Characterisation of cord blood and its potentiality regarding leukaemia: The haematopathological condition

A Thesis

Submitted by

Sayak Manna

for the award of the degree

DOCTORATE OF PHILOSOPHY (SCIENCE)

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Dedicated

To all the Universal Souls who will read this thesis

Certificate from the Supervisor

This is to certify that the thesis entitled "**Characterisation of cord blood and its potentiality regarding leukaemia: The haematopathological condition**" submitted by **Sayak Manna**, who registered on **24.09.2019** for the award of **Ph.D. (Life Science and Biotechnology)** degree of **Jadavpur University**, is absolutely based up their work under my supervision and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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Declaration

I hereby declare that the research work presented in this thesis, entitled ***"Characterisation of Cord Blood and Its Potentiality Regarding Leukaemia: The Haematopathological Condition"*** was carried out at the Calcutta School of Tropical Medicine, Kolkata, under the supervision of Dr. Sujata Law, Assistant Professor, Calcutta School of Tropical Medicine. The work described in this thesis is original and is being submitted to Jadavpur University, Kolkata, in partial fulfilment of the requirements for the degree of Doctor of Philosophy. To the best of my knowledge, the work presented here has not been submitted, either in part or in full, for any other degree or diploma at this or any other institution.

Signature of the scholar with date

'Oh! My Supremely Beloved, it is Thy loving Grace that has drawn me to Thy Lotus Feet and put into my heart the Divine Seed of the Light of Thy Realization that this 'I of mine' is verily 'Thou and Thine' and 'Thine and Thine' alone. Let this seed sprout, grow and bear the fruit- the fruit of Divine Ananda, the unfailing 'Awareness of Thou' in everything and everywhere and everlasting as the 'Thou of I' and 'I of Thou', the Pure Consciousness, Existence, Bliss, Love and Peace Absolute.'

-Prajnanpurush Sri Sri Babathakur

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Abstract

Umbilical cord blood (UCB), often considered a biological waste, is rich in hematopoietic stem and progenitor cells, having unique morphological and functional attributes when compared to the adult peripheral blood (PB). Recently, the therapeutic potential of UCB has ventured and its components are increasingly used in experimental biology and clinical research focussing mainly on translational and regenerative medicine. Cord blood (CB) cellular components have been explored for treating myocardial infarction, neurodegenerative diseases, diabetes, liver cirrhosis, and autoimmune conditions whereas the plasma component is widely used in treating eye anomalies, aging-related issues, neurodegenerative diseases and in cell cultures. The study aimed to establish haematological reference values for UCB of South Asian populations like India specific to the West Bengal cohort, and to examine variations in haematological parameters between urban and rural populations considering different modes of delivery, thus exploring UCB's distinctive cellular characteristics through comparison with adult PB. We also sought to investigate the potential therapeutic property of the plasma component of cord blood when administered as a treatment in leukemic scenarios.

With the advent of industrialisation, carcinogens such as N-nitroso compounds (NOCs) are radially being released into the environment through processed food, contaminated water bodies, tobacco and pesticides and chemical manufacturing units. We used N-ethyl-n-nitrourea (ENU), a potent carcinogenic NOC to induce leukaemia in Swiss Albino mice followed by the induction of cord blood plasma factor (CBPF) as a therapeutic measure to ameliorate the induced leukemic condition in the in-vivo model. The study also aimed to evaluate the efficacy of biologically procured, natural concoction like CBPF as a treatment for leukaemia, while minimising side effects on leukemic profile, which are evident in chemotherapeutic drugs, without claiming it as a 'safer alternative therapy.' Its focus was solely on assessing its potential benefits and not advocating it as a replacement for current treatments.

Through this study, haematological parameters of the regional population were compared to global standards. The morphological and protein variations were assessed between UCB and PB erythrocytes and leukocytes using scanning electron microscopy (SEM), SDS-PAGE for protein profiling, and flow cytometry for membrane protein expressions and cellular characteristics. Following the characterisation, ENU-induced leukemic mice received CBPF intravenously for 30 days. Pre- and post-treatment effects were analysed across hematopoietic profiles, immune cell cytotoxicity, tissue architecture, bone marrow cellularity, and

transcription factor profiling through biochemical staining, cell culture, histology, SEM and flow cytometry.

The cellular components from the cord blood of the West Bengal population showed unique haematological profiles, with elevated WBC and lowered monocyte counts compared to the global scenario. Rural groups expressed higher neutrophil counts, whereas urban groups showed higher lymphocyte and platelet counts. Minor differences in haematological profile were also noted between the vaginal and caesarean delivery modes amidst the urban and rural population. Cellular analysis revealed unique morphological traits in UCB erythrocytes with variations in indentation and altered membrane textures compared to PB cells. Unique metabolic characteristics including low osmotic fragility and reduced lipid peroxidation along with overexpression of GLUT1 and reduced Band 3 proteins were observed in UCB. In the leukaemia study, CBPF administration as a treatment significantly reduced leukemic blasts and abnormal neutrophils in peripheral blood and bone marrow, decreased notable blast infiltration in extra medullary tissues like spleen and liver and enhanced cytotoxic efficacy through increased mature immune cells. Histological analysis verified improved bone marrow architecture and reduced blast cells with significant changes in transcriptional profile further supporting CBPF's role in modulating immune and hematopoietic pathways and reinforcing the anti-leukemic activity of cord blood.

In conclusion, this study established the first UCB haematological reference range for the West Bengal population, revealing notable urban-rural differences along with the reporting of the distinct cellular and protein expression differences in the erythrocyte population of UCB and adult PB. This highlights UCB’s unique hematopoietic profile and its potential use in neonatal treatment like transfusion. This work is possibly the first study that reports the use of CBPF in an ENU-induced leukemic mice model. Through this research, we established that standalone CBPF administration may yield promising anti-leukemic effects, including reduced blast cell burden, increased hematopoietic differentiation, and improved tissue architecture with minimal toxic effect, indicating its potential use in haematopathological and regenerative medicine. This research accentuates UCB’s clinical relevance and encourages further exploration of CBPF's role, potentially in combination with standard therapies, to improve therapeutic outcomes in haematological disorders.

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Abbreviations

ALL	:	Acute Lymphoblastic Leukaemia
AML	:	Acute Myeloid Leukaemia
APB	:	Adult Peripheral Blood
BSA	:	Bovine Serum Albumin
CB	:	Cord Blood
CBC	:	Complete Blood Count
CBP	:	Cord Blood Plasma
CBPF	:	Cord Blood Plasma Factors
CLL	:	Chronic Lymphoblastic Leukaemia
CML	:	Chronic Myeloid Leukaemia
CNS	:	Central Nervous System
CREC	:	Clinical Research Ethics Committee
DC	:	Differential Count
ELISA	:	Enzyme-Linked Immunosorbent Assay
ENU	:	N-Ethyl-N-Nitroso Urea/ N-N-Ethyl Nitroso Urea
FBS	:	Foetal Bovine Serum
Fr	:	Fresh
FSC	:	Forward Scatter
Fx	:	Fixed
GVHD	:	Graft-Versus-Host Disease
GVL	:	Graft-Versus-Leukaemia
Hb	:	Haemoglobin concentration
HLA	:	Human Leukocyte Antigen

HSPC	:	Haematopoietic Stem Progenitor Cell
hUCB	:	Human Umbilical Cord Blood
i.p.	:	Intraperitoneal
i.v.	:	Intravenous
IFNγ	:	Interferon γ
MCH	:	Mean Corpuscular Haemoglobin
MCHC	:	Mean Corpuscular Haemoglobin concentration
MCV	:	Mean Corpuscular Volume
MNC	:	Mononuclear Cell
OFT	:	Osmotic Fragility Test
PAS	:	Periodic Acid-Schiff
PB	:	Peripheral Blood
PBS	:	Phosphate Buffer Solution
PCV	:	Packed Cell Volume
PFA	:	Para Formaldehyde
PPP	:	Platelet Poor Plasma
PRP	:	Platelet Rich Plasma
RBC	:	Red Blood Cells
RC	:	Rural Caesarean
RN	:	Rural Normal
RT	:	Room Temperature
SEM	:	Scanning Electron Microscopy
SSB	:	Sudan Black B
SSC	:	Side Scatter

TC	:	Total Count
TF	:	Transcription Factor
TGFβ	:	Transforming Growth Factor β
TNFα	:	Tumour Necrosis Factor α
UC	:	Urban-Caesarean
UCB	:	Umbilical Cord Blood
UCBT	:	Umbilical Cord Blood Transplant
UN	:	Urban-Normal
WB	:	Whole Blood
WBC	:	White Blood Cells
WCB	:	Whole Cord Blood
WPB	:	Whole Peripheral Blood

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1. Introduction

Cancer is a persistent challenge that pushes the limits of human endurance, strength and resilience. However unyielding it may appear to be, it inspires mankind to strategize fight for survival and hope by investigating time in ground breaking research work. Curing cancer in an individual is a hurdle. To overcome such an obstacle, it involves a constant collaborative effort between the medical professionals and the cancer research community who thrives on innovative measures to combat this relentless adversary. In the past two decades, advancements in biological research and sequencing technologies have transformed our knowledge on cancer. Scientists have unearthed critical aspects of how cancer develops, speculated relapse and remission, identified disrupted signalling pathways, intervened genetic profiling of malignant cells and investigated cellular mechanisms eventually progressing in the therapeutic field to tailor personalised treatments. These findings have enriched our understanding about the disease progression and how they can be classified for specific treatment measures. Personalised medicine though holds great promise for more targeted and effective therapies but modernised treatment interventions come with unexpected devastating results viz toxicity, treatment failure, immune-suppression etc. To combat such adverse situations, the cancer research community plays an essential role in providing clinicians with the tools and insights needed to better treat patients with less toxicity and equal effectiveness, linking scientific breakthroughs to real-world care.

Through this study, we aimed to establish a standard reference range for cord blood parameters, specific to the southern region of West Bengal, our studied cohort. The unique differences observed in cellular and non-cellular components, compared to adult blood, highlight the potential of cord blood as an important treatment regime for certain anomalies. We tried to build a dialogue that how cord blood often regarded as a biological waste, has immense potential in therapeutic and translational applications. The content of this thesis's findings also focuses on leukaemia as a whole and does not specifically address any lineage-specific leukaemogenesis that are either lymphoid or myeloid in origin and a possible therapeutic measure using cord blood plasma factor to ameliorate the haematopathological condition. Introducing readers to the broader classification of leukaemia and its entities through this chapter is essential, hence a short overview of leukaemia focussing on its classification, epidemiology, aetiology, symptoms and treatment measures are presented along with a gestalt of cord blood so that the narrative of this study can be delivered in the subsequent chapters.

1.1 Biology and classification of leukaemia

Leukaemia, a haematopathological condition, represents a group of non-epithelial neoplasms that originates from haematopoietic stem cells within tissues, responsible for blood-formation like bone marrow, or from immune cells, having the unique innate potential to migrate and invade unlike solid tumours that require complex cellular reprogramming and genetic modification for effective metastatic dissemination (Whiteley et al., 2021). The broad categorisation differentiates the types according to the i) stage at which haematopoiesis gave rise to the malignancy, ii) the location where tumour cells have started to accumulate and eventually produce symptoms, iii) the severity of the disease, and iv) the molecular biomarkers identified in the cells that confirms leukaemia. Leukaemia can be broadly classified based on the affected lineage (lymphocytic or myeloid) and proliferative potential (chronic or acute) (**Figure 1 and 2**). The complexity and ambiguity of this haematological malignancy is discussed in the detailed, up-to-date classification published by WHO (Alaggio et al., 2022; Khoury et al., 2022; Xiao et al., 2024), though it is subject to improvement as new facts are discovered in the upcoming time.

1.1.1 Lymphoid Neoplasms

Lymphoblastic leukaemia represents a clonal proliferation of lymphoid precursor cells having B- cells, T-cells and sometimes rarely NK-cells.

B-cell and T-cell Neoplasms

B lymphocytes also known as B-cells, play an integral role in the acquired immune system by producing antibodies upon antigenic stimulation. B-cell development and differentiation starts from a common lymphoid progenitor (CLP) of haematological lineage in the bone marrow and in secondary lymphoid organs like spleen and lymph nodes. A mature (or naïve) B-cell express the B-cell receptor (BCR) on its surface, differentiates either into a plasma cell or a memory B-cell. B-cell neoplasms may develop at any stage within the process of B-cell differentiation. Another type of lymphocyte, the T lymphocytes or T-cells, are white blood cells that act through cell-mediated immunity or develop through acquired immune response. Activated T-cells identify antigens and destroy them through destructive processes or by activation of cells in response to antigens. Similar to all blood cells, T-cells though develop in bone marrow from common lymphoid progenitors, but their maturation happens in the thymus, where they develop the capability to distinguish between non-self-antigens or invading antigens and the

body's healthy cells. Mature T-cells circulate in the blood with T-cell receptor (TCR) on their surface and wait for activation. Like B-cells, T-cells can also undergo neoplastic transformation at any of the developmental stages either at the apex or at the terminal end of differentiation.

1.1.1.1 Acute Lymphoid Leukaemia

Acute lymphoblastic leukaemia (ALL) is characterised by the malignant transformation and expansion of lymphoid progenitor cells (B or T cells) within the bone marrow, peripheral bloodstream and extra-medullary locations like in central nervous system (CNS) and in testes. Majority of ALL cases (80-85%) involves B-cell precursor, whereas only 15-20% are of them are of T-cell lineage (Graux, 2011). The incidence of this blood disorder shows a bimodal distribution with the first peak in childhood and a second peak at around the age 50 (Paul et al., 2016). ALL predominantly affects children (nearly 80%) with favourable outcomes; however, in adults, the disease is more severe. Despite significant improvements in paediatric outcomes is observed due to dose-intensification strategies, prognosis for older patients remains poor. Although induction chemotherapy yields a high initial response rate, it is only 30–40% of adult ALL patients who achieve lasting remission (Terwilliger & Abdul-Hay, 2017).

1.1.1.2 Chronic Lymphoid Leukaemia

Chronic lymphocytic leukaemia (CLL) is considered as the most common type of leukaemia in adults, this lymphoproliferative disorder marked by the expansion of monoclonal, mature B cells in the bone marrow, peripheral blood and secondary lymphoid tissues. CLL is common in USA and Europe with 4.7 new cases per 1,00,000 individuals in contrasts to individuals in Caribbean, Africa and Asia who estimate around 1.4 cases per 1,00,000 individuals, suggesting the role of genetic factors having to play a significant role in this racial disparity (Bosch & Dalla-Favera, 2019). CLL is prevalent is males with a median age of 70 years in all ethnic sub groups (Sant et al., 2011) and it remains an incurable disease with a heterogeneous clinical course, where treatment decisions still depend on conventional factors. CLL is considered as an indolent disease where asymptomatic people may never need the treatment (Shadman, 2023).

1.1.2 Myeloid Neoplasms

Myeloid cells originate from common myeloid progenitor cells (CMP) in the marrow which include erythrocyte, granulocyte (neutrophils, eosinophils, and basophils), and monocyte population. Granulocytes and monocytes play an essential role in the innate immune system.

They combat inflammation by phagocytosing bacteria, larger organisms, or damaged cells. Myeloid cells destroy parasites and secret inflammatory mediators (Stienstra et al., 2017). Myeloid neoplasms result from the out numbering of abnormal cells derived from common myeloid progenitors.

1.1.2.1 Acute Myeloid Leukaemia

25% of leukaemia in adults is diagnosed with acute myeloid leukaemia (AML) (Kumar, 2011) and is associated with poor outcomes. In about 15% of all cases, AML is linked to prior chemotherapy, radiation therapy, or environmental exposures like pesticides or benzene. Studies revealed, the disease is highly diverse genetically, with abundant of driver mutations like NPM1, RUNX1, ASXL1, and CEBPA, as well as chromosomal abnormalities like t(15;17)(q22;q12), t(8;21)(q22;q22), and t(X;11q23) (Papaemmanuil et al., 2016).

1.1.2.2 Chronic Myeloid Leukaemia

Chronic Myeloid Leukaemia (CML) is a myeloproliferative neoplasm occurring in 1–2 per 100,000 adults, accounting for about 15% of adult leukemia cases. It is caused by the Philadelphia chromosome, a genetic translocation [t(9;22)(q34;q11.2)] that creates the BCR::ABL1 fusion gene and oncoprotein, driving the disease (Jabbour & Kantarjian, 2022). Symptoms of CML varies from none to severe leucocytosis in advanced stages. The blast phase is similar to AML or ALL. Diagnosis of different phases of CML involves blood tests and identifying chromosomal abnormalities including identifying clinical symptoms and blast counts (Rinaldi & Winston, 2023). Of late CML has evolved into a model for targeted therapy, as discovery of tyrosine kinase inhibitors (TKIs) have changed the treatment landscape (Senapati et al., 2023). Once a fatal disease, is now a blood disorder with a life expectancy comparable to the general population.

1.1.3 Mixed Leukaemia

Mixed phenotype acute leukaemia (MPAL) is a rare and aggressive form of leukaemia seen in 2–5% of all leukaemia cases. It is also known as mixed lineage leukaemia, bilineal leukaemia, hybrid leukaemia, or biphenotypic leukaemia, MPAL is a heterogeneous disorder characterized by the presence of both myeloid and lymphoid blasts or a single blast population co-expressing myeloid and lymphoid markers (Weinberg & Arber, 2010). Approximately 0.35 cases per 1,000,000 person-years is documented and being a rare disease it is challenging to diagnose

and treat. Chemotherapeutic approaches typically involve ALL-like treatments or hematopoietic stem cell transplantation. The haematological disorder is more common in males, with a median age at diagnosis of around 50 years (Khan et al., 2018).

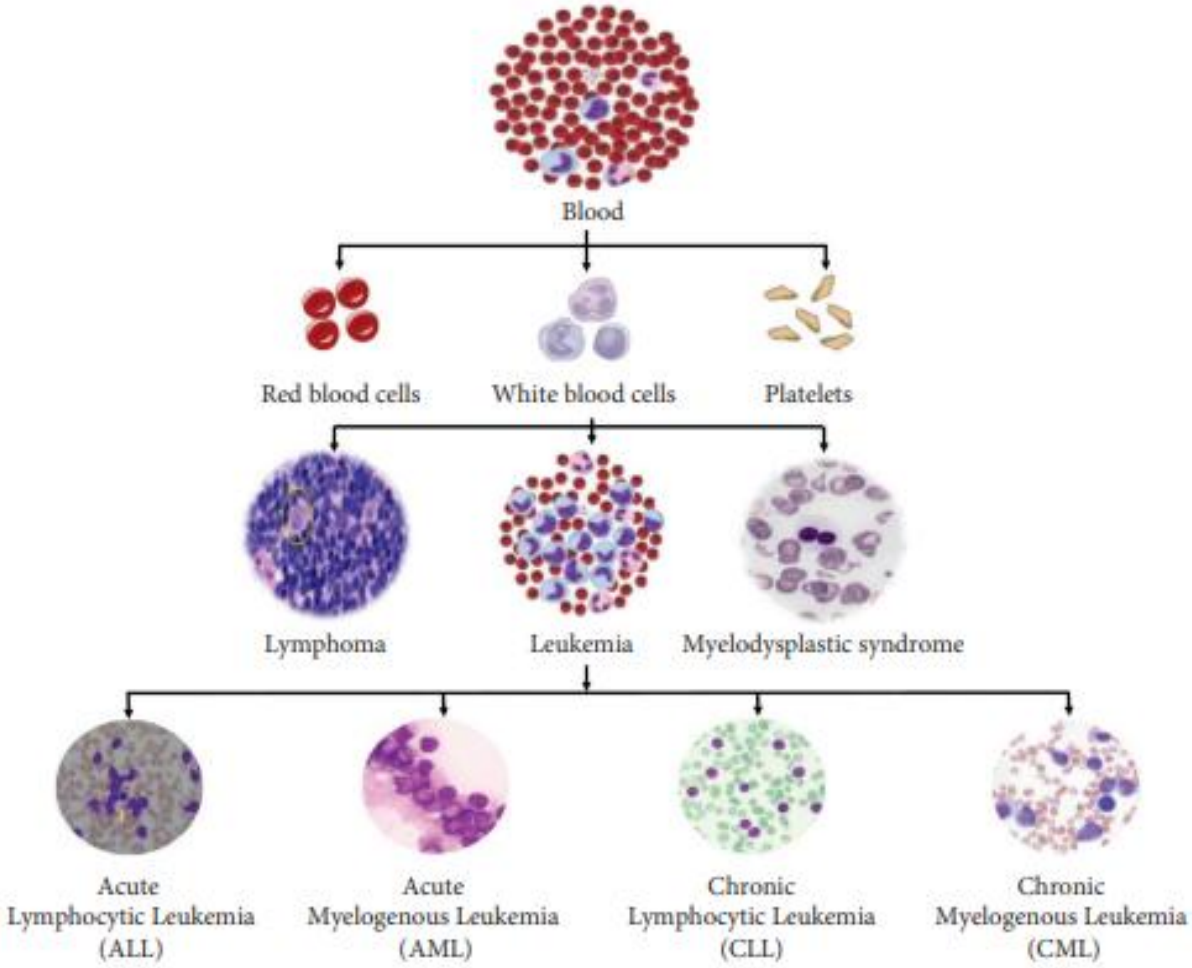


Figure 1: Types of leukaemia adapted from (Bibi et al., 2020)

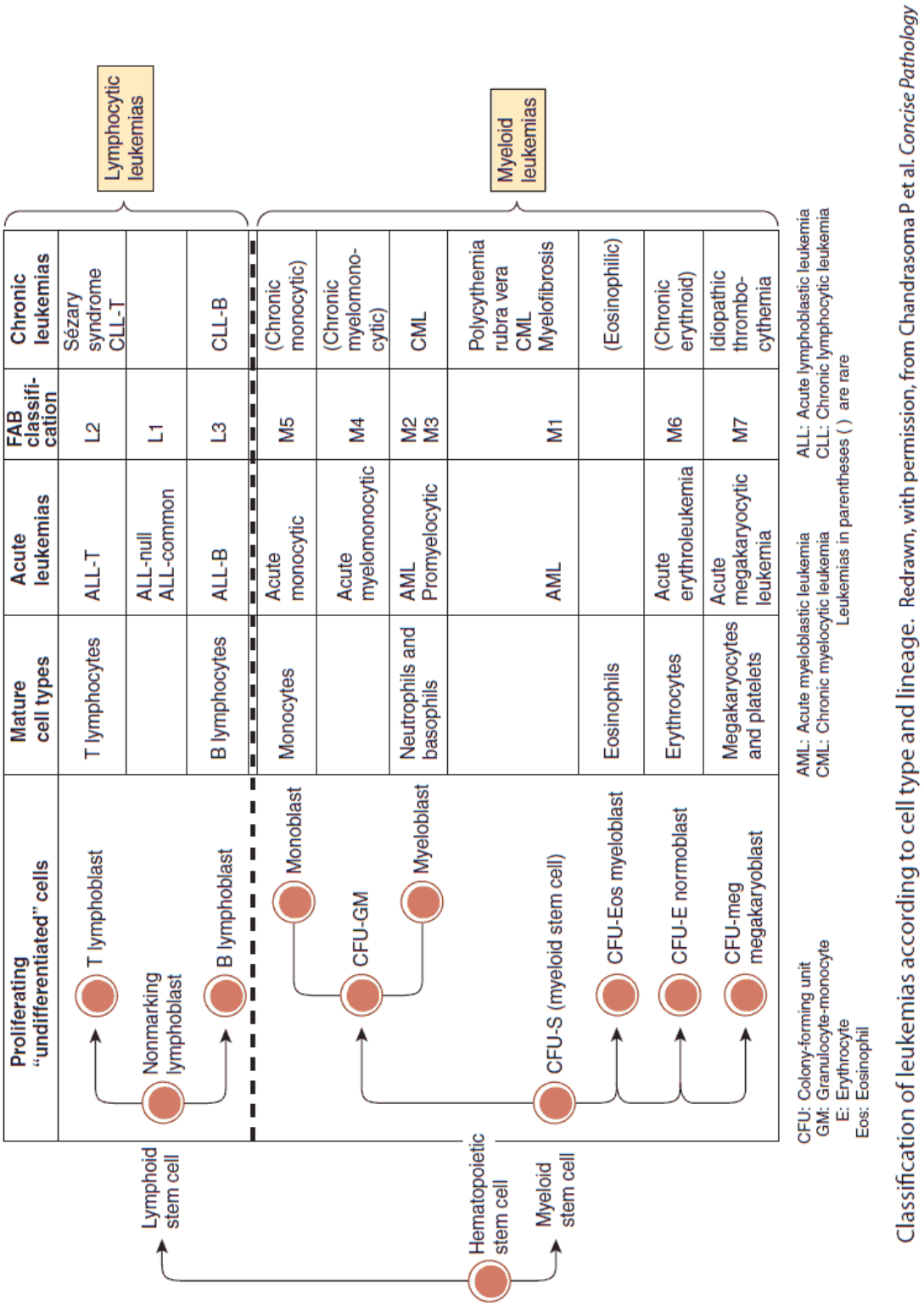


Figure 2: Classification of leukaemia, adapted from All about leukemia: A diagnosis to medicine [A comprehensive overview], Manual of Medicine.

