodoulou et al., 2020). Alongside sinusoids, HSCs are adjacent to BM arterioles, and this was recognised by 3D BM confocal imaging of HSCs (Kunisaki et al., 2013). Similarly, the localisation of α-

catulin^{+}KIT^{+} HSCs showed that approximately 10% of HSCs were associated with arteriole blood vessels, whereas 80% of HSCs were close to sinusoids (Acar et al., 2015). These findings about HSCs localisation commend that most HSCs inhabit near the sinusoids, while a minor frequency of HSCs lodge near the arterioles in the BM.

2.4.2. Constituents of the HSC niche

2.4.2.1. Growth factors

Growth factors (GFs) are widespread groups of small, secreted proteins that manoeuvre on specific target cell receptors and activate intracellular signalling cascades that basically control cellular behaviours (Bazan, 1990). Several niche-derived GFs in the BM niches determine HSC behaviour, including retention, maintenance, proliferation, and mobilisation (Pinho & Frenette, 2019). **Table 1** provides an overview of the primary niche GFs, their cellular origins, and their functions within the niche. Generally, the growth factors arbitrate response through the binding on specific receptors of HSCs. For instance, CXCL12 can bind to CXCR4-expressing HSCs, and SCF determines its effects through binding and stimulating KIT receptors on HSCs (Ikuta & Weissman, 1992; Zou et al., 1998; Lai et al., 2014). Investigations on GF-receptor binding via antibody blocking or deleting expression using genetic approaches have demonstrated that the BM niche gets significantly modified and impairs normal haematopoiesis (Ding & Morrison, 2013; Tzeng et al., 2011). Various GFs regulate different HSC behaviours; for example, SCF mainly promotes HSC maintenance, whereas Notch ligands induce HSC proliferation. Vascular cell adhesion protein 1 (VCAM1) and CXCL12 modulate HSC retention and mobilisation (Asada et al., 2017; Butler et al., 2010). This data indicates that the balance of HSC activities is firmly managed by the contributions of growth factors. In addition, niche GF homeostasis and biological functions modified by stress, such as ageing, irradiation and chemotherapy. During homeostasis, specific

GFs are replaced to enhance the regeneration of blood cells after an injury. These involve transforming growth factor (TGF) β1 and fibroblast growth factor 1 (FGF1), which elevate HSC expansion and restore HSC quiescence after chemotherapy treatment (Hérault et al., 2017; Zhao et al., 2014). Therefore, an elaborate growth factor profile of the BM niche, how these GFs alter in response to varying stresses, and how they control haematopoietic recovery post-irradiation remains to be explored.

2.4.2.2. Cellular components

The hassle of reconstituting the BM niche *in vitro* is associated with its significant cellular diversity. Genetic approaches helped in understanding the cellular architecture of the BM niche, including Cre-mediated lineage tracing, the deletion of molecular markers and imaging or functional readouts. Marker-based approaches are inadequate to classify the heterogeneous populations (Al-Sabah et al., 2020). Lately, single-cell RNA-sequencing (scRNA seq) subjugates such shortcomings providing more detailed and systematic information in BM cellular architecture. Generally, stromal cellular components of the BM niche are unique and highly heterogeneous, mainly containing endothelial cells (ECs), fibroblasts, pericytes, osteoblasts, mesenchymal stromal cells, nerve cells and smooth muscle cells (Baccin et al., 2019; Baryawno et al., 2019; Tikhonova et al., 2019). Various MSC subpopulations have been discovered, including leptin receptor $(LepR)^{+}$cells, $nestin^{+}$ cells, CXCL12-abundant reticular cells (CAR cells), and neural–glial antigen 2 $(NG2)^{+}$ cells (Ding et al., 2012; Kunisaki et al., 2013; Méndez-Ferrer et al., 2010; Sugiyama et al., 2006). The significant growth factors and their impact on HSCs in regulating the BM niche are mentioned in **table 2.4.2.2.** The activities of HSCs in the BM niche are regulated by their progenies. For instance, conditional deletion of megakaryocytes subsequently induces HSCs activation and proliferation, denoting that megakaryocytes may be

significantly associated with the maintenance of HSCs quiescence. This is regulated by the secreted chemokines of the differentiated cells, including CXCL4, TGFβ1 and FGF1 (Bruns et al., 2014; Jiang et al., 2018). Megakaryocytes have a significant impact on myeloid-biased von Willebrand factor (vWf)$^{+}$ HSCs, while lymphoid-biased vWf^{-} HSCs remain unaffected by their influence. The expansion of vWf+ HSCs is contributed by the conditional deletion of megakaryocytes and increases their self-renewal capacity along with reconstitution potential, thereby having no effect on vWf^{-} HSCs (Pinho et al., 2018). These investigations suggest that prominent niches for HSC subpopulations and their progeny may prevail, which have definite developmental potential. Overall, the regulation of HSCs by their progeny occurs in a feedback loop.

Table 2.4.2.2: Locally secreted factors associated with HSC regulation in the BM niche
(Xiao et al., 2022)

Growth Factor	HSC Receptor	Niche cellular Source	Impact on HSCs
SCF	c-Kit	ECs	Induce HSC maintenance and promote HSC recovery after myeloablation
		CAR cells	Direct HSC engraftment
		Nestin^{+} cells	Induce HSC maintenance
		LepR^{+} cells	Promote HSC maintenance and enhance HSC regeneration after irradiated.
		NG2^{+} cells	Promote HSC maintenance
CXCL12	CXCR4	CAR cells	Promote HSC maintenance
		Nestin^{+} cells	Induce HSC maintenance and retention
		LepR^{+} cells	Promote HSC retention
		ECs	Maintain HSC quiescence, self-renew and retention
		NG2^{+} cells	Maintain HSCs quiescence and retention

Growth Factor	HSC Receptor	Niche cellular Source	Impact on HSCs
TPO	MPL	OBs	Maintain HSC quiescence
OPN	CD44	OBs	Restrict HSC pool
Angiopoietin-1	Tie-2	OBs	Maintain HSC quiescence and self-renewal, and enhance survival under stressed
		$LepR^+$ cells	Promote HSC recovery after irradiation
Vcam1	VLA-4/Integrinα4β1	ECs	Direct HSC homing
		Macrophage	Promote HSC retention
G-CSF	G-CSF receptor	OBs	Maintain HSC quiescence
		Macrophages	Maintain HSC retention
TGF-β1	TGF-β receptors	Schwann cells	Maintain HSC quiescence and self-renewal
		Megakaryocytes	Maintain HSC quiescence and enhance HSC expansion under stress
Notch ligand Jagged-1	Notch receptor	OBs	Support HSC self-renewal
		ECs	Support HSC self-renewal and proliferation
WNT ligands	Fizzled receptors	OBs	Maintain HSC quiescence and enhance HSC recovery under stress
Pleiotrophin	RPTP-β/ζ	ECs	Enhances self-renewal and BM retention, and accelerates haematopoietic recovery following myelosuppression
Netrin-1	Neogenin-1	ECs	Maintain HSC quiescence and self-renewal
DARC/TGFβ	CD82	Macrophage	Promote HSC quiescence
FGF1	FGF receptor	Megakaryocytes	Promote HSC proliferation and enhance HSC recovery under stressed
FGF2		Unknown	Promote HSC recovery after stress

2.5. Bone marrow - Mesenchymal stromal cells

In bone marrow, mesenchymal stromal cells (MSCs) play a vital role in furnishing physical support and secreting soluble factors to maintain and control haematopoietic stem progenitor cells.

Mesenchymal stem cells, also referred to as non-haematopoietic stem cells, were first identified in bone marrow (Friedenstein et al., 1976). They are spindle-shaped, adherent, and possess the capability to differentiate into fully mature cells of various mesenchymal tissues, including fat and bone (**Figure 2.5**). After years of research, there is now a comprehensive understanding of these cells. MSCs can be collected from various tissues, including bone marrow, umbilical cord, placenta, fat, lung, liver, and skin. MSCs obtained from adult bone marrow are most diligently studied and represent 0.001–0.01% of the cell numbers in BM and further drops in the umbilical cord and peripheral blood (Wexler et al., 2003).

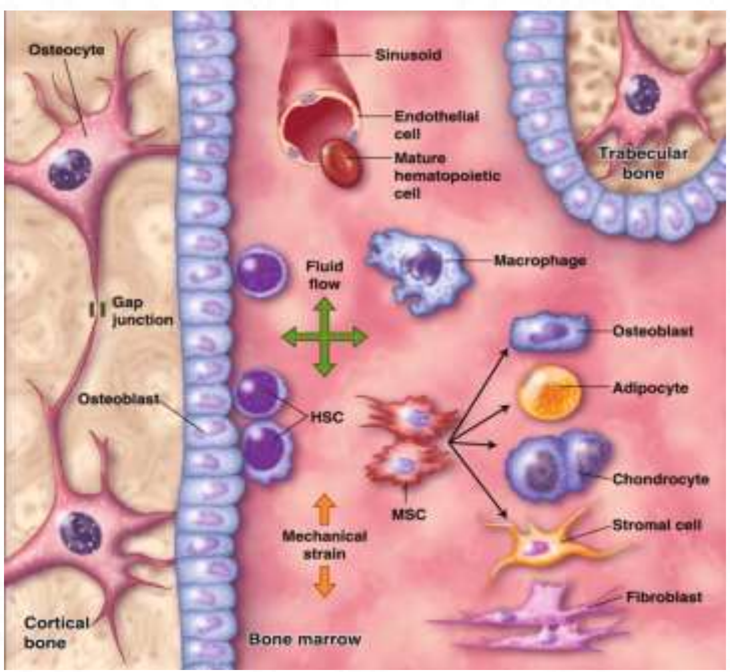


Figure 2.5.: Bone marrow mesenchymal stem cell niche
(Castillo & Jacobs, 2010)

Within the bone marrow cavity exists a dynamic and intricate environment which houses numerous cell types from various lineages. These cells communicate through both local cell-to-cell interactions and the release of soluble factors into the marrow, facilitating communication across greater distances. Experts believe that the MSC niche in the bone marrow is situated near the sinusoid network and is affected by various cell types, such as haematopoietic stem cells in their endosteal niche, mature haematopoietic cells, macrophages, and fibroblasts. Mesenchymal stem cells possess the unique capability of transforming into multiple cell types, such as osteoblasts, adipocytes, chondrocytes, and stromal support cells.

2.5.1. Cell surface markers of MSCs

The International Society for Cellular Therapy (ISCT) states that under normal culture conditions, BM MSCs are adhesive to plastic and express certain surface molecules like CD105, CD73, and CD90. However, they do not express CD45, CD34, CD14, CD11b, CD79α, CD19, or HLA-DR. Further, BM-MSCs differentiate into adipocytes, osteocytes, and chondrocytes in vitro (Dominici et al., 2006). It is essential to distinguish cell surface markers of MSCs to isolate and characterize MSCs in vivo. The expression of PDGFRa and Sca-1 in the non-haematopoietic and endothelial compartments of adult mice's bone marrow was first determined in MSCs (Morikawa et al., 2009). Through the utilisation of a transgenic mouse model, cells that express Nestin, Leptin-Receptor (LepR), and Grem1 have been identified and serve as the primary source for HSC niche factors like CXCL12 and SCF in the bone marrow (Zhou et al., 2014; Worthley et al., 2015). On the other hand, establishing human MSCs has involved the use of monoclonal antibodies by flow cytometry analysis. One of the commonly recognised surface markers for MSCs derived from human BM is CD271, and CFU-Fs are noted to be significantly enriched in both $CD271^{+}CD146^{+}$ and $CD271^{+}CD146^{-/low}$ cells. Surface markers CD90, CD73, CD105, CD49a, CD140b, SSEA-4, MSCA-1, and STRO-1 were used for sorting human MSCs (Ëuhring et al., 2007; Tormin et al., 2011). Despite the vast amount of information obtained about human MSCs through experiments conducted in culture, the intricacy and physiological characteristics of these cells in vivo are still poorly understood. The investigations thus far have shown that the function of MSCs in vivo cannot be accurately reflected by their in vitro features. Regrettably, the current understanding of human BMSCs has primarily been developed through in vitro culture systems. Therefore, it would be greatly beneficial to develop strategies for identifying the human BM-MSCs in vivo in order to address the complexity of the "stem-cell mess of MSCs" (Sipp et al., 2018).

2.5.2. Immunomodulatory potential of MSCs

Mesenchymal stem cells detect the location of the injury and activate the immune response, both innate and adaptive, when necessary. Conversely, they can also inhibit the activity of immune cells in cases where the response is excessive (Bernardo & Fibbe, 2013). MSCs exhibit either pro-inflammatory or anti-inflammatory functions based on the secretion level of factors in the inflammatory environment. When IFN-g and TNF-a levels are deficient, MSCs take on a pro-inflammatory phenotype. This causes MSCs to produce chemokines and factors like CXCL9, CXCL10, CXCL-11, MIP-1a/b, and RANTES, which activate T cells and produce PGE-2 to disrupt dendritic cell precursors. Furthermore, when there is a lack of IL-6 but the presence of IFN-g and IL-1, MSCs encourage the growth and stimulation of macrophages, which produce more IFN-g and TNF-a within the damaged tissue (Shi et al., 2018). MSCs possess anti-inflammatory properties by suppressing the immune response in an environment where there is a significant production of inflammatory cytokines. When exposed to elevated levels of IFN-g and TNF-a, MSCs generate cytokines like TGF-b and HGF and release soluble factors, including IDO, PGE2, and NO, thereby stimulating the activation of regulatory T cells (Tregs) (Jiang & Xu, 2020; Mishra et al., 2020). Therefore, MSCs exhibiting an anti-inflammatory phenotype can help restore immune balance by inhibiting the activation and proliferation of T lymphocytes while promoting the activation of Tregs.

2.5.3. MSCs as a vital element of the Bone Marrow Niche

Mesenchymal stem cells have a crucial function in regulating haematopoiesis within the bone marrow niche, starting from embryonic stages. At E11, MSCs were first found in the aorta-gonad-mesonephros area during haematopoietic system development (Mendes et al., 2005). In the bone marrow of mice, MSCs are in endosteal and vascular niches (Birbrair & Frenette, 2016). MSCs

specifically reside in the endosteal niche, where they line the surface of bones and establish physical interactions with osteoblasts and HSPCs (Asada et al., 2017). Endosteal MSCs are a valuable source of osteoprogenitors and play a vital role in osteogenesis by secreting various growth factors and cytokines (Majumdar et al., 2000). In the vascular niche, MSCs are situated near blood vessels and play a role in maintaining the balance of HSPCs through direct communication or by releasing supportive factors (Corselli et al., 2013). The findings support the idea of a bone marrow stroma made up of various subgroups of MSCs, situated in distinct interconnected regions. This arrangement enables communication between different cell types and molecular signalling. In the human BM environment, MSCs are found in the endosteum and surrounding sinusoidal vessels. The $CD271^{+}$ MSCs exhibit strong clonogenic abilities, along with a higher potential for proliferation and differentiation into mesodermal tissues, as compared to the $CD271^{-}$ MSCs. The analysis of $CD271^{+}$ MSCs showed increased gene expression related to cell adhesion and extracellular matrix, as well as higher levels of early osteogenesis, chondrogenesis, and adipogenesis genes (Ganguly et al., 2019).

2.6. Bone marrow adipocytes

In the 19th century, Franz Ernst Christian Neumann observed the presence of red and yellow regions in the bone marrow (BM), which were later identified as bone marrow adipocytes (BMA). A century later, it was discovered that adipocytes make up the primary cellular element of the yellow bone marrow (Zakaria & Shafrir, 1967). Among adults, BMA accounts for approximately 50 to 70 percent of bone marrow volume and around 5 percent of total body fat. The fat found in bone marrow is unlike the subcutaneous and visceral fat. It exists in two separate groups - constitutive marrow adipose tissue (cMAT) and regulated marrow adipose tissue (rMAT). Research suggests that cMAT undergoes a particular temporal and spatial development pattern before the age of 25

and remains resilient against stress challenges. In contrast, rMAT is believed to develop gradually over the course of one's lifetime (Scheller & Rosen, 2014). The cMAT is characterised by larger adipocytes and minimal active haematopoiesis and is situated in the distal skeletal regions. rMAT consists of individual adipocytes scattered throughout and is situated in regions with high bone turnover. This location allows rMAT to impact both haematopoiesis and skeletal remodelling effectively (Scheller et al., 2015). Bone marrow fat accumulates at birth and occurs faster in distal skeletal sites compared to proximal sites. The BM fat cells make up the largest group of cells in the bone marrow cavity. Their connection to haematopoiesis has gained interest recently, but the exact relationship is still unclear.

2.6.1. Role of BMA in Bone marrow niche

As a component of the haematopoietic niche, BMA influences the proliferation and differentiation of HSCs through the secretion of various factors, including adiponectin, leptin, prostaglandins, IL-6, and other related factors derived from adipocytes (Dias et al., 2015; Poloni et al., 2013). At present, it remains unclear if these factors originate from sources beyond BM fat and play a role in impacting HSCs. The protein hormone Adiponectin is essential in regulating glucose levels and breaking down fatty acids. The ADIPOQ gene in humans encodes its production in the adipose tissue (K et al., 2012). Adiponectin plays a crucial role in promoting the proliferation of HSCs and helps in maintaining their undifferentiated state. Adiponectin-induced HSCs demonstrated greater effectiveness in haematopoietic reconstitution in lethally irradiated mice due to AdipoR1-mediated signalling (DiMascio et al., 2007). Leptin, a 16-kDa protein, is produced by adipocytes and can also be secreted by BMF in the bone marrow microenvironment, leading to elevated concentrations of the protein in the bone marrow. Leptin can boost HSC growth, while

prostaglandins can inhibit it by causing apoptosis, and IL-6 promotes HSC differentiation (Poloni et al., 2013). (**Figure 2.6.1**).

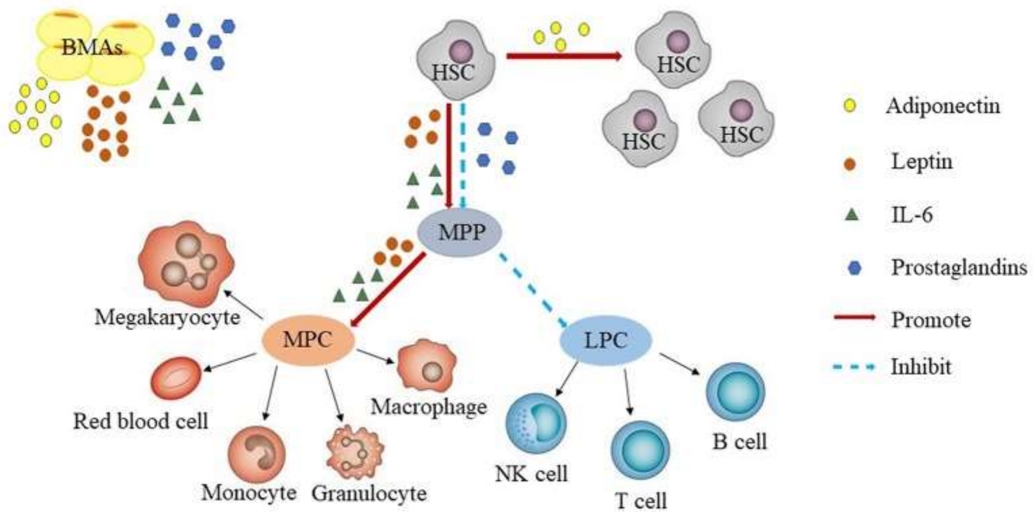


Figure 2.6.1.: Bone marrow adipocytes and haematopoiesis
(H. Wang et al., 2018)

The BMAs secrete various substances, including adiponectin, leptin, prostaglandins, and IL-6. Adiponectin plays a role in promoting the proliferation of HSCs. The differentiation of HSCs is encouraged by Leptin and IL-6, while the proliferation of HSCs is inhibited by prostaglandins. Typically, BMAs tend to encourage the differentiation of HSCs into myeloid progenitors rather than B-lineage progenitors.

BMA is essential for promoting HSC survival, as shown by a 5-week coculture study demonstrating its support for HSC proliferation and differentiation. Moreover, it was discovered that BMA exhibited similar levels of expression for CXCL12, IL-8, colony-stimulating factor 3 (CSF3), and leukaemia inhibitory factor (LIF) as primary human BM MSC. However, BMA showed higher levels of IL-3 expression (Mattiucci et al., 2018). Additional research is needed to clarify the extent to which BMA produces inflammatory cytokines in comparison to other significant sources in BM and their contribution to haematopoiesis in steady-state conditions in vivo. (Spindler et al., 2014). BMA may potentially regulate HSC through cell-to-cell contact mechanisms, despite the lack of a complete definition for these mechanisms. Mice studies found

that troglitazone-induced pre-adipocytic and BM stromal cells supported HSC better than preosteoblastic cells and control-treated BM stromal cells in long-term serial dilution assays. When the preadipocyte cell line OP9 reaches complete adipocyte differentiation, its capacity to support primitive HSC decreases in comparison to its undifferentiated state or under osteogenic conditions. In cell-to-cell contact cultures, preadipocyte OP9 cells exhibit improved HSC support compared to transwell, with multiple pathways involved, such as Notch, Wnt, and Hedgehog (Spindler et al., 2014). Adipocytes from bone marrow MSCs cannot support haematopoietic progenitors but can induce myeloid and B lymphoid differentiation (Corre et al., 2004). On the contrary, some studies have reported that the proportion of adipocytes produced from BM MSCs during in vitro differentiation promotes the survival of $CD34^{+}$ cells while hindering their differentiation. BMA inhibits granulocyte formation in $CD34^{+}$ cells through cell-to-cell contact mechanisms in vitro. Fibroblast-like fat cells containing neuropilin-1 cause this effect, which is not observed in transwell cocultures (Belaid-Choucair et al., 2008). It is crucial to have in vivo data to make informed conclusions about the possibility of cell-to-cell communication between BMA and HSC and whether such communication is bidirectional.

2.7. Telomere

Telomeres, present at the ends of chromosomes, are repetitive sequences of non-coding DNA. The telomeres in mammals consist of repetitive $(TTAGGG)_n$ DNA sequences and a shelterin complex made up of six proteins. This complex helps create a t-loop structure that protects the exposed chromosome ends of telomeric DNA from damage caused by DNA machinery (Shay & Wright, 2019). They play various essential roles in protecting genome stability and function. These roles include discerning chromosome ends from DNA double-stranded breaks (DSBs) and maintaining chromosome length. During DNA replication, conventional DNA polymerases cause

the shortening of chromosome ends, known as the end replication problem. Failure to maintain telomeres can lead to replicative senescence and apoptosis, as there are no specific measures to prevent their shortening (Capkova & Mason, 2013).

2.7.1. History of Telomere

Telomeres were first discovered in the 1930s when McClintock and Muller observed a distinct structure at the chromosome ends of *Zea mays* and *Drosophila melanogaster*. They speculated that this unique structure played a vital role in preventing chromosome end fusion (Creighton & McClintock, 1931; Muller, 1938). The term "telomere" was coined by Muller, derived from the Greek words "telos" meaning "end" and "meros" meaning "part"; therefore, it means "end part" (**Figure 2.7.1**). In 1961, research in human fetal cells showed a limited capacity for replication, reaching a maximum of 50 to 60 doublings. This phenomenon was known as the "Hayflick limit" or replicative senescence (Hayflick, 1965; Hayflick & Moorhead, 1961). During the early 1970s, an observation was made regarding the asymmetry in linear DNA replication, which led to the introduction of the "end replication problem". It was predicted that with each cell division, there would be a loss of chromosomal DNA from the termini of the lagging strand due to the removal of the terminal RNA primer and cause progressive chromosomal shortening (Olovnikov, 1973; Watson, 1972).

The ciliated protozoan *Tetrahymena thermophila* was studied by Blackburn and Gall, who sequenced its rDNA. They found that the termini of the DNA were made up of tandem repeats of hexanucleotide sequences of 5'-CCCCAA-3' and 3'-TTGGGG-5' on the complementary strand (Blackburn & Gall, 1978). In 1985, Greider and Blackburn discovered a new enzyme activity called telomerase, which can add DNA repeat sequences to the ends of chromosomes and prolong telomere length (Greider & Blackburn, 1985). The RNA component was cloned from *Tetrahymena*

thermophila. Through this journey of discovery, it was established that telomere attrition co-occurs with the replicative lifespan of primary human cells in culture (Greider & Blackburn, 1989; Harley et al., 1990). This discovery proved that short telomeres activate the Hayflick limit. Telomere attrition and damage are shown to cause permanent cell cycle arrest. This was demonstrated using human fibroblasts that were overexpressing a mutant form of TRF2 which activated DNA damage checkpoints (d'Adda di Fagagna et al., 2003).

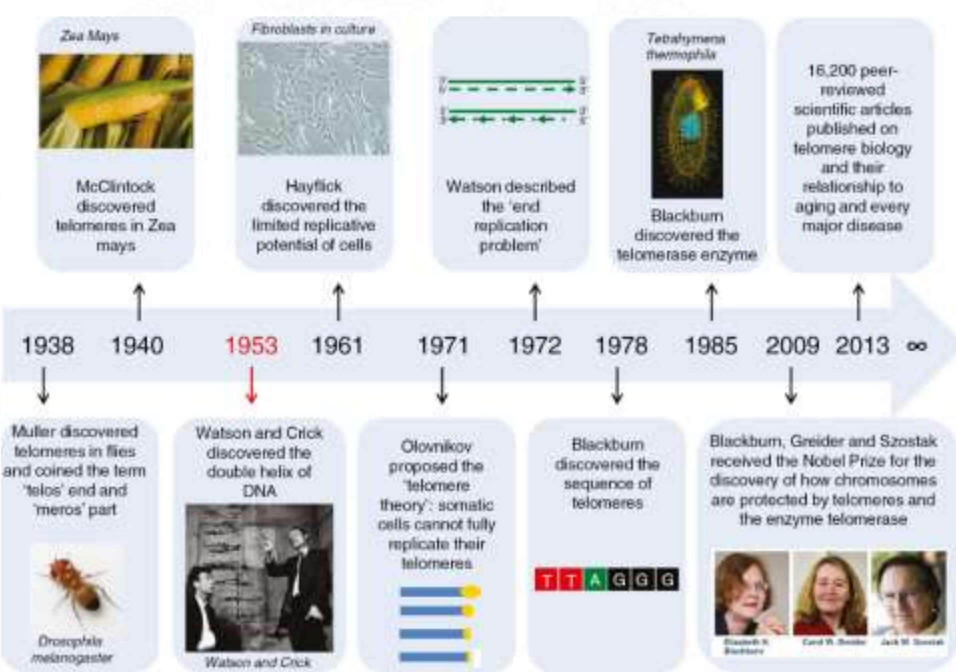


Figure 2.7.1.: Timeline of discoveries in the human telomere field (Herrmann et al., 2018)

One of the main functions of telomeres is to safeguard and maintain the integrity of DNA. This is achieved by shielding chromosomes from degradation, preventing end-to-end fusion and erosion, and stabilizing broken chromosome ends. Telomere also plays a role in regulating the DNA repair process, especially in cases of double-strand break (DSB) damage with non-homologous end-joining (NHEJ) (T. De Lange, 2005). *Additionally, they aid in determining the replicative potential of cells by tracking the number of cell divisions. TLs are also involved in the proper segregation of chromosomes during both mitosis and meiosis, as well as in attaching them to the nuclear envelope. As individuals age, their telomere length inevitably decreases, serving as a significant biomarker for the nine hallmarks of ageing. The processes that preserve telomere length prevent rapid telomere erosion and are closely linked to the functions of specific enzymes, with the most popular one being telomerase.*

2.7.2. Telomere structure

Animals have highly conserved telomere sequences of $(TTAGGG)_n$, with subtle variations present in specific phyla such as Arthropoda (Gomes et al., 2010). It is interesting to note that sequence conservation leads to the occurrence of similar telomere sequences in both heterothermic invertebrates, such as the snail *Cantareus aspersus*, and homeothermic vertebrates like *Homo sapiens*. However, most insects possess a pentameric motif (TTAGG), except for Coleoptera (Gomes et al., 2011; Vítková et al., 2005). Various species have had their telomere sequences determined, with some being utilized as bioindicators in environmental toxicology, including *Cantareus aspersus, Daphnia pulex*, and *Eisenia foetida*. Telomere sequences can vary in repetition number depending on the species or age, and in Metazoa, they often reach several thousands of nucleotides. For example, in the lymphocytes of humans, the size can vary between 2.5 and 15 kbp, which is dependent on the patient (O'Callaghan & Fenech, 2011).

2.7.2.1. Shelterin complex

Maintaining chromosomal integrity is crucial for the sustainability of a species' health span and propagation, and telomeres play a vital role in this process. The function of protecting telomere ends is conserved throughout evolution, from lower multicellular organisms like *Tetrahymena thermophila* to higher-order organisms such as *Homo sapiens* (Roake & Artandi, 2020). Telomeres have a structural composition of tandem repeat sequences of TTAGGG, which can measure several to tens of kilobases. At their 3' end, they have a single-stranded overhang of 75 to 300 nucleotides that are enriched in guanine nucleotides. It was established by seminal work that the overhang folds back onto itself, forming a structure similar to a lariat, known as the T loop (Griffith et al., 1999).

The six-member shelterin complex, which includes repressor and activator protein 1 (RAP1), act as adaptor, telomeric repeat-binding factor 1 (TRF1), TRF2, TRF1-interacting nuclear protein 2 (TIN2), TIN2-interacting protein (TPP1), and protection of telomeres protein 1 (POT1) bind to DNA, serves as the primary protector of chromosome ends. Shelterin not only protects the end of the telomere, but it also senses the telomere length and regulates the telomerase activity (Van Steensel et al., 1998; Colgin et al., 2003; Li & De Lange, 2003). The binding of telomeric dsDNA is done by TRF1 and TRF2 homodimers, while the telomeric 3' tail is capped by the POT1-TPP1 heterodimer (Loayza & De Lange, 2003; Van Steensel & De Lange, 1997). The shelterin complex is formed by connecting TRF1 and TRF2 dimers with POT1-TPP1 through TIN2. RAP1 serves as an additional component of TRF2, aiding in protecting telomeres, particularly those that are severely shortened (Lim et al., 2017; Rai et al., 2016).

At the base of the telomere-loop (T-loop), there is a complex structure known as the displacement loop (D-loop). This involves the telomeric single-strand invading and displacing a portion of the G-rich strand, resulting in proximity of both telomeric ssDNA and dsDNA. To facilitate D-loop formation, it is often suggested and depicted that an assembled shelterin complex (including TRFs and POT1 proteins) would provide stability (**Figure 2.7.2.1**). Recent studies have demonstrated that TRF2 plays a crucial role in T-loop (or D-loop) formation in vivo. This is supported by the fact that TRF2 alone is capable of driving T-loop formation in vitro (Timashev & De Lange, 2020). Mutations in the components mentioned may lead to the disturbance of the shelterin-telomere complex, which can cause premature senescence and end fusions (Van Steensel et al., 1998). When TRF1 is overexpressed or POT1 is deregulated, telomerase binding to telomere ends is negatively affected, resulting in telomere shortening. Additionally, the absence of TRF1 can cause the creation of common fragile sites in telomeric DNA (Loayza & De Lange, 2003).

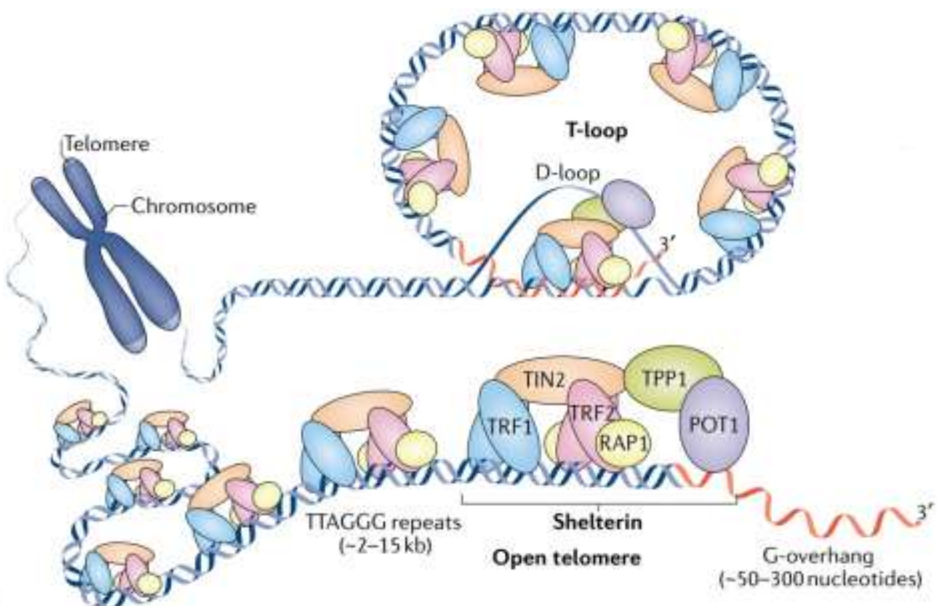


Figure 2.7.2.1: Structure of telomere - shelterin complex at the chromosome end

(Lim & Cech, 2021)

Telomeres are structures made of DNA and protein located at the ends of linear chromosomes. In vertebrates, the telomeric DNA is composed of both double-stranded and single-stranded DNA, featuring a repetitive sequence of TTAGGG. The presence of this duality enables the single-stranded 3' tail to penetrate the double-stranded DNA region, resulting in the formation of a displacement loop (D-loop) and a telomere loop (T-loop). The formation of T-loops is controlled by shelterin complexes, which can limit the access of telomerase to the 3' tail. Telomerase can bind to the 3' tail and add telomeric repeat once the telomere is opened, most likely during the S phase of the cell cycle.

2.8. Telomere length

2.8.1. Telomere end replication process

With each cell division, the end replication problem occurs due to DNA polymerase's inability to fully replicate the chromosomal ends, leading to the gradual shortening of telomeres (O'Sullivan & Karlseder, 2010). In the process of DNA replication, which is semi-conservative, each strand of the double helix serves as a template for generating a new complementary strand (Pfeiffer & Lingner, 2013). The process of synthesizing a new strand in the 5' to 3' direction moving towards the replication fork is initiated by DNA polymerase Polα, using a single RNA primer. This strand

is then elongated further by Polε, replacing the primer and forming what is known as the leading strand (Daigaku et al., 2015). To synthesize the lagging strand in the 5' to 3' direction, multiple primers must be annealed, which elongate into short Okazaki fragments opposite to the replication fork. This process is less efficient than the synthesis of the leading strand. After the replication process is complete, the degradation of the primer causes gaps within the DNA strand. These gaps are then filled by the polymerase, known as Polδ, and then ligated together in order to form a continuous strand. Due to primer degradation, a gap is left at the terminal end, resulting in a reduced 5′ end of the lagging strand (Turner et al., 2019). During the lagging strand synthesis, it is believed that the absence of DNA Polα-primase at the end of linear DNA is responsible for the inability to replicate approximately 250 nucleotides of an average length at the end of linear templates (**Figure 2.8.1**). According to a hypothesis, the size of the 3' overhang determines the rate of telomere shortening. This is caused by the loss of nucleotides at the chromosomal end, resulting in a G-rich single strand at the end of the telomeres (Huffman et al., 2000).

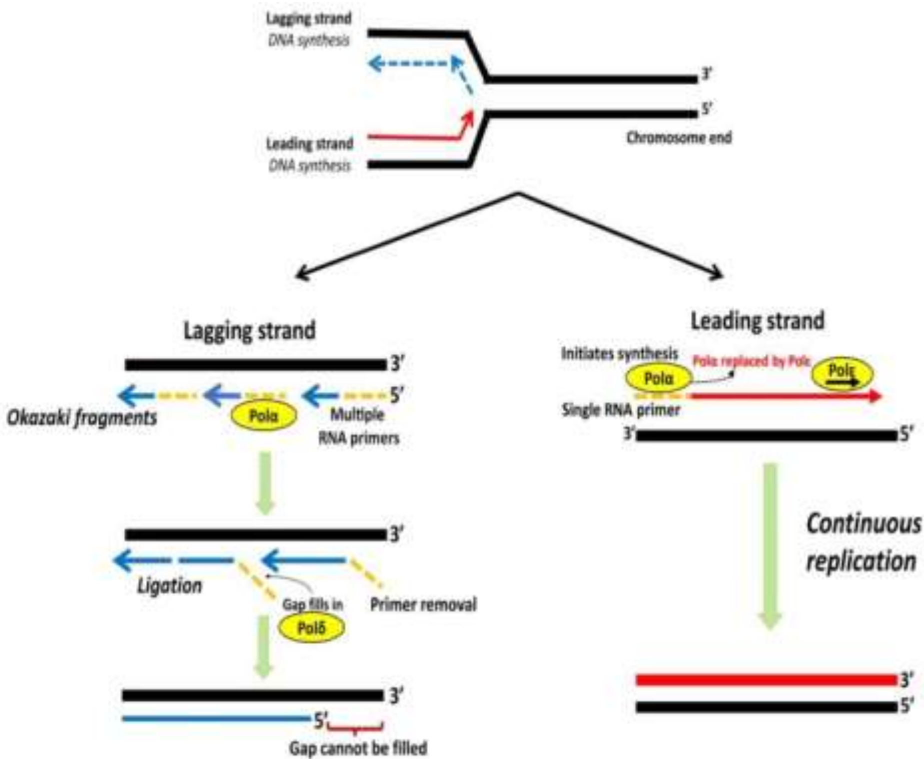


Figure 2.8.1.: Schematic representation of lagging and leading strand replication (Srinivas et al., 2020)

The leading strand synthesis is initiated by DNA polymerase Polα using a single RNA primer, which is then elongated further by Polε. The replication of the lagging strand occurs in fragments called Okazaki fragments, which are created from multiple primers in a discontinuous manner. The RNA primers undergo degradation, and Polδ fills in the gaps before discontinuous fragments are ligated together. The terminal region is non-replicated due to the unfilled gap at the 50 end.

In a culture, typical human cells cease dividing after 40 to 60 passages due to the Hayflick limit, which was discovered by Leonard Hayflick. This limit occurs as telomeres gradually shorten during incomplete replication, ultimately resulting in replicative senescence (Shay & Wright, 2011). *It has been estimated that a human telomere typically contains enough repeats to counteract the impact of telomere erosion in the absence of maintenance mechanisms, with the loss per mitosis estimated to be between 50 and 250 base pairs* (Heidenreich & Kumar, 2017).

2.8.2. Telomere length maintenance

In certain self-renewing tissues like haematopoietic cells, skin cells, and gastrointestinal epithelium, telomerase can help reduce the shortening of telomeres in proliferating cells (Shay & Wright, 2019). As we age, telomeres in adult stem cells and somatic tissues tend to shorten due to insufficient telomerase, which cannot maintain their length indefinitely (Blasco, 2005).

Telomerase, a ribonucleic protein, helps prevent telomere attrition caused by replication. In over 90% of cancer cases, telomerase is upregulated to maintain telomere length. In about 10% to 15% of tumours, telomeres are elongated through an alternative lengthening of telomeres (ALT) process based on homologous recombination (Gaspar et al., 2018).

2.9. Telomerase

2.9.1. Discovery of telomerase

Scientists have proposed that short telomeres may limit the number of times normal cells can divide. However, there must be a solution to the telomere end replication problem in immortal organisms, as well as in the germline cells of higher organisms and immortal cancer cells. A graduate student named Carol Greider, working in Elizabeth Blackburn's laboratory, found the solution through further research on *T. thermophila*. They discovered an enzymatic activity that could synthesize and elongate telomeres and named it terminal transferase, and this was later termed telomerase (Greider & Blackburn, 1985). The RNase led to the discovery of an RNA species that co-purified with telomerase activity, proving that telomerase exists as a ribonucleoprotein complex, and the RNA template sequence was identified in *T. thermophila*. Telomerase activity found in different species creates species-specific telomere repeat sequences in an RNA-dependent manner, similar to *T. thermophila* (Greider & Blackburn, 1987). A significant advancement was made when telomerase, a ribonucleoprotein, was observed to function on a 3' overhang, and this was followed by the cloning of human telomerase reverse transcriptase (TERT) and RNA component (TERC), respectively (Lingner et al., 1997). Over the years, studies on mice lacking TERC and TERT genes, either alone or in combination with progeria or cancer-related genes, have shown that telomere dysfunction can cause premature ageing, cancer, and degenerative diseases. These experiments have demonstrated that intact telomeres play a crucial role in maintaining genome

stability, tissue stems cell reserves, organ system homeostasis, and a healthy lifespan (Jaskelioff et al., 2011). A study conducted in 1998 found that enforced hTERT expression gives primary human cells, such as retinal pigment epithelial cells, fibroblasts, and vascular endothelial cells, the ability to replicate indefinitely. It is worth noting that these cell cultures maintain a normal karyotype and do not exhibit any malignant properties (Bodnar et al., 1998).

2.9.2. Telomerase structure

Telomerase consists of two parts, TERT and TERC, with TERC acting as a template to extend telomeric nucleotide repeats (Blackburn et al., 2006). Several accessory molecules are involved in regulating telomerase biogenesis, subcellular localization, and function (Arndt & MacKenzie, 2016). The 3' end of TERC has a H/ACA domain that is conserved. This domain binds to a protein complex consisting of dyskerin (DKC1), non-histone protein 2 (NHP2), nucleolar protein 10 (NOP10), and encoding H/ACA ribonucleoprotein complex subunit 1 (GAR1) (Egan & Collins, 2012). Dyskerin is bound by NOP10 and GAR1, whereas NHP2 binds to the RNA directly. The TERC component found in the nucleolus combines with TERT to create a complete telomerase complex. This complex then identifies the Cajal body (CAB) box and is further aided by the involvement of telomerase cajal body protein 1 (TCAB1), which helps to recruit the mature telomerase complex to the Cajal body. In the S-phase of the cell cycle, Cajal bodies play a role in aiding in employing mature telomerase complex to the telomeres (Venteicher et al., 2009). Moreover, additional proteins like ATPase reptin and pontin have played a role in telomerase assembly by interacting with TERT and dyskerin (**Figure 2.9.2**). Pontin and reptin aid in the formation of TERT with TERC and dyskerin, as well as the remodelling of the mature telomerase complex. Their interaction with dyskerin also contributes to the assembly and stability of TERC (Venteicher et al., 2008).

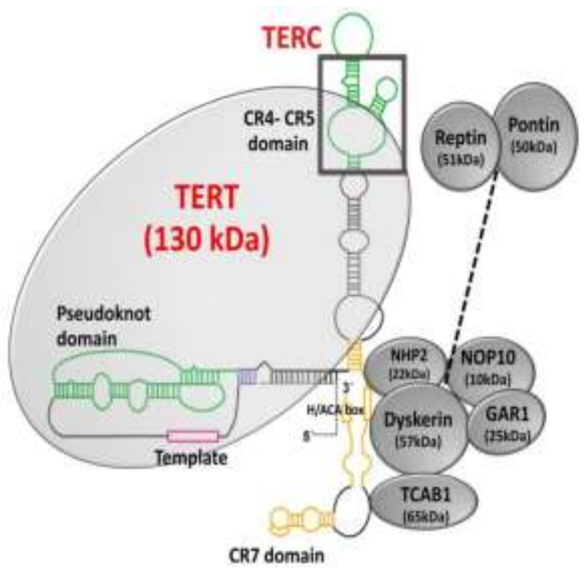


Figure 2.9.2.: Structure of telomerase complex
(Srinivas et al., 2020)

Telomerase consists of two components, the catalytic subunit known as TERT (telomerase reverse transcriptase) and the RNA component, TERC (telomerase RNA component). Dyskerin, along with GAR1, NHP2, and NOP10, interacts with TERC by binding to the H/ACA box. They play a role in regulating telomerase biogenesis, subcellular localization, and function.

2.9.3. Telomerase function

Telomerase primarily functions to elongate telomeres through RNA-dependent mechanisms. This process involves multiple stages in the telomerase catalytic cycle, with the addition of one telomeric repeat occurring after substrate binding. After the enzyme's active site, the end product can either dissociate or go through translocation and then elongation. Telomerase has the unique ability to move the DNA it synthesizes to the template starting point, which can be classified into two processivity types. The addition of nucleotides (type I processivity) is a common function of all polymerases, while repeat addition (type II processivity) is specific to telomerase and determines the enzyme's ability to repeatedly copy an RNA template region by elongating only one substrate molecule (Autexier & Lue, 2006; Lue, 2004). During the initial stage of the telomerase reaction cycle, primer binding occurs through complementary interaction with the

template region. The TERT active site's structural components control the efficiency of duplex formation and the translocation of the newly synthesized product during the processive synthesis of telomeric repeats. The most significant aspect of telomerase is its capacity to add repeats continuously (Wallweber et al., 2003).

2.10. Alternative lengthening of telomeres (ALT)

Immortal human cell lines utilize the ALT pathway as an alternative method for telomere synthesis, activated by epigenetic aberrations and mutations in genes encoding chromatin remodelling proteins at telomere and other genomic sites, as well as tumour suppressors such as p53 (Bryan et al., 1995, 1997; Conomos et al., 2013). The ALT pathway involves several mechanisms, such as telomere elongation through homologous recombination. Specific proteins, including HR protein complexes (MRN, MRE11, RAD50, NSB1, NBN), SMC5, SMC6, FANDCD2, FANCA, and FEN1, play a role in the ALT pathway by assisting in the restart of telomeric DNA replication or maintaining the structural integrity of telomere (Cesare & Reddel, 2010). The ALT pathway has been observed in human somatic cells, particularly those lacking TP53, which are genetically unstable and are known to promote the growth of cancerous cells in humans, accounting for 15% of cancers (Roake & Artandi, 2016).

The ALT pathway, which does not rely on telomerase, was initially discovered in a telomerase-deficient strain of *S. cerevisiae* and later studied in human cancer cell lines and tumours. The process of synthesizing telomeric DNA in ALT+ cells involves a combination of intra- and inter-telomeric recombination and replication (Lundblad & Blackburn, 1993; Muntoni et al., 2009). At telomeres, ALT+ cells exhibit various characteristics, such as (1) the presence of ALT-associated PML bodies (APBs), (2) heterogeneous telomere length, (3) abundant extrachromosomal telomere repeat (ECTR), and (4) elevated levels of telomere sister chromatid

exchange (T-SCE) (Cesare & Griffith, 2004; Yeager, 1999). A distinctive type of nuclear structure called APBs can be found exclusively in ALT+ cells, composed of both the PML protein and telomeric DNA (Yeager et al., 1999).

2.10.1. Framework of the ALT pathway

Initially, the ALT pathway was identified in a mutant budding yeast that lacked functional telomerase. Rad51 plays a crucial role as the recombinase in the homologous recombination (HR) pathway of yeast. The binding of Rad51 to single-stranded DNA is dependent on the presence of Rad52, which also possesses the capability to anneal complementary ssDNA (McEachern & Haber, 2006; New et al., 1998). Yeast has two different types of ALT; type I survivors, depend on Rad51 and Rad52 for maintaining telomeres by amplifying subtelomeric sequences. Type II survivors rely only on Rad52 to maintain telomeres by expanding telomeric repeats. The extension of telomeres in human cells through the ALT pathway is believed to be more comparable to type II survivor behaviour (Le et al., 1999; Teng & Zakian, 1999). Until recently, there was limited knowledge of the functioning of the ALT pathway in human cells that are ALT+. This is because there were no direct monitoring methods available for ALT DNA synthesis. However, several assays have been developed to model or monitor ALT DNA synthesis, such as break-induced telomere synthesis (BITS). With the help of MiDAS technology at telomere and the ATSA approach for ALT telomere synthesis in APBs, we have successfully uncovered the molecular mechanisms behind ALT. With the help of MiDAS technology at telomeres and ATSA approach for ALT telomere synthesis in APBs, molecular mechanisms behind ALT have been successfully uncovered (Dilley et al., 2016; Özer et al., 2018).

When a replication fork collapses due to a single-ended double-strand break, a repair process called Break-induced replication (BIR) is triggered. This process involves conservative

DNA replication to extend the repair. Pol32 yeast and POLD3/4 human proteins serve as crucial accessory subunits for DNA polymerase, playing a significant role in BIR (Anand et al., 2013; Lydeard et al., 2007). Telomeres undergo conservative DNA replication in a POLD3/4-dependent manner in ALT+ human cells, which links ALT to BIR. The presence of human RAD52 is crucial for BIR at collapsed forks and MiDAS at fragile sites (Sotiriou et al., 2016). In human cells, the ALT pathway relies on RAD52, unlike RAD51, which is similar to yeast type II survivors. The fusion of TRF1-FOK1 nuclease can cause Telomeric DSBs, which lead to telomere clustering and BITS (**Figure 2.10.1**). The absence of RAD51 affects telomere clustering but not BITS. Telomeric MiDAS appears to be more effective in ALT+ cells due to the high replication stress experienced at their telomeres, as compared to ALT- cells. Conservative DNA replication is observed through Telomeric MiDAS, which mimics the phenotype of ALT+ cells. When RAD52 is depleted or inhibited, there is a decrease in telomeric MiDAS. Conversely, RAD51 depletion leads to an increase in telomeric MiDAS, fragile telomeres, and the formation of telomere dysfunction-induced foci (TIF) (Min et al., 2017). Studies show that in vitro, RAD52 is more effective than RAD51 in promoting D-loop formation on telomeric DNA when RPA is present (Zhang et al., 2019). Additionally, increasing the expression of RAD52 has been found to enhance telomere synthesis in both G2 and mitotic cells. Based on the findings, it appears that RAD52 plays a crucial role in ALT in human cells, while RAD51 does not have direct involvement. Instead, RAD51 may aid in preventing telomere fragility by safeguarding stalled replication forks. It is important to note that RAD51 has been proven to be necessary for telomere extension in cells with overexpressed BLM, so it is possible that RAD51 may have a specific role in certain instances of ALT (Sobinoff et al., 2017).

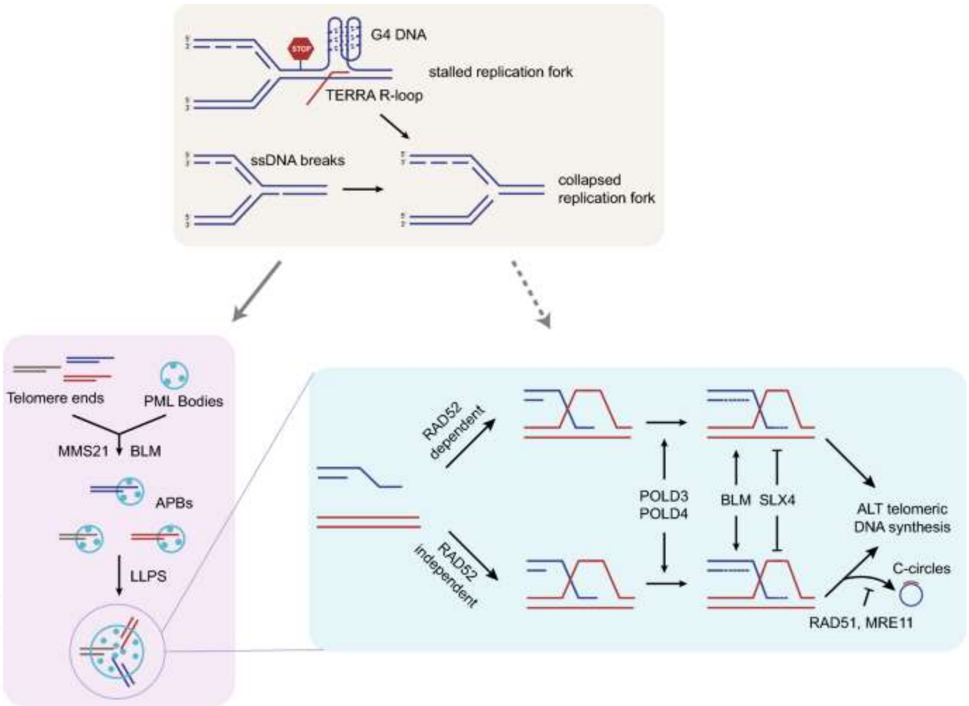


Figure 2.10.1.: Framework of the ALT pathways

(Zhang & Zou, 2020)

The stress caused by telomere replication could initiate the activation of ALT. The build-up of R-loops, G-quadruplexes, and single-strand breaks in DNA at telomeres can disrupt DNA replication, resulting in the breakdown of replication forks and the creation of one-ended double-strand breaks (DSBs) (Upper section). When there is replication stress or DNA damage at telomeres, telomere proteins may undergo SUMOylation. This process recruits PML and triggers APB formation through SUMO/SIM-mediated LLPS. Efficient ALT may be driven by the clustering of telomeres and the enrichment of DNA repair, recombination, and replication proteins in APBs. (Lower left section). The initiation of BIR in APBs is caused by one-ended DSBs occurring at telomeres. ALT can occur through both RAD52-dependent and -independent BIR pathways. During BIR, the conservative DNA replication relies on POLD3/POLD4, facilitated by BLM and impeded by SLX4. The RAD52-independent BIR pathway produces C-circles, which are suppressed by RAD51 and MRE (Lower right section).

2.11. Telomere length measurement

Telomere attrition is a widely recognized cell-intrinsic event linked to normal cellular ageing (Mayer et al., 2006). Furthermore, research has demonstrated that telomere attrition and dysfunction significantly contribute to the development of age-related ailments such as atherosclerosis, myocardial infarction, Alzheimer's dementia, and heart failure. There is evidence

linking telomere shortening to certain lifestyle factors. This has led to speculation that biological age may be a crucial factor in identifying those at risk for health issues typically associated with advancing age (Shammas, 2011). Determining the correlation between telomere length and biobehavioral traits and health conditions may prove helpful as a biomarker for assessing health risks and improving outcomes. To accurately measure telomere length, it is crucial to utilize valid and reliable techniques. This understanding is essential for comparing results among various investigative teams and creating effective experiments for future research. As cells divide, their telomeres shorten progressively. This happens because of incomplete lagging strand DNA synthesis, oxidative damage, and exonucleolytic processing events, among other factors. Eventually, this shortening leads to growth arrest in the cells, which is an initial barrier to tumour formation in humans. The shortest telomere length plays a crucial role in signalling the beginning of senescence. Nevertheless, the typical methods for assessing telomere length primarily offer insights into the average telomere length (Aubert et al., 2012).

2.11.1. Terminal Restriction Fragmentation

The gold standard method for determining telomere length is Terminal Restriction Fragment (TRF) analysis, which was the original technique developed for this purpose. This involves thoroughly digesting genomic DNA using a combination of restriction enzymes that frequently cut DNA but do not recognize sites in the telomeric and subtelomeric regions, thus preserving the integrity of telomeric DNA. To determine the length of intact telomeres on each chromosome, they are separated based on size using agarose gel electrophoresis. The resulting telomeric fragments are then examined either through southern blotting or in-gel hybridization using a probe designed for telomeric DNA. The sizes of telomeres will appear as a smear, which

is evaluated by referencing a DNA ladder made up of known fragment sizes to determine the intensity and size of the smear (Allshire et al., 1989; Harley et al., 1990).

2.11.2. Polymerase Chain Reaction-based Techniques

Several PCR-based methods have been developed to overcome the challenge of requiring vast amounts of DNA for assessing telomere lengths. These processes comprise quantitative PCR (qPCR), absolute telomere length (aTL) quantitation and monochrome multiplex quantitative PCR (MMqPCR). PCR magnifies a DNA sequence of interest over 20-40 cycles by using custom-designed primers, with the amount of the PCR product (the amplicon) doubling with each cycle. In qPCR, DNA sequence quantity is usually measured using a fluorophore that emits a fluorescent signal upon intercalation with double-stranded DNA (such as SYBR green) or a probe with a fluorophore that is released upon amplification of the sequence of interest (like TaqMan probes). The fluorescence emitted after each cycle is measured to determine the starting material quantity (Ding & Cantor, 2004).

A primer set and protocol for determining telomere length through qPCR technology were developed by Cawthon et al. in 2002. Telomere length estimation using qPCR was previously unsuccessful due to the challenge of designing primers that effectively target the repeating TTAGGG sequence of the telomere without forming primer dimers. Primer dimers occur when two primers bind to each other and amplify the primer sequence instead of the target DNA from the patient/cell line. To solve the issue of primer dimerization, Cawthon implemented two strategies. Firstly, they utilized primers that only bind to the C- and G-rich segments and have mismatches in other areas. Secondly, they performed the first two cycles at lower temperatures, allowing the primers to bind with the telomeric DNA. Subsequently, the remaining cycles were conducted at higher temperatures to amplify the desired products from the initial two cycles, which

were specific to the patient/cell line DNA rather than the primer DNA. In order to measure the length of telomeres, the quantity of the telomere amplification product (T) was compared to that of a single-copy gene (S) in different wells or tubes. This resulted in the calculation of the T/S ratio, which is correlated with the average telomere length but is not an exact measure in base pairs (Cawthon, 2002). Variability in DNA levels between wells/tubes due to pipetting can affect the accuracy of assays. A revised method called monochrome multiplex quantitative PCR (MMqPCR) amplifies both telomeric and single-copy DNA from the same tube, improving precision and eliminating shortcomings. (Cawthon, 2009). O'Callaghan and Fenech developed the aTL qPCR method, a modified version of the basic qPCR-based technique. It involves using a standard curve with known telomere lengths to estimate the length in base pairs of specimen telomere lengths instead of a relative T/S value (O'Callaghan & Fenech, 2011).

2.11.3. Single Telomere Length Analysis (STELA)

Current telomere tests only show the average length and not individual telomere lengths. This can be a problem since critically short telomeres can trigger cellular ageing. Using an assay that identifies individual telomere lengths may be helpful in research design. Baird et al. developed a method called single telomere length analysis (STELA) to assess the length of individual telomeres on specific chromosomes. A modified version of qPCR, this technique involves using specific primers and ligation to amplify telomeric DNA from one end of a chromosome's subtelomeric sequence (Abdallah et al., 2009; Baird et al., 2003). However, due to the complexity and lack of specificity of certain chromosomal subtelomeric regions, only a select few chromosomes (XpYp, 2p, 11q, 12q, and 17p) meet the necessary criteria for successful chromosome-specific/chromosome arm-specific telomeric DNA amplification. It is possible that not all critically short telomeres can be detected using the STELA method due to variations in the

rate at which specific telomeres reach this stage (Britt-Compton et al., 2006). This is because there is diversity in the length of heritable telomeres among different telomeres in an individual. A modified version of the STELA method called "Universal STELA" has been developed. This adaptation enables the identification of any critically short telomere, irrespective of its position on the chromosome (Bendix et al., 2010).

2.11.4. Quantitative Fluorescence in situ Hybridization (Q-FISH)

Quantitative Fluorescence in Situ Hybridization (Q-FISH) of telomeric repeats can be performed only on metaphase chromosomes or interphase nuclei by labelling with a fluorescent (CCCTAA)3 probe. Q-FISH uses cells as a substrate instead of DNA, which sets it apart from TRF and PCR-based assays. The substrate for this method can be fresh cells, frozen cells, formalin-fixed paraffin-embedded cells, or permeabilized ones for chromosome-specific analyses (Montpetit et al., 2014).

2.11.5. Metaphase Chromosome Q-FISH

The Q-FISH method, developed by Peter Lansdorp and his team, provides a way to assess telomere length (Lansdorp et al., 1996). This technique involves using a specific probe for the telomeric repeat sequence (CCCTAA)3 to visualize telomeres, while the rest of the chromosome is visualized using a nonspecific DNA stain like 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide. An early adaptation of this method was reported by Krejci and Koch. In this assay, a synthetic peptide nucleic acid (PNA) probe is used, which has been proven to have higher hybridization efficiency for telomeric repeat sequences compared to DNA probes (Krejčí & Koch, 1998). This is because the PNA probe has a neutral (uncharged) backbone. One benefit of using the Q-FISH method is the ability to estimate the size of each of the 92 individual telomeres in humans rather than just average or small telomeres (Egholm et al., 1993). In addition, this approach

is the sole means of evaluation that can identify telomere-free chromosome endings where there is not a sufficient telomere sequence for successful hybridization. Metaphase Q-FISH investigations have proven crucial in disclosing the variability of telomere length among various chromosomes and illuminating the frequency of chromosomal instability linked to telomere-free ends (Vera & Blasco, 2012).

2.11.6. Interphase Q-FISH

Adaptations of the Q-FISH procedure have overcome its limitations, including the use of interphase cells instead of metaphase chromosomes. Interphase Q-FISH is helpful in assessing telomere lengths in various specimen types, such as blood cells, formalin-fixed or paraffin-embedded tissues, and frozen tissues. To compare the fluorescent signals of telomere-specific and centromeric probes, many investigators using interphase Q-FISH evaluates a ratio of signal between the targeted sequences, employing different colour fluorophores (Aubert et al., 2012). One notable benefit of utilizing interphase Q-FISH methodology is its ability to gather data on both telomere length and histological information simultaneously. This approach can also be paired with immunostaining techniques to pinpoint particular cells of interest, also known as telomapping (Vera & Blasco, 2012).