1.2 Epidemiology of leukaemia

The World Health Organization's International Agency for Research on Cancer (IACR) and the Global Burden of Disease (GBD) provide us global estimation of cancer that includes leukaemia, using GLOBOCAN database to procure data from cancer registries worldwide. GDB estimated collated data through modelling techniques from high-income countries, who generally provide higher-quality data as well as low- and middle-income countries, who contribute through less reliable sources.

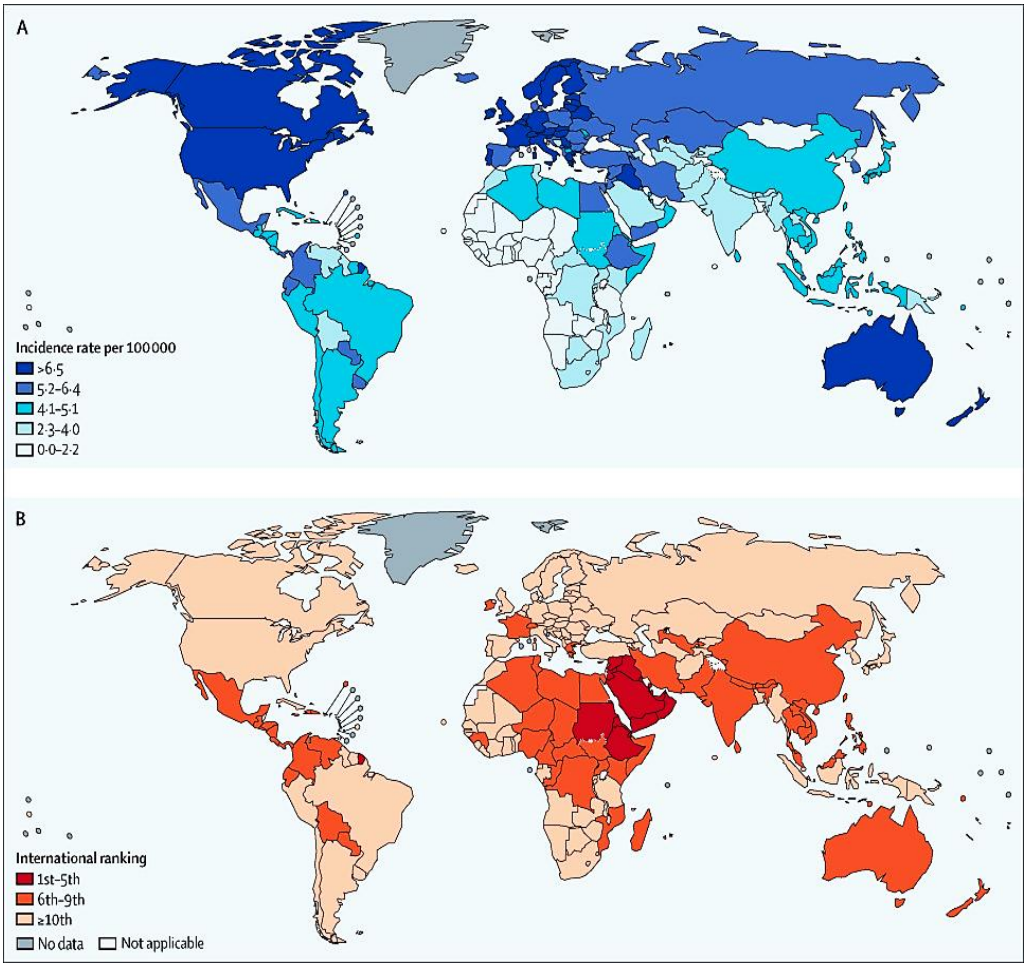


Figure 3: Leukaemia epidemiological patterns in 184 countries, adapted from (Miranda-Filho et al., 2018) where A) illustrates incidence rate/100000 individuals and B) illustrates the international ranking.

In 2018, GLOBOCAN estimated, leukaemia to be the 15th most common cancer and 11th leading cause of cancer deaths worldwide, with 437,033 new cases and 309,006 deaths. It is estimated from the age-standardised incidence rate that males had higher rates of both diagnosis (6.1 per 100,00) and mortality (4.2 per 100,000) compared to women (4.3 per 100,000 and 2.5 per 100,00). Leukaemia occurrence increases with age, though some types, like acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML), also affect the children.

The leukaemia rates are higher in more developed countries. In high-development countries, the incidence and death rates are significantly higher than in low-development countries, likely due to differences in healthcare access, diagnosis, and reporting. From 2018 report, incidence in high/very high HDI countries substantially exceeded that of low/medium HDI countries (7.5 vs. 4.0 per 100,000 for males; 5.3 vs. 3.0 per 100,000 for females). The same was true for mortality in high/very high HDI countries and low/medium countries (4.5 vs. 3.2 per 100,000 for males; 2.9 vs. 2.4 per 100,000 for females) (Bray et al., 2018). Between 2005 and 2015, leukaemia cases rose by 26%, driven mostly by population growth and aging (Baeker Bispo et al., 2020). In 2022 report, leukaemia was accounted for 2.5% of all cancers and 3.1% of mortality related to cancer (Huang et al., 2022) (**Figure 3**)

1.3 Aetiology of leukaemia

1.3.1 Age and Race

(Baeker Bispo et al., 2020) reports, leukaemia frequency varies significantly by age and race. In the UK, 42.8% of cases occurrence is seen in individuals over 65 years. In the U.S., the age-adjusted incidence per 100,000 individuals is highest among Whites (15), followed by Blacks (11), Hispanics (10.6), American Indian/Alaskan Natives (8.3), and Asian/Pacific Islanders (7.8). Studies also revealed, childhood leukaemia, affecting 4.6 per 100,000 under 20, comprises 20–30% of cancers in this group. Acute lymphoblastic leukaemia (ALL) peaks at ages 2–5, accounting for 75% of childhood leukaemia cases. Acute myeloblastic leukaemia (AML) is more common in adults, with a median diagnosis age of 66 and 54% of cases after age 65.

1.3.2 Genetic Factors

Incidence of certain leukaemia (especially acute ones) is more likely to develop in people with specific hereditary and genetic abnormalities. Autosomal inherited dominant genes like CEBPA, RUNX1, and GATA2 are important genes linked to leukaemia. C/EBPa, which is necessary for granulocytic development, is encoded by CEBPA at chromosome 19q13.1. GATA2, located at 3q21.3, preserves hematopoietic stem cell integrity and phagocytosis, while RUNX1, located at 21q22.12, controls haematopoiesis. ETV6-RUNX1 fusion gene, IKZF1 deletion, BCR::ABL1 fusion are found in ALL cases in the paediatric cohort. Mutations in GATA2 have been connected to congenital neutropenia and MonoMAC syndrome. Leukaemia risk is enhanced by genetic disorders such as Li-Fraumeni syndrome, Down syndrome, Fanconi

anaemia, and Diamond-Blackfan anaemia. Acute myeloid leukaemia, chronic myelomonocytic leukaemia, and myelodysplastic syndrome are often caused by disorders such as monosomy 7 and inherited bone marrow failure syndromes (Papaemmanuil et al., 2014; Stieglitz & Loh, 2013)

1.3.3 Environment and Occupation

Susceptibility to leukaemia increases with exposure to various environmental factors, including exposure to chemicals, infections, and radiation. Increased leukaemia risk is associated with occupations that include agricultural work (pesticides, fertilizers), construction, animal slaughtering, oil/gas industries (benzene exposure), petrochemicals, automotive repair, nuclear power (ionizing radiation), and electrical utilities (magnetic fields). Other high-risk professions include furniture manufacturing, healthcare (infectious agents), painting, hairdressing, dry-cleaning (chemicals), teaching, and transportation. Exposure to alkylating agents, formaldehyde, hydrocarbons (benzene, gasoline, trichloroethylene), and parental occupations is also involved. Despite the diversity of associated occupations, a unifying cause remains unclear which leads to the development of leukaemia, having both direct and indirect chemical exposures play key roles in the advancement of this haematological malignancy (Tebbi, 2021)

1.3.4 Effect of Radiation

An observational study as documented by (Moloney, 1955) (Cardis et al., 2006) where survivors of Hiroshima, Nagasaki, and Chernobyl disaster experienced ionizing radiation exposure at many life stages, including preconception and postnatal have developed leukaemia. There is conflicting evidence about the relationship between diagnostic X-rays and leukaemia; some research indicates a higher risk, especially with computed tomography (CT) scans, while other studies find no association at all (Tebbi, 2021). Studies also revealed exposure to solar UV radiation lead to the incidence of childhood leukaemia (Little et al., 2024).

1.3.5 Prior exposure to Chemotherapy and Immunosuppression

Cancer patients who receive chemotherapy, especially alkylating agents, platinum derivatives, or topoisomerase II inhibitors, experience a higher risk of leukaemia that get amplified by radiation therapy (Tebbi et al., 2007). In a study of 82,700 breast cancer patients, combined chemotherapy and radiation increased leukaemia risk 17.4-fold. The risk of leukaemia development depends on the drug dose and treatment procedure. It is reported that melphalan

is ten times more leukaemogenic than cyclophosphamide. Cyclophosphamide doses below 20,000 mg showed only a slight increase in secondary leukaemia risk.

1.3.6 Parental and Residential Factors

Certain paternal occupations and hobbies involving chemicals like gasoline, solvents, pigments paints, pesticides, and plastics could be linked to the increasing risk of leukaemia in children (Freedman et al., 2001; Glass et al., 2003; Lin et al., 2020). Industrial jobs like metalworking, textiles, pharmaceuticals, engine repair and petroleum may contribute to this risk. Children can also be indirectly or directly exposed to chemical agents through breastfeeding, contaminated clothing, or their environment. Additionally, household use of pesticides and insecticides has been associated with a higher likelihood of developing leukaemia (Menegaux et al., 2006). These insights emphasise the need to understand how environmental and occupational factors impact children's health, thus measures should be taken by families and communities to reduce such risks and provide safer living conditions.

1.3.7 Infections

Infections caused by viruses, bacteria or fungi, either alone or in combination with genetic mutations, have contributed to the development of leukaemia. Researchers have extensively explored the roles of viruses like Epstein–Barr, Herpesvirus, HIV, SARS, COVID-19, and HTLV-1, but no single infectious agent has been consistently connected to leukaemia across all patient groups (Da Rocha Paiva Maia & Filho, 2013; Gold et al., 1989; Greaves, 2020; Tebbi et al., 2021)

1.4 Symptoms of leukaemia

Leukaemia diagnosis typically follows symptoms like fever, fatigue, joint pain and recurrent infections. Symptomatic presentation varies by subtype: CLL (47%), CML (71%), AML (77%), and ALL (78%). Diagnosis of acute leukaemia (AL) may present with chest pain; AML often includes bruising, bleeding, shortness of breath, and cough; ALL and CLL can present with masses. In CLL cases, infection (32%), fatigue (17%), and lymphadenopathy (7%) are reported, while in CML patients' symptoms like fatigue (34%), bleeding (21%), and weight loss (20%) is evident. Acute leukaemia is frequently present with fatigue, fever, infection, and haemorrhage and 95% of all paediatric ALL cases are associated with peripheral blood blast infiltration, enlarged lymph nodes, and/or spleen (Friese et al., 2011; Howell et al., 2013; Shephard et al., 2016)

1.5 Treatment and management of leukaemia

Acute Lymphoblastic Leukaemia: ALL is divided into B or T lymphocyte variants. Treatment is based on the lymphoblast origin and the presence of >20% blasts in peripheral blood smear or bone marrow aspirate. The presence or absence of the Philadelphia (Ph) chromosome is the one of the most important molecular markers for ALL treatment. For Ph-positive ALL, treatment often combines chemotherapy with tyrosine kinase inhibitors (TKIs) like dasatinib or ponatinib. Paediatric cohort experiences paediatric-regime peg-asparaginase intervention, while hyperCVAD or targeted therapies like blinatumomab are common for those under 65. Elderly patients may receive TKIs with steroids or reduced-dose chemotherapy. Chemotherapy-free options are also showing success (Slayton et al., 2018) (Foà et al., 2020). Chemotherapy is the key treatment for Ph-negative patients. Paediatric patients are treated under peg-asparaginase regimens, rituximab-based options are for CD20-positive adults under 65, and corticosteroids or low-dose hyperCVAD are for older patients (Chennamadhavuni A et al., 2023; DeAngelo et al., 2015; Stock et al., 2019).

Acute Myeloid Leukaemia: The standard treatment for AML includes '7+3' regimen, where a 7-day continuous infusion of cytarabine is combined with a 3-day course of an anthracycline like daunorubicin or idarubicin. Advancement in cytogenetics and NGS testing now allow patients to be classified by molecular markers, aiding prognosis and treatment decisions (Chennamadhavuni A et al., 2023; Döhner et al., 2017).

Chronic Lymphoblastic Leukaemia: For CLL patients with an IGVH mutation, chemotherapy with FCR (fludarabine, cyclophosphamide, rituximab) or BR (bendamustine, rituximab) is preferred as a treatment procedure over a decade of disease-free survival. High-risk patients with del17p/TP53 mutations benefit from targeted therapies like venetoclax or BTK inhibitors (ibrutinib, acalabrutinib) which are often combined with rituximab or obinutuzumab. Reports revealed, BTK inhibitors suit older patients well (Chennamadhavuni A et al., 2023; Costello et al., 2021).

Chronic Myeloid Leukaemia: Treatment with TKI targeting the Ph chromosome in CML patients revolutionized the cancer care. High-risk patients benefit from second-generation TKIs like nilotinib, dasatinib, or bosutinib, while low-risk cohort often start with imatinib as first line therapy. Risk assessment uses tools like the Sokal, EUTOS, or ELTS scores to guide therapy and achieve effective, deeper and faster responses (Chennamadhavuni A et al., 2023; Pfirrmann et al., 2016; Yun et al., 2016).

Mixed Phenotype Acute Leukaemia: MPAL is a rare and complex form of leukaemia, making up just 2–5% of all leukemic cases. MPAL diagnosis can be difficult because it is not extensively studied also the trial data to guide the treatment is limited. Doctors often use ALL-like regimens to manage the disease. The rarity of this blood disorder underscores the need for more research to improve the knowledge and treatment options (Khan et al., 2018).

1.6 Treatment toxicity of leukaemia

Chemotherapy associated with childhood acute lymphoblastic leukaemia affects all organs in the body- causing severe side effects such as infections (viral and fungal), mucositis, neuropathy, bone toxicities (including osteonecrosis), thromboembolism, endocrinopathies (steroid induced adrenal insufficiency and hyperglycemia), nephrotoxicity, hypersensitivity, pancreatitis, and hyperlipidemia. Non-infectious toxicities though poorly characterised but are reported diversely enabling clinicians to assess risk factors across protocols (Rokkanen et al., 2024; Schmiegelow et al., 2017). Although, advanced chemotherapy treatments have increased the 5-year survival rates by 90% but assessing and reducing toxicity post treatment still remains a challenge. Chemotoxicity is linked to systemic DNA damage and inflammation in healthy cells thus direct drug-induced cell death is evident. Emerging research suggests chemotoxicity arises due to cell-free chromatin particles (cfChPs) released by dying cells (**Figure 4**), which cause DNA damage, activating and inflammatory cascades and apoptotic pathways in bystander healthy cells (Juthani et al., 2024).

To reduce the effect of chemotherapy toxicity, cancer researchers across the globe are exploring alternative therapeutic approaches including herbal extracts or natural concoctions, having anti-cancerous, anti-oxidative and anti-inflammatory properties and effectively targeting cancer while reducing harmful side effects. These strategies focus on enhancing treatment measures and safety, ultimately improving patient quality of life and increasing survival rates with reduced toxicity. Here we tried to assess the therapeutic potential of a naturally obtaining, biological concoction like cord blood plasma factor (CBPF) on a carcinogenic drug induced leukemic mice model.

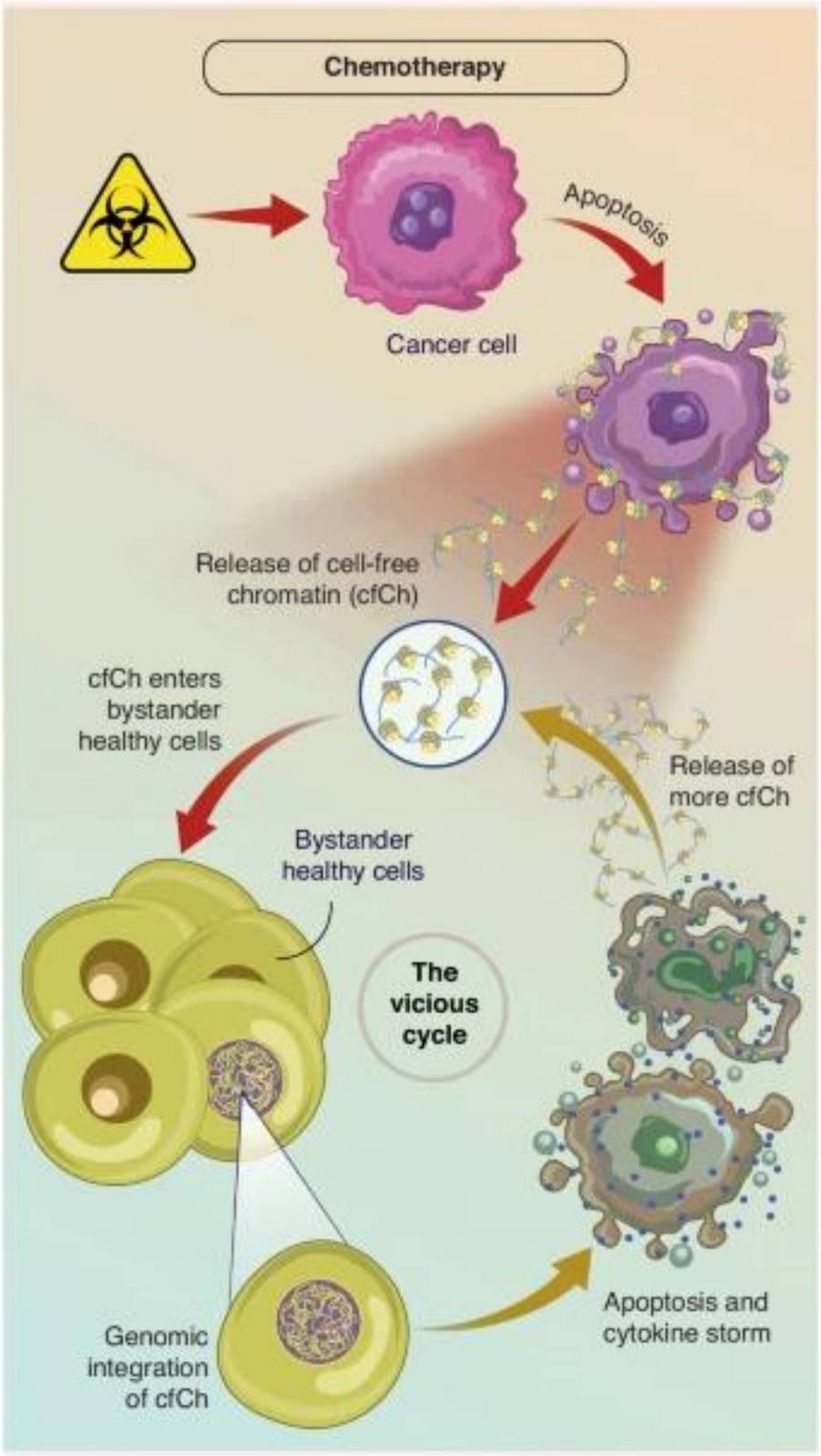


Figure 4: Schematic illustration of a vicious cycle of bystander cell death, adapted from (Juthani et al., 2024)

1.7 Cord blood and its therapeutic properties

The human umbilical cord is a thin, tube-like structure that connects the growing foetus to the placenta in the mother's uterus. The cord contains one vein that delivers oxygen-rich blood and two arteries that carry deoxygenated blood. These three blood vessels are helically coiled to form the structure of the umbilical cord. The venation in cord ensures proper blood circulation to support the growing foetus and holds a treasure of unexplored properties that could be investigated for future use (Nand Jha et al., 2021). Umbilical cord blood (UCB) is enriched with stem cells like mesenchymal stem cells (MSCs) and hematopoietic stem and progenitor cells (HSPCs). Its plasma component contains cytokines, growth factors, and immune-modulating factors that support the growth and function of naïve immune cells and MSCs, which holds multipotent properties. These beneficial constituents make UCB plasma a promising option for the treatment of various diseases. Additionally, therapies using UCB plasma pose no risk of transmission of animal pathogens thus enhancing their safety and appeal (Yoo et al., 2016).

Platelet-rich plasma (PRP) is recognised as a rich source of trophic factors (Parazzi et al., 2010), that contribute in healing various conditions including ocular surface disorders like severe dry eye disease and corneal ulcers and injuries, skin wounds, and orthopaedic anomalies (Samarkanova et al., 2020). PRP can be derived from adult peripheral blood and umbilical cord blood (CB) and studies indicated significant differences in their composition (Buzzi et al., 2018; Tian et al., 2023). CB-PRP has shown therapeutic advantages due to its higher levels of anti-inflammatory molecules, whereas adult PRP contains more pro-inflammatory factors, potentially intensifying certain conditions (Belderbos et al., 2013), this anti-inflammatory trait instigated us to use it for our leukemic study.

The therapeutic efficacy of cord blood plasma (CBP) has been verified in animal models like in rats with acute ischemic stroke, where CBP administration reduced inflammation and enhanced neurogenesis, resulting in significant functional recovery (Yoo et al., 2016). CBP contains tissue inhibitor metalloproteinase 2 protein which stimulated hippocampal function and memory restoration in 18-month-old mice post treatment (Castellano et al., 2017). Recent research also highlighted functional improvements in Alzheimer's mice after injecting a specific fraction of cord blood serum when compared to adult blood serum (Habib et al., 2018). Moreover, umbilical cord serum has been successfully used in humans to treat corneal defects and neurotrophic keratitis (Yoon et al., 2007).

1.8 Cytokine and growth factor profiling in cord blood

(Dhillon et al., 2012) and (Romanov et al., 2019) reported the concentration of cytokines and growth factors in cord blood plasma which can be applied in various regenerative medicines. Study documented, when cord plasma was compared to adult plasma or sera, there were significantly low concentrations of pro-inflammatory cytokines (IL-2, IL-6, IFN-γ, and TNF-α) and elevated chemokine IL-8 in CBP with significantly higher levels of VEGF, G-CSF, EGF and FGF-basic growth factors (Ehrhart et al., 2018a). Human megakaryoblasts, erythroblasts and granulocyte-macrophage precursors can be cultured using specific cytokine cocktails. Growth factors such as GM-CSF, SCF, Flt3, IL-11, IL-3, and IL-6 induce cell proliferation, whereas M-CSF, G-CSF, Epo, and Tpo initiate differentiation and are responsible for maturation (Hordyjewska et al., 2015). In another study by (Garanina et al., 2017), shown in (**Figure 5**) an extensive profiling of cord blood plasma was done by polar plot, where it is reported that the detected cytokines (pg/ml; log scale) in hUCB plasma were IL-1β, IL-6, IL-1ra, IL-4, IL-10, IL-13, IL-2, IL-7, IL-12, IL-17, IFN-g, TNF-a, IL-5, IL-9, IL-15, G-CSF, GM-CSF, PDGF-bb, FGF-2, VEGF, IL-8, Eotaxin, MCP-1, IP-10, MCP-1, MIP-1a, MIP-1b, RANTES, 6Cine, BCA-1, CTACK, ENA-78, Eotaxin2, Eotaxin-3, Fractalkine, GCP, Gro-a, Gro-b, I-309, I-TAC, MCP-2, MCP-3, MCP-4, MDC, MIF, MIG, MIP-3a, MIP3-b, MPIF-1, SCYB16, SDF-1ab, TARC, TERC. Building on this understanding of cytokines and growth factors, we utilised cord blood plasma factor (CBPF) to induce blast differentiation and maturation of arrested leukemic cells with an intention to develop functional immune cells and reduce the blast burden.