2.11.7. Flow-FISH

Flow-FISH is a variation of the Q-FISH method that merges flow cytometry with the hybridization of a pan-telomeric probe to cells in suspension. Unlike metaphase and interphase Q-FISH, which hybridize to cells fixed to slides, Flow-FISH hybridizes to cells flowing one by one past laser. Using the same telomeric (CCCTAA)3 PNA probe as other Q-FISH approaches, Flow-FISH measures the average amount of fluorescence present in cells to determine the average telomere length for the cell population being studied (Hultdin et al., 1998). This technique is its

ability to categorize cells into smaller groups based on their size, granulation, and/or antibody labelling. This makes flow-FISH a popular choice for measuring average telomere length in different haematopoietic cell subtypes. Additionally, flow-FISH is the first telomere assay that is employed as a diagnostic tool in the clinical setting, assisting in the identification of patients with dyskeratosis congenital - a condition associated with shortened telomeres. Another advantage of this method is its ability to determine the 3D distribution of telomeric signals within cells (Alter et al., 2007; Samassekou et al., 2010). A summary of the pros and cons of primary methods used for telomere length measurements is listed in **Table 2.11.7.**

Table 2.11.7.: Comparison of advantages/limitations of methods used to assess telomere length

Methods	Advantages	Limitations
TRF	Gold standard method	Requires large (>1µg) amount of DNA
	Numerous studies for comparisons Numerous studies for comparisons	Labour intensive
	Does not require specialized equipment	Subtelomeric polymorphisms can impact data.
		Provides mean length measure but not recognition of individual short telomeres or ends lacking a telomere
qPCR	Can use small (ng) amounts of DNA	Variation between and within batches
	Less labour intensive	Reference standards lacking
	Referenced to standard single copy gene	Requires qPCR equipment
	Multiplex controls for DNA amount added.	Does not provide an absolute kilobase length estimate unless coupled with standard oligo
		Provides mean length measure but does not allow recognition of individual short telomeres or ends lacking a telomere
STELA	Allows for detection of critically short telomeres	Only provides information for a small subset of specific chromosome ends
	Does not require viable cells	Does not provide mean telomere data
	Does not require specialized equipment	Does not recognize ends lacking a telomere

		Limited in the ability to detect long telomeres
		Labour intensive
Q-FISH	Can identify single telomere changes (higher resolution)	Labour intensive
	Can assess telomere lengths in specific cell types	Requires high skill level for chromosome assessment
	When used on metaphase chromosomes, it can identify individual telomeres (long or short), signal free ends, end-to-end telomeres, and a mean telomere length measure	Requires microscope (typically fluorescent)
		"Length" expressed as relative fluorescence unit (often compared to standard [centromeric] value
		Requires mitotically active cells for metaphase chromosomes but not for interphase nuclei
Flow-FISH	Can determine the mean length for specific cell populations	Labour intensive
	When coupled with antibodies can provide cell type specific information	Requires high skill level
	Potential for automation	Requires flow sorting equipment
		Length expressed as relative fluorescence unit
		Provides mean length measure but not recognition of chromosome-specific individual short telomeres or ends lacking a telomere

2.12. Bone marrow failure

Bone marrow failure (BMF) occurs when there is a decrease or complete stop in the production of blood cells, which can affect one or more types of cells. In cases of BMF, especially in severe or advanced stages pancytopenia is observed, which is characterized by reduced levels of white blood cells (WBCs), red blood cells (RBCs), and platelets circulating in the body. BMF's pathophysiology involves several factors. Basically, there is the destruction of haematopoietic stem cells due to injury from various sources, such as drugs, chemicals, radiation, viruses, or

autoimmune mechanisms. And then there is premature senescence and apoptosis of haematopoietic stem cells due to genetic mutations. While another factor involves ineffective haematopoiesis can result from stem cell mutations, as well as vitamin B12 or folate deficiency. The disruption of the bone marrow microenvironment that supports haematopoiesis can also contribute to BMF. The decreased production of haematopoietic growth factors or related hormones can be a factor. Lastly, the marrow space is infiltrated by abnormal cells leading to the loss of normal haematopoietic tissue. The severity and duration of cytopenias determine the clinical implications of bone marrow failure. If left untreated, severe pancytopenia can quickly lead to fatality. In some cases, patients may not display any symptoms initially, and their cytopenia may be discovered during a routine blood test. Thrombocytopenia leads to increased bruising and bleeding. Decrease in erythrocytes lead to fatigue, paleness, and cardiovascular issues. Prolonged neutropenia increases the risk of life-threatening infections (Drexler et al., 2022).

2.12.1. Bone marrow failure syndromes

Bone marrow failure syndromes encompass a diverse range of non-malignant haematological disorders, both inherited and acquired, that result in low levels of blood cells in either one or multiple lineages. These conditions can occur independently of any other factors that may impact the function of the bone marrow. Inherited germline mutations in HSCs are responsible for constitutional syndromes that result in specific diseases such as Fanconi anaemia (FA), Shwachman–Diamond syndrome (SDS), dyskeratosis congenita (DKC), neutropenia (Kostman Disease), congenital amegakaryocytic thrombocytopenia (CAMT), and familial telomerase diseases. On the contrary, acquired BMF syndromes occur when external factors such as chemicals, drugs, and viruses directly or indirectly damage the HSC pool (**Figure 2.12.1**). The harm caused to HSCs is mainly due to immune effector mechanisms, which may be activated by

viruses or drug metabolites (Young et al., 2006). Regardless of the cause of damage to the bone marrow (BM), acquired and constitutional BMF syndromes result in qualitative and/or quantitative impairments of HSCs or their progenies, leading to reduced self-renewal ability. There have been numerous reports of cellular and cytokine dysregulation in various acquired BMF syndromes, including acquired aplastic anaemia, myelodysplastic syndromes, and chronic T and natural killer (NK) granular lymphocyte disorders.

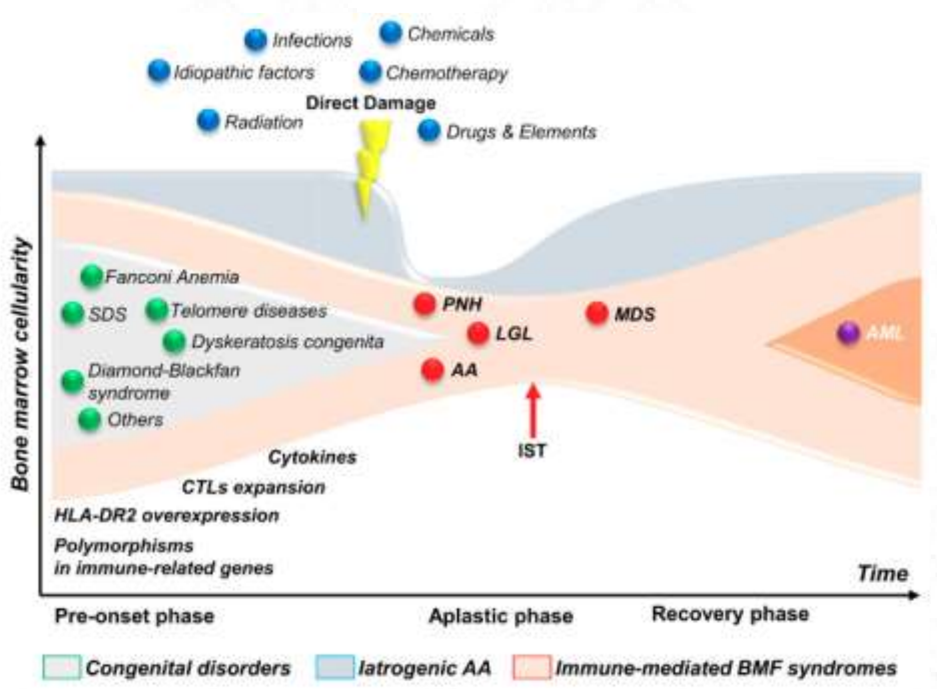


Figure 2.12.1.: Occurrence of bone marrow failure syndromes with respect to marrow cellularity and onset time

(Giudice et al., 2021)

BMF syndromes refers to a group of conditions characterized by the absent or reduced haematopoietic precursors in the bone marrow and lead to peripheral blood cytopenia. These syndromes can be categorized into three types: congenital disorders, iatrogenic aplastic anaemia and immune-mediated BMF. In some congenital disabilities like Shwachman-Diamond syndrome or Fanconi anaemia, the haematopoietic stem cells have genetic mutations that affect the normal functioning of hemopoiesis. This leads to inadequate maintenance of normal levels of circulating cells over time. In cases of iatrogenic AA, HSCs are harmed by external stressors like chemicals and radiation. Immune-mediated BMF, on the other hand, occurs when dysregulated immune responses lead to an autologous immune attack of cytotoxic T lymphocytes (CTLs) against HSCs or suppress hemopoiesis by altering the BM microenvironment. The way BMF syndromes present clinically may vary depending on the disease. Nevertheless, the use of immunosuppressive

therapies can frequently help to recover bone marrow cellularity. This is a crucial indication that BMF has an immune-mediated pathogenesis.

2.12.1.1. Inherited bone marrow failure syndromes

Inherited Bone Marrow Failure Syndromes are a rare group of heterogenous disorders that are diverse and have varying phenotypes and can affect one or multiple lineages. The classic disorders are Fanconi anaemia (FA), dyskeratosis congenita (DC), Shwachman-Diamond syndrome (SDS), Diamond-Blackfan anaemia (DBA), congenital amegakaryocytic thrombocytopenia (CAMT), and severe congenital neutropenia (SCN). In addition to their physical traits, these syndromes are closely linked to mutations in specific pathways. FA is caused by mutations in DNA damage response genes, DC is linked to telomere maintenance genes, and DBA is associated with mutations in ribosome function genes. SDS is currently being recognized as a disorder related to ribosome maturation and proteostasis (Alter et al., 2009; Da Costa et al., 2018; Finch et al., 2011; Rosenberg et al., 2008) (**Table 2.12.1.1**). Constitutional syndromes are visible in childhood, with physical abnormalities affecting multiple organs, and family history is also observed. However, genetic testing has shown deficiencies can also arise in adulthood, with implications for family members. In some cases, the use of graft from an affected sibling has resulted in fatal graft rejection. Additionally, patients with mutations in the gene responsible for producing the growth factor thrombopoietin may experience persistent marrow failure after transplantation (Fogarty et al., 2003; Seo et al., 2017).

The scientific understanding of how these conditions, which vary in both physical manifestation and genetic makeup, lead to single or multiple cytopenias is still lacking. Although a definitive pathway has not been identified, research conducted on zebrafish indicates that TP53 responses may play a role. The activation of the TP53 pathway has been observed in bone marrow

failure cases involving DC, FA, and other syndrome (Carrillo et al., 2014; Ceccaldi et al., 2012). It has been proposed that the TP53 pathway plays a role in causing bone marrow failure in inherited neutropenias like SCN and SDS. Exposure to environmental factors can also speed up this process, such as aldehydes that create DNA crosslinks in FA (Glaubach et al., 2014). However, the exact ways in which epigenetics and genetic co-modifiers contribute to these diseases are not yet fully comprehended. Studying the molecular basis of IBMFS can enhance our comprehension of haematopoiesis and the growth and maintenance of non-haematological tissues. As IBMFS has been associated with increased risk of developing leukaemia, gaining insights into their pathophysiology can also aid in our understanding, prevention, and potentially treating cancer and genetic alterations associated with ageing.

Table 2.12.1.1.: Inherited bone marrow failure syndromes

(Oyarbide et al., 2019)

Disease	Prevalence Per 1,00,000	Male to Female ratio	Symptoms	Genes and their estimated frequency	Cancer predisposition
Diamond-Blackfan anaemia (DBA)	5-7	1:1	Erythroid failure, congenital malformations, growth retardation, short stature. Thumbs, upper limbs, hands, craniofacial, urogenital, and cardiovascular anomalies are also common.	RPS19 (25%), RPL5 (7%), RPS26 (6.6%), RPL11 (5%), RPL35a (3%), RPS10 (3%), RPS24 (2.4%), RPS17 (1%), RPL15, RPS28, RPS29, RPS7, RPS15, RPS27a, RPS27, RPL9, RPL18, RPL26, RPL27, RPL31, TSR2, GATA1, EPO	AML, MDS, ALL, Hodgkin and non-Hodgkin lymphomas, osteogenic sarcoma, breast cancer, hepatocellular carcinoma, melanoma, fibrohistiocytoma, gastric cancer, colon cancer
Dyskeratosis congenita (DC)	1	3:1	Abnormal skin pigmentation, nail dystrophy, mucosal leukoplakia, pulmonary fibrosis and BMF	DKC1 (17-36%), TERC (6-10%), TERT (1-7%), NHP2 (<1%), NOP10 (<1%), WRAP53 (3%) and TINF2 (11-24%), ACD, PARN, RTEL1, USB1,	AML, solid tumours

				TCAB1, POT1, TPP1, WRD79, TR, NOLA2,NOLA3	
Disease	**Prevalence Per 1,00,000**	**Male to Female ratio**	**Symptoms**	**Genes and their estimated frequency**	**Cancer predisposition**
Fanconi anaemia (FA)	3	1.2:1	Developmental abnormalities in a number of organ systems and BMF	FANCA (65%), FANCB (<1%), FANCC(14%), FANCG (10%), FANCD1/BRCA2 (<1%), FANCD2 (<1%), FANCE (4%), FANCF (4%), RAD51, FANCC1, FANL, FANCL, FANC, PALPB2, RADC51C, SLX4, FANCCQ, BRCA1, FANCT	AML, solid tumours
Shwachman-Diamond syndrome (SDS)	13	1.7:1	Exocrine pancreatic insufficiency, BM dysfunction and skeletal abnormalities	SBDS (90%), DNAJC21, EFL1, SRP54	AML, MDS
Congenital amegakaryocytic thrombocytopenia (CAMT)	Unknown (less than 100 cases reported)		Thrombocytopenia and megakaryocytopenia	MPL	AML, MDS
Severe congenital neutropenis (SCN)	5		Neutropenia	ELANE, GFI1, HAX1, G6PC3, VPS45, JAG1, CSF3R, WAS, SRP54	AML, MDS

2.12.1.2. Acquired bone marrow failure syndromes

Acquired BMF syndromes are a distinct group of disorders that are identified by a decrease in the production of mature cells in the bone marrow. In most cases, these syndromes occur due to the immune system destroying haematopoietic stem cells or their progenitors at different stages of

development. Examples of acquired BMF syndromes include acquired aplastic anaemia, myelodysplastic syndromes, and chronic T and natural killer (NK) granular lymphocyte disorders.

2.13. Aplastic anaemia

Aplastic anaemia is a disorder of the haematopoietic stem cells that leads to reduced bone marrow cellularity and pancytopenia. In most cases, the cause of acquired aplastic anaemia is due to immune-mediated pathophysiology. This occurs when autoreactive lymphocytes attack and destroy haemopoietic stem cells. While environmental factors like drugs, viruses, and toxins may trigger abnormal immune responses in some patients, most cases remain classified as idiopathic. Aplastic anaemia, like other autoimmune diseases, can have a diverse clinical course. While some patients experience mild symptoms that require minimal or no treatment, others may exhibit life-threatening pancytopenia, which is a medical emergency. It is common for patients with aplastic anaemia also to develop paroxysmal nocturnal hemoglobinuria and myelodysplastic syndrome, indicating a connection between these disorders at a pathophysiological level. Acquired aplastic anaemia can be effectively treated through allogeneic bone marrow transplantation and immunosuppressive therapy with antithymocyte globulin and cyclosporine (Brodsky & Jones, 2005).

2.13.1. History

A severe form of aplastic anaemia, which was first described by German pathologist Paul Ehrlich in the late 19th century, has been linked to high mortality rates. Prior to the 1920s, BM evaluations were not commonly conducted and were only occasionally performed post-mortem in previous cases. AA is a rare condition that was initially thought to be a variation of other blood disorders. However, post-mortem examinations showed that it was a different condition due to its quick decrease in blood counts and watery, yellow, and acellular marrow (Brodsky & Jones, 2005).

In the past, treating AA was mainly focused on providing supportive care due to a lack of understanding of the condition. The diagnosis of a pan-marrow failure was made based on a BM with a low cell count and a decrease in all three blood cell types. The underlying causes of the hypo-functioning marrow were unknown, but it was suspected to be linked to exposure to chemicals, drugs, or toxins, resulting in damage to BM cells and their removal (Kaufman et al., 1996; Young & Kaufman, 2008). The potential involvement of the immune system in AA was not discovered until 1964. An experiment conducted by Barnes and Mole involved injecting lymph node cells from C3H/H mice into sublethally irradiated CBA/H mice, which resulted in the latter dying from pancytopenia a few weeks later. The number of effector cells infused was found to be directly linked to the severity of the disease, indicating a potential linkage between the immune system and AA (Scheinberg & Chen, 2013). Studies suggest autoimmune causes of AA, with evidence from autologous recovery after immunosuppressive therapy and the absence of rejection in syngeneic transplants. Anti-lymphocytic serum and androgens have shown effectiveness in treatment, while high-dose corticosteroids had moderate effectiveness with excessive toxicity (Bacigalupo et al., 1979).

Back in 1972, a person with aplastic anaemia was the first to undergo a successful allogeneic bone marrow transplant. This breakthrough, along with the advancement of potent immunosuppressive therapy during the 1970s, greatly enhanced the outlook for a disease that had previously resulted in fatalities within a year of diagnosis. During the 1980s, the addition of CSA improved the anti-lymphocyte serum treatment, resulting in a further 10-20% improvement in response rates. This led to an overall haematologic response rate of 60-70% (Frickhofen et al., 1991). Various laboratory experiments supported the idea that immunology played a role in AA-related marrow destruction (Young, 2006). Androgens are commonly used in AA treatment, while

corticosteroids are only used for serum sickness prophylaxis with ATG. The hematologic response is the most effective measure of survival following IST, with robust responders having better outcomes (Rosenfeld et al., 2003).

2.13.2. Epidemiology and Etiology

AA is a rare disease that is typically acquired during the first three decades of life. Its incidence in Western countries is two cases per million per year, while it is 2-3 times higher in Asia (Kaufman et al., 1991). Although there is an epidemiological association between benzene and pesticides, they only account for a small fraction of the cause. In rural Thailand, various exposures to non-bottled water, certain animals, animal fertilizer, and pesticides seem to suggest an infectious cause (Issaragrisil et al., 2006). The occurrence of AA differs significantly across the globe and also varies geographically within India. The bimodal age distribution at presentation in western countries is 15-25 years or ≥60 years (Vaht et al., 2017). Most studies from India report a younger age at presentation, with a median age of 25 years (range 2-83 years) and 36.5 years (range 19-77 years) unlike western countries (Mahapatra et al., 2015; Nair et al., 2013). This variability in incidence may be due to dissimilarity in exposure to environmental factors including viruses, drugs and chemicals, diagnostic criteria, genetic background, and study designs. The incidence of AA shows gender variation. In developed countries, the sex ratio is nearly equal (Young & Kaufman, 2008), while in countries like India, male patients are more common due to social bias (Mahapatra et al., 2015; Nair et al., 2013).

The last century's data on AA showed idiosyncratic reactions to drugs or chemical hazards. Studying idiosyncratic drug reactions is challenging due to their extreme rarity. The only apparent predisposition to abnormal drug metabolism found so far is a single study from over 20 years ago, which examined an individual exposed to carbamazepine. Some series have observed an

overrepresentation of drug-metabolizing glutathione-S-transferase gene deletions, whereas there is no mechanism reformed for chloramphenicol. An infectious agent in 5-10% of cases of AA with seronegative hepatitis has not been noted (Gerson et al., 1983; Sutton et al., 2004).

2.13.3. Pathophysiology

2.13.3.1. Direct damage to the marrow

The pathophysiological of AA can be majorly classified into three main mechanisms that cause an empty bone marrow (**Figure 2.13.3.1**). The direct damage to marrow mainly occurs iatrogenically due to radiation therapy or chemotherapy. Benzene's impact depends on the dosage and is temporary in normal amounts. It can affect several parts of the body but usually resolves on its own. While it can negatively impact blood cell production and has been linked to anaemia, exposure poses minimal risk and only accounts for a small percentage of bone marrow failure cases in most countries (Issaragrisil et al., 2006). China's regulation of workplace toxins like benzene is slow, despite rapid industrialization. Cytopenia severity is dosage and duration dependent. Milder symptoms may recover after exposure ends, but marrow failure is a direct effect of benzene exposure (Gross et al., 2010).

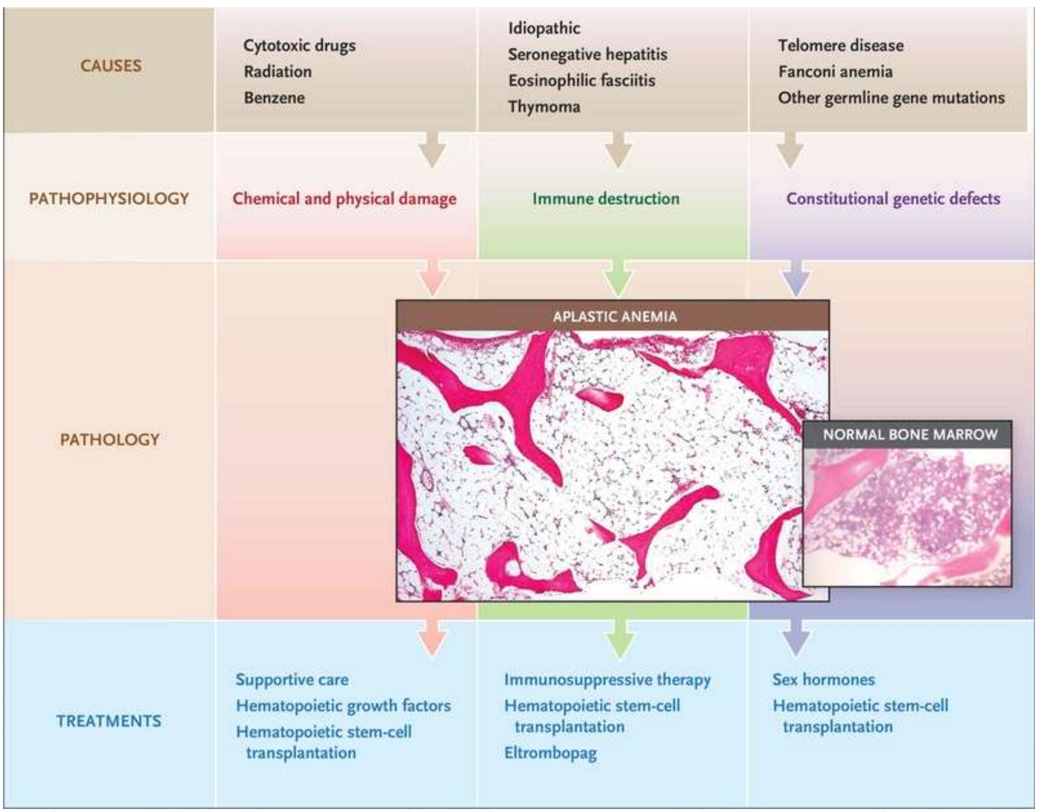


Figure 2.13.3.1.: Diverse pathophysiological features of aplastic anaemia that lead to a common pathologic process in bone marrow failure

(Young, 2018)

Aplastic anaemia is characterized by the replacement of haematopoietic cells with fat. This can be caused by chemical or physical damage, with the most common causes being iatrogenic - such as after cytotoxic chemotherapy or irradiation - or exposure to benzene, typically in industrial settings. An outcome of this is hypocellular marrow. In some cases of constitutional genetic defects that impact the function of haematopoietic stem cells and the immune system, marrow failure and marrow with lower cell count are noticeable. Aplastic anaemia, a disease that affects the immune system, is primarily caused by the destruction of haematopoietic stem cells and progenitor cells by T cells. The treatments vary depending on the underlying cause, whether it is marrow damage, genetic defects with multiorgan effects, or an immune-related condition.

2.13.3.2. Immune-mediated pathophysiology

During diagnosis, the immune system has been altered in AA patients, eventually eliminating marrow progenitor cells. One of the crucial factors is the augmented production of interferon-gamma and tumour necrosis factor-alpha in the blood and bone marrow, resulting in the

elimination of early progenitor cells through a Fas-dependent process. The increase in oligoclonal $CD8^+$ cells suggests an unknown antigen is driving the immune response (Young et al., 2006). There is also evidence of immune pressure, as one human leukocyte antigen haplotype on chromosome 6p is lost in myeloid cells, providing a potential escape mechanism from $CD8^+$ -mediated HSC destruction seen in some cases of leukaemia relapse (Ogawa, 2016). The lack of immune regulation is also a contributing factor to the development of AA. During diagnosis, there is a decrease in regulatory cells like T and B cells, which leads to a more pro-inflammatory environment, and is evident by a high Th17/Treg ratio (Solomou et al., 2007a). However, after responding to IST, this ratio tends to normalize. Acquired somatic mutations in the transcription STAT3 signalling pathway may be pathogenic in AA and are also in large granular lymphocytosis, leading to overactive T cells (Jerez et al., 2013). It has been suggested that immune escape could be the reason behind the clonal expansion of PNH cells, whereas the absence of HLA, acquired by 6p loss of heterozygosity (LOH) or some somatic mutations, sustains haematopoiesis in AA (Imi et al., 2018). These cells lack glycosylphosphoinositide (GPI)-anchored proteins in their red and white blood cells, which happens due to a mutation in the PIGA (phosphatidylinositol glycan class A) gene of a stem cell (Gargiulo et al., 2017).

In a model of immune-mediated marrow failure, there is a significant increase in donor $CD4^+$ and $CD8^+$ T cells within the recipient's marrow. This excessive activation results in the elimination of stem and progenitor cells. The development of BM failure involves both Th1 and Th17 immune responses, with the Fas/Fas ligand pathway playing an important role in the target cell destruction. Studies have shown that infusion of natural Tregs can suppress effector T-cell expansion and preserve marrow HSCs, as well as block IL-17 (**Figure 2.13.3.3**). There has also

been an observed upregulation of proinflammatory and adhesion signalling (NOTCH, CXCR4) in models that could serve as potential therapeutic targets (Scheinberg, 2021).

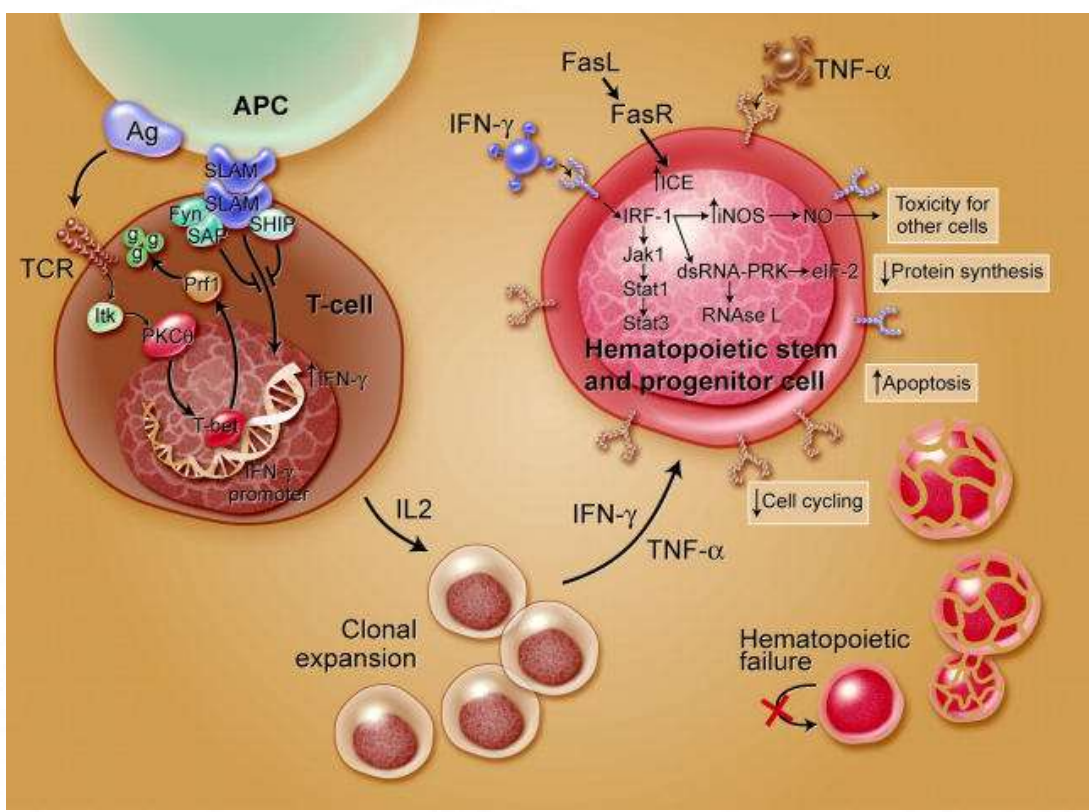


Figure 2.13.3.2.: Immune destruction of haematopoiesis in aplastic anaemia (Young et al., 2006)

When antigen-presenting cells (APCs) present antigens to T lymphocytes, it prompts the activation and multiplication of T cells. The transcription factor T-bet is responsible for binding to the promoter region of interferon-γ (INF-γ) and initiating gene expression. When SAP binds to Fyn, it results in the modulation of SLAM activity, leading to a decrease in gene transcription of IFN-γ expression. Individuals diagnosed with aplastic anaemia exhibit constant T-bet expression and decreased SAP levels. Additionally, IFN-γ and TNF-α enhance the cellular receptors of other T cells, as well as the Fas receptor. When interleukin-2 production is increased, it causes the expansion of T cells. Meanwhile, the activation of the Fas receptor through its ligand results in the apoptosis of target cells. Certain impacts of IFN-γ are conveyed through interferon regulatory factor 1 (IRF-1), which hinders the transcription of genes within cells and the initiation of the cell cycle. The cellular genes, such as inducible nitric oxide synthase (NOS), are powerfully stimulated by IFN-γ. The production of nitric oxide (NO), a toxic gas, may also diffuse toxic effects. As a consequence, cell cycling is reduced, and apoptosis occurs, resulting in cell death.

2.13.3.3. Role of telomere in AA

Individuals with dyskeratosis congenita often have a short telomere length (TL), which is linked to mutations in the telomerase complex (TERT, TERC, DKC1, RTEL1, TINF2, NOP10) (Alter et al., 2007). Sometimes, infrequent AA can be linked to telomeropathy, which may be caused by a mutation in the telomerase complex genes or a very short telomere (<1st percentile) (Calado & Young, 2012; Wang et al., 2020). The interpretation of these findings may differ due to the varying degree of penetrance exhibited by genetic defects. In cases of low penetrance, mutations may not accurately predict the phenotype, severity of disease, or response to IST. Having a short telomere length is linked to genomic instability, leading to a higher risk of haematologic relapse and clonal evolution after immunosuppressive therapy (Scheinberg et al., 2010). Sex hormones boost telomerase activity both in vivo and in vitro by elevating the expression of the TERT gene, and this may help elucidate the effect of androgens on unselected cases of AA. During a clinical trial, it was found that danazol was successful in lengthening telomeres and producing a positive hematologic response in most patients with an underlying telomeropathy (Jouneau et al., 2016). Approximately 33% of individuals diagnosed with AA exhibit shortened telomeres in peripheral blood. This finding supports the theory that there may be a connection between telomere dysfunction and the development of AA.

2.13.3.4. Role of mesenchymal stromal cells in AA

According to studies conducted on mice, acquired AA is linked to a deficiency of HSCs (Scheinberg & Chen, 2013). For individuals diagnosed with AA, both PB and BM exhibit significantly lower levels of CD34+ cells, as well as long-term culture initiating cells (LT-CIC), which serve as the functional equivalent of HSC-enriched populations in humans (J. Marsh et al., 1990). Microenvironment dysfunction may cause AA, as proven by unsuccessful bone marrow

transplants from identical twins despite immunosuppression (Hinterberger et al., 1997). The haematopoietic and non-haematopoietic components of the bone marrow work closely together to maintain and regulate haematopoiesis. (**Figure 2.13.3.4**). It is not unexpected for AA to be linked with abnormalities in the nonhaematopoietic BM microenvironment components, given the firm interplay and regulatory feedback loops between resident haematopoietic and niche cells. In comparison to the control group, individuals with AA displayed a significant decrease in vascular cells, endosteal cells, and perivascular cells (Young, 2018). Studies have found that patients with AA and BMF have abnormal functions and disordered components in their BM microenvironment. Specifically, research has shown that BM stromal cells from AA patients have a reduced ability to support haematopoiesis in long-term cultures (Chao et al., 2010; Hamzic et al., 2015; Li et al., 2012; Shipounova et al., 2009). While few other studies showed contrary results, stating that there is no difference in AA MSCs supporting haematopoiesis (Bueno et al., 2014; Caruso et al., 2017).

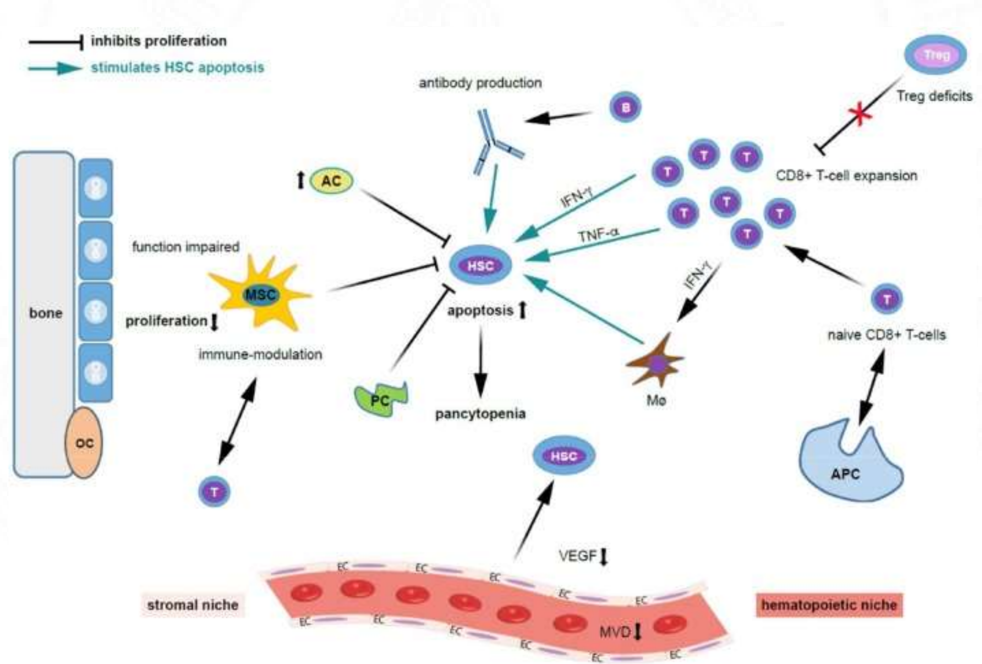


Figure 2.13.3.4.: Possible mechanisms contributing to bone marrow niche modulation and immune destruction of haematopoiesis in acquired aplastic anaemia
(Medinger et al., 2018)

Patients with acquired aplastic anaemia display not only low numbers of haematopoietic stem cells (HSC) but also an altered haematopoietic niche. On the left side of the figure, the effect of stromal cells ("stromal niche") and their interaction with HSC and on the right side, the effects of the immune cells on HSC ("haematopoietic niche") are shown. Regarding the stromal niche, impairments in osteoblastic, vascular, and perivascular HSC niches might contribute to defective haematopoiesis in patients with AA. MSC function is impaired in AA, HSCs cannot adequately proliferate, and activated T-cells are not suppressed. MSC aberrant alteration impairs the maintenance of immune homeostasis. Adipocytes (AC) are increased, pericytes are decreased (PC) and suppress haematopoiesis. Further, the microvessel density (MVD) and vascular endothelial growth factor (VEGF) expression are decreased in AA. Given the close interaction and regulatory feedback loops between resident haematopoietic and niche cells, it is not surprising that besides immune destruction, AA also associates with defects in non-haematopoietic BM microenvironment components.

When compared to MSCs from healthy donors, those derived from patients with AA exhibited abnormal morphology, reduced proliferation and clonogenic potential, as well as heightened apoptosis (Chao et al., 2010; Huo et al., 2020; Li et al., 2012). However, there were noticeable variations among the samples taken from individual patients. Studies reported that AA MSCs tend to differentiate into adipogenic rather than osteogenic lineages and have lower suppression of T cells proliferation by AA MSCs when co-cultured with MNCs (Li et al., 2012; Chaturvedi et al., 2018; Huo et al., 2020). Contrarily, the latest studies have shown that MSCs obtained from patients with acquired AA do not show any significant differences in differentiation potential when compared to healthy individuals. It has been found that MSCs obtained from individuals with acquired AA are capable of forming HSC niches in vivo that are comparable to those created by MSCs from healthy individuals (Bueno et al., 2014; Michelozzi et al., 2017; Sharma et al., 2021). The results of these studies have been a topic of controversy. One possible explanation for the discrepancies observed could be the heterogeneity among individual patients, as the studies involved a limited number of patients. Additionally, technical differences in the employed assays could also have had an impact. It is worth noting that some older studies may

have included patients with unidentified congenital genetic defects in their analysis, which could have contributed to conflicting results. This is especially relevant as advanced genetic testing was not used to differentiate between immune-mediated acquired AA and aplasia that develops from congenital genetic syndromes. In the latter case, stromal and other non-haematopoietic microenvironmental cells may carry a congenital genetic lesion and could be affected by it (Medinger et al., 2018).

2.13.4. Diagnosis of AA

Acquired AA can periodically be traced to a definitive cause like drugs, toxins, seronegative hepatitis, or pregnancy, but extensively considered to be idiopathic. The symptoms experienced are linked to the level of peripheral blood cytopenias and can consist of shortness of breath when active, tiredness, quickly getting bruises or petechiae, nosebleeds, bleeding gums, heavy menstrual periods, headaches, and fever (Dezern & Brodsky, 2011). To diagnose the condition, a thorough examination of the complete blood count, leukocyte differential, reticulocyte count, BM aspirate and biopsy. It is also recommended to perform peripheral blood flow cytometry to detect cells missing GPI anchored proteins, bone marrow karyotyping, and FISH to help exclude hypoplastic MDS (Mukhina et al., 2001). To diagnose AA, there must be at least two of the following: (i) haemoglobin <100 g/l, (ii) platelet count $<50 \times 10^9$/l, (iii) neutrophil count $<1{\cdot}5 \times 10^9$/l (Guinan, 2011). The severity was classified as per the modified Camitta criteria, as mentioned in **Table 2.13.4**.

Table 2.13.4: Severity criteria of aplastic anaemia

(Guinan, 2011)

Classification	**Criteria**
Severe (SAA)	BM cellularity <25% (or <50% if <30% of BM is haematopoietic cells) and ≤2 of the following: Peripheral blood neutrophil count < 0.5 x 10^9/L Peripheral blood platelet count < 20 x 10^9/L Peripheral blood reticulocyte count < 20 x 10^9/L
Very Severe (VSAA)	As mentioned above, but peripheral blood neutrophil count must be < 0.2 x 10^9/L
Non-Severe (NSAA)	Hypocellular BM with peripheral blood values not meeting criteria for SAA

When diagnosing AA, it is essential to rule out other potential causes, such as infections, autoimmune diseases, drug reactions, or other marrow disorders like leukaemias or MDS, since AA is described by pancytopenia and hypocellular marrow. It is crucial to differentiate between these disorders and inherited types of AA in younger patients. Additionally, investigating telomere, chromosome fragility, and ribosomopathies is essential (Sieff, 2018). It can be challenging to distinguish between hypoplastic MDS and AA in older patients, which can be aided by quantifying CD34+ cells, intracellular and cell surface markers like p53, Hb F and telomere length (Matsui et al., 2006). In around 40-50% of AA cases, a PNH clone may coexist, but it is generally small and has no significant clinical impact upon diagnosis. Interestingly, a PNH clone has been linked to a positive response to IST and is infrequently observed in cases of inherited AA (Narita et al., 2015). It is not common practice to conduct myeloid gene panel testing or next-generation sequencing (NGS) during diagnosis as it has not been proven to have prognostic value on its own. As a result, these tests are not presently recommended for assessing disease or planning treatment at the time of diagnosis (Yoshizato et al., 2015). It has been discovered through routine cytogenetic testing

that around 10% of patients who seem to have AA based on other criteria may actually have clonal chromosomal abnormalities (Afable et al., 2011).

2.13.5. Treatment of AA

Prior to the 1970s, the approach to managing SAA focused mainly on providing supportive care. This involved the use of transfusions and antibiotics, but their long-term efficacy was limited. The safety of blood products, resistance to transfusions, excessive iron accumulation, and limited options for antimicrobial treatment were all significant challenges, while androgens were found to be effective in some patients (Sanchez-Medal et al., 1969). In the 1980s, growth factors were tested for their effectiveness in treating SAA but showed limited activity. However, the natural history of SAA was altered by haematopoietic stem cell transplantation (HSCT) and IST, both of which have been linked to comparable long-term survival rates (Bacigalupo et al., 2016). The use of HSCT has been particularly successful in curing AA, with engraftment and the absence of transplant-related complexities resulting in aid for most patients. Initially, only match-related donors were available, but this expanded over time to include histocompatible matched unrelated donors (MUD) due to improved outcomes (Bacigalupo & Sica, 2016). In practice, various factors such as age, the existence of a histocompatibility donor, and comorbidities need to be considered. For children and young adults with a matched sibling donor, a related HSCT is typically the first treatment option and for older patients, IST is commonly utilized. In cases where a histocompatibility donor is not available, it is essential to screen all younger patients for potential donors in BM registries when initiating IST (Scheinberg, 2012).

2.13.5.1. Immunosuppressive therapy

Although ATG has been extensively documented, it remains one of the least comprehended immunosuppressive therapies. The fundamental concept behind creating various ATGs is by

immunizing an animal with lymphoid antigens and collecting the polyclonal sera. The horse-ATG preparations consist of diverse antibodies that recognize human T-cell epitopes. Most of these antibodies target activated T-cells or activation antigens (Mohty, 2007). While the decrease in circulating lymphocyte levels is temporary, the number of activated T-cells remains reduced for a longer duration. This decrease can also be observed in the production of IFN-γ and possibly TNF-α after horse-ATG (Sloand et al., 2002). CSA has a targeted effect on T lymphocytes, reducing their response to stimuli and cytokine release. It has been combined with horse-ATG for severe AA treatment (Frickhofen & Rosenfeld, 2000).

It has been more than a century since Ilya Mechnikov created the first antiserum by injecting rat spleens and emulsions of rabbit lymph nodes into guinea pigs. However, the inconsistent outcomes in the past posed challenges to the standardization and purity of antiserums, hindering their use (Kaufmann, 2008). During the early 1960s, researchers observed a notable increase in the survival rate of skin allografts in rats treated with anti-lymphocytic serum (ALS), even when the donor and recipient had different tissue types (Woodruff & Anderson, 1963). Additionally, a few years later, dogs who received kidney transplants showed improved survival rates after being administered ALS and can prolong kidney allograft survival and reverse hyperacute rejection, which other agents cannot achieve (Starzl et al., 1967). Paul Ehrlich and Ilya Mechnikov won the Nobel Prize in 1908 for their contributions to immunology. Their work laid the foundation for the development of immunology as a separate field of study (Kaufmann, 2008).

According to NIH criteria, the response to IST was classified as mentioned in **Table 2.13.5.1** (Scheinberg, et al., 2009). Clinical trials conducted across the United States, Europe, and Asia have revealed a consistent hematologic response rate of 60-70%, with long-term survival rates exceeding 80-85%, while younger patients showed better improvement. IST has a 30%

relapse rate and a 10-15% clonal evolution rate. Like HSCT, the outcome is worse for older patients. However, eligible older patients should consider h-ATG/CsA as IST (Contejean et al., 2018; Scheinberg & Young, 2012).

Table 2.13.5.1: Response criteria to immunosuppressive therapy of AA

(Scheinberg, et al., 2009)

Response	**Criteria**
Complete response (CR)	If there was transfusion independence with Hb ≥ 11 g/dL Neutrophils > 1.5×10^9/L Platelets > 100×10^9/L
Partial response (PR)	Transfusion independence with Hb ≥ 8 g/dL Neutrophils > 0.5×10^9/L Platelets > 20×10^9/L
No response (NR)	When there is transfusion dependence

Rabbit-ATG is similar to horse-ATG but is made by vaccinating rabbits with human thymocytes. It appears to be more effective at reducing lymphocytes for a more extended period of time. The increased lymphocytotoxicity of r-ATG can be attributed to the IgG subtype's higher affinity for human lymphocytes, less variability between batches, longer half-life, and superior efficacy in depleting lymphocytes (Scheinberg et al., 2007). Rabbit-ATG may improve immune regulation by converting CD4+CD25- to CD4+CD25+ regulatory T cells in vitro, only in the presence of r-ATG and not h-ATG (Feng et al., 2008). For some patients, h-ATG/CsA treatment does not work. Repeat immunosuppression courses have shown response rates between 30-70%. R-ATG/CsA as the first course may result in a hematologic response of around 50%, with lower initial and salvage rates of recovery (Trcuelu et al., 1998).

A study was conducted on patients using eltrombopag as a single agent. The study involved gradually increasing the dosage, starting at 50 mg and increasing it by 25 mg every two weeks for a total daily dose of 150 mg over three to four months. This was done to tackle the issue of high endogenous Tpo levels in the patient population (Olnes et al., 2012). Out of the 43 individuals observed, a haematologic response was observed in 17 of them (40%). Surprisingly, seven of these responses were multilineage, which was unexpected. As a result, eltrombopag was approved for use in treating SAA for individuals who did not respond well to first-line IST. The response rate of eltrombopag is generally around 50%, with a range of 40% to 60% in the second line of treatment. Romiplostim, an alternative Tpo-RA, has been studied as a second-line therapy for AA and has demonstrated efficacy (Lee et al., 2019). According to a study, the response of platelets at week 9 varied based on the dosage. Lower doses of 1 and 3 μg/kg did not elicit any responses, occasional responses were seen at 6 μg/kg, and the majority of participants responded positively to higher doses of 10 μg/kg. In the extension phase, as the dosage increased to 20 μg/kg, the response continued to improve, and this was similar to that observed with eltrombopag (Jang et al., 2021). Other retrospective studies have also shown the activity of romiplostim in refractory SAA but at a lower rate (Zhao et al., 2019).

A study examined the use of eltrombopag in combination with h-ATG/CsA for first-line treatment. Specifically, the three-drug combination of ATG/CSA/eltrombopag (ACE) was studied prospectively in three separate groups. After six months, the hematologic and CR rate was found to be 87% and 39%, respectively. This CR rate is 39% which was 3-4X higher than reported with h-ATG/CsA alone, and the primary endpoint of achieving a CR ≥ 30% at six months was met. Interestingly, the best outcomes were observed in cohort 3, with overall and CR rates of 94% and 58%, respectively. They started drug treatments on day 1 improved their quality of life, with faster

recovery of blood counts, fewer transfusions, and hospital visits. Lab experiments showed an increase in marrow progenitor cells from the therapy (Townsley et al., 2017).

2.13.5.2. Haematopoietic stem cell transplant

The replacement of failed BM has been found to be a curative treatment for the underlying disease. In the past, transplantation has faced challenges such as graft rejection and graft-versus-host disease, as well as a shortage of compatible donors. However, there have been significant recent advancements in expanding donor options. When dealing with immune AA in a young patient, transplantation is always the most preferred course of treatment. If a transplant is done promptly after diagnosis, using a graft from a compatible sibling donor, the outcomes are remarkable. Young children have a survival rate exceeding 90%, while adolescents have a rate of over 80% (Dufour et al., 2014, 2015). In addition, the occurrence of both short- and long-term complications is low. The standard conditioning regimen for patients involves using Cyclophosphamide (Cy) alone or in combination with ATG, while unstimulated bone marrow is the graft source. According to a previous randomized trial, the incidence of graft failure was roughly 16% (Champlin et al., 2007).

Several factors have been identified as being linked to lower survival rates after undergoing MRD HSCT. These factors include the individual's age, the source of the graft, the duration between the transplantation and diagnosis, the number of transfusions received prior to transplantation, previous immunosuppressive therapy, and the existence of any infections prior to transplantation. Despite the increasing frequency of transplants using grafts from sibling donors in older adults, success rates have remained stagnant for many years (Gupta et al., 2010). In registry data, recipients older than 40 years old are three times more likely to die than children, with a survival rate of 50% for them. As peripheral blood poses a higher risk of GVHD, marrow is

preferred. It is often difficult for patients to find histocompatible sibling donors, but thankfully, large donor registries can offer the possibility of an HLA match for children in need (Gupta et al., 2010).

2.14. Fanconi anaemia

Fanconi Anaemia is a genetic defect that affects multiple systems. It is characterized by a combination of symptoms and signs, such as BMF and somatic malformations, epithelial cancers of the head and neck, a tendency to develop cancers, primarily acute myeloid leukaemia and sensitivity to pro-inflammatory cytokines and alkylating agents. FA, as an inherited BMF syndrome, has been extensively studied and is the most common among its counterparts (Barankin et al., 2005; Mathew, 2006).

2.14.1. Pathophysiology of FA

FA is attributed to a germline mutation developed from a defective DNA damage response. With the exception of FANCB mutations which are X-linked, all other mutations are inherited as an autosomal recessive disease. Mutations in eight FA subtypes, namely FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM, lead to a loss of FANCD2 and FANCI monoubiquitylation, which is the primary regulatory event in the FA pathway (**Figure 2.14.1**). It has been found that a minimum of 95% of patients possess germline mutations in any of the 16 genes responsible for encoding proteins that participate in the shared DNA repair pathway (Khincha & Savage, 2013). In addition, these functions also include signalling for stress and initiating apoptosis as a response to oxidative damage and inflammatory cytokines. Due to abnormal DNA repair, individuals with FA have cells that are highly sensitive to the clastogenic effect of DNA. The discovery that the breast cancer susceptibility gene BRCA2 is also an FA gene

(FANCD1) underscores the strong collaboration between the FA pathway and the BRCA1 and BRCA2 proteins in preventing breast and ovarian tumours through DNA repair mechanisms (Garcia-Higuera et al., 2001). The identification of FANCJ (also known as BRCA1-interacting helicase1 [BRIP1]), FANCN (also referred to as partner and localizer of BRCA2 [PALB2]), and FANCO (RAD51C; a previously known homologous repair factor) confirms the vital link between breast and ovarian susceptibility genes with FA (Meindl et al., 2010; Xia et al., 2007). Patients with FA subtype FANCA usually experience a less severe form of the disease and have a later onset of bone marrow failure. However, those with subtypes FANCC and FANCG tend to have a more severe form of the disease and require earlier intervention, such as BM transplantation from an unrelated donor. Patients having FANCD1 mutation are bound to increased incidence of leukaemia and solid tumours with early onset (Myers et al., 2012). It is crucial to diagnose and identify FANC mutations early on to provide informed genetic counselling for parents of FA patients regarding future pregnancies.

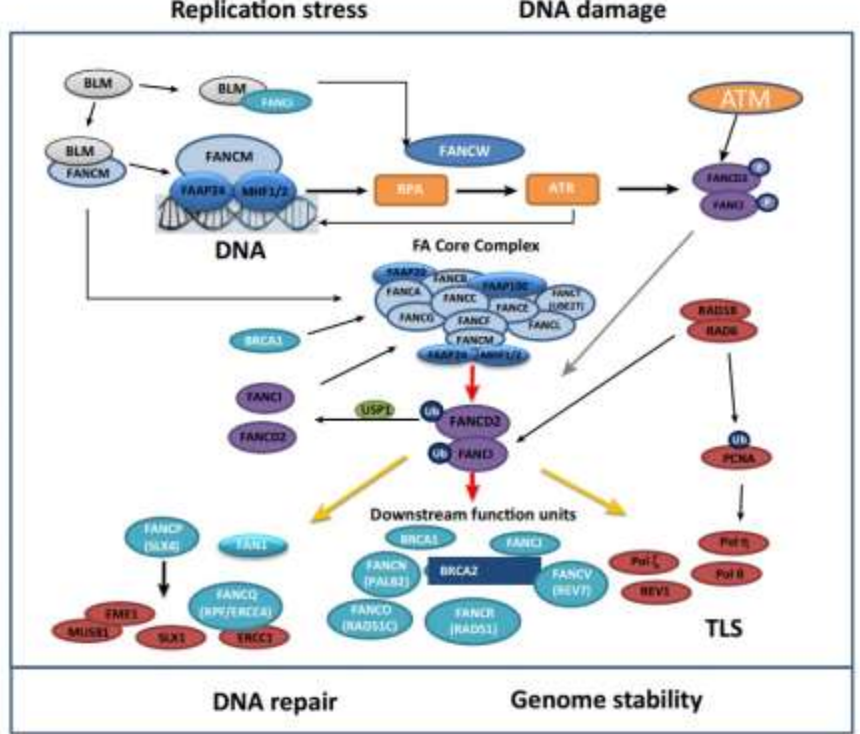


Figure 2.14.1.: Signaling pathway involved in Fanconi anaemia

(Nepal et al., 2017)

FA pathway refers to the signalling transductions that involve one or more FA proteins. In FA signalling, a group of proteins, including FANCA, B, C, E, F, G, L, M, T, and possibly I, along with FAAPs such as FAAP 20/24/100 and MHF1/2, work together to ensure the proper functioning of the ubiquitin E3 ligase. This results in the monoubiquitination of FANCD2 and FANCI. When FANCD2 and FANCI are monoubiquitinated, they work together to coordinate the repair of DNA damage through various mechanisms such as BER, NER, TLS, HR, and NHEJ. This ensures the stability of the genome with the involvement of other FA proteins and proteins outside of the FA pathway. Furthermore, the enzyme USP1 has the ability to remove ubiquitin from FANCD2/I, ultimately rendering the FA-BRCA pathway inactive. Several proteins, including ATM, ATR, and HHR6, as well as others like FANCM and FAAPs that are either known or yet to be discovered, play a crucial role in regulating checkpoint responses during DNA damage or replicative stresses. The canonical FA pathway is indicated by the red arrowheads. In FA signalling, an example of pathway independent roles is FANCM-centered sensing upstream, which may involve FANCW's modulation of RPA functions.

2.14.2. Diagnosis of FA

To diagnose the FA, the current criteria involve the detection of elevated chromosomal breakage in cells that are cultured using DNA-crosslinking agents. The presentation of FA varies

according to the age of the patient. During the early stages of life, the most prominent characteristic is the presence of physical abnormalities. It is common for 60% of patients to have a physical anomaly, such as short stature, hypo- and hyperpigmented skin lesions, café au lait spots, or abnormalities of the radial ray (30%), which may affect the thumb. About 20-25% of cases present with microphthalmia, structural renal abnormalities, microcephaly, and hypogonadism. Many individuals with this condition experience endocrine abnormalities, comprising approximately two-thirds of cases. These abnormalities may include growth hormone deficiency and short stature, midline brain abnormalities, hypothyroidism, obesity, abnormal glucose and insulin metabolism, and dyslipidemia. Moreover, most FA patients develop BMF between ages 5 and 15 years, with 6.5 years being the median age of diagnosis. FA often presents with hematologic abnormalities such as pancytopenia accompanied by macrocytosis and the prolonged presence of fetal haemoglobin. It is common for thrombocytopenia to occur before anaemia and neutropenia. Currently, in clinical practice, if there is still suspicion of FA diagnosis despite a negative chromosomal fragility test on peripheral blood, it is recommended to perform the test on skin fibroblasts. Patients with mosaic FA can be identified through a positive skin fibroblast test, which is reported to make up to 25% of all cases. (Lo Ten Foe et al, 1997). The bone marrow can exhibit a range of findings, from normal cellularity to complete marrow aplasia, with an increase in lymphocytes, plasma cells, and mast cells, or complete aplasia that is indistinguishable from idiopathic aplastic anaemia. It is possible for patients with FA to exhibit varying degrees of dyshaematopoiesis, and they may also have cytogenetic clones like monosomy 7 or add 3q. The impact of these clones on prognosis remains uncertain, but the growth of clones with enhanced proliferation does contribute to the development of MDS/AML.

2.14.3. Treatment of FA

For a long time, androgens have been utilized to manage BMF in patients with FA. In 7 out of 8 patients, a starting dose of 5 mg/kg per day of Danazol resulted in a response within six months. Some subjects maintained this response for up to 3 years, even with a reduction to half of the dose. There were no severe or unacceptable side effects that led to the discontinuation of therapy (Scheckenbach et al., 2012). When it comes to treating BM failure in FA patients, androgens and haematopoietic growth factors are often successful, but some patients become unresponsive to these treatments. In these cases, HSCT is a likely option as long as a donor is available.

In the last two decades, there has been a significant improvement in the outcome of HSCT. This is due to the reduction of preparative regimens that previously caused excessive toxicities in FA patients, who are highly sensitive to DNA alkylating agents and irradiation. Survival rates significantly improved following the introduction of fludarabine (Flu) in the conditioning regimen (Wagner et al., 2007; De Latour et al., 2013). Many medical professionals prefer using bone marrow cells over peripheral blood stem cells due to the latter's association with lower survival rates and a higher incidence of graft-versus-host disease. To avoid acute posttransplant morbidity, it is essential to steer clear of GvHD due to the heightened risk it poses as a result of the underlying DNA repair defect and increased cell apoptosis tendency, mortality and may lead to late post-transplant malignancies (De Latour et al., 2013). Now that many patients with FA survive their haematopoietic problems, prevention, surveillance, and treatment of solid tumours are becoming more critical. Because radiotherapy and chemotherapy may cause severe side effects, surgery is the primary therapy for solid tumours in FA. Gene therapy is emerging as a new form of therapy that could be considered an option. Recently, there have been advancements in gene therapy

protocols that involve lentiviral vectors and do not require a conditioning regimen. The initial reports are positive and are anticipating the conclusion of these trials to understand better the potential of this therapeutic tool (Adair, 2016; Rio, 2016).

2.15. Myelodysplastic syndrome

Myelodysplastic syndromes refer to stem cell malignancies that are clonal in nature. They are recognized by cytopenias, dysplasia in one or more myeloid cell lineages, inefficient haematopoiesis, and a higher likelihood of developing AML. The classification of this condition is determined by various factors such as the percentage of bone marrow and peripheral blood blasts, the type, degree, and number of dysplastic cell lineages, the presence or absence of ring sideroblasts, and the occurrence of specific chromosomal abnormalities (Zeidan et al., 2019). At diagnosis, the median age of diagnosis is approximately 70 years old, with an annual incidence rate that climbs to 25 per 100,000 in those aged 65 years or older. The estimated incidence of MDS is 4.3 and 1.8 per 100,000 individuals per year in the United States and Europe, respectively. Some Asian countries report lower rates, while other parts of the world have less well-estimated figures. Other risk factors like older age, male sex, obesity, smoking, and previous subjection to toxins of radiation therapy or chemotherapy. It is observed that MDS is more prevalent in men as compared to women, with yearly incidence rates of around 5.4 and 2.9 per 100,000, respectively. In 10-15% of MDS patients, there is a possibility of progression to acute myeloid leukaemia (Visconte et al., 2014). The Revised International Prognostic Scoring System (R-IPSS) has been updated to include more distinct cytogenetic prognostic subgroups into low and high-risk MDS and enhance the accuracy of prognostic stratification in MDS. The IPSS-R considers the severity and quantity of cytopenias, the percentage of blasts in the BM, and the likelihood of particular cytogenetic abnormalities being present. The intermediate-risk IPSS-R group is varied, with certain patients

exhibiting a slower progression similar to lower-risk MDS while others experience a more aggressive form of the disease (Greenberg et al., 2012).

2.15.1. Pathophysiology of MDS

MDS typically exhibits genetic defects, including chromosomal aberrations, gene mutations, copy-number alterations, and abnormal gene expressions. Abnormal clones can emerge during the course of a disease, exacerbating the patient's condition. Though they may be present at the onset of the disease, they can also appear later in the course of the illness (Solé et al., 2000). It is crucial to consider genomic instability, including deletions and translocations, as they may include areas that hold tumour suppressor genes (TSGs) that are significant to MDS biology (**Figure 2.15.1**). It has been noted that around 50% of primary and 80% of therapy-related MDS (t-MDS) cases have unbalanced translocations (Le Beau et al., 1986). Complex karyotype (3 or more defects) is usually correlated with dismal outcomes and can be seen following chemo/radiotherapy and toxic chemicals exposure (Greenberg et al., 2012).

Somatic mutations in multipotent stem cells play a role in the development of MDS, although an evident defect has yet to be identified. Research suggests that AML risk is higher in individuals with genomic instability caused by genetic defects or mutations. In fact, it has been estimated that around 78% of MDS patients have at least one somatic mutation (Papaemmanuil et al., 2013). Recurrent mutations hold significant clinical value, particularly for prognosis, diagnosis, risk stratification, and treatment response. Certain mutations, such as TP53, EZH2, ETV6, RUNX1, and ASXL1, have been linked to low overall survival rates (Bejar et al., 2011, 2012). Certain mutations in TP53, TET2, DNMT3A, and SF3B1 can indicate progression or predict response to treatment in low-risk MDS. SF3B1 mutations are also helpful in distinguishing between clonal and non-clonal causes of MDS (Visconte et al., 2015).

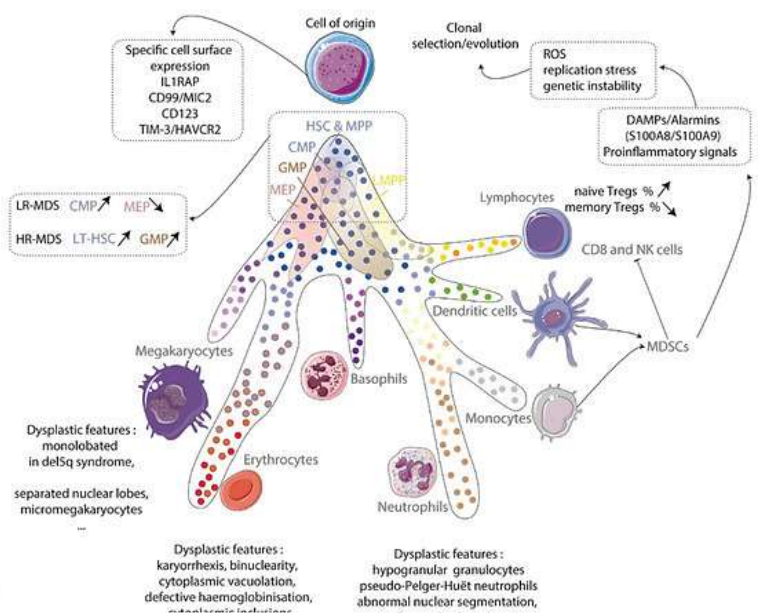


Figure 2.15.1.: Cells of origin in MDS

(Fontenay et al., 2021)

Myelodysplastic syndrome begins with a clonal haematopoietic stem cell. This particular cell exhibits distinct surface markers and promotes haematopoiesis that favours myeloid cells over lymphoid cells. The haematopoietic stem and progenitor compartments are disrupted, leading to the production of abnormal megakaryocytes, erythroblasts, and granulocytes. The production of alarms by myeloid-derived suppressive cells (MDSCs) within an inflammatory microenvironment leads to the generation of ROS, replication stress, and genetic instability, ultimately driving clonal evolution.

2.15.2. Diagnosis of MDS

MDS often exhibits chromosomal abnormalities such as -5/5q-, -7/7q-, +8, 20q-, +21, 12p, 13q-, and 17p-. These abnormalities are detected in 40-60% of cases and play a crucial role in the prognostic scoring systems used (Bejar, 2013; Estephan & Tiu, 2014). Conventional metaphase cytogenetics (MC) has limited sensitivity, reaching only 10%. It is informative in only 46-59% of MDS patients due to non-viable cells or non-informative karyotypes, resulting in some subtle chromosomal abnormalities being undetectable or masked. In cases where defects are undetectable

and there is no growth, FISH has proven to be a helpful complement to MC for chromosomes 5, 7, 8, 11, 13, and 20. However, studies comparing FISH to MC have not determined if FISH is superior. Spectral karyotyping has been used in combination with FISH to enhance the detection of minimal chromosomal rearrangements and monosomies (Imataka & Arisaka, 2012). SNP-A karyotyping, a DNA hybridization and fluorescence technique, is used clinically as a diagnostic tool to improve detection rates of MC with limited sensitivity. In MDS patients with normal karyotype, SNP-A has the ability to identify cryptic lesions such as copy neutral-loss of heterozygosity (CN-LOH) or acquired somatic uniparental disomy in 50% of cases. Certain lesions may be harmful, and the use of MC and SNP-A is important in identifying them, as the number of SNP lesions in MDS can impact prognosis (Gondek et al., 2008). Lately, whole exome or whole genome sequencing and deep sequencing have proven useful in detecting germline and somatic variants in MDS.

2.15.3. Treatment of MDS

The treatment options available for patients with MDS depend on the severity of the disease. Through clinical characterization, it has been determined that these patients are categorized into various prognostic risk groups, thereby requiring distinct treatment approaches (Greenberg et al., 2012). Low-risk MDS patients are typically recommended to receive supportive care, which may include transfusions, growth factors, and antibiotic therapy. To delay the transformation to acute myeloid leukaemia, medical professionals often use chemotherapy regimens such as hypomethylating therapy (HMT), which involves decitabine and 5-azacytidine, as well as immunomodulatory agents like lenalidomide, cytarabine, idarubicin, and daunorubicin. These treatments are commonly administered to high-risk MDS patients. Azacitidine and decitabine, which are hypomethylating agents, exhibit response rates of over 40% for higher-risk

(HR) patients. However, lenalidomide has a response rate of 60% to 70% for del(5q) patients and 20% to 30% for non-(del5q) patients (Fenaux et al., 2009; Kantarjian et al., 2007; List et al., 2006). It is crucial to conduct clinical trials for new and effective drugs as a significant number of MDS patients do not respond to treatment or experience relapse after the initial response. Treating these patients can be challenging due to their biological heterogeneity to different cytogenetic and myeloid cell mutational profiles (Bejar & Greenberg, 2017). Eventually, achieving long-term remission involves the utilization of high-dose chemotherapy and transplantation of haematopoietic stem cells (HSCs Over the past decade, significant progress has been made in molecularly characterizing MDSs (Bejar et al., 2011; Haferlach et al., 2014). New studies show that specific mutations in MDSs cause signalling pathway dysfunction and affect disease prognosis and drug effectiveness. Furthermore, emerging data show that immune-related mechanisms can hinder the body's capability to eradicate malignant clones in MDSs (Yang et al., 2014). Currently, many biospecific agents are being studied in clinical trials to target the pathogenetic mechanisms in MDS. Promising findings from ongoing research studies indicate the possibility of utilizing agents that target specific disease mechanisms for the treatment of this category of disorders.

2.16. National and International status

Aplastic anaemia has an incidence of 1.5 to 7 cases per million inhabitants per year with a median age of diagnosis of 25 to 60 years, varying by geography (Vaht et al., 2017). A comprehensive and precise data on the epidemiology of AA is unavailable. The variation in occurrence of AA could be due to distinct pathophysiology and various environmental factors (Dolai et al., 2023).

Mesenchymal stromal cells play an important role in support, maintenance, and regulation of HSCs. In several haematological disorders, aberrant MSCs may create a microenvironment that

inhibits normal cell function and promotes disease progression. AA derived MSCs have shown abnormal morphology, reduced proliferation, clonogenic potential, and increased apoptosis than normal healthy MSCs (Chao et al., 2010). Studies have reported that AA MSCs have higher adipogenic than osteogenic differentiation. Co-culture with MNCs results in lower suppression of T cell proliferation by AA MSCs (Chaturvedi et al., 2018; Huo et al., 2020). Contrarily, the latest studies have shown that MSCs obtained from patients with acquired AA do not show any significant differences in differentiation potential when compared to healthy individuals. (Bueno et al., 2014;Sharma et al., 2021). The findings of these studies have been contentious. Further investigations of stromal component in AA patients will help in better understanding the mechanism.

Several studies have shown that patients with AA have varying telomere lengths in leukocytes, with a higher proportion of patients having shorter telomeres compared to healthy individuals (Ball et al., 1998; Brümmendorf et al., 2001). This suggests that telomere length may be a factor in the pathophysiology of AA. Lately, studies have been reported on telomere length in AA in Indian population (Adhikari et al., 2021; Mandal & Dutta, 2020). A high risk of relapse, poor response to treatments and the development of MDS was observed in AA patients with shorter telomeres at diagnosis (Baerlocher et al., 2005; Sakaguchi et al., 2014). The length of leukocyte telomeres in the donor has a positive correlation with survival after allogenic unmatched haematopoietic cell transplantation, while the recipient's telomere length does not seem to have an impact (Gadalla et al., 2015). Studies have reported shorter telomere length in FA compared to age-matched controls(Leteurtre et al., 1999,Hanson et al., 2001). Similarly MDS patients have shorter telomere lengths than normal controls, but the association of telomere length to other clinical parameters in MDS has been inconsistent (Göhring et al., 2012; Lange et al., 2010;

Rollison et al., 2011). Although the pathophysiology of AA has been discussed extensively, a thorough understanding of the condition and how it affects the Indian population is essential.

The observance of recovery after failed bone marrow transplant in aplastic anemia led to the treatment with immunosuppressive therapy. About half of the patients treated with antithymocyte globulin showed improvement in their blood cell counts (Young & Maciejewski, 1997). The most widely utilized method of immunosuppressive for patients with SAA is the combination of ATG and CSA. It has been observed that a significant percentage, ranging from 60% to 70% of patients show positive hematologic improvement in response to immunosuppression (Rosenfeld et al., 1995; Rosenfeld et al., 2003). Despite successful treatment, around one-third of responders experience a relapse. Also, myelodysplasia may manifest in 10% to 15% of cases due to clonal evolution, which typically occurs later (Socié et al., 2000). Earlier studies conducted in India have reported ATG (ATGAM) response rates at sixth month ranging from 40% to 87.9%, in both children and adult AA patients (Nair et al., 2012; Nair et al., 2013; Mahapatra et al., 2015; George et al., 2015; Lionel et al., 2023). Since the understanding of aberrant immune homeostasis causing haematopoiesis destruction in AA, it has led to studies investigating on immune cells, subsets, and cytokines produced (Feng et al., 2011; Liu et al., 2020; Lu et al., 2015; Shi et al., 2012). Few studies in India have investigated observed aberrant expression profile of IL-8, IFN-γ, TNF-α and IL-2 cytokines in BM T-cells and their levels in BM plasma of AA patients (Bhargawa et al., 2022; De et al., 2019).

3.0. MATERIALS AND METHODS

3.1. Study participants

Patients diagnosed to have bone marrow failure syndromes at the Department of Haematology, Christian Medical College, Vellore, India, between 2001 and 2022 were included in the study. At diagnosis, PB samples were collected from BMF patients after obtaining informed consent, and bone marrow samples were collected from AA patients through BM aspiration. The telomere study included retrospectively stored DNA samples during diagnostic procedures before transplant and prospectively collected PB samples. PB and BM samples were collected prospectively for cell-culture analysis. Further, PB blood samples of AA patients treated with ATG at baseline and post-treatment were collected prospectively. Patients below 15 years of age were considered to belong to the pediatric group.

3.2. Ethical Review

The Institutional Review Board of Christian Medical College, Vellore, approved this study (IRB Min. No. 9491 dated 24.06.2015 and IRB Min. No. 12929 dated 24.06.2020).

3.3. Patient recruitment

The study cohort comprised three patient groups along with a cohort of healthy controls, and the inclusion criteria are listed below for each group. Only patients willing to sign the informed consent and fulfilling the inclusion criteria were included in the study.

3.3.1. Aplastic anaemia

The diagnosis of aplastic anaemia was made based on standard criteria, i.e., there must be at least two of the following: (i) haemoglobin <100 g/l (ii) platelet count <50 × 10^9/l (iii) neutrophil

count $<1.5 \times 10^9$/l. The severity of AA was categorised according to the modified Camitta criteria (Camitta et al., 1975, Bacigalupo et al., 1988). Severe AA (SAA) was defined as having marrow cellularity <25% (or 25–50% with <30% residual haematopoietic cells), with at least 2 of (1) neutrophils $<0.5\times0^9$/l, (2) platelets $<20\times10^9$/l or (3) reticulocyte count $<20\times10^9$/l. Very severe AA (VSAA), as for SAA but neutrophils $<0.2\times10^9$/l, and non-severe AA (NSAA) as AA not fulfilling the criteria for SAA or VSAA. Clinical data of all these patients were collected from individual medical records and institutional databases.

3.3.2. Fanconi anaemia

The diagnosis of Fanconi anaemia was confirmed by a positive chromosomal breakage analysis using mitomycin C. In patients where the results of chromosomal breakage analysis were ambiguous, the diagnosis was confirmed using skin biopsy fibroblast culture analysis of the ubiquitination of the FANC-D2 gene.

3.3.3. Myelodysplastic syndrome

The diagnosis criteria of MDS include (i) one or more cytopenias that cannot be explained by a drug, toxin, vitamin deficiency, infection, or other condition: haemoglobin <100 g/l, absolute neutrophil count $<1.8 \times 10^9$/l, and platelets $<100 \times 10^9$/l, (ii) dysplasia in ≥10% of cells in a given hematopoietic lineage (erythroid precursors, granulocytes, megakaryocytes) in bone marrow or peripheral blood, (iii) presence of 5-19% blast cells, and (iv) presence of a specific MDS linked chromosomal abnormalities like del (5q), del (20q), +8 or -7/del (7q) (Arber et al., 2016). MDS patients have been classified according to the International Prognostic Scoring System (IPSS) risk score into lower-risk MDS and higher-risk MDS, based on the percentage of marrow blasts, number and extent of blood cytopenias, and marrow cell karyotype. Further, the revised version of the IPSS (IPSS-R) stratified patients into five risk groups with different outcomes regarding

AML evolution and survival of the same factors (Greenberg et al., 2012). Although there was no age cut-off for all the patient cohorts, we included patients from 1 to 60 years of age for analysis.

3.3.4. Hematopoietic stem cell transplantation cohort

Patients with aplastic anaemia who underwent matched-related sibling donor transplants during 2001-2019 were included. Patients undergoing transplants for known inherited bone marrow failure syndromes and patients having matched unrelated donors or haploidentical transplants were excluded. Transplant outcomes were recorded, which included graft failure, time to neutrophil recovery, acute and chronic graft incidence versus host disease, and overall survival. An absolute neutrophil count $\geq 0.5 \times 10^9/l$ achieved for three consecutive days was defined as neutrophil recovery. Acute graft versus host disease was graded per Glucksberg criteria, while chronic GVHD was described according to NIH consensus criteria. Clinical data of HSCT patients were collected from individual medical records and institutional databases.

3.3.5. Immunosuppressive therapy cohort

Aplastic anaemia patients treated with ATG and signing informed consent between 2018 to 2022 were included. This cohort comprised patients who had received treatment prior to ATG and other therapies at the time of diagnosis. Patients were given ATG (ATGAM, USA) at 40 mg/kg/day for four days. The classification of response to treatment was according to NIH criteria (Scheinberg, Scheinberg, et al., 2009b) as complete response (CR) having Hb $\geq$ 11 g/dl with transfusion independence, neutrophils $> 1.5 \times 10^9/l$ and platelets $> 100 \times 10^9/l$, partial response (PR) having Hb $\geq$ 8 g/dl with transfusion independence, neutrophils $> 0.5 \times 10^9/l$ and platelets $> 20 \times 10^9/l$ and no response (NR) when there is transfusion dependence. Red blood cells were transfused when Hb decreased to less than 7 g/dl and platelets while bleeding or fever were less than 20,000/ccmm.

3.4. Healthy controls cohort

Subjects considered healthy controls included HSCT donors, excluding those with beta-thalassemia and aplastic anaemia from 2001 to 2022. Other controls included healthy relatives of patients from whom peripheral blood samples were collected prospectively for the study. Bone marrow collected during the diagnostic procedure from lymphoma patients with absent marrow involvement was considered normal marrow samples for cell-culture experiments.

3.5. Haematological analysis

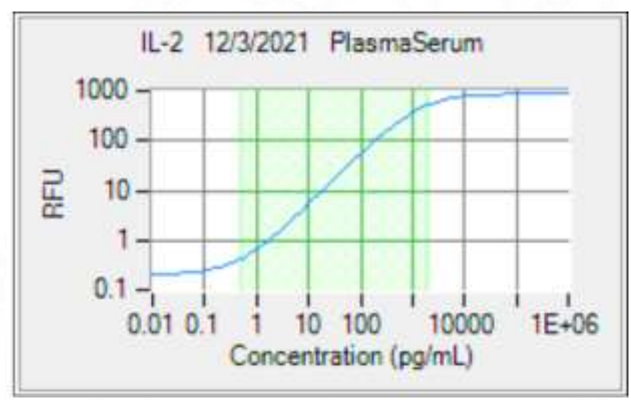
An automated Haematology analyzer (SYSMEX KX21) was used to measure the complete blood count. The morphology of bone marrow cells was determined microscopically using cytochemical stain.

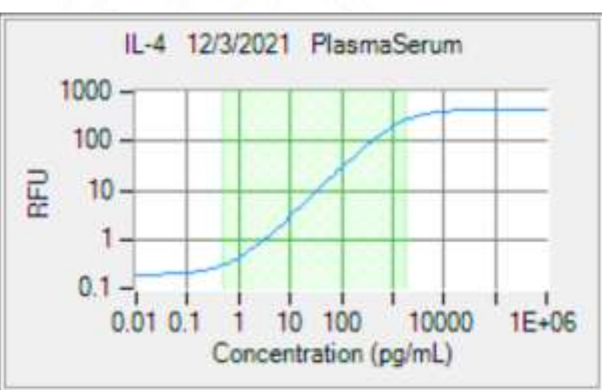
3.6. Biochemical analysis

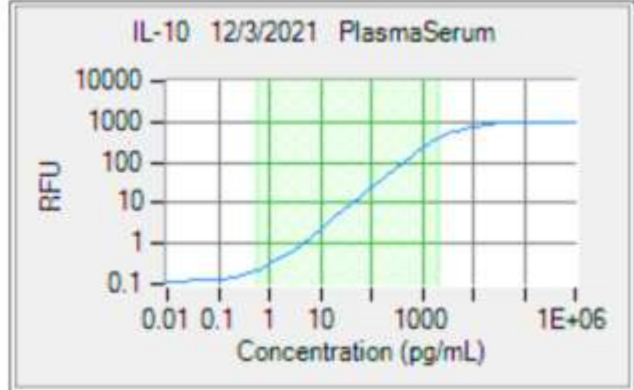
3.6.1. Cytokines analysis

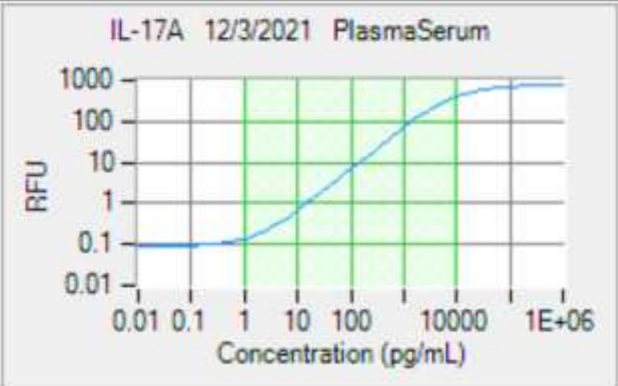
Serum/plasma was isolated from peripheral blood and stored at -80ºC until analysis. Cytokine expression levels were determined in serum/plasma using Simple Plex, an integrated immunoassay system for rapidly and sensitively detecting the targeted protein antigens. To conduct Simple Plex assays, a disposable microfluidic cartridge and an automated analyzer called the Ella instrument are used, following the instructions provided by the manufacturer (ProteinSimple, San Jose, CA, USA). Briefly, human serum samples were diluted 1:2 for interleukin (IL)-2, IL-4, IL-10, IL-17A, IL-22, TNF-α, IFN-γ and TGF-β in the sample diluent. The diluted, quality control, and buffer samples were loaded into each cartridge. To identify the cartridge, a barcode scanner was utilised, which automatically loads lot-specific standard curves that are factory-calibrated and embedded in each cartridge's barcode. SimplePlex Runner software was used for identifying

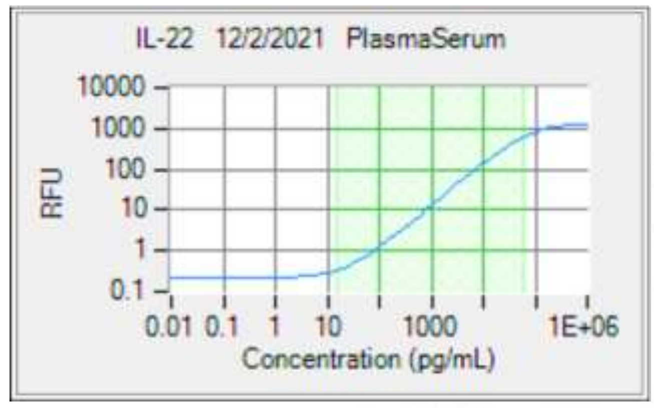
samples and dilution factors. The assay provides triplicate results (one per glass nano-reactor) for every analyte in each sample that was automatically displayed. Raw (background-subtracted) signal levels are reported in relative fluorescence units (RFU) for each glass nano-reactor. Mean RFU signal values, standard deviation, and coefficient of variation are provided for triplicate glass nano-reactors. The RFU values were automatically back-fit to barcode-embedded standard curves. Back-fit concentrations were multiplied by user-defined dilution factors to provide calculated concentrations in picograms per millilitre for each analyte and every sample.

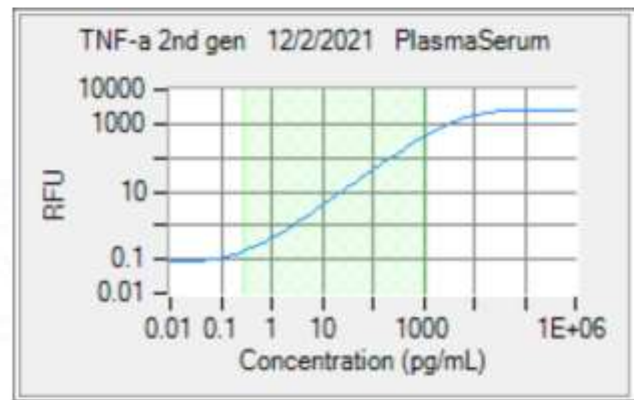


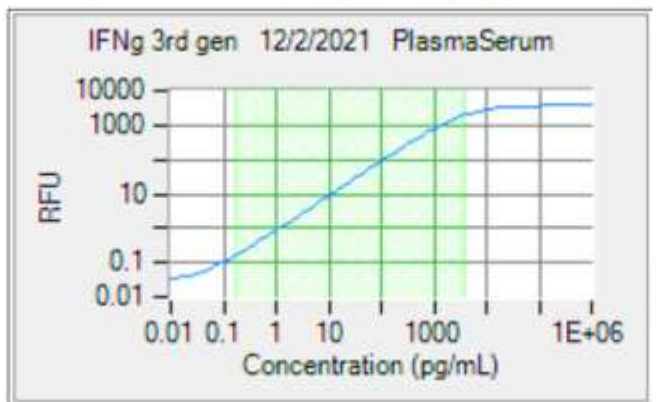












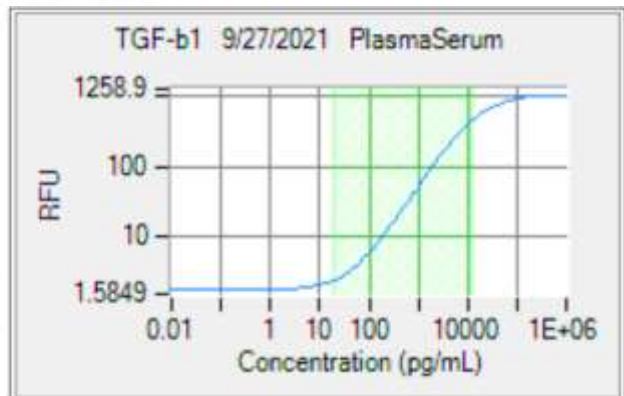


Figure 3.6.1.: Standard curve generated in a lot-specific factory-calibrated cartridge

Table 3.6.1.: Coefficients of the standard curve

Analyte	a	b	c	d	g	LLOQ	ULOQ	units
IL-2	0.20	1.06	1268.41	872.84	1.00	0.540	2050	pg/ml
IL-4	0.19	1.06	1258.46	458.23	1.00	0.500	1920	
IL-10	0.12	1.06	3417.62	1030.43	1.00	0.580	2212	
IL-17A	0.09	1.07	8130.59	751.75	1.00	1.05	10000	
IL-22	0.21	1.10	69516.81	1374.47	1.00	16.60	63300	
TNF-A	0.08	1.05	4905.05	2676.29	1.00	0.30	1160	
IFN-G	0.03	1.02	3616.26	3772.30	1.00	0.17	4000	
TGF-B	1.80	1.09	15191.90	1033.39	1.00	20.8	12684	

3.7. Molecular analysis

3.7.1. DNA extraction

Peripheral blood samples were collected from study participants in an anticoagulated EDTA tube**.** Genomic DNA was extracted from whole blood, MSCs and $CD34^+$ cells using Puregene gentra Kit (Qiagen, Maryland, USA) and stored at 4^0C till analysis. A NanoDrop spectrophotometer was utilised to determine the quality and quantity of DNA (Thermo Scientific, USA). The 260/280 absorbance ratio was determined to evaluate the purity, and samples within the range of 1.8-2.0 were further processed.

3.7.2. RNA extraction

Total RNA was extracted after 21 days from the osteogenic and adipogenic differentiated cells using the Trizol reagent (Invitrogen, CA, USA) as per standard protocol and stored at -80ºC for further use. Briefly, the cells were lysed in trizol reagent and added 0.2ml chloroform per 1ml of trizol reagent. The mixture was incubated for 15 minutes and centrifuged at 13000g for 15 minutes. The aqueous phase was collected slowly and transferred into a fresh tube while an equal volume of isopropanol was added. This mixture was incubated for 10 minutes and centrifuged at 13000g for 10 minutes, during which RNA was precipitated and washed with 75% ethanol. The RNA pellet was air-dried, suspended in RNase-free water, and stored at -80ºC until further use. The concentration of extracted RNA was assessed using a nanodrop microvolume UV-Vis spectrophotometer (Thermo Scientific, USA). The RNA purity was analysed by calculating the absorbance ratio of 260/280, ranging from 1.9-2.2.

3.7.3. cDNA synthesis

The RNA (0.5 µg) was reverse transcribed with the High-Capacity cDNA Reverse Transcription (RT) Kit (Invitrogen, CA, USA) using the manufacturer's protocol. The following PCR conditions were followed: 25ºC for 10 minutes, 37ºC for 120 minutes, and 85ºC for 5 minutes, and the cDNA samples were stored at -20ºC.

3.7.4. Polymerase chain reaction (PCR)

The expression of the housekeeping gene ACTIN was assessed using PCR, and PCR products were analysed using 2% agarose gel (**Figure 3.7.4.**). The primer sequence (**Table 3.7.4.**), master mix and PCR conditions are given in **Table 3.7.4.1**.

Table 3.7.4.: Primer sequence of ACTIN gene

Gene Name	Primer sequence
ACTIN	Forward - GGCGGCACCACCATGTACCCT
	Reverse - AGGGGCCGGACTCGTCATACT

Table 3.7.4.1.: The composition of Mastermix and PCR conditions to amplify ACTIN gene from synthesised cDNA

Sl no	Reagents	Volume (µl)
1	Emerald Mastermix	7.5
2	Forward primer	0.5
3	Reverse primer	0.5
4	Water	5.5
5	cDNA	1
	Total volume	15

Sl no	Temperature and time	Cycles
1	95^0C – 5 minutes	1
2	94^0C – 30 seconds	35
3	60^0C – 1 minute	
4	72^0C – 1 minute	
5	72^0C – 15 minutes	1
6	4^0C – Hold	

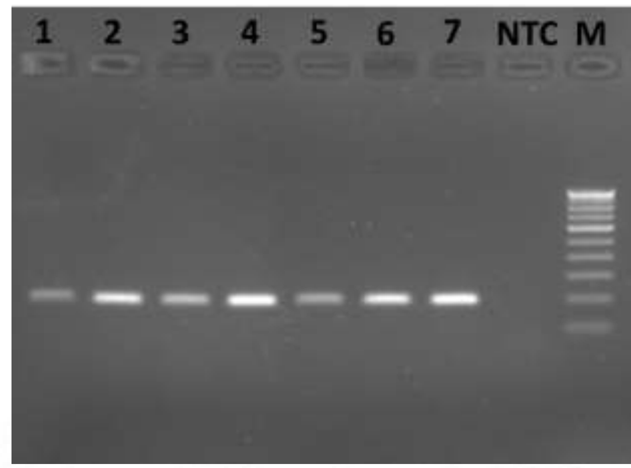


Figure 3.7.4.: Amplification of ACTIN from cDNA in agarose gel electrophoresis
(Lane M: 100bp marker, Lane 1-7: cDNA samples showing amplification of ACTIN, Lane NTC: Negative control)

3.7.5. Quantitative real-time PCR

The PCR reactions were performed on a **7500 Fast Real-time PCR System** (Applied Biosystems, USA), using Takyon SYBR Green PCR Master Mix (Eurogentec, Belgium). After each run, a melting curve stage was set up to affirm product specificity. The delta-delta-Ct method was employed to determine the relative gene expression level of the gene of interest (ALP, RunX2 for osteogenic lineage and LPL, FABP4 for adipogenic lineage) normalised to the ACTIN gene as the endogenous control. The primer sequence used for osteogenic and adipogenic lineage genes is mentioned in **Table 3.7.5**.

Table 3.7.5.: Primer sequence of osteogenic and adipogenic lineage genes

Sl No	Primer name	Primer sequence (5' – 3')
1	ALP Forward	GGAACTCCTGACCCTTGACC
2	ALP Reverse	TCCTGTTCAGCTCGTACTGC
3	Runx2 Forward	CCCGTGGCCTTCAAGGT
4	Runx2 Reverse	CGTTACCCGCCATGACAGTA
5	LPL Forward	TCATTCCCGGAGTAGCAGAGT
6	LPL Reverse	GGCCACAAGTTTTGGCACC
7	FABP4 Forward	ACTGGGCCAGGAATTTGACG
8	FABP4 Reverse	CTCGTGGAAGTGACGCCTT

3.7.6. Telomere length measurement using real-time qPCR

The relative telomere length (rTL) was measured using quantitative real-time polymerase chain reaction from the extracted DNA, as described by Cawthon 2002. Briefly, reference DNA at concentrations of 40, 20, 10, 5 and 2.5 ng/µl by serial dilution were prepared from standard genomic DNA. Two master mixes of PCR reagents were prepared, one with telomere (TEL, T) primer pair and another with single copy gene (RPLP0, also termed 36B4; S) primer pair for standard curve [The 36B4 gene, encoding acidic ribosomal phosphoprotein PO, is located on chromosome 12]. The telomere and single-copy gene-specific primer sequences are given in **Table 3.7.6**. A master mix of 10µl volume (**Table 3.7.6.1.**) was prepared containing Takyon SYBR Green PCR Master Mix (Eurogentec, Belgium), lambda DNA (New England Biolabs, USA), the assay-specific mix of primers and genomic DNA normalised to 10ng/µl. One non-template control, one positive control, and standard genomic DNA were amplified in duplicates in each run. The PCR

thermal conditions for relative telomere length assay using telomeric primers and single copy gene primers consisted of an initial denaturation of 10 min at 95ºC, followed by a total of 40 cycles at 95ºC for 15 seconds, 58ºC for 60 seconds, and subsequently melt curve stage. All telomere and 36B4 reactions were performed in duplicates, conducted in 96-well plates, and the experiment was conducted in a **7500 Fast Real-time PCR System** (Applied Biosystems, USA).

Table 3.7.6.: Telomere and single copy gene-specific primer sequences

Sl no	Primer Name	Primer sequence
1	TEL Forward	5' GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT 3'
2	TEL Reverse	5' TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA 3'
3	36B4 Forward	5' CAGCAAGTGGGAAGGTGTAATCC 3'
4	36B4 Reverse	5' CCCATTCTATCATCAACGGGTACAA 3'

Table 3.7.6.1.: The composition of the master mix for telomere length measurement

Sl no	Reagents	Volume (µl)
1	SYBR Mastermix	5
2	Forward primer	0.125
3	Reverse primer	0.125
4	Lambda DNA	0.5
5	Water	2.25
6	Genomic DNA	2
	Total volume	10

3.7.6.1. Calculation of relative telomere length using qPCR

Relative telomere length was expressed as a ratio of the quantity of telomeric DNA (T) normalised to the copy number of a single-copy nuclear gene (S), yielding the T/S ratio. The ratio between telomeric DNA (T) and single copy gene (S) was enumerated for each DNA sample. The relative T/S ratio was calculated using a reference genomic DNA sample in all plates. The relative T/S ratio was achieved using the formula $2^{-(\Delta Ct-\Delta Cs)} = 2^{-\Delta\Delta Ct}$. The value of the T/S ratio is directly proportional to the average telomere length.

3.7.6.2. Calculation of relative age-adjusted telomere length

The rTL values were log-transformed to ensure normality. Age-adjusted telomere length for each patient was computed as follows: [(observed log rTL-linear predicted rTL)/root mean square error (RMSE)]. The linear predicted rTL and RMSE were derived from a linear regression model of log rTL and age from healthy controls. The age-adjusted relative telomere length was then normalised (Y. Wang et al., 2020).

3.8. Targeted gene panel using Next-generation sequencing

The quality and quantity of the DNA were assessed by agarose gel electrophoresis and fluorometer (Qubit, Invitrogen), respectively. The targeted gene capture was performed from DNA using a custom capture kit covering 2196 genes associated with various inherited haematological disorders, haematological malignancies, and inherited bone marrow failure syndromes. Among the 2196 genes, the analysis was restricted to 96 genes related to IBMFS. The panel included exons, UTRs, and exon-intron junctions with a minimum of 100 intronic bases at the flanking ends. The sequencing process utilised the Illumina HiSeq sequencer, which produced 2 x 150 bp paired sequence reads at a depth > 80x.

3.9. Whole Exome Sequencing and Analysis

The extracted DNA samples were quantified using Qubit DNA BR Assay (Invitrogen). The purity of DNA was examined using QIAxpert and 1% Agarose gel to approve DNA integrity. After confirmation, the samples that passed the quality check passed was taken for the library preparation and sequencing. Whole genome libraries were prepared using SureSelect XT Library prep kit (Agilent Technologies) and Whole Exome capture (using AGV5+UTR probe). After pooling and diluting the libraries, they were loaded to achieve the optimal concentration for cluster amplification on the Illumina flow cell. After the completion of cluster generation, the clustered flow cell was sequenced using the HiSeq X instrument (Illumina Inc) to generate 13GB of 150bp paired end reads. Further, the comprehensive WES data were also manually investigated for potential novel IBMF-causing genes. The pathogenic effect of the variants was assessed using guidelines from the American College of Medical Genetics and Genomics.

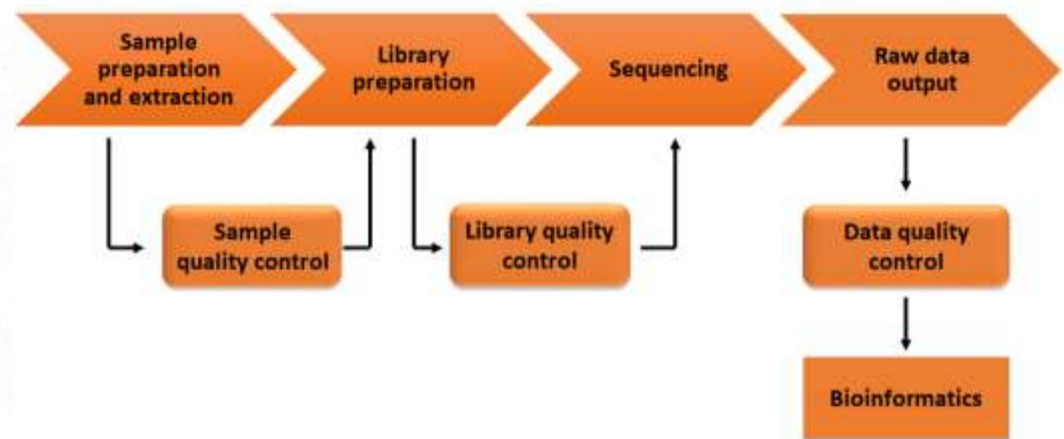


Figure 3.9.: Next-generation sequencing workflow

3.9.1. NGS Data Analysis

Genome Analysis Toolkit (GATK) best practices pipeline was carried out to perform bioinformatics analysis. Briefly, FASTQ files generated from the sequencer were converted into

binary alignment map (BAM) files following alignment with the human reference genome (GRCh37/hg19) and eventually to variant call format (VCF) files. The generated VCF files were converted to Excel-compatible, comma-separated values (CSV) files using web-based annotate variations (ANNOVAR) software (Chang & Wang, 2012). The non-coding variants other than splice site variants were removed from the data. Common variants were filtered based on the minor allele frequency (MAF) in geographically matched population databases like 1000 Genome, genome aggregation database (gnomAD), exome aggregation consortium (ExAC), and single nucleotide polymorphism database (dbSNP), where variants identified in more than 1% of the population (MAF>0.01) were filtered out. The functional implication of the missense variants was evaluated using various computational algorithms, namely sorting intolerant from tolerant (SIFT), protein variation effect analyzer (PROVEAN), polymorphism phenotyping (PolyPhen2), likelihood ratio test (LRT), functional analysis through hidden Markov models (FATHMM), MutationTaster, and MutationAssessor. The pathogenicity of the non-coding variants was reported based on MutationTaster, MutationAssessor, database splicing consensus single nucleotide variants (dbscSNV), and FATHMM. Human genome mutation database (HGMD), online Mendelian inheritance in man (OMIM), and clinically relevant variant (ClinVar) database records were looked at to determine the clinical significance of reported variants. Based on these parameters and the American College of Medical Genetics and Genomics (ACMG) guidelines, the variants were labelled as pathogenic/likely pathogenic/variant of uncertain significance (VUS)/likely benign/benign (Richards et al., 2015). The VCF files were validated on a platform (Varminer-variant interpretation tool), and the VEP program against the Ensembl release 91 human gene model was used to analyse gene annotation of the variants.

3.10. DNA Sanger Sequencing

Sanger sequencing was performed using the established protocols in our laboratory (Arun et al., 2018). Gentra Puregene Blood kit (Qiagen, Germany) was used to extract genomic DNA from peripheral blood cells. The quality and quantity of the extracted were assessed using a DNA Microvolume spectrophotometer (Nanodrop, Thermo Scientific) and agarose gel electrophoresis. Polymerase chain reaction (PCR) amplified in-house designed primers targeting all the exons and exon-intron junctions (**Table 3.10.**). The variants were analysed using bidirectional Sanger sequencing in Big Dye Terminator version 3.1 chemistry and analysed in an ABI3130 DNA analyzer (Applied Biosystems, Foster City, California). The sequences were aligned and analysed using the bioinformatics software Geneious (Kearse et al., 2012).

Table 3.10.: List of primer sequences for Sanger sequencing

Sl. No.	Primer name	Exon No.	Primer sequence (5' - 3')
1	MPL	EXON 7 F	CAGGACTACAGACCCCACAG
2	MPL	EXON 7 R	GGGTGGGAACTATGTGGGAA
3	MPL	EXON 11 F	TGCTTCTCTTCCTTCTCCCC
4	MPL	EXON 11 R	CAAGGATCCAGTACCAGGCA
5	MPL	EXON 10 F	GTGACCGCTCTGCATCTAGT
6	MPL	EXON 10 R	ACAGAGCGAACCAAGAATGC
7	MPL	EXON 5 F	GTGGAAGCTGCCTCATCTCA
8	MPL	EXON 5 R	TTGACTCACCTGCATCTCCA
9	TERT	EXON 11 F	GCACGGCTTTTGTTCAGATG
10	TERT	EXON 11 R	TCTGACCCTTTGGGATTGG
11	TERT	EXON 9 F	CCCGTCTCTCACCTGTGTCT
12	TERT	EXON 9 R	CAGAGCAGTCATGGTCTCCA
13	TINF2	INTRON 8 F	TCTGTGCAGCTCCGTCATTA

Sl. No.	Primer name	Exon No.	Primer sequence (5' - 3')
14	TINF2	INTRON 8 R	CATTCCCTGAACCCTCTGAA
15	TINF2	INTRON 6 F	GACCATCTTCAAGGACGCAC
16	TINF2	INTRON 6 R	TCTTCCTTGCTCTCAGGCTT
17	RTEL1	EXON 30 F	GCAGCAGTTTGAGGAGGTCT
18	RTEL1	EXON 30 R	CTTCCACGCAGGAGTCTGA
19	RTEL1	EXON 29 F	TGCAGGACTACAAGGGTTCC
20	RTEL1	EXON 29 R	AGACCACCTTGGACCCAGA
21	BRCA2	EXON 27 F	GGGGAGGGAGACTGTGTGTA
22	BRCA2	EXON 27 R	TTTGAAGTCATCTGGGCTGA
23	BRCA2	EXON 19 F	CTAACAGTACTCGGCCTGCTC
24	BRCA2	EXON 19 R	CCTGTATAGGGTATGCTCTTTGAA
25	ATM	EXON 45 F	CAGCAAAGAAGTAGAAGGAACCA
26	ATM	EXON 45 R	TTTCAGAAAAGAAGCCATGACA
27	FANCA	INTRON 27 F	AATGTGGGGTTGTGTGTGTG
28	FANCA	INTRON 27 R	GGGCCTCTGAGAACAATCTG
29	WRAP53	EXON 10 F	GAGCCCGTGTTGAGTTTTCT
30	WRAP53	EXON 10 R	ACTCTCTGTGGGCTCAGGAA
31	ITGA2B	INTRON 15 F	TATCCCATCAGAGCTCAGCC
32	ITGA2B	INTRON 15 R	GAGGAATGTTGTGCCCAGTG
33	DNAJC21	INTRON 7-8 F	TGCTCTGATCAAAGAGGGTTC
34	DNAJC21	INTRON 7-8 R	CATGATGGCAGGTTCCTGTA
35	GFI1	EXON 7 F	CATTCAGCCAGAGCTCCAAC
36	GFI1	EXON 7 R	GAGGTAAGGCGAAGGAGGAG
37	PRF1	EXON 3 F	GTCTGGGATCAGGACTCTGG
38	PRF1	EXON 3 R	GGACATAGTCCAGGCAGGTG
39	PRF1	EXON 3 F	CATGTGACCTTGAGCAGTCC
40	PRF1	EXON 3 R	GGGTGCCGTAGTTGGAGATA

Sl. No.	Primer name	Exon No.	Primer sequence (5' - 3')
41	NLRP12	EXON 3 F	TGCAAGACCTCATCTTCAGC
42	NLRP12	EXON 3 R	CGTGTGGTGATGAGCAAAGA
43	ERCC6L2	EXON 12 F	CGTGGGCTCTAATGACCAGT
44	ERCC6L2	EXON 12 R	TCTGAGATGGATGAAAAATGGCT

3.11. Cell culture

3.11.1. Mononuclear cells isolated from bone marrow

Mononuclear cells (MNCs) were separated by gradient centrifugation using lymphocyte separation medium (LSM) (MP Biomedicals, Irvine, CA, USA). The bone marrow is first diluted with 1X PBS and slowly layered onto LSM (1:2 ratio) and centrifuged at 400g for 30 minutes with slow acceleration and deceleration. The MNCs were taken and subjected to RBC lysis followed by PBS wash and used for further use.

3.11.2. $CD34^+$ cells isolation

The cells were sorted using EasySep Human CD34 positive selection kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocol. Briefly, the isolated BM MNCs using gradient centrifugation were suspended in a conditioning medium (PBS containing 2% FBS and 1mM EDTA) at a concentration of $1x10^7$ cells in 0.1ml. The EasySep™ Human CD34, Positive Selection Cocktail, was added to the cells and incubated at room temperature for 10 minutes. Then, EasySep™ Dextran RapidSpheres™ 50100 was added and incubated for 5 minutes. The tube volume was made up to 2.5 ml with conditioning medium, mixed well and placed in EasySep™ magnet for 3 minutes. The magnet was inverted in one continuous motion to discard the supernatant. After removing the tube from the magnet (the tube contains the

isolated cells), washing was performed three more times. In the final wash, cells were suspended in PBS and centrifuged to obtain the cell pellet. Cell numbers were determined using the cell counter.

3.11.2.1. Purity of CD34$^+$ cells

The purity of enriched CD34$^+$ cells was assessed by flow cytometer. The cells were suspended in 100µL of PBS, 2 µL of APC linked CD34 antibody (BD Pharmingen, San Diego, CA, USA) was added, and incubated in the dark for 20 mins. The cells were washed with PBS and acquired on a Navios flow cytometer (Beckman Coulter), and Kaluza software (Beckman Coulter) was used to analyse the results.

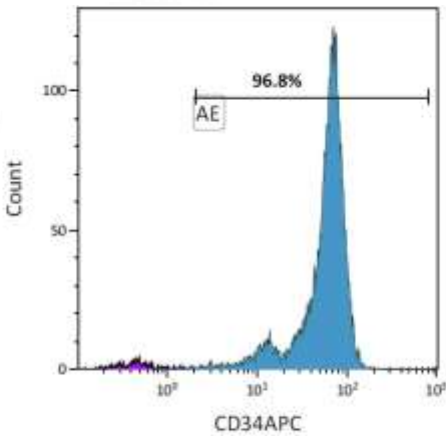


Figure 3.11.2.1: Purity assessment of CD34$^+$ cells by flow cytometry

3.11.2.2. CD34$^+$ cells expansion

CD34$^+$ cells were cultured for seven days in serum-free StemSpan SFEM Medium (StemCell Technologies, Vancouver, Canada), which was supplemented with StemSpan CD34$^+$ expansion supplement (StemCell Technologies), which contains Flt-3L, SCF, IL-3, IL-6 and TPO.

3.11.3. Mesenchymal stromal cells expansion

The separated bone marrow MNCs from AA and lymphoma patients were suspended in low glucose DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 units/ml penicillin and 100 ug/ml streptomycin in T25 flask. Cells were incubated in a humified atmosphere with 5% CO_2. The media was changed when cells were adherent, reaching approximately 80% confluence, and harvested using 0.05% trypsin/ ethylenediaminetetraacetic acid (EDTA). The cells in passages 2 or 3 were used for further experiments. The morphology of MSCs was observed using an inverted light microscope (Zeiss, Axiovert) (Hamzic et al., 2015).

3.11.3.1. Immunophenotyping of MSCs

Flow cytometry assessed the MSCs harvested at passage 2 for cell surface expression. The cells ($1\text{-}5\times10^5$) were incubated for 20 minutes with the following mouse anti-human pre-conjugated antibodies (BD Pharmingen, San Diego, CA, USA): CD73-peridinin chlorophyll (PerCP)- Cy5.5, CD90-phycoerythrin (PE)- Cy7, CD105-PE, CD14-fluorescein isothiocyanate (FITC), CD34-allophycocyanin (APC) and CD45-PerCP. The cells were washed in PBS supplemented with 0.5% NaN3 (Sigma-Aldrich) and 0.1% FBS and acquired on a Navios flow cytometer (Beckman Coulter). The data analysis was performed using the Kaluza software (Beckman Coulter).

3.11.3.2. Proliferative potential of MSCs

Approximately 5×10^4 MSCs of passage 2 were plated in a 24 well-plate, and the cells were counted after 96 hours. Population doubling (PD) was calculated with the equation:

$PD = \log_2 (N_t / N_o)$, N_o - initial cell count ($5x10^4$ cells), and N_t - final cell count after 96 hours. Population doubling time (PDT) was calculated using $PDT = (t - t_0) (\log_2)/ (\log N_t - \log N_o)$, t_0 - time of seeding, t - time of harvesting (96h) (Hamzic et al., 2015; Sharma et al., 2021).

3.11.3.3. Differentiation potential of MSCs

Approximately $5\text{-}8\times10^4$ MSCs were seeded in DMEM medium in 24 well plates until the cells reached 70-80% confluence. The osteogenic and adipogenic lineage induction using the osteogenic and adipogenic differentiation media (Hi Media Laboratories Pvt. Ltd) was performed for 21 days, with media change every three days. On day 21, cells were stained with Alizarin Red and Oil Red O. Alizarin red assay was quantified by eluting the stain in ethanol, and OD was measured at 595nm. The stain was eluted in isopropanol to quantify the oil red O, and OD was measured at 510nm (Chao et al., 2010).

3.11.3.4. Immunosuppression property of MSCs

MSCs were seeded at $1\times10^{\wedge 5}$ cells/mL in 24-well plates in DMEM supplemented with 10% FBS. Meanwhile, $1\times10^{\wedge 6}$ human peripheral blood mononuclear cells (PB-MNCs) were isolated by gradient centrifugation and stained with cell division tracking dye (Chao et al., 2018). The cells were incubated for 20min at 37°C 5μM with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen-Gibco) and washed with RPMI media to remove unbound cells. The MSCs and MNCs were co-cultured in the ratio of 1:10. The CFSE-stained MNCs were suspended in RPMI media and added to each well-containing MSCs and stimulated with 1 μg/ml phytohemagglutinin (PHA) to activate the T-cells proliferation. The intensity of CFSE dye with PHA-induced proliferating T-cells was evaluated after 96 hours using flow cytometry (Navios, BC), and data were analysed using Kaluza software.

3.12. Immunophenotypic analysis using flow cytometry

Peripheral blood collected from ATG treated AA patients was analysed for T-cells subsets and NK cells using flow cytometry. The cells were incubated for 20 minutes with the following anti-human pre-conjugated antibodies (BD Pharmingen, San Diego, CA, USA) CD3-PerCP Cy5.5, CD4-FITC, CD8-PE, CD16-PE, CD56-APC and Human regulatory T-cell cocktail (BD Pharmingen, San Diego, CA, USA). The cells were washed in PBS supplemented with 0.5% NaN3 (Sigma-Aldrich) and 0.1% FBS and acquired on a Navios flow cytometer (Beckman Coulter). The data analysis was performed using the Kaluza software (Beckman Coulter).

3.13. Statistics

3.13.1. Analysis of the entire patients' cohort

Data were represented as the mean of values ± SD or median values with the range indicated in the tables. For the analysis of continuous variables, Student's t-test or Mann-Whitney U test was used, while chi-square test was used for categorical data. Kruskal Wallis test was used to compare the continuous variables among the groups statistically. Pearson’s correlation coefficient evaluated the association between relative telomere length and age. All *p*-values were 2-sided, with values of 0.05 or less indicating statistical significance. Statistical analysis was performed using the IBM SPSS 24.0 Software (IBM Corp. Armonk, NY, USA) and GraphPad Prism 8.4 (GraphPad Software, Inc., San Diego, CA).

3.13.2. Analysis of HSCT cohort

Data were represented as the mean of values ± SD or median values with the range indicated in the tables. Student’s *t*-test or Mann–Whitney *U* test was used for continuous variables, whereas the chi-square test was for categorical data. The telomere length on patient characteristics

and transplant parameters were evaluated using logistic regression analysis**.** For multivariate analysis, Cox proportional hazard model was used to compare various categories of telomere length. For survival analysis, the product-limit method of Kaplan–Meier to calculate the probability of overall survival (OS) and 95% confidence intervals (CIs) was performed. The log-rank test compared the survival distribution across groups. The associations between patient or donor relative telomere length and outcomes of interest have been tested in separate models. The same was performed for the analysis of age-adjusted telomere length data. The association between age and relative telomere length was evaluated using Pearson's correlation coefficient. All survival estimates are reported as ±1SE. All *p*-values were 2-sided, with values of 0.05 or less indicating statistical significance. Statistical analysis was conducted using the IBM SPSS 24.0 Software (IBM Corp. Armonk, NY, USA).

4.0. RESULTS

4.1. Mechanism of action of mesenchymal stromal cells in Aplastic anaemia

In aplastic anaemia, haemopoietic activity is significantly reduced and attributed to the failure of haemopoietic stem cells within the bone marrow. The interaction between HSCs and various cells in the BM microenvironment, including mesenchymal stromal cells, helps regulate haematopoiesis. The involvement of MSCs in the functional regulation of HSCs in AA is not clear; therefore, we studied the physical and functional properties of AA MSCs in vitro and compared them with normal BM MSCs.

4.1.1. Baseline Characteristics of Subjects

Bone marrow was collected from AA patients (N = 50) and normal controls (N = 25) during the diagnostic procedure. The mononuclear cells isolated by gradient centrifugation were cultured in DMEM media for 3-4 weeks to obtain mesenchymal stromal cells. Of the total collected samples, MSCs were generated in 20 AA and 15 normal samples. The median age of patients with AA was 22 (6-67) years, and of normal controls was 19 (5-54) years. Based on severity criteria, there were 12 patients with SAA and 4 each with NSAA and VSAA, respectively (**Table 4.1.1.**).

Table 4.1.1.: Baseline characteristics of Subjects

Parameters		**AA MSCs**	**Normal MSCs**
N		20	15*
Age Median (Range)		22 (6-67)	19 (5-54)
Severity	**NSAA** **SAA** **VSAA**	4 12 4	NA
Pediatric **Adult**	**N**	7 13	3 12
Age Median (Range)	Pediatric Adult	8 (6-14) 36 (20-67)	8 (5-10) 22 (16-54)

*Normal MSCs include (N = 3) normal BM and (N = 12) lymphoma BM (BM report: Cellular marrow with no evidence of lymphoma).

4.1.2. Morphology and Immunophenotypic Profile of MSCs

The MSCs in passage 2 showed a homogeneous population of fibroblast-like morphology and confluent nature, confirmed by inverted light microscopy. No morphological difference was observed between MSCs derived from AA and normal BM (**Figure 4.1.2.**). Further, the adherent stromal layers were determined at passage 2 for their expression of CD73, CD90, and CD105 surface markers and the lack of expression of CD14, CD34 and CD45 markers. MSCs obtained from BM aspirates when characterised for expression of surface markers by flow cytometric analysis; the cells were positive for CD73, CD90, and CD105 in both AA and normal BM samples (>85%) (**Figure 4.1.2.1.a**). The surface markers CD34 and CD45 were expressed on a small percentage (<3%) of cells, specifying the presence of very few haemopoietic and epithelial cells in the cultures (**Figure 4.1.2.1.b**).

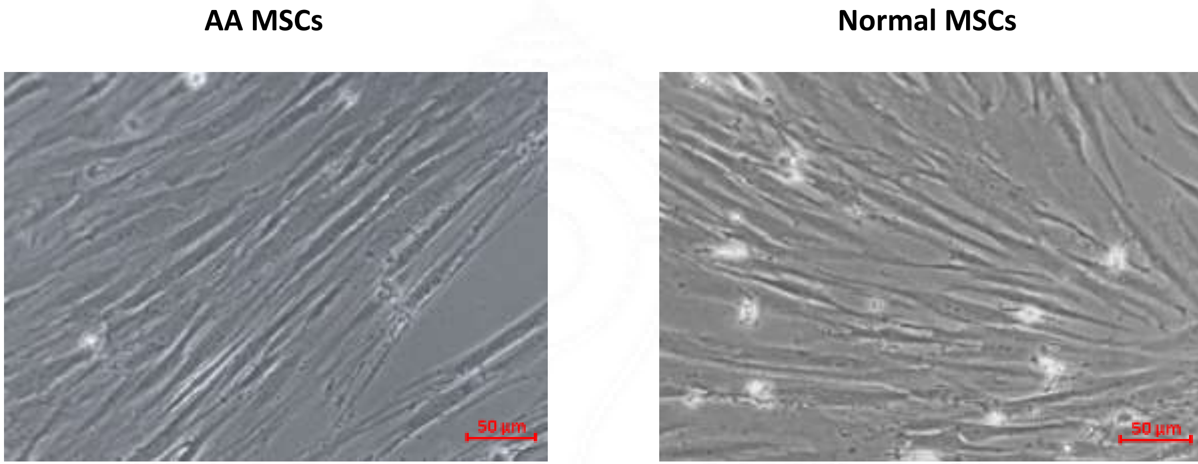
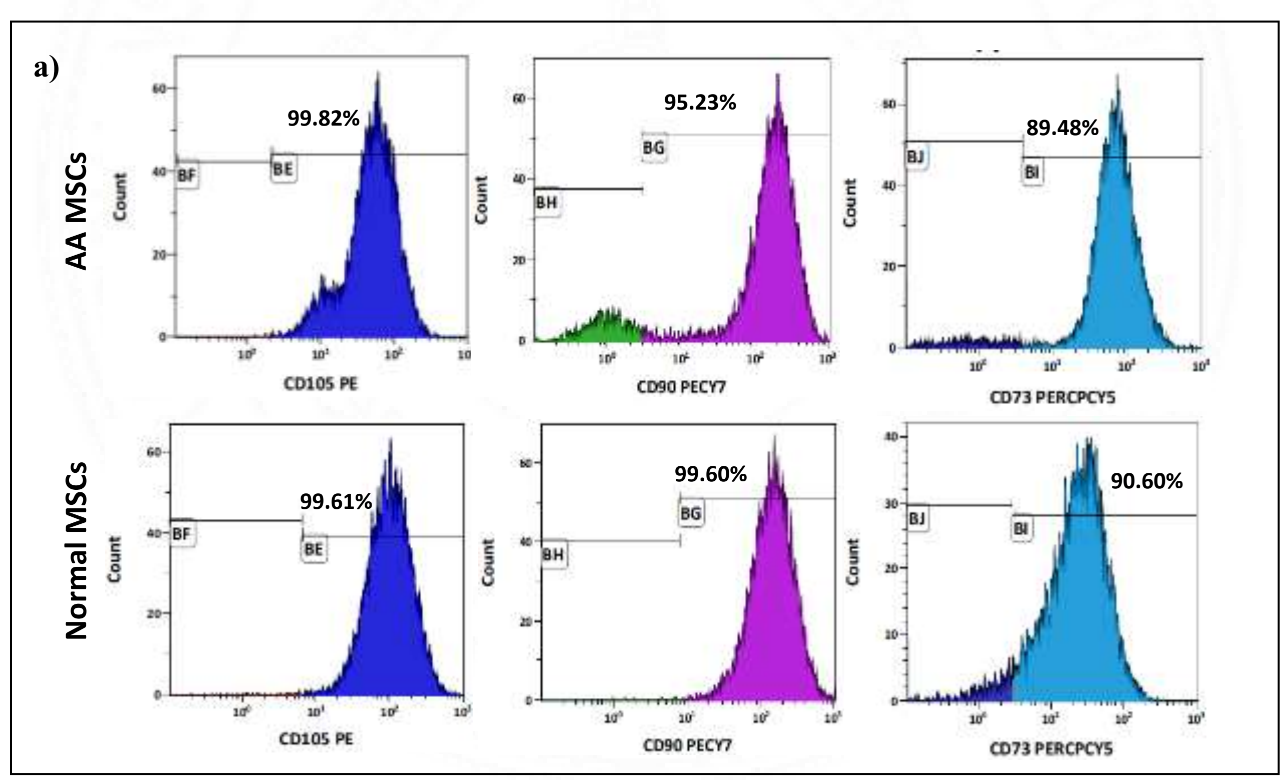


Figure 4.1.2.: The representative morphology of BM-MSCs from AA patients and normal controls. Magnification of micrographs (40X)



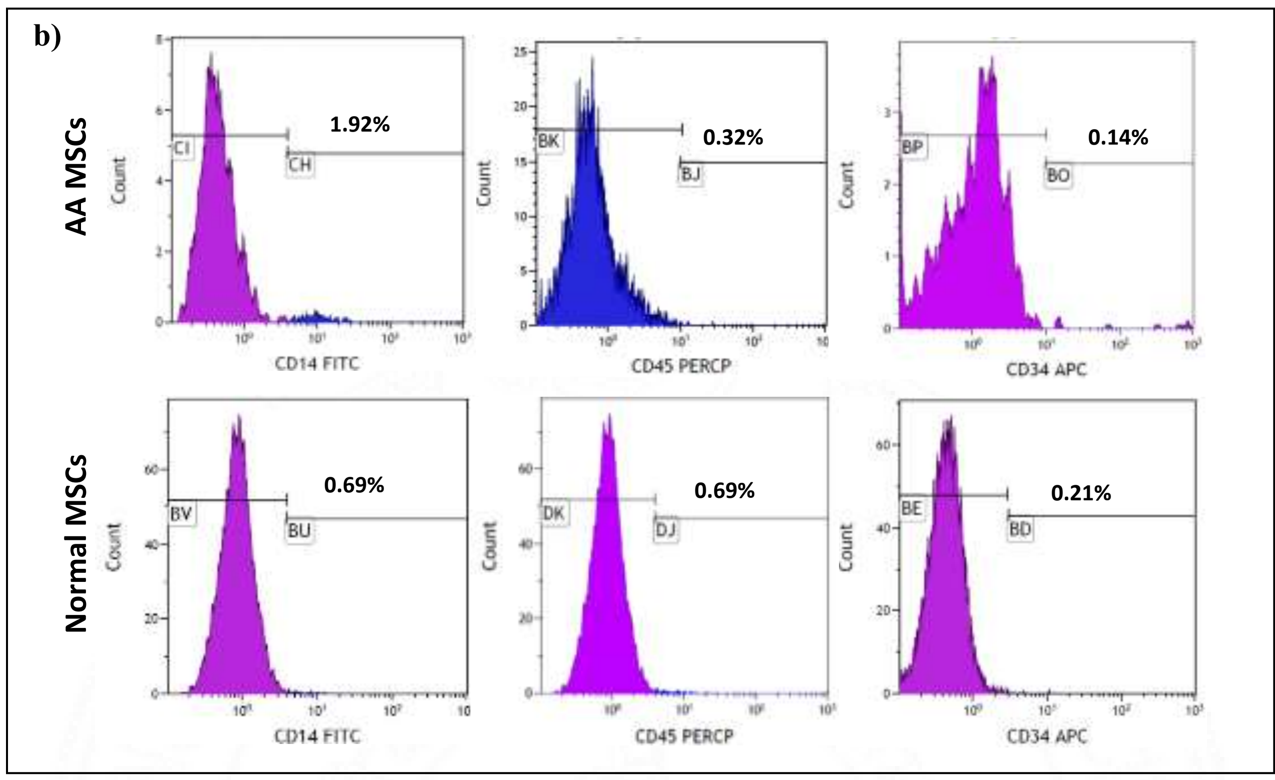


Figure 4.1.2.1.: Immunophenotypic profile of BM-MSCs of AA patients and normal controls. The immunophenotype of MSCs was assessed with monoclonal antibodies a) Positive markers (CD105, CD90, CD73) and b) Negative markers (CD14, CD45, CD34) using flow cytometry.