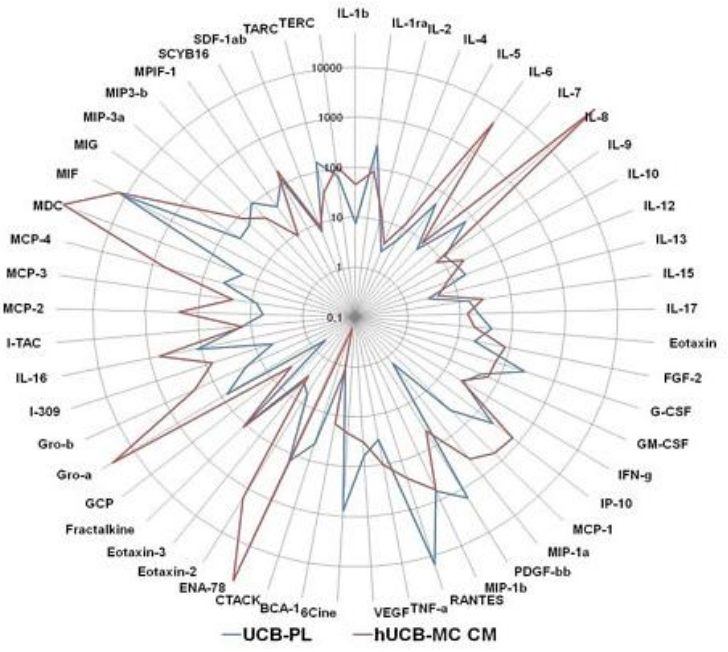


Figure 5: An extensive profiling of cord blood plasma was done by polar plot,

2. Literature Review

This chapter provides readers with a concise review on how nitroso compounds (NOC) or chemicals cause disruptions in the normal pathway of haematopoiesis, which eventually result in hematopoietic neoplasms. Leukaemia, lymphoma, and myelodysplasia are such haematological malignancies that belong to a class of blood disorders caused by genetic mutations in the bone marrow hematopoietic stem and progenitor cells (HSPCs). Uncontrolled proliferation and accumulation of immature or partially matured white blood cells (WBCs), also known as blasts, in the bone marrow, peripheral circulation, and other organs are distinct characteristics of several haematopathological disorders. A brief understanding of haematopoiesis and leukemic stem cell with the involvement of transcription factors is discussed with the readers. To study the effects of mutagens and comprehend the neoplastic nature of cancer formation due to the disruption in transcription factors, it is essential to develop leukemic models in rodents, especially mice, using strong mutagenic and carcinogenic alkylating agents such as N-ethyl-N-nitrosourea (ENU), a nitroso chemical.

2.1 Haematopoiesis

Haematopoiesis is a process by which the cellular components of blood are continuously renewed over the course of an organism's lifetime. Numerous populations of highly specialized cells with distinct roles, including immunological defence and oxygen transport, make up the hematopoietic system. In vertebrates, blood stem cells are produced through the allocation and specialization of specific embryonic cells across various sites that change along the developmental progresses. In mammals, normal haematopoiesis occurs sequentially in the yolk sac, the aorta-gonad mesonephric (AGM) region near the dorsal aorta, the foetal liver, and ultimately the bone marrow. According the recent research, another location that is active during the shift from the AGM to the foetal liver is the placenta. The developmental stage of the cells and distinct habitats that promote haematopoietic stem cell (HSC) proliferation or differentiation affect the characteristics of HSCs at each location, as shown in (**Figure 6**) where the haematopoietic development in a mouse is exhibited (S. H. Orkin & Zon, 2008). Within the mammalian haematopoietic system, rare, low immunogenic, naïve haematopoietic stem cells (HSCs) sit at the top of the hierarchy and are of two sub types- the long-term haematopoietic stem cell (LT-HSC), capable of indefinite self-renewal and a short-term haematopoietic stem cell (ST-HSC) that usually self-renew for a defined interval and undergoes differentiation. HSC gives rise to non-self-renewal progenitor cells that in turn give

rise to differentiated, multipotent, specialised cells which eventually develops into fully functioning matured blood cells (Passegué et al., 2003). In the bone marrow, the fine balance between differentiation and self-renewal is strictly controlled to maintain haematopoietic homeostasis throughout life (**Figure 7**). An imbalance in HSC activity can impose serious consequences. If HSCs differentiate too much or don't self-renew enough then their numbers can decrease. On the other hand, if differentiation is insufficient or self-renewal becomes unchecked, it can lead to leukaemia or myeloproliferative disorders. Cell extrinsic cues, such as long-range humoral and neural signals or local cues from the BM microenvironment, also known as the "stem cell niche," interact intricately with cell intrinsic factors, such as transcriptional and epigenetic regulators and metabolic pathways, to control HSC activity (Pinho & Frenette, 2019).

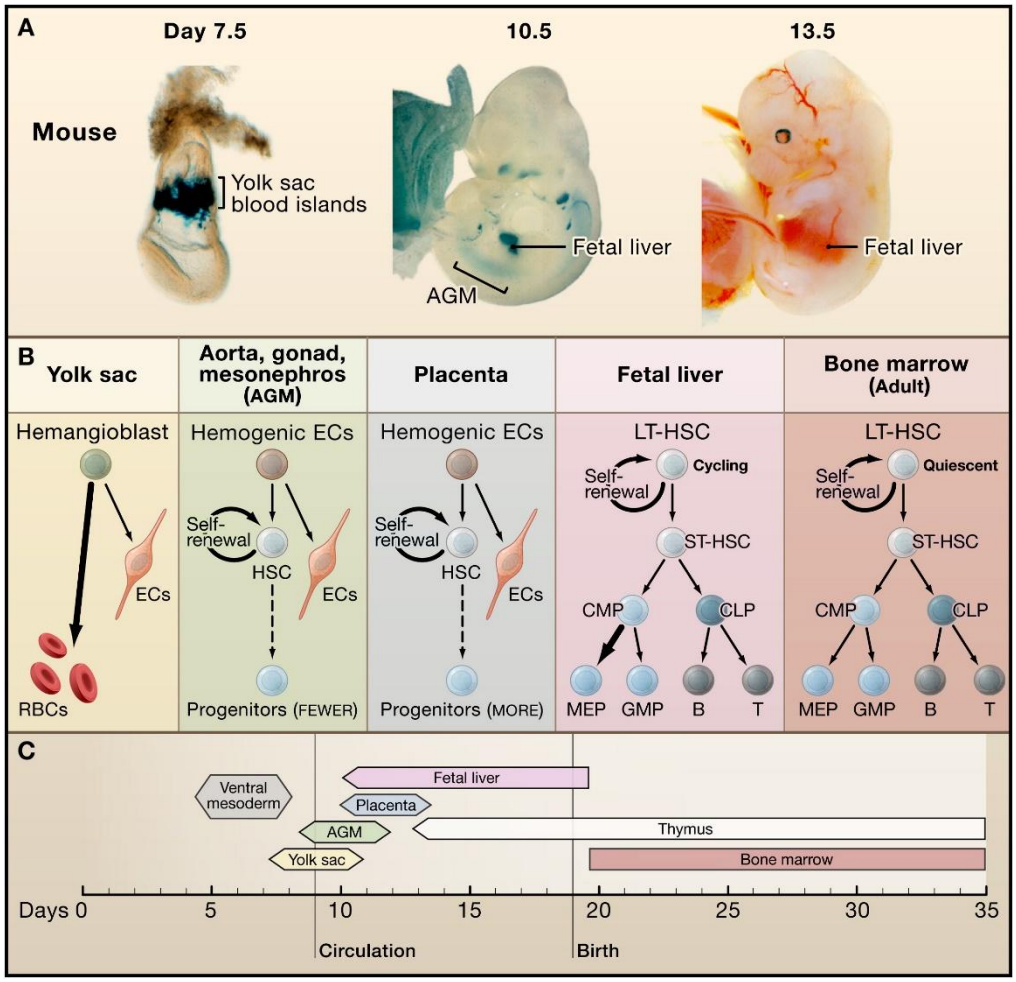


Figure 6: Haematopoiesis in mouse embryo, adapted from (S. H. Orkin & Zon, 2008)

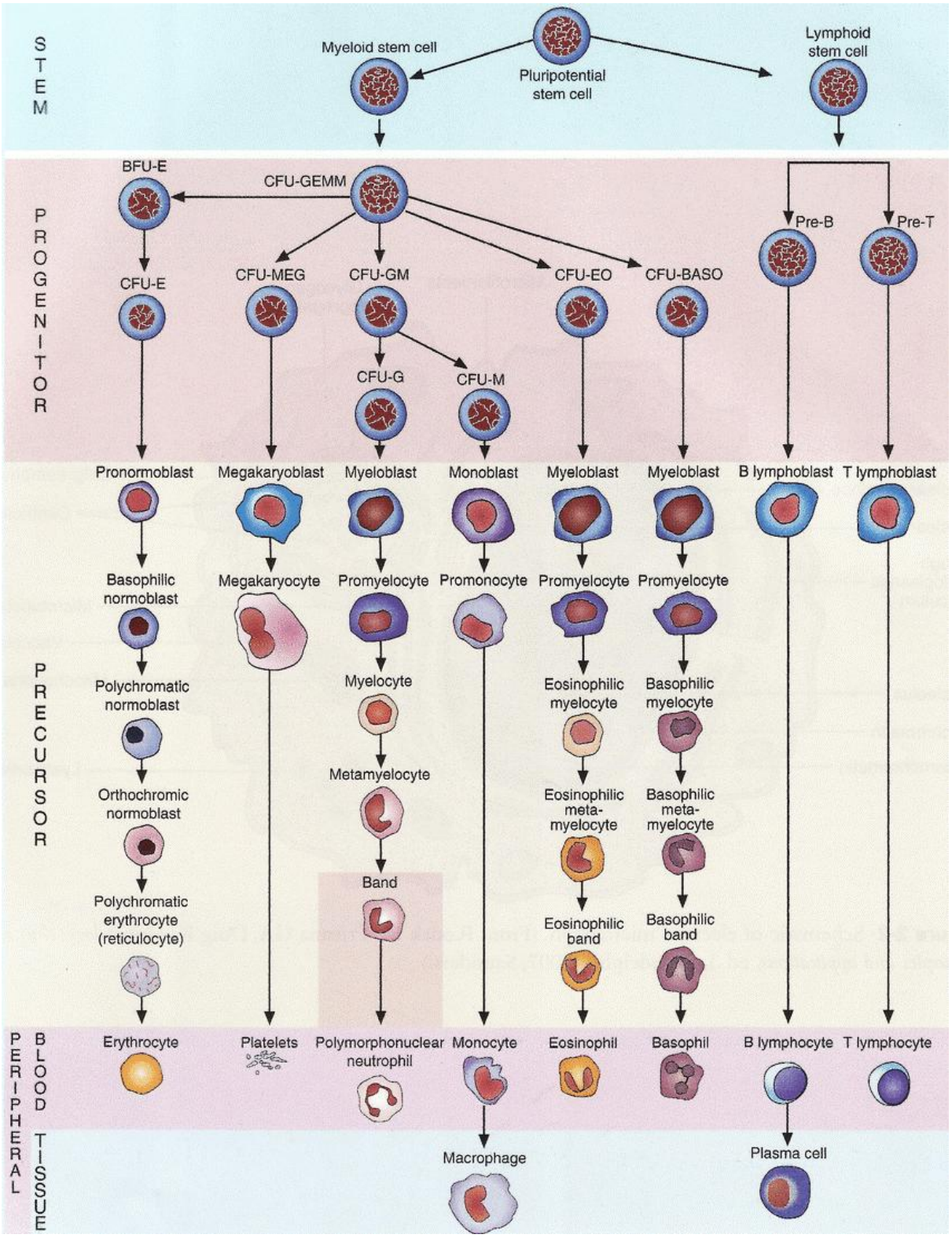


Figure 7: Haematopoiesis in bone marrow adapted from (Carr & Rodak, 2012)

2.2 Disruption of normal haematopoiesis and progression of leukemic stem cells

Normal haematopoiesis, which is necessary for the production of healthy blood cells, is disrupted in leukaemia (Duarte et al., 2018) where in leukemic condition, the healthy blood cells in bone marrow is overtaken by leukemic cells. In leukemic malignancy, the normal cell differentiation process is disrupted, halting at any stage of differentiation, leading to a continuous proliferation and self-renewal of cells. This leads to the production of a large number of abnormal cells, as most fail to mature into fully functional cells or progress towards terminal differentiation (Sell, 2005). These malignant leukemic cells gain a competitive benefit over the healthy population by inhibiting the proliferation of hematopoietic stem cells (HSCs) and progenitor cells, either directly or through the release of humoral factors leading to marrow failure (Choi et al., 2024; Ferrao Blanco et al., 2023). Blocked differentiation, increased proliferation without maturation, prolonged survival due to apoptosis resistance, and improved self-renewal capacities are some of the key characteristics of leukemic cells that are transformed in the bone marrow (Passegué et al., 2003).

Leukaemia progression occurs in a sequential manner almost similar to other malignancies, where a clonal population of blast cells is produced by accumulating somatic mutations (Ravandi & Estrov, 2006). Cancer is maintained by a small subset of such leukemic cells, also known as leukemic stem cells (LSCs) or leukaemia-initiating cells and they share key properties with normal HSCs, including self-renewal, unlimited proliferation, and resistance to apoptosis which play crucial point in neoplastic transformation (**Figure 8**). These similarities suggest that leukaemia may originate from HSCs that increases their longevity and ability to accumulate mutations over time (Filipek-Gorzała et al., 2024). Alternatively, LSCs can arise from differentiated progenitor cells that reacquire self-renewal capabilities through oncogenic mutations. Knowing the processes underlying the development and operation of LSCs emphasizes how crucial it is to target these cells in order to treat leukaemia effectively. Researchers all over the world are striving hard to discover therapies to restore normal haematopoiesis and slow the course of leukaemia which could tackle the mechanisms that give LSCs their competitive advantage. There has been encouraging improvement in leukaemia therapy techniques. However, resolving its intricacies remains a huge problem for scientists and clinicians worldwide. To properly treat the disease's resistance and recurrence mechanisms, persistent efforts are needed.

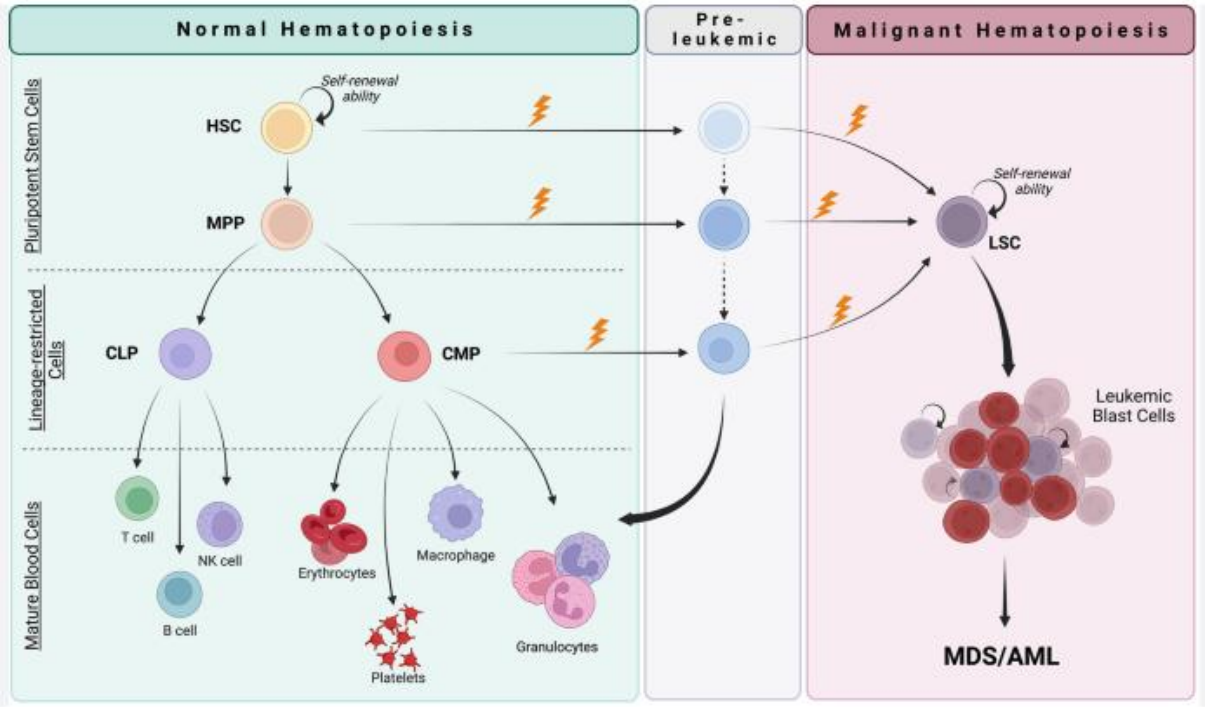


Figure 8: Illustration of normal haematopoietic stem and progenitor cells undergoing genetic mutations leading to the formation of leukemic stem cells and subsequent clonal expansion as seen in leukemic condition, adapted from (De Stefano et al., 2024)

2.3 Transcription factors involved in normal haematopoiesis and leukaemia

Transcription factors (TFs) play a crucial role in the orchestration of the haematopoietic development. Throughout the embryogenesis, the formation of specialised blood cells through lineage specific differentiations from haematopoietic stem cells is significantly influenced by the TFs (**Figure 9**). These factors belong to various DNA-binding protein families and not from any specific group, acting in coordination with other transcription factors, chromatin modifiers and co-factors to regulate gene expression and determining cell fate (Ravasi et al., 2010; Vaquerizas et al., 2009).

In haematopoietic development, erythroid, lymphoid, or myeloid differentiation is strictly regulated by TFs, for an instance, SCL transcription factor is encoded by Tal1 gene that regulates erythroid and megakaryocytic lineage differentiation (Hall et al., 2003).

The Gfi1 gene encodes a transcriptional repressor necessary for granulocytic differentiation, and if there is disruption in Gfi1 takes place then it can block neutrophil maturation. Additionally, Gfi1 plays a vital role in maintaining HSC quiescence, warranting their long-term stability and function. These findings highlight the importance of transcription factors in both normal blood cell development and disease (Zeng et al., 2004).

RUNX1 is a key transcription factor required for definitive haematopoiesis during embryonic development and absence of it will lead to the death of mouse in embryonic stage. But downregulation of RUNX1 expression is essential during erythroid differentiation thus emphasising its stage-specific role (Ichikawa et al., 2013).

Transcription factor CEBPα plays an essential role in myeloid differentiation and its expression is crucial for neutrophil maturation. Aberration in the expression of CEBPα due to the alterations in the CEBPA gene, can contribute to the development of acute myeloid leukaemia (AML), often alongside other haematological abnormalities (Avellino & Delwel, 2017).

The transcription factor PU1, belonging from the Ets family, is essential for the growth of myeloid and lymphoid cells. Recent research updates, differing PU.1 levels affect cell differentiation fate. Lower levels of PU1 facilitates granulocyte or B-cell differentiation, whereas higher concentrations favours macrophage growth. This highlights the dose-dependent significance of PU1 in haematopoiesis (Dahl & Simon, 2003). Notably, many transcription factors critical for haematopoiesis are associated with chromosomal translocations or somatic mutations in human blood malignancies.

In murine models, genetic manipulation of these intrinsic transcription factors often leads to malignancies, highlighting the relation between HSC development and leukaemia scenario. Important "HSC transcription factors," like MLL, Runx1, TEL/ETV6, SCL/tal1, and LMO2, are frequently involved in leukaemia-associated genetic changes. As already mentioned rampant changes in these factors, may disrupt gene expression, as observed with SCL/tal1 and LMO2 in T-cell acute leukaemia (Palomero et al., 2006), or production of fusion proteins with MLL, Runx1, and TEL/ETV6 in myeloid and lymphoid leukaemia. Though disruption in these factors lead to haematopathological outcomes but they also play vital roles in blood cell differentiation, showing their versatility in both diseased and normal conditions. Other lineage-specific factors like PU.1, Gfi-1, and C/EBPα also influence HSC function and differentiation, signifying the overlapping roles of transcription factors.

Studying transcription factors' roles in blood cell development and haematological disease is challenging due to their changing expressional patterns. To elucidate the importance of their functionality, a requirement of targeted inactivation at specific stages or times is essential (S. H. Orkin & Zon, 2008; Rosenbauer et al., 2005; Rosenbauer & Tenen, 2007)

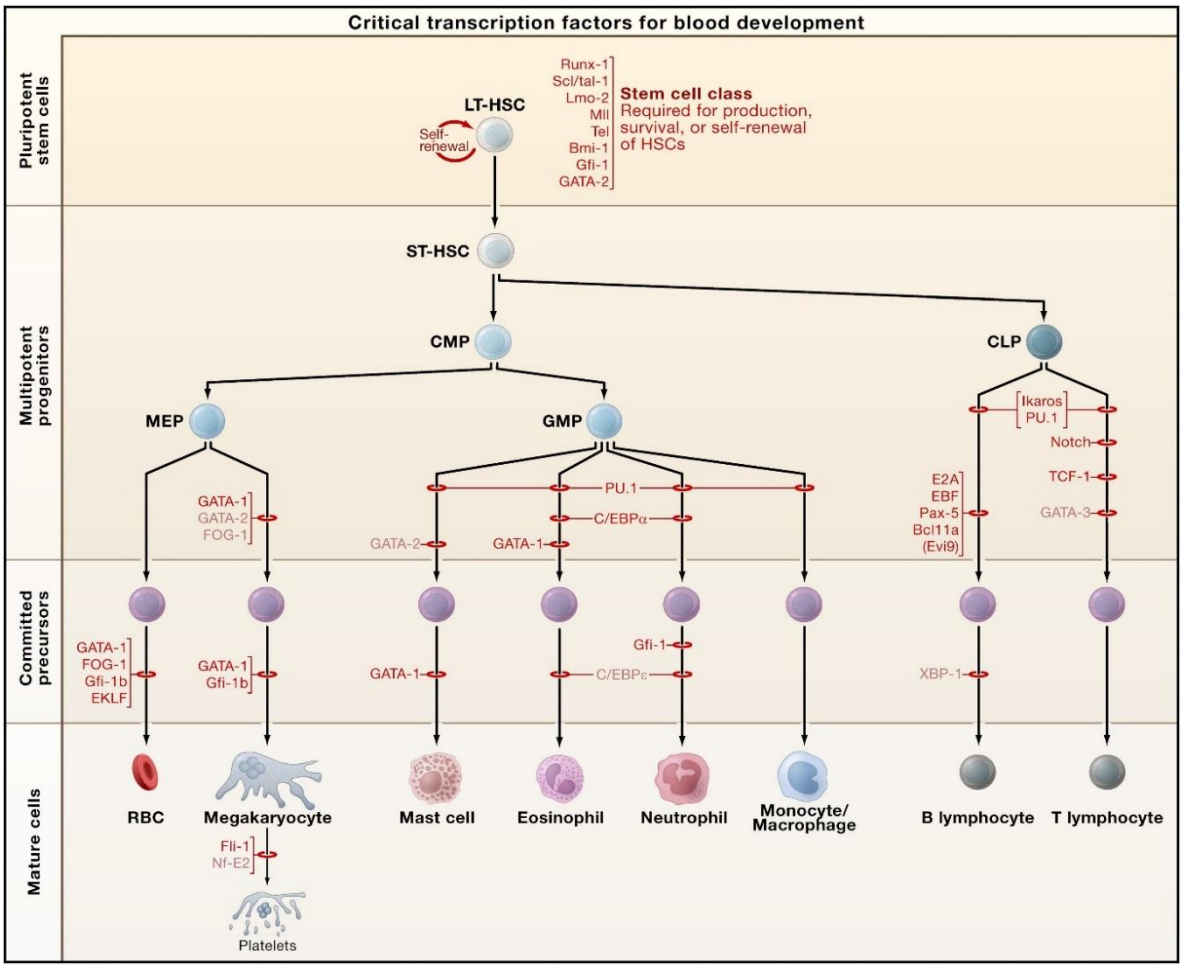


Figure 9: Transcription factors responsible for haematopoiesis, adapted from (S. H. Orkin & Zon, 2008)

2.4 Use of ENU as mutagen in leukemic mice models

N-ethyl-N-nitrosourea (ENU), a known to be a potent mutagen, an effective carcinogen and a detrimental teratogen that causes congenital abnormalities and have the potential to increase the risk of tumours in central nervous system (CNS) and bring genetic disorders (Hou et al., 2024). ENU is often used in research to develop tumour models, including leukaemia. Studies show that intravenous ENU administration (dose 40-80 mg/Kg) during pregnancy can increase the chances of CNS malignancies. Notably, it has been discovered that most infants get cancer as a result of receiving ENU via trans-placental injections during the first week of postnatal development (PN7) or the second-to-last week of gestation (E15 to E21). ENU triggers leukaemia in mice by inducing mutations at every 700 locations. A single intraperitoneal (i.p) administration of 80 mg/kg ENU results in the formation of leukaemia in 6–8 months with an increased VEGF-mediated angiogenesis and blocked apoptosis via Bcl-2 and Bax. ENU-induced leukaemia is associated to an excess accumulation of neoplastic lymphocytes, infiltrating organs like kidneys and lungs, along with an increased blast population in peripheral

blood (Aliyu et al., 2020). (R. Chatterjee, Chattopadhyay, & Law, 2016a) reported that leukaemogenesis is linked to the dysregulation of the p53 signalling pathway, featuring diminished p21 expression and downregulation of the p53-Ndn-Gfi axis. (Basak et al., 2010) highlighted in long term bone marrow cultures from ENU-induced leukemic mice, stromal fibroblast cells were observed that supported the hyper proliferative bone marrow cell growth. The Wnt signalling pathway studied by (Chattopadhyay et al., 2019) showed signs of deregulation, supported by significant overexpression of Wnt3a, Fzd7, β-catenin, and cyclinD1, along with decreased Wnt antagonist protein, Dkk1. In the same study, the leukemic phenotype displayed characteristics of both lymphoid and myeloid lineages, which was further validated by blood profile analysis. In experimental investigation involving the study of disease model- dosage, frequency, and mode of administration determine the outcome of ENU exposure, that invariably impact the target cells, organs, and leukaemia induction. These results highlight how crucial dose accuracy is when using ENU is used to investigate the development of cancer and its effects.