4.1.3. Proliferative capacity of MSCs

The proliferative capacity of MSCs was examined by reporting the cumulative population doubling (PD) of over three passages. The average cumulative PD of AA and normal MSCs was 1.13 ± 0.46 and 1.11 ± 0.41, respectively ($p = 0.809$) (N = 10, **Figure 4.1.3.a**). Subsequently, the population doubling time after analysing for 96 hours in AA MSCs was 60 ± 22 hours and in normal MSCs was 59 ± 18 hours, respectively ($p = 0.810$) (N = 10, **Figure 4.1.3.b**). The data showed no difference in the proliferative potential of MSCs obtained from AA and normal BM.

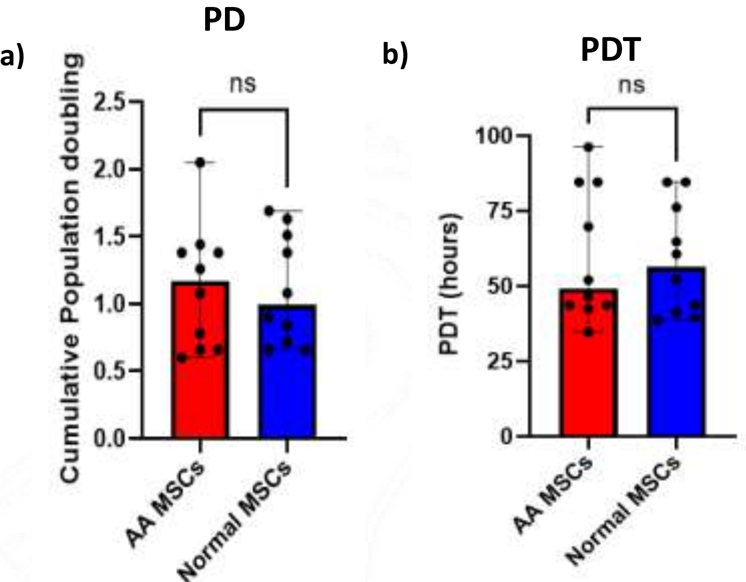
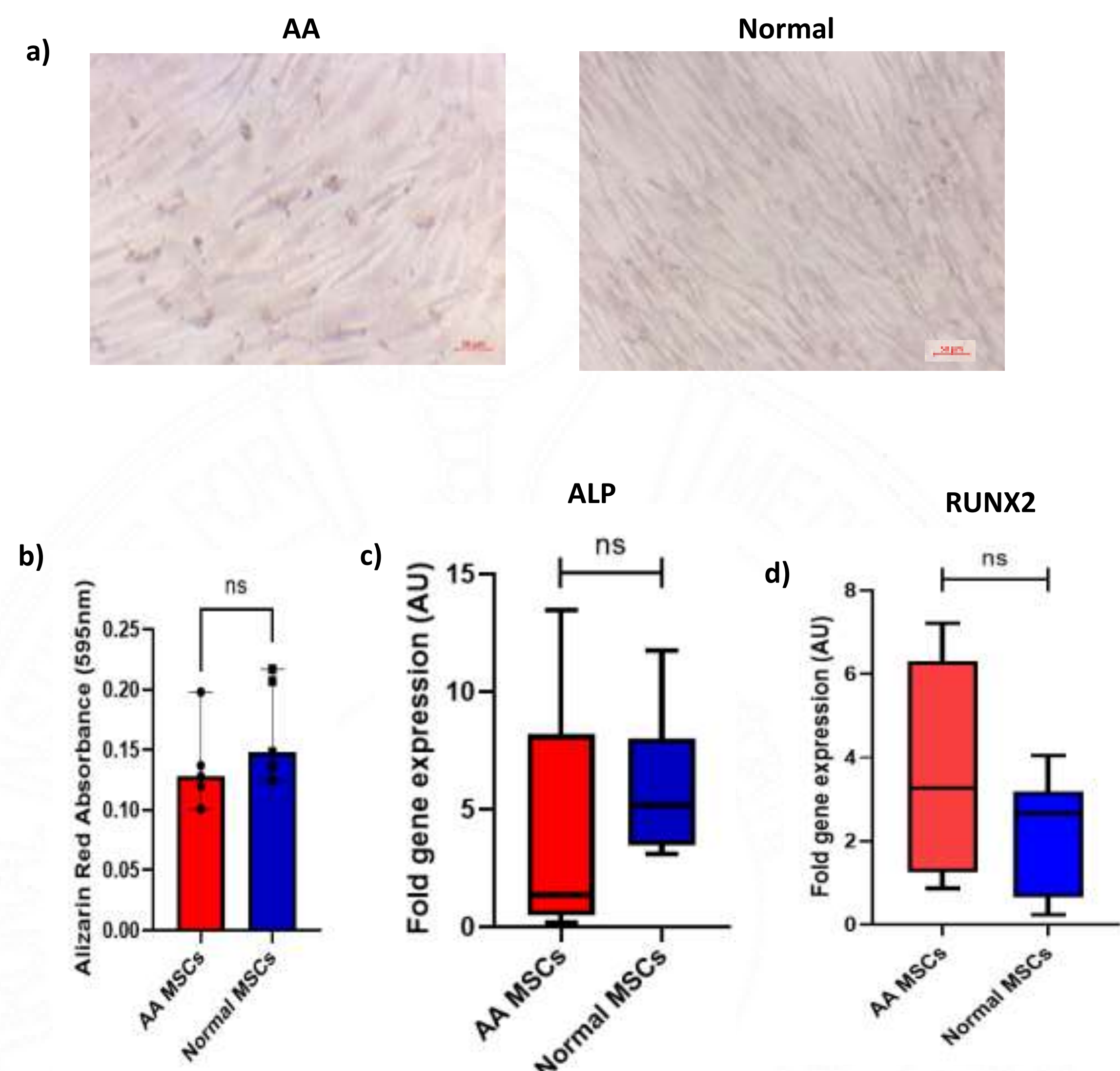


Figure 4.1.3.: Proliferation capacity of MSCs from AA and normal. a) Population Doubling and b) Population Doubling Time

4.1.4. Differentiation potential of MSCs

The differentiation ability of MSCs was performed using osteogenic differentiation and adipogenic differentiation assays. To induce osteogenic differentiation, BM-MSCs were seeded in 24-well plates and cultured in an osteogenic medium for 21 days, with the medium changed every three days. After osteogenesis, calcification was observed when stained with alizarin red (**Figure 4.1.4.a**). To quantify the alizarin red stain, it was diluted using ethanol, and the absorbance (OD) was read at 595nm (**Figure 4.1.4.b**). RNA extracted from differentiated cells showed the presence of osteogenic markers (ALP and RUNX2) when analysed using qPCR (**Figure 4.1.4.c,d**).



4.1.4.: The capacity of osteogenic differentiation was detected by (a) positive staining of Alizarin Red, magnification of micrographs (40X) and (b) quantification of Alizarin stain (AA: N = 5, Normal: N = 5). The osteogenic lineage specific gene expression (c) ALP and (d) RUNX2 were detected by qPCR (AA: N = 10, Normal: N = 10)

Similarly, MSCs were seeded in an adipogenic medium for adipogenic differentiation, and the differentiated cells were stained. The lipid droplet formation was demonstrated by Oil Red O staining on day 21 (**Figure 4.1.4.1.a**). The Oil red O stain was quantified by diluting the stain from

the cells using isopropanol, and OD was read at 510nm (**Figure 4.1.4.1.b**). Upon analysis of adipogenic markers from the extracted RNA, similar levels of (LPL and FABP4) were observed in both AA and normal MSCs (**Figure 4.1.4.1.c,d**). In both osteogenic and adipogenic differentiation, there was no significant difference observed between AA and normal MSCs.

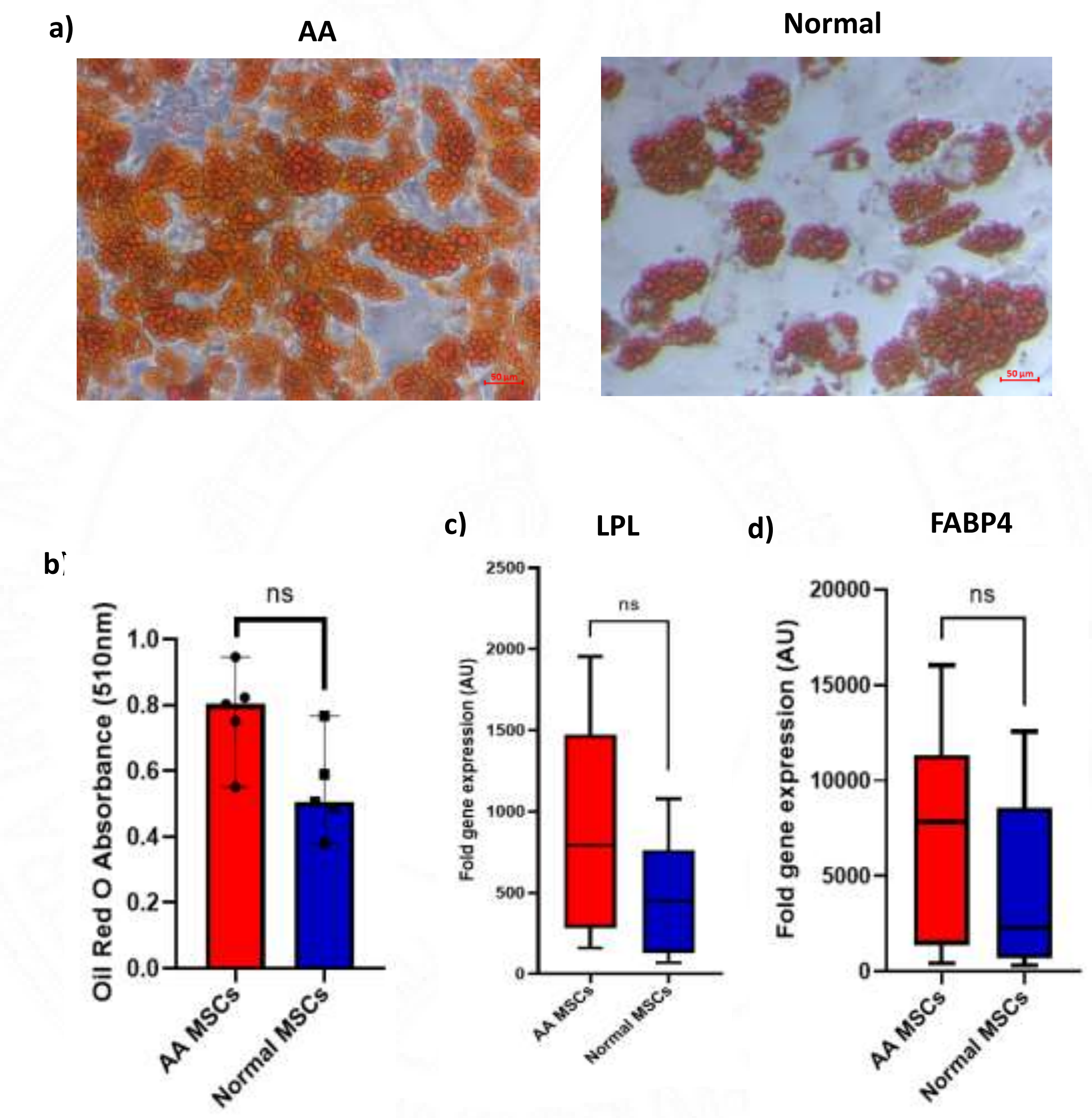


Figure 4.1.4.1.: The capacity of adipogenic differentiation was detected by (a) positive staining of Oil Red O, magnification of micrographs (40X) and (b) quantification of Oil Red O stain (AA: N = 5, Normal: N = 5). The adipogenic lineage specific gene expression (c) LPL and (d) FABP4 (AA: N = 10, Normal: N = 10) were detected by qPCR

4.1.5. The immunosuppressive potential of MSCs

To test the immunosuppressive properties of MSCs, the proliferation of CFSE-labelled human T lymphocytes stimulated by PHA was analysed. The stimulation of PBMCs in co-culture assay resulted in reduced $CD3^+$ T-cell proliferation after 96 hours. The median percentage of T-cells proliferation when co-cultured with AA MSCs was 45% (21 - 61) and 51% (40 - 86) with normal MSCs. Therefore, the median percentage of T-cells suppressed by AA MSCs was comparable with normal MSCs [55% (39 - 79) and 49% (14 - 60), respectively] ($p = 0.224$) (N = 10, **Figure 4.1.5.a,b**).

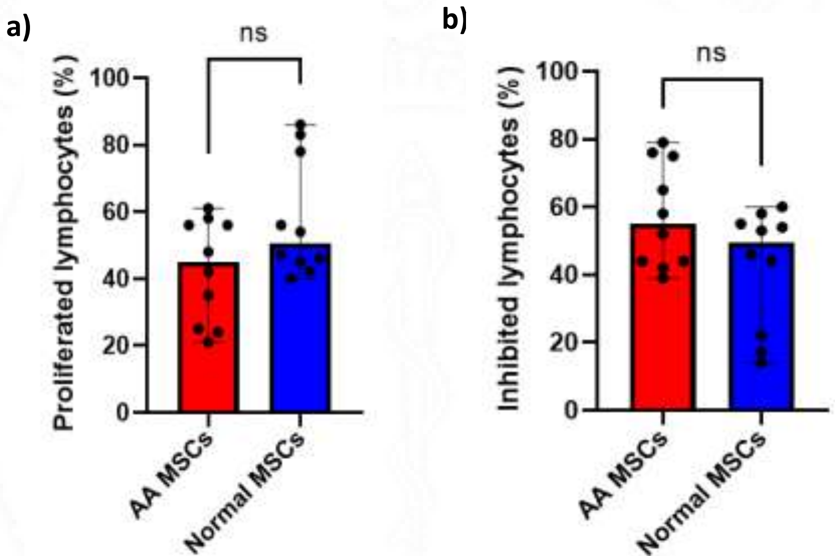


Figure 4.1.5.: Comparison of percentage T-cells (a) proliferated and (b) inhibited cells when co-cultured with AA MSCs and normal MSCs

4.1.6. Telomere length measurement in MSCs

The extracted DNA from AA and normal MSCs at passage 2 were analysed for relative telomere length (rTL) using the qPCR method. The median rTL of AA (N = 17) and normal MSCs (N = 10) were 1.50 (0.34 - 3.83) and 0.94 (0.69 - 2.41), respectively. There was no difference

between the median rTL of AA-MSCs and normal MSCs [AA: 1.50 (0.34 - 3.83) vs Normal: 0.94 (0.69 - 2.41), (p = 0.138)] (**Figure 4.1.6.**). Upon comparing the rTL in MSCs of patients with NSAA and (SAA + VSAA) group was comparable [NSAA: 1.84 (0.62 - 2.78) vs SAA+VSAA: 1.38 (0.34 - 3.83), p = 0.412] (**Figure 4.1.6.1.**). Since there was no difference in telomere length between AA and normal MSCs using qPCR, we compared rTL between pediatric and adults in each group. The rTL was observed to be significantly different between pediatric and adult AA patients [Ped: 1.79 (0.95 - 3.83) vs Adult: 0.93 (0.34 - 2.26), p = 0.033], whereas in normal controls rTL was comparable [Ped: 1.12 (0.80 - 1.23) vs Adult: 0.92 (0.69 - 2.41), p = 0.667] (**Figure 4.1.6.2.a,b**). Additionally, there was no difference in rTL when comparing rTL in pediatric AA and normal MSCs, and similar outcomes were also observed with adults in both groups (**Figure 4.1.6.3.**). The MSCs rTL in AA patients was significantly higher when compared to their PB rTL [MSCs: 1.50 (0.34 - 3.83) vs PB: 0.64 (0.04 - 1.33), p = 0.006] (**Figure 4.1.6.4.a**) while there was no difference in rTL of MSCs and PB in normal controls [MSCs: 0.94 (0.69 - 2.41) vs PB: 0.98 (0.38 - 2.10), p = 0.981] (**Figure 4.1.6.4.b**).

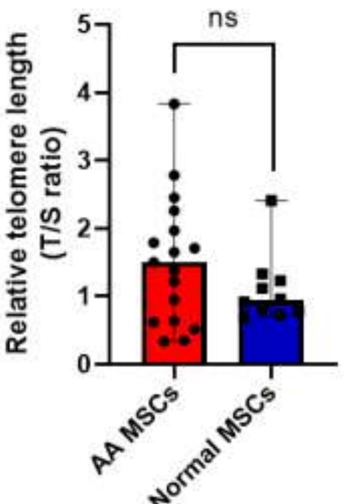


Figure 4.1.6.: Comparison of relative telomere length between AA MSCs (N = 17) and Normal MSCs (N = 10)

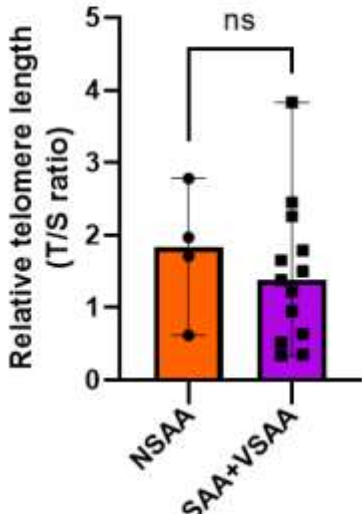


Figure 4.1.6.1.: Comparison of relative telomere length in MSCs with respect to the severity of AA (NSAA: N = 4, SAA + VSAA: N = 13)

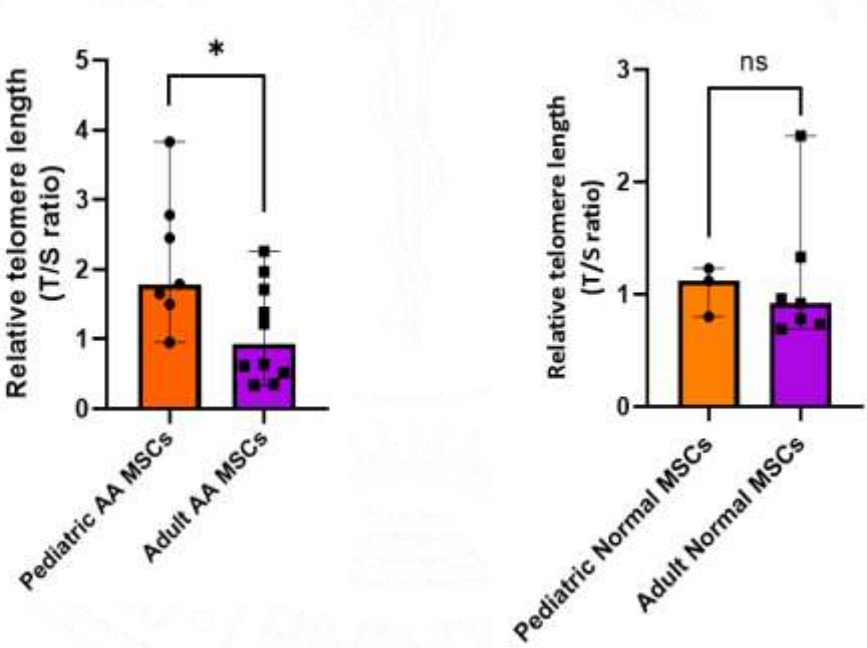


Figure 4.1.6.2.: Relative telomere length in pediatric (AA: N = 7, Normal: N = 3) and adult (AA: N = 11, Normal: N = 7) in (a) AA and (b) normal MSCs

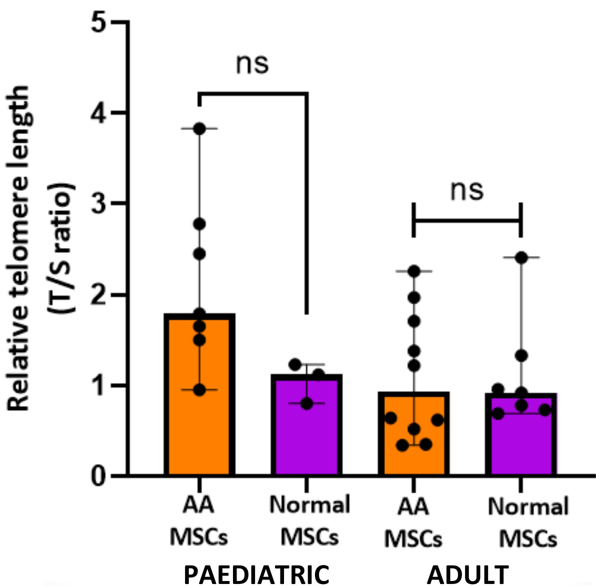


Figure 4.1.6.3.: Relative telomere length in pediatric (AA: N = 7, Normal: N = 3) and adult (AA: N = 11, Normal: N = 7) in AA and normal MSCs

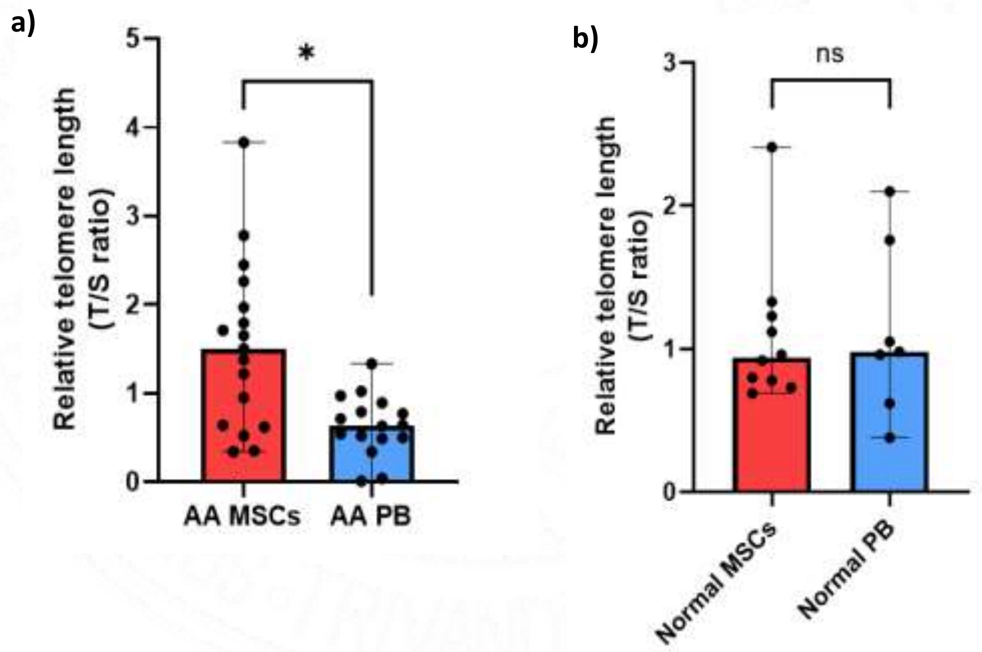


Figure 4.1.6.4.: Relative telomere length in MSCs (AA: N = 16, Normal: N = 10) and peripheral blood (AA: N = 16, Normal: N = 7) of (a) AA and (b) normal

4.2. Role of telomere length in bone marrow failure syndromes

4.2.1. Measurement of telomere length by qPCR - experiment parameters

Telomere length measurement using the qPCR method was first reported by Cawthon (Cawthon, 2002), and we standardized the same experiment in our laboratory. Standard genomic DNA was serially diluted to acquire a standard curve, and this DNA sample was used in all experiments as a calibrator along with a positive control sample. The quality and quantity of extracted DNA were measured using NanoDrop, and the purity of DNA determined by the A260/A280 ratio lay between 1.8-2.0 for all samples.

The standard curve was used to determine the copy number of telomere (T) and 36B4 (S), respectively. These standard curves were used to determine the T/S ratio or relative TL. The qPCR coefficient of determination and slope of standard curves of TEL and 36B4 were ($R^2 = 0.99$, slope = -3.623; $R^2 = 0.94$, slope = -3.726, respectively) (**Figure 4.2.1.a,b**). The melt curve and melt peak show the homogeneity and specificity of the amplified product of telomere and 36B4. After thermocycling in qPCR assay, melt curve test determines the homogeneity of the amplified product. SYBR Green dye is a dye that can detect any double-stranded DNA and non-specific binding during a dissociation curve and as a result, can produce multiple peaks. The occurrence of a melt peak demonstrates the precise amplification of the product, resulting in a singular peak. The results of our assay indicate a high level of specificity in the amplification of telomere and 36B4 products, as shown in **Figure 4.2.1.c,d**. The absence of any peak, apart from telomere and 36B4, indicates that there was no occurrence of primer-dimer formation or non-specific binding. The mean coefficient of variation for the telomere assay was 0.9% and 0.3% for the 36B4 assay. The

intra-assay CV and inter-assay CV for the reference sample from duplicates were 3.44% and 6.33%, respectively.

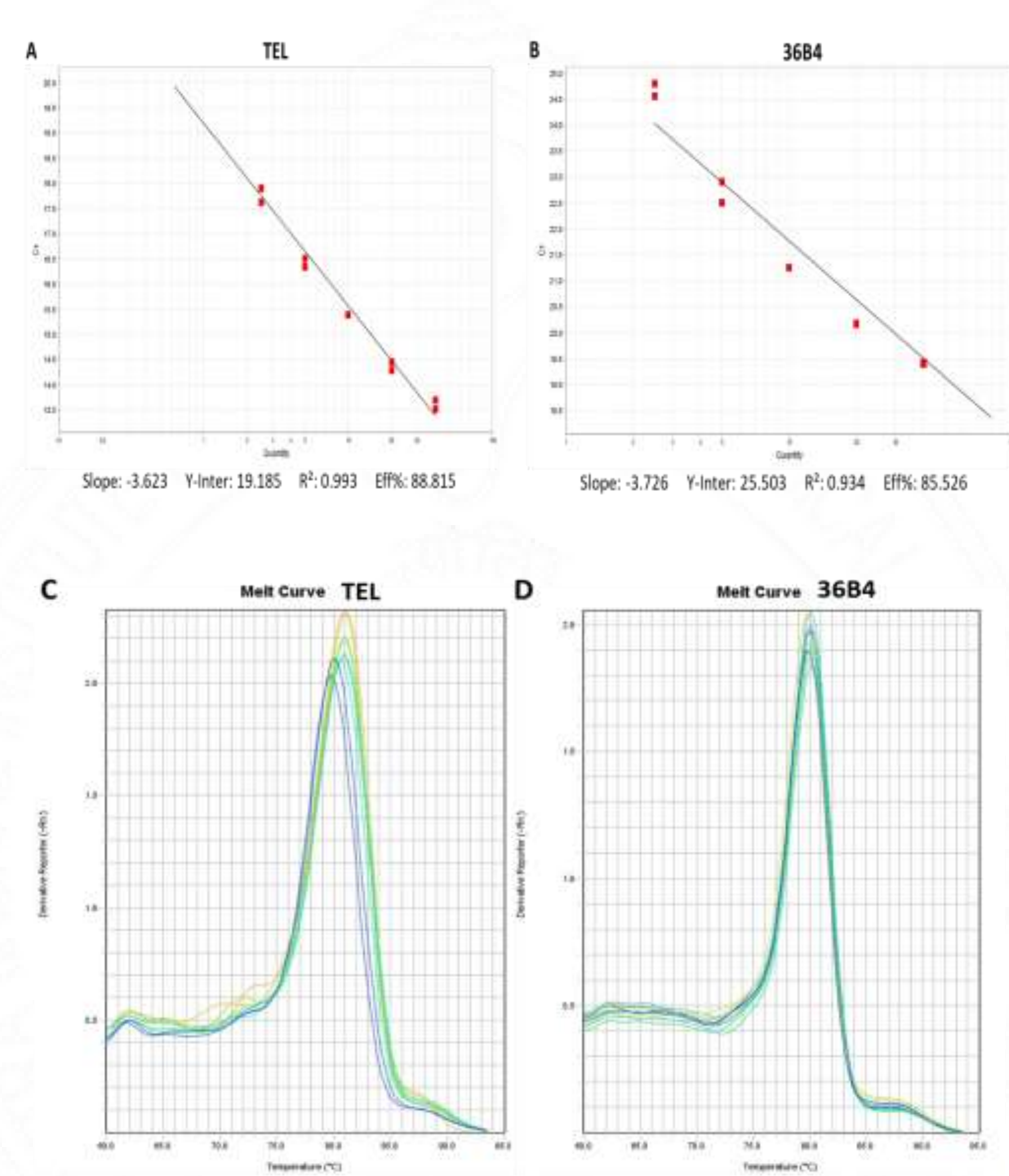


Figure 4.2.1.: Standard curve and melt curve of TEL and 36B4 gene

In the semi-log plot of DNA concentration versus cycle threshold, both standard curves (A & B) were linear concentration ranges of standard DNA of TEL and 36B4 (R^2 = 0.993 & R^2 = 0.934, respectively). The efficiency and slope of standard curves of TEL (E = 88.8%, slope = -3.623) and 36B4 (E = 85.5%, slope = -3.726) were noted. The melt curve/peak (C & D) shows good homogeneity and specificity of the amplified product of TEL and 36B4.

4.2.2. Telomere Length of Study Participants

4.2.2.1. Telomere Length Measurement in Healthy Controls

We studied the telomere length in eight hundred (N = 800) healthy controls. The median age of healthy controls was 31 (0 - 60) years and included 471 males (59%) and 329 females (41%). The median rTL of the cohort was 0.78 (0.11-3.65), and rTL for males and females were [0.79 (0.11 - 3.65) and 0.78 (0.19 - 3.57), p=0.984] respectively. The median age-adjusted rTL was 0.36 (0.20 - 0.54). A significant correlation was observed between age distribution and rTL (r = -0.319, p = 0.000, **Figure 4.2.2.1**); lower rTL was seen with increasing age. Further, rTL was categorized age-wise into decades (**Figure 4.2.2.1.1.**), and rTL between males and females was compared age-wise (**Table 4.2.2.1, Figure 4.2.2.1.2.)**.

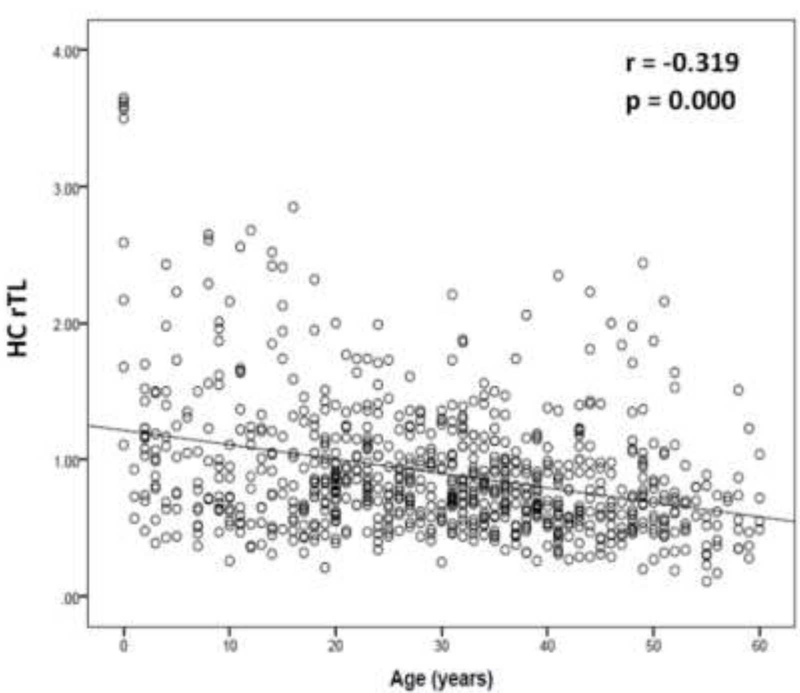


Figure 4.2.2.1.: Correlation of relative telomere length with age in healthy controls

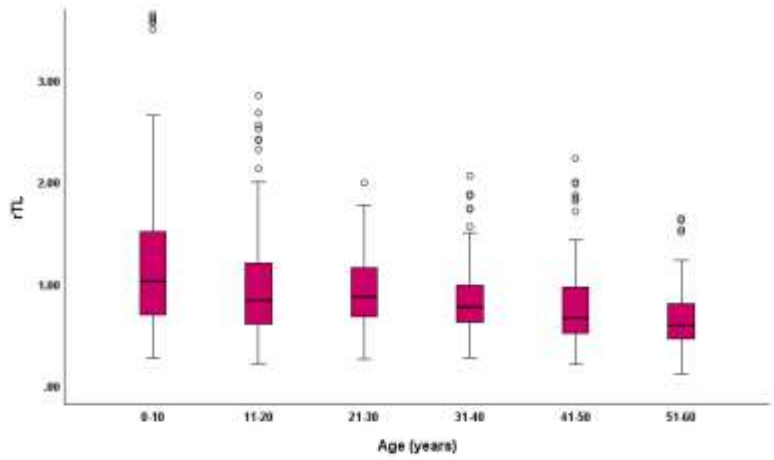


Figure 4.2.2.1.1: Age-wise (decades) distribution of relative telomere length in healthy controls

Table 4.2.2.1.: Comparison of relative telomere length between males and females in the healthy controls categorised age-wise (decades)

		Age (years)	0 to 10	11 to 20	21 to 30	31 to 40	41 to 50	51 to 60
HC [800]	M	N	42	85	103	124	86	31
		rTL Median (Range)	1.04 (0.26-3.65)	0.82 (0.31-2.85)	0.91 (0.34-1.99)	0.81 (0.26-2.06)	0.65 (0.20-2.23)	0.58 (0.11-1.64)
	F	N	58	55	47	76	53	40
		rTL Median (Range)	1.02 (0.39-3.57)	0.89 (0.21-2.68)	0.85 (0.25-1.64)	0.75 (0.32-2.21)	0.67 (0.27-2.44)	0.62 (0.19-2.16)
	p value		0.793	0.439	0.345	0.282	0.652	0.706

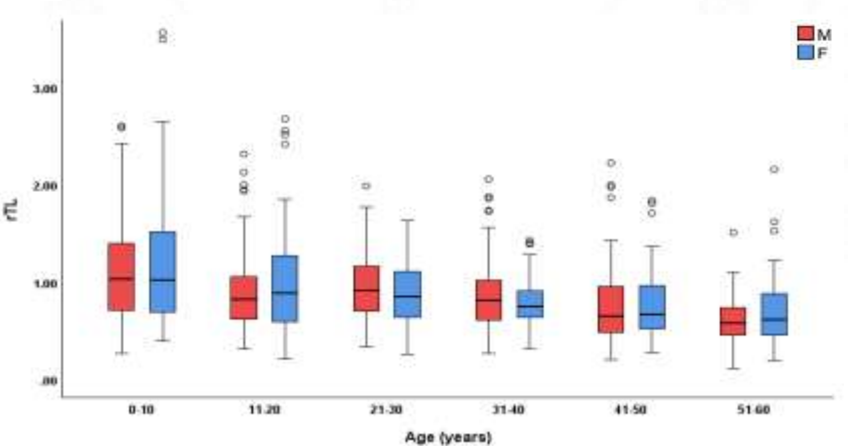


Figure 4.2.2.1.2.: Comparison of relative telomere length between males and females in the healthy controls categorised age-wise (decades)

4.2.2.2. Telomere length analysis in patients with Aplastic anaemia

We analysed 650 patients with AA having a median age of 30 (1 - 60) years, including 404 (62%) males and 246 (38%) females. Based on disease severity, 157 (24%) patients had NSAA with a median age of 34 (4-60) years, 384 (59%) SAA having 28 (1-60) years as median age and 109 (17%) had VSAA with median age of 25 (1-59) years. The overall median telomere length of AA was 0.73 (0.10 - 3.00), and there was no difference in median rTL between males and females [0.71 (0.10 - 3.00) vs 0.76 (0.12 - 2.98), p = 0.930] respectively. A significant negative correlation was observed between age and rTL (r = -0.168, p = 0.000; **Figure 4.2.2.2.**). The median rTL and AA patient based on severity were observed to be significantly different among the three groups [NSAA: 0.70 (0.10 - 3.00), SAA: 0.73 (0.11 - 2.98), and VSAA: 0.79 (0.14 - 2.53), p = 0.023] (**Figure 4.2.2.2.1.a)**. While comparing (SAA + VSAA) with NSAA, the rTL was not significantly different between the groups [0.74 (0.11 - 2.98) vs 0.70 (0.10 - 3.00), p = 0.069] (**Figure 4.2.2.2.1.b**). The median rTL of AA patients was stratified based on age into decades (**Figure 4.2.2.2.2.**). A similar analysis was performed to compare rTL between males and females (**Table 4.2.2.1, Figure 4.2.2.2.3.)**, and for the severity of AA (**Figure 4.2.2.2.4.**).

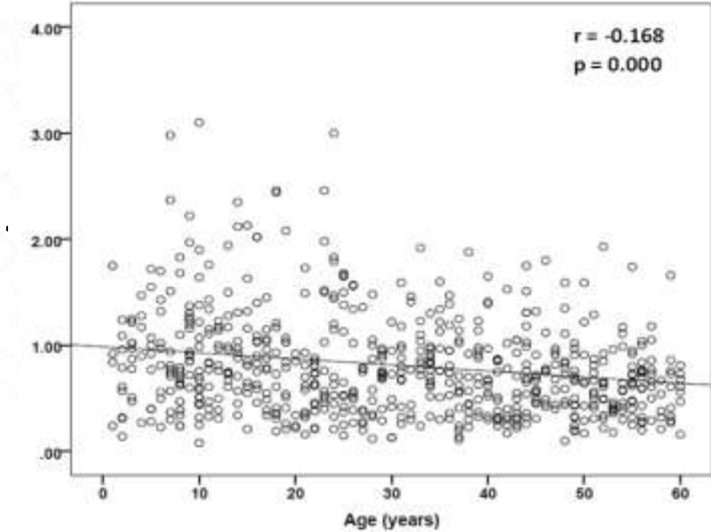


Figure 4.2.2.2.: Correlation of relative telomere length with age in patients with aplastic anemia

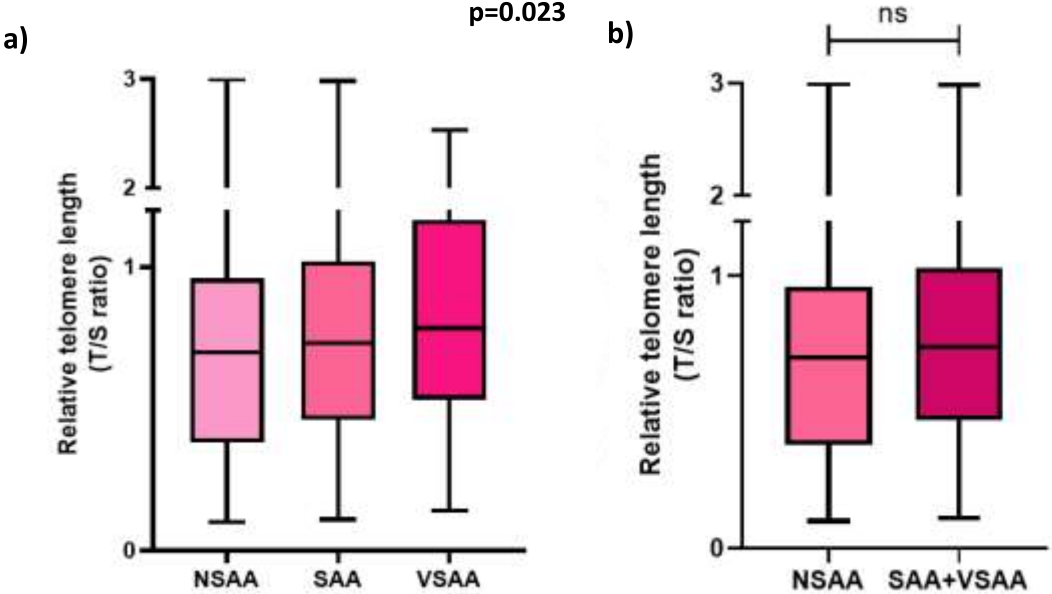


Figure 4.2.2.2.1.: Relative telomere length in AA patients categorised based on disease severity: (a) all three groups, and (b) combined (SAA + VSAA) with NSAA

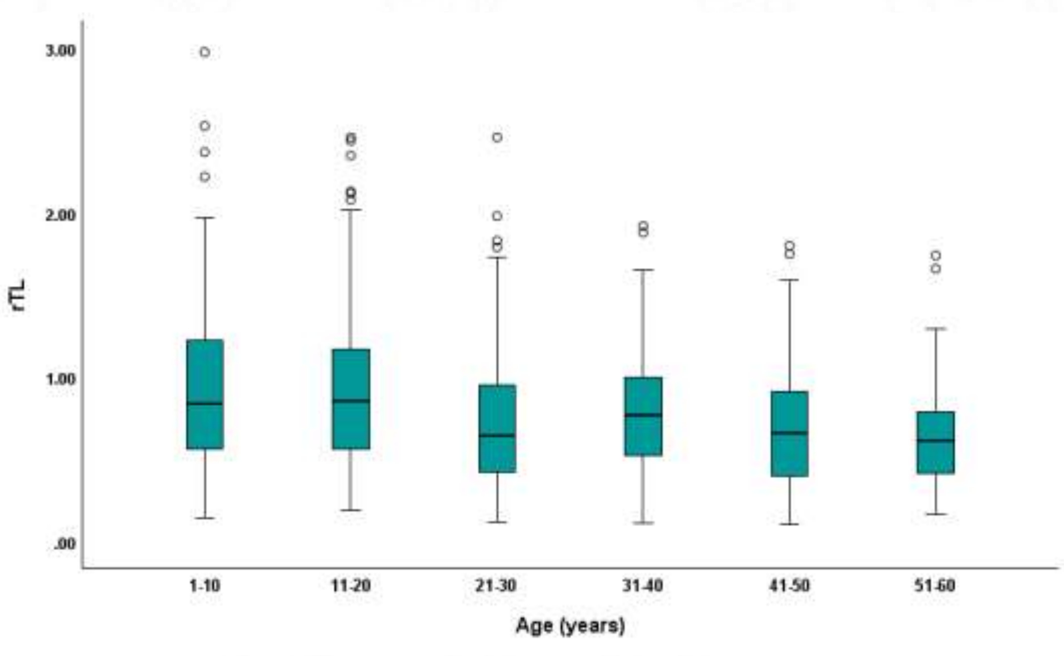


Figure 4.2.2.2.2.: Age-wise (decades) distribution of relative telomere length in patients with aplastic anaemia

Table 4.2.2.2.: Comparison of relative telomere length between males and females in Aplastic anaemia patients categorised age-wise (decades)

		Age (years)	0 to 10	11 to 20	21 to 30	31 to 40	41 to 50	51 to 60
AA [650]	M	N	63	76	70	72	67	56
		rTL Median (Range)	0.77 (0.14-1.97)	0.92 (0.19-2.44)	0.67 (0.12-3.00)	0.69 (0.14-1.65)	0.64 (0.17-1.80)	0.59 (0.16-1.74)
	F	N	47	34	40	38	43	44
		rTL Median (Range)	0.96 (0.26-2.98)	0.82 (0.21-2.46)	0.62 (0.13-1.83)	0.95 (0.11-1.92)	0.68 (0.10-1.59)	0.63 (0.29-1.93)
	p value		**0.023**	0.544	0.699	**0.006**	0.175	0.382

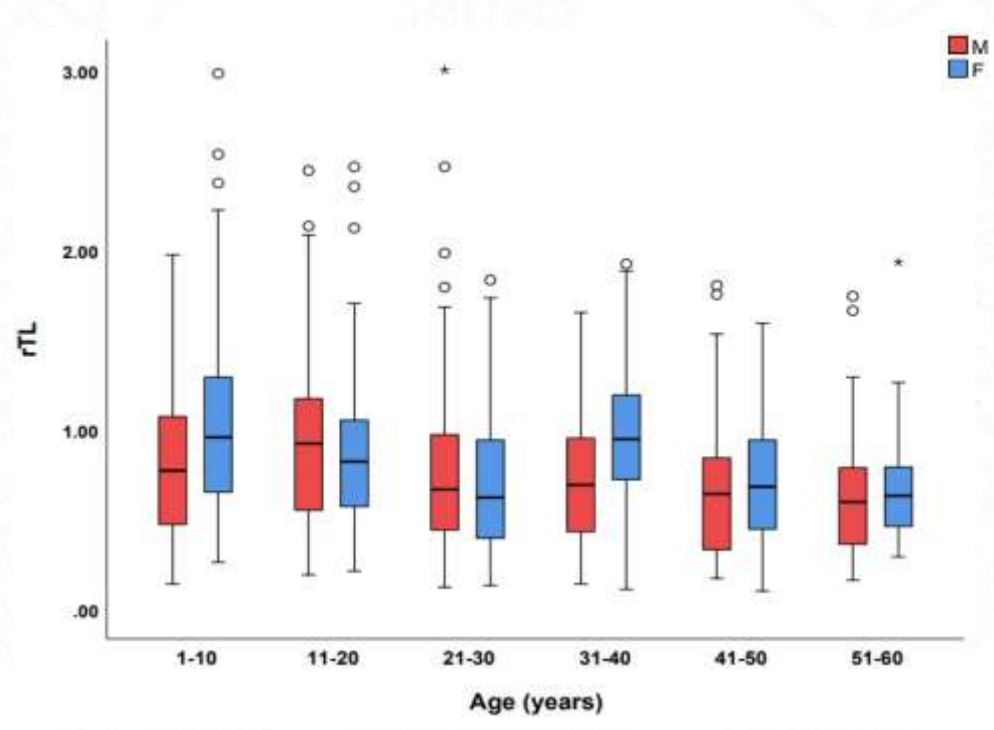


Figure 4.2.2.2.3.: Comparison of relative telomere length between males and females in Aplastic anaemia patients categorised age-wise (decades)

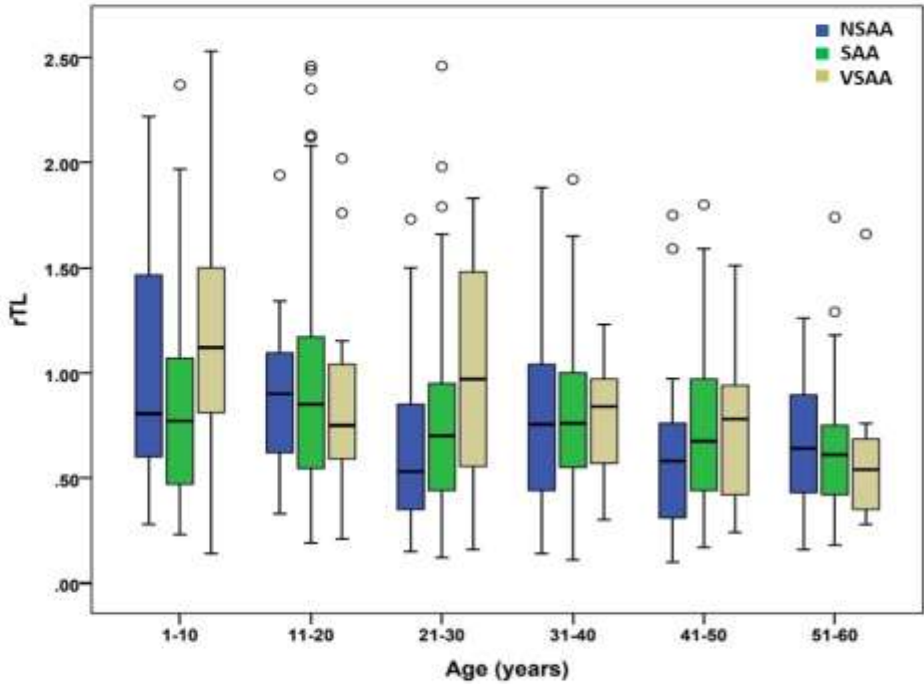


Figure 4.2.2.2.4.: Comparison of relative telomere length in the severity of Aplastic anaemia patients categorised age-wise (decades)

The overall median relative telomere length in patients with aplastic anaemia was significantly shorter compared to healthy controls [0.73 (0.10 - 3.00) vs 0.78 (0.11 - 3.65), p = 0.000] (**Figure 4.2.2.2.5.**). When classified age into decades, patients aged (0 - 10) and (21 - 30) years showed significantly shorter rTL in AA than healthy controls. The comparison of rTL in AA patients and healthy controls with age (decade-wise) is shown in **Figure 4.2.2.2.6**. Based on the median rTL of healthy controls, we classified the number of AA patients as having low telomere length in each age group. We observed that about 344 AA patients (53%) in our cohort had shorter relative telomere lengths compared to age-matched healthy controls (**Table 4.2.2.2.1.)**.

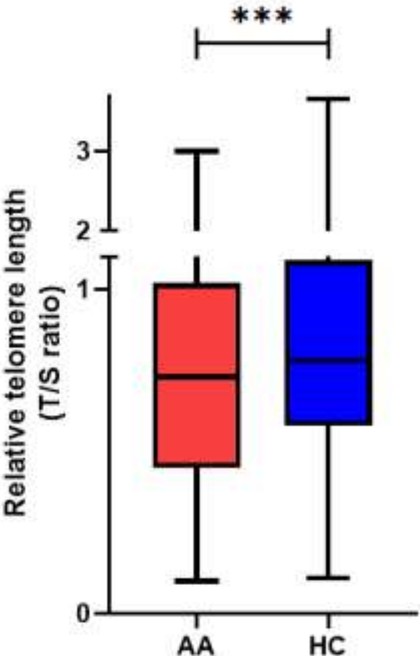


Figure 4.2.2.2.5.: Comparison of relative telomere length in patients with aplastic anaemia with healthy controls

Table 4.2.2.2.1.: Comparison of relative telomere length in patients with Aplastic anaemia with healthy controls age-wise in decades

	Age (years)	0-10	11-20	21-30	31-40	41-50	51-60
HC [800]	**N**	100	140	150	200	140	70
rTL	**Median (Range)**	0.97 (0.26-3.65)	0.83 (0.21-2.85)	0.87 (0.25-1.99)	0.77 (0.26-2.21)	0.66 (0.20-2.44)	0.58 (0.11-2.16)
AA [650]	**N**	110	110	110	110	110	100
rTL	**Median (Range)**	0.83 (0.14-2.98)	0.84 (0.19-2.46)	0.70 (0.12-3.00)	0.76 (0.11-1.92)	0.66 (0.10-1.80)	0.61 (0.16-1.93)
	p value	**0.003**	0.703	**0.000**	0.447	0.054	0.849
N [344]	**Patients with shorter rTL (N)**	67	51	73	55	54	44

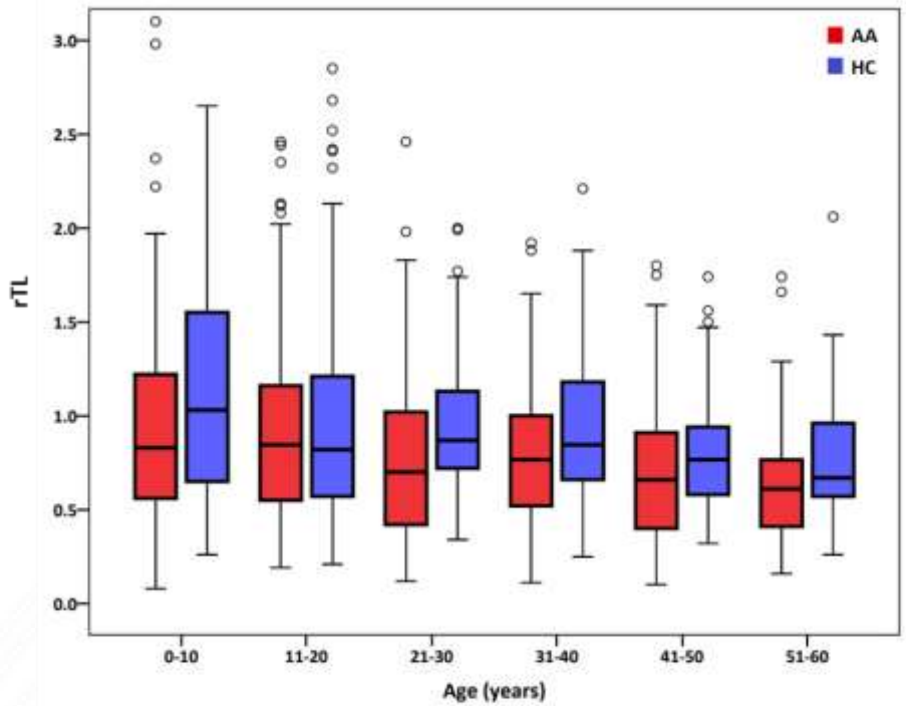


Figure 4.2.2.2.6.: Comparison of relative telomere length in age-wise (decades) in aplastic anaemia patients with healthy controls

The median age-adjusted telomere length of patients for the entire cohort was 0.36 (0.20 - 0.54), and median age-adjusted TL between males and females were [0.37 (0.20 - 0.54) & 0.34 (0.20 - 0.54)] respectively. The median age-adjusted telomere length based on severity were NSAA: 0.33 (0.20 - 0.52), SAA: 0.37 (0.20 - 0.54), and VSAA: 0.39 (0.20 - 0.54). The telomere length was classified as percentiles, and about one-fourth of patients with AA (N=156, 24%) were observed to have rTL <10th percentile (median age=37 years), and of them, 42 patients (27%) had rTL <1st percentile.

4.2.2.3. Analysis of telomere length in Fanconi anaemia patients

The study included 100 patients with FA with a median age of 11 (2 - 32) years and included 65 males and 35 females. Based on disease severity, 63 (63%) patients had NSAA, 32 (32%) SAA and 5 (5%) had VSAA. The median telomere length in patients with FA was 0.78 (0.10 - 3.72). The median rTL between males and females was not significantly different [0.75 (0.10 - 3.72) & 0.80 (0.26 - 2.08), p = 0.779]. No significant correlation was observed between age distribution and rTL (r = -0.190, p = 0.059, **Figure 4.2.2.3**). The median rTL of FA patients based on severity were not significantly different [NSAA: 0.89 (0.10 - 3.72), SAA: 0.73 (0.26 - 2.08), and VSAA: 0.84 (0.18 - 0.91), p = 0.148; **Figure 4.2.2.3.1.a**]. While comparing (SAA + VSAA) with NSAA, the rTL was significantly different between the groups [0.74 (0.18 - 2.08) vs 0.89 (0.10 - 3.72), p = 0.044] (**Figure 4.2.2.3.1.b**). The median age-adjusted telomere length of patients was 0.48 (0.33 - 0.55). The median age-adjusted rTL of males and females was 0.49 (0.34 - 0.55) and 0.48 (0.33 - 0.54), respectively. The median rTL of patients with FA was significantly shorter compared to age-matched healthy controls [0.78 (0.10-3.72) vs 0.88 (0.21 - 3.65), p = 0.028] and rTL with age decade-wise (**Figure 4.2.2.3.2.a,b**). We observed that about 56 FA patients (56%) in our cohort had shorter relative telomere lengths compared to age-matched healthy controls.

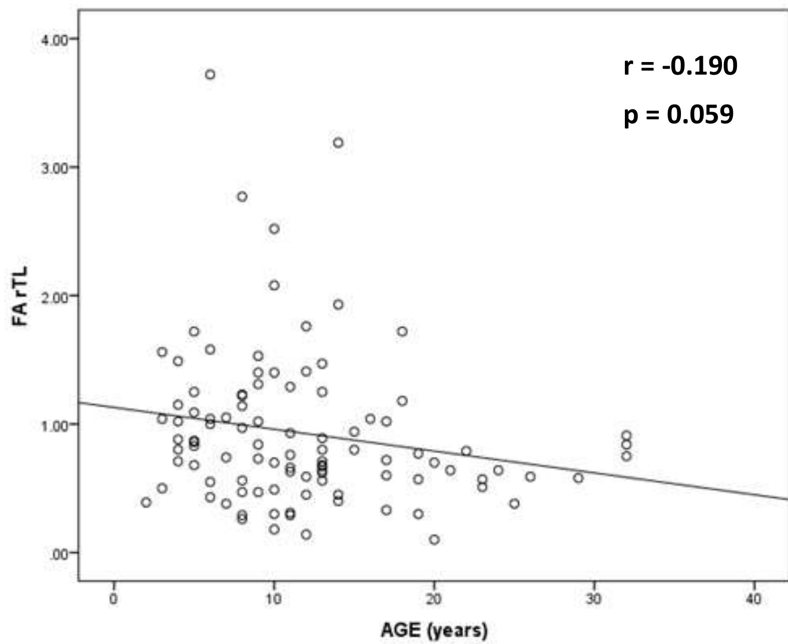


Figure 4.2.2.3.: Correlation of relative telomere length with age in patients with Fanconi anaemia

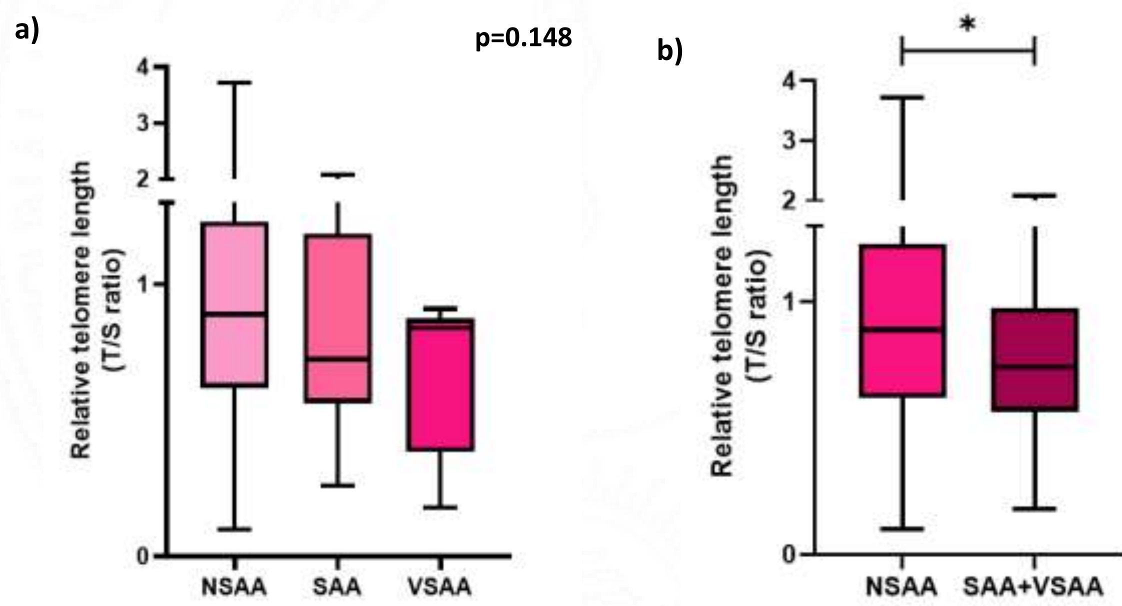


Figure 4.2.2.3.1.: Relative telomere length in FA patients categorised based on disease severity: (a) all three groups, (b) combined (SAA + VSAA) with NSAA

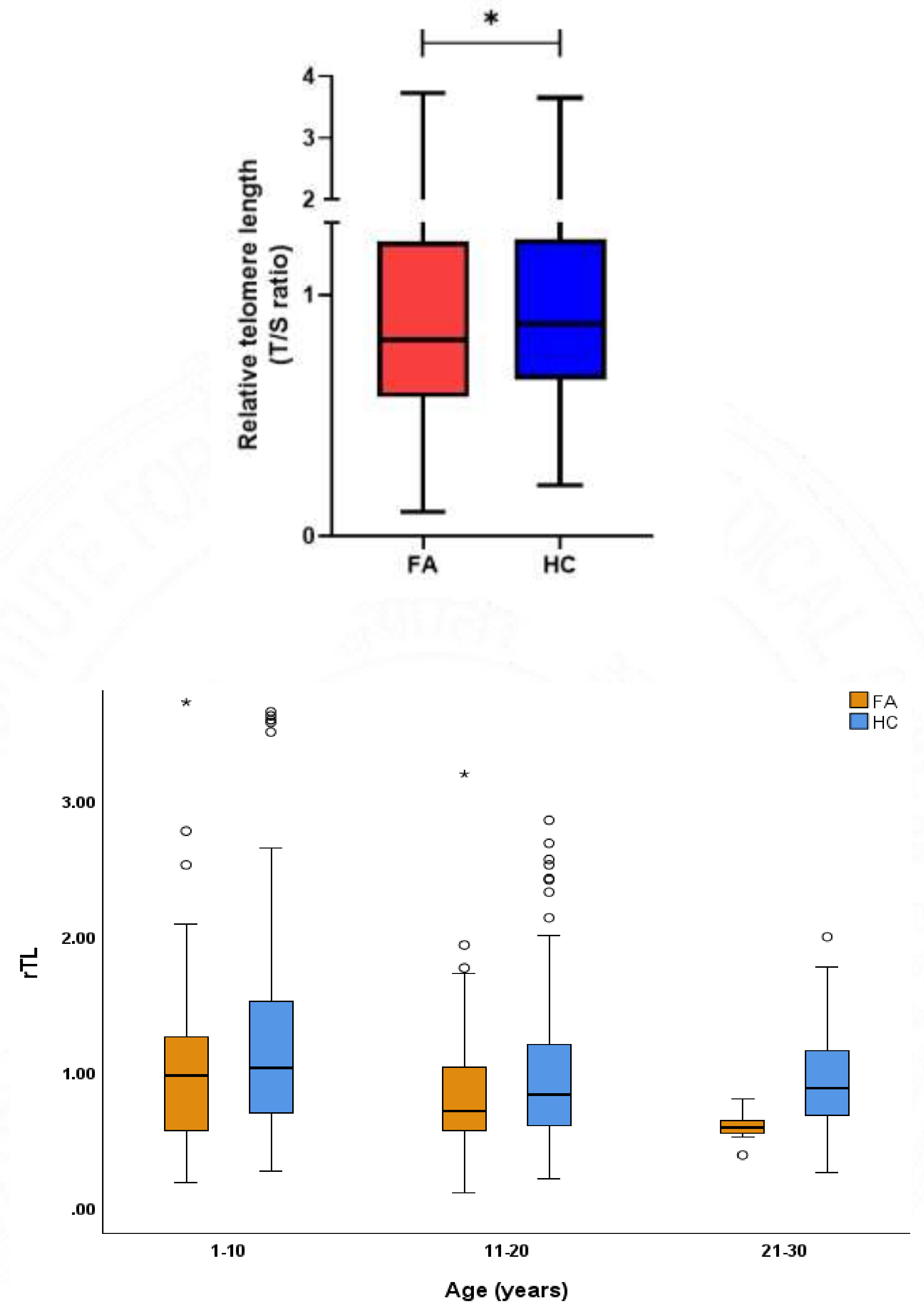


Figure 4.2.2.3.2.: Comparison of relative telomere length in patients with Fanconi anemia with (a) healthy controls and (b) age-wise (decades) distribution

While comparing age-matched AA and FA patients, there was no significant difference between the median rTL [0.79 (0.12 - 3.00) vs 0.78 (0.10 - 3.72), p = 0.727] (**Figure 4.2.2.3.3.a**), but the median age-adjusted rTL was observed to be significantly higher in FA [0.45 (0.36 - 0.54) vs 0.48 (0.33 - 0.55), p = 0.001]. Patients with AA and FA were classified into decades (age-wise); there was no correlation between the median rTL of FA and AA in the (0 - 10 years) age group (p = 0.121) (**Figure 4.2.2.3.3.b)**. While comparing rTL based on severity between AA and FA groups, a significant difference was observed in NSAA [0.70 (0.10 - 3.00) vs 0.89 (0.12 - 3.72), p = 0.000], whereas there was no significance in (SAA + VSAA) [0.74 (0.11 - 2.98) vs 0.71 (0.18 - 2.08) ,p = 0.810) (**Table 4.2.2.3.**). About 10 (10%) FA patients had rTL <10th percentile, and two patients (2%) with rTL <1st percentile.