2.5 Sources of N-nitroso compounds and their exposure

With the advancement of industrialisation an increase in exposure to carcinogenic pollutants from industries are significantly affecting the biotic and abiotic components of nature. Reports from recent analysis estimate, total human exogenous exposure to N-nitrosamines compounds at approximately 1.10 μmol/day. The primary exposure sources are dietary intake (0.79 μmol/day; 72%), occupational environments (0.15–0.30 μmol/day; 25%), cigarette smoking (0.02 μmol/day; 2%), and minor sources such as pharmaceuticals, cosmetics, and environmental air (0.001 μmol/day; 1%). In healthy adults, excretion of apparent total N-nitroso compounds (ATNC) is 1.30 ± 1.05 μmol/day in urine and 1.56 ± 1.56 to 3.17 ± 2.58 μmol/day in faeces. Specific urinary N-nitroso compound excretions include volatile N-nitrosamines (0.03% of urinary ATNC) and N-nitrosamino acids and their derivatives, which constitute 16.0% of urinary ATNC. Metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its O-glucuronide conjugate, contribute 0.2% of urinary ATNC, accounting for >1.5% of identifiable urinary N-nitroso compounds (Haorah et al., 2001; Jakszyn et al., 2006). (Bowles et al., 2024) explains how nitrate enters the body through the enterosalivary nitrate-nitrite-nitric oxide pathway when consumed foods with high nitrate content. The majority of nitrate is eventually eliminated from the body by urine, although some is absorbed in the stomach and enters the bloodstream. Fraction of nitrites goes back to the mouth, where bacteria convert into nitrite, which is

subsequently swallowed and transformed into nitric oxide in the acidic environment of the stomach. This process though produces advantageous molecules, but it can also produce toxic nitroso compounds when red meat is present since it contains a lot of heme and certain amines. According to a crude mass balance, 45–75% of the overall amount of N-nitroso compound exposure in humans happens through in vivo production rather than direct external sources. This highlights the intricacy of nitrosoamine exposure pathways and the necessity of more investigation into the unknown substances and their possible effects on health (**Figure 10**)

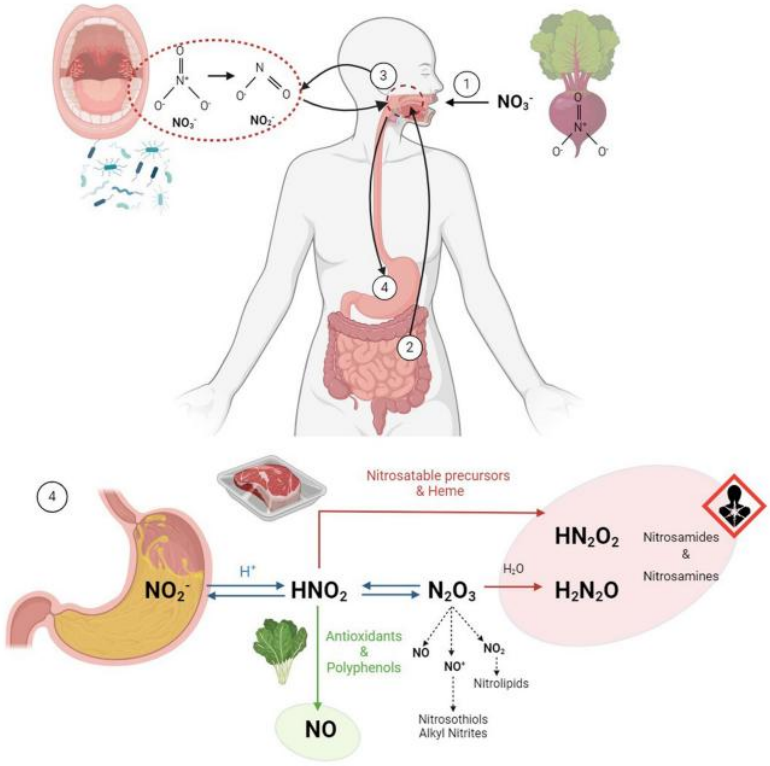


Figure 10: Nitrosoamine pathway in humans, adapted from (Bowles et al., 2024)

2.6 Effect of ENU on biological system

A genotoxicant like ENU is a potent nirosoamine toxin and mutagen that can disrupt biological systems by alkylating DNA, modifying proteins through amino acid carbamoylation, and by inducing oxidative stress which can cause adverse effects (Nath & Maiti, 2022).

2.6.1 ENU as an alkylating agent

ENU is known to be a highly potent DNA alkylating agent that directly modifies nucleotide bases in nucleic acids without any need for metabolic activation. It ethylates adenine (N1, N3, N7), guanine (O6, N3, N7), thymine (O2, O4, N3), and cytosine (O2, N3) thus forming DNA adducts. These modifications in DNA structure cause base mispairing and nucleotide substitutions, resulting in mutations in single-locus eventually leading to an overall mutation in the genome. ENU exposure can induce change in one base pair per million nucleotide base-pairs in the genomic DNA, giving it a status as one of the most effective and notorious

carcinogenic agents to bring about genetic mutations. This detrimental potential of ENU is so widely studied and established that it is used as a crucial tool to develop mouse mutant models (Angelis et al., 2000; Concepcion et al., 2004; Nolan et al., 2000).

2.6.2 Nature of ENU-induced gene mutation

ENU's strong DNA-alkylating property induces frequent point mutations in exposed genomic DNA, primarily caused by nucleotide base substitutions. GC→AT transitions are the most frequent mutation, followed by AT→GC, AT→CG, AT→TA, GC→CG, and GC→TA alterations. GC→AT and AT→GC transitions, and AT→TA transversions are especially common in bone marrow cells. Although point mutations make up the majority of ENU-induced alterations, some deletions were also studied by scientists. Moreover, by creating 7-ethyl deoxyguanosine and ethyl phosphotriester complexes inside the DNA strands, ENU-induces DNA ethylation, decreases DNA's template activity during replication and prevents transcription, further disrupting genetic processes (Jump et al., 1980; Keiji Marushige; Yasuko Marushige, 1983)

2.6.3 ENU-induced amino acid carbamoylation

ENU's potential not only limits to alkylation of DNA but it can also alter protein structure and function through amino-acid carbamoylation. ENU, inside the cells, decomposes to produce cytotoxic cyanate ions and cause short-term adverse effects. These cyanate forms isocyanic acid, which in turn reacts with protein amino groups, thus disrupting their structure and functional properties. Isocyanic acid is more stable than ENU's alkylating component and significantly impacts cellular proteins, including histones. By destabilizing chromatin through altered histone interactions, it disrupts DNA-histone binding. Similar effects are observed with methyl-nitrosourea (MNU). Studies show that ENU-induced carbamoylation renders proteins inactive, impairing growth promotion and altering key cellular processes, including cell cycle progression (Hitotsumachi et al., 1985; Jump et al., 1980; P. Knox, 1976).

2.7 ENU-induced mutagenicity in tissues and organs

ENU's chemical properties enable it to be highly soluble in body fluids. After its administration in the biological system, ENU is evenly distributed throughout the body except in the central nervous system, where its uptake is limited by the brain blood barrier. ENU is mutagenic to nearly all tissues, though its effects vary across organs, it can exert detrimental effect and damage major organs like testis, liver, kidneys, brain, spleen, and bone marrow. Studies documented, ENU's potential to cross the placental barrier and bring havoc changes in nucleic

acid of a developing embryo. In neonatal mice, brain cells exposed to ENU have shown to transform into malignant cells expressing oncogenes in long-term cultures (A. Mahlke et al., 2011; Frei et al., 1978).

2.8 ENU-induced haematological malignancies in mice

ENU, a powerful mutagen widely used by researchers to model human diseases and study haematological malignancies like myelodysplasia and leukaemia, in laboratory mice (Stottmann, Rolf W.; Beier, 2015). The mutagenic effects of ENU affect an organism's genetic make-up, and it is studied that different strains of organism exhibit varying degrees of mutagenic susceptibility (Fenske et al., 2006). ENU induces tumours in various tissues and hematopoietic malignancies are frequent amongst the diseases. Studies have identified strains of mice like SWR/J, DBA/2J, and Balb/cByJ are particularly susceptible to leukaemia, while AKR/J and C57BL/6J are prone to have both myeloid and lymphoid malignancies (Fenske et al., 2006). As mentioned earlier, primary mechanism of ENU involves DNA alkylation, leading to gene mutations, translocations, and inversions that causes leukemogenesis, depending on this, myeloid malignancies were observed in 80% of SWR/J, 50% of DBA/2J, and 33% of Balb/cByJ mice, while AKR/J mice exhibited 70% lymphoid and 20% myeloid malignancies. Mice with ENU-induced AML and MDS exhibit cytopenia and abnormalities of the bone marrow, which are similar to the characteristics of human diseases. High ENU doses increase toxicity and increase the rate of leukaemia development, highlighting the occurrence of dose dependent mutagenesis (Jonas et al., 2016). According to research (Fei et al., 2018), ENU-induced leukaemia is caused by mutations in important genes like Tet2, Gata2, Idh1, and Ikzf1, which are frequently linked to haematological malignancies in humans. As confirmed in AML1-ETO fusion models (Nimer & Moore, 2004; Yuan et al., 2001), ENU also gives genetically predisposed mice a "second hit" that increases their leukemogenic potential. ENU-induced leukaemia can also be transplanted confirming its use in the study of severe illnesses. Taking into consideration, ENU's potential as a helpful tool for researching leukaemia, particularly in models of genetically engineered mice, has opened up several general and clinical research avenues. ENU makes it possible to investigate the genetic alterations that cause leukaemia, such as single-gene mutations and intricate processes like translocations.

2.9 Mechanism behind ENU-induced leukaemogenesis

Nitrosoureas like ENU induce leukaemia by altering the bone marrow environment (Basak et al., 2010). Though the exact mechanisms behind the triggering of leukaemogenesis remain unclear but ENU-induced random gene mutations as primary cause in DNA genome are widely

accepted. Intraperitoneal administration of ENU at doses of 80–400 mg/kg in mice resulted in approximately one-point mutation per 1,000 loci, resulting in 20–30 mutations per genome (Nguyen et al., 2011). These mutations carry significant potential to cause phenotypic changes, including cancer. Key genes like TP53, NF-1, RUNX1, CEBPA, PU1, PTPN-11, RAS, FANCA, WT-1, GATA-1, and U2AF1 (S34F) are often affected and contribution to leukaemia and other haematological malignancies are widely reported (Di Nardo & Cortes, 2016; Lindsley et al., 2015). In addition to point mutations, ENU-induced genetic alterations like translocations and chromosomal inversions (Olney et al., 2002; Reilly, 2005), are known to bring in myeloid malignancies as seen in t(8;21) translocation, which leads to AML through the AML1-ETO fusion (Tijchon et al., 2019). These genetic disruptions, triggered by ENU-induced DNA alkylation, are central to leukemic development in the bone marrow. ENU causes leukaemia by oxidative stress, immunological suppression, and bone marrow destruction in addition to its mutagenesis effects. These combined effects demonstrate ENU's potency as a leukemogenic drug and provide information on the genetic and environmental variables that affect the course of leukaemia (**Figure 11**).

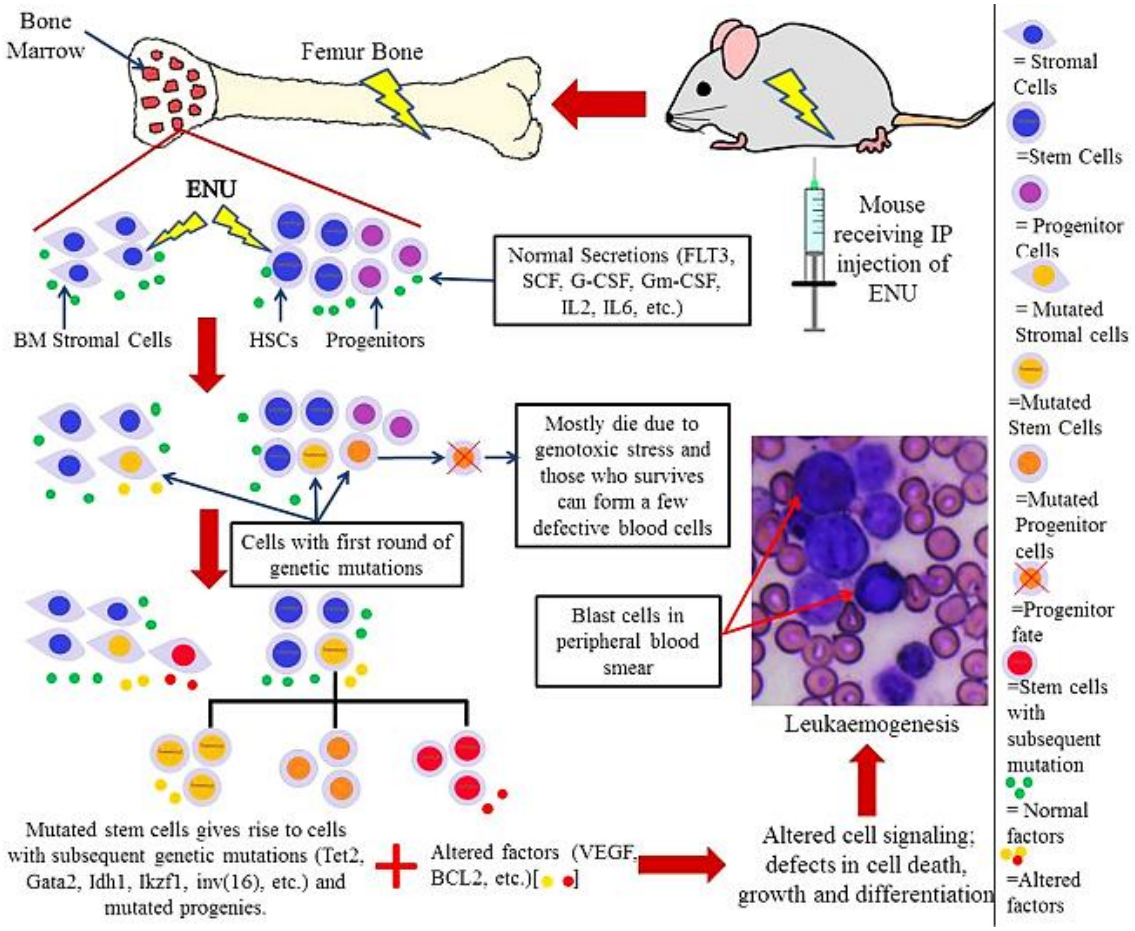


Figure 11: Schematic illustration of ENU induced leukaemogenesis in murine model with disruption in genetic profiling, adapted from internet

2.9.1 Genetic mutation in ENU-induced leukaemogenesis

According to reports, bone marrow is more susceptible to nitrosourea-mediated injury because it typically contains less alkyltransferase enzyme than other bodily tissues. Excision repair is the main method used to remove the changed bases because BM has low levels of the repair enzyme alkyltransferase. This results in a DNA strand break, which starts a cascade reaction downstream. When DNA breaks, a nuclear enzyme called poly [ADP-ribose] polymerase (PARP) is catalytically activated. Activated PARP then uses NAD+ as a substrate to initiate the production of poly [ADP ribose]. Poly [ADP-ribose] reduces the likelihood of recombination events and the corresponding change in chromosome structure by repelling DNA pieces due to their negative charge (Rhun et al., 1998; Satoh et al., 1994).

2.9.2 ENU-induced bone marrow suppression

The administration of ENU elicits bone marrow suppression causing a reduction in blood cell count, leading to pancytopenia. The inhibitory effect of nitrosoureas is evident between 3 to 6 weeks after the administration and sustains for an extra 2-3 weeks or beyond. This inhibition of bone marrow function frequently raises the chance of infection due to the presence of insufficient circulating immune cells post ENU treatment. Nitrosoureas exhibit harmful effects on the initial, non-renewable stem cells in the bone marrow suppressing the rapidly multiplying mature bone marrow progenitors in an individual. Examining from the molecular level, the delivered nitrosoureas, like ENU, cause the formation of altered guanine (O^6-methylguanine) that later pairs incorrectly or forms cross-links with deoxycytidine leading to marrow toxicity (Breeden et al., 1982; Todd H. Wasserman, Milan Slavik, 1975).

2.9.3 ENU-induced immune suppression

Nitrosoureas, such as ENU, are recognized for their capacity to induce considerable immunosuppression, mainly due to their potent effects on bone marrow suppression. These compounds damage early hematopoietic stem and progenitor cells, resulting in a significant decrease in circulating normal blood cells. It is seen, that typically the lowest blood cell counts happen around 4–6 weeks after treatment (Caignard et al., 2014). Myelosuppression, which is frequently observed with nitrosourea toxicity, particularly impacts the production of granulocytes and monocytes, diminishing the immune system's response and heightening the risk of infections (Ordoñez-Rueda et al., 2012). The production of healthy blood cells and their immune protection functionality are further hampered by mutagenesis caused by ENU by modifying important genes, that control haematopoiesis and immunological functions. Genetic

alterations in Gfi1, Stat4, Flt3, and Map3k8 impair granulocyte and natural killer (NK) cell function, preventing cytokine transmission and inflammatory immune responses (Caignard et al., 2014). ENU-induced mutations in genes such as Unc13d and Gimap5, blocking the action of cytotoxic T lymphocytes (CTLs) and increasing susceptibility to infections. Additionally, these genetic changes impair "cancer immunosurveillance," and compromise the immune system's ability to identify and eradicate newly developing cancer cells. ENU creates an environment that allows mutant bone marrow stem and progenitor cells to proliferate by impairing normal immune function, which may raise the risk of leukaemia and other malignancies. This dual effect of ENU—immunosuppression and mutagenesis—underscores its significant influence on haematopoiesis and immune regulatory processes.

2.10 Clinical presentations in ENU-induced leukemic animals

The manifestation of ENU-induced leukaemia varies in animals and their strains. The doses, experimental setup and other confounding and biological factors may also contribute to the presentation of the haematological diseases. The most common reported clinical symptoms are anaemia (less haemoglobin, ranging from 1.2 to 13.1 g/dl), abnormally low thrombocyte levels, and an abnormally increased WBC count (20,000–130,000 cells/μL). In cases of myeloid leukaemia, PB and BM smear analysis revealed an abundance of immature myeloid precursors or myeloblasts with distinctive granulated cytoplasm and a large round nucleus with many nucleoli. Leukaemia that grows repeatedly affects various body tissues, including the brain, liver, kidney, and spleen. Splenomegaly, or enlarged spleen is evident, and myeloblast build-up or blast infiltration in the red pulp are the main symptoms of splenic distress. The erythrogenic cells and platelet-producing megakaryocytes from the mouse spleen are replaced by these infiltrating blast cells. Myeloblasts are also reported to infiltrate into the portal triads of the hepatic lobules in liver, where they begin extramedullary hematopoiesis and characteristic hepatomegaly. (Fenske et al., 2006; Jonas et al., 2016). It is widely believed that the hematopoietic cells original mutations brought in by ENU may change their differentiation and survival pathways in leukemic animals, providing numerous advantages for the neoplastic survival in bone marrow and other extra medullary organs. It is possible that some alterations could make the progenitor cells more active in self-renewal and these mutations may give the damaged HSC a selective growth and survival advantage, resulting in completely converted leukemic stem cells (LSC) (Gilliland et al., 2004). The hallmark of leukaemia is the transformation of normal haematopoietic stem/progenitor cells, undergoing mutations into leukemic stem cells and entering into unlimited self-renewal events eventually giving rise to aberrant and poorly regulated organogenesis (Passegué et al., 2003).

3. Aims and Objectives

1. To characterise and establish a standardised reference range of cord blood cells in West Bengal population
2. To assess the ethnic difference in Indian population (focussing Bengal) when compared to other populations of the world
3. To analyse the difference in cellular and non-cellular components of cord blood, procured from urban and rural population of West Bengal
4. Characterization of isolated cord blood plasma factors (CBPF)
5. Development of leukemic murine model
6. Introduction of CBPF in leukemic treated group as a therapeutic intervention
7. Haematological profiling, bone marrow cell profiling, cell culture analysis and histopathological scenarios of experimental groups
8. Flow cytometric analysis of the transcription factors to study leukemic blast differentiation
9. Delineation of cord blood microenvironment: Scanning electron microscopy

4. Materials and Methods

4.1 General equipment and reagents

4.1.1 Instruments/Equipment

Instruments/Equipment	Supplier Company
Binocular Bright Field Light Microscope	Olympus, INDIA
Biological Incubator	Biological Enterprises, Delhi, INDIA
Camera	Canon, JAPAN
Clinical Centrifuge	Remi, INDIA
Colorimeter	EES-DIGI-8F, INDIA
Cooling Centrifuge	Remi , INDIA
Common Refrigerator	LG , INDIA
CO_2 Incubator (HF212 UV)	Heal Force R, GERMANY
Digital Photoelectric Colorimeter	Systronics , INDIA
Digital Weighing Machine	Wensar , Chennai , INDIA
Electronic Balance	Afcoset, India
ELISA Reader	
Flow cytometer (FACS-Calibur)	Becton, Dickinson and Company, USA
Filtration Unit	Milipore, USA
Hemocytometer	Rohem , INDIA
Horizontal Laminar Air Flow	Ultra Biotech , INDIA
Hot Air Oven	Biological Enterprises, Delhi, INDIA
Hot Water Bath	Bio Technics India , INDIA
Incubator with Shaker	Naugra Export, Ambala, INDIA
Manual Rotary Microtome (HM 325)	Leica Biosystems,India
Micropipettes	Glaxo, UK
Microwave Machine	LG , INDIA
Mini Table Top Centrifuge	Remi, INDIA
pH Meter	
-20°C Refrigerator	Vest frost , DENMARK
-80°C Refrigerator	Remi, INDIA; Celfrost, India
Scanning Electron Microscope (S-5330)	Zeiss EVO18, Jena, GERMANY
Spectrophotometer	Shimadzu UV-1800, JAPAN
Syringe Destroyer	Edutek Instrumentation , Ambala , INDIA
Trinocular Inverted Microscope with Phase Contrast Facility	Dewinter Technologies , ITALY
Vertical Autoclave Machine	Advanced Technocracy Inc., Ambala, INDIA
Vortex	Accurate Scientific Instruments, Thane, INDIA
Water Distillation Plant	Borosil , INDIA

4.1.2 Plastics

Plastic Equipment	Supplier Company
Beakers- 250ml	US Plastic Corporation, USA
Beakers- 500ml	US Plastic Corporation, USA
Centrifuge Tube-15ml	Falcon, USA
Centrifuge Tube-50ml	Falcon, USA
Centrifuge Tube-50ml	Falcon, USA
Cryo Box	Eppendorf, GERMANY
0.5 ml Cryo vials	Eppendorf, GERMANY
35mm Disposable Tissue Culture Plate	Corning, USA
60mm Disposable Tissue Culture Plate	Corning, USA
FACS Tubes	BD-Bioscience, USA
Micro-centrifuge Tube- 1.5ml	Eppendorf, GERMANY
Micro-centrifuge Tube- 2ml	Eppendorf, GERMANY
Micropipette Tips- 0.1-10µl, 200µl, 1000µl	Eppendorf, GERMANY
Micropipette Tips- 1-200µl	Eppendorf, GERMANY
Micropipette Tips- 100-1000µl	Eppendorf, GERMANY
Polystyrene Membrane Filter Unit	Millipore, USA
Tip Box	Eppendorf, GERMANY
75 sq.cm Tissue Culture Flasks	Corning, USA
Six Well Disposable Tissue Culture Plate	Falcon, USA
Squeeze Bottles	US Plastic Corporation, USA
(Disposable) syringe- 0.5ml	DispoVan, INDIA
(Disposable syringe)- 5ml	DispoVan, INDIA
(Disposable) syringe- 10ml	DispoVan, INDIA
Test-tube Racks	Naugra Export, Ambala, INDIA
Microcentrifuge Racks	Tesca Technologies Pvt. Ltd., Jaipur, INDIA
Graduated Measuring Cylinder	US Plastic Corporation, USA
Coplin Jar (for histology staining)	Sigma, USA
Drinking Water Bottle for Mice	Sigma, USA
Plastic Box	Sigma, USA