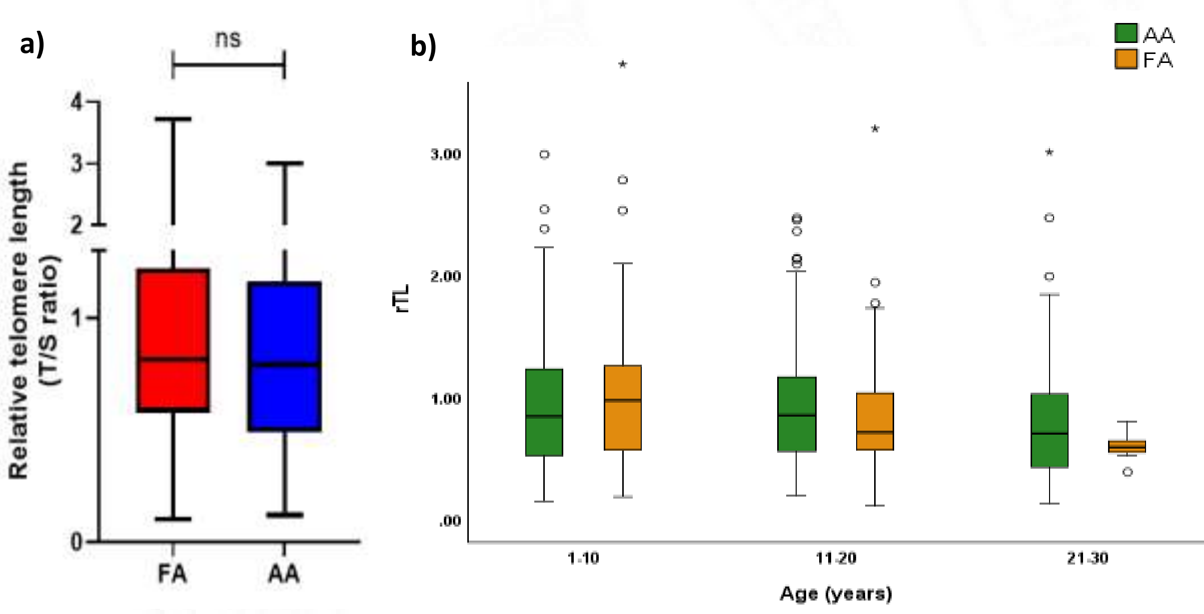


Figure 4.2.2.3.3.: Comparison of relative telomere length in patients with aplastic anemia and (a) Fanconi anemia and (b) age-wise (decades) distribution

Table 4.2.2.3.: Comparison of baseline characteristics and relative telomere length between Aplastic anemia and Fanconi anemia patients

			AA		FA		p value
N			330		100		
Age (years)	Median (Range)		15 (1-30)		11 (2-32)		0.000
Gender	M [N (%)] F		209 (63) 121 (37)		65 (65) 35 (35)		0.998 0.997
Relative telomere length	Median (Range)		0.79 (0.12-3.00)		0.78 (0.10-3.72)		0.727
Age-adjusted telomere length	Median (Range)		0.45 (0.36-0.54)		0.48 (0.33-0.55)		0.001
Relative telomere length (Gender)	Median (Range)	M F	0.77 (0.10-3.00) 0.81 (0.12-2.98)		0.75 (0.10-3.72) 0.80 (0.26-2.08)		0.845 0.760
Age-adjusted telomere length (Gender)	Median (Range)	M F	0.45 (0.20-0.54) 0.46 (0.20-0.54)		0.49 (0.34-0.55) 0.48 (0.33-0.54)		0.001 0.194
Relative telomere length Median (Range)	Severity	NSAA SAA VSAA	0.70 (0.10-3.00) 0.73 (0.11-2.98) 0.79 (0.14-2.53)		0.89 (0.10-3.72) 0.73 (0.26-2.08) 0.84 (0.18-0.91)		0.000 0.535 0.477
Relative telomere length (age-wise in decade; years) Median (Range)	0-10 11-20 21-30	N	0.83 (0.14-2.98) 0.84 (0.19-2.46) 0.70 (0.12-3.00)	110 110 110	0.97 (0.18-3.72) 0.71 (0.10-3.19) 0.58 (0.38-0.79)	49 40 8	0.121 0.133

4.2.2.4. Analysis of telomere length in Myelodysplastic syndrome patients

The median age of 100 patients with MDS was 46 (6 - 82) years and included 58 males and 42 females. Thirty and seventy patients had high-risk and low-risk MDS, respectively. The overall median telomere length of patients with MDS was 0.58 (0.19 - 2.42). No significant correlation was observed between age distribution and rTL ($r = -0.102$, $p = 0.311$, **Figure 4.2.2.4.**).

The median rTL of males and females was observed to be [0.57 (0.19 - 2.42) & 0.59 (0.30 - 1.60), p = 0.304], respectively. There was no difference between median rTL of high-risk and low-risk MDS patients [0.57 (0.30 - 1.89) vs 0.59 (0.19 - 2.42), p = 0.228] (**Figure 4.2.2.4.1.**). The median rTL of MDS patients compared with age-matched healthy controls was significantly shorter [0.58 (0.19 - 2.42) vs 0.78 (0.11 - 3.65), p = 0.000] (**Figure 4.2.2.4.2.**). We observed that about 59/83 MDS patients (71%) in the age (1-60) years had shorter relative telomere lengths compared to age-matched healthy controls.

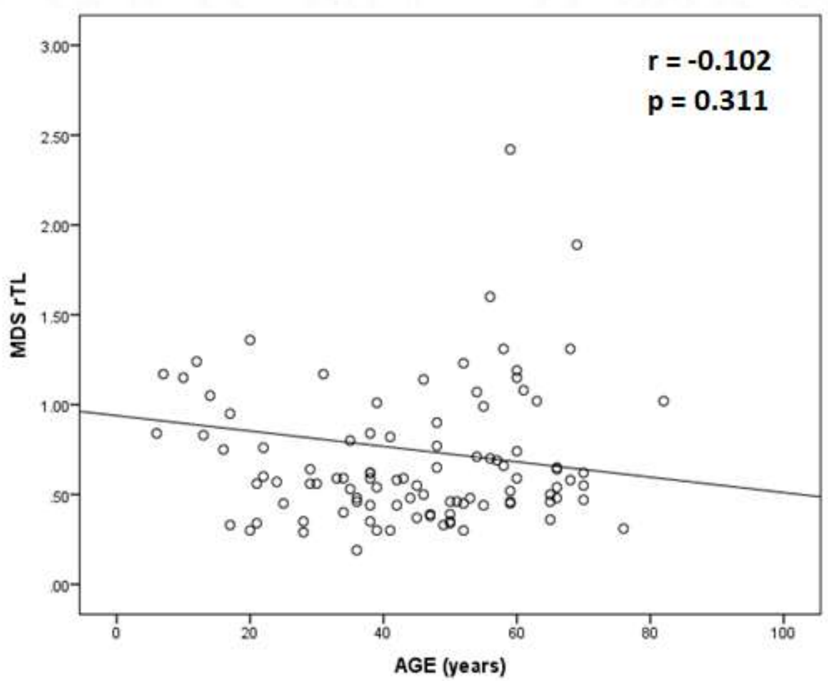


Figure 4.2.2.4.: Correlation of relative telomere length with age in patients with myelodysplastic syndrome

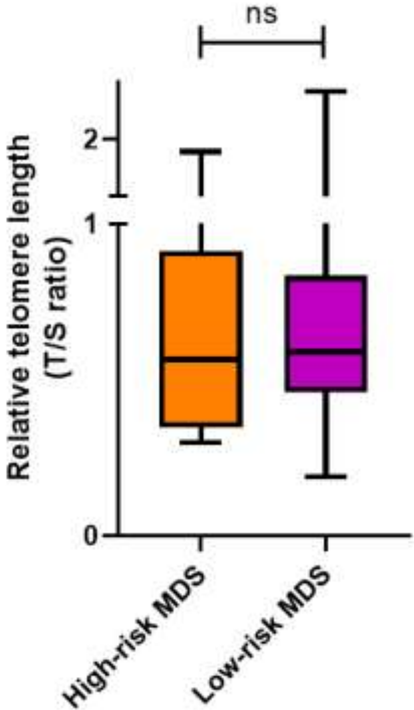


Figure 4.2.2.4.1.: Comparison of relative telomere length between high-risk and low-risk MDS patients

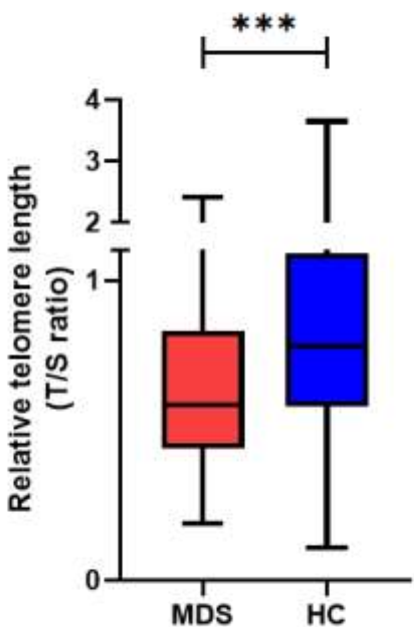


Figure 4.2.2.4.2.: Comparison of relative telomere length in patients with myelodysplastic syndrome with healthy controls

The median age-adjusted telomere length of patients was 0.24 (0.09 - 0.56), and the median age-adjusted rTL between males and females were 0.23 (0.09 - 0.56) & 0.25 (0.15 - 0.48), respectively. We observed a significant difference in the median rTL [0.73 (0.10 - 3.00) vs 0.58 (0.19 - 2.42), p = 0.031] (**Figure 4.2.2.4.3.**) and age-adjusted rTL between patients with age-matched AA and MDS [0.36 (0.20 - 0.54) vs 0.24 (0.09 - 0.56), p = 0.000]. The patients were classified age-wise (<30 & >30 years), and there was no significant difference in median rTL between AA and MDS (**Table 4.2.2.4.**). About 23 (23%) MDS patients had rTL <10th percentile, and one patient (1%) with rTL <1st percentile.

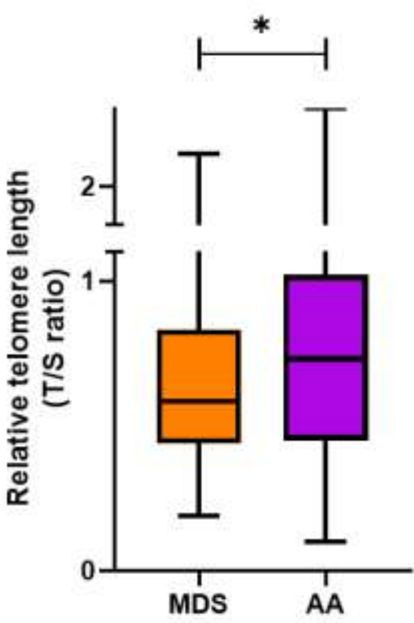


Figure 4.2.2.4.3.: Comparison of relative telomere length in patients with myelodysplastic syndrome with aplastic anaemia

Table 4.2.2.4.: Comparison of baseline characteristics and relative telomere length in patients with Aplastic anaemia and Myelodysplastic syndrome

			AA		MDS		p value
N			650		100		
Age **(years)**	**Median (Range)**		30 (1-60)		46 (6-82)		0.000
Gender	**M [N (%)]** **F**		404 (62) 246 (38)		58 (58) 42 (42)		0.000 0.000
Relative telomere length	**Median (Range)**		0.73 (0.10-3.00)		0.58 (0.19-2.42)		0.031
Age-adjusted telomere length	**Median (Range)**		0.36 (0.20-0.54)		0.24 (0.09-0.56)		0.000
Relative telomere length (Gender)	**Median (Range)**	**M** **F**	0.71 (0.10-3.00) 0.76 (0.12-2.98)		0.57 (0.19-2.42) 0.59 (0.30-1.60)		0.274 0.028
Age-adjusted telomere length (Gender)	**Median (Range)**	**M** **F**	0.37 (0.20-0.54) 0.35 (0.20-0.54)		0.23 (0.09-0.56) 0.25 (0.15-0.48)		0.000 0.000
Relative telomere length (years) Median (Range)	**0-30** **31-60**	**N**	0.79 (0.12-3.00) 0.67 (0.10-1.93)	330 320	0.64 (0.29-1.89) 0.57 (0.19-2.42)	23 60	0.349 0.208

4.3. Impact of telomere length in Aplastic anaemia patients undergoing hematopoietic stem cell transplant

4.3.1. Patient characteristics of the transplant cohort

A total of 281 patients underwent MSD transplants during this period of which 194 DNA samples of patients and donors were available for telomere length measurement. Eight patients having failed the telomere length assay, and 11 patients who received bone marrow grafts were also excluded. The data for telomere length (T/S ratio) was normally distributed, and percentile distribution was obtained. The lowest and highest 2 percentile were omitted as outliers in both the

recipients and donors (Recipient <2nd percentile, T/S ratio: 0.06, 0.09, 0.1; N=3 and >98th percentile, T/S ratio: 3.1, 5.98, 7.52; N=3). Donor < 2nd percentile, T/S ratio: 0.04, 0.06, 0.06; N=3 and >98th percentile, T/S ratio: 5.61, 5.64, 5.76; N=3 and thus 12 more patients were excluded. Therefore, the final analysis had 163 patients, which included 157 patients with fully matched sibling donors and 6 with fully matched family donors.

One hundred and sixty-three patients (106 males and 57 females) who underwent MSD HSCT were included in this analysis. The median age of AA patients and their donors at the time of HSCT were 24 (3 - 58) and 26 (3 - 60) years, respectively (**Table 4.3.1.**). The majority (76%) had either severe or very severe aplastic anaemia. All patients with non-severe aplastic anaemia (N = 45) were transfusion dependent despite treatment with either stanazolol or danazol (N = 26), Cyclosporine (N = 13) or Anti-thymocyte globulin (N = 6). Donors were HLA-matched sibling donors in 157 (96%), while 6 (4%) were fully matched family donors.

The main conditioning regimen used for HSCT was a combination of cyclophosphamide (60 mg/kg/day x 2 days) and fludarabine (30 mg/m^2/day x 6 days) (88%), and the graft source was peripheral blood stem cells (PBSC) in all patients. None of the patients received ATG as part of conditioning or GVHD prophylaxis. Neutrophil engraftment occurred in 89% at a median of 15 days (range: 9 - 28), while 14 expired prior to engraftment due to sepsis and graft failure was observed in 10 (6%) patients. Of the 10 patients with graft failure, 3 (2%) had primary graft failure, while 7 (4%) had secondary graft failure (**Table 4.3.1.**).

Of the 149 patients who were evaluated for chimerism analysis on day 28 post-HSCT, 142 had complete chimerism (CC), 4 had mixed chimerism (MC), and 3 had primary graft failure. Four patients with CC and all 4 with MC subsequently developed secondary graft failure. The incidence of grade 2-4 acute GVHD was 28%, while the incidence of grade 3-4 acute GVHD was 12%. The

incidence of chronic GVHD was 24% and was mild to moderate in most patients. At a median follow-up of 37 months (1-161), 117 (72%) patients are alive.

The baseline characteristics of the patients included in this analysis (N = 163) were compared with those who were excluded (N = 23), and they were similar with respect to age, gender distribution and telomere length, but there was a higher incidence of using a non-Flu/Cy regimen (p = 0.001) and higher incidence of graft failure (p = 0.047) (**Table 4.3.1.1.**).

Table 4.3.1.: Baseline characteristics of patients undergoing MSD transplant for aplastic anaemia

Parameters	Patients (N = 163)	Donors (N = 163)
Age (years) **Median (Range)**	 24 (3-58)	 26 (3-60)
Relative TL Median (Range)	0.76 (0.12-3.00)	0.92 (0.15-5.24)
Age-adjusted Relative TL Median (Range)	0.45 (0.0032-0.97)	0.54 (0.0052-0.99)
Gender N (%) **Male** **Female**	 106 (65) 57 (35)	 87 (54) 76 (46)
Disease status N (%) **NSAA** **SAA** **VSAA**	 39 (24) 93 (57) 31 (19)	 NA
Conditioning Regimen N (%) **Fludarabine + Cyclophosphamide** **Others**	 144 (88) 19 (12)	 NA
Graft N (%) **PBSC**	 163 (100)	 NA
Cumulative incidence of Graft Failure N (%) **Yes** **No**	 10 (6) 139 (85)	 NA
Cumulative incidence of Acute GVHD N (%)	46/146 (28%)	NA
Cumulative incidence of Chronic GVHD N (%)	40/128 (24%)	NA
Final Outcome N (%) **Alive** **Expired**	 117 (72) 46 (28)	 NA

Table 4.3.1.1.: Comparison of baseline characteristics and outcomes between patients recruited in the study (N = 163) versus patients excluded (N = 23)

Parameters	Patients (N = 163)	Patients (N = 23)	p value	Donors (N = 163)	Donors (N = 23)	p value
Age (years) **Median (Range)**	24 (3-58)	17 (5-48)	0.151	26 (3-60)	20 (1-48)	0.660
Relative TL Median (Range)	0.76 (0.12-3.00)	0.84 (0.06-7.52)	0.833	0.92 (0.15-5.24)	0.67 (0.04-6.11)	0.114
Age-adjusted Relative TL Median (Range)	0.45 (0.003-0.97)	0.48 (0.15 – 0.82)	0.711	0.54 (0.005-0.99)	0.44 (0.02-0.92)	0.186
Gender N (%) **Male** **Female**	 106 (65) 57 (35)	 13 (56) 10 (44)	0.426	 87 (54) 76 (46)	 16 (70) 7 (30)	0.143
Disease status N (%) **NSAA** **SAA** **VSAA**	 39 (24) 93 (57) 31 (19)	 2 (9) 17 (74) 4 (17)	0.210	NA	NA	NA
Conditioning Regimen N (%) **Flu/Cy** **Others**	 144 (88) 19 (12)	 14 (60) 9 (40)	0.001	NA	NA	NA
Graft N (%) **PBSC**	 163 (100)	 23 (100)		NA	NA	NA
Graft Failure N (%) **Yes** **No**	 10 (6) 139 (85)	 5 (22) 18 (78)	0.047	NA	NA	NA
Acute GVHD N (%)	46/146 (28%)	3/23 (13%)	0.252	NA	NA	NA
Chronic GVHD N (%)	40/128 (24%)	5/23 (22%)	0.677	NA	NA	NA
Final Outcome N (%) **Alive** **Expired**	 117 (72) 46 (28)	 12 (52) 11 (48)	0.095	NA	NA	NA

4.3.2. Telomere length analysis in patients and transplant donors

The median relative telomere length of patients (PTL) was 0.76 (0.12 - 3.00) and 0.92 (0.15 - 5.24) of donors (DTL). The median and range for recipient-donor age difference in sibling donors were 0 (-25 to 13), and for family donors were -24 (-42 to 28). The relative telomere length was seen to decrease with age in AA patients and transplant donors (**Figure 4.3.2.**).

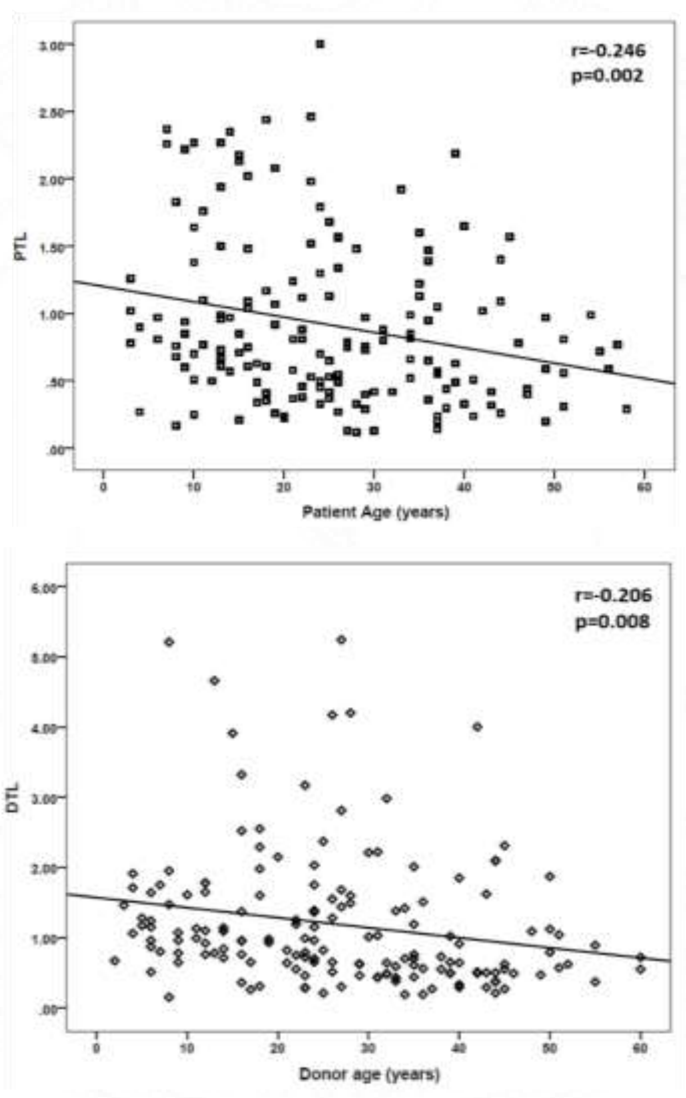


Figure 4.3.2.: Scatter plots of the correlation between relative telomere length and age in (a) AA patients and (b) transplant donors

4.3.3. Relative telomere length of patients and donors categorised into median and quartiles

The relative telomere length for both patients and donors was categorised into short (PTLm < 0.76, DTLm < 0.92) and long (PTLm > 0.77, DTLm > 0.93) based on their respective median rTL. Eighty-two patients and 82 donors had a shorter rTL. In addition, the rTL of both patient and donor was classified into quartiles [PTL: <0.45, 0.45 - 0.76, 0.77 - 1.17, >1.17, DTL: <0.55, 0.55 - 0.92, 0.93 - 1.55, >1.55]. Finally, for the analysis, rTL for patients and donors was categorised as longer (comprising the highest quartile; HQ) and shorter (consisting of the combination of the 1st, 2nd, and 3rd quartile; LQ) telomere lengths.

Telomere length as a continuous variable was not found to be significant, and therefore rTL was categorised into median and quartiles. Baseline and transplant characteristics based on median values of patient and donor relative telomere length are summarised in **Table 4.3.3.** The median patient telomere length was not found to be significantly associated with either baseline characteristics or transplant outcomes. A short median donor telomere length (DTLm) was associated with increasing donor age (p = 0.001) and a higher incidence of acute graft versus host disease (p = 0.021) but not eith survival outcome (p = 0.117).

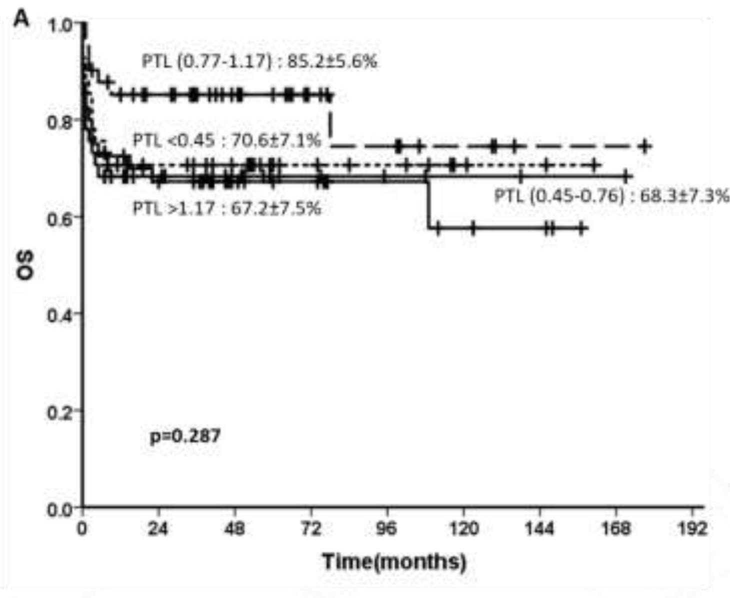
Table 4.3.3.: Characteristics of patients and donors based on median relative telomere length

Parameters	PTL Median {PTLm}			DTL Median {DTLm}		
	Short <0.76	Long >0.77	p value	Short <0.92	Long >0.93	p value
	N=82	N=81		N=82	N=81	
P Age (years)			0.162			
<20	26 (32)	37 (46)		NA		
20-40	42 (51)	35 (43)				
>40	14 (17)	9 (11)				
P Sex			0.101			
Male	48 (58)	58 (72)		NA		
Female	34 (42)	23 (28)				
D Age (years)						0.001
<20	NA			17 (21)	39 (48)	
20-40				45 (55)	33 (41)	
>40				20 (24)	9 (11)	
D Sex						0.876
Male	NA			43 (52)	44 (54)	
Female				39 (48)	37 (46)	
Disease subtype			0.441			
NSAA	23 (28)	16 (20)		NA		
SAA	45 (55)	48 (59)				
VSAA	14 (17)	17 (21)				
Graft Failure	4 (5)	6 (8)	0.746	6 (8)	4 (6)	0.747
Neutrophil recovery N (%)	72 (88)	74 (91)	0.610	74 (90)	72 (89)	0.803
Acute GVHD	24 (33)	22 (30)	0.722	30 (41)	16 (22)	0.021
Chronic GVHD	17 (27)	23 (35)	0.446	19 (32)	21 (31)	1.000
Final Outcome			0.602			0.117
Alive	57 (69)	60 (74)		54 (66)	63 (78)	
Expired	25 (31)	21 (26)		28 (34)	18 (22)	

The baseline and transplant characteristics based on quartiles of patient and donor relative telomere length are summarised in **Table 4.3.3.1**. When divided into quartiles, overall survival was not different between the 4 quartiles for patient telomere length (p = 0.287) (**Figure 4.3.3.a**); however, for donor telomere length, the highest quartile (> 1.55) showed significantly better survival compared to the other 3 quartiles (p = 0.022) (**Figure 4.3.3.b**). After combining the lower 3 quartiles into a single group among the patients as well as of donors (LQ), a longer telomere length (HQ) in donors was significantly associated with better 5-year overall survival (95% vs 65%; p = 0.002) (**Figure 4.3.3.1.a,b**). Age-adjusted telomere length for both patients and donors did not show any correlation with patient or donor age, graft failure, GVHD or survival.

Table 4.3.3.1.: Characteristics of patients and donors based on quartiles relative telomere length

Parameters	PTL Quartile (PTL Q}			DTL Quartile {DTL Q}		
	Low <1.17	High >1.18	p value	Low <1.55	High >1.56	p value
	N=123	N=40		N=123	N=40	
P Age (years)			0.123			
<20	44 (36)	19 (47)		NA		
20-40	58 (47)	19 (48)				
>40	21 (17)	2 (5)				
P Sex			1.000			
Male	80 (65)	26 (65)		NA		
Female	43 (35)	14 (35)				
D Age (years)						0.130
<20	NA			37 (30)	19 (47)	
20-40				63 (51)	15 (38)	
>40				23 (19)	6 (15)	
D Sex						0.588
Male	NA			64 (52)	23 (58)	
Female				59 (48)	17 (42)	
Disease subtype			0.502			
NSAA	31 (25)	8 (20)		NA		
SAA	71 (58)	22 (55)				
VSAA	21 (17)	10 (25)				
Graft Failure	8 (7)	2 (6)	1.000	7 (6)	3 (8)	0.729
Neutrophil recovery N (%)	112 (91)	34 (85)	0.370	107 (87)	39 (97)	0.074
Acute GVHD	38 (34)	8 (24)	0.297	38 (35)	8 (21)	0.108
Chronic GVHD	30 (31)	10 (33)	0.824	24 (27)	16 (41)	0.147
Final Outcome			0.313			0.002
Alive	91 (74)	26 (65)		81 (66)	36 (90)	
Expired	32 (26)	14 (35)		42 (34)	4 (10)	



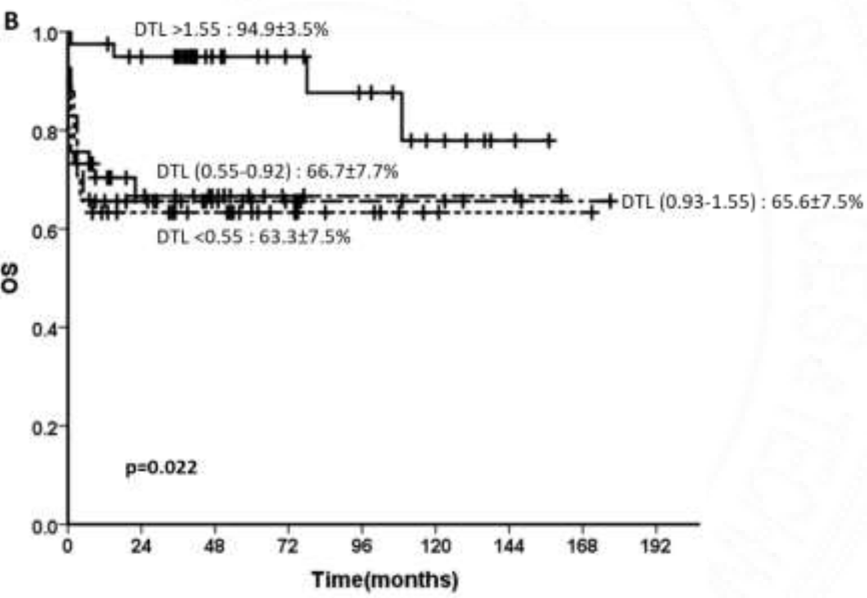
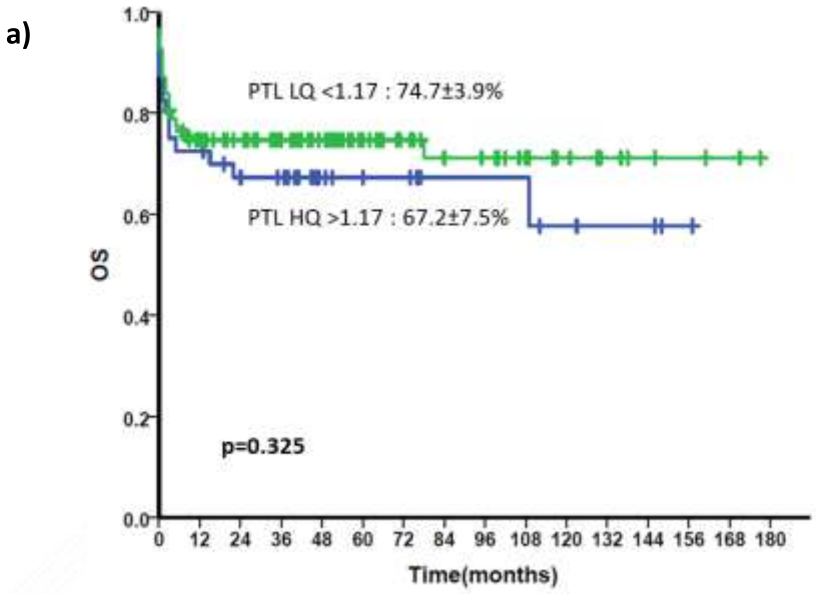


Figure 4.3.3.: Overall survival based on quartiles of (A) patient telomere length and (B) donor telomere length

a)



b)

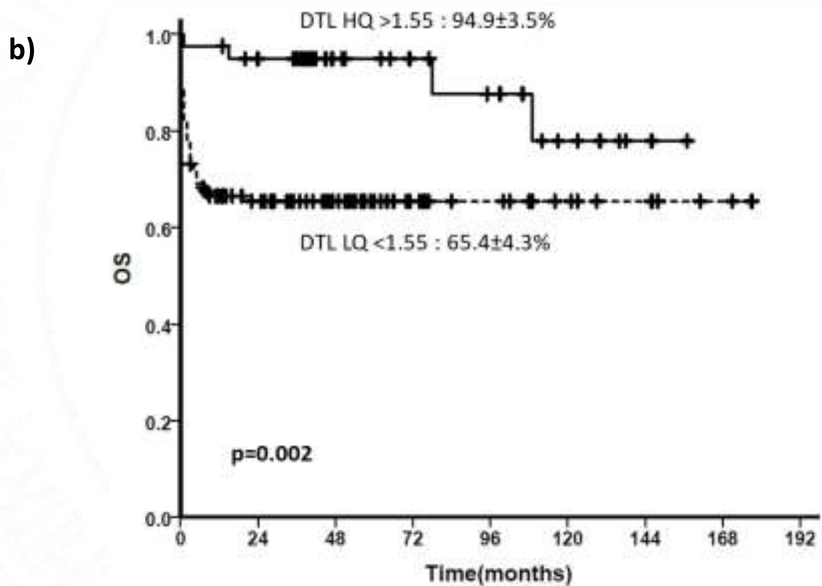


Figure 4.3.3.1.: 5-year overall survival comparing the highest quartile of the (a) patient and (b) donor telomere length with the combination of lower 3 quartiles

4.3.4. Association of patient telomere length with transplant outcomes

Logistic regression analysis showed that HSCT outcomes were not influenced by the pre-transplant relative telomere length of the recipient. The median patient relative telomere length (PTLm) did not influence graft failure (HR = 0.7, p = 0.558), neutrophil recovery post-HSCT (HR = 1.5, p = 0.460), the incidence of acute GVHD (HR = 1.2, p = 0.639), chronic GVHD (HR = 0.7, p = 0.366) or overall survival (HR = 0.8, p = 0.518). Both long and short patient relative telomere length was significantly associated with older age (>40 years) but not with transplant outcomes, including acute GVHD (HR = 1.7, p = 0.256), neutrophil recovery (HR = 0.6, p = 0.281), graft failure (HR = 1.2, p = 0.788) or overall survival (HR = 1.5, p = 0.275) (**Table 4.3.4.**).

Table 4.3.4.: Logistic regression analysis of patient telomere length with demographic characteristics and HSCT outcomes

	PTLm	p value	PTL LQ	p value	PTL HQ	p value
	HR (95% CI)		HR (95% CI)		HR (95% CI)	
Patient age (years)						
<20						
20-40	1.7 (0.87-3.35)	0.119	2.1 (0.92-4.89)	0.079	1.3 (0.63-2.78)	0.469
>40	2.2 (0.83-5.88)	0.111	3.4 (1.16-9.99)	0.026	4.5 (0.96-21.29)	0.055
Patient gender	0.3 (0.29-1.01)	0.082	1.4 (0.65-3.04)	0.377	1.0 (0.47-2.12)	0.996
Disease subtype						
NSAA						
SAA	0.7 (0.31-1.39)	0.268	1.0 (0.45-2.38)	0.924	0.8 (0.33-2.07)	0.694
VSAA	0.6 (0.22-1.49)	0.252	0.3 (0.07-1.08)	0.065	0.5 (0.18-1.59)	0.267
Graft failure	0.7 (0.18-2.50)	0.558	1.3 (0.31-5.19)	0.736	1.2 (0.25-6.16)	0.788
Neutrophil recovery	1.5 (0.53-4.07)	0.460	1.3 (0.42-3.86)	0.670	0.6 (0.19-1.62)	0.281
Acute GVHD	1.2 (0.59-2.38)	0.639	1.3 (0.59-2.92)	0.494	1.7 (0.69-4.04)	0.256
Chronic GVHD	0.7 (0.33-1.50)	0.366	1.0 (0.42-2.37)	1.000	0.9 (0.37-2.11)	0.779
Survival outcome	0.8 (0.40-1.58)	0.518	0.9 (0.43-2.03)	0.863	1.5 (0.71-3.28)	0.275

4.3.5. Association of donor telomere length with transplant outcomes

The median relative telomere length of the donor (DTLm) was significantly associated with donor age (HR = 3.1, p = 0.002 for age 20 - 40 years and HR = 5.1, p = 0.001 for age > 40 years) and acute GVHD (HR = 2.4, p = 0.019). Median donor telomere length (DTLm), however did not influence graft failure (HR = 1.4, p = 0.587) or neutrophil recovery (HR = 0.9, p = 0.777) but had a trend towards overall survival (HR = 0.5, p = 0.093). Longer donor relative telomere length (DTL HQ) was found to be significantly associated with overall survival compared to shorter donor rTL (DTL LQ) (HR = 0.2, p = 0.006) (**Table 4.3.5.**).

Table 4.3.5.: Logistic regression analysis of donor telomere length with demographic characteristics and HSCT outcomes

	DTLm	p value	DTL LQ	p value	DTL HQ	p value
	HR (95% CI)		**HR (95% CI)**		**HR (95% CI)**	
Donor age (years)						
<20						
20-40	3.1 (1.51-6.46)	0.002	4.0 (1.41-11.37)	0.009	2.2 (0.98-4.75)	0.056
>40	5.1 (1.93-13.46)	0.001	9.5 (2.95-30.74)	0.000	1.9 (0.69-5.65)	0.208
Donor gender	0.9 (0.50-1.72)	0.810	1.3 (0.65-2.70)	0.444	0.8 (0.39-1.63)	0.547
Graft failure	1.4 (0.38-5.31)	0.587	0.7 (0.15-3.53)	0.681	0.8 (0.21-3.45)	0.816
Neutrophil recovery	0.9 (0.32-2.36)	0.777	1.3 (0.42-3.86)	0.670	5.8 (0.75-45.45)	0.092
Acute GVHD	2.4 (1.16-4.92)	0.019	2.1 (0.98-4.66)	0.057	2.1 (0.89-5.11)	0.089
Chronic GVHD	1.0 (0.49-2.19)	0.924	1.8 (0.76-4.22)	0.184	0.5 (0.24-1.17)	0.117
Survival outcome	0.5 (0.27-1.10)	0.093	0.6 (0.27-1.25)	0.171	0.2 (0.07-0.64)	0.006

The survival probability for patients who received HSCT from donors with longer (DTL HQ) *versus* shorter (DTL LQ) telomeres was 95% vs 65% (p = 0.002) (**Figure 4.3.3.1.b**). Older

patients (age > 40 years: HR = 3.3, p = 0.034) and donor age (age > 40 years: HR = 5.2, p = 0.004, (20 – 40) years: HR = 3.9, p = 0.004) and shorter median donor (DTLm) (HR = 2.3, p = 0.019) were significantly associated with acute GVHD (**Table 4.3.5.1.**). There were no factors that were found to be associated with graft failure.

Table 4.3.5.1.: Association of acute GVHD and graft failure with pre and posttransplant parameters

	Graft Failure		**Acute GVHD**	
Parameters	**HR (95% CI)**	**p value**	**HR (95% CI)**	**p value**
Patient Age (years) **<20** **20-40** **>40**	 0.1 (0.01-0.75) 3.3 (0.85-12.90)	 0.997 0.084	 1.9 (0.89-4.45) 3.3 (1.09-9.95)	 0.092 0.034
Patient Gender	0.7 (0.21-2.94)	0.727	0.8 (0.39-1.65)	0.548
Donor Age (years) **<20** **20-40** **>40**	 0.3 (0.06-1.91) 0.5 (0.49-9.34)	 0.218 0.313	 3.9 (1.54-9.89) 5.2 (1.68-16.13)	 0.004 0.004
Donor Gender	0.9 (0.24-3.08)	0.809	1.8 (0.87-3.71)	0.110
AA type **NSAA** **SAA** **VSAA**	 0.8 (0.19-3.49) 0.4 (0.04-4.31)	 0.794 0.468	 2.07 (0.81-5.31) 2.50 (0.79-7.85)	 0.130 0.117
Neutrophil recovery	0.2 (0.09-1.16)	0.999	0.4 (0.05-3.68)	0.432
Acute GVHD	0.4 (0.05-3.68)	0.432		
PTLm	0.6 (0.18-2.50)	0.558	1.2 (0.58-2.37)	0.639
DTLm	1.4 (0.38-5.31)	0.587	2.3 (1.16-4.92)	0.019
PTL HQ	1.2 (0.25-6.16)	0.788	1.7 (0.69-4.04)	0.256
DTL HQ	0.8 (0.21-3.45)	0.816	2.1 (0.89-5.11)	0.089

Since the highest quartile for donor telomere length was significantly associated with better survival, we divided all transplant recipients into 4 groups based on the combination of lower (LQ) and higher quartiles (HQ) of patient and donor telomere length. The highest quartile of donor telomere length (DTL HQ), irrespective of its combination with either high (PTL HQ) or low patient telomere length (PTL LQ), showed survival ranging from 88 – 100% (p = 0.003) (**Figure 4.3.5.**).

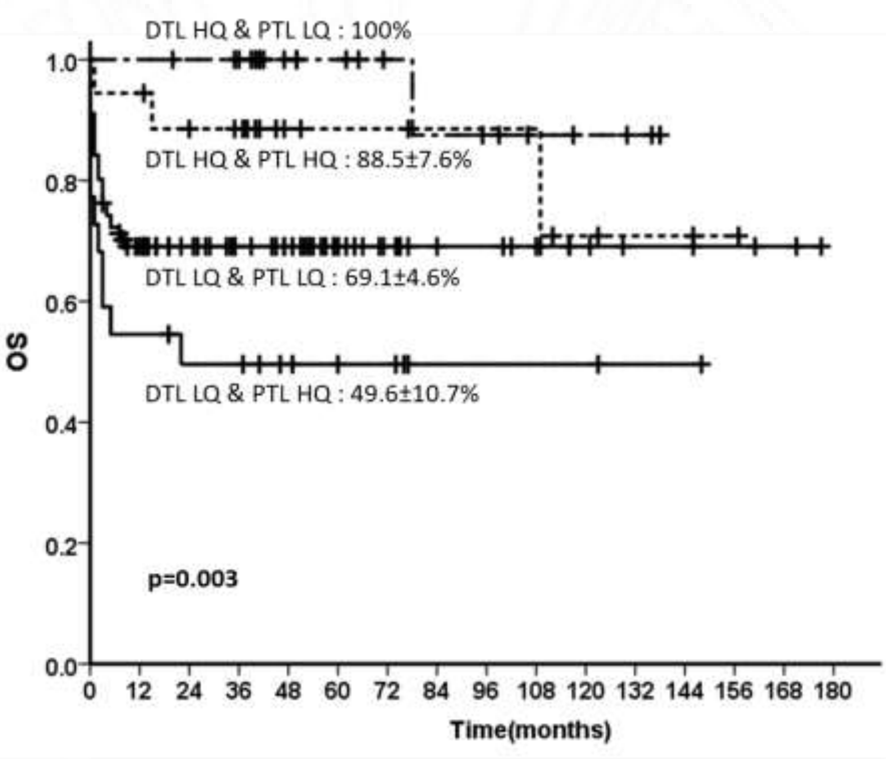


Figure 4.3.5.: Overall survival based on the combination of telomere length quartiles of patient and donor

4.3.6. Factors influencing overall survival

The factors that would affect overall survival in patients undergoing matched sibling donor transplants for aplastic anaemia were studied. On univariate analysis, patients aged (20 – 40) years (HR = 2.1, p = 0.045), older patients (HR = 3.4, p = 0.005), older donors (HR = 3.0, p=0.011), presence of graft failure (HR = 4.5, p=0.001), presence of acute GVHD (HR = 3.4, p = 0.001) adversely affected overall survival while longer donor relative telomere length (DTL HQ) (HR = 4.3, p = 0.006) were found to be significantly associated with better overall survival. The presence of chronic GVHD and patient telomere length (rTL) did not have a significant impact on overall survival. On multivariate analysis, patient age between (20 – 40) years (HR = 4.4, p = 0.050) and acute GVHD (HR = 3.0, p = 0.006) continued to be associated with poorer overall survival while longer donor relative telomere length (DTL HQ) continued to be associated with improved survival outcome (HR = 0.3, p = 0.049) (**Table 4.3.6.**).

Table 4.3.6.: Factors influencing overall survival in patients undergoing matched sibling donor HSCT for aplastic anaemia

	Univariate analysis		Multivariate analysis	
Parameters	**Survival probability (%)**	**p value**	**HR (95% CI)**	**p value**
Patient Age (years)		0.011		
<20	84%			
20-40	68%		4.4 (0.99-19.44)	0.050
>40	52%		4.3 (0.80-23.26)	0.088
Donor Age (years)		0.028		
<20	84%			
20-40	69%		1.1 (0.23-4.90)	0.948
>40	55%		1.4 (0.29-7.49)	0.631
Graft Failure		0.000	2.4 (0.49-12.07)	0.275
Yes	40%			
No	81%			
Acute GVHD		0.001	3.0 (1.37-6.57)	0.006
Yes	63%			
No	80%			
Chronic GVHD		0.354		
Yes	93%			
No	90%			
PTLm		0.440		
Short < 0.76	70%			
Long > 0.77	74%			
PTL HQ		0.325		
Low < 1.17	74%			
High > 1.18	65%			
DTLm		0.090		
Short < 0.92	66%			
Long > 0.93	78%			
DTL HQ		0.006	0.3 (0.09-0.99)	0.049
Low < 1.55	65%			
High > 1.56	95%			

The causes of death were analysed in patients who had donors with the highest quartile (DTL HQ) versus the lower 3 quartiles (DTL LQ). There were 46 deaths, including 4 in the DTL HQ group and 42 in the DTL LQ group. Deaths in the DTL HQ group were mainly due to chronic GVHD (N =3) and primary graft failure (N = 1). Deaths in the DTL LQ group were mainly due to multi-drug resistant bacterial infections (N = 21), grade IV acute GVHD (N = 9), invasive fungal infection (N = 6), graft failure (N = 2) and other miscellaneous causes (N = 4).

4.4. Analysis of Germline genetic variants in AA patients using NGS

4.4.1. Patient characteristics of the cohort

In this cohort, 120 young patients (≤40 years of age) diagnosed to have AA between the years January 2006 to August 2021 were included. The median age of patients was 10 (0 - 40) years, including 67 males (56%) and 53 females (44%). There were 18 patients with NSAA (15%), 74 with SAA (62%) and 28 with VSAA (23%). The median relative telomere length of the entire cohort was 0.84 (0.21 - 3.10). Genetic variants were identified in 26 patients (22%), which included germline pathological (PAT) genetic variants in 10%, variants of limited significance in 6% and variants of uncertain significance (VUS) in 6% of patients. The characteristics of patients according to variants and telomere length were categorised age-wise (**Table 4.4.1.**).

4.4.2. Analysis of genetic variants in patients with AA

Using targeted gene capture sequencing, in 26 patients, we detected 12 pathogenic variants (7 missense, 1 nonsense, 2 frameshifts, and 2 splice site) of known causative IBMFS genes, including MPL (N = 5), NLRP12 (N = 1), DNAJC21 (N = 1), PRF1 (N=1), TINF2 (N = 2), and RTEL1 (N = 2).

Table 4.4.1.: Characteristics of AA patients categorised age-wise

Age (years)		<=5	6-10	11-15	16-30	31-40
Total (N=120)		30	40	22	23	5
Pathogenic variants (N=12)		7 (23.3%)	1 (2.5%)	1 (4.5%)	3 (13.0%)	0
Variants of unknown significance (VUS) (N=14)	Heterozygous variants (N=7)	2 (6.7%)	2 (5.0%)	1 (4.5%)	2 (8.7%)	0
	Others* (N=7)	3 (10.0%)	2 (5.0%)	1 (4.5%)	1 (4.3%)	0
No variants (N= 94)		17 (60.0%)	35 (87.5%)	19 (86.5%)	17 (74.0%)	5 (100%)
Relative TL (N=120) Median/(Range)		0.81 (0.21-1.47)	0.97 (0.24-3.10)	0.82 (0.31-1.63)	0.82 (0.21-2.02)	0.68 (0.42-0.90)
No Variants (N=94) Variants (N=26)		0.71 (0.24-1.40) 1.17 (0.21-1.81)	0.97 (0.24-3.10) 1.11 (0.76-1.37)	0.81 (0.31-1.70) 1.11 (0.73-1.63)	0.83 (0.55-2.02) 0.42 (0.21-1.29)	
p value		0.126	0.756	0.575	0.025	

Homozygous variants were found in 6 patients (5 in MPL and 1 in DNAJC21), compound heterozygous variant in one patient (NLRP12), and heterozygous mutations in 5 patients (2 in TINF2, 2 in RTEL1, and 1 in PRF1). Seven VUS variants were present in the following genes, ATM (N = 1), WRAP53 (N = 1), PRF1 (N = 1), FANCA (N = 1), ERCC6L2 (N = 1) and TERT (N = 2). The other variants (N = 7) were classified as either the association of the gene with bone marrow failure or the pathogenicity of the variant is uncertain. The genes in this category include ITGA2B (N = 1), BRCA2 (N = 1), PRF1 (N = 2), MPL (N = 2), and GFI1 (N = 1) (**Table 4.4.2.**). The variants identified in NGS were validated using Sanger sequencing. The samples from the patients in whom VUS were identified and patients within 5 years of age in no variants were considered for whole exome sequencing. There were no pathogenic variants observed in these samples.

Table 4.4.2.: Information on variants identified in patients with AA

Sample ID	Gene	dbSNP ID	DNA change	Type of mutation	cDNA change	Amino acid change	Zygosity	ACMG classification	Mode of Inheritance
AA 369	MPL	rs775704066	NC_000001.11:g.43352239C>T	missense	NM_005373.3:c.1589C>T	p.Pro530Leu	Homozygous	likely pathogenic	AR
N 716	NLRP12	rs770257961	NC_000019.10: g.53810702-53810703ins	frameshift	NM_144687.4:c.956_957insTCTTAA	p.Pro319_Thr320insLeuAsn	Compound heterozygous	VUS	AD
		rs745918068	NC_000019.10: g.53810704-53810705ins	frameshift	c.958_959insGCTTAATT	p.Thr320SerfsTer11		Pathogenic	
AA 987	DNAJC21	rs368148362	NC_000005.10:g.3494118 4G>A	splice site	NM_001012339.3:c.983+1G>A		Homozygous	pathogenic	AR
P 485	MPL	rs587778514	NC_000001.11:g.43338564_43338565del	frameshift	NM_005373.2:c.235_236del	p.Leu79Glu fsTer84	Homozygous	Pathogenic	AR
12/583	MPL	rs775704066	NC_000001.11:g.43352239C>T	missense	NM_005373.3:c.1589C>T	p.Pro530Leu	Homozygous	likely pathogenic	AR
AA 730	TINF2	rs121918544	NC_000014.9:g.24240635C>T	missense	NM_001099274.3):c.845G>A	p.Arg282His	Heterozygous	Pathogenic	AD
AA718	MPL	rs775704066	NC_000001.11:g.43352239C>T	missense	NM_005373.3:c.1589C>T	p.Pro530Leu	Homozygous	Pathogenic	AR/AD
9/412	MPL	rs2153918662	NC_000001.11:g.43346629G>A	missense	NM_005373.3:c.1165G>A	p.Val389Met	Homozygous	likely pathogenic	AR
AA 958	TINF2	rs1414353472	NC_000014.9:g.24240059C>T	Splice site	NM_001099274.3:c.1221+5G>A		Heterozygous	likely pathogenic	AD
P 469	PRF1	rs28933973	NC_000010.11:g.70599048G>A	missense	NM_005041.5:c.673C>T	p.Arg225Trp	Heterozygous	Pathogenic	AD/AR
AA 1042	RTEL1	rs1449687529	NC_000020.11:g.63692964del	frameshift	NM_032957.5:c.2884del	p.Leu962Ser fsTer34	Heterozygous	Pathogenic	AD
M 638	RTEL1	rs373740199	NC_000020.11:g.63693247C>T	nonsense	NM_032957.5:c.3028C>T	p.Arg1010Ter	Heterozygous	Pathogenic	AD
AA 894	ITGA2B	rs773464534	NC_000017.11:g.44380302C>T	splice site	NM_000419.5:c.1545-1G>A		Heterozygous	Pathogenic	AD/AR
SB	BRCA2			nonsense	NM_000059.4:c.9675T>G	p.Tyr3225Ter	Heterozygous	Likely Pathogenic	AR
AA 898	PRF1	rs779855980	NC_000010.11:g.70598202C>A	nonsense	NM_005041.5:c.1519G>T	p.Glu507Ter	Heterozygous	likely pathogenic	AR
AA 479	MPL	rs1328242642	NC_000001.11:g.43338229G>A	missense	NM_005373.3:c.1565G>C	p.Arg522Thr	Heterozygous	likely pathogenic	AR
4/577	BRCA2	rs587782785	NC_000013.11:g.32370487C>G	nonsense	NM_000059.4:c.8417C>G	p.Ser2806Ter	Heterozygous	Pathogenic	AR
13/917	MPL	rs993195285	NC_000001.11:g.43338229G>A	missense	NM_005373.3:c.770G>A	p.Arg257His	Heterozygous	likely pathogenic	AR
AA 1056	GFI1			missense	NM_001127216.2:c.1234C>G	p.Arg412Gly	Heterozygous	VUS	AD
P 497	ATM	rs1173542225	NC_000011.10:g.108321366T>A	missense	NM_000051.4:c.6518T>A	p.Leu217His	Heterozygous	VUS	AR
14/743	WRAP53	rs748109782	NC_000017.11:g.7703247C>T	missense	NM_001143990.1:c.1408C>T	p.His470Tyr	Heterozygous	VUS	AR
L 788	PRF1	rs150156593	NC_000010.11:g.70599158G>T	missense	NM_005041.5:c.563C>T	p.Pro188Leu	Heterozygous	VUS	AR
N 829	*ERCC6L2*	rs368747018	NC_000009.12:g.95941482G>A	missense	NM_001010895.4:c.1780G>A	p.Gly605Ser	Heterozygous	VUS	AR
N 710	TERT			missense	NM_198253.3:c.2840C>G	p.Ser947Cys	Homozygous	VUS	AR
AA 454	TERT	rs771514853	NC_000005.10:g.1268618G>A	missense	NM_198253.3:c.2484C>G	p.Cys828Trp	Heterozygous	VUS	AD
16/196	FANCA	rs577636020	NC_000016.10:g.89765078_89765079del	Splice site	NM_000135.4:c.2602-9_2602-8del		Heterozygous	VUS	AR

4.4.3. Telomere length in AA patients identified with genetic variants

The median rTL in AA patients with variants 0.97 (0.21 - 1.81) and no variants 0.83 (0.24 - 3.10) had no significant difference (p = 0.685, **Figure 4.4.3.**). While a significant difference in median rTL between patients with variants and no variants in the age group 11-15 years (p = 0.043). Further, we compared rTL among the telomere-associated pathogenic variants (N=4), other pathogenic variants (N = 8), and heterozygous VUS (N = 7) and observed significant differences between the telomere-associated and other pathogenic variants (p = 0.008, **Figure 4.4.3.1.**).

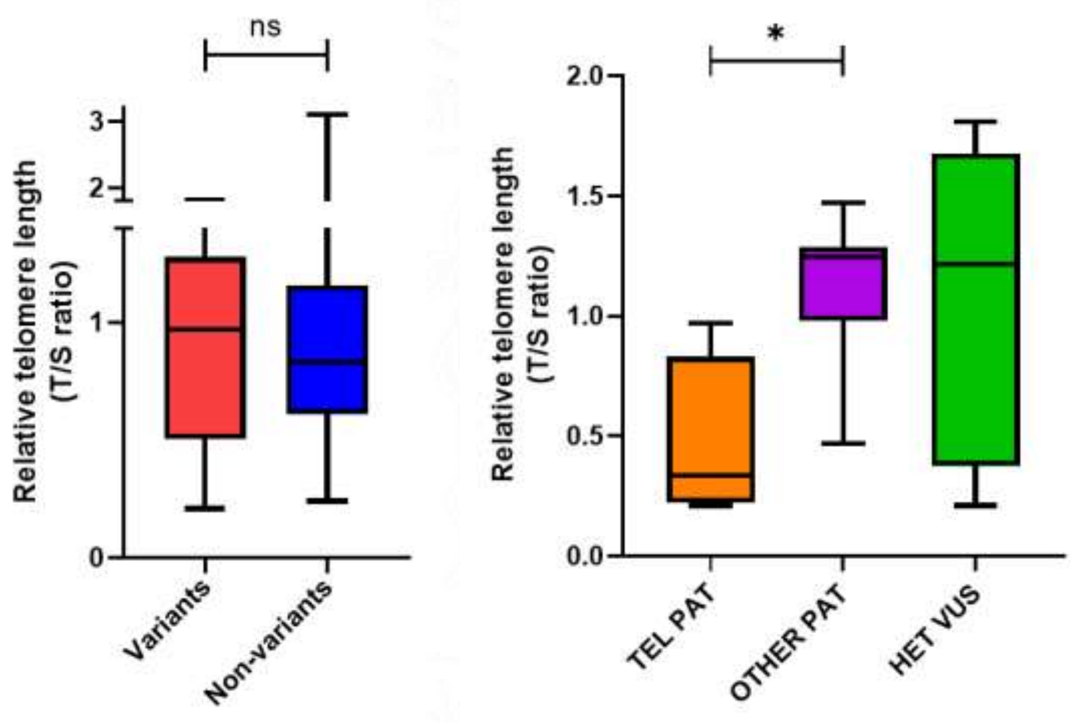
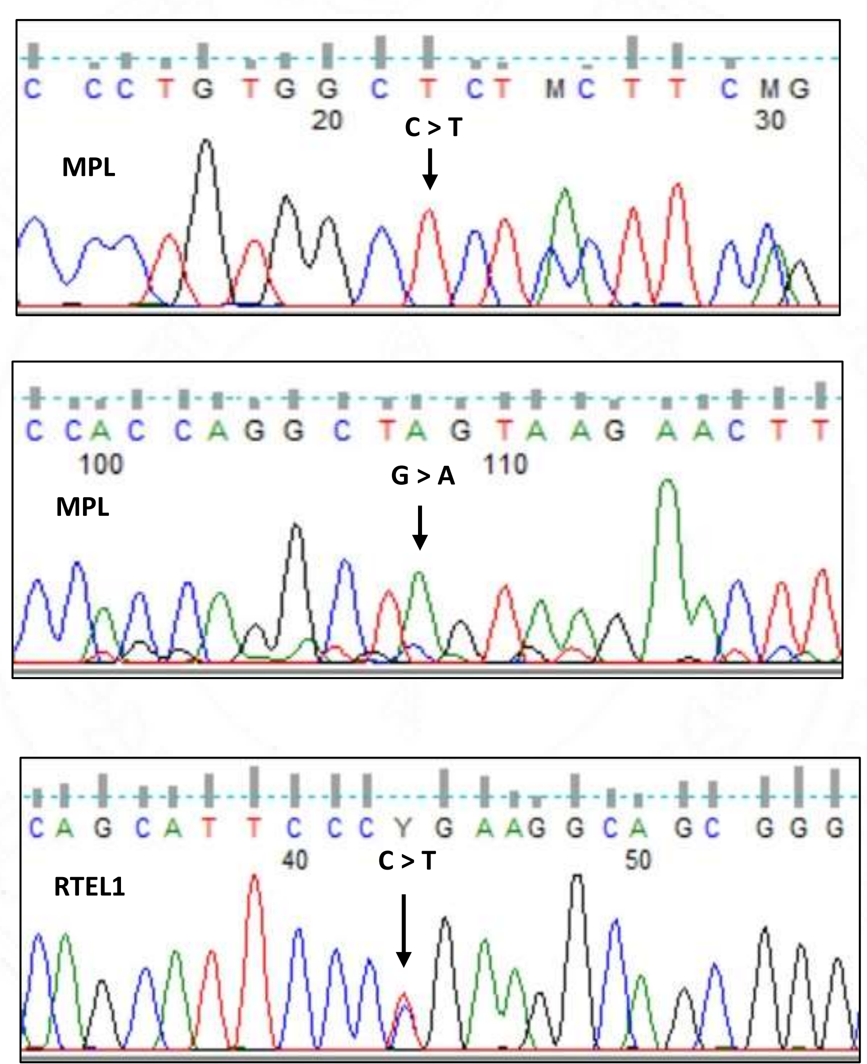


Figure 4.4.3.: Comparison of relative telomere length in variants and non-variants AA patients

Figure 4.4.3.1.: Comparison of relative telomere length in AA patients among the telomere-associated and other pathogenic variants and heterozygous VUS

4.4.4. Validation of variants observed in NGS using Sanger sequencing

Sanger sequencing was performed to validate the single nucleotide variants identified from targeted gene panel sequencing. The electropherogram of the six pathogenic variants in the MPL gene (homozygous c.1589C > T), MPL gene (homozygous c.1165G > A), RTEL1 (heterozygous c.3028C > T), TINF2 (heterozygous c.845G > A), and TINF2 (heterozygous c.1221+5G > A) (**Figure 4.4.4.**).



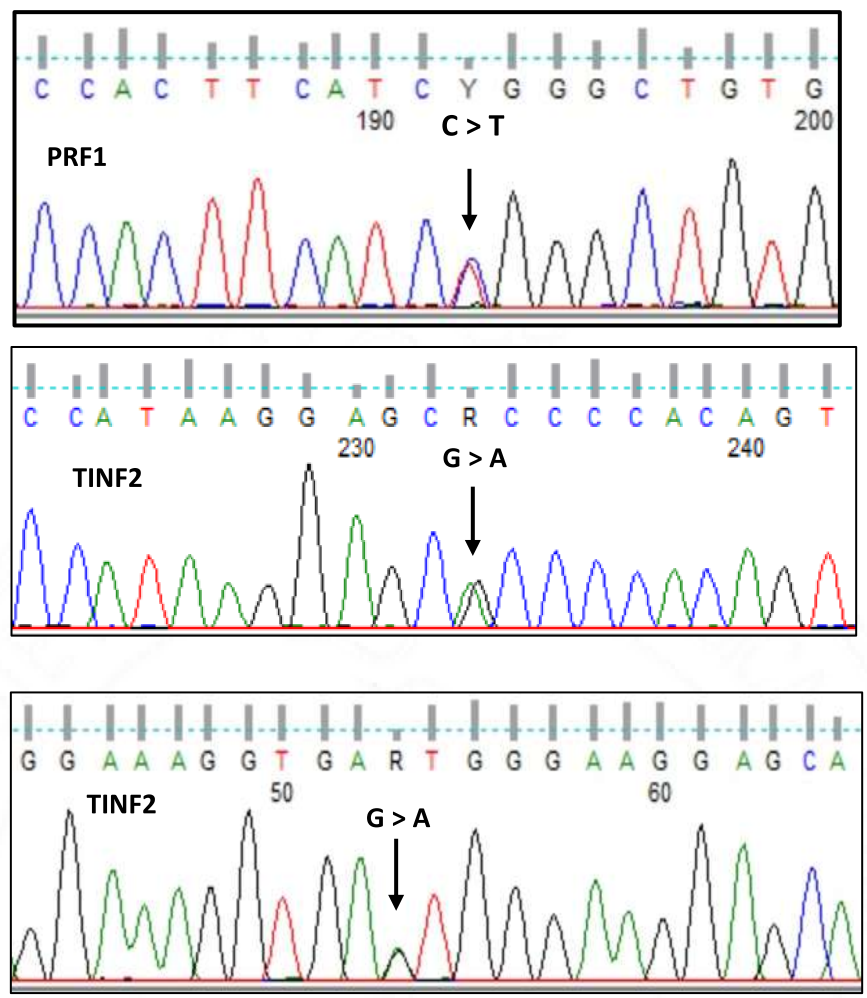


Figure 4.4.4.: Sanger sequencing electropherogram showing single nucleotide variant in pathogenic variant genes: (a) MPL (Homozygous c.1589C > T), (b) MPL gene (Homozygous c.1165G > A), (c), RTEL1 (Heterozygous c.3028C > T), (d) PRF1 (Heterozygous c.673C > T), (e) TINF2 (Heterozygous c.845G > A), and (f) TINF2 (Heterozygous c.1221+5G > A). All sequences are of forward strand (5' - 3')

4.5. Telomere length analysis in AA and normal CD34$^+$ cells

The CD34$^+$ cells were isolated from bone marrow of aplastic anemia (N = 5) and normal controls (N = 5). The cells were cultured invitro in Stemspan media for expansion. The cells were collected, and DNA was extracted to determine the relative telomere length using qPCR. The characteristics of patients and controls are mentioned in Table **4.5.** Since the purity of DNA in AA

CD34$^+$ cells (N=2) were low, telomere length assay was excluded. The rTL of CD34$^+$ cells between AA and normal BM were comparable [0.90 (0.55-1.73) vs 0.76 (0.57-1.89), p = 0.179] (**Figure 4.5.**).

Table 4.5.: Characteristics of patient and normal controls

Parameters	AA CD34$^+$	Normal CD34$^+$
N	5	5
Age (years) **Median (Range)**	32 (4-51)	20 (10-29)
Severity SAA **VSAA**	3 2	NA
Relative telomere length	0.90 (0.55-1.73)	0.76 (0.57-1.89)

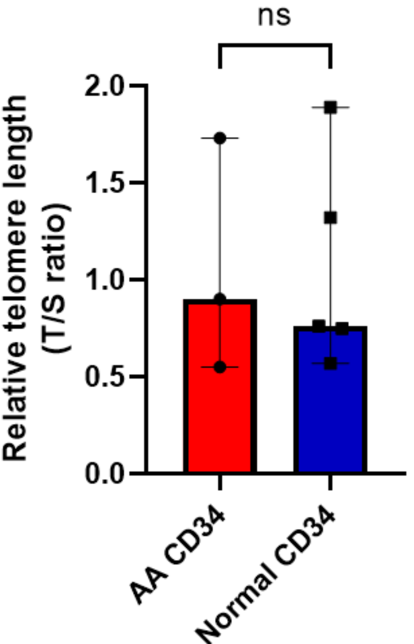


Figure 4.5.: Comparison of relative telomere length in CD34$^+$ cells aplastic anemia and normal bone marrow

4.6. Analysis of the immune and molecular mechanism in Aplastic anemia patients treated with Immunosuppressive therapy (IST)

4.6.1. Baseline characteristics of the AA patients treated with IST

A total of 120 AA patients treated with ATG from the duration of March 2018 to October 2022 were enrolled in this study. Out of 120, (N = 30) were lost to follow-up and (N=18) patients expired prior to 3rd month post treatment. Eventually, seventy-two (N = 72) patients were analyzed who had samples available at baseline and 3rd month post ATG treatment. The median age was 36 (7 - 64) years and included 42 males and 30 females. Based on the response criteria, (N = 37) patients responded to ATG, and (N = 35) were non-responders (**Table 4.6.1.**).

Table 4.6.1.: Characteristics of AA patients who underwent ATG treatment

Parameters	All (N=72)	Responders (N=37)	Non-responders (N=35)	p value
Age (years) Median (Range)	36 (7-64)	38 (7-64)	42 (9-60)	0.509
Gender N (%) M	42 (58)	21 (57)	20 (57)	0.974
F	30 (42)	16 (43)	15 (43)	0.972
Severity				
NSAA	15 (21)	9 (23)	8 (22)	0.890
SAA	50 (68)	26 (69)	24 (68)	0.890
VSAA	7 (1)	3 (8)	4 (10)	0.701

4.6.2. Telomere length analysis in patients with AA treated with ATG

All 72 patients were included in the telomere length analysis. The median relative telomere length at each time point is mentioned in Table **4.6.2**. Since there was no change in median telomere length at baseline and post-treatment (3rd month), we calculated the percentage change in post-treatment time points with respect to baseline values.

Table 4.6.2.: Telomere length analysis in patients with AA treated with ATG

ATG	Baseline	1st month	2nd month	3rd month
N	72	69	67	72
Median rTL	0.62	0.64	0.60	0.65
Range	0.25-2.23	0.09-2.71	0.17-2.42	0.07-1.47
% increase Median (Range)		4.01 (-83.33 to 226.51)	-12.40 (-82.10-175.75)	-7.03 (-81.08-187.09)

Further, a similar analysis was carried out in response to ATG at 3rd month post-treatment at each time point in AA patients. There was no significant difference in median relative telomere length or percentage change between responders (N = 37) and non-responders (N = 35). Further, we categorized the patients' age in decades and observed that rTL at 3rd month was not significant between responders and non-responders (**Figure 4.6.2.a,b,c**).

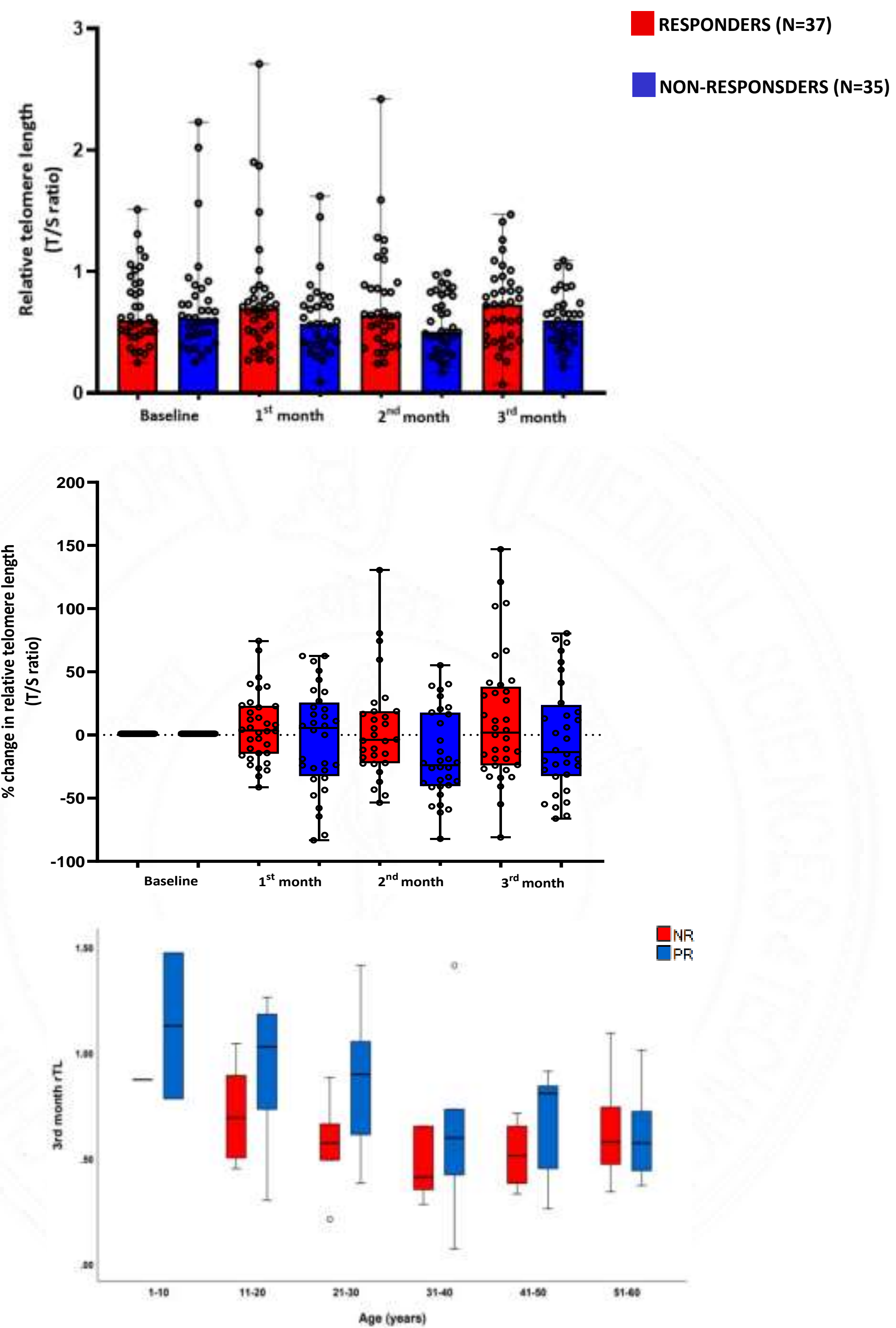


Figure 4.6.2.: Median relative telomere length (a) and percentage change (b) of AA patients in response to IST post-treatment (c) 3rd month rTL between responders and non-responders

4.6.3. Immunophenotyping of immune cells in AA patients treated with ATG

From the total 72 AA patients included, immunophenotype data were available at baseline and 3rd month post-treatment for (N = 60) patients. The median age of these patients was 42 (9 - 64) years and included 35 males and 25 females. Based on the severity, 14 were NSAA, 40 were SAA, and 6 were VSAA. The percentage of $CD3^+$ T cells and the T-cell subsets ($CD4^+$ and $CD8^+$) and NK cells ($CD16^+CD56^+$) from peripheral blood collected at baseline and post-treatment (1st, 2nd, 3rd, and 6th month) were investigated by flow cytometry. The median percentage of these cells at each time point is mentioned in Table **4.6.3**.

Table 4.6.3.: Percentage of T-cells subsets and NK cells at different time points in patients with AA treated with IST

Timeline	N	$CD3^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)	NK cells (%) ($CD16^+CD56^+$)
		Median (Range)			
Baseline	**60**	56 (1-87)	41 (20-83)	35 (13-77)	7 (1-57)
1st month	**58**	65 (2-88)	44 (34-81)	35 (18-57)	6 (1-45)
2nd month	**56**	58 (1-88)	36 (3-86)	35 (12-74)	8 (1-45)
3rd month	**60**	54 (2-88)	33 (6-87)	36 (12-72)	4 (1-46)

The percentage of T cells and NK cells were analysed with respect to response at 3rd month post-treatment to ATG. There was no significant difference observed in the percentage of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in AA patients compared with the response (N = 29) and non-response (N = 31) to ATG treatment in 3rd month post-treatment (**Figure 4.6.3.a,b; 4.6.3.1.a,b; 4.6.3.2.a,b**). Similar results were observed with respect to NK cells as well (**Figure 4.6.3.3.a,b**). Seeing that

there was no difference in T cells and NK cells between responders and non-responders in the 3rd month, we calculated percentage change post-treatment time points with respect to baseline values. Although a slight decrease was observed in T cell subsets in responders in the 3rd month, it was not significantly different.

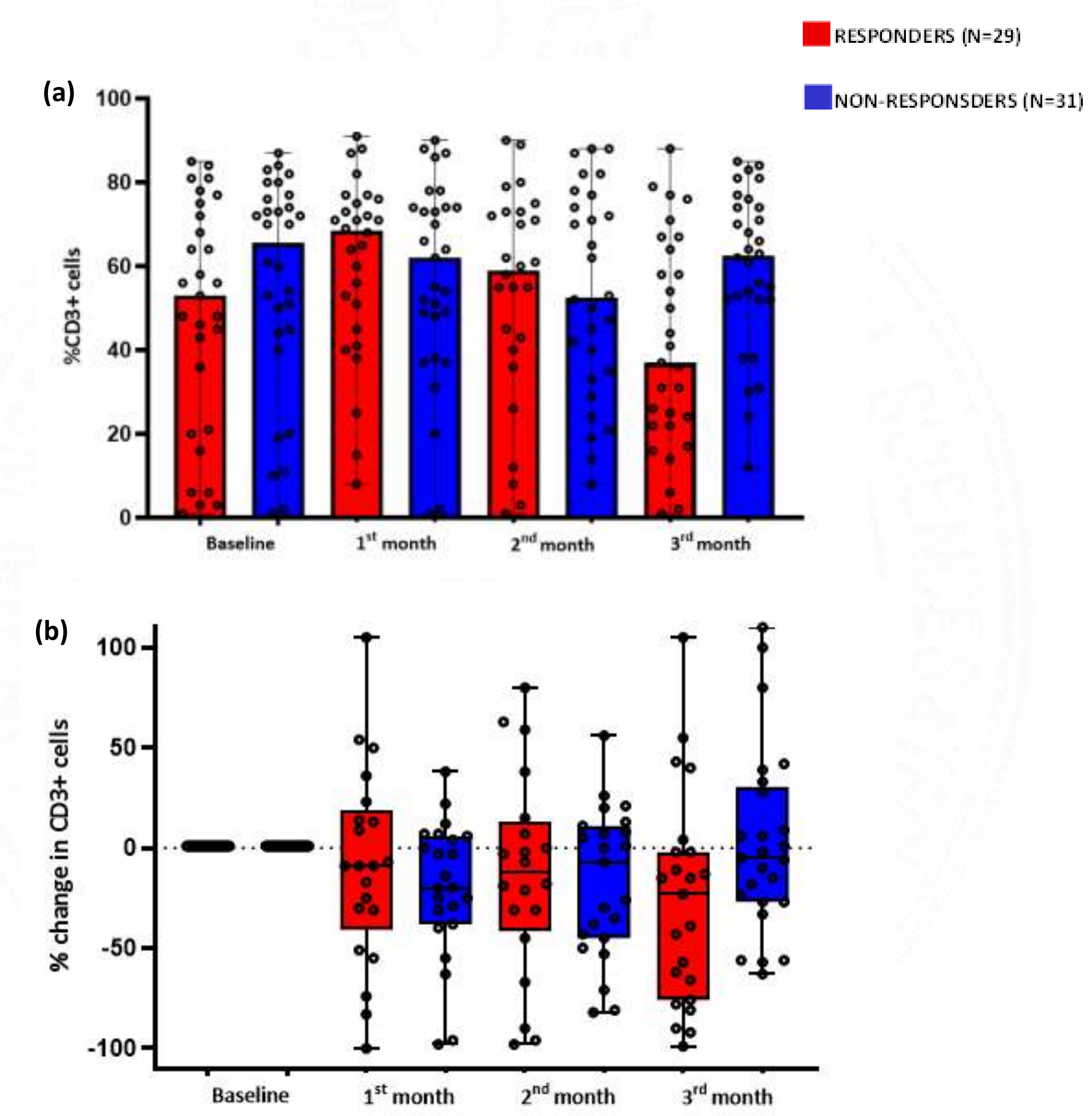
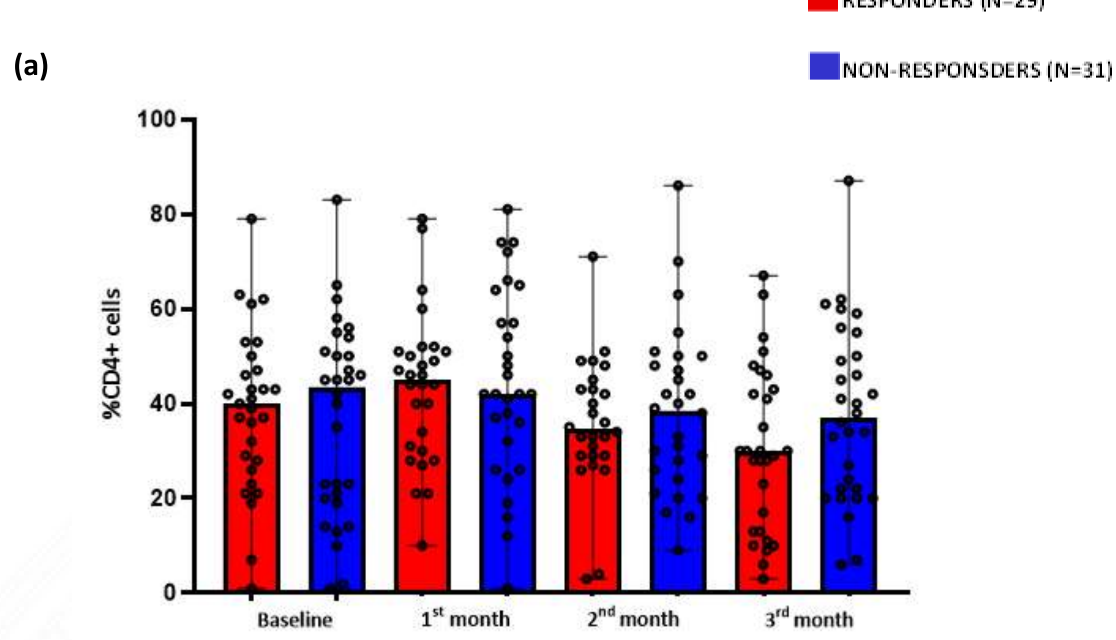


Figure 4.6.3.: Percentage of $CD3^+$ cells in patients with AA in response to IST at 3rd month
(a) Overall percentage and (b) percentage change



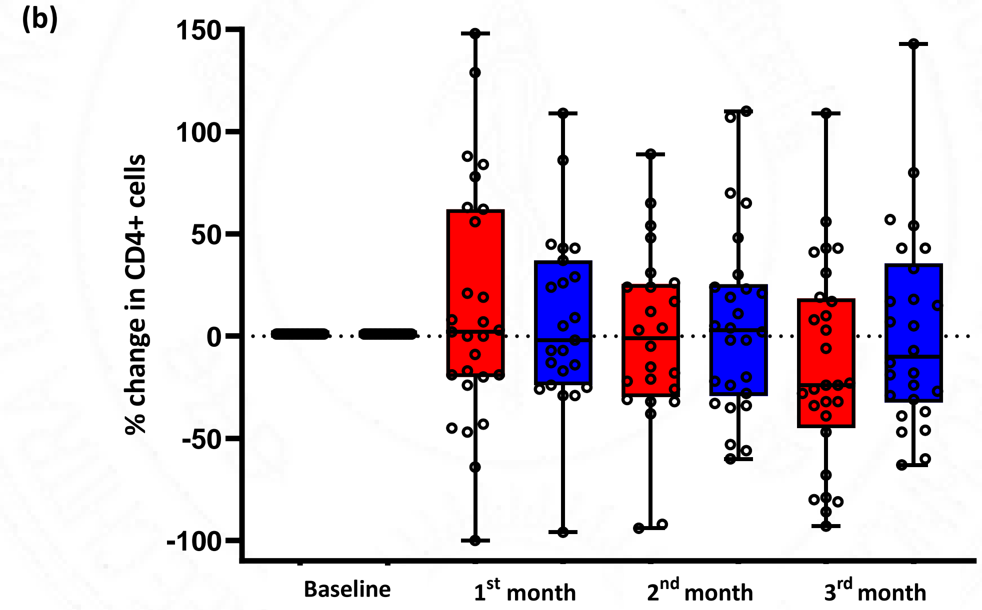
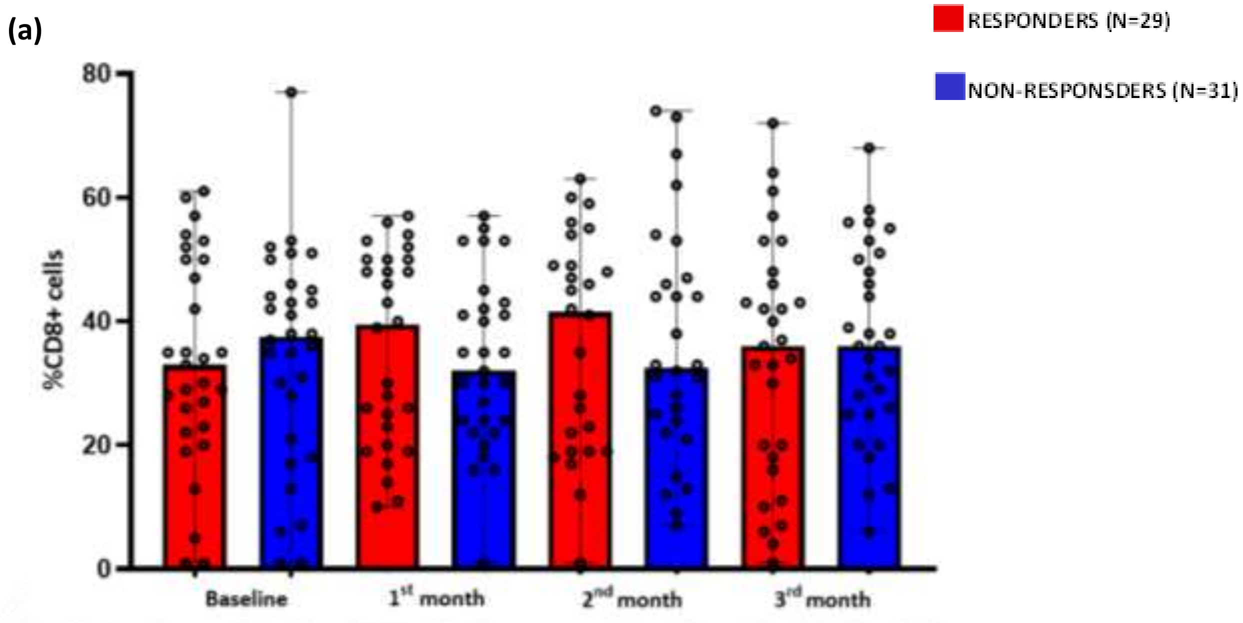


Figure 4.6.3.1.: Percentage of $CD4^+$ cells in patients with AA in response to IST at 3rd month (a) Overall percentage and (b) percentage change



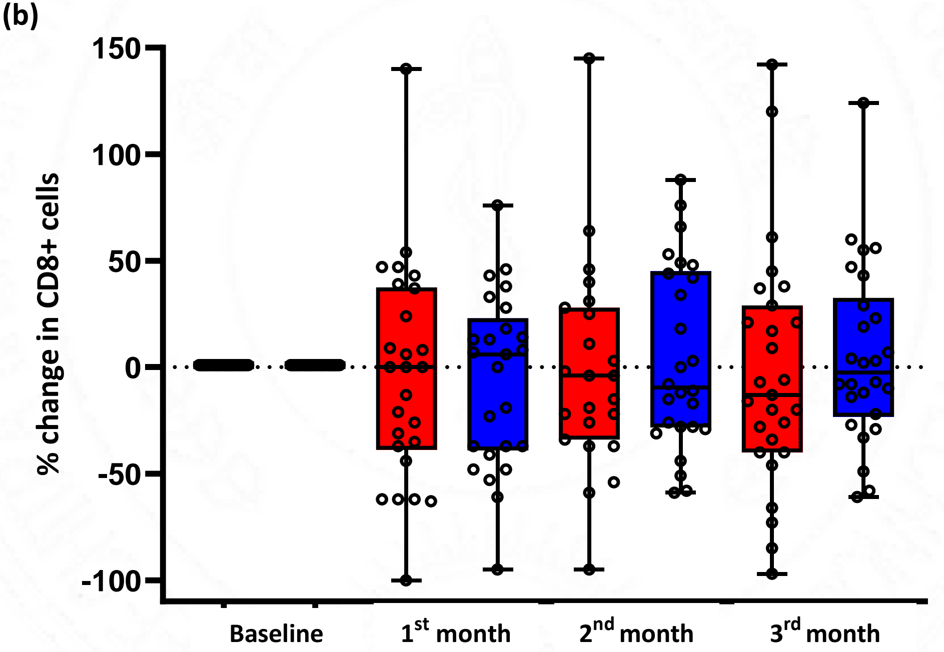
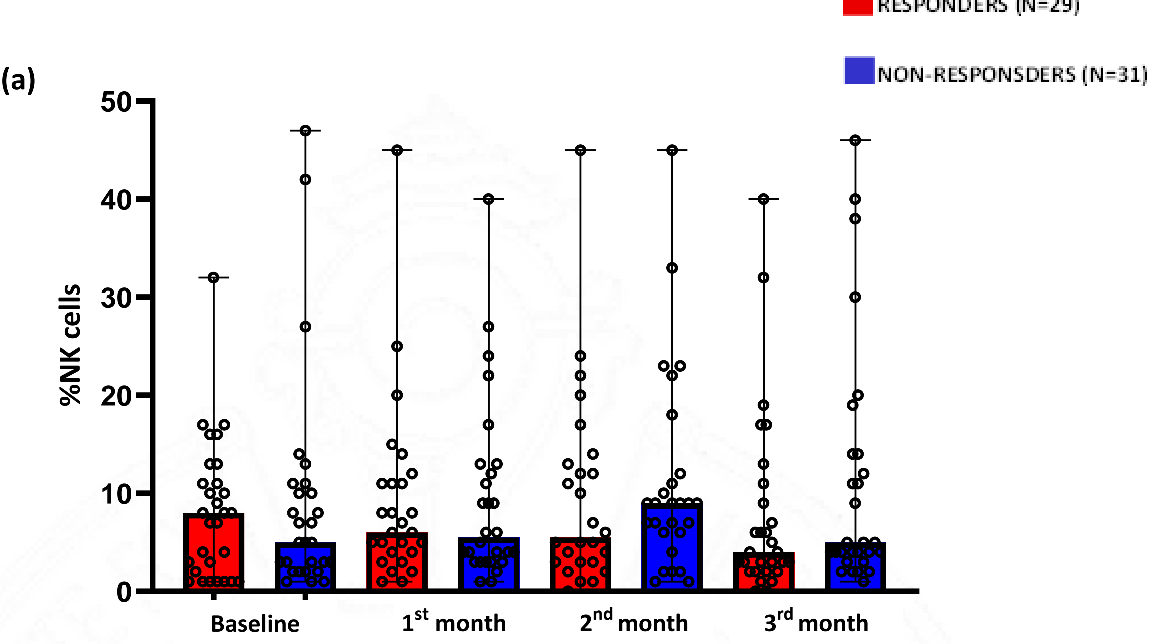


Figure 4.6.3.2.: Percentage of $CD8^{+}$ cells in patients with AA in response to IST at 3rd month (a) Overall percentage and (b) percentage change



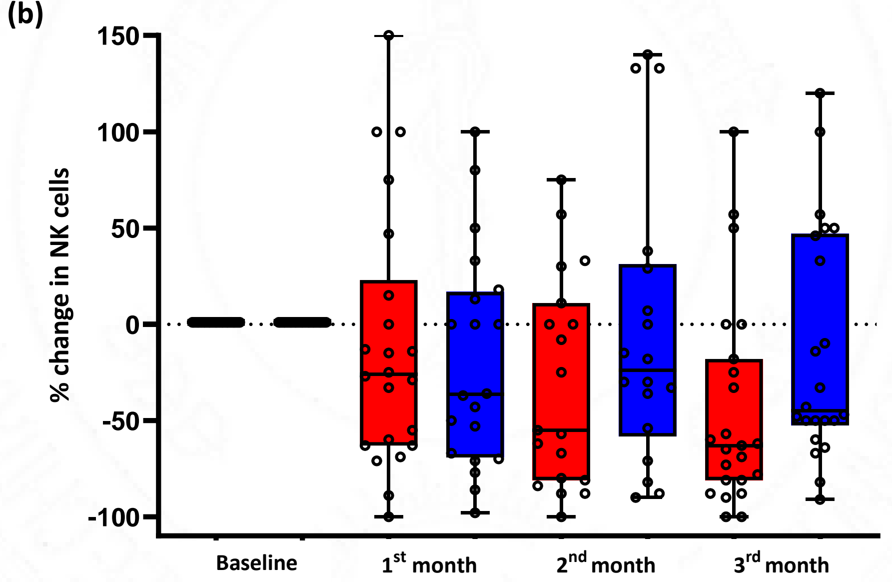


Figure 4.6.3.3.: Percentage of NK cells in patients with AA in response to IST at 3rd month (a) Overall percentage and (b) percentage change

The percentage of Tregs was analysed in (N = 48) AA patients and was observed to be significantly increased at 3rd month with treatment to ATG compared to pretreatment [1.62 (0.22 - 3.73) vs 2.72 (1.42 - 6.04), p = <0.001; **Figure 4.6.3.4.**]. In patients with non-severe and severe AA at diagnosis, the percentage of Tregs was significantly higher at 3rd month post-treatment than baseline in SAA [2.65 (1.42 - 6.04) vs 1.63 (0.22 - 3.73), p = <0.001; **Figure 4.6.3.5.**]. When compared at 3rd month response to ATG, a significant increase in Tregs was observed from baseline in responders (N = 23) and non-responders (N = 25) (**Figure 4.6.3.6.**).

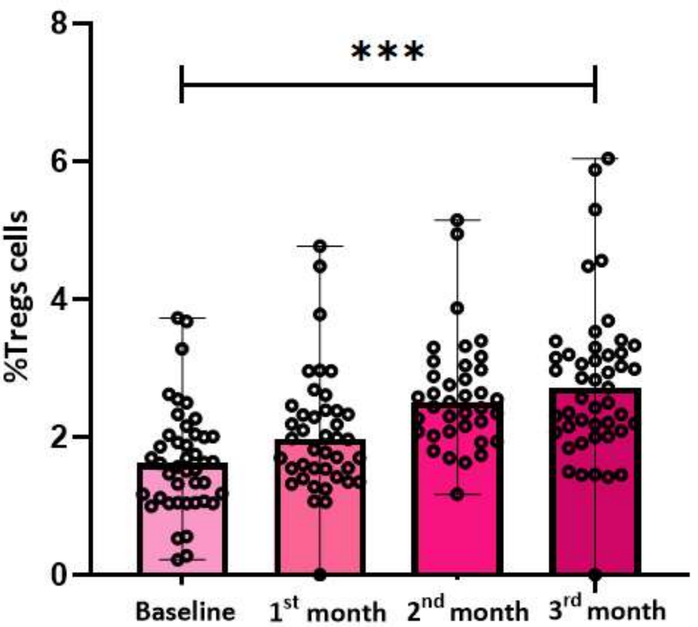


Figure 4.6.3.4.: Percentage of Tregs in patients with AA in response to IST at 3rd month post-treatment

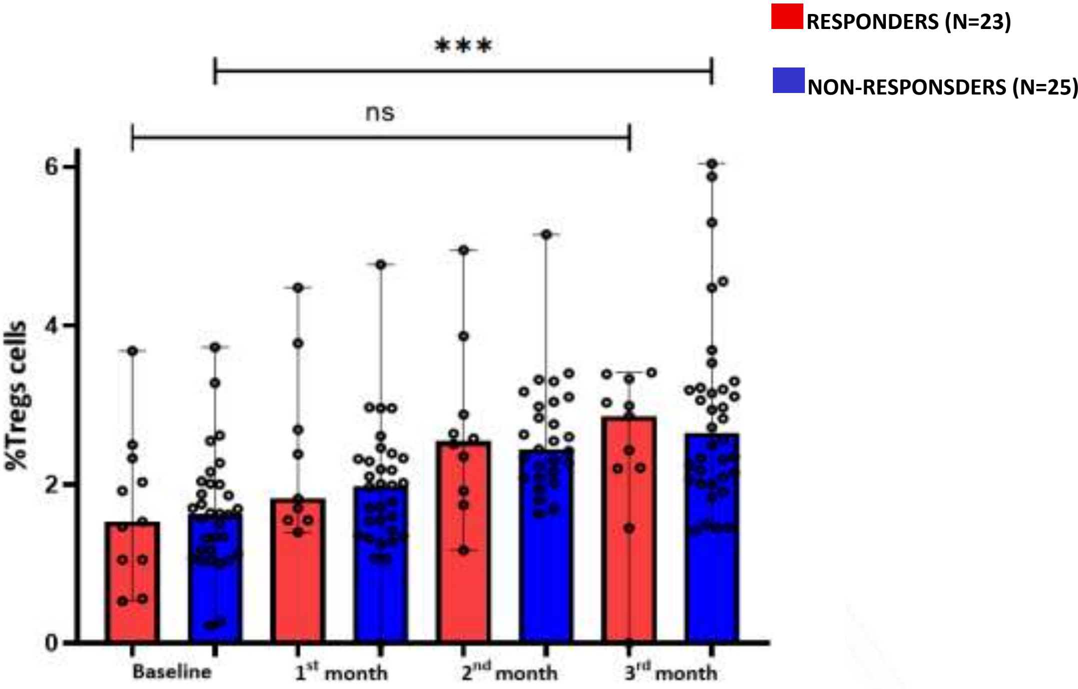


Figure 4.6.3.5.: Percentage of Tregs with respect to the severity in IST-treated AA patients

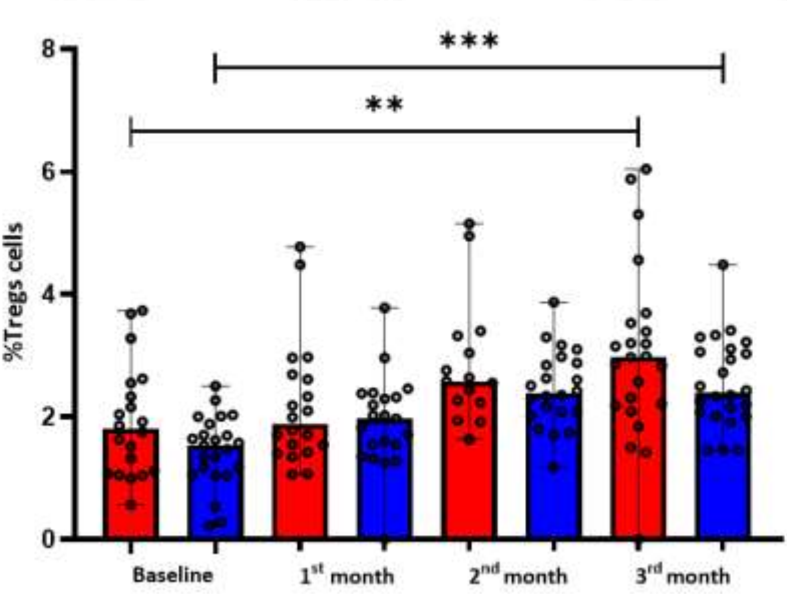


Figure 4.6.3.6.: Percentage of Tregs in patients with AA in response to IST at 3rd month

4.6.4. Cytokine levels in serum/plasma of AA patients treated with ATG

Serum/plasma levels of eight cytokines (1L-10, TGF-β, IFN-γ, TNF-α, IL-17A, IL-22, IL-2, IL-4) from 29 AA patients treated with ATG at baseline and post-treatment up to 3rd month were analysed using simple plex assay in AA patients. Levels of IL-2, IL-4 and TGF-β were undetectable in all patients at each time point. Therefore, data from the other 5 cytokines at each time point were considered for analysis (**Table 4.6.4.**).

Table 4.6.4.: Cytokines levels in the serum/plasma of AA patients treated with ATG at baseline and post-treatment

Timeline N=29	IL-10	IL-17A	IL-22	IFN-G	TNF-A
	Median (Range) (pg/ml)				
Baseline	5.620 (0.927-23.9)	0.704 (0.000-22)	1.820 (0.000-67)	1.390 (0.319-24.2)	6.860 (4.06-32)
1st month	6.930 (0.154-19.9)	0.166 (0.000-10.7)	1.710 (0.000-60)	2.600 (0.264-15.1)	9.070 (3.31-11.1)
2nd month	5.035 (1.95-34.2)	0.309 (0.000-16.2)	0.942 (0.000-37.4)	1.635 (0.419-12.9)	9.820 (4.66-20.5)
3rd month	4.890 (1.81-31.6)	1.242 (0.000-7.19)	0.339 (0.000-30.9)	1.380 (0.532-14.4)	9.735 (5.05-31.3)

Further, we analysed the 5 cytokines at each time point in response to ATG at 3rd month post-treatment. There was no significant difference observed in all the cytokines levels in response (N = 15) or non-response (N = 14) to ATG treatment at 3rd month in patients with AA (**Figure 4.6.4.a,b,c,d,e**)

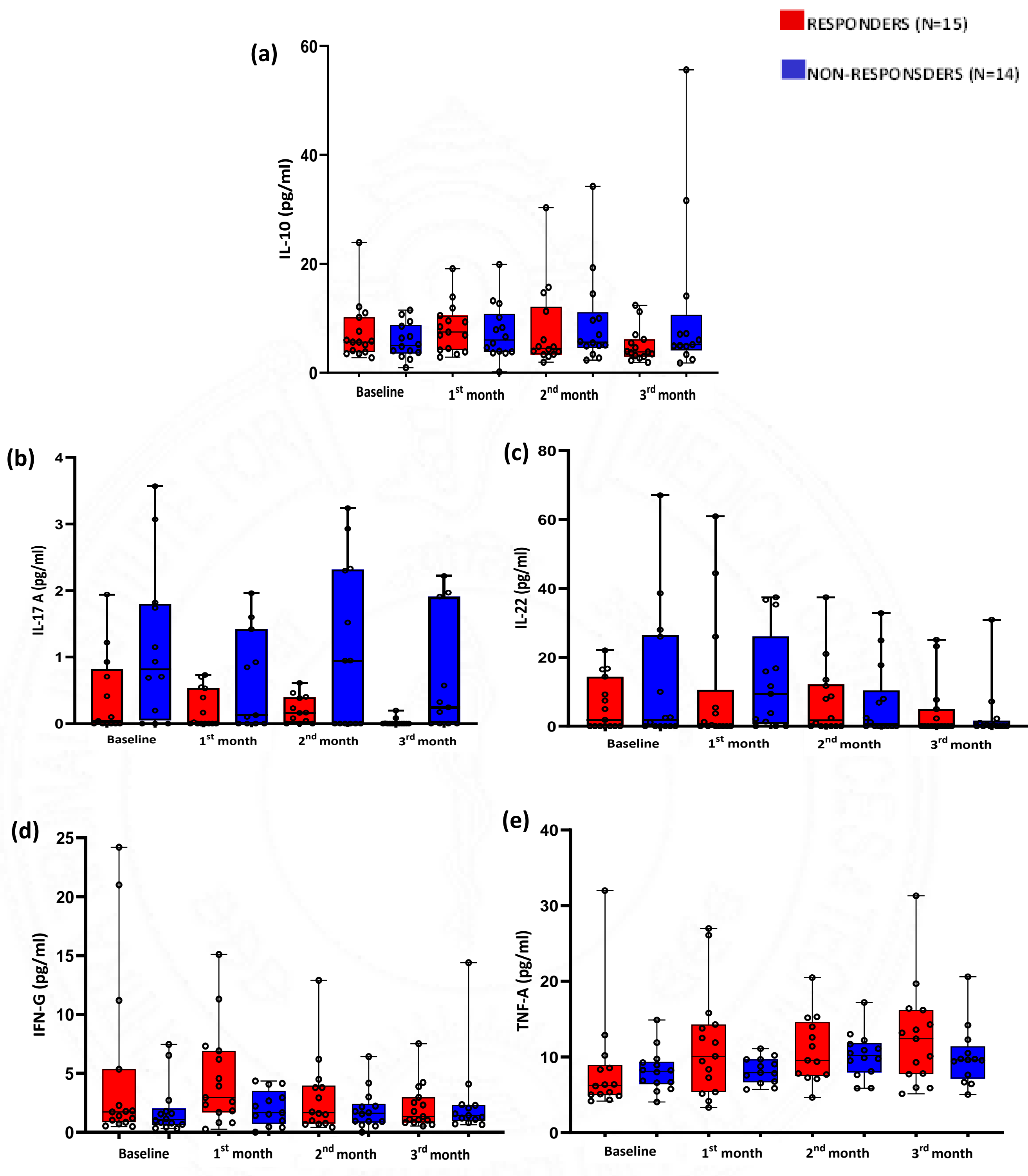


Figure 4.6.4.: Cytokines levels in the serum/plasma of AA patients in response to ATG treatment using Ella simplex system (a) IL-10, (b) IL-17A, (c) IL-22, (d) IFN-G and (e) TNF-A

5.0. DISCUSSION

Haematopoiesis is the process of forming blood cells and occurs throughout embryonic development and adulthood to maintain the blood system. The blood system serves as a model for comprehending the biology of stem cells and their involvement in disease, ageing, and oncogenesis. Haematopoietic stem cells in adult mammalian bone marrow are capable of self-renewal and are at the top of a hierarchy of progenitors that progressively become restricted to specific lineages. Bone marrow failure syndromes result in reduced HSCs and hematopoietic precursor production, leading to cytopenia in peripheral blood. There are two types, inherited and acquired. Inherited bone marrow failure encompasses a wide range of heterogeneous diseases, including Fanconi anaemia, telomere biology disorders, Shwachman-Diamond syndrome, Diamond-Blackfan anaemia, and congenital cytopenia. The acquired form, likely caused by an autoimmune reaction, can be treated with immunosuppressants and HSCT.

Aplastic anaemia is a serious condition that advances into bone marrow failure and needs prompt treatment to avoid severe consequences. The main feature of AA is hypocellularity in the bone marrow, which leads to low levels of peripheral pancytopenia. The exact cause of AA molecular pathogenesis remains unclear, and it is possible that different processes may be responsible for each case. It is believed that an antigen-triggered and potentially autoimmune dysfunction of T-cell regulation plays a role in damaging hematopoietic stem cells and is a highly favoured pathological hallmark. In certain cases, mutations in telomerase complex genes like TERT and TERC may indicate the involvement of abnormal telomerase function and repair. There is growing interest in understanding the involvement of bone marrow mesenchymal stromal cells in the pathophysiology of AA. Despite advancements in understanding the pathophysiology of

aplastic anaemia, it is still incomplete. With the advent of advanced genomic sequencing and other techniques, there has been a gradual enhancement in comprehending the pathogenic mechanisms of AA, identifying the disease, and ultimately, providing better patient outcomes.

5.1. Characteristics of mesenchymal stromal cells in aplastic anaemia

Mesenchymal stromal cells located in the bone marrow have a crucial role in creating a favourable environment for HSCs and are responsible for regulating and maintaining haemopoiesis. Patients with various haematological diseases have shown significant changes in their MSCs. However, it is still unclear how these alterations contribute to the progression of the disease. Results from in vitro investigations involving microenvironmental MSCs in AA have been contradictory. Therefore, to understand the potential involvement of MSC in reduced haemopoiesis in AA, we compared the basic properties of MSCs from AA patients with normal subjects. We observed that AA and normal MSCs displayed typical fibroblast-like morphology and surface markers, like the previously reported studies (Hamzic et al., 2015; Michelozzi et al., 2017). Both MSCs expressed distinct mesenchymal markers according to the criteria of the ISCT for human MSCs.

5.1.1. AA MSCs exhibited normal proliferation and telomere length

There was no discrepancy in the proliferative potential or population doubling time of AA of normal MSCs. Michelozzi and colleagues found no difference in population doubling time between AA and normal MSCs (Michelozzi et al., 2017), while other studies showed decreased proliferation and lower PDT in AA MSCs (Chao et al., 2010; Hamzic et al., 2015). Cell behaviour can be greatly affected by the growth surface and expansion media, leading to variability in different studies. Several studies reported telomere length in normal MSCs (Samsonraj et al.,

2013), whereas there are no data available on AA MSCs. In the present study, we measured telomere length in AA MSCs using qPCR. When compared with normal MSCs, there was no noticeable difference in rTL between the groups. However, a significantly longer rTL in paediatric AA MSCs compared to adults was observed. Also, there was no variation in the rTL of MSCs between non-severe and severe AA patients. These results suggest that MSCs may not play a role in the pathogenesis of AA and are normal. This corroborates with the fact that in HSCT, MSCs are recipient in origin and can support normal haematopoiesis with donor HSCs.

5.1.2. The differentiation and immunomodulatory properties of AA MSCs are not distinguishable from normal

The potential for variability in differentiation can indeed be affected by specific lineage's altered transcription factors. We assessed the differentiation potential of MSCs and noted that though AA MSCs displayed enhanced lipid droplets during adipogenic differentiation, the lineage-specific gene expression was not distinguishable from that of normal BM counterparts. Similarly, there was no variation in osteogenic potential between AA and normal MSCs. Some studies have found impaired or decreased osteogenic and adipocyte differentiation in AA MSCs compared to controls (Chao et al., 2010; Shipounova et al., 2009), while others did not find any difference (Bueno et al., 2014; Michelozzi et al., 2017; Sharma et al., 2021). On the other hand, certain studies reported enhanced differentiation of AA MSCs into adipocytes than normal, but osteocyte differentiation was unchanged or impaired (Li et al., 2012; Chaturvedi et al., 2018; Huo et al., 2020). The osteocytes and osteoblasts derived from BM-MSCs play a crucial role in maintaining skeletal homeostasis. They regulate haematopoiesis directly or indirectly via soluble factors (Divieti Pajevic & Krause, 2019). With age, or after undergoing chemotherapy, adipocytes in the BM microenvironment tend to increase. Recent studies suggest that bone marrow adipocytes suppress

the growth and differentiation of hematopoietic stem cells (Naveiras et al., 2009). Despite the regenerative capabilities of BM-MSCs, they display reduced differentiation potential when exposed to inflammatory environments. It can be inferred that AA MSCs have no impact on haematopoiesis based on our findings.

During inflammation, MSCs modulate the immune response through the release of immunomodulatory factors, growth factors, chemokines, and anti-inflammatory cytokines. Therefore, we analysed the immunomodulatory property of MSCs by co-culturing MNCs and MSCs and observed the suppression of T-cells by AA MSCs did not vary from that of normal. Our data suggests that BM MSCs obtained from AA patients possess intact functional and immunological properties. Certain studies showed comparable functional immunomodulatory properties of AA-MSCs with normal (Bueno et al., 2014; Xu et al., 2009), while some found abnormal immunoregulation of AA MSCs (Shipounova et al., 2009; Huo et al., 2020). This variability may be due to differences in cell metabolic activity in BM, which can affect the degree of suppression. Studies on BM MSCs in AA patients compared to normal have reported aberrant morphology, impaired adipogenic and osteogenic potential, changes in gene expression, and a reduced ability to support haematopoiesis in vitro. However, our data are in alignment with a study indicates otherwise (Bueno et al., 2014).

There may be a significant amount of heterogeneity among various studies, which could be the reason for the discrepancy in the data available on AA MSCs. The results of studies may have been influenced by the severity of AA, age, gender and whether a patient treated with IST is in remission, refractory to treatment, or still in remission. We collected our BM samples during the diagnosis phase; hence we could not address these points in our study. In addition to heterogeneous population groups, there were inconsistencies in research methods across studies, including

differences in cell numbers, materials used, and techniques used to measure specific outcomes. This can also clarify the conflicting outcomes observed in various studies or why some distinctions were statistically insignificant.

5.2. Telomere length assessment

Comparing telomere length measurements in studies can be difficult due to variations in laboratory techniques and pre-measurement factors (Aubert et al., 2012). It is crucial to understand the advantages and drawbacks of different telomere length measurement methods. This is because long-term stem cell divisions, which are essential for tissue renewal, are limited by short telomeres rather than average telomere length. Genetic disorders (telomeropathies) can cause early-onset diseases due to short telomeres (Holohan et al., 2014). Robust and reproducible telomere length measurements may predict genetic and age-related pathologies in humans and animals. The Terminal Restriction Fragment is the gold standard method which requires a large amount of DNA. While recently, Flow-FISH is the most widely used method, requires intact cells for TL measurement. As aplastic anaemia is characterized by peripheral blood pancytopenia, we used qPCR which can be performed using a low amount of DNA.

It is important to note that the qPCR relative telomere length values can be influenced by various pre-assay technical factors, such as the DNA extraction method. There may also be other factors that have not yet been fully identified. The variations observed could be attributed to DNA quality, sample preparation techniques, and telomere length range. A more comprehensive range of telomere length values tends to result in a higher R^2 correlation coefficient. It has been reported that qPCR exhibits greater inter-laboratory variability and is mainly used for epidemiological studies in telomere length measurement compared to Southern blot.

In this study, we standardised qPCR assay in our laboratory to measure relative telomere length in DNA extracted from blood samples. Relative TL measured using qPCR is said to be inferior because of its relatively lower precision compared with the gold standard of Southern blots or flow-FISH telomere length assays. A correlation between the qPCR and flow-FISH methods has been reported (R^2=0.42 (Khincha et al., 2017), 0.33 (Gutierrez-Rodrigues et al., 2014)). In our study, a comparison of qPCR assay with flow-FISH was not performed. However, the correlation coefficient was examined to be R^2=0.50, using an absolute human telomere length kit (ScienCell) for a few samples compared with qPCR assay.

5.2.1. Telomere length in the normal Indian population

Telomeres shorten over time due to incomplete DNA replication after each cell division. Maintaining the stability of the human genome depends on the regulation of telomere length and biological ageing is significantly affected by telomere shortening. It has been observed that telomere length varies greatly among people of the same age, and this variability is also present at birth. In the present study, we included eight hundred normal subjects to measure telomere length. As normal physiology suggests, we observed a gradual decrease in median telomere length with an increase in age in decades. Our data had about a hundred subjects in each age group, and the distribution of the subjects was not restricted to a particular region in India. Studies on the general population have revealed a strong correlation between older age and shorter leukocyte telomere length, which was in alignment with our data. On the other hand, research has shown that children tend to have longer mean rTL than adults (Helby et al., 2017; Nguyen et al., 2018).

According to studies, there is a significant difference in telomere length between males and females. Studies have shown that the length of telomeres is generally longer in females than in

males. However, some studies, including ours, have not found any significant difference in telomere length between male and female subjects (Yadav & Maurya, 2022). Telomere research has mainly examined the influence of environmental and specific genetic factors, establishing links between telomere length, morbidity, and mortality. Most population studies have concentrated on investigating healthy adults, leaving little information about telomere lengths in healthy children. Our study established the median relative telomere length in a large normal Indian population, including children and adults.

5.2.2. Shorter telomere length is observed in bone marrow failure syndromes compared to normal population

The discovery based on the genetic basis of telomere erosion in dyskeratosis congenita may suggest abnormal telomerase function in patients with acquired marrow failure, potentially causing telomere shortening. In our study, we measured telomere length in six hundred and fifty AA patients. There was a gradual decline in rTL with age, and no difference was observed in rTL in males and females. In our analysis, 54% of aplastic anaemia patients had shorter median relative telomere length than age-matched healthy controls. Research has shown that patients with AA have shorter telomeres in their leukocytes compared to healthy individuals (Ball et al., 1998; Brümmendorf et al., 2001). Similar findings were noted from recent studies in India (Adhikari et al., 2021; Mandal & Dutta, 2020). We also observed that rTL in combining patients with severe and very severe AA was comparable to the non-severe group and this result was in alignment with a Chinese study (Song et al., 2013).

In patients with FA, we observed that telomere length was significantly lower than the age-matched healthy controls. Moreover, while categorised based on severity, the severe group had significantly shorter rTL than the non-severe. According to a study, telomere length was compared

in inherited BMFS such as FA, Shwachman-Diamond syndrome, and Diamond-Blackfan anaemia with dyskeratosis congenita and determined that, on average, the telomeres in these conditions were in the lower half of the normal range (Alter et al., 2015). Furthermore, we noted that rTL was not distinct when compared between FA and age-matched AA patients. A study evaluated the diagnostic value of telomere length in patients with BMF who were classified as patients with DC, non-DC IBMFS, and AA. No significant difference in telomere length was observed when non-DC IBMFS (which included FA) compared to AA, which correlated with our data (Miwata et al., 2021). This could be because patients with FA are below 30 years of age in our data, and generally telomere length is longer in young individuals. The ALT could modulate telomere attrition through a homologous recombination mechanism, and the FA core complex is involved in telomere maintenance to synthesise new telomeric DNA (Fan et al., 2009).

Our data showed MDS patients have significantly shorter telomere length than age-matched healthy controls and with aplastic anaemia patients. Telomere length was comparable between low-risk and high-risk MDS patients. The shorter TL in patients with MDS may be related to the older age of the cohort (median age of 46 years), as TL deteriorates as the age progresses. Several studies have reported that patients with MDS have short telomeres compared to healthy controls (Hwang et al., 2016; Myllymäki et al., 2020; Rollison et al., 2011). The study's strength includes a large sample size and comprehensive clinical information. This is the first study to directly compare telomere length in AA, FA, or MDS patients.

5.2.3. Donor telomere length impacts overall survival in patients with AA undergoing HSCT

Telomeres are markers for biological ageing, and telomere shortening with age is well-characterised (Zhao et al., 2014). Our study showed that the telomere length in transplant donors significantly impacted transplant outcome, as opposed to the recipient telomere length. Our data

determined that individuals in the highest quartile of donor relative telomere length (DTL HQ) experience better overall survival rates, which is consistent with findings from AA patients who have undergone unrelated donor transplants (Gadalla et al., 2015; Gadalla et al., 2016). The influence of donor telomere length in a study had a greater effect on patients under 40 years of age who had undergone HSCT. Our study did not observe any correlation between patient telomere length and factors such as acute or chronic GVHD, neutrophil recovery, or overall survival. According to a study from NIH, telomere length did not affect response to immunosuppressive therapy with ATG and CSA, but it did impact relapse, clonal evolution, and overall survival (Scheinberg et al., 2010). Currently, limited data is available regarding the correlation between telomere length and the results of HSCT for AA. Moreover, this data is confined to transplants from matched unrelated donors (Gadalla et al., 2016; Wang et al., 2020).

We compared mortality causes between the high donor telomere group and the combined other three groups (DTL LQ). Notably, within the DTL HQ group, mortality was primarily caused by chronic graft versus host disease and graft failure. Conversely, in the DTL LQ group, infective complications (specifically gram-negative sepsis) and acute graft versus host disease were identified as the leading causes of mortality. Confirming these discoveries in a more extensive group of patients is necessary. However, if the results remain consistent in a larger sample, effective measures could be implemented to lessen the mortality rate.

A logistic regression analysis found that the shorter telomere length of the donor was an independent risk factor for survival, in addition to older age, the presence of acute GVHD, and graft failure. According to prior reports, there seems to be a significant correlation between the age of the donor and the outcomes of HSCT (Schrezenmeier et al., 2007). Peripheral blood stem cells are commonly used as the graft source in our population to reduce the risk of graft failure. This is

because over 90% of patients with graft failure do not have the financial resources to undergo a second transplant and often succumb to infections. The utilisation of PBSCs grafts has been linked to a higher mortality rate due to the potential occurrence of acute or chronic GVHD (Bacigalupo et al., 2012). Most of the AA patients in our cohort received fludarabine and cyclophosphamide conditioning regimens. A study showed that patients over 40 years tend to have a lower overall survival rate, but using fludarabine-based regimens improves outcomes (Shin et al., 2016).

Our analysis in causes of death revealed that around 50-60% of patients expired due to bacterial or fungal infections. We further divided the cohorts based on donor telomere length into higher and lower quartiles. Also, bacterial sepsis and other infections (fungal and viral) were only observed in the low quartile cohort and not in the high telomere length group. It is worth mentioning that extensive research on the general population has shown a correlation between shorter telomeres and an increased likelihood of infections (Shin et al., 2016). Recently, a study was conducted on AA patients who underwent an unrelated donor transplant and analysed the correlation between donor telomere length and mortality. The findings indicated that longer telomere lengths in B, NK, and fully differentiated T cells were linked to a lower risk of infection-related death. Donor telomere length significantly reduced the rate of infection-related deaths after HCT for SAA (Gadalla et al., 2018).

For our observations to have a further impact on clinical practice, a significantly greater number of patients must be studied. Performing a more comprehensive analysis will enable us to confirm the disparities in mortality causes observed between the DTL-HQ and DTL-LQ groups. If these variations are confirmed in a bigger group, we could consider adjusting the conditioning regimen and antimicrobial prophylaxis to decrease the death rate linked to infections and acute

graft versus host disease. In a matched sibling donor transplant, we are often restricted by donor availability (usually single donor availability), unlike in unrelated donor transplants.

The strengths of this cohort include matched sibling donors, the availability of pre-transplant samples, the post-transplant follow-up, and comprehensive clinical and outcome information, all contributing to the research's thoroughness. The correlation between the donor's telomere length and the patient's survival after transplant was only significant in the longest telomeres. A larger sample size would be beneficial in reevaluating this association across more telomere length categories. This is the first study that determined the influence of recipient and donor telomere lengths on transplant outcomes of AA patients undergoing matched sibling donor transplantation.

5.2.4. Germline variants identified in young patients with AA

Since approximately half of the AA patients in our study had shorter rTL compared to age-matched healthy controls, we analysed inherited BMF gene variants using NGS in young AA patients (age $\leq$ 40 years). Our study identified germline variants in 22% of AA patients in inherited BMF genes by genetic screening. Most patients had pathogenic variants in the genes involved in haematopoiesis (MPL) and telomere biology (TINF2, RTEL1), and other genes were NLRP12, DNAJC21 and PRF1 (**Figure 5.1.**). A previous investigation observed that children and young adults who underwent HSCT to treat AA had 5.1% pathogenic mutations in inherited BMF/MDS genes (Keel et al., 2016).

Ten patients with severe AA unresponsive to immunosuppressive therapy were screened using a targeted next-generation sequencing panel of 72 inherited BMF genes, and results showed that 3 out of 10 AA patients (30%) had mutations. One patient had a heterozygous TERT mutation, another patient carried compound heterozygous mutations in RTEL1, and the third patient had a

heterozygous mutation in microtubule-associated serine/threonine kinase-like (MASTL), which was reported in autosomal dominant thrombocytopenia (Ghemlas et al., 2015). The studies indicate that although mutations in individual genes are uncommon, mutations in the inherited BMF gene group are present in a notable portion of patients.