4.1.3 Glassware

Glass Equipment	Supplier Company
Beaker- 25ml	Borosil, INDIA
Beaker- 50ml	Borosil, INDIA
Beaker- 250ml	Borosil, INDIA
Beaker- 500ml	Borosil, INDIA
Coplin Jar (Xylene container)	Borosil, INDIA
Conical Flask- 50ml	Borosil, INDIA
Conical Flask- 100ml	Borosil, INDIA
Conical Flask- 250ml	Borosil, INDIA
Cover Slips	Blue Star, INDIA
Cuvette	Greiner, USA
Funnel	Borosil, INDIA
Graduated Centrifuge Tube	Borosil, INDIA
Graduated Measuring Cylinder	Borosil, INDIA
Graduated Measuring Flask	Borosil, INDIA
60mm Petri Plate	Borosil, INDIA
Reagent Bottle- 100ml	Borosil, INDIA
Reagent Bottle- 250ml	Borosil, INDIA
Reagent Bottle- 500ml	Borosil, INDIA
Glass Slides	Blue Star, INDIA
Watch Glass	Borosil, INDIA

4.1.4 Chemicals/Reagents

Chemicals/Reagents	Supplier Company
Acetone	Loba Cheme, INDIA
Bovine Serum Albumin	Sigma, USA
Cedarwood oil for Microscope	Merck, GERMANY
3,3'-Diamino Benzidine Tetrachloride	Sigma, USA
Drabkin's Solution	Organo Biotech Lab Pvt. New Delhi, INDIA
DPX	Sigma, USA
EDTA	Sigma, USA
Ethanol	Bengal Chemicals, INDIA
FACS Fluid	BD-Bioscience, USA
Formaldehyde	Merck, INDIA
Glacial Acetic Acid	Merck, INDIA
Glutaraldehyde (25% aqueous solution)	Lancaster, UK
Heparin	Braun, USA
Hi-Sep LSM-1077	Himedia, INDIA
H_2O_2	Merck, INDIA
Isopropyl alcohol	Merck, INDIA
Methanol	Loba Cheme, INDIA
Mounting media (Xylene cyanole)	Merck, USA
N-N' Ethyl nitrosourea (ENU)	Sigma, USA
Paraformaldehyde	Sigma, USA
Periodic Acid	Sigma, USA
Potassium dihydrogen phosphate	Merck, INDIA
Potassium Chloride	Merck, INDIA
RBC diluting fluid	International Biological Laboratories, INDIA
Sodium Chloride	Merck, INDIA
Sodium hydroxide	Merck, INDIA
di-Sodium hydrogen phosphate	Merck, INDIA
TritonX-100	Sigma, USA
WBC diluting fluid	International Biological Laboratories, INDIA
Xylene	Merck, INDIA

4.1.5 Stains and Fluids

Stains	Supplier Company
Brilliant Cresyl Blue Stain (Composition: Brilliant Cresyl Blue-1.0 gm{ Merck}, Sodium Citrate-0.4 gm and Sodium chloride solution-100ml)	Merck, USA
Eosin	Merck, USA
Giemsa	Sigma-Aldrich, USA
Hematoxylin- Mayer‘s	Sigma-Aldrich, USA
(Romanowsky stains) Lesihman	Sigma-Aldrich, USA
Schiff’s Reagent	Sigma-Aldrich, USA
Trypan Blue	Merck, INDIA

4.1.6 Cell Culture Media and supplements

Media and Supplements	Supplier Company
Fetal Bovine Serum (South American origin)	Lonza, USA
Penicillin G	Sigma, USA
RPMI-1640	Sigma, USA
Streptomycin	Sigma, USA

4.1.7 Others

Materials	Supplier Company
Animals: Inbred Swiss Albino Mice (Mixed Sexes)	Institutional Animal house
Cotton	Amalgamated Suppliers, Kolkata, India
De-ionised water	“
Filter paper	“
Gloves	Sigma, USA
Ice-bucket	Amalgamated Suppliers, Kolkata, India
Scissors	“
Surgical Blade	“
Tissue paper	“
Watch/Timer	“

4.2 Antibodies

4.2.1 Primary Antibodies

Primary Antibodies	Supplier Company
AhR	Cell Signaling Technologies, USA
CD-235a	Cell Signaling Technologies, USA
CEBPa	Cell Signaling Technologies, USA
GFI1	Cell Signaling Technologies, USA
Oct-4	Cell Signaling Technologies, USA
PU1	Cell Signaling Technologies, USA
RUNX	Cell Signaling Technologies, USA

4.2.2 Secondary Antibodies

Secondary Antibodies	Supplier Company
Goat anti rabbit IgG -AlexaFluor-488	Invitrogen, USA
Goat anti rabbit IgG-PE	Santa Cruz Biotechnology, USA

4.3 ELISA

Cytokines/Growth Factors	Supplier Company
IFNa2	RayBiotech, GA
IFNy	RayBiotech, GA
TGFb	RayBiotech, GA
TNFa	RayBiotech, GA

4.4 Cell Line

Cell Line	Supplier Company
Sarcoma-180	Chittaranjan National Cancer Institute, Kolkata, India

4.5 Software

Software	Supplier Company
Adobe photoshop	USA
Cell Quest Pro (v9.1) for FACS data analysis	Becton, Dickinson and Company , USA
FlowJo (v10.8.1) for FACS data analysis	Becton, Dickinson and Company , USA
Graph Pad Prism for Statistical analysis	USA
Microsoft Windows Office 2013	USA
SPSS (v16)	USA

4.6. Composition of Important Buffers and Media

4.6.1. 1X Phosphate Buffer Solution (PBS)

Constituents: NaCl – 8 g; KCl – 0.2 g; Na2HPO4 – 1.44 g; KH2PO4 – 0.24 g

Salts were dissolved in 800 ml of deionized water and pH was adjusted to 7.4 by HCl. Thereafter, the remaining volume was adjusted up to 1 liter.

4.6.2. 2% Paraformaldehyde Solution

Constituents: Paraformaldehyde -2g and PBS (1X) – 100ml

PBS solution containing 2g Paraformaldehyde was heated in a 56°C water bath until the paraformaldehyde dissolved properly (approximately 60 min). Thereafter, the solution was allowed to cool to room temperature and the pH was adjusted to 7.4 using 0.1M NaOH or 0.1M HCl as needed.

4.6.3. Primary Antibody Dilution Buffer

Constituents: BSA – 1g; Triton X-100 – 0.5ml; 1X PBS (0.01M) -100ml.

Adjusted pH- 7.2-7.4. Mixed well and stored at 4°C.

4.6.4. Secondary Antibody dilution Buffer

Constituents: Tween 20 - 0.5ml; 1X PBS (0.01M) – 100ml. Adjusted pH-7.2

4.6.5. RPMI-1640

Constituents: RPMI-1640 -1.0400g; Streptomycin - 0.010g; Penicillin G- 0.005g; Autoclaved deionized water – 100ml.

FBS was added following the decomplementation and as per requirement.

Note: The detailed methods of various experiments are available in respective chapters.

5. Standardization of Cord Blood Haematological Reference Ranges: A Comparative Study of Urban and Rural Populations in West Bengal Against Global Standards

Chapter Summary

The present chapter will help us understand the importance of standardisation of umbilical cord blood reference values in West Bengal. Across the nation, cord blood banking is growing its importance, and cord blood with its immense potential may offer clinical advantages in urgent medical situations. The haematological values of cord blood may differ depending on several factors like age, altitude, lifestyle, gestation period during pregnancy, ethnicity, and regional and genealogical groups, so we primarily focussed our study on the international and intra-national differences of cord blood haematological profile. Comparisons were also drawn between the cord blood values of urban and the rural population along with the mode of delivery- normal and caesarean deliveries. A new angle in terms of urban and rural population study is introduced in cord blood analysis, which was not recorded before. The standardisation of the cord blood reference range for the West Bengal population will not only help clinicians in taking decisions during the critical neonatal assessment and transplantations but will also open avenues for clinical research and translational medicine.

5.1 Introduction

Human umbilical cord blood (hUCB) is rich in hematopoietic stem/progenitor cells (HSPC) (Mayani & Lansdorp, 1998) and has immense clinical potential to be used as an appealing alternative source of hematopoietic stem cell transplantation for patients having a wide variety of diseases (Rocha et al., 2004) for its depressed or reduced immunological responses (Chalmers et al., 1998) and high availability. Post-partum cord blood was once considered biological waste product is now emerging as a potentially curative therapy in the amelioration of several haemato-pathological conditions like cancers and inherited non-malignant diseases, including bone marrow failure syndromes, inborn errors of metabolism and haemoglobinopathies (O'Brien et al., 2006). As mentioned, umbilical cord blood (UCB) offers extensive logistic and clinical advantages like significantly faster availability of banked cryopreserved UCB units, with patients receiving umbilical cord blood transplantation (UCBT) in a median of 25 to 36 days earlier than those receiving bone marrow (Barker et al., 2002)(Dalle et al., 2004); extension of the donor pool despite 1 to 2 HLA mismatch; lower

incidence and severity of acute graft-versus-host disease (GVHD); lack of risk to the donor and higher frequency of rare haplotypes compared with bone marrow registries; lack of donor attrition; and lower risk of transmitting infections by latent viruses- cytomegalovirus and Epstein-Barr virus (Davey et al., 2004). The disadvantages of UCBT include the low number of hematopoietic stem/progenitor cells in UCB compared to bone marrow or mobilised adult peripheral blood, which translates into an increased risk of graft failure and delayed hematopoietic engraftment. The impossibility of using donor lymphocyte transfusion for immunotherapy is another concern for using UCB HSPC.

Reports have shown that children who received cord blood for transplantation from unrelated donors were able to reconstitute haematopoiesis and achieve sustained engraftment in most cases, there were low incidences of GVHD, and did not result in a higher relapse risk (Dalle et al., 2004) (Davey et al., 2004) (Gluckman et al., 1997) (Michel et al., 2003) (Rubinstein et al., 1999) (Wagner et al., 2002) (Staba et al., 2004). Studies reveal the comparison of the results of UCBT and Bone Marrow Transplantation (BMT) from unrelated donors in children is of paramount importance because for many patients the search process will identify both Unrelated Bone Marrow (UBM) donors and UCB units. Some published studies have reported retrospective analyses of the comparing outcomes after UCBT and UBMT in children (Rocha et al., 2001)(Barker et al., 2001)(Eapen et al., 2007). Details of patients, their disease conditions, and transplant characteristics are enlisted in (**Figure 12**) as reported by (Rocha et al., 2004). These published data strongly suggest the consideration of UCB as an acceptable alternative to matched unrelated BM in children and verily support the start of a simultaneous search for BM and UCB-unrelated donors. The decision on the final selection of unrelated donor BM vs UCB must be based on the urgency of the transplant and the characteristics of the BM and UCB unrelated donor such as cell dose and HLA compatibility etc. Children who are requiring the urgency of transplantation, UCB will be advantageous.

The UCB HSPCs with their self-renewal and differentiation ability differentiate into multi-hematopoietic lineages (Lu et al., 1993) thus leading to the opening of numerous opportunities in the field of translational research and other therapeutic interventions (Mahla, 2016; Weiss & Troyer, 2006). UCB being the reservoir of HSPCs evolve into a valuable source of immature cells that may be engineered and manipulated to obtain advanced cell therapy products such as in tissue repair (Peterson, 2004) and gene therapy (Kohn et al., 1995). Complete Blood Count (CBC) provides us with the haematological status and health status of the newborns. However, conducting blood tests has some negative aspects, especially in newborns, such as ensuring a

minimally invasive procedure, difficulty in finding the vein to collect peripheral blood, not enough blood can be taken for the test, and some possible local complications. For these reasons, some studies have been conducted on whether or not to use UCB instead of venous blood for CBC screening in newborns (Prakash et al., 2017) (Imam et al., 2011). The advantages of procurement of umbilical cord blood for HSPCs include an easy and harmless collection procedure, which is safe for both the mothers and the newborns, possibly reduced graft-versus-host reactivity, low viral contamination (Tse & Laughlin, 2005) and a painless procedure (Costakos et al., 2009). When cord blood is procured through proper guidelines availability of samples is high and the risk of transmissible infections is low (Lanfranchi et al., 2009). Studies revealed umbilical cord blood can also be considered an excellent alternative to neonatal blood for the evaluation of sepsis in infants (Carroll et al., 2012a; Hansen et al., 2005).

Studies revealed extensive research work is already done to standardise the reference values of human peripheral blood but work on the haematological reference values of umbilical cord blood is in progression (Pranke et al., 2001a; Walka et al., 1998). Ethnicity differs based on geographical, socio-cultural, and genealogical aspects and one can certainly expect differences in haematological profile in people belonging to different races and nationalities. Some of the previous works were done to establish a reference range of cord blood values focussing mostly on Caucasian populations (Forestier et al., 1991; Noguera et al., 1999; Redźko et al., 2005) and a few South East Asian populations (Jan et al., 2007; Lee et al., 2009). Since ethnicity ensures a difference in blood parameters we expected to unearth the difference in the cord blood haematological profile of the Indian population.

The target of the present study was to obtain important information by analysing cellular components of cord blood and to assess the ethnic difference in the Indian population (focussing Bengal) when compared to the Caucasian and other ethnic populations of the world. Emphasis was also given to studying the haematological difference based on area i.e. urban and rural population of Bengal and mode of delivery i.e. normal and caesarean. To avoid overlapping of data, four groups were made consisting of urban and rural population as two major divisions and mode of delivery as sub-divisions. Thus the groups formed were urban-normal, urban-caesarean, rural normal and rural caesarean. Through this study, we reported both international and intra-national differences in cord blood parameters and anticipated establishing a reference value for further scientific progressions.

Characteristic	Unrelated Cord-Blood Transplant (N=98)	Unrelated Bone Marrow Transplant (N=584)	P Value†
Patient-related			
Age — yr			
Median	24.5	32	<0.001
Range	15–55	15–59	
Male sex — no. (%)	50 (51)	318 (54)	0.53
Weight — kg			
Median	58	68	<0.001
Range	38–92	40–108	
Positive cytomegalovirus serologic status before transplantation — no./total no. (%)	63/94 (67)	161/288 (56)	0.05
Disease-related			
Type of disease — no. (%)			0.12
Acute myeloblastic leukemia	45 (46)	317 (54)	
Acute lymphoblastic leukemia	53 (54)	267 (46)	
Status at transplant			<0.002
First complete remission	26 (27)	197 (34)	
Second complete remission	21 (21)	191 (33)	
More advanced phase	51 (52)	196 (34)	
Previous autologous transplant	19 (19)	44 (8)	<0.001
Disease classification — no./total no. (%)			
Acute lymphoblastic leukemia			
Phenotype			0.08
T-cell	12/43 (28)	26/161 (16)	
B-cell	31/43 (72)	135/161 (84)	
Cytogenetics			0.17
t(9;22)	16/26 (62)	36/79 (46)	
t(4;11)	1/26 (4)	3/79 (4)	
Others	9/26 (35)	40/79 (50)	
Acute myeloblastic leukemia			
French–American–British			0.98
M5, M6, M7	8/40 (20)	60/297 (20)	
Others	32/40 (80)	237/297 (80)	
Cytogenetics‡			0.27
Favorable	7/25 (28)	20/123 (16)	
Intermediate	17/25 (68)	90/123 (73)	
Poor	1/25 (4)	13/123 (11)	
Donor-related			
Age — yr			
Median	—	36	
Range	—	19–57	
Positive cytomegalovirus serologic status before transplantation — no./total no. (%)	—	117/287 (41)	
HLA compatibility — no./total no. (%)§			<0.001
6 of 6	6/95 (6)	584 (100)	
5 of 6	48/95 (51)	—	
4 of 6	37/95 (39)	—	
3 of 6	4/95 (4)	—	
Transplantation-related			
Year of transplantation			
Median	2000	1999	0.001
Range	1998–2002	1998–2002	
Conditioning regimen — no. (%)			
Regimen based on total-body irradiation	64 (65)¶	426 (73)	0.10
Regimen based on busulphan	34 (35)¶	158 (27)	0.10
Antithymocyte or antilymphocyte globulin	75 (77)	216 (37)	<0.001
Prophylaxis against graft-versus-host disease — no. (%)			<0.001
Cyclosporine alone	6 (6)	25 (4)	
Cyclosporine and corticosteroids	69 (70)	2 (0.3)	
Cyclosporine and methotrexate	9 (9)	554 (95)	
Other	14 (14)‖	3 (0.5)	
No. of nucleated cells infused — $\times 10^8$/kg			
Median	0.23	2.9	<0.001
Range	0.09–0.6	<1.0–9	

* Percentages may not sum to 100 because of rounding.
† The chi-square test was used for categorical variables, and the Mann–Whitney nonparametric test for continuous variables.
‡ Cytogenetic features associated with a favorable risk were t(8;21), t(15;17), or inv(16), and with a poor risk were monosomy 7, 11q23 abnormalities, monosomy 5, del (5q), abnormal 3q, t(6;9), or a complex karyotype. The remaining cytogenetic abnormalities were classified in the intermediate-risk group.
§ HLA compatibility was defined by HLA-A and B by means of serology or low-resolution DNA typing and by HLA-DRB1 by means of high-resolution typing.
¶ In the cord-blood group, the regimen based on total-body irradiation included cyclophosphamide (22 patients), melphalan (3), or fludarabine alone (5) or in combination with two or more drugs (34); the regimen based on busulfan included cyclophosphamide (13 patients), melphalan (3), or cyclophosphamide plus thiotepa (17) or other drugs (1).
‖ Other prophylaxes included regimens based on tacrolimus.

Figure 12: Details of the recipients of cord blood and bone marrow transplants from unrelated donors, adapted from (Rocha et al., 2004). Table considered as a figure.

5.2 Materials and methods

5.2.1. Procurement of Cord Blood

Umbilical cord blood was collected from consenting mothers (average age: 26.1 and 38-40 weeks' gestation period) as per the ethical guidelines and permission obtained from Clinical Research Ethics Committee (CREC) (**Ethical Approval Number: CREC-STM/52**) of Calcutta School of Tropical Medicine and Medical College, Kolkata. An average of 40-45 ml of cord blood was directly collected from the transected cord (**Figure 13**) in sterile heparinized 50ml Falcon Tube (Falcon, USA) (**Figure 14**), immediately after the separation of the infant from the mother at Eden Hospital, Medical College with the help of gynaecologists and obstetricians in both labour room and operation theatre. Mothers having issues like diabetes mellitus, hepatitis, respiratory problems, blood infections, genitourinary disease, eclampsia and previous cases of miscarriages were not taken into consideration for this study. After the blood collection, the samples were taken to the Dept. of Biochemistry and Medical Biotechnology Laboratory, School of Tropical Medicine within 10-15 mins in 4°-8°C ice containers. Cord blood procurement was followed by the informed consent of the mothers and patient parties. Patient details were collected from the daily medical reports of the hospital for scientific analysis. The mothers were from different districts of the southern part of West Bengal that includes major cities, towns and rural areas. Along with the cord blood, adult peripheral blood (APB) was also collected in sterile heparinized vials from a population of normal consenting adults (average age: 23.5), these consenting adults were not the delivering mothers. An average of 10 ml of peripheral blood was collected by venepuncture at the blood sample centres of School of Tropical Medicine, Kolkata.

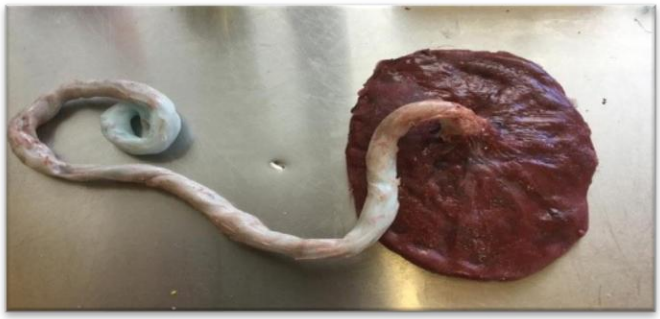


Figure 13: Umbilical cord attached to placenta

Figure 14: Umbilical cord blood post delivery

5.2.2. Analysis of cord blood for haemogram and biochemical parameters

Cord Blood haematological profiling

A total of 10-12 ml of the collected undiluted cord blood sample was used for the blood haemogram and biochemical parameter analysis at the laboratory. Haematological analyses were performed using both the manual and automatic multiparameter haematology analyser, Beckman Coulter. Complete Blood Count (CBC) was done including Haemoglobin concentration (Hb), Red Blood Cells (RBC), White Blood Cells (WBC), Packed Cell Volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin concentration (MCHC), Platelets Count, WBC Differential Count.

The haemoglobin level estimation was conducted both manually and in an automated analyser whereas the glucose level estimation was strictly done using an automated analyser for biochemical assay from both peripheral blood of mothers' and cord blood samples.

Haemoglobin estimation

The haemoglobin estimation of umbilical cord blood was performed colorimetrically by diluting 10µl of blood with 2.5ml of Drabkin's solution and taking the reading at 540nm after 10 mins incubation at room temperature (RT).

Total red blood cell count

The total red blood cell (RBC) count was estimated by diluting 10 µl of umbilical cord blood with 1990 µl of Dacie's fluid and incubating for 10 mins at RT. After incubation, 150 µl of the mixed solution was charged into the hematocytometer chamber and counting was done in the RBC chamber.

Total white blood cell count

The total white blood cell (WBC) count was estimated by diluting 10 µl of umbilical cord blood with 190 µl of Turk's fluid and incubating for 10 mins at RT. After incubation, 150 µl of the mixed solution was charged into the haemocytometer chamber and counting was done in the WBC chamber.

Reticulocyte count

Reticulocyte count was done by staining unfixed umbilical cord blood with Brilliant Cresyl Blue (Himedia S066, India) solution in 1:1 ratio and incubated for 45 minutes at room

temperature. After incubation, the stained blood was smeared on a glass slide and observed under the binocular microscope. The juvenile red cells, or reticulocytes contain remnants of basophilic ribonucleoproteins. The supra-vital stain reacts with remnants of riboproteins to form a bluish black precipitate of granules or filaments. Approximately 1000 cells were counted under the microscope (Olympus CH20i, Japan) and among them, the total number of reticulocytes was determined. (**Figure 15 A**)

WBC Differential count

10 µl of umbilical cord blood was smeared onto a glass slide and stained with Leishman (Himedia, India) stain to examine the cytology and differential count of cells. The smeared slide was flooded with Leishman stain and incubated for 3 minutes with a cover (to avoid drying and evaporation of alcohol present in the stain), after incubation, the smear was flooded with distilled water for 7 minutes and finally washed with tap water and air dried before being studied under the microscope (Olympus CH20i, Japan) (**Figure 15 B**).

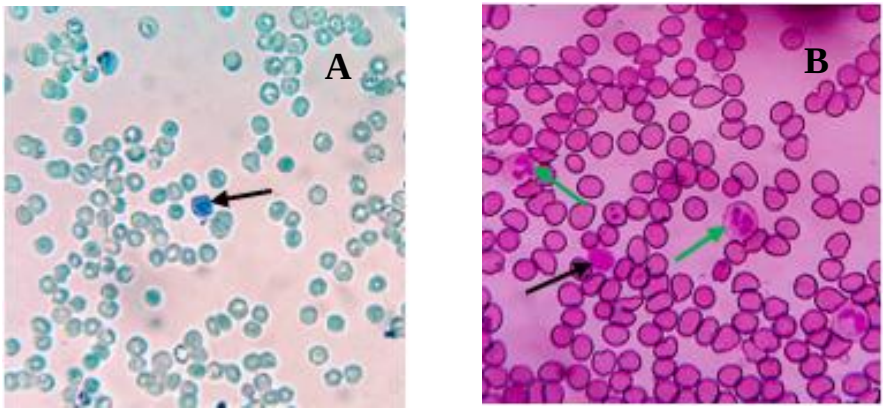


Figure 15: Cytological evaluation of cold blood cells: A. Reticulocyte stain using Brilliant Cresyl Blue (shown with black arrow). B) Differential staining by Leishman stain depicting different WBC populations like large lymphocyte (shown with black arrow) and neutrophils (shown with green arrows). Images captured at 40X.

5.2.3 Isolation of Cord Blood and Adult Peripheral Blood Plasma

For the isolation of cord blood and adult peripheral blood plasma, the whole blood from two groups was centrifuged at 200g for 10 min at 4°C, the plasma was carefully pipetted out and stored at -80°C till further analysis. The frozen CBP aliquots were thawed in a 37°C water bath before being used in the experiment. An individual lot of CBP is prepared from a single cord blood sample. CBP samples were pooled in as “pooled CBP”. Analysis to determine the

number of platelets in each sample was not performed; hence, the isolated and pooled plasma cannot be specifically characterised as “platelet rich” or “platelet-poor” plasma.

5.2.4. Isolation of Mononuclear Cells (MNC)

The isolation of MNC from umbilical cord blood was done using density-gradient centrifugation within 30 minutes of procurement. Preparation of the blood for centrifugation was performed in a highly sterilised environment, within the Laminar Air Flow hood. The cord blood (preferably stored in 4°C) was first diluted with equal volume (1:1 ratio) of cold sterilised 1X Phosphate Buffered Saline Solution (PBS) (pH: 7.4) or 0.9% Sodium Chloride Solution (NaCl). A total of 6ml of cold diluted cord blood was carefully topped or layered over 3ml of HiSep LSM 1077 solution (Himedia, India) (room temperature), a ratio of 1:2 in a 15ml centrifuge tube (Falcon, USA). Careful measures were taken so that the HiSep solution was not disturbed and the blood was topped on the solution without mixing. The centrifuge tubes were then placed in (REMI R8C, India) centrifuge machine and centrifuged at 500g for 25 minutes (Bieback, 2004) without brakes.

After centrifugation the content of the tube was separated in 4 broad layers. The topmost supernatant contains plasma, consisting of platelets and cord blood plasma factors (CBPF). At the interphase of plasma and clear HiSep solution was a thin cloudy buffy coat consisting of mononuclear cells. The bottom-most layer right below the HiSep layer consists of erythrocytes and granulocytes. The plasma layer was pipetted out and discarded without disturbing the buffy coat. The MNC layer was prudently pipetted out and was washed twice with chilled 1X PBS or 0.9% NaCl by centrifugation for 5 mins at 400g. Post washing the cells were resuspended using 1X PBS or 0.9% NaCl, cell counting and viability were done using Tuerk and Trypan Blue stains (**Figure 16**).

5.2.5. Staining of Isolated Mononuclear Cells

The freshly isolated MNCs were stained using Giemsa stain. The Geimsa stain was diluted using distilled water in the ratio 1:9 (1ml stain with 9ml distilled water). 10 µl of the isolated single-celled mononuclear cell suspension was smeared on a clean glass slide, Giemsa solution was added after air-drying. The stained cells were incubated at room temperature, covered and away from the air for 10 mins, and then washed with tap water. The stained slide was then observed under the microscope (Olympus CH20i, Japan) to study the cellular morphology.

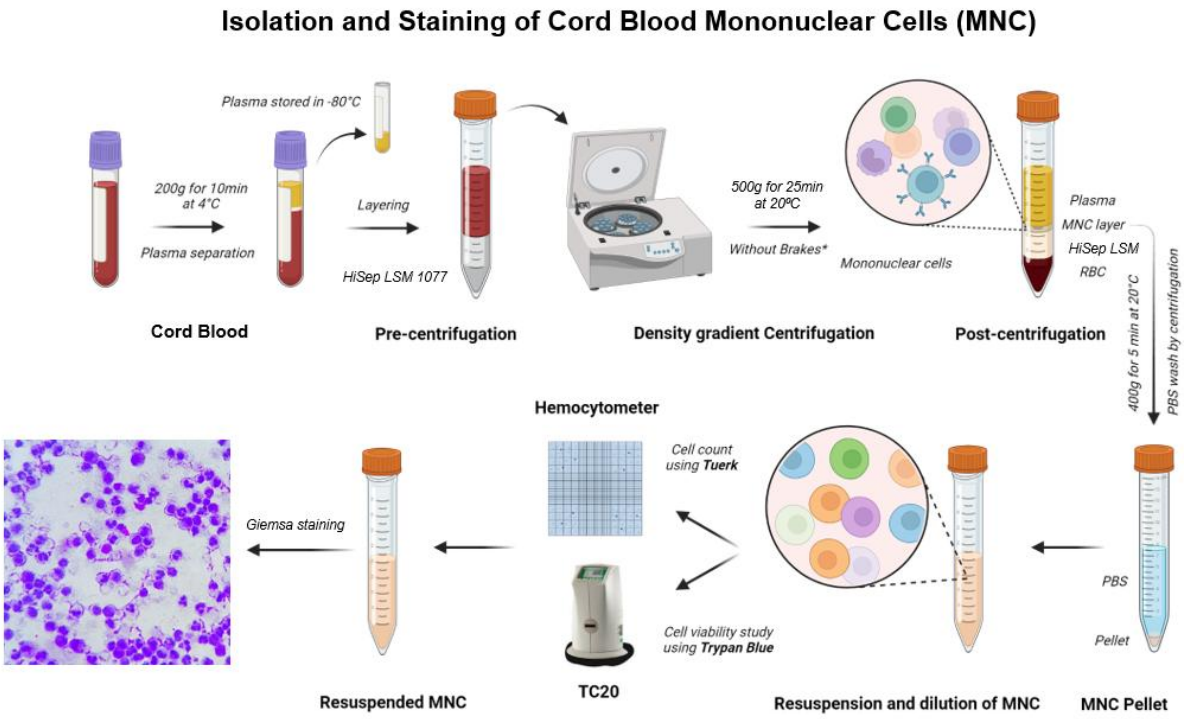


Figure 16: Schematic illustration of isolation procedure and staining of cord blood mononuclear cells

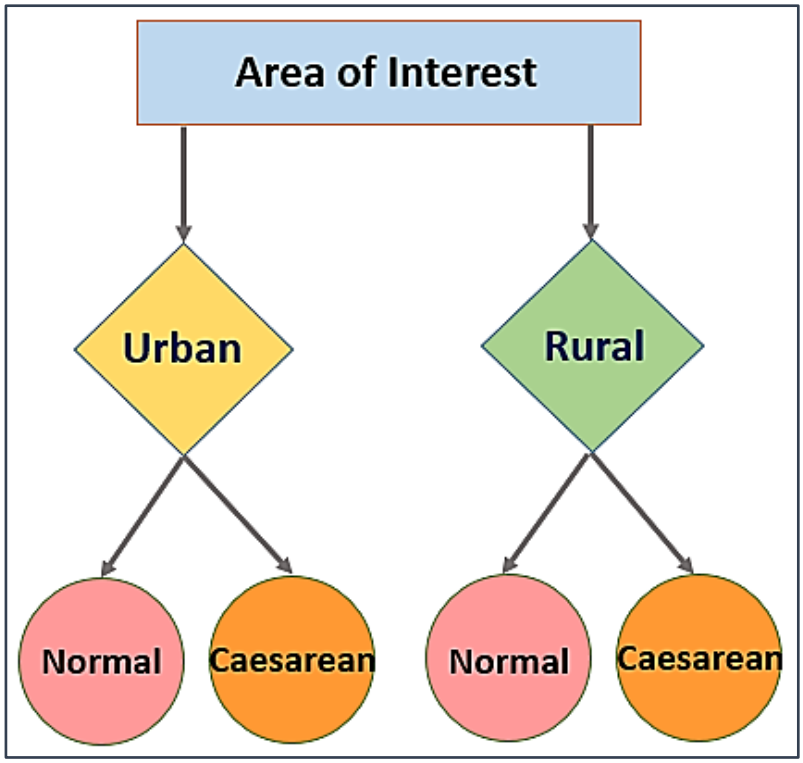


Figure 17: Schematic diagram of the experimental design

5.2.6 Determination of cytokines and growth factors in blood plasma

To quantify the cord blood plasma factor (growth factors and cytokines) levels, the stored plasma aliquots were thawed and TGFβ, TNFα, IFNγ and IFNα2 were evaluated using the standard ELISA kits (RayBiotech, GA). The enzyme-linked immunosorbent assay (ELISA) tests were performed as per the manufacturer's instructions (RayBiotech). The plasma samples were analysed and the median value was taken for the analysis. The lower limits of detection for cytokines and growth factors were different for each experiment (Patra et al., 2021). Pooled APB plasma was used as a control to evaluate the cytokine levels of cord blood plasma.

5.3 Statistical Analysis

Differential statistics and Independent Student's t-test with the normal distribution of different variables in SPSS version 16 was used. Statistical significance was considered at $P < 0.05$.

5.4 Results

The Mean±SD of the consenting mothers' peripheral blood data was estimated. The glucose level (mg/dL) of the mothers' peripheral blood was 84.0±16.65 and was significantly high than cord blood which was 71.52±23.22 (*p*-value = 0.03). The maternal haemoglobin (g/dL) was 11.2±1.2 which was significantly less than the cord blood haemoglobin value, 15.2±1.4 (*p*-value = <0.001) (**Figure 18 A and 18 B**). We noticed the cord blood haemoglobin value of our study was within the standard European range 11.0-18.0 g/dL but more than the South East Asian population value, which is within 11.2g/dL.

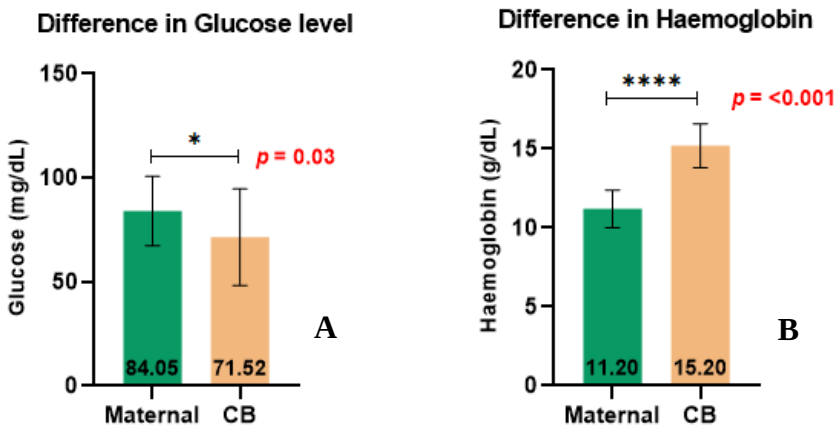


Figure 18: Graphical representation of biochemical components of blood: A. Graphical representation of difference in glucose level (mg/dL) in mother's peripheral blood and cord blood. B. Graphical representation of difference in haemoglobin concentration (g/dL) in mother's peripheral blood and cord blood

The estimated Mean±SD values of total umbilical cord blood RBC (10^6/mm^3), PCV (%), MCV (fL), MCH (Pg) and MCHC (g/dL) were 4.3±0.6, 46.7±4.8, 105.8±5.3, 34.3±2.3 and 32.4±0.9. The analysed data estimated the values of total cord blood WBC (10^3/mm^3), neutrophil (%), lymphocyte (%), eosinophil (%) and monocyte (%) to be 16.2±4.7, 62.1±12.8, 31.1±13.8, 3.7±1.8 and 1.8±0.9. The total cord blood platelet count (10^3/mm^3), was 226.8±73 (Figure). We separately analysed urban-normal, urban caesarean, rural-normal and rural caesarean groups and compared the observed values of their cord blood RBC (10^6/mm^3), Hb (g/dL), PCV (%), MCV (fL), MCH (Pg), MCHC (g/dL), WBC (10^3/mm^3), neutrophil (%), lymphocyte (%), eosinophil (%), monocyte (%) and platelet count (10^3/mm^3) (**Figure 19**).

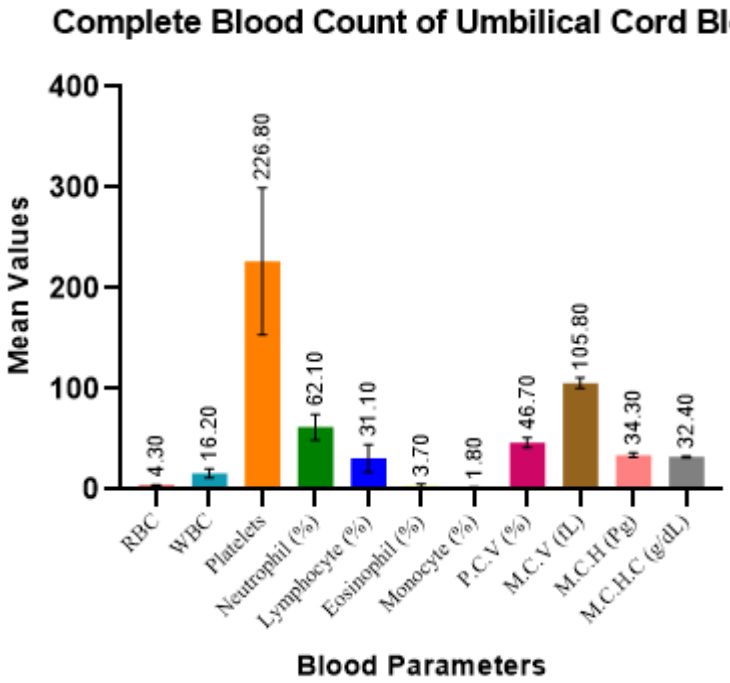


Figure 19: Graphical representation of cord blood parameters

Since our study primarily focussed on the difference in haematological parameters of urban and rural population along with the mode of delivery, we estimated the values of MCV (fL), WBC (10^3/mm^3), neutrophil (%), lymphocyte (%), eosinophil (%), monocyte (%) and platelets count of urban and rural groups separately and normal and caesarean groups separately. It was observed that there were no statistical differences found in normal vs caesarean group (**Table 1**). The estimated MCV value showed a statistical difference in urban population 105.1± 5.0 vs the rural estimated range 102.7± 6.6 with a *p*-value of 0.043 (<0.05). The mean value of neutrophil in urban population of West Bengal was 60.0±13.3 which was statistically less than the rural population's estimated mean 66.3±9.0 with a *p*-value of 0.04. As expected the mean

value of lymphocyte in urban population of West Bengal was 33.6±14.5 which was statistically high than the rural population's estimated mean of 26.8±10.4 (p = 0.047). The platelet count (10^3/mm^3) of the urban was 236.3±73.5 and the rural population was 203.7±69.6 and showed no statistical differences. There were no statistical differences noticed in the observed values of eosinophil between the two groups but monocytes of the rural population were statistically high than the urban population with a p-value of 0.01. Parameters like RBC and WBC didn't show any statistical differences between the groups (**Figure 20 A to 20 G**).

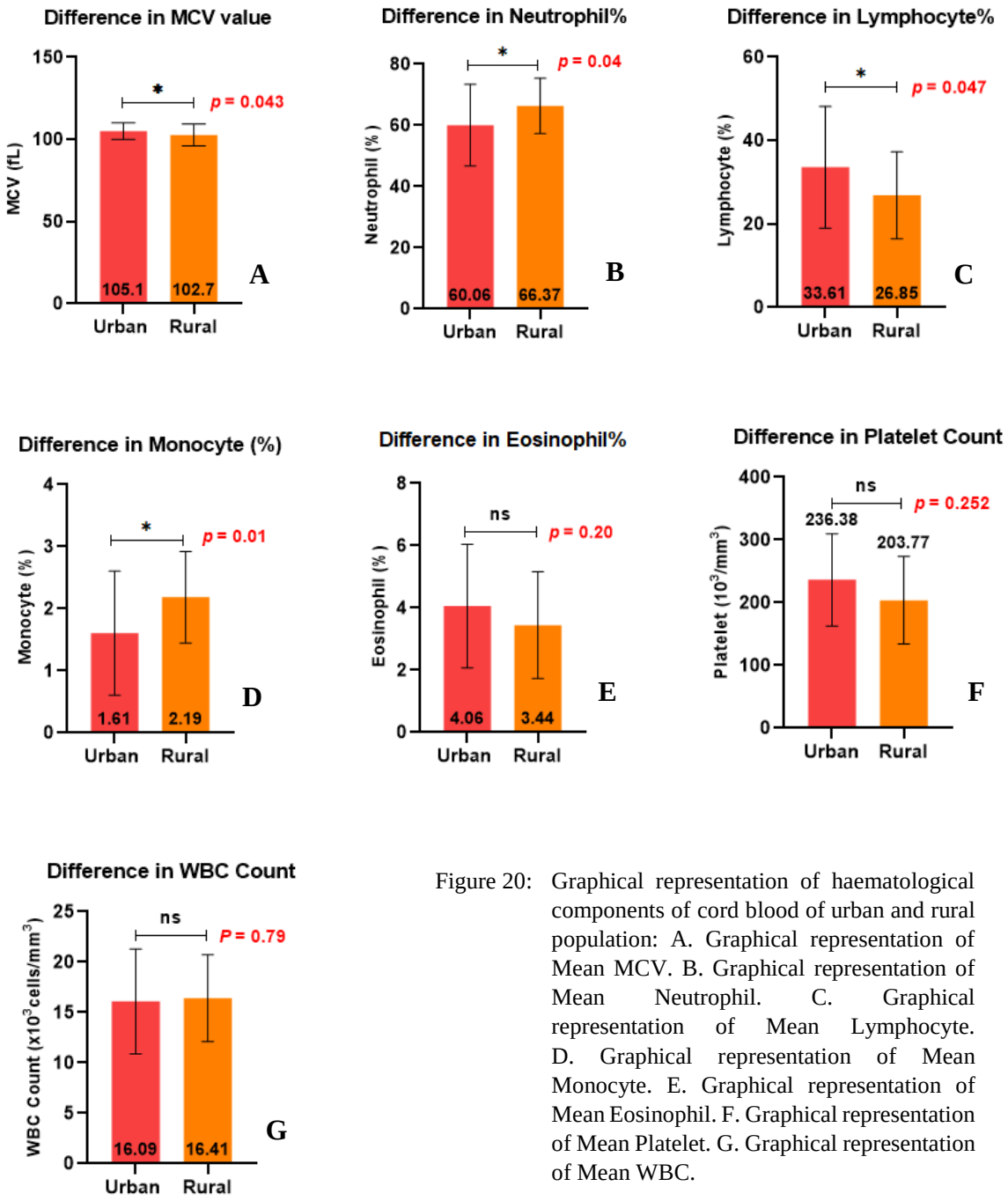


Figure 20: Graphical representation of haematological components of cord blood of urban and rural population: A. Graphical representation of Mean MCV. B. Graphical representation of Mean Neutrophil. C. Graphical representation of Mean Lymphocyte. D. Graphical representation of Mean Monocyte. E. Graphical representation of Mean Eosinophil. F. Graphical representation of Mean Platelet. G. Graphical representation of Mean WBC.

The estimated values of each haematological parameter and glucose level from the four groups urban-normal (UN), urban caesarean (UC), rural-normal (RN) and rural caesarean (RC) were analysed and we observed some interesting results. There were no statistical differences observed in WBC count, neutrophil (%), lymphocyte (%), and glucose level but interestingly the UN showed statistically higher eosinophil than UC and RN showed statistically higher value than RC, we also observed the eosinophil value of UN was statistically 2 fold higher than RC with a *p*-value of 0.001. In monocyte population UC was statistically higher than RN by 2 folds with a *p*-value of 0.009. Graphical estimation of the blood parameters from four groups is given in (**Figure 21 A to 21 F**).

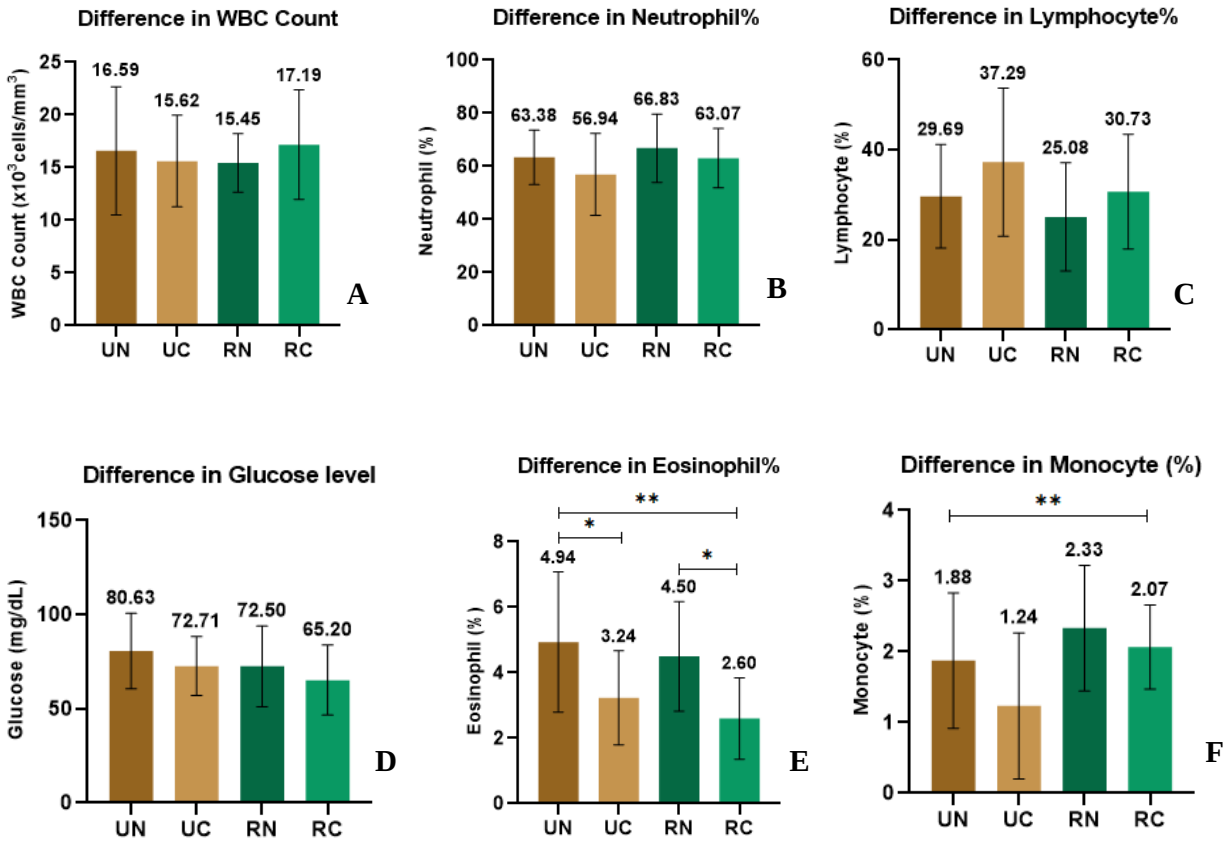


Figure 21: Graphical representation of biochemical and haematological parameters of Urban-Normal (UN), Urban-Caesarean (UC), Rural-Normal (RN) and Rural Caesarean (RC). A. Graphical representation of WBC. B. Graphical representation of Neutrophil. C. Graphical representation of Lymphocyte. D. Graphical representation of Glucose level. E. Graphical representation of Eosinophil. F. Graphical representation of Monocyte.

The results showed difference in haematological parameters of West Bengal when studied internationally or in perspective to global scenario. It was noticed that the estimated mean value of total WBC population was higher when compared to Caucasian population like USA and Greece. We observed the value was also higher for our study than the South East Asian population like Korea and Taiwan, African population like Nigeria, Middle Eastern like Iraq, and East Asian population like Pakistan and Nepal (**Figure 22 A**). When intra-national comparison was drawn, it was noticed that the value of West Bengal's WBC was similar to that of Chennai's but was higher than Chandigarh (**Figure 22 B**). The neutrophil count in West Bengal was found to be higher than USA, Korea, Taiwan as well as Chennai and Chandigarh of India. The estimated value of lymphocyte in West Bengal was found to be as equal to USA but lesser than Taiwan and slightly lesser than Korea and much lesser in values when compared to other countries like Iraq, Pakistan and Nepal. It was also less when compared to other Indian regions like Chennai and Chandigarh. The eosinophil mean value of West Bengal was equal to USA but higher than other international countries as well as higher than the two Indian cities stated in our study. The Interesting observation was the mean value of monocyte population in West Bengal was marginally more than Nepal but far less than countries like USA, Greece, Taiwan, Korea, Iraq and Pakistan even lesser than Chennai. (**Table 2)**

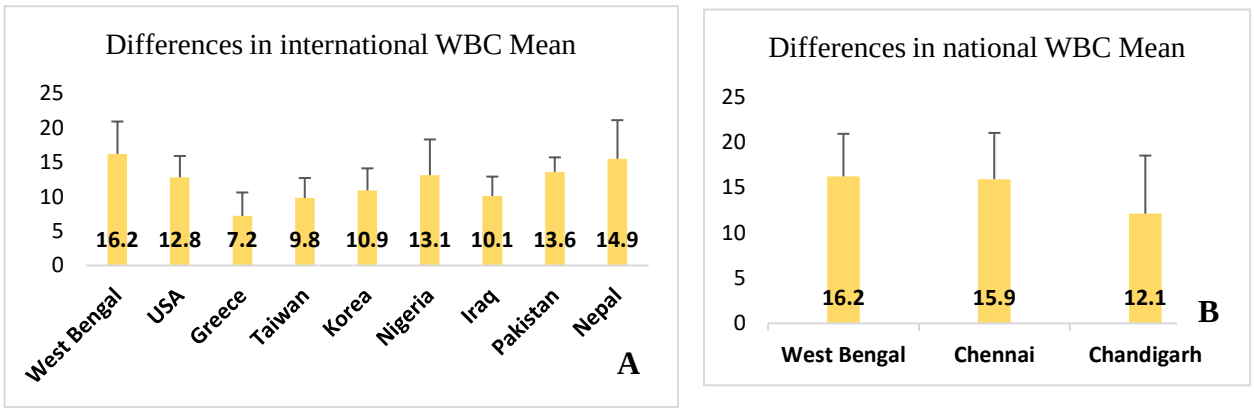


Figure 22. Graphical representation of international and intra-national total WBC: A. Graphical representation of WBC count from different countries when compared to West Bengal. B. Graphical representation of WBC count of Indian cities.