The previous studies examining genetics were restricted to single genes or a restricted number of gene sets. A study screened patients with seemingly acquired AA to detect mutations in telomerase-associated genes. Their findings showed that about 7 out of 205 (3.4%) AA patients, who were unresponsive to IST, had heterozygous mutations in TERT (Yamaguchi et al., 2005). The study involved individuals ranging from 2 to 83 years of age, with a median age of 34 years, which is higher than the median age of our group of AA patients, which is 10 years.

Our study identified pathological genetic variants in 10% of patients, VUS in 12%. Telomere length was analysed in this cohort, and there was no difference between patients with genetic variants or without variants. Moreover, AA patients having telomere-associated variants had significantly shorter telomere length compared to other pathogenic variant groups. A study performed exome sequencing on 732 SAA patients who received HSCT and revealed 218 single-nucleotide variants or small insertions/deletions in 59 genes related to IBMFS. Of these, 113 were pathogenic and detected in 16.5% (121/732) patients, while 91 (12.4%) patients had 105 in silico predicted deleterious variants of uncertain significance (dVUS) (Mcreynolds et al., 2022).

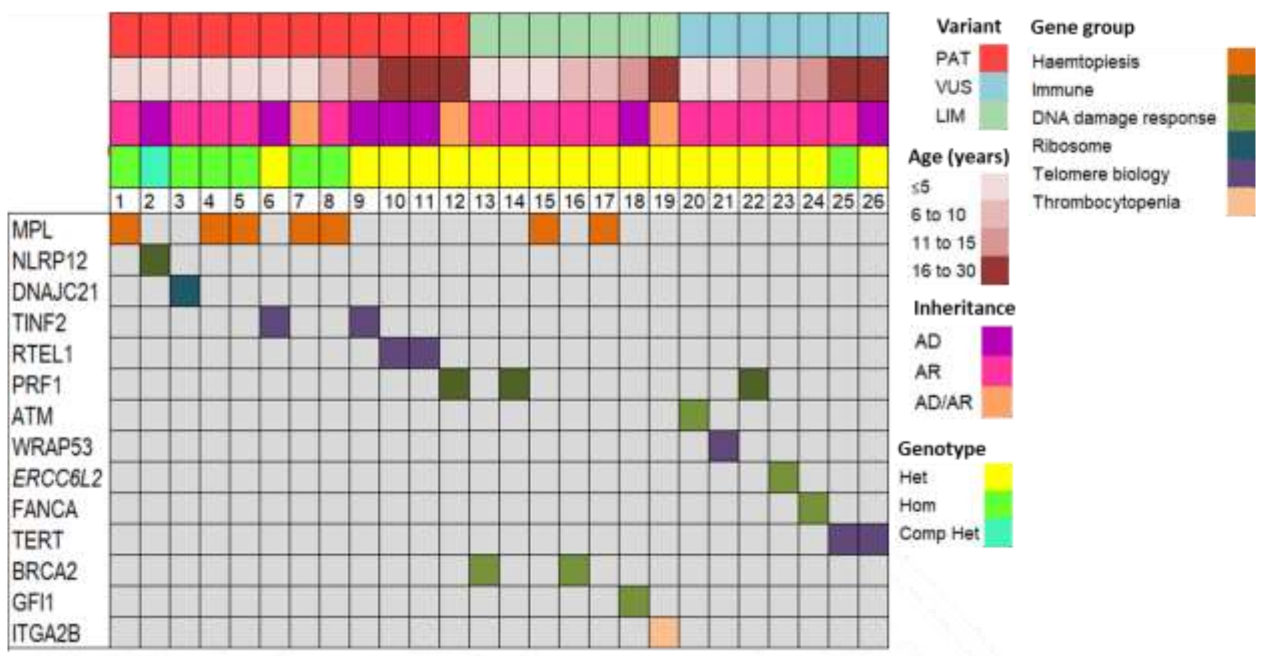


Figure: 5.1. Genetic information by age in aplastic anaemia patients having germline variants

5.2.5. Telomere length in CD34+ cells

Telomere length is one of the factors in determining the self-renewal property of HSCs. Therefore, in a subset of AA patients we enriched, cultured, and expanded CD34+ cells from BM and measured telomere length using qPCR. Although a small sample was analysed due to the strenuous process of acquiring CD34+ cells from aplastic marrow, we observed no difference in telomere length between AA and normal marrow CD34+ cells. Studies have reported a culture and measurement of telomere length in CD34+ cells from healthy controls from different sources such as the umbilical cord, fetal liver, or HSCs (Gammaitoni et al., 2004). In aplastic anaemia, having hypoplastic marrow, there is difficulty in isolating CD34+ cells and culturing them. According to a study, hematopoietic progenitors derived from iPSCs in SAA cell lines exhibit an abnormal shortening of telomeres during the hematopoietic differentiation process (Melguizo-Sanchis et al., 2018). The HSCs we obtained from AA patients maybe the HSCs which have escaped the immune

attack and surviving; hence displaying a normal telomere length. A clear understanding of telomere length in CD34+ cells of AA patients would be obtained by analysing bigger samples and probably having similar age and severity groups.

5.3. The immune-mediated pathogenesis in AA patients

The most widely utilized method of immunosuppressive treatment for patients with SAA is the combination of ATG and CSA. New drugs have recently been introduced, and it has been reported that when eltrombopag is added to standard IST, the treatment of SAA yields highly encouraging results. The complete response rate can reach up to 39%, and the overall response rate can reach 82% after six months (Townsley et al., 2017; Zaimoku et al., 2022). After undergoing the new treatment regimen, patients were monitored for a long-term period. Unfortunately, it was observed that the cumulative relapse rate in the responders was 39%, and all subjects exhibited a clonal evolution rate of 15% after four years (Scheinberg, Wu, et al., 2009). Our centre conducted a study on 677 AA patients to determine the prevalence and predictors of specific infections in immunosuppressive therapy. The study investigated the response to ATG and CSA after six months. Of the total participants, 58.6% (397) showed a response, with 22.9% (155) achieving a complete response and 35.7% (242) having a partial response (Lionel et al., 2023). Numerous studies have confirmed that abnormal T-cell immunity plays a critical role in the pathogenesis of AA. This includes hyperactive $CD8^+$ cytotoxic T cells, abnormally polarized Th1 and Th17 cells, and regulatory T cells with reduced function. As a result, various immune-related markers have been identified as predictors of response to IST, including subsets of regulatory T and B cells.

5.3.1. Association of telomere length in response to ATG treatment in AA patients

In addition to the HSCT cohort, we investigated the relationship between telomere length and hematopoietic response in AA patients treated with ATG. About 50% of patients responded at the third month post ATG treatment. There was no association between leukocyte telomere length and response to IST in the third month, and similar results were observed when rTL was categorised age-wise into decades. Further, we analysed the percentage change of rTL in 3rd month, though there was a decrease in non-responders it was not significantly different compared to responders. Our data was in alignment with the NIH study that reported no correlation between telomere length and response to IST with ATG but was associated with clonal evolution, risk of relapse, and poor overall survival (Scheinberg et al., 2010). Another study on 64 paediatric patients with AA by a Japanese group revealed that lymphocyte TL plays a significant role in predicting the response to IST (Sakaguchi et al., 2014). In our study, we have analysed data up to the third-month response from the administration of ATG. A better understanding of the association of telomere length in response to ATG treatment would be obtained when analysed at a later time at the sixth month or one-year post-treatment. The reason why certain patients do not respond is still unclear. Some possible factors include a scarcity of hematopoietic stem cells, insufficient immunosuppression, and a non-immune cause for bone marrow dysfunction. Shorter telomeres did not play a role in non-immune causes, as there was no link between telomere length and response to immunosuppression.

5.3.2. Immunotyping of immune cells and cytokine analysis in response to ATG treatment

Abnormal T-cell immunity plays a critical role in the pathophysiology of AA. Our study included a cohort of AA patients treated with ATG and analysed the immune cells prior to and post-treatment using flow cytometry. In our observations, there was no correlation in the

percentage of T cells (including $CD3^+$, $CD4^+$, and $CD8^+$ cells) before administering ATG or during the third month after treatment in patients with AA. Our data aligned with the findings published by the Japanese group (Narita et al., 2019). Additionally, we analysed the percentage change in each cell population ($CD3^+$, $CD4^+$, and $CD8^+$), but there was no significant difference in T-cells in response to ATG. There was no difference when immune cells were analysed with severity or age. Studies reported the distribution of lymphocyte subsets was compared between AA patients and healthy controls. It was discovered that patients with AA had a higher percentage of CD8+ T cells and a lower percentage and an absolute number of CD4+ T cells. Interestingly, AA patients exhibit a significant decrease in the absolute number of NK cells. Although the presence of lymphocytes was higher in AA patients (likely due to a decrease in the number of neutrophils), the overall count of lymphocytes in AA patients was significantly lower than that of the control group (Yu et al., 2022).

Immunomodulatory properties have been discovered in NK cells, which suggests their involvement in developing autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus. Evidence suggests that in the pathophysiology of AA, NK cells primarily serve an immunoregulatory function rather than a cytotoxic one. Therefore, we analysed NK cells ($CD56^+CD16^+$) in AA patients treated with ATG and observed that there was no change in NK cells post-treatment. A recent study showed that patients who received ATG treatment for SAA had a significant increase of NK cells in responders than in non-responders receiving ATG, both in proportion and absolute count (Yu et al., 2022). However, our results were contrary with respect to NK cells in AA patients post-ATG treatment. So far, there have been limited studies on the clinical correlation between NK cells and IST response in individuals with SAA. To comprehend

the function of NK cells in AA, additional research is necessary given the intricate and multifaceted nature of NK subsets function.

Tregs, a specialised T cell lineage, play a crucial role in regulating immune responses to autoantigens and harmful foreign antigens. The role of Tregs and their communication with other immune system components is viewed more as an environmental alteration rather than simply turning off a select few effector cells. In our study, we observed that percentage of Tregs increased significantly from pretreatment to the patients who responded to ATG treatment at third month. The elevation of Tregs levels post ATG treatment in our data indicates that Tregs play an essential role in inducing immune tolerance and managing the growth and advancement of autoimmunity by suppressing autoreactive T cells. Recent findings suggest that the imbalance between regulatory T cells and effector T cells is a significant factor in AA, impacting immune homeostasis. The decline of $CD4^{+}$ $CD25^{+}$ $FOXP3^{+}$ Tregs in AA patients may be the reason for increased autoreactive T cells and AA phenotypes (Solomou et al., 2007). In individuals with AA, the quantity and occurrence of Tregs were notably lower compared to those of age-matched healthy donors (Kordasti et al., 2012; Shi et al., 2012). Little data is available on the role of Tregs in AA, especially in patients treated with immunosuppressive therapy.

Immune cells secrete soluble low-molecular-weight proteins called cytokines and chemokines. These proteins regulate haematopoiesis and mediate inflammatory responses by modifying the bone marrow microenvironment. Some of these factors are crucial for haematopoietic stem cells survival, differentiation, and proliferation. Even though there have been many studies on bone marrow failure, there is limited knowledge regarding the specific cytokines and chemokines in the blood. In our analysis, we included a combination of pro- and anti-inflammatory cytokines produced by immune cells in BM that are associated with the

pathophysiology of aplastic anaemia. We observed undetectable levels of cytokines IL-2, IL-4 and TGF-β. Whilst no difference in the cytokines levels (IL-10, IFN-γ, TNF-α, IL-17A, and IL-22) was observed in AA patients' PB plasma/serum in response to post-ATG at the third month. Our data was contrary to the study published by the Chinese group; however, their analysis was carried out in bone marrow plasma (Liu et al., 2020). A study found that IST is ineffective in clearing excess TNF-α and IFN-γ from the marrow of AA patients (Dufour et al., 2009). A more intense IST approach that targets TNF-α might reduce the harmful potential on the marrow, increase response to ATG, and reduce relapse risk. IL-10 is known to act as a suppressor cytokine in T-cell proliferation, affecting both Th1 and Th2 cells. TGF-β suppresses proinflammatory cytokine production from macrophages, B cells, and T cells and strongly inhibits T cell-mediated immune responses. TNF-α and IFN-γ induce Treg polarization in AA without affecting IL-4, IL-10, or IL-17 production (Giudice et al., 2021). Therefore, by blocking pro-inflammatory cytokines can trigger anti-inflammatory activation that exacerbates the disease.

The assessment of soluble circulating mediating factors in marrow failure has been restricted to only a couple of cytokines in AA. Assessing alterations in disease based on the measurement of a single cytokine is likely insufficient due to the complex array of proteins involved in an immune response. A comprehensive analysis of 31 cytokines, chemokines, and growth factors in the plasma of healthy volunteers, as well as AA and MDS patients, was conducted in a study (Feng et al., 2011). They observed certain chemokine levels increased sixth month post IST compared to pretreatment in AA patients. In contrast, most of the cytokine levels except IL-1, IL-6 and TNF-α were undetectable in the pretreatment of AA patients. The administration of transfusions, steroids, CsA, and G-CSF may affect the expression of inflammatory cytokines (Jun et al., 2005).

Most studies have compared these cytokine levels in AA and healthy controls, while very few data are on AA patients treated with IST. It is unclear why we did not observe a significant decrease in the anti-inflammatory cytokine levels reported by other studies. The study has limitations, such as a heterogeneous study population, a relatively small patient sample size, and a short follow-up period (third month) considered post-ATG treatment. Perhaps, a larger sample size and data collected at longer follow-up would give a clear understanding of the immune cells and pro- and anti-inflammatory cytokine levels produced in AA patients treated with ATG.

Various environmental factors contribute to the destruction of HSCs caused by antigen-driven and autoimmune processes. There is compelling data to suggest that an imbalance in T-cell homeostasis in the marrow environment connects these two claims. In addition, the maintenance of telomeres is closely related to the self-renewal ability of HSCs. Our research findings summarise, that the attrition of telomere length in leukocytes, communication between the microenvironment with the impaired haematopoietic compartment and the immune response, along with the presence of germline genetic variants, contribute to the pathophysiology of AA (**Figure 5.2.**).

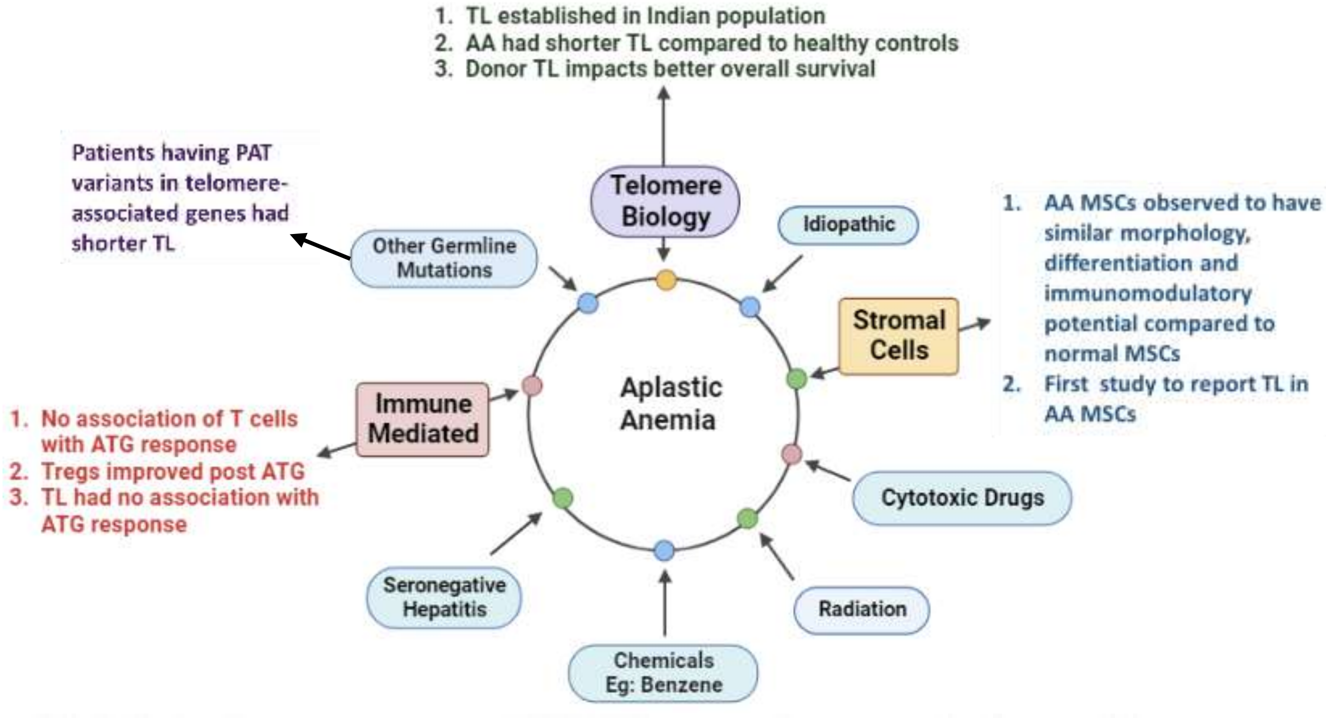


Figure: 5.2. Summary of salient findings on factors involved in the pathophysiology of aplastic anaemia

5.4. Limitations of the study

Limitations of the study include a study population with varying characteristics, a relatively small sample size of patients, and a short duration of follow-up in the ATG treated cohort. Additionally, the measurement of telomere length was not carried out in specific subpopulations of blood cells for patients who received ATG treatment. An additional number of bone marrow samples and uniformity in age and disease severity would further explain the function of AA MSCs and telomere length in AA $CD34^+$ cells. We measured telomere length using the qPCR method since it requires a minimum amount of DNA. While recently, the most reliable method was demonstrated to be Flow-FISH, as telomere length can be measured in cell subtypes.

5.5. Future directions

Over the past few years, there has been significant progress in understanding the intricate pathophysiology of AA. The use of deep gene-sequencing techniques, SNP arrays, multicolour flow cytometry, and fluorescently conjugated monoclonal antibodies has aided in identifying T-cell subpopulations. Single-cell RNA sequencing, a novel technique, analyzes transcriptomes of individual cells, providing detailed characteristics of gene expression and enabling direct comparison of heterogeneous cells. This approach could provide fresh perspectives on the pathophysiology of AA, particularly regarding the recurrent mutations and genetic instability that contribute to clonal evolution.

A major obstacle in studying AA molecular pathophysiology has been the lack of available progenitor cells for conducting various biotechniques. Induced pluripotent stem cells (iPSCs) show promise in AA biology by developing into hematopoietic cell lineages, offering a solution to existing barriers. Using iPSC in combination with RNA splicing and gene editing technologies can uncover genetic and epigenetic occurrences related to telomere shortening. Over time, the process should enhance its capacity to accurately differentiate founder mutations from other acquired subclonal populations and those that occur naturally with ageing. This could potentially enable the prediction of clinical outcomes for patients with AA, leading to improved algorithms for therapeutic decisions, such as allogeneic stem cell transplantation. In the future, the advancement of new technologies and techniques may uncover the definite genetic source of the tolerance for the inappropriate and persistent immune response observed in AA.

6.0. Summary and Conclusion

The maintenance of HSC regeneration and differentiation involves the regulation of MSCs, immune mechanisms, and telomeres. Bone marrow failure is characterized by profound impairment of the hematopoietic stem cell pool, leading to bone marrow aplasia. It can be inherited due to genetic variants in genes involved in various aspects of haematopoiesis or acquired for which the causative factors are heterogeneous. We used BMF as a model to understand factors which influence HSC loss. Towards this, we analysed the characteristics of MSCs, its differentiation property and immunosuppressive properties. Telomere length analysis was carried out in BMF patients and healthy controls; in peripheral blood, HSCs and in MSCs by qPCR. Profiling of T cells was done by flow cytometry in patients receiving immunosuppressive therapy (ATG). Cytokine profiling and telomere length were also analyzed to understand factors influencing HSC recovery and response after treatment.

We demonstrated that bone marrow MSCs, from individuals with aplastic anaemia as compared to healthy individuals, exhibit typical morphology, standard proliferation and differentiation potential, and changes in gene expression related to their osteogenic and adipogenic potential and immunomodulatory properties. Additionally, the telomere length of aplastic anaemia BM MSCs exhibited no discernible difference from healthy BM controls.

Telomere length decreases with age in the normal Indian population, and there is no variation in the length of telomeres between males and females. The median relative telomere length in patients with aplastic anaemia, Fanconi anaemia and myelodysplastic syndrome was significantly shorter than in age-matched healthy controls. There was no significant association of telomere length with the disease severity in patients with aplastic anaemia and Fanconi anaemia.

When compared to age-matched AA, patients with FA had no difference in telomere length; conversely, rTL was significantly lower in MDS patients.

Telomere length of matched sibling donors significantly influenced the transplant outcome, contrary to the telomere length in recipients. We found no significant correlation of transplant associated factors, including neutrophil recovery, acute or chronic GVHD, or overall survival with telomere length in AA patients. Shorter telomere length in donors is an exclusive risk factor for overall survival, along with older age, graft failure, acute and chronic GVHD in logistic regression analysis. After analysing the causes of death in our group, we found that around 50-60% of patients expired due to bacterial or fungal infections. Donors with longer telomere lengths were unaffected by bacterial sepsis and other infections (fungal and viral), which only occurred in donors with shorter telomeres. Therefore, from our data, it can be inferred that telomeres play a role in HSC self-renewal property in BMF.

We identified that 22% of aplastic anaemia patients had germline variants in inherited BMF genes using targeted gene panel sequencing. According to our data, 10% of patients have pathological genetic variants, and 12% have variants of uncertain significance. The cohort was analysed for telomere length; no discernible difference was found between patients with and without genetic variants. Patients with pathogenic IBMFS variants exhibited mainly in genes related to haematopoiesis disorders and telomere biology disorders. We cultured and expanded CD34+ cells from patients with AA and measured telomere length through qPCR. After analysing a small sample, which was a complex process of obtaining CD34+ cells from aplastic marrow, we found no variation in the telomere length between CD34+ cells of AA and normal marrow.

Our data shows no correlation between the length of leukocyte telomeres and the response to IST within the third month. Telomere length did not influence response to IST when categorising individuals by age in decades. There was no change in the percentage of T cells (including $CD3^+$, $CD4^+$, and $CD8^+$ cells) before and after treatment with ATG in patients with AA, and the same was also observed in NK cells. There was a significant increase in the percentage of Tregs after treatment in patients who responded three months after ATG treatment.

We measured a mixture of pro- and anti-inflammatory cytokines generated by immune cells in BM, which are linked to the pathophysiology of aplastic anaemia. We have noticed deficient levels of cytokines, specifically IL-2, IL-4, and TGF-β, that were undetected. No variations in cytokine levels (IL-10, IFN-γ, TNF-α, IL-17A, and IL-22) were detected in the plasma/serum of AA patients after receiving ATG treatment for three months.

The study suggests that telomere biology in HSCs, the involvement of microenvironment mesenchymal stromal cells with the hematopoietic system, and the interplay of immune mechanisms within the marrow contribute to the loss of HSCs regeneration and differentiation (**Figure 6.0.**).

6.1. Significant findings of the study

- Establishment of telomere length in a diverse age group in a significant number of normal individuals in the Indian population.
- Telomere length in 54% of patients with aplastic anaemia was shorter compared to age-matched healthy controls.
- Telomere length in other bone marrow failure (FA and MDS) syndromes was lower than in age-matched healthy controls.
- Donor telomere length impacts overall survival in patients with AA undergoing HSCT.

- Telomere length and immune cells such as T cells and NK cells were not associated with response to ATG in patients with AA.
- A subset of T-cells, Tregs that mediate immunosuppression showed improvement in response to ATG in AA patients.
- Cytokines produced by immune cells did not correlate with response to ATG in patients with AA.

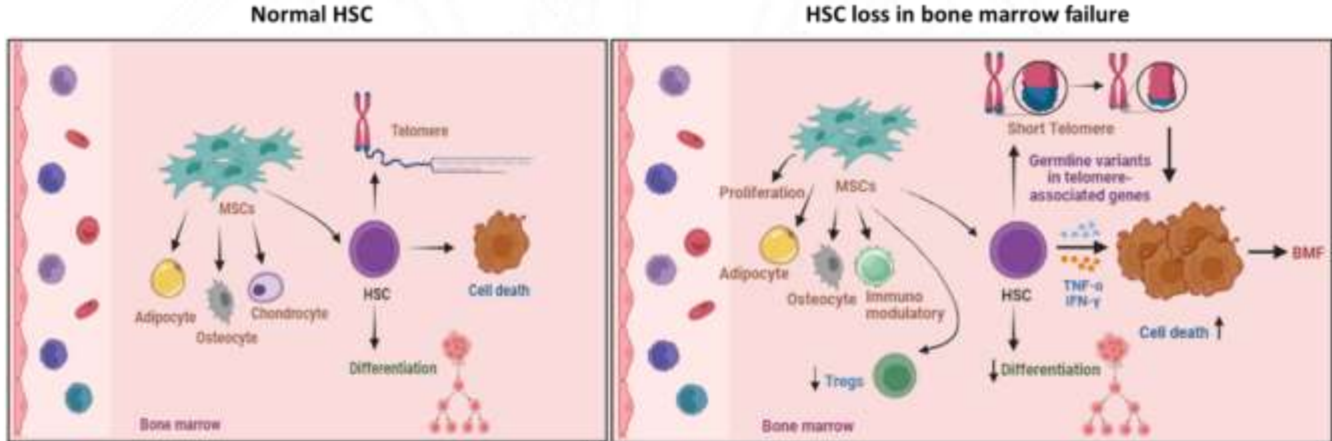


Figure 6.0.: Summary of factors influencing HSC regeneration and differentiation leading to bone marrow failure based on our study

In normal homeostasis, MSCs in the BM microenvironment play a role in supporting HSC. Telomere maintenance is essential for protecting self-renewal capacity while the immune mechanism maintains a balanced HSC differentiation. The immune mechanism in the case of BMF is altered, while the MSCs remain normal. Due to germline variants in telomere-associated genes, telomere length is shortened, leading to accelerated cell death and bone marrow failure.

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Description: Aplastic anemia (AA) is a bone marrow failure caused by the immune system attacking hematopoietic stem cells, mainly by autoreactive T-cells. The doctoral study conducted a comprehensive evaluation of the factors related to the pathophysiology of AA in the Indian population. The relative telomere length using qPCR was measured in the normal Indian population and currently is used as a diagnostic procedure prior to transplant. The relative telomere length in patients with aplastic anemia, Fanconi anemia, and myelodysplastic syndrome was evaluated and compared with age-matched healthy controls. Ours was the first Indian study to report the impact of donor telomere length on transplant outcomes in patients undergoing matched sibling donor transplantation for AA. We also analyzed the correlation of telomere length in leukocytes with response to ATG therapy. Reports on MSCs in AA have been controversial, although some studies indicated that MSCs derived from patients with AA differ from healthy control, few studies suggested otherwise. We examined the characteristics of bone marrow MSCs in individuals with aplastic anemia and healthy individuals and determined the telomere length of MSCs.

2014 – 2016: Master of Technology (Biotechnology), Vellore Institute of Technology, Vellore, India. Score:92%
Courses: Molecular biology, Genetic engineering, Genomics, Biochemistry, Biodiversity, Bioethics, Food biotechnology, Environmental biotechnology, Pharmaceutical biotechnology
Dissertation Title: Injectable, shape memory cryogels for tissue engineering of human induced pluripotent stem cells.
Supervisor: Dr. Murugan Ramalingam, Associate Professor, Centre for Stem Cell Research (CSCR), Vellore, India.

2010 – 2014: Bachelor of Engineering (Biotechnology), New Horizon College of Engineering, Outer Ring Road, near Marathahalli, Kaverappa Layout, Kadubeesanahalli, Bengaluru, India
Score: 86%.
Basic areas of Molecular Biology, Biochemistry, Cell Biology, Microbiology, Biochemistry, Bioinformatics, Animal biotechnology, and Immunology.
Dissertation Title: Optimization of Solid-state Fermentation Process for Enhancing the Yield of Terreic Acid.
Supervisor: Mr. R.S. Upendra Raju, Senior Assistant Professor, Department of Biotechnology, New Horizon College of Engineering, Bengaluru, India.

Experience:

1. Research Fellow, Department of Haematology, Christian Medical College, Vellore, working in the pathophysiology of Aplastic anemia under the supervision of Dr. Eunice Sindhuvi and Dr. Biju George (July 2016 - April 2024)
2. Intern, Centre for Stem Cell Research, Vellore (July 2015- December 2015)
   Project on "Injectable, shape memory cryogels for tissue engineering of human induced pluripotent stem cells"
   Under the supervision of Dr. Murugan Ramalingam, Centre for Stem Cell Research, Vellore.

Other Experience:

1. Involved in teaching postgraduate diploma students briefing them on molecular biology and PCR-based techniques (2019-2022).
2. Handling of laboratory animals' workshop at Christian Medical College, Vellore, June 2019.
3. Basic flow cytometry course organized by 'BD-NCBS of Centre of Excellence in Cytometry' at National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru, August 2019.

Technical expertise:

- Polymerase chain reaction (PCR), Real-time quantitative PCR (qPCR), Southern blotting, Sanger sequencing, Gene scan, NGS data analysis
- Multiparametric flow cytometry (BD GAVIOS, NAVIOS), data analysis – Kaluza software, Flow-FISH method for telomere length measurement
- Handling of primary samples and enrichment of target cell population (HSCs and MSCs)
- Animal handling, Western blotting, ELISA, cell-culture assays
- Biomaterial synthesis, iPSC culture, tissue culture

Awards and Achievements:

1. Indian Journal of Medical and Paediatric Oncology (IJMPO) award received for outstanding research conducted by a Postgraduate for the article "Impact of donor telomere length on survival in patients undergoing matched sibling donor transplantation for aplastic anemia." Christian Medical College, Vellore, 2022.
2. Indian Council of Medical Research - Senior Research Fellowship 2022.
3. Abstract Achievement Award in "63rd Annual American Society of Hematology meeting and exposition" held in Atlanta, Georgia, USA, Dec 2021.
4. Travel Grant awarded by the Department of Science and Technology (DST-SERB, India) to attend the 63rd Annual American Society of Hematology meeting and exposition held in Atlanta, Georgia, USA, Dec 2021.
5. Abstract Achievement Award in "60th Annual American Society of Hematology meeting and exposition" held in San Diego, California, USA, Dec 2018.

6. Travel Grant awarded by the Department of Science and Technology (DST-SERB, India) to attend the 60th Annual American Society of Hematology meeting and exposition held in San Diego, California, USA, Dec 2018.
7. Qualified Graduate Aptitude Test for Engineering (GATE) 2016.
8. University 7th rank in Master of Technology (Biotechnology), Vellore Institute of Technology (VIT), Vellore, India, 2016.
9. University 8th rank (Gold medal) in Bachelor of Engineering (Biotechnology), Visvesvaraya Technological University, Karnataka, India, 2014.
10. Endowment award for best academic performance in High School, 2008.

Publications:

1. **Aruna Barade**, Kavitha M Lakshmi, Anu Korula, Fouzia N Aboobacker, Uday P Kulkarni, Aby Abraham, Vikram Mathews, Biju George, Eunice S. Edison. Comparison of telomere length in patients with bone marrow failure syndromes and healthy controls. ***European Journal of Haematology*****, 2024, 1-9.**

2. **Aruna Barade**, Fouzia Aboobacker, Anu Korula, Kavitha Lakshmi, Anup Devasia, Aby Abraham, Vikram Mathews, Eunice Edison, and Biju George. Impact of donor telomere length on survival in patients undergoing matched sibling donor transplantation for aplastic anemia. ***British Journal of Haematology*****, 2022, 196(3): 724-734.**

3. Upendra R.S., Pratima Khandelwal, Zeinab Raftani Amiri, Rahila Banu, **Aruna Barade**, Veena.K, Gayathri.V and Yamini.D.E. Artificial Neural Network: A novel method for optimization of Bioproducts and Bioprocesses: A critical review**.** ***MSR Journal of Sciences*****, 2014, 1(1): 21-34.**

Book Chapter:

1. Maria Leena, **Aruna Barade**, Deepti Rana, Chetna Dhand, Seeram Ramakrishna and Murugan Ramalingam. Nanofiber Composites in Biomolecular Delivery. Nanofiber Composite Materials for Biomedical Applications, Murugan Ramalingam and Seeram Ramakrishna (1st Edition), Elsevier Publication, UK (2017).

Peer-Reviewed Meeting Abstracts:

American Society of Hematology: Poster presentations

1. **Aruna Barade**, Arun Kumar A, Deborah A, Anu Korula, Anup J Devasia, Fouzia N Aboobacker, Kavitha M Lakshmi, Aby Abraham, Vikram Mathews, Biju George, Eunice S. Edison. Germline variants contribute significantly to the pathogenesis of Aplastic anemia in India. "63rd Annual American Society of Hematology meeting and exposition" (Atlanta, Georgia, USA, December 2021).

2. **Aruna Barade**, Sreenithi Santhakumar, Biju George, and Eunice Sindhuvi Edison. Decreased Iron Utilization Leads to Dysregulation of Iron Homeostasis in Aplastic anemia. "60th Annual American Society of Hematology meeting and exposition" (San Diego, California, USA, December 2018).

3. **Aruna Barade**, Anu Korula, Nisham PN, Kavitha M Lakshmi, Uday Prakash Kulkarni, Anup Devasia, Aby Abraham, Alok Srivastava, Vikram Mathews, Eunice Sindhuvi Edison, and Biju George. Donor Telomere Length influences engraftment and outcome in Aplastic anemia patients undergoing matched-related stem cell transplantation. "59th Annual American Society of Hematology meeting and exposition" (Atlanta, Georgia, USA, December 2017).

HAEMATOCON: Indian Society of Haematology and Blood Transfusions

1. **Aruna Barade**, Deborah Arul, Sreenithi S, Kavitha M Lakshmi, Anu Korula, Fouzia, Uday Prakash Kulkarni, Anup Devasia, Aby Abraham, Alok Srivastava, Vikram Mathews, Eunice Sindhuvi Edison, and Biju George. Relevance of telomere biology and immune cells in the treatment outcome of patients with aplastic anemia. Dr. J. C. Patel Oration. HAEMATOCON 2019, New Delhi, India.

2. Deborah A, **Aruna Barade**, Eunice S Edison, Biju George. Effect of telomere length on outcome in patients with aplastic anemia treated with anti-thymocyte globulin. HAEMATOCON 2019, New Delhi, India. (Poster)

3. **Aruna Barade**, Eunice Sindhuvi, Biju George. Significance of genetic variants in TERT and TERC genes in aplastic anemia. HAEMATOCON 2018, Kochi, India. (Oral)

International Bone marrow failure symposium: Poster presentations

1. **Aruna Barade**, Kavitha M Lakshmi, Anu Korula, Fouzia N Aboobacker, Uday P Kulkarni, Aby Abraham, Vikram Mathews, Biju George, Eunice S. Edison. Comparison of telomere length in different bone marrow failure syndromes. 2nd International Bone marrow failure symposium, Christian Medical College, Vellore, April 8th & 9th, 2022.

2. **Aruna Barade**, Anu Korula, Nisham PN, Kavitha M Lakshmi, Uday Prakash Kulkarni, Anup Devasia, Aby Abraham, Alok Srivastava, Vikram Mathews, Eunice Sindhuvi Edison, and Biju George. Incidence of Telomere Length in patients with Aplastic anemia in the Indian population. 1st International Bone marrow failure symposium, Christian Medical College, Vellore, Feb 3rd & 4th, 2020.

References:

1. Dr. Eunice Sindhuvi E, Professor, Department of Haematology, Christian Medical College, Vellore, Ranipet Campus, Kilminnal Village - 632 517, Tamil Nadu, India, email - eunice@cmcvellore.ac.in, Ph: 0417-2224575

2. Dr. Biju George, Professor, Department of Haematology, Christian Medical College, Vellore, Ranipet Campus, Kilminnal Village - 632 517, Tamil Nadu, India, email – biju@cmcvellore.ac.in

3. Dr. Pratima Kandelwal, Professor of Practice, Faculty of Life & Allied Health Sciences, RUAS, Bengaluru – 560054, email – pratima2k1@gmail.com

Recd [illegible] 04/09/15

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Ethics Committee Registration No: ECR/326/INST/TN/2013 issued under Rule 122D of the Drugs & Cosmetics Rules 1945, Govt. of India

Dr. George Thomas, D Ortho Ph.D.
Chairperson, Ethics Committee

Dr. B. Antonisamy, M.Sc., Ph.D., FSMS, FRSS.
Secretary, Research Committee

Prof. Keith Gomez, B.Sc., MA (S.W), M.Phil.
Deputy Chairperson, Ethics Committee

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Nihal Thomas
MD, MNAMS, DNB (Endo), FRACP (Endo), FRCP (Edin) FRCP (Glasg)
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

September 2, 2015

Dr. Biju George
Professor
Department of Haematology
CMC, Vellore

Sub: **External Research grant project NEW PROPOSAL: (DBT)**
Relevance of telomere biology in the pathogenesis and treatment outcome of patients with bone marrow failure disorders.
Dr. Biju George (Employment Number: 30156), Haematology, Dr. Eunice Sindhuvi (Emp. No: 30925), Haematology, Dr. Vikram Mathews (E. No 14972), Haematology.

Ref: IRB Min No: 9491 [OBSERVE] dated 24.06.2015

Dear Dr. Biju George,

The Institutional Review Board (Silver, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Relevance of telomere biology in the pathogenesis and treatment outcome of patients with bone marrow failure disorders" on June 24th 2015.

The Committee reviewed the following documents:

1. IRB Application format
2. Healthy volunteer information sheet
3. Patient Information Sheet and Informed Consent Form
4. No of documents 1 - 3

The following Institutional Review Board (Silver, Research & Ethics Committee) members were present at the meeting held on June 24th 2015, at 9.45 am in the CREST/SACN Conference Room Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Silver, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788 E-mail: research@cmcvellore.ac.in

Dr. George Thomas, D Ortho Ph.D.
Chairperson, Ethics Committee

Dr. B. Antonisamy, M.Sc., Ph.D., FSMS, FRSS.
Secretary, Research Committee

Prof. Keith Gomez, B.Sc., MA (S.W), M.Phil.
Deputy Chairperson, Ethics Committee

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Nihal Thomas
MD, MNAMS, DNB (Endo), FRACP (Endo), FRCP (Edin) FRCP (Glasg)
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. B. Antonisamy	M. Sc, PhD, FSMS, FRSS	Professor, Biostatistics, CMC, Vellore, Member Secretary, Research Committee, IRB.	Internal, Statistician
Dr. Suresh Devasahayam	BE, MS, PhD	Professor of Bio-Engineering, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Anuradha Bose	MBBS, DCH, MD,MRCP, FRCPCH	Professor, Child Health, CMC, Vellore	Internal, Clinician
Dr. B. Poonkuzhali	MSC, PhD	Professor, Haematology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Molly Jacob	MBBS, MD, PhD	Professor, Biochemistry, CMC, Vellore	Internal, Clinician
Dr. Thambu David	MBBS, MD, DNB	Professor, Medicine, CMC, Vellore	Internal, Clinician
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, CMC, Vellore	Internal, Clinician
Dr. Anil Kuruvilla	MBBS, MD, DCH	Professor, Child Health, CMC, Vellore	Internal, Clinician
Dr. George Thomas	MBBS, D Ortho, PhD	Orthopaedic Surgeon, St. Isabella Hospital, Chennai, Chairperson, Ethics Committee, IRB, Chennai	External, Clinician
Prof. Keith Gomez	BSc, MA (S.W), M. Phil (Psychiatry Social Work)	Student counselor, Loyola College, Chennai, Deputy Chairperson, Ethics Committee, IRB	External, Lay Person & Social Scientist
Dr. Sathya Subramani	MD, PhD	Professor, Physiology, CMC	Internal, Clinician

IRB Min No: 9491 [OBSERVE] dated 24.06.2015

Ethics Committee Silver, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788 E-mail: research@cmcvellore.ac.in

Dr. George Thomas, D Ortho Ph.D.
Chairperson, Ethics Committee

Dr. B. Antonisamy, M.Sc., Ph.D., FSMS, FRSS.
Secretary, Research Committee

Prof. Keith Gomez, B.Sc., MA (S.W), M.Phil.
Deputy Chairperson, Ethics Committee

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Nihal Thomas
MD, MNAMS, DNB (Endo), FRACP (Endo), FRCP (Edin) FRCP (Glasg)
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Vinitha Ravindran	PhD (Nursing)	Professor & Addl. Deputy Dean, College of Nursing, CMC, Vellore	Internal, Nurse
Mrs. Ruma Nayak	MSc (Nursing)	Professor, Head of Paediatric Nursing & Deputy Nursing Superintendent, College of Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Legal Expert, Vellore	External, Legal Expert
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay person
Dr. Shirley David	MSc, PhD	Professor, Head of Fundamentals Nursing Department, CMC, Vellore	Internal, Nurse
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMC, Vellore	Internal, Legal Expert
Rev. Dr. T. Arul Dhas	MSc, BD, DPC, PhD(Edin)	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Dr. Binu Susan Mathew	MBBS, MD	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Jayaprakash Muliyil	BSc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, Vellore	External, Scientist &Epidemiologist
Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology, Additional Vice Principal (Research), Deputy Chairperson, IRB, Member Secretary (Ethics Committee), IRB	Internal, Clinician

IRB Min No: 9491 [OBSERVE] dated 24.06.2015

Ethics Committee Silver, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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Dr. George Thomas, D Ortho Ph.D.
Chairperson, Ethics Committee

Dr. B. Antonisamy, M.Sc., Ph.D., FSMS, FRSS.
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Prof. Keith Gomez, B.Sc., MA (S.W), M.Phil.
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Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Nihal Thomas
MD, MNAMS, DNB (Endo), FRACP (Endo), FRCP (Edin) FRCP (Glasg)
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Administrative Committee's approval is to be obtained for opening the account-head, employing any personnel or purchasing any equipment. The investigator also needs to present to Administrative Committee, the terms and condition of the Funding agency for approval.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo),FRACP(Endo) FRCP(Edin) FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 9491 [OBSERVE] dated 24.06.2015

Ethics Committee Silver, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Ethics Committee Registration No: ECR/326/INST/TN/2013 Re Reg-2019 Issued under Rule 122D of the Drugs & Cosmetics Rules 1945, Govt. of India

Dr. George Thomas, D. Ortho., Ph.D.,
Chairperson, Ethics Committee

Dr. Prasanna Samuel, M.Sc., Ph.D.,
Secretary, Research Committee

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee

Dr. Biju George, MD., DM.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

October 14, 2020

Ms. Aruna Barade,
Department of Haematology,
Christian Medical College,
Vellore – 632 004.

Sub: Fluid Research Grant: New Proposal:
stem cell Characterization of Mesenchymal Stromal Cells in Aplastic anemia.
Ms. Aruna Barade, Employment Number: 81807, Senior Research Fellow, Department of Haematology, Dr. Eunice Sindhuvi Edison, (Emp. No. 30925) Haematology, Dr. Biju George (Emp No: 30156),

Ref: IRB Min. No. 12929 (OBSERVE) dated 24.06.2020

Dear Ms. Aruna Barade,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Suceena Alexander, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Suceena Alexander
Secretary (Ethics Committee)
Institutional Review Board

Dr. Suceena Alexander, MD.,DM.,FASN.
Secretary - (Ethics Committee)
Institutional Review Board
Christian Medical College,
Vellore - 632 002, Tamil Nadu, India.

Ethics Committee Silver, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788 E-mail: research@cmcvellore.ac.in

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Ethics Committee Registration No: ECR/326/INST/TN/2013 Re Reg-2019 Issued under Rule 122D of the Drugs & Cosmetics Rules 1945, Govt. of India

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Chairperson, Ethics Committee

Dr. Prasanna Samuel, M.Sc., Ph.D.,
Secretary, Research Committee

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee

Dr. Biju George, MD., DM.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

October 14, 2020

Ms. Aruna Barade,
Department of Haematology,
Christian Medical College,
Vellore – 632 004.

Sub: Fluid Research Grant: New Proposal:
stem cell Characterization of Mesenchymal Stromal Cells in Aplastic anemia.
Ms. Aruna Barade, Employment Number: 81807, Senior Research Fellow, Department of Haematology, Dr. Eunice Sindhuvi Edison, (Emp. No. 30925) Haematology, Dr. Biju George (Emp No: 30156),

Ref: IRB Min. No. 12929 (OBSERVE) dated 24.06.2020

Dear Ms. Aruna Barade,

The Institutional Review Board (**Silver**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed your project titled "stem cell Characterization of Mesenchymal Stromal Cells in Aplastic anemia" on June 24, 2020. I am quoting below the minutes of the meeting.

The Committee reviewed the following documents:

1. IRB Application Format
2. Patient Information Sheet
3. Informed Consent Form
4. No. of Documents 1 – 3.

Due to the extra-ordinary situation caused by the COVID - 19 pandemic and the subsequent government lockdown, the members of the Institutional Review Board (**Silver**, Research & Ethics Committee), Christian Medical College, Vellore - 632002, listed below performed an online review for the month of June 2020.

Ethics Committee Silver, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788 E-mail: research@cmcvellore.ac.in

Name	Qualification	Designation	Affiliation
Dr. George Thomas	D Ortho, PhD	Orthopaedic Surgeon, St. Isabella Hospital, Chennai, Chairperson, Ethics Committee, IRB, Chennai	External, Clinician
Dr. Suceena Alexander	MD, DM, FASN.	Professor, Nephrology, Additional Vice Principal (Research), Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore.	Internal, Clinician
Dr. Biju George	MBBS, MD, DM	Professor, Haematology,	Internal, Clinician
Dr. RV. Shaji	M.Sc, PhD	Professor, Heamatology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Suresh Devasahayam	MS, PhD	Professor of Bio-Engineering, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Sathya Subramani	MD, PhD	Professor, Physiology, CMC, Vellore	Internal, Clinician
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Dr. D. J. Christopher	DTCD, DNB, FRCP(Glasg), FCCP(USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Thomas V Paul	MD, DNB, PhD	Professor, Endocrinology, CMC, Vellore	Internal, Clinician
Dr. Prasanna Samuel	M. Sc, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Sridhar Gibikote	DMRD, DNB	Professor, Radiology, CMC, Vellore	Internal, Clinician
Dr. Abhay Gahukamble	MS, D Ortho, DNB(Ortho)	Associate Professor, Paediatric Orthopaedics, CMC, Vellore	Internal, Clinician
Dr. Laxmi Govindaraj	MBBS., MD., Pharmacology	Sr. Resident, Clinical Pharmacology, CMC, Vellore	Internal Pharmacologist
Dr. Ajith Sivadasan	MD, DM	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert

Ethics Committee Silver, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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Chairperson, Ethics Committee

Dr. Prasanna Samuel, M.Sc., Ph.D.,
Secretary, Research Committee

Prof. Keith Gomez, MA (S.W), M.Phil.,
Deputy Chairperson, Ethics Committee

Dr. Biju George, MD., DM.,
Chairperson, Research Committee

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Santhanam Sridhar	DCH, DNB	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Dr. Manoranjitham.S	M Sc (N) P.hd in Psychiatric Nursing.	Professor and Head Psychiatric Nursing, College of Nursing, CMC, Vellore	Internal, Nurse
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, Vellore	External Legal Expert
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay person

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of Withdrawals for the study entitled: "stem cell Characterization of Mesenchymal Stromal Cells in Aplastic anemia" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of 3,00,000/- INR (Rupees Three Lakh Only) will be granted for 2 years. 1,50,000/- INR (Rupees One Lakh fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 1,50,000/- INR (Rupees One Lakh fifty Thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Suceena Alexander
Secretary (Ethics Committee)
Institutional Review Board.

Dr. Suceena Alexander, MD.,DM.,FASN.
Secretary - (Ethics Committee)
Institutional Review Board
Christian Medical College,
Vellore - 632 002, Tamil Nadu, India.

IRB Min. No. 12929 (OBSERVE) dated 24.06.2020

bjh research paper

Impact of donor telomere length on survival in patients undergoing matched sibling donor transplantation for aplastic anaemia

Aruna Barade, Fouzia Aboobacker, Anu Korula, Kavitha Lakshmi, Anup Devasia, Aby Abraham, Vikram Mathews, Eunice Edison and Biju George

Department of Haematology, Christian Medical College Vellore, Vellore, India

Received 12 July 2021; accepted for publication 22 September 2021

Correspondence: Biju George, Department of Haematology, Christian Medical College, Vellore-632004, Tamilnadu, India.
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Summary

Although telomere shortening is seen frequently in patients with aplastic anaemia (AA), there are no data on its association in matched sibling donor (MSD) transplants. We evaluated the effect of pre-transplant telomere length of patients and donors, measured by quantitative real-time polymerase chain reaction in 163 recipients undergoing MSD transplants. The median age of patients and donors was 24 and 26 years, respectively. Fludarabine and cyclophosphamide was the main conditioning regimen used and all received peripheral blood stem cell grafts. Engraftment occurred in 89% with graft failure (primary and secondary) in 6%. Acute and chronic graft-*versus*-host disease (GVHD) occurred in 28% and 24%, respectively. At a median follow-up of 37 months, 117 patients (72%) were alive. All patients and donors were divided into short and long telomere length based on their median and quartile values. Patient telomere length was not associated with severity of AA, neutrophil recovery, graft failure, acute GVHD or chronic GVHD. Longer donor telomere length was associated with better overall survival [hazard ratio (HR) = 0·2, *P* = 0·006] but did not influence neutrophil recovery, graft failure, acute or chronic GVHD. The five-year overall survival was significantly better (94·9 ± 3·5% vs 65·4 ± 4·3%, *P* = 0·002) for donors with long (highest quartile, DTL-HQ) versus short (lower three quartiles, DTL-LQ) telomeres, respectively. On multivariate analysis, longer donor telomere length, recipient age and acute GVHD continued to remain significant. This is the first study demonstrating an association of donor telomere length on overall survival following MSD transplant for AA but it needs to be confirmed in larger studies.

Keywords: aplastic anaemia, telomere length, haematopoietic stem cell transplantation, matched sibling donor, overall survival.

Introduction

Aplastic anaemia (AA) is a bone marrow failure disorder characterized by pancytopenia and hypocellular marrow and can be fatal if not treated early.[1] Haematopoietic stem cell transplantation (HSCT) in the presence of a matched related donor or immunosuppressive therapy using a combination of anti-thymocyte globulin (ATG) and ciclosporin +/- eltrombopag are the mainstays of therapy.[2] HSCT is associated with cure rates of over 80–90% in patients below the age of 20 years and over 70% in patients between 20 and 40 years of age.[3] Several pre-and post-transplant factors have been shown to influence the outcome of HSCT including age, time from diagnosis to HSCT, number of prior transfusions, graft source and the presence of acute graft-*versus*-host disease (GVHD).[4,5] At our centre, we have been using a combination of fludarabine and cyclophosphamide as our conditioning regimen along with peripheral blood stem cells (PBSC) as the graft source since 2004, which had helped improve overall survival to 75% along with low rates of graft failure of 1·8%.[6]

Telomere shortening has been shown to occur in 30–50% of patients with AA and short telomeres are associated with poor outcomes following immunosuppressive therapy with

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ATG.[7,8] Initial studies on unrelated donor transplant recipients in AA suggested a linear correlation between telomere length in the donor and overall survival but subsequent studies suggest a correlation more with recipient telomere length than donor telomere length.[9–11] There are no published studies looking at the association of telomere length in patients undergoing a matched sibling donor transplant for AA. In this analysis, we tried to study the association between donor and patient telomere length and transplant outcomes in patients undergoing MSD transplants for AA.

Methods

Study participants

This is a retrospective analysis of patients who underwent matched sibling donor transplant at Christian Medical College, Vellore, India between 2001 and 2019. Patients undergoing transplant for inherited bone marrow failure syndromes and patients undergoing either a matched unrelated donor transplant or haplo-identical transplant were excluded from this analysis. A total of 281 patients underwent MSD transplant during this period of which 194 DNA samples of patients and donors were available for telomere length measurement. Eight patients were excluded due to failed telomere length assay while 11 patients who received bone marrow grafts were also excluded. The data for telomere length [telomeric DNA (T) to single copy gene (S) ratio, T/S] was normally distributed and percentile distribution was obtained. The lowest and highest two percentiles were omitted as outliers in both the recipients and donors (recipient < second percentile, T/S ratio: 0·06, 0·09, 0·1; $n = 3$ and >98th percentile, T/S ratio: 3·1, 5·98, 7·52; $n = 3$; donor < second percentile, T/S ratio: 0·04, 0·06, 0·06; $n = 3$ and >98th percentile, T/S ratio: 5·61, 5·64, 5·76; $n = 3$) and thus 12 more patients were excluded. Therefore, the final analysis had 163 patients, which included 157 patients with fully matched sibling donors and six with fully matched family donors. We also included data on telomere length analysis for 700 persons representing the age-matched normal healthy population that we had previously used for establishing age references for telomere length in the Indian population (Fig S1). The median age of healthy controls was 31 (1–60) years. The number of healthy controls for each decade of age were: 0–10 years, $n = 85$; 11–20 years, $n = 110$; 21–30 years, $n = 135$; 31–40 years, $n = 199$; 41–50 years, $n = 110$; and 51–60 years, $n = 61$, respectively. This study was approved by the Institutional Review Board and the Ethics Committee of the institution.

Methodology

Clinical data on all patients were collected from individual medical records and institutional databases. Transplant outcomes such as graft failure, time to neutrophil recovery, incidence of acute and chronic GVHD and overall survival were recorded. Neutrophil recovery was defined as an absolute neutrophil count $\geq 0{\cdot}5 \times 10^9$/l for three consecutive days. Acute GVHD (aGVHD) was graded as per Glucksberg criteria while chronic GVHD (cGVHD) were defined according to NIH consensus criteria.

Telomere length measurement

Genomic DNA was extracted from the peripheral blood of patients and donors using the QIAGEN Gentra Puregene Kit (Gentra Puregene Blood kit, Qiagen, Hilden, Germany) and stored at 4°C till analysis. Purity of the DNA samples was assessed using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) by estimating the absorbance ratio at 260/280 nm and samples within the range of 1·8–2 were considered as pure. The relative telomere length (rTL) was measured from the extracted DNA using the quantitative real-time polymerase chain reaction (qPCR), as previously described.[12,13]

Briefly, for the standard curve, two master mixes of PCR reagents were prepared, one with the TEL primer pair and another with the S (36B4) primer pair. One reference DNA sample was diluted serially to produce five concentrations of DNA ranging from 10 to 0·625 ng/ml. PCR was performed using 10 µl reaction volumes consisting of: 5 µl of SYBR Green PCR Master Mix (Takyon, Eurogentec, Belgium), 0.5 µl of lambda DNA, 2·25 µl of sterile water, and 0·125 µl of the assay-specific mix of primers. For each DNA sample, the T/S ratio is computed. The relative T/S ratio is calculated in relation to a reference genomic DNA sample. The relative T/S ratio was obtained using the formula $2^{-(\Delta Ct\ -\ \Delta Cs)} = 2^{-\Delta\Delta Ct}$. This T/S ratio is directly proportional to average telomere length. All telomere and 36B4 reactions were performed in duplicate. The laboratory personnel conducting the telomere length assay was blinded to patient characteristics and clinical outcomes before the statistical analysis. The qPCR efficiency of the TEL and 36B4 assay were ($R^2 = 0{\cdot}99$, slope $= -3{\cdot}6$) and ($R^2 = 0{\cdot}94$, slope $= -3{\cdot}6$). The mean coefficient of variation (CV) was 0·9% for the telomere assay and 0·3% for the 36B4 assay. The intra-assay CV and inter-assay CV for the reference sample from duplicates was 3·44% and 6·33% respectively, and the intraclass correlation coefficient (ICC) was 96%.

Calculation of age-adjusted telomere length

The rTL values were log-transformed to ensure normality. Age-adjusted telomere length for each patient was computed as follows: [(observed log rTL − linear predicted rTL)/root mean square error (RMSE)].[11] A similar analysis was performed for donor values. The linear predicted rTL and RMSE were derived from a linear regression model of log rTL and age from 700 healthy controls for whom rTL was measured and included in this analysis. The age-adjusted rTL was then normalized and used to evaluate the probabilities of engraftment, aGVHD, cGVHD or overall survival.

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Statistical analysis

Data were represented as mean of values ± SD or as median values with range as indicated in the tables. Student's *t*-test or the Mann–Whitney *U*-test were used to statistically compare the continuous variables and the chi-squared test for categorical data. The telomere length on patient characteristics and transplant parameters were evaluated using logistic regression analysis. For multivariate analysis, the Cox proportional hazard model was used to compare different categories of telomere length. For survival analysis, the product-limit method of Kaplan–Meier to calculate the probability of overall survival (OS) and 95% confidence intervals (CIs) was performed. The log-rank test was used to compare the survival distribution across groups. The associations between patient or donor rTL and outcomes of interest have been tested in separate models. The same was performed for the analysis of age-adjusted telomere length data. Pearson's correlation coefficient was used to evaluate the association between rTL and age. All survival estimates are reported as ±1SE. All *P*-values were two-sided, with values of 0·05 or less indicating statistical significance. Statistical analysis was done using the IBM SPSS 24·0 Software (IBM, Armonk, NY, USA).

Results

Patient characteristics

In all, 163 patients (106 males and 57 females) who underwent MSD HSCT were included in this analysis. The median age of patients and donors at the time of HSCT was 24 (range: 3–58) and 26 (range: 3–60) years, respectively (Table I). The majority (76%) had either severe or very severe aplastic anaemia. All patients with non-severe aplastic anaemia (n = 45) were transfusion-dependent despite treatment with either stanazolol or danazol (n = 26), Ciclosporin (n = 13) or ATG (n = 6). Donors were human leukocyte antigen (HLA)-matched sibling donors in 157 (96·3%) while six (3·7%) were fully matched family donors. The main conditioning regimen used for HSCT was a combination of fludarabine (30 mg/m^2/day × 6 days) and cyclophosphamide (60 mg/kg/day × 2 days) (88%) and the graft source was PBSCs in all patients. None of the patients received ATG as part of conditioning or GVHD prophylaxis. Neutrophil engraftment occurred in 89% at a median of 15 days (range: 9–28) while 14 expired prior to engraftment due to sepsis and graft failure was observed in 10 (6%) patients. Of the 10 patients with graft failure, three (1·8%) had primary graft failure while seven (4·2%) had secondary graft failure. Of the 149 patients who were evaluable for chimaerism analysis on day 28 post HSCT, 142 had complete chimaerism (CC), four had mixed chimaerism (MC) and three had primary graft failure. Four patients with CC and all four with MC subsequently developed secondary graft failure. The incidence of grade 2–4 acute GVHD was 28% while the incidence of grade 3–4 acute GVHD was 12%. The incidence of chronic GVHD was 24% and was mild to moderate in most patients. At a median follow-up of 37 months, 117 (72%) patients are alive (Fig 1). We compared the baseline characteristics of the patients included in this analysis (n = 163) with those who were excluded (n = 23) and they were similar with respect to age, gender distribution and telomere length but there was a higher incidence of using a non-Flu/Cy regimen (P = 0·001) and higher incidence of graft failure (P = 0·047) (Table SI).

Table I. Baseline characteristics of patients undergoing MSD transplant for aplastic anaemia.

Parameters	Patients (n = 163)	Donors (n = 163)
Age		
Median (range)	24 (3–58)	26 (3–60)
Relative TL		
Median (range)	0·76 (0·12–3·00)	0·92 (0·15–5·24)
Age-adjusted relative TL		
Median (range)	0·45 (0·0032–0·97)	0·54 (0·0052–0·99)
Gender n (%)		
Male	106 (65)	87 (54)
Female	57 (35)	76 (46)
Disease status n (%)		
NSAA	39 (24)	NA
SAA	93 (57)	
VSAA	31 (19)	
Conditioning regimen n (%)		
Fludarabine + cyclophosphamide	144 (88)	NA
Others	19 (12)	
Graft n (%)		
PBSC	163 (100)	NA
Cumulative incidence of graft failure n (%)		
Yes	10 (6)	NA
No	139 (85)	
Cumulative incidence of acute GVHD n (%)	46/146 (28%)	NA
Cumulative incidence of chronic GVHD n (%)	40/128 (24%)	NA
Final outcome n (%)		
Alive	117 (72)	NA
Expired	46 (28)	

GVHD, graft-*versus*-host disease; MSD, matched sibling donor; NA, not available; NSAA, non-severe aplastic anaemia; SAA, severe aplastic anaemia; PBSC, peripheral blood stem cells; TL, telomere length; VSAA, very severe aplastic anaemia.