Quantification of Growth factors and Cytokines

The concentrations of CBP and APB plasma cytokines and growth factors are shown in (**Figure 23 A to 23 D**). The ELISA kits had a lower and a higher detection limit of 18 pg/ml- 4000 pg/ml for TGFβ; 30 pg/ml-6000 pg/ml for TNF-α; 1 ng/ml-400 ng/ml for IFNγ and 5 ng/L-1000 ng/L for IFNα2. The concentration of TGFβ (pg/ml) in CBP (3445±168.1) was significantly higher than adult (838.9±112.8), with p = <0.0001. The expression of TNF-α (pg/ml), IFNγ (ng/ml) and IFNα2 (ng/L) in CBP (249±26.57; 33.49±2.2; 38.71±1.47) showed significantly lower expression than their equivalent adult peripheral blood plasma (756.2±10.25; 81.57±8.57 and 92.35±1.91), with p values <0.0001; 0.0015 and <0.0001.

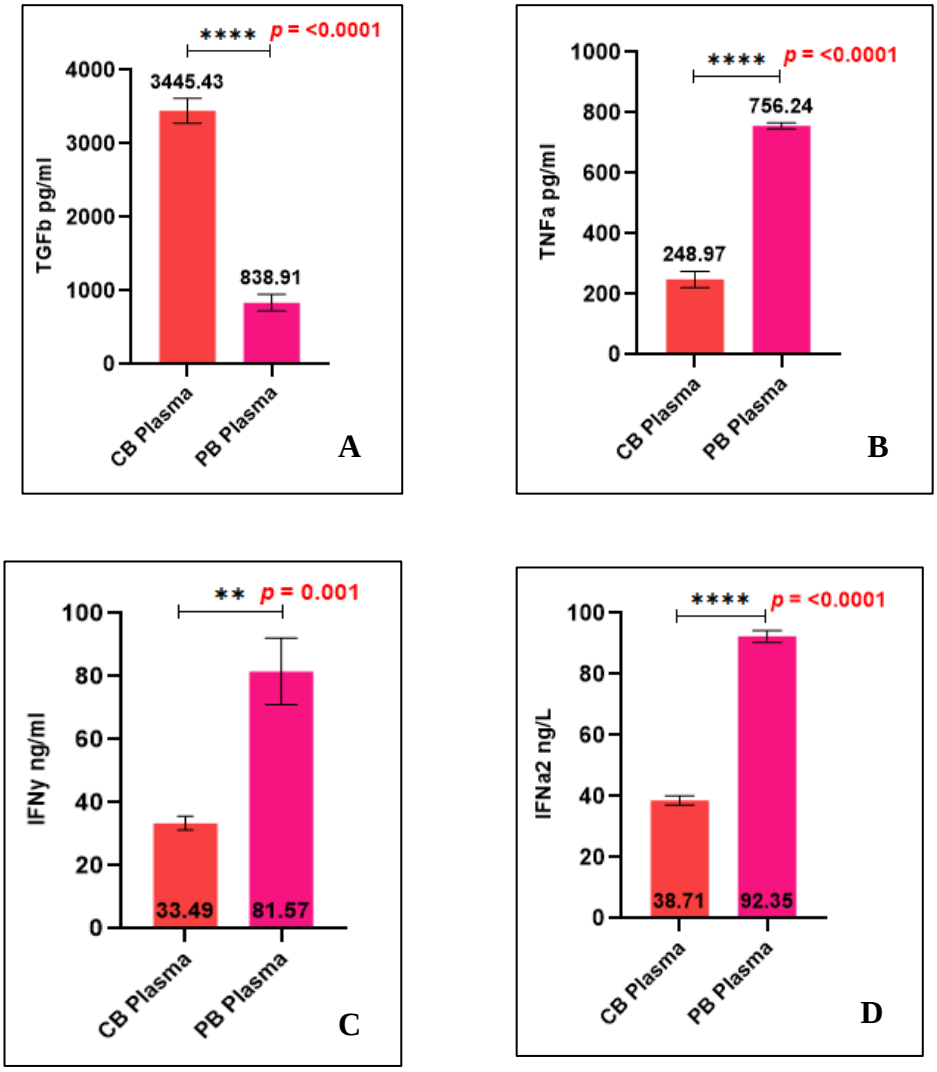


Figure 23: Cytokine profiling of cord and peripheral blood plasma. A. Concentration of TGFβ pg/ml; B. Concentration of TNFα pg/ml; C. Concentration of IFNγ ng/ml; D. Concentration of IFNα2 ng/L.

5.5 Discussion

In government and private hospitals, neonates often undergo clinical evaluations to assess their post-partum health status. In order to undergo a haematological assessment, neonates are often subjected to potentially painful phlebotomy procedures for laboratory testing. The use of cord blood laboratory values has been suggested as a means to decrease the risk of painful venipuncture and anaemia. Cord blood is the remaining blood of the placenta and the long and helical cord that connects the foetus with the mother for substances exchange (Spurway et al., 2012) that is collected to study the profound quantitative and qualitative hematologic differences of neonates when compared to older children and adults (Proytcheva, 2009). Studies documented, haematological parameters of term new-borns (at birth) have significantly higher values than those of older children and adults (Jacob, 2016). Thus, it is inappropriate if adult reference ranges are being used for the assessment of paediatric blood (Glasser et al., 2015). On the other hand, considering utilising a reference interval obtained from some other population to study the population of interest could potentially lead to inappropriate patient management and unnecessary use of resources (Berg & Lane, 2011).

The present study was conducted for the characterisation and standardisation of reference range of cord blood parameters in West Bengal population and to assess the ethnic difference in Indian population (focussing West Bengal) when compared to various other populations of the world. We also analysed the difference in cellular components of cord blood, procured from urban and rural population of West Bengal to bring a new angle to the study. Previous studies already documented works on cord blood in respect of gender, weight, mode of delivery and gestation period (CLSI, 2008) but little or no work is done on the difference in cord blood parameters when compared to urban and rural populations. Since ethical variations play a vital role in constructing socio-cultural aspects that might affect the differences in haematological parameters, the design of the experiment was framed in a manner where comparison can be drawn on both international and intra-national levels (**Figure 17**).

In both the urban and rural population there was an increase in neutrophil and monocyte mean values in normal delivery when compared to caesarean delivery. In the case of lymphocyte count the mean values in caesarean delivery was more when compared to normal delivery. Our observed mean differences were in sync with a previous study which showed statistically significant results (Glasser et al., 2015) but no statistical differences were observed. The total WBC values in the case of the urban population were similar to the previous work but the WBC

of the rural population showed a slightly different result. It was observed both eosinophil and platelet count showed higher values in normal delivery when compared to caesarean delivery. Our study also showed a statistically high value of neutrophil count in rural areas and a statistically high lymphocyte count in urban areas which is an interesting result. The range of PVC was higher than Greece and Taiwan but almost similar to other countries and two Indian states. The MCH, MCV, and MCHC of the Indian population were within the European and South East Asian standard reference range.

The Mean and SD value of RBC was within the standard reference range 4.0-6.2 (10^6 cells/ul) but the value for WBC was more than the usual Caucasians, South East Asians (Taiwan and Korea), and Nigerian population (Adewumi et al., 2014), Ethiopia (Angelo et al., 2021), Turkey (Gunduz & Temel, 2021), Iraq (Al-Marzoki et al., 2012), Pakistan (Qaiser et al., 2009), Nepal (Basnet et al., 2016) and even higher when compared to other Indian cities (Marwaha et al., 1992; Suman et al., 2015), which is a unique finding. The standard European reference value is 4.00-11.0 (10^3 cells/ul) which is similar to South East population and Nigerian but lesser than the estimated Indian value (Y. H. Chang et al., 2011; Katsares et al., 2009; Lee et al., 2009). Our studies showed the values of Neutrophils, Lymphocytes and Monocytes in the Bengal population were within the reference range of the South Asian population which are 11.0-69.0 (Neutrophils), 17.0-56.0 (Lymphocytes) and 1-17 (Monocytes) but when the values of urban and rural groups were observed with the normal and caesarean delivery, the ranges within the groups showed differences. The mean neutrophil value of the rural-caesarean group was lesser than the urban-normal, rural-normal and rural caesarean groups, eventually making the lymphocyte value higher than the three mentioned groups. The eosinophil value of urban-normal and rural-normal was recorded to be higher than the other groups and they showed statistical differences within the groups too. As we succeeded to standardise a local reference range and reported the difference in the urban and rural population in cord blood parameters for the first time focussing on Bengal, we assume this can be an important study in the Indian clinical scenario. Though there is no obvious explanation for the higher difference in WBC population in Bengal as compared to the global scenario and differences in cord blood parameters between urban and rural populations, we expect further studies can unveil the possible reasoning and give a better clinical perspective.

Blood Parameters	West Bengal			
	Urban		Rural	
	Normal	Caesarean	Normal	Caesarean
RBC	4.5±0.3	4.3±0.6	4.6±0.5	4.5±0.6
Hb (g/dL)	15.9±1.2	15.1±1.9	15.2±1.0	14.4±0.8
P.C.V (%)	48.5±4.1	45.5±5.8	47.9±3.7	45.0±3.9
M.C.V (fL)	106.1±1.9	106.7±5.9	104.8±8.0	104.4±6.2
M.C.H (Pg)	34.3±1.0	34.5±2.4	34.0±3.6	33.9±2.9
M.C.H.C (g/dL)	32.4±0.7	32.4±0.8	32.1±1.4	32.3±1.2
WBC	16.5±6.0	15.6±4.3	15.4±2.7	17.1±5.1
Neutrophil (%)	63.3±10.2	56.9±15.3	66.8±12.8	63.0±11.2
Lymphocyte (%)	29.6±11.5	37.2±16.4	25.0±12.0	30.7±12.7
Eosinophil (%)	4.9±2.1	3.2±1.4	4.5±1.6	2.6±1.2
Monocyte (%)	1.8±0.9	1.2±1.0	2.3±0.8	2.0±0.5
Platelets	254.7±66.8	218.0±78.2	216.8±103.0	195.6±45.3

Table 1: Table representing cord blood parameter differences in urban and rural population and mode of delivery via normal and caesarean delivery.

	West Bengal	USA (38-41 weeks)	Greece	Taiwan	Korea	Nigeria	Iraq	Pakistan	Nepal	Chennai	Chandigarh
RBC	4.3 ± 0.6	4.2	2.4 ± 0.8	3.2 ± 0.4	_	4.0 ± 0.5	4.2 ± 0.4	4.2 ± 0.4	4.3 ± 0.6	4.1 ± 0.4	_
Hb (g/dL)	15.2 ± 1.4	14.4	8.8 ± 2.9	11.2 ± 1.5	11.8 ± 1.4	13.2 ± 1.5	13.7 ± 1.4	14.9 ± 1.4	15.2 ± 1.9	14.9 ± 1.7	_
P.C.V (%)	46.7 ± 4.8	45.9	25.9 ± 8.8	36.9 ± 4.6	_	44.8 ± 5.7	44.4 ± 4.7	45.6 ± 4.8	48.7 ± 6.8	44.6 ± 5.3	_
M.C.V (fL)	105.8 ± 5.3	109	105 ± 6	115 ± 6.8	_	110.3 ± 11.8	111.5 ± 6.0	105.8 ± 6.2	101.2 ± 6.0	108.1 ± 4.8	_
M.C.H (Pg)	34.3 ± 2.3	34.2	35.8 ± 3.1	34.9 ± 1.9	_	32.6 ± 4.1	34.4 ± 2.3	34.9 ± 2.1	33.9 ± 2.2	36 ± 1.7	_
M.C.H.C (g/dL)	32.4 ± 0.9	31.4	34.3 ± 7.3	30.3 ± 1.2	_	29.7 ± 1.6	30.9 ± 1.9	32.4 ± 2.1	33.2 ± 1.5	33.3 ± 0.8	_
WBC	16.2 ± 4.7	12.8	7.2 ± 3.4	9.8 ± 2.9	10.9 ± 3.2	13.1 ± 5.2	10.1 ± 2.8	13.6 ± 4.2	14.9 ± 4.4	15.9 ± 5.1	12.1 ± 6.4
Neutrophil (%)	62.1 ± 12.8	54.2	_	44.9 ± 14.7	57.2 ± 8.0	_	51 ± 11.24	50.1 ± 12.4	63.0 ±11.6	50.3 ± 12.2	52.5 ± 16
Lymphocyte (%)	31.1 ± 13.8	31.2	_	33.8 ± 10.9	30.9 ± 7.4	_	39.8 ± 10.17	39.8 ± 12.2	35.2 ± 11.5	35.9 ± 12.2	41.6 ± 16.2
Eosinophil (%)	3.7 ± 1.8	3	1.22 ± 0.97	2.6 ± 2.3	2.9 ± 1.7	_	1.22 ± 0.97	3.3 ± 2.1	1.2 ± 1.5	2.9 ± 2	2.3 ± 1.9
Monocyte (%)	1.8 ± 0.9	10.5	7.85 ± 2.77	8.8 ± 4.4	8.5 ± 2.0	_	7.8 ± 2.77	6.5 ± 2.8	0.48 ± 0.7	8.9 ± 3.1	_
Platelets	226.8 ± 73	173	160 ± 59	217 ± 45	208 ± 39	225.0 ± 72.2	267.6 ± 60.6	256.2 ± 76.5	226.8 ± 61.2	215 ± 67	199.2 ± 56.6

Table 2: Table representing cord blood parameter differences of different countries when compared to West Bengal.

5.6 Conclusion

The analyses ensure a difference in haematological parameters in Indian population as and when compared to the usual standard reference values of other ethnic communities like Caucasians, South East Asians and Nigerians. It is also noticed that urban and rural population of Southern part of West Bengal shows difference in neutrophil, lymphocyte and eosinophil count. The study ascertains its contribution to the standardisation of reference value of cord blood parameters in Indian population, focusing West Bengal.

6. Comparative Analysis of Cord and Peripheral Blood Erythrocytes Using Scanning Electron Microscopy and Flow Cytometry

Chapter Summary

The human umbilical cord blood (hUCB) is rich in hematopoietic stem/progenitor cells (HSPC) and a complete understanding of haematological status and physiological distinction of the cord blood cells can help clinicians to take proper curative regimens as and when neonates undergo clinical investigation. Through this chapter, we aimed to focus on the importance of the morphological distinction of erythrocytes, isolated from cord and adult peripheral blood (ABP) using techniques like high-resolution scanning electron microscopy (SEM) and surface protein expression using flow cytometry. Some conventional biochemical analyses like osmotic fragility of the cell membrane, haemoglobin co-oxidation study, and lipid peroxidation assay were also performed along with membrane protein content using gel electrophoresis (SDS-PAGE). The images from our SEM study indicated clear morphological variations in the cord erythrocyte population with a higher degree of cellular deformities and differences in membrane texture when compared to APB erythrocytes. Flow cytometric analysis of cord erythrocyte revealed a significant difference in CD235a expression when compared to the adults and we also observed overexpression of GLUT1 and decreased expression of Band 3 in cord erythrocyte membrane. As supporting evidence to the arguments made in this study, we also showed cord erythrocytes have low osmotic fragility, a slower rate of co-oxidation of cord haemoglobin, and a lesser lipid peroxidation level than that of adults. Therefore, from this study, we concluded that cord blood erythrocytes have deeper indentations leading to higher flexibility or pliability, more oxygen-carrying capacity and less osmotic fragility in comparison to adult erythrocytes.

6.1 Introduction

Foetal and neonatal anaemias are some of the major complications of pregnancy and postnatal development that have been reported in various research studies. Some of the documented reasons for foetal anaemia include- immune haemolytic disease (Liley, 2003), fetomaternal or twin-to-twin haemorrhages, defects in haemoglobin structure and synthesis and viral infections like parvovirus B19 (C. Brugnara and O. S. Platt, 2003). Contrariwise neonatal anaemia can result from foetal anaemia, impaired erythrocyte production by bone marrow, post-partum haemorrhage due to obstetric accidents (C. Brugnara and O. S. Platt, 2003) and frequent

drawing of blood for laboratory testing from new-borns (Colombatti et al., 2016; Jopling et al., 2009). The standard practice for the treatments of foetal and neonatal anaemia is transfusions of red blood cells (RBCs), either by intrauterine or by intravenous procedure (Hume, 1997), to help replace the lost RBCs of the foetus or neonate. In the present-day world healthcare system, blood transfusions play an important role in clinical practice. Records indicate, up to 90 % of extremely prematurely born infants receive at least one red blood cells transfusion in their early life (Bianchi et al., 2021; Heeger et al., 2019; Howarth et al., 2018; Strauss, 2010)

Blood transfusion is an important and frequent component of neonatal intensive care and like any other scientific field, it is a continuously evolving discipline. The frequent requirement of blood transfusions is for the acute treatment of perinatal haemorrhagic shock in neonates and the recurrent correction of the anaemic foetus and infants (Whyte et al., 2014). Foetus and neonates with anaemia usually receive intrauterine (Moise, 1993) and intravenous transfusions (the RBC components of the blood) derived from the adult donors (Zhurova et al., 2012). In recently investigated studies, umbilical cord blood (UCB) is considered an excellent alternative source of blood transfusions (Bianchi et al., 2018). In the past 20 years collection and processing of umbilical cord blood for allogenic and autologous transfusions have been extensively studied (Garritsen et al., 2003; Khodabux et al., 2011; Orlando et al., 2020; Teofili et al., 2020; Widing et al., 2007). Adult peripheral red blood cells are morphologically and biophysically different from foetal or neonatal blood (S. K. Jain, 1989; Matovcik & Mentzer, 1985; Oski, 1972, 1973). The umbilical cord blood erythrocyte component, containing the haemoglobin molecule, plays an important role in blood transfusion that involves- the transportation of oxygen from the blood to tissues, transportation of carbon dioxide from tissues to the lungs and maintenance of the physiological pH of the blood by being a buffer system in the body (Högman & Meryman, 2006). The foetal and umbilical cord haemoglobin consists of two alpha and two gamma subunits ($\alpha_2\gamma_2$) which have a higher affinity for oxygen as compared to the adult that have two alpha and two beta subunits ($\alpha_2\beta_2$) (De Halleux et al., 2002). Due to the presence of gamma subunits, umbilical cord blood differs from adult peripheral blood haemoglobin content. The haemoglobin variants distribution in the adult peripheral blood is >95 % HbA, ~2.5 % HbA_2, and <1 % HbF, whereas in the new-borns the distribution is 30 % HbA, 0 % HbA_2 and almost 70 % HbF (Barreto Henriksson et al., 2022). Gathering information from the aforementioned studies we can conclude that instead of using blood units from adult donors, umbilical cord erythrocyte units could be used for transfusion

as an alternative to reduce anaemia, hyperoxemia, and fluctuating oxygenation which are prominent risk factors for neonatal morbidities.

Though umbilical cord blood is considered a biological waste it is an excellent source of low immunogenic hematopoietic stem and progenitor cells (HSPC) (Mayani & Lansdorp, 1998) and is gradually getting recognition in the field of diagnostic and translational medicines. As cord blood's cellular parameters are similar to that of the foetal or neonatal venous blood (Carroll et al., 2012b) it can be considered to be a safer and painless alternative source for various haematological studies (Costakos et al., 2009; Lanfranchi et al., 2009) and blood transfusion in case of hematopathological conditions and therapies. During the isolation of stem cells from the procured cord blood, the RBC component of neonates is often discarded and less importance is given due to its high rate of haemolysis when compared to adult RBC (Zhurova et al., 2012). The membrane composition and biophysical properties of cord blood RBC greatly differ from that of adult RBC, thus making them unique for further studies in the field of haematology and cell biology (S. Orkin, 2015). As discussed, the erythrocyte haemoglobin structure is one of the unique physiological differences found between umbilical cord blood and adult blood. The difference is due to the presence of high concentration of foetal haemoglobin (Stiene-Martin et al., 1998; Zijlstra et al., 1991). In our study, we concentrated on the morphological differences and cytochemical variations in cord and adult peripheral blood RBC using high-resolution Scanning Electron Microscopy, gel electrophoresis, flow cytometric, and other biochemical analyses. Our observation led to the analysis of some unique features of cord erythrocytes that varied from that of adult peripheral blood. The studied results of this chapter indicated morphological variations, the difference in size and structure through scanning electron microscopy; the difference in membrane composition and proteins, cell count and surface protein expression using flow cytometry.

Previous studies paved the way to ensure qualitative and quantitative differences in the blood parameters between an adult and a new-born focussing their arguments on the developmental changes of foetal haematopoiesis (Proytcheva, 2009), our study focussed majorly on the red blood cells of the cord blood when compared to the venous blood of the adults. Several studies reported considerable changes in the haematological profile of one-day-old neonates and that of babies who were three days old and surprisingly, there were changes in values observed as the children grew with years. It was studied that there is a steady decline in blood parameters due to the decrease in erythropoietin level (Christensen et al., 2012; Saleh et al., 2013). Profound changes in haematological parameters are recorded in pre-term babies and post-term

babies when compared to the adults (Jacob, 2016) but the little angle is drawn on the very subject that involves umbilical cord blood of newborns and not the venous blood of the neonates. Through our study, we could indicate the morphological differences and cytochemical variations in cord blood and adult peripheral blood RBC which would ensure great importance in clinical diagnosis and research.

6.2 Materials and methods

6.2.1 Procurement of adult peripheral blood and cord blood

The peripheral blood was collected in sterile heparinised vials from a population of normal consenting adults (average age: 23.5), who volunteered for the study. An average of 8-10 ml of blood was collected by venepuncture at the sample collection centres of Medical College, Kolkata and Calcutta School of Tropical Medicine, Kolkata. The umbilical cord blood was collected in sterile heparinised Falcon Tubes (Falcon, USA) from consenting mothers who delivered full-term new-borns (average age of mothers: 26.1 years and gestation period: 38-40 weeks) who underwent both caesarean and normal deliveries. In our study, both the participating groups comprised non-smoking individuals with no history of recreational drug intake, and a minimum of 12.5g/dL of blood haemoglobin were considered. Details of cord blood collection is elaborated in chapter 5 materials and methods. Consenting adult participants and delivering mothers with hypertension, diabetes mellitus, hepatitis, respiratory problems, blood infections like HPV and HIV and genitourinary diseases were not taken into consideration. Pregnant mothers having eclampsia and previous miscarriage cases were also avoided at the time of sampling. Blood samples with clot formation and haemolysis were excluded from the study. After the collection of blood from the participants, the samples were taken to the Dept. of Biochemistry and Medical Biotechnology laboratory, School of Tropical Medicine, Kolkata within 10-15 mins in 4°-8°C ice containers. The study ensured written informed consent to be taken from both the adult participants and delivering mothers before the procurement of peripheral and cord blood samples.

6.2.2 Morphological studies of erythrocytes under high resolution scanning electron microscopy (SEM)

The heparinised uncoagulated whole blood from adults and cord blood from delivering mothers were washed by centrifugation three times with phosphate-buffered saline (PBS) to remove the plasma and buffy coat. After washing, the erythrocytes were collected from the base of the

pellet and were fixed with 2.5% glutaraldehyde in PBS for 1 h. The samples were again washed with PBS for 5 min × three times followed by a serial dehydration technique, with 30%, 50%, 70%, 90% and three times with 100% (absolute) ethanol. The cells were then dried, mounted, and coated with platinum, and observed under a high-resolution scanning electron microscope (Zeiss EVO18 special edition SEM, Jena, Germany). The micrographs were taken at 15 kV accelerating voltage (Buys et al., 2013).

6.2.3 Osmotic fragility test (OFT)

Twenty microliters of erythrocyte suspension (25%) from both the participating groups were gently transferred into tubes containing 2 ml of graded concentrations of NaCl i.e., 0.85 g%, 0.44 g% and 0 g% respectively. All the tubes were incubated at 37ºC for 30 min and then centrifuged at 1300 g for 5 min (Sikdar et al., 2017; Van Der Walt & Russell, 1978). The obtained supernatant thus contained various concentrations of haemoglobin derived from haemolysed erythrocytes. The percentage of haemolysis was detected by monitoring the concentrations of haemoglobin in the supernatant spectrophotometrically (Shimadzu UV-1800, Kyoto, Japan) at 540 nm. The percentage of haemolysis estimated in each concentration of NaCl was calculated assuming 100% haemolysis in the case of 0 g% NaCl.

6.2.4 Haemoglobin co-oxidation study

Wallace and team stated human haemoglobin oxidizes spontaneously at a slower rate (auto-oxidation); but in the presence of an oxidizing agent (co-oxidation), the rate of oxidation increases due to nucleophilic displacement of superoxide by anions (Wallace et al., 1974). In our study, we used nitroblue tetrazolium (NBT), containing two tetrazole moieties, as a strong oxidizing agent (Abugo & Rifkind, 1994). Here we studied the co-oxidation process of haemoglobin in cord blood as compared to adult blood. Briefly, 4×10^{-5} M purified HHb in 0.01 M PB, pH 7.4 was titrated by adding 1×10^{-4}M NBT and monitored spectrophotometrically at 630 nm. The formation of methaemoglobin was measured using molar extinction co-efficient, 3.7 mM^{-1} cm^{-1} at 630 nm (Elmer et al., 2009).