Telomere length analysis

The median rTL of patients (PTLm) was 0·76 (0·12–3·00) and of donors (DTLm), 0·92 (0·15–5·24). The median and

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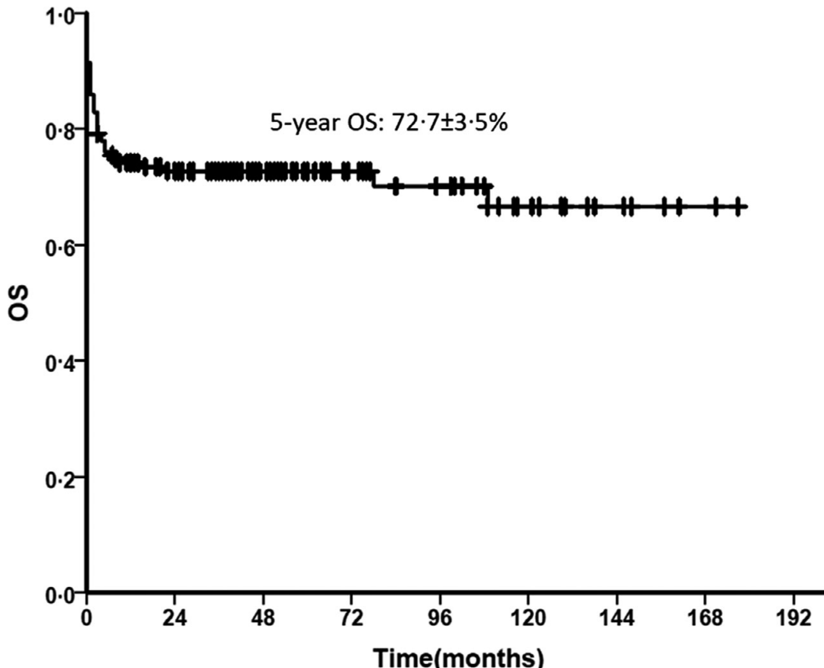

Fig 1. Overall survival (OS) following haematopoietic stem cell transplantation (HSCT) of patients with aplastic anaemia.

range for recipient–donor age difference in sibling donors was 0 (−25 to 13) and for family donors was −24 (−42 to 28). The rTL was seen to decrease with age in healthy controls, patients and donors (Fig S1).

The rTL for both patients and donors was categorized into short (PTLm < 0·76, DTLm < 0·92) and long (PTLm > 0·77, DTLm > 0·93) based on their respective median rTL. Eighty-two patients and 82 donors had a shorter rTL. In addition, rTL of both patients and donors was classified into quartiles [PTL: <0·45, 0·45–0·76, 0·77–1·17, >1·17, DTL: <0·55, 0·55–0·92, 0·93–1·55, >1·55]. Finally, for the analysis, rTLs for patients and donors were categorized as longer (comprising the highest quartile; HQ) and shorter (comprising a combination of the first, second and third quartile; LQ) telomere lengths.

Baseline and transplant characteristics based on median values and quartiles of patient and donor rTL are summarized in Table II. Telomere length as a continuous variable was not found to be significant and therefore rTL was categorized into median and quartiles. The median patient telomere length was not found to be significantly associated with either baseline characteristics or transplant outcomes. A short median donor telomere length (DTLm) was associated with increasing donor age ($P = 0{\cdot}001$) and a higher incidence of acute GVHD ($P = 0{\cdot}021$). When divided into quartiles, OS was not different between the four quartiles for patient telomere length ($P = 0{\cdot}287$; Fig 2A); however, for donor telomere length, the highest quartile (>1·55) showed a significantly better survival compared to the other three quartiles ($P = 0{\cdot}022$; Fig 2B). After combining the lower three quartiles into a single group among the donors (DTL-LQ), a longer donor telomere length (DTL-HQ) was significantly associated with better five-year OS (94·9 ± 3·5% vs. 65·4 ± 4·3%, $P = 0{\cdot}002$; Fig 3). Age-adjusted telomere length for both patients and donors did not show any correlation with patient or donor age, graft failure, GVHD or survival (data not shown).

Association of patient telomere length with transplant outcomes

Logistic regression analysis showed that HSCT outcomes were not influenced by pre-transplant rTL of the recipient (Table III). The median patient rTL (PTLm) did not influence neutrophil recovery post HSCT [hazard ratio (HR) = 1·5, $P = 0{\cdot}460$], graft failure (HR = 0·7, $P = 0{\cdot}558$), incidence of acute GVHD (HR = 1·2, $P = 0{\cdot}639$), chronic GVHD (HR = 0·7, $P = 0{\cdot}366$) or OS (HR = 0·8, $P = 0{\cdot}518$). Both long and short patient rTL (PTL-HQ and PTL-LQ) were significantly associated with older age (>40 years) but not with transplant outcomes including neutrophil recovery (HR = 0·6, $P = 0{\cdot}281$), graft failure (HR = 1·2, $P = 0{\cdot}788$), acute GVHD (HR = 1·7, $P = 0{\cdot}256$) or OS (HR = 1·5, $P = 0{\cdot}275$).

Association of donor telomere length with transplant outcomes

The median rTL of donors (DTLm) was significantly associated with donor age (HR = 3·1, $P = 0{\cdot}002$ for age 20–40 years and HR = 5·1, $P = 0{\cdot}001$ for age > 40 years) and acute GVHD (HR = 2·4, $P = 0{\cdot}019$; Table III). Median donor telomere length (DTLm) however did not influence neutrophil recovery (HR = 0·9, $P = 0{\cdot}777$) or graft failure

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Table II. Characteristics of patient and donor based on categories of relative telomere length.

	PTL median {PTLm}			DTL median {DLTm}			PTL quartile (PLT Q}			DTL quartile {DLT Q}		
Parameters	Short <0·76 *n* = 82	Long >0·77 *n* = 81	*P* value	Short <0·92 *n* = 82	Long >0·93 *n* = 81	*P* value	Low <1·17 *n* = 123	High >1·18 *n* = 40	*P* value	Low <1·55 *n* = 123	High >1·56 *n* = 40	*P* value
P age (years)			0·162						0·123			
<20	26 (32)	37 (46)		NA			44 (36)	19 (47)		NA		
20–40	42 (51)	35 (43)					58 (47)	19 (48)				
>40	14 (17)	9 (11)					21 (17)	2 (5)				
P sex			0·101						1·000			
Male	48 (58)	58 (72)		NA			80 (65)	26 (65)		NA		
Female	34 (42)	23 (28)					43 (35)	14 (35)				
D age (years)						0·001						0·130
<20	NA			17 (21)	39 (48)		NA			37 (30)	19 (47)	
20–40				45 (55)	33 (41)					63 (51)	15 (38)	
>40				20 (24)	9 (11)					23 (19)	6 (15)	
D sex						0·876						0·588
Male	NA			43 (52)	44 (54)		NA			64 (52)	23 (58)	
Female				39 (48)	37 (46)					59 (48)	17 (42)	
Disease subtype			0·441						0·502			
NSAA	23 (28)	16 (20)		NA			31 (25)	8 (20)		NA		
SAA	45 (55)	48 (59)					71 (58)	22 (55)				
VSAA	14 (17)	17 (21)					21 (17)	10 (25)				
Graft failure	4 (5)	6 (8)	0·746	6 (8)	4 (6)	0·747	8 (7)	2 (6)	1·000	7 (6)	3 (8)	0·729
Neutrophil recovery *n* (%)	72 (88)	74 (91)	0·610	74 (90)	72 (89)	0·803	112 (91)	34 (85)	0·370	107 (87)	39 (97)	0·074
Acute GVHD	24 (33)	22 (30)	0·722	30 (41)	16 (22)	0·021	38 (34)	8 (24)	0·297	38 (35)	8 (21)	0·108
Chronic GVHD	17 (27)	23 (35)	0·446	19 (32)	21 (31)	1·000	30 (31)	10 (33)	0·824	24 (27)	16 (41)	0·147
Final outcome			0·602			0·117			0·313			0·002
Alive	57 (69)	60 (74)		54 (66)	63 (78)		91 (74)	26 (65)		81 (66)	36 (90)	
Expired	25 (31)	21 (26)		28 (34)	18 (22)		32 (26)	14 (35)		42 (34)	4 (10)	

DTL, donor telomere length; GVHD, graft-*versus*-host disease; NA, not available; NSAA, non-severe aplastic anaemia; SAA, severe aplastic anaemia; PTL, patient telomere length; VSAA, very severe aplastic anaemia.

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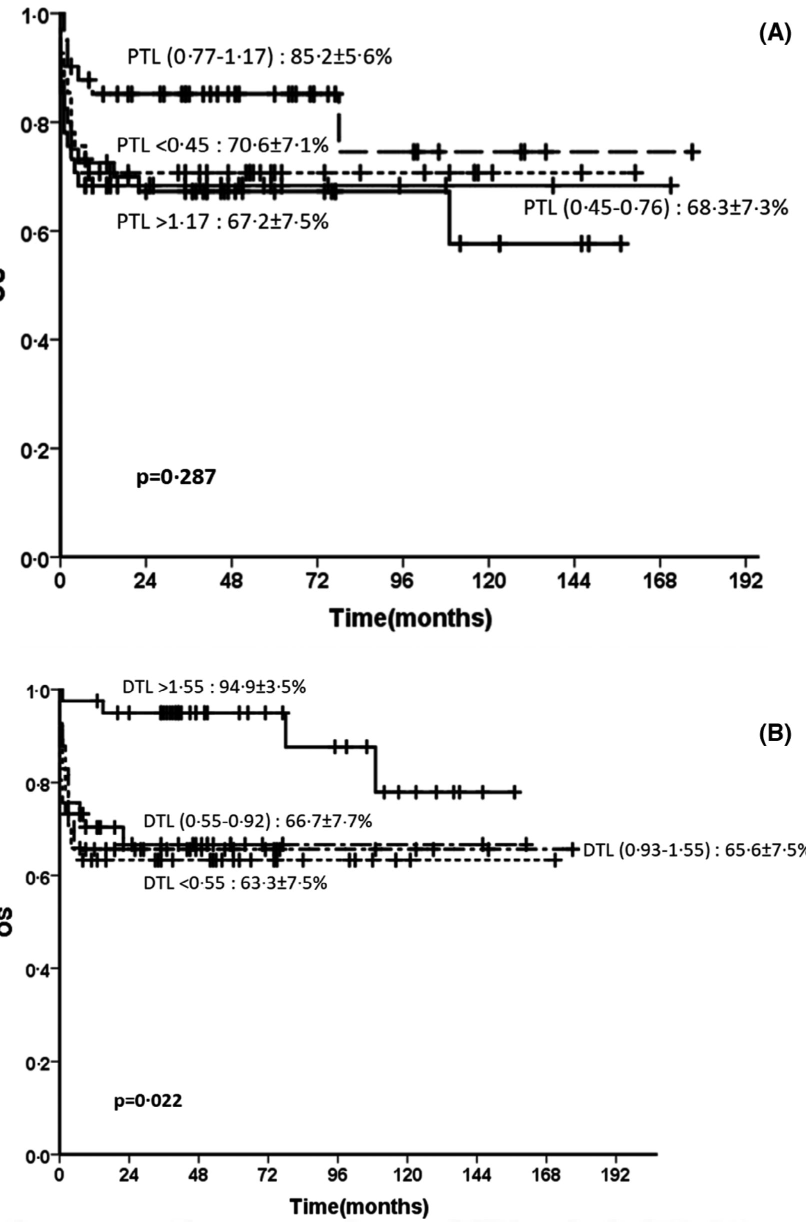

Fig 2. Overall survival (OS) following haematopoietic stem cell transplantation (HSCT) for aplastic anaemia with respect to each quartile of (A) patients (PTL) and (B) donor relative telomere length (DTL).

(HR = 1·4, $P = 0{\cdot}587$) but had a trend towards OS (HR = 0·5, $P = 0{\cdot}093$). Longer donor rTL (DTL-HQ) was found to be significantly associated with OS compared to shorter donor rTL (DTL-LQ; HR = 0·2, $P = 0{\cdot}006$). The survival probability for patients who received HSCT from donors with longer (DTL-HQ) *versus* shorter (DTL-LQ) telomeres were 94·9 ± 3·5% vs 65·4 ± 4·3% ($P = 0{\cdot}002$; Fig 3). Older patient (age > 40 years: HR = 3·3, $P = 0{\cdot}034$) and donor age (age > 40 years: HR = 5·2, $P = 0{\cdot}004$, 20–40 years: HR = 3·9, $P = 0{\cdot}004$) and shorter median donor rTL (DTLm; HR = 2·3, $P = 0{\cdot}019$) were significantly associated with acute GVHD (Table SII). There were no factors that were found to be associated with graft failure.

Since the highest quartile for donor telomere length was significantly associated with better survival, we divided all transplant recipients into four groups based upon combination of lower (LQ) and higher quartiles (HQ) of patient and donor telomere length. The highest quartile of donor telomere length (DTL-HQ), irrespective of its combination with either high (PTL-HQ) or low patient telomere length (PTL-LQ), showed survival ranging from 88% to 100% ($P = 0{\cdot}003$; Fig S2).

Factors influencing overall survival

We studied factors that would affect OS in patients undergoing matched sibling donor (MSD) transplant for AA (Table IV). On univariate analysis, patients aged 20–40 years (HR = 2·1, $P = 0{\cdot}045$), older patients (HR = 3·4, $P = 0{\cdot}005$), older donors (HR = 3·0, $P = 0{\cdot}011$), presence of graft failure

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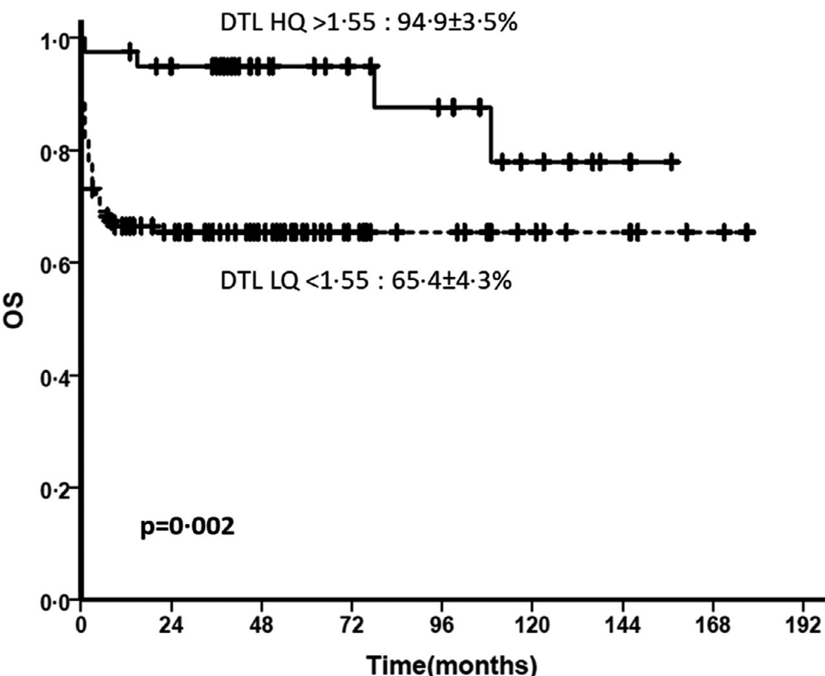

Fig 3. Overall survival (OS) following haematopoietic stem cell transplantation (HSCT) for aplastic anaemia observed based on lowest quartile (LQ) and highest quartile (HQ) of donor relative telomere length (DTL).

(HR = 4·5, P = 0·001), and presence of acute GVHD (HR = 3·4, P = 0·001) adversely affected OS while longer donor rTL (DTL-HQ; HR = 4·3, P = 0·006) was found to be significantly associated with better OS. Presence of chronic GVHD and patient telomere length (rTL) did not have a significant impact on OS. On multivariate analysis, patient age between 20 and 40 years (HR = 4·4, P = 0·050) and acute GVHD (HR = 3·0, P = 0·006) continued to be associated with poorer OS while longer donor rTL (DTL-HQ) continued to be associated with improved survival outcome (HR = 0·3, P = 0·049).

We analysed the causes of death in patients who had donors with the highest quartile (DTL-HQ) *versus* the lower three quartiles (DTL-LQ). There were 46 deaths including four in the DTL-HQ group (two died beyond five years) and 42 in the DTL-LQ group. Deaths in the DTL-HQ group was mainly due to chronic GVHD (n = 3) and primary graft failure (n = 1). Deaths in the DTL-LQ group were mainly due to multi-drug-resistant bacterial infections (n = 21), grade IV acute GVHD (n = 9), invasive fungal infection (n = 6), graft failure (n = 2) and other miscellaneous causes (n = 4).

Discussion

Telomeres are markers for biological ageing and telomere shortening with age is well characterized. Studies have suggested that telomere shortening is active during infancy and slows down thereafter.[14] Early investigations on telomere lengths of leukocytes in patients with AA have demonstrated that patients have shorter telomeres compared to healthy individuals.[15–17] In a study of 183 patients from the NIH, investigators showed that telomere length did not influence response to immunosuppressive therapy with ATG and ciclosporin but influenced haematological relapse, clonal evolution and overall survival.[8] There are minimal data on the association of telomere length and the outcomes of HSCT for AA and these have been restricted to matched unrelated donor transplants.[9,10] This study was therefore conducted to assess whether telomere length had an association with the outcome of matched sibling donor transplants for aplastic anaemia.

In the present study, donor telomere length rather than recipient telomere length was found to be significantly associated with transplant outcomes. In our study, the highest quartile of donor rTL (DTL-HQ) was associated with better OS similar to observations seen in patients undergoing unrelated transplants.[9,10] In one study, the impact of donor telomere length was seen more in patients less than 40 years of age receiving bone marrow grafts.[10] We did not find any impact of patient telomere length (median or age-adjusted) on neutrophil recovery, acute or chronic GVHD or OS (data not shown) in our study. A more recent analysis of 490 unrelated donor transplants for SAA by Wang *et al.* using rTL suggested that recipients having telomere lengths less than the 10th percentile for age had a higher mortality and were associated with worse survival following unrelated donor transplantation.[11] We compared the causes for mortality in the high quartile donor telomere group (DTL-HQ) with the other three groups combined (DTL-LQ). Interestingly in the DTL-HQ group, mortality was mainly due to chronic GVHD and graft failure while infective complications (gram-negative sepsis) and acute GVHD were the major

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Table III. Logistic regression analysis of patient and donor telomere length with demographic characteristics and HSCT outcomes.

	PTLm HR (95% CI)	*P* value	DTLm HR (95% CI)	*P* value	PTL-LQ HR (95% CI)	*P* value	PTL-HQ HR (95% CI)	*P* value	DTL-LQ HR (95% CI)	*P* value	DTL-HQ HR (95% CI)	*P* value
Patient age (years)												
<20												
20–40	1·7 (0·87–3·35)	0·119	NA									
>40	2·2 (0·83–5·88)	0·111			2·1 (0·92–4·89)	0·079	1·3 (0·63–2·78)	0·469	NA		NA	
					3·4 (1·16–9·99)	0·026	4·5 (0·96–21·29)	0·055				
Donor age (years)												
<20												
20–40	NA		3·1 (1·51–6·46)	0·002	NA		NA		4·0 (1·41–11·37)	0·009	2·2 (0·98–4·75)	0·056
>40			5·1 (1·93–13·46)	0·001					9·5 (2·95–30·74)	0·000	1·9 (0·69–5·65)	0·208
Patient gender	0·3 (0·29–1·01)	0·082	NA		1·4 (0·65–3·04)	0·377	1·0 (0·47–2·12)	0·996	NA		NA	
Donor gender	NA		0·9 (0·50–1·72)	0·810	NA		NA		1·3 (0·65–2·70)	0·444	0·8 (0·39–1·63)	0·547
Disease subtype												
NSAA												
SAA	0·7 (0·31–1·39)	0·268	NA		1·0 (0·45–2·38)	0·924	0·8 (0·33–2·07)	0·694	NA		NA	
VSAA	0·6 (0·22–1·49)	0·252			0·3 (0·07–1·08)	0·065	0·5 (0·18–1·59)	0·267				
Graft failure	0·7 (0·18–2·50)	0·558	1·4 (0·38–5·31)	0·587	1·3 (0·31–5·19)	0·736	1·2 (0·25–6·16)	0·788	0·7 (0·15–3·53)	0·681	0·8 (0·21–3·45)	0·816
Neutrophil recovery	1·5 (0·53–4·07)	0·460	0·9 (0·32–2·36)	0·777	1·3 (0·42–3·86)	0·670	0·6 (0·19–1·62)	0·281	1·3 (0·42–3·86)	0·670	5·8 (0·75–45·45)	0·092
Acute GVHD	1·2 (0·59–2·38)	0·639	2·4 (1·16–4·92)	0·019	1·3 (0·59–2·92)	0·494	1·7 (0·69–4·04)	0·256	2·1 (0·98–4·66)	0·057	2·1 (0·89–5·11)	0·089
Chronic GVHD	0·7 (0·33–1·50)	0·366	1·0 (0·49–2·19)	0·924	1·0 (0·42–2·37)	1·000	0·9 (0·37–2·11)	0·779	1·8 (0·76–4·22)	0·184	0·5 (0·24–1·17)	0·117
Survival outcome	0·8 (0·40–1·58)	0·518	0·5 (0·27–1·10)	0·093	0·9 (0·43–2·03)	0·863	1·5 (0·71–3·28)	0·275	0·6 (0·27–1·25)	0·171	0·2 (0·07–0·64)	0·006

CI, confidence interval; DTL, donor telomere length; GVHD, graft-*versus*-host disease; HQ, highest quarter; HR, hazard ratio; HSCT, haematopoietic stem cell transplantation; LQ, lowest quarter; m, median; NA, not available; NSAA, non-severe aplastic anaemia; SAA, severe aplastic anaemia; PTL, patient telomere length; VSAA, very severe aplastic anaemia.

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Table IV. Factors influencing overall survival in patients undergoing matched sibling donor HSCT for aplastic anaemia.

	Univariate analysis		Multivariate analysis	
Parameters	Survival probability (%)	*P* value	HR (95% CI)	*P* value
Patient age (years)		0·011		
<20	84%			
20–40	68%		4·4 (0·99–19·44)	0·050
>40	52%		4·3 (0·80–23·26)	0·088
Donor age (years)		0·028		
<20	84%			
20–40	69%		1·1 (0·23–4·90)	0·948
>40	55%		1·4 (0·29–7·49)	0·631
Graft failure		0·000	2·4 (0·49–12·07)	0·275
Yes	40%			
No	81%			
Acute GVHD		0·001	3·0 (1·37–6·57)	0·006
Yes	63%			
No	80%			
Chronic GVHD		0·354		
Yes	93%			
No	90%			
PTLm		0·440		
Short < 0·76	70%			
Long > 0·77	74%			
PTL-HQ		0·325		
Low < 1·17	74%			
High > 1·18	65%			
DTLm		0·090		
Short > 0·92	66%			
Long > 0·93	78%			
DTL-HQ		0·006	0·3 (0·09–0·99)	0·049
Low < 1·55	65%			
High > 1·56	95%			

CI, confidence interval; DTL, donor telomere length; GVHD, graft-*versus*-host disease; HQ, highest quarter; HR, hazard ratio; HSCT, haematopoietic stem cell transplantation; m, median; PTL, patient telomere length.

causes of mortality in the DTL-LQ group. These findings will need to be validated in a larger cohort of patients but if the data are consistently observed in a larger series, appropriate interventions could be used to reduce this mortality.

In a logistic regression analysis, shorter donor telomere length was an independent risk factor for survival along with older age, presence of acute GVHD and graft failure. The association between donor age and outcomes after HSCT has been previously reported.[5] OS is lower in patients above the age of 40 years though outcomes seem to be improving with the use of fludarabine-based regimens.[18] Similarly, the presence of acute or chronic GVHD with the use of PBSC grafts is associated with a higher mortality.[19] In our population, PBSCs are used as the graft source to reduce the risk of graft failure since >90% of patients with graft failure do not have the economic sources to proceed for a second transplant and therefore die due to infection.

We analysed the causes of death in this cohort and approximately 50–60% of patients expired due to either bacterial or fungal infection; when divided by cohorts based upon donor telomere length i.e., into DTL-HQ and DTL-LQ, bacterial sepsis and other infections (fungal and viral) were seen only in the DTL-LQ but not in the cohort with high telomere length (DTL-HQ). It is interesting to note that large studies in the general population have suggested an association between short telomere length and higher risk of infections.[20] In a recent study of 197 patients undergoing unrelated donor transplant looking at the association between donor telomere length and mortality, it was found that longer TL in B cells, natural killer (NK) cells and fully differentiated T cells was associated with lower risk of infection-related death and it was concluded that donor TL was associated with reduced rate of infection-related deaths after haematopoietic cell transplantation for SAA.[21]

How will these data influence clinical practice? We will need to study a much larger number of patients to confirm the validity of our observations. A larger analysis will also help us to validate the differences in the causes of mortality that we see between the DTL-HQ and DTL-LQ group. If these differences are validated in a larger cohort, we could

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potentially look at changes in conditioning regimen, antimicrobial prophylaxis, etc. to reduce the mortality associated with infections and acute GVHD since in a MSD transplant, we are generally limited by donor choice (usually a single donor in a majority) unlike unrelated donor transplants.

The strengths of our study include the analysis of a MSD cohort, post-transplant follow-up, availability of pre-transplant samples and comprehensive clinical and outcome information. The statistically significant association between donor telomere length and patient survival after transplant was limited to the longest quartile. Therefore, a larger sample size will help in reassessing this association in more categories of telomere length. In this study, we used a qPCR assay to measure rTL in DNA extracted from blood samples. Relative TL measured using qPCR is said to be inferior because of its relatively lower precision compared with the gold standard of Southern blots or flow cytometry with fluorescence *in-situ* hybridization (flow-FISH) TL assays.[22–24] In our study, comparison of qPCR assay with other techniques such as flow-FISH was not performed; however, correlation between the two methods has been reported (R^2 = 0·42,[22] 0·33[23]).

This is the first study that has looked at the impact of donor and recipient telomere lengths on transplant outcomes in patients undergoing MSD transplantation for AA. In this analysis, we report a significant impact of donor telomere length on OS and larger studies are needed to validate these findings.

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Author contributions

Contribution: AB performed the research, analysed the data and wrote the paper; KML performed the statistical analysis, analysed the data and edited the paper; FNA, AK, UPK, AJD, AA and VM recruited and treated the patients, provided critical advice and reviewed the paper. BG and ES designed the study, organized the data, supervised the research and edited the paper. All authors have read and approved the final manuscript.

Conflicts of interest

None of the authors have any relevant conflict of interest.

Supporting Information

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

Fig S1. Scatter plots of the correlation between relative telomere length and age in: (A) healthy controls; (B) aplastic anaemia (AA) patients; and (C) transplant donors.

Fig S2. Overall survival based on combination of telomere length quartiles of patient and donor.

Table SI. Comparison of baseline characteristics and outcomes between patients recruited in the study (n = 163) *versus* patients excluded (n = 23).

Table SII. Association of acute chronic graft-*versus*-host disease (GVHD) and graft failure with pre- and post-transplant parameters.

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ORIGINAL ARTICLE

Comparison of telomere length in patients with bone marrow failure syndromes and healthy controls

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Abstract

Introduction: During normal aging, telomeric DNA is gradually lost in dividing somatic cells, and critically short telomeres lead to replicative senescence, apoptosis, or chromosomal instability. We studied telomere length in bone marrow failure syndromes (BMFS) compared to normal healthy population.

Methods: Peripheral blood was collected from the participants, and genomic DNA was extracted. Relative telomere length was measured using a quantitative polymerase chain reaction. Statistical analysis was performed using SPSS and GraphPad Prism 8.2 software.

Results: The median age of normal Indian population was 31 (0–60) years. As expected, telomere length (TL) showed a decline with age and no difference in TL between males and females. The median age of 650 patients with aplastic anemia (AA) was 30 (1–60) years. TL was significantly shorter in patients with AA compared to healthy controls ($p < .001$). In FA and MDS patients, TL was significantly shorter than age-matched healthy controls ($p = .028$; $p < .001$), respectively. There was no difference between the median TL in age-matched AA and FA patients ($p = .727$). However, patients with MDS had shorter TL than age-matched AA ($p = .031$).

Conclusion: TL in BMF syndrome patients was significantly shorter than age-matched healthy controls.

KEYWORDS
aplastic anemia, bone marrow failure, quantitative real-time polymerase chain reaction, telomere length

1 | INTRODUCTION

Protection of genetic information and genomic stability is crucial for cells, and the discovery of telomeres in the 1970s was a breakthrough.[1–3] Telomeres are DNA-protein structures consisting of tandem TTAGGG repeats that protect the ends of linear chromosomes from degradation.[4] Human telomeres range from 5 to 15 kb and are remarkably heterogeneous among individuals and vary with age, organ, and proliferative history of each cell.[5] Telomeric erosion results in critically short telomeres, leading to cellular senescence, including apoptosis and genomic instability, or minimizes the cellular proliferative potential.[6] In stem cells and germline cells, telomere erosion is counteracted by a ribonucleoprotein enzyme, telomerase which is encoded by the TERT (telomerase reverse transcriptase) and TERC (telomerase RNA component), along with additional factors such as DKC1, NOP10, NHP2, and GAR1. Telomerase prevents replication-dependent loss of telomere and cellular senescence in highly proliferative cells.[7]

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Aplastic anemia is a bone marrow failure (BMF) disorder resulting in peripheral pancytopenia and marrow hypoplasia, and its incidence is believed to be 2–3 times higher in Asia.[8,9] The pathophysiology of acquired aplastic anemia is believed to be T-cell mediated destruction of bone marrow hematopoietic cells.[10] Studies have demonstrated that the telomere length of leukocytes in patients with AA varies, with an increased proportion of the patients having shorter telomeres.[11,12] In addition, lower telomere length has been shown to be associated with frequent relapses, clonal evolution to MDS, and poor survival in patients undergoing treatment with anti-thymocyte globulin (ATG).[13] Donor and patient telomere length also influences outcomes in patients undergoing unrelated stem cell transplantation for aplastic anemia.[14,15] Recent studies from India have shown that telomere length is low in aplastic anemia patients compared to healthy controls.[16,17] Inherited bone marrow failure disorders like Fanconi anemia are associated with genomic instability, bone marrow failure, and cancer predisposition.[18] Studies have reported shorter telomere length in FA compared to age-matched controls.[19,20] Myelodysplastic syndromes are acquired disorders characterized by pancytopenia, bone marrow hyperplasia, dysplasia, and progression to secondary acute myeloid leukemia (AML).[21] Studies have shown that MDS patients have shorter telomere lengths than normal controls, but the association of telomere length to other clinical parameters in MDS has been inconsistent.[22–24]

In this study, we established relative telomere length in a healthy Indian population and compared telomere length in patients with Aplastic anemia, Fanconi anemia, and Myelodysplastic syndromes.

2 | METHODS

2.1 | Study participants

This study included patients diagnosed with AA, FA, and MDS at Christian Medical College, Vellore, India, between 2001 and 2021. The Institutional Review Board and the Ethics Committee of the institution approved the study. A total of 650 patients with AA and 100 patients each with FA and MDS, whose DNA samples were available, were included. Clinical data of all these patients were collected from individual medical records and institutional databases. Patients with AA were classified into very severe (VSAA), severe (SAA), and non-severe aplastic anemia (NSAA) using the standard Camitta criteria.[25] As healthy controls (HC), 800 transplant donors were selected, except donors with Thalassemia and aplastic anemia. Due to a shortage of HC in the age 51–60 years, samples were collected from transplant patients' relatives with consent.

The inclusion criteria are listed below for each group. Only patients willing to sign the informed consent and fulfilling the inclusion criteria were included in the study. The diagnosis of aplastic anemia was made based on standard criteria, that is, there must be at least two of the following: (i) hemoglobin < 100 g/L (ii) platelet count < 50×10^9/L (iii) neutrophil count <1.5×10^9/L. Known inherited bone marrow failure patients were excluded from this group. The diagnosis of Fanconi anemia was confirmed by clinical presentation and a positive chromosomal breakage analysis using mitomycin C. In patients where the results of chromosomal breakage analysis were ambiguous, the diagnosis was confirmed using skin biopsy fibroblast culture analysis of the ubiquitination of the FANC-D2 gene. The diagnosis criteria of MDS include (i) one or more cytopenias that cannot be explained by a drug, toxin, vitamin deficiency, infection, or other condition: hemoglobin < 100 g/L, absolute neutrophil count <1.8×10^9/L, and platelets < 100×10^9/L, (ii) dysplasia in ≥ 10% of cells in a given hematopoietic lineage (erythroid precursors, granulocytes, megakaryocytes) in bone marrow or peripheral blood, (iii) presence of 5%–19% blast cells, and (iv) presence of a specific MDS linked chromosomal abnormalities like del (5q), del (20q), +8 or −7/del (7q).[26] MDS patients have been classified according to the International Prognostic Scoring System risk score into lower-risk MDS and higher-risk MDS, based on the percentage of marrow blasts, number and extent of blood cytopenias, and marrow cell karyotype.

2.2 | DNA extraction

Peripheral blood samples were collected in an anticoagulated EDTA tube from participants. Genomic DNA was extracted from whole blood using Puregene gentra Kit (Qiagen, Maryland, USA), and samples were stored at 4°C till analysis. DNA samples were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). The 260/280 absorbance ratio was determined to evaluate the purity, and samples within the range of 1.8–2.0 were qualified for analysis.

2.3 | Relative telomere length measurement using quantitative real-time polymerase chain reaction

The relative telomere length (rTL) was measured using quantitative real-time Polymerase Chain Reaction (qPCR) from the extracted DNA, as previously described.[15,27] By serial dilution, reference DNA at concentrations of 40, 20, 10, 5, and 2.5 ng/μL was prepared from standard genomic DNA. Two master mixes of PCR reagents were prepared, one with telomere (TEL, T) primer pair and another with single copy gene (RLP0, also termed 36B4; S) primer pair for standard curve. (The 36B4 gene, which encodes acidic ribosomal phosphoprotein PO, is located on chromosome 12.) The telomere and single copy gene-specific primer sequences are given in Table S1. A 10 μL volume of the master mix was prepared, which consists of 5 μL of Takyon SYBR Green PCR Master Mix (Eurogentec, Belgium), 2.25 μL of sterile water, 0.5 μL of lambda DNA (New England Biolabs, USA), 0.25 μL of an assay-specific mix of primers, and 2 μL of genomic DNA normalized to 10 ng/μL. In each run, one non-template control, one positive control, and a standard genomic DNA (calibrator) were amplified in duplicates. The PCR thermal conditions for relative telomere length assay using telomeric primers and single copy gene primers consisted of an initial denaturation of 10 min at 95°C, followed by a total of

40 cycles at 95°C for 15 s, 58°C for the 60s and subsequently melt curve stage. All telomere and 36B4 reactions were performed in duplicates, carried out in 96-well plates, and the experiment was performed in a 7500 Fast Real-time PCR System (Applied Biosystems, USA). The laboratory personnel conducting the telomere length assay were blinded to patient characteristics and clinical data before the statistical analysis.

2.4 | Calculation of relative telomere length using qPCR

Relative telomere length was expressed as a ratio of the quantity of telomeric DNA (T) normalized to the copy number of a single-copy nuclear gene (S), yielding the T/S ratio. The ratio between telomeric DNA (T) and single copy gene (S) was enumerated for each DNA sample. The relative T/S ratio was calculated in relation to a reference genomic DNA sample used in all plates. The relative T/S ratio was achieved using the formula $2^{-(\Delta Ct\text{-}\Delta Cs)} = 2^{-\Delta\Delta Ct}$. The value of the T/S ratio is directly proportional to the average telomere length.

2.5 | Calculation of relative age-adjusted telomere length

The rTL values were log-transformed to ensure normality. Age-adjusted telomere length for each patient was computed as follows: ([observed log rTL-linear predicted rTL]/root mean square error [RMSE]).[15,28] The linear predicted rTL and RMSE were derived from a linear regression model of log rTL and age from 800 healthy controls. The age-adjusted relative telomere length was then normalized using STATA version 16.1 software.

2.6 | Statistical analysis

Data were represented as the mean of values ± SD or median values with the range indicated in the tables. Student's *t*-test or Mann–Whitney *U* test was used to statistically compare the continuous variables and the chi-square test for categorical data. Kruskal Wallis test was used to statistically compare the continuous variables among the groups. Pearson's correlation coefficient was used to evaluate the association between relative telomere length and age. All *p*-values were 2-sided, with values of .05 or less indicating statistical significance. Statistical analysis was done using the IBM SPSS 24.0 Software (IBM Corp. Armonk, NY, USA) and GraphPad Prism 8.2 (GraphPad Software, Inc., San Diego, CA).

3 | RESULTS

3.1 | Characteristics of study participants

Participants in the study included 800 healthy controls, 650 with AA, 100 with FA, and 100 with MDS. The median age of healthy controls was 31 (0–60) years and included 471 males (59%) and 329 females (41%). The median age in BMF syndromes was 30 (1–60) years in AA, 11 (2–32) years in FA, and 46 (6–82) years in MDS. There was an excess of males in AA (62%) and FA (65%), respectively, whereas 58% of males were in the MDS group. Based on severity criteria, 157 (24%) patients had NSAA, 384 (59%) SAA, and 109 (17%) had VSAA among the AA patients, while 63 (63%) patients had NSAA, 32 (32%) SAA, and 5 (5%) had VSAA in patients with FA. About 30 and 70 patients had high-risk and low-risk MDS,[26] respectively.

3.2 | Telomere length measurement in healthy controls

The median rTL was 0.78 (0.11–3.65) with a similar median rTL for males (0.79 [0.11–3.65]) and females (0.78 [0.19–3.57]). A significant correlation was observed between age distribution and rTL ($r = -0.319$, $p < .001$, Figure S2A), with lower TL observed with increasing age. The number of healthy controls in each decade of age (years) was (0–10) $N = 100$, (11–20) $N = 140$, (21–30) $N = 150$, (31–40) $N = 200$, (41–50) $N = 140$, (51–60) $N = 70$, respectively. The median rTL for each decade of age among healthy controls is mentioned in Table 1.

3.3 | Telomere length analysis in patients with Aplastic anemia

The overall median telomere length of AA was 0.73 (0.10–3.00) with no difference in median rTL between males and females (0.71 [0.10–3.00] & 0.76 [0.12–2.98], $p = .930$). The overall median relative telomere length in patients with aplastic anemia was significantly shorter than in healthy controls ($p < .001$) (Figure 2B). The median rTL of AA patients based on severity were not significantly different (NSAA: 0.70 [0.10–3.00], SAA: 0.73 [0.11–2.98], and VSAA: 0.79 [0.14–2.53], $p = .023$) (Figure 1A). Further, we categorized the severity of AA based on age decade-wise (Figure 1B). While comparing (SAA + VSAA) with NSAA, the rTL was not significantly different between the groups ($p = .069$, Figure 1C).

Age and rTL were significantly correlated in AA patients ($r = -0.168$, $p < .001$, Figure S2B). When classified into decades (age), patients aged 0–10 and 21–30 years showed significantly shorter rTL in AA than healthy controls (Table 1). The comparison of rTL in AA patients and healthy controls with age (decade-wise) is shown (Figure 1D).

The median age-adjusted telomere length of patients for the entire cohort was 0.36 (0.20–0.54), and median age-adjusted TL between males and females were (0.37 [0.20–0.54] & 0.34 [0.20–0.54]), respectively. The median age-adjusted telomere length based on severity were NSAA: 0.33 (0.20–0.52), SAA: 0.37 (0.20–0.54), and VSAA: 0.39 (0.20–0.54). About 344 (53%) AA patients in our cohort had shorter relative telomere lengths compared to the median rTL of age-matched

TABLE 1 Telomere length parameters in patients with Aplastic anemia and healthy controls.

			AA		HC		*p*-Value
N			650		800		
Age (years)	Median (Range)		30 (1–60)		31 (0–60)		.729
Gender	M (*N* [%])		404 (62)		471 (59)		.024
	F		246 (38)		329 (41)		.001
Relative telomere length	Median (range)		0.73 (0.10–3.00)		0.78 (0.11–3.65)		<.001
Age-adjusted telomere length	Median (range)		0.36 (0.20–0.54)				
Relative telomere length (gender)	Median (range)	M	0.71 (0.10–3.00)		0.79 (0.11–3.65)		<.001
		F	0.76 (0.12–2.98)		0.78 (0.19–3.57)		.083
Age-adjusted telomere length (gender)	Median (range)	M	0.37 (0.20–0.54)				
		F	0.35 (0.20–0.54)				
Relative telomere length (age-wise in decade; years)	0–10	N	0.83 (0.14–2.98)	110	0.97 (0.26–3.65)	100	.003
	11–20		0.84 (0.19–2.46)	110	0.83 (0.21–2.85)	140	.703
	21–30		0.70 (0.12–3.00)	110	0.87 (0.25–1.99)	150	<.001
Median (range)	31–40		0.76 (0.11–1.92)	110	0.77 (0.26–2.21)	200	.447
	41–50		0.66 (0.10–1.80)	110	0.66 (0.20–2.44)	140	.054
	51–60		0.61 (0.16–1.93)	100	0.58 (0.11–2.16)	70	.849

healthy controls. The telomere length was classified as percentiles, and about one-fourth of patients with AA ($N = 156$, 24%) were observed to have rTL <10th percentile (median age = 37 years), and of them, 42 patients (27%) had rTL <1st percentile.

3.4 | Analysis of telomere length in Fanconi anemia patients

The median telomere length in patients with FA was 0.78 (0.10–3.72), and no significant correlation was observed between age distribution and rTL ($r = -0.190$, $p = .059$, Figure S2C). The median rTL between males and females was not significantly different (0.75 [0.10–3.72] & 0.80 [0.26–2.08], $p = .779$). The median age-adjusted telomere length of patients was 0.48 (0.33–0.55). The median age-adjusted rTL of males and females was 0.49 (0.34–0.55) and 0.48 (0.33–0.54), respectively. The median rTL of FA patients based on severity were not significantly different (NSAA: 0.89 [0.10–3.72], SAA: 0.73 [0.26–2.08], and VSAA: 0.84 [0.18–0.91], $p = .148$). The overall median rTL of patients with FA was significantly shorter compared to age-matched healthy controls (0.88 [0.21–3.65], $p = .028$) (Figure 2B).

There was no significant difference between the median rTL of patients with AA and FA age-matched ($p = .727$) (Figure 2A), while the median age-adjusted rTL was observed to be significantly different ($p = .001$). Patients with AA and FA were classified into decades (age-wise); there was no association between the median rTL of FA and AA in the (0–10 years) age group ($p = .121$) (Table 2). While comparing rTL based on severity between AA and FA groups, a significant difference was observed in NSAA ($p < .001$), whereas there was no significance in (SAA + VSAA) ($p = .810$) (Table 2). About 10 (10%) FA patients had rTL <10th percentile, and two patients (2%) with rTL <1st percentile.

3.5 | Analysis of telomere length in Myelodysplastic syndrome patients

The overall median telomere length of patients with MDS was 0.58 (0.19–2.42), and no significant correlation was observed with age distribution and rTL ($r = -0.102$, $p = .311$, Figure S2D). The median rTL of males and females was observed to be [0.57 (0.19–2.42) & 0.59 (0.30–1.60), $p = .304$], respectively. The median rTL of high-risk and low-risk MDS patients were (0.57 [0.30–1.89] and 0.59 [0.19–2.42], $p = .228$), respectively. The median age-adjusted telomere length of patients was 0.24 (0.09–0.56), and the median age-adjusted rTL between males and females were 0.23 (0.09–0.56) & 0.25 (0.15–0.48), respectively. The median rTL of MDS patients compared with age-matched healthy controls was significantly shorter ($p < .001$) (Figure 2B).

We observed a significant difference in the median rTL and age-adjusted rTL between patients with AA and MDS when matched for age ($p = .031$; $p < .001$), respectively (Figure 2A). The patients were classified age-wise (<30 & >30 years), and there was no significant difference in median rTL between AA and MDS (Table 3). About 23 (23%) MDS patients had rTL <10th percentile, and one patient (1%) with rTL <1st percentile. A consolidated comparison of baseline characteristics and rTL of AA, FA, MDS, and HC is mentioned in Table S2.

We further evaluated the rTL in quartiles within each group. When comparing the rTL in quartiles of AA patients with the other groups, there was a significant difference in all quartiles except for

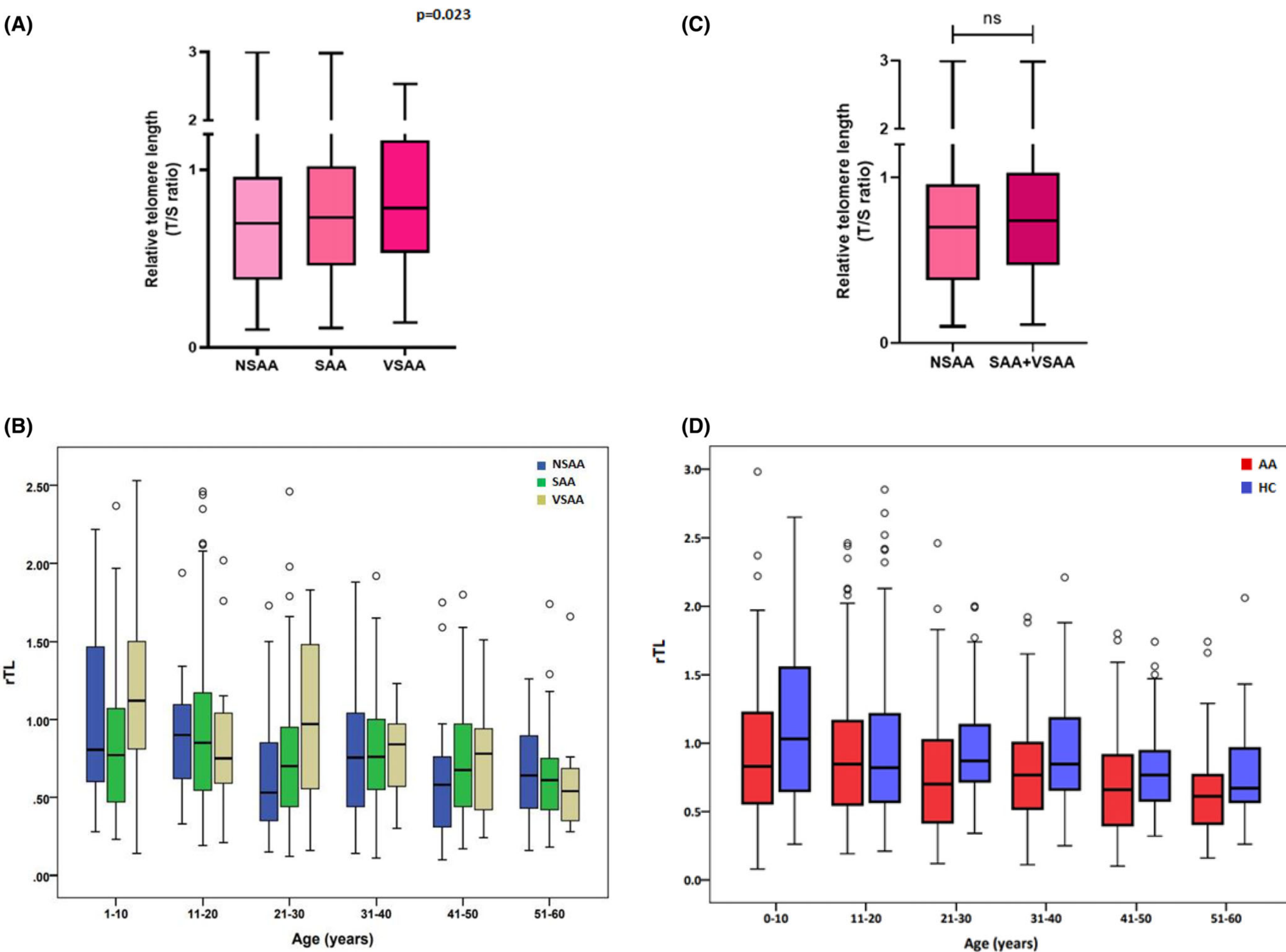


FIGURE 1 Relative telomere length in AA patients categorized based on disease severity. (A) All three groups, (B) classified based on age decade-wise, and (C) combined (SAA + VSAA) with NSAA (D) Comparison of age-wise (decades) distribution of relative telomere length in patients with aplastic anemia with healthy controls.

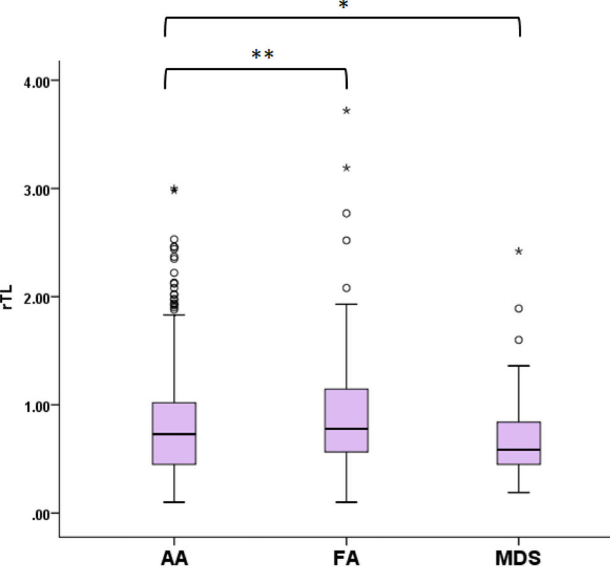
the 4th quartile in comparisons between AA with HC and MDS groups (Table S3).

4 | DISCUSSION

After each cell division, DNA replication cannot be fully completed, which shortens telomeres over time. Telomere length regulation vital in maintaining genome stability in humans, and telomere shortening plays a crucial role in biological aging. Oxidative stress and inflammation induce increased erosion at telomeric ends resulting in attrition of telomere length.[29] There is high telomere length variability even in same-aged individuals and comparable variability at birth.[30] A study with 75 309 individuals from the general population reported that higher age was strongly associated with shorter leukocyte rTL ($p < 1*10^{300}$; $r^2 = 0.073$).[31] Another interesting study from Australia included 1874 families, of which telomere length data were available for 2549 individuals (1206 children and 1343 adults). The mean rTL of children was observed to be longer than adults (1.09 vs. 0.81, $p < .001$).[32]

An Indian data of 98 normal adult individuals with ages ranging from 35 to 70 years were analyzed for telomere length and observed a negative correlation with age and rTL. There was no significant difference between gender and telomere length.[33] A recent study with 105 healthy subjects of both sexes between the ages of 20–77 years showed a significant ($p < .001$) age-dependent decline in rTL.[34] There was no significant difference between telomere length in males and females in alignment with the reported data. Ours is the first Indian study to establish age-wise relative telomere length in a large normal population.

Early investigations on telomere lengths of leukocytes in patients with AA have demonstrated patients having shorter telomeres compared to healthy individuals.[11,12,35] The analysis of telomere length in aplastic anemia is comparable with Indian findings.[16,17] Our last reported data affirms that rTL is shorter in AA patients compared to their transplant donors.[28] In this analysis, we noticed a similar pattern while comparing median relative telomere length in patients with aplastic anemia and healthy controls. We also observed that patients with SAA + VSAA had shorter rTL compared to the non-severe group,



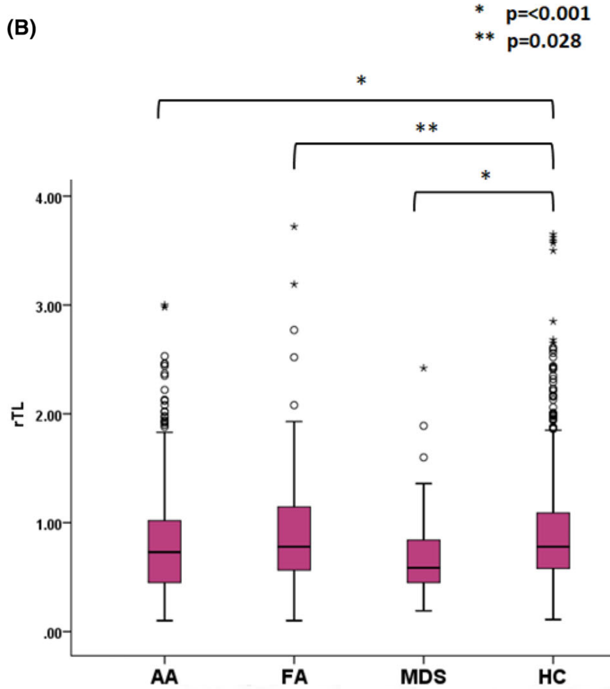


FIGURE 2 Comparison of relative telomere length (A) among bone marrow failure and (B) bone marrow failure with normal controls.

although not significant ($p = .069$). A Chinese study of 71 cases with AA showed similar results; no significant difference was observed between NSAA and SAA + VSAA groups ($p = .573$).[36]

A study comparing telomere length in other inherited BMFS with dyskeratosis congenita (DKC) showed that patients with FA, Diamond-Blackfan anemia (DBA), and Shwachman-Diamond syndrome (SDS) on average, had telomeres that were in the lower half of the normal range.[37] Further, the diagnostic value of TL in 133 patients with BMF was evaluated and compared in patients with DKC, non-DKC IBMFS, and AA. This study observed no significant difference in telomere length between non-DC IBMFS (FA: 9/15) compared to AA,[38] aligning with our data. This may be due to the lower age group (1–32) years in patients with FA where generally TL is longer. Several studies have reported that patients with MDS have short telomeres compared to healthy controls.[24,39,40] Our data showed MDS to have significantly shorter telomere length than healthy controls and patients with aplastic anemia. The shorter TL in patients with MDS may be related to the older age of the cohort (median age of 46 years), wherein TL deteriorates as the age progresses.

Shorter telomeres are linked to a higher risk of age-related diseases such as cardiovascular disease, diabetes, and certain cancers. Additionally, individuals with shorter telomeres may experience a reduced lifespan.[41] Shorter telomere lengths in BMFS have been reported compared to age-matched healthy controls. This study comprehensively analyses telomere length in a sizable population of healthy or BMFS patients. More than half of the population of patients with BMF in our study had shorter telomere lengths compared to the healthy controls. Clinically, telomere length data from healthy controls can be used to identify patients with shorter rTL in prospective cases. Furthermore, our data emphasizes the association of rTL with the categorization of median rTL based on age decades, the difference between males and females, the severity of the disease, and rTL among the BMFS groups.

Also, a shorter telomere length can be considered a prognostic factor for patients undergoing hematopoietic stem cell transplantation (HSCT). NGS sequencing was conducted in 75/650 patients with aplastic anemia using a targeted gene panel for inherited bone marrow failure (IBMF). The analysis identified nine pathogenic (PAT) variants and eight variant of uncertain significance (VUS) in IBMF genes. Among these, three PAT variants (2 RTEL1 and 1 TINF2) and one VUS (TERT) were in telomere-associated genes (Table S4). Notably, AA patients with these variants exhibited shorter telomere lengths than those with pathogenic variants in other genes associated with IBMF. Mutations in telomere-associated genes or malignant progression have been demonstrated to be associated with accelerated telomere length shortening in BMFS. However, an investigation into this association has not been conducted in other groups. The occurrence of very short telomere length in a patient having a VUS variant in the telomere-associated gene might suggest a potential pathogenicity.

Research indicates that patients with AA who have shorter telomeres are less likely to respond to immunosuppressive treatment and, if they do respond, have a higher likelihood of relapse.[42] A decrease in telomere length is evident in a specific subgroup of newly diagnosed MDS patients with more aggressive disease and complex karyotypic abnormalities. Additionally, patients with short telomere length may develop AML post-HSCT.[24] The limitation of this study includes that these aspects were not investigated in patients who fell within the category of less than the 1st percentile. Studies have categorized rTL into median, quartile, or percentiles to determine the number of patients having short telomere lengths. The value of rTL varies from study to study based on the method used to measure rTL. Patients

TABLE 2 Comparison of baseline characteristics and telomere length in patients with Aplastic anemia and Fanconi anemia.

			AA		FA		p-Value
N			330[a]		100		
Age (years)	Median (range)		15 (1–30)		11 (2–32)		<.001
Gender	M (N [%])		209 (63)		65 (65)		.998
	F		121 (37)		35 (35)		.997
Relative telomere length	Median (range)		0.79 (0.12–3.00)		0.78 (0.10–3.72)		.727
Age-adjusted telomere length	Median (range)		0.46 (0.36–0.54)		0.48 (0.33–0.55)		.001
Relative telomere length (gender)	Median (range)	M	0.77 (0.10–3.00)		0.75 (0.10–3.72)		.845
		F	0.81 (0.12–2.98)		0.80 (0.26–2.08)		.760
Age-adjusted telomere length (gender)	Median (range)	M	0.45 (0.20–0.54)		0.49 (0.34–0.55)		.001
		F	0.46 (0.20–0.54)		0.48 (0.33–0.54)		.194
Relative telomere length	Severity	NSAA	0.70 (0.10–3.00)		0.89 (0.10–3.72)		<.001
		SAA	0.73 (0.11–2.98)		0.73 (0.26–2.08)		.535
Median (range)		VSAA	0.79 (0.14–2.53)		0.84 (0.18–0.91)		.477
Relative telomere length (age-wise in decades; years)	0–10	N	0.83 (0.14–2.98)	110	0.97 (0.18–3.72)	49	.121
	11–20		0.84 (0.19–2.46)	110	0.71 (0.10–3.19)	40	.133
Median (range)	21–30		0.70 (0.12–3.00)	110	0.58 (0.38–0.79)	8	

[a]Only age-matched AA patients were compared to FA patients.

TABLE 3 Comparison of baseline attributes and telomere length in patients with Aplastic anemia and Myelodysplastic syndrome.

			AA		MDS		p-Value
N			650		100		
Age (years)	Median (Range)		30 (1–60)		46 (6–82)		<.001
Gender	M (N [%])		404 (62)		58 (58)		<.001
	F		246 (38)		42 (42)		<.001
Relative telomere length	Median (range)		0.73 (0.10–3.00)		0.58 (0.19–2.42)		.031
Age-adjusted telomere length	Median (range)		0.36 (0.20–0.54)		0.24 (0.09–0.56)		<.001
Relative telomere length (Gender)	Median (range)	M	0.71 (0.10–3.00)		0.57 (0.19–2.42)		.274
		F	0.76 (0.12–2.98)		0.59 (0.30–1.60)		.028
Age-adjusted telomere length (Gender)	Median (range)	M	0.37 (0.20–0.54)		0.23 (0.09–0.56)		<.001
		F	0.35 (0.20–0.54)		0.25 (0.15–0.48)		<.001
Relative telomere length (years)	0–30	N	0.79 (0.12–3.00)	330	0.64 (0.29–1.89)	23	.349
Median (range)	31–60		0.67 (0.10–1.93)	320	0.57 (0.19–2.42)	60	.208

below median rTL, in the 1st or 10th percentile, or in the lower quartile are considered to have very short telomere length. Furthermore, DNA has been extracted from leukocytes sourced from the peripheral blood of patients. Since bone marrow failure patients have low WBC counts, it is challenging to get sufficient cells for measuring telomere length in subsets. Hence, we utilized the qPCR method for measuring telomere length. The study analysis did not include any patients with low outliers in rTL. Our study had data on NGS for 11.5% of AA patients, but NGS was unavailable for the other patient groups.

The study's strength includes a large sample size and comprehensive clinical information. Using qPCR assay, we measured relative telomere length in DNA extracted from blood samples. The usage of qPCR assay may be limited due to its relatively lower precision compared with the gold standard of Southern blots or flow cytometry with fluorescence in-situ hybridization (flow-FISH) TL assays. We did not compare qPCR assay with other techniques, such as flow-FISH; however, the correlation between the two methods has been reported ($R^2 = 0.42$,[43] 0.33[44]). To the best of our knowledge, this is the first study to directly compare telomere length in patients with AA, FA, or MDS.

AUTHOR CONTRIBUTIONS

Aruna Barade performed the research, analyzed the data, and wrote the paper. Kavitha M Lakshmi performed the statistical analysis and analyzed the data. Fouzia N Abubacker, Anu Korula, Uday P Kulkarni, Aby Abraham, and Vikram Mathews recruited and treated the

patients, provided critical advice, and reviewed the paper. Biju George and Eunice Sindhuvi designed the study, organized the data, supervised the research, and edited the paper. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any relevant conflict of interest.

DATA AVAILABILITY STATEMENT

Data may be made available upon contact with the corresponding author.

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SUPPORTING INFORMATION

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

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1.0. Introduction

The marrow produces billions of blood cells daily; however, the function of bone marrow was not understood in ancient times and was experimentally defined in the late 19th century. Hippocrates, a renowned Greek physician (460 to 375 BC), formulated his conclusions through observation and reasoning rather than mysticism. He believed that bone tissue derives its nutrients from the marrow, a prominent view shared by Galen (130 to 200 AD). Aristotle (384 to 322 BC) held a different perspective on the matter; he believed that the marrow was a waste product of bones, referred to as "excrementum ossium". During the 18th century, anatomists were fascinated by the vascularity of the marrow and supported Hippocrates' belief that it was the vascular element of bone. Charles Robin, in 1872 observed that the marrow is formed after the bone during development, which further implies that it is unlikely to be a source of bone nutrition (Robin, 1875).

The existence of red blood cells was first documented in the 17th century, but it wasn't until the 19th century that scientists began investigating their origins. Cell theory in 1838 by Schleiden and Schwann identified the cell as the basic building block of life, enabling the idea of red blood cell production. Neumann and Bizzozero first observed bone marrow as the site of blood formation. In the late 1800s, there were significant advancements in morphologic methods, mainly due to Paul Ehrlich's innovations in aniline dyes and heat-fixed films for blood smears and bone marrow analysis. Through this discovery, an understanding of blood production was obtained and eventually confirmed the haematopoietic stem cell, as hypothesised by Neumann. The bone marrow microenvironment significantly influences the expansion of haematopoietic stem cells (HSCs) (Cooper, 2011).