6.2.5. Preparation of erythrocyte membrane

About 1 ml of venous blood was collected in a vial containing heparin. It was then mixed thoroughly to avoid clotting. About 3 ml of PBS was added to it. After gentle mixing, it was centrifuged at 1800–2000 rpm for 3 min. Then the supernatant was discarded carefully and the process was repeated 3–4 times. The remaining packed cell volume (PCV) was resuspended in

1.5 ml hypotonic buffer solution (1×10^{-2} M Tris HCl, 1.44×10^{-3} M K_2EDTA, Cocktail protease inhibitor), pH 7.4 for complete lysis of cells. After 15 min, the hemolysate was centrifuged at 20,000 × g for 30 min at 4°C. The RBC membrane pellet thus obtained was carefully washed with isotonic buffer (1×10^{-2} M Tris HCl, 1.44×10^{-3} M K_2EDTA, 1.7×10^{-2} M NaCl), pH 7.8 several times for complete removal of haemoglobin. Then it was resuspended in buffer (1×10^{-2} M Tris HCl, pH 7.4) and kept in several aliquots, and preserved at –20°C for further uses (Rocha-Pereira et al., 2004).

6.2.6 Quantification of erythrocyte membrane proteins

Quantification of the isolated membrane protein was performed by the standard Lowry method using bovine serum albumin (BSA) as the standard protein. The quantified protein was then subjected to various experiments (Lowry, 1951)

6.2.7 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of membrane proteins

SDS-PAGE of the quantified membrane protein was done using 12% running gel solution and 4% stacking gel solution. The gel was stained using Coomassie blue staining (Agre et al., 1985; Chevallet et al., 2006; Jovanovic et al., 2007).

6.2.8 Lipid peroxidation assay

Lipid peroxidation is being studied extensively concerning disease, modulation by antioxidants and other contexts. In lipid peroxidation, oxidants like free radicals or nonradical species attack the lipid molecules containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs), which involve hydrogen abstraction from a carbon molecule, with oxygen insertion resulting in lipid peroxyl radicles (Ayala et al., 2014). A large number of by-products are formed during this process. The most common method used is the estimation of the aldehydic products by their ability to react with the thiobarbituric acid (TBA) that would yield 'thiobarbituric acid reactive substances' (TBARS), which can be measured spectrophotometrically. One millilitre (ml) of LPO reagent (TBA-0.375%, TCA-15%, 0.25N HCl) was mixed with 50 µl of protein sample. The mixture was boiled at a temperature of 80ºC in the water bath for 20 minutes. After cooling, the mixture was then centrifuged for 5 min at 2000 rpm. The supernatant was measured at 532 nm (Sawas & Pentyala, 2004).

6.2.9 Isolation of red blood cells (RBC) for Flow cytometry

The isolation of red blood cells (RBC) from both the adult peripheral and the cord blood samples was done by repeated centrifugation using 1X PBS solution within 30 minutes of procurement. Around 2 ml of whole undiluted blood samples were centrifuged at 2000 rpm at room temperature for 10 minutes using REMI (R8C) centrifuge machine. Post centrifugation, the plasma layer was pipetted out and discarded. To the pellet, 2 ml of 1X PBS solution was added and thoroughly resuspended. The resuspended pellet was then washed by centrifugation at 2000 rpm at room temperature for 10 minutes, this centrifugation, resuspension and aspiration steps were repeated 3-4 times. After the final centrifugation wash, the supernatant was discarded and from the bottom-most layer of the pellet (packed cell volume) 200ul of the sample containing RBC is carefully pipetted out.

6.2.10 Flow cytometry preparation

Flow cytometric analysis of erythrocyte or red blood (RBC) cell samples was done after preparation and isolation from adult peripheral blood (PB) and cord blood (CB). The isolated RBC from peripheral and cord blood were prepared for flow cytometric analysis by fixing in 1.5% paraformaldehyde (PFA) for 30 mins in complete darkness followed by a thorough wash with 1X PBS solution. The fixed erythrocytes were suspended in sheath fluid (BD Bioscience, USA) and divided into polystyrene FACS tubes (BD Bioscience, USA) at a concentration of $1x10^6$ cells/ml. For the analysis of surface protein marker expression, the fixed cells, at a concentration of $1x10^6$ cells/ml were stained with 2µL primary antibody (Cell Signalling Technology, USA) against CD235a (raised in mouse and reactive against human) and were incubated for 30 mins at 37°C in an incubator. Incubated cells were then counter-stained with a secondary antibody (anti-mouse IgG) conjugated with Alexa flour 488 (Invitrogen, USA) and further incubated in the dark at 37°C for 30 mins. Finally, the stained cells were washed with 1X PBS and prepared for flow cytometry. The unstained samples and stained samples of CB and PB were assessed using BD FACS Calibur flow cytometer (Becton Dickinson, USA) and analysed using CellQuest Pro software (v9.1 Becton Dickinson).

Gating was established to compare the differences in the population of adult peripheral blood and umbilical cord blood erythrocytes. Gating strategies involved the grouping of cellular components, emphasising red blood cells in similar positions of the dot plots of both PB and CB samples. Differences in the percentage of cells and the expression of CD235a from the cellular distribution pattern are taken into consideration. The side scatter (SSC) and forward

scatter (FSC) settings were represented in the Logarithmic scale for the dot plot analysis of the percentage of unstained CB RBC and PB RBC cells (**Figure 26 A and 26 B**). The cell percentage difference and Mean Fluorescence Intensity difference were estimated accordingly in the **Table 3**. The histogram overlay plot (**Figure 26 D**) was done to express the Mean Fluorescence Intensity (MFI) difference **Table 4** in CB RBC and PB RBC stained with antibody marker CD235a Alexa 488.

6.3 Statistical Significance

Independent Student's t-test with the normal distribution of different variables was used in SPSS version 16. Statistical significance was considered at $P < 0.05$.

6.4. Results

6.4.1 Scanning Electron Microscopy (SEM) analysis of erythrocyte

To investigate the morphological alteration of cord blood's erythrocytes, scanning electron microscopy (SEM) analysis was carried out which implicates different degrees of torus or dent formation on the surface of erythrocytes of cord blood as compared to normal adult blood. It was observed some cord blood erythrocytes have deeper indentations and some expressed little or no dents when compared to adult peripheral blood erythrocytes (**Figure 24 A and 24 B**). Study reveals that dent formation in erythrocytes is due to the increased viscous property of blood (Baskurt & Meiselman, 2007) and the contractility of NMIIA (Non-muscle myosin II A) motor proteins (Smith et al., 2018). It was also noticed the cord erythrocytes with deeper indentions showed rougher surfaces (**Figure 24 C**) when compared to the ones with lesser dents (**Figure 24 D**). Through this study, another observation surfaced that cord erythrocytes with shallow dents have pits on them (**Figure 24 E**) whereas the adult erythrocytes seldom showed any rough surfaces or pits on them (**Figure 24 F**).

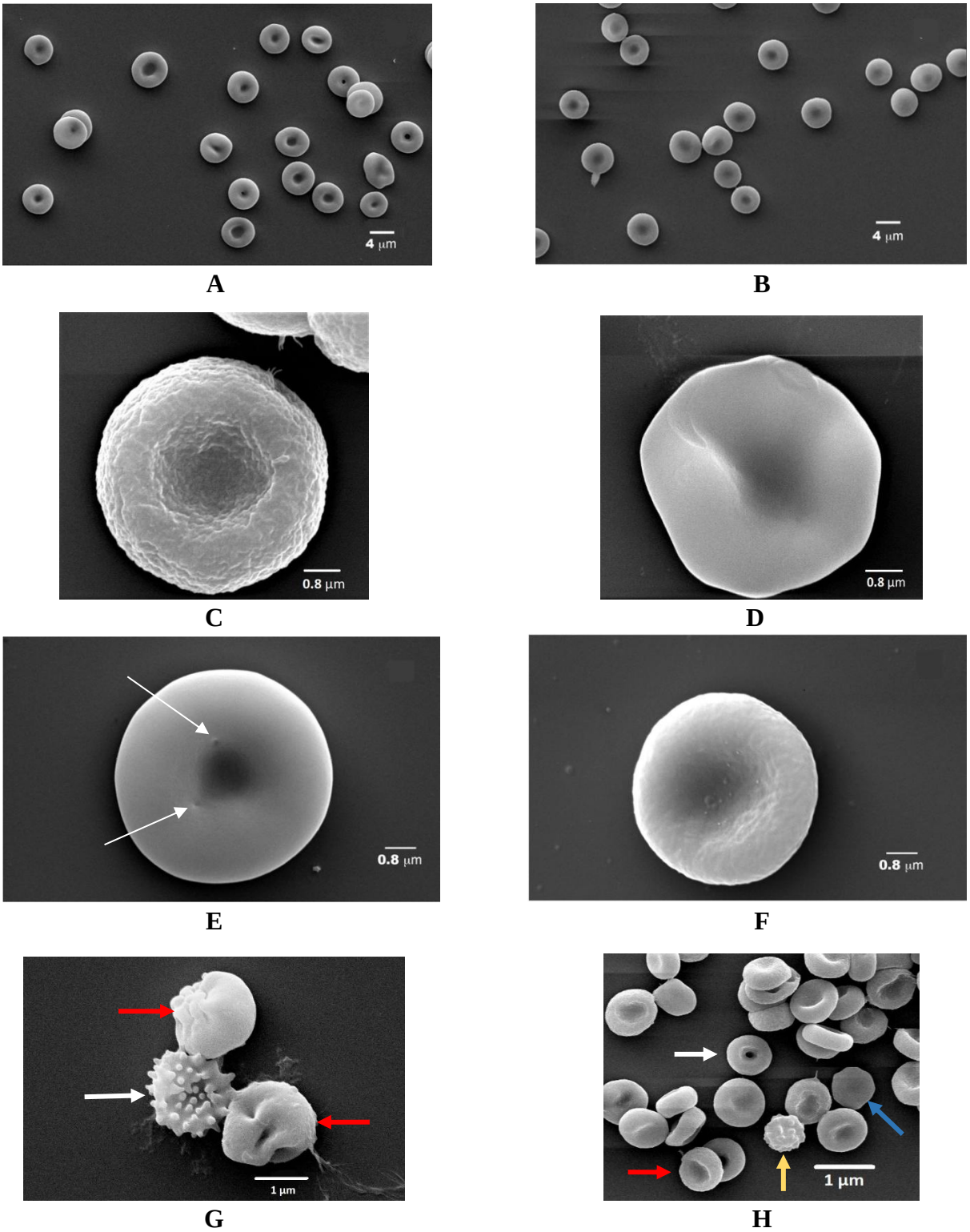


Figure 24: Morphological study of erythrocytes under Scanning Electron Microscope (SEM). A. Erythrocytes from cord blood, magnification in 2500X; B. Erythrocytes from adult peripheral blood, magnification in 2500X; C. Single erythrocyte of cord blood showing deeper indentation with rough membrane surface, magnification in 20000X; D. Single erythrocyte of cord blood showing shallow indentation with smooth membrane surface, magnification in 20000X; E. Single erythrocyte of cord blood showing smooth membrane surface with prominent pits (white arrows), magnification in 20000X; F. Single erythrocyte of adult peripheral blood, magnification in 20000X; G. Reticulocytes (red arrows) and echinocyte (white arrow) isolated from cord blood, magnification in 13000X and H. Cluster of deformed erythrocytes from cord blood showing an acanthocyte (yellow arrow), donut shaped erythrocyte (white arrow), shallow dented smooth erythrocyte (blue arrow) and deep dented erythrocyte (red arrow)

6.4.2 Osmotichemolysis of erythrocyte

The osmotic haemolysis study implicated that erythrocytes of cord blood were less prone to haemolysis by hypotonic stress compared to normal adult erythrocytes. This highlights the possible pliability of cord erythrocyte membrane and also indicates that the erythrocyte membrane integrity of cord blood remains more stable than the normal adult erythrocyte (**Figure 25 A**)

6.4.3 Co-oxidation study of haemoglobin

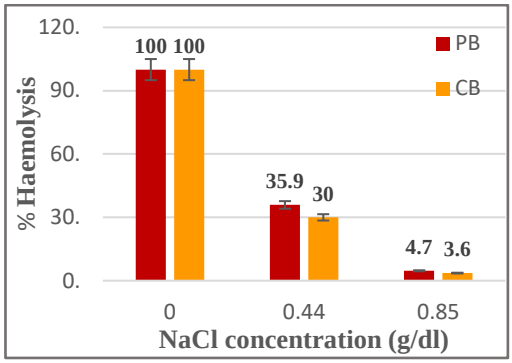
In normal physiological conditions, haemoglobin (oxy-form) oxidizes spontaneously to its met-haemglobin form along with the production of superoxide radicals at a slow rate by the process of auto-oxidation. However, in the presence of an oxidizing agent, such as nitro blue tetrazolium (NBT), the rate of oxidation increases (co-oxidation). The superoxide molecule, produced in such a reaction augments the oxidative load and eventually degrades different biomolecules. Here through this experiment, we studied the co-oxidation of haemoglobin and found that the rate of co-oxidation of haemoglobin was slower in cord blood than the normal blood (**Figure 25 B**).

6.4.4 SDS-PAGE analysis of erythrocyte membrane proteins

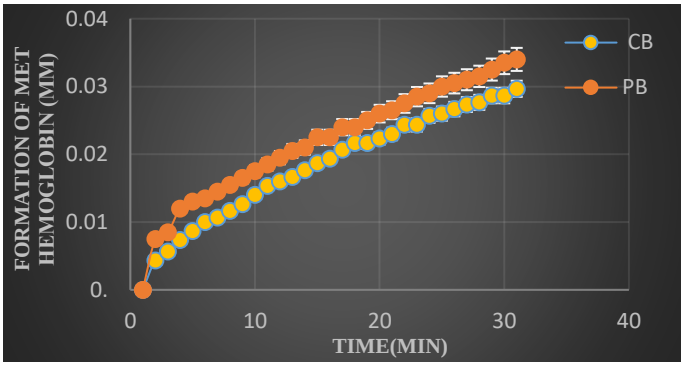
From the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, we observed alteration in the expression of various paramount cell-membrane associated proteins, namely Spectrin, Band 3, Band 4.1, and Band 4.5 (GLUT1) in cord blood erythrocyte as compared to normal adult peripheral blood. The most noticeable result is that there is overexpression of GLUT1 protein (**Figure 25 C**) and the appearance of reddish-violet coloration of the band upon Coomassie blue staining.

6.4.5 Lipid peroxidation assay of erythrocyte membrane

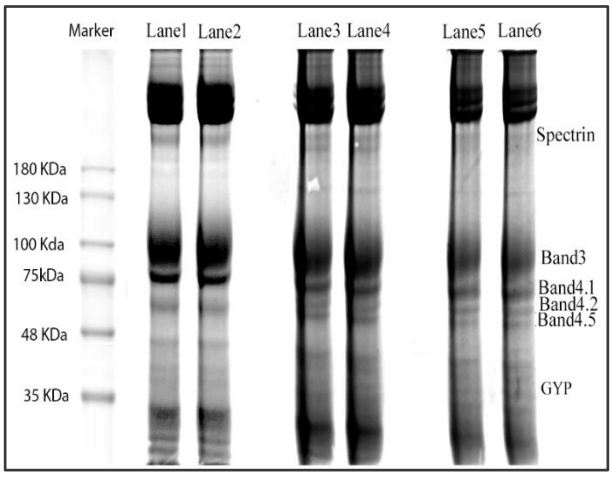
Lipid peroxidation as indicated by the amount of TBARS formation in cord blood erythrocyte membrane was less as compared to normal adult peripheral erythrocyte membrane, indicating that the cord blood erythrocyte might be exposed to less exogenous and endogenous oxidative insults than that of normal adult erythrocytes (**Figure 25 D**).



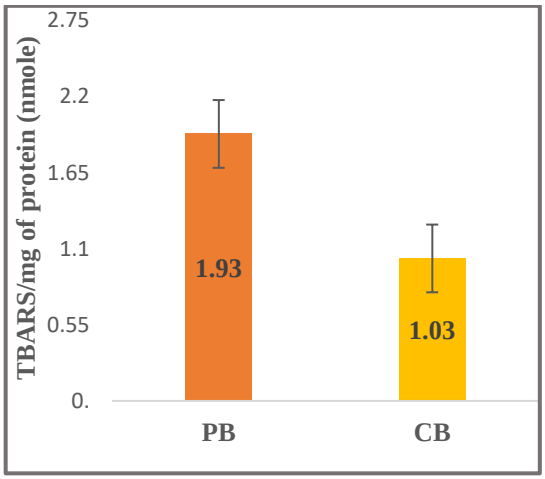
A. Osmotic fragility erythrocytes



B. Co-oxidation study of haemoglobin



C. SDS PAGE analysis of erythrocyte membrane proteins

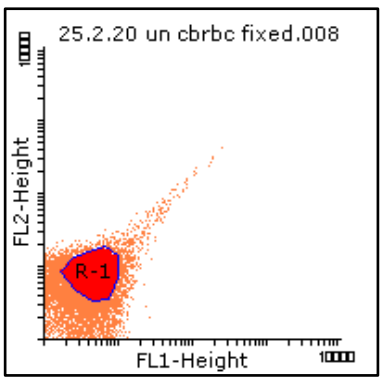


D. Lipid peroxidase assay

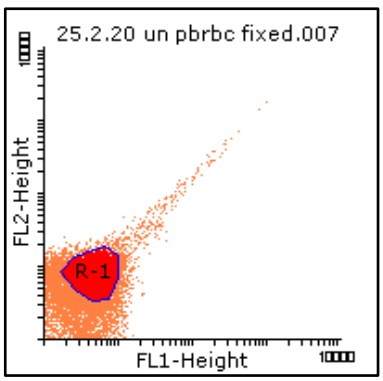
Figure 25: Biochemical assays of cord and peripheral blood erythrocytes. A. Osmotic haemolysis of erythrocyte of adult peripheral blood (PB) and cord blood (CB) in presence of three different concentrations of NaCl (0, 044 and 0.85 g/dl); B. Formation of methaemoglobin by purified haemoglobin from normal adult blood and cord blood in presence of NBT in 0.01 M phosphate buffer, pH- 7.4. CB- Cord Blood, PB- Peripheral Blood; C. SDS-PAGE of erythrocyte membrane proteins followed by Coomassie brilliant blue staining. Each lane of the gel contains 100 µg of protein. Lane 1 and 2 represent erythrocyte membrane proteins of adult blood. Lane 3 and 4 represent erythrocyte membrane proteins of cord blood (sample 1), Lane 5 and 6 represent erythrocyte membrane proteins of cord blood (sample 2); D. Formation of TBARS/mg of proteins as a result of lipid peroxidation in adult peripheral blood (PB) and cord blood (CB)

6.4.6 Flow cytometric Analysis

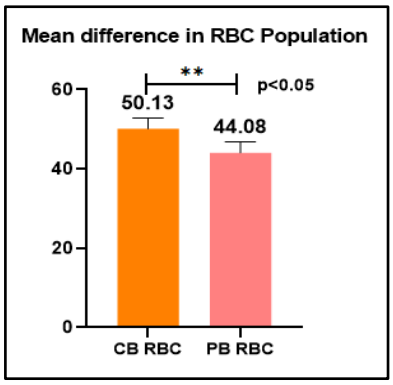
The graphical representation of Mean±SD values of unstained cord and adult peripheral blood erythrocyte percentages was estimated to be 50.13±2.6 and 44.08±2.7. There was a significant difference in cell percentages recorded as the t-value was 3.873 and *P*- value was 0.0031 (**Figure 26 C**). To confirm our reading, we also performed a Mann-Whitney U test of the unstained CB and PB erythrocyte cell percentage and found significant differences. The U-value was 2 and *P*- value was 0.0087. The graphical representation of MFI values of CD235a Alexa 488 expression in cord and adult blood was estimated to be 237.3±4.8 and 215±3.9. The t-value was 8.689 and *P*- value was 0.0001 which ensured a significant difference in MFI expression (**Figure 26 E**).



A Dot plot of CB RBC

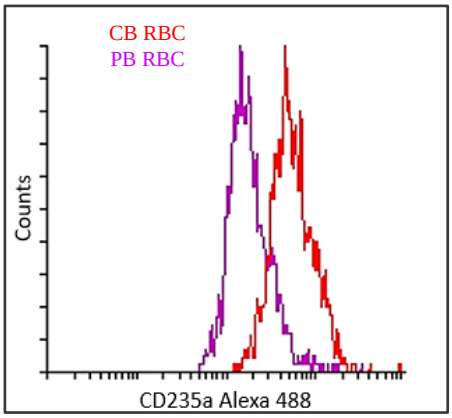


B Dot plot of PB RBC

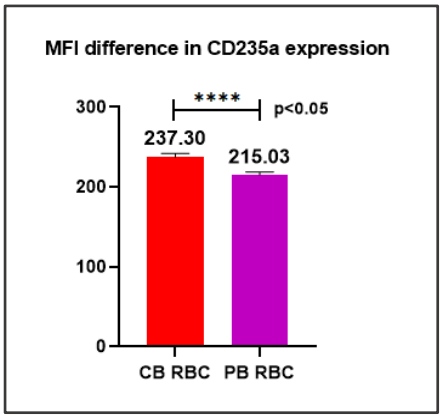


C Graphical representation of cord and peripheral RBC

Figure 26: Flow cytometry analysis of cord and peripheral red blood cells. A. Dot plot of cord red blood cells (CB RBC); B. Dot plot of peripheral red blood cells (PB RBC); C. Graphical representation of RBC percentage difference in cord blood (CB) and peripheral blood (PB)



D Histogram plot of CD235a Alexa 488 expression in CB and PB RBC



E Graphical representation of MFI difference in CD235a expression

Figure 26: Flow cytometry analysis of cord and peripheral red blood cells. D**.** Histogram plot of CD235a Alexa 488 expression in CB RBC and PB RBC; E**.** Graphical representation of MFI difference in CD235a expression

Cell Population	Region/Gate	Gate %		MFI		Total events
		CB RBC	PB RBC	CB RBC	PB RBC	
RBC	**R1**	**48.01**	**44.56**	**5.0**	**4.9**	**10000**

Table 3: Table representing flow cytometric differences in cord blood red blood cell (CB RBC) and adult peripheral blood red blood cell (PB RBC) percentage and MFI

Antibody Marker	Gate %		MFI		Total events
	CB RBC	PB RBC	CB RBC	PB RBC	
CD 235a	**31.6**	**26.8**	**236.6**	**213.4**	**10000**

Table 4: Table representing flow cytometric differences in MFI values of CD235a expression in cord blood red blood cells (CB RBC) and adult peripheral blood red blood cells (PB RBC)

6.5 Discussion

After the delivery of babies, cord blood is found to be left in the postpartum placenta and procurement of cord blood is relatively easy involving no risk to the mothers or the babies. Umbilical cord blood, a rich source of hematopoietic stem cells (HSCs) is thought to be helpful for the treatment of several genetic diseases, blood malignancies, and immune deficiencies (Hordyjewska et al., 2015).

In this particular study, we tried to elucidate the recorded changes in morphological and biochemical facets of cord blood erythrocyte collected from the post-partum umbilical cord when compared to adult peripheral blood. First, we viewed the morphological alteration of cord erythrocyte through scanning electron microscopy. The imaging gave evidence of various depths of indent formation in cord erythrocytes, leading to a higher degree of deformations unlike the torus formed in normal adult erythrocytes, which are mostly having similar dents on them. Human erythrocyte itself has a dimple or dent in the middle thus maintaining its biconcave shape. In normal physiological conditions, the dent which is related to alteration or modification in contractility of NMIIA (Non-muscle myosin II A) motor proteins, liable for promoting membrane stiffness and exerting force on diverse F-actin networks to control cell shapes (Smith et al., 2018) and flexibility while they flow in the blood through microvasculature network for the transport of oxygen in tissues. We tried to come up with a possible explanation that the reduced shear stress of cord blood, depending on factors like higher blood viscosity, lower pH and high temperature might impact cord erythrocytes deeper dent formation (Y. Kim et al., 2012). Another study concerning sickle cell anaemia (Renoux et al., 2016) confirms that foetal haemoglobin (HbF) concentration in new-born favours deformation in neonate erythrocytes but as HbF decreases with age, HbF is replaced by sickle cell haemoglobin (HbS) which makes the erythrocyte permanently deformed and rigid, thus losing the flexibility to regain the original structure, which is quite possible in foetal erythrocyte. This study is in sync with our argument that tries to rationale the reason behind the deeper indentations in cord RBC. Our SEM images have further shown some changes in the membrane surface texture of cord erythrocytes, differences in their cell sizes with a notable increase in reticulocyte number and other RBC deformities. Cord RBCs showed different sizes with a higher number of reticulocyte counts (**Figure 24 G**). The hypoxic condition in the cord and placenta triggers the synthesis of erythropoietin which in turn stimulates the production of reticulocytes (K. & F., 2011), the difference in reticulocyte is seen as the gestational age increases (Maconi et al., 2010). We even noticed several acanthocytes and echinocytes. On the

contrary adult blood had little to no reticulocytes and almost no cellular deformities. One of the studies (Jacob, 2016) confirmed, neonates have morphologic differences of biconcave discs, a higher percentage of stomatocytes compared with adults, at least 3-5% of the red cells may be distorted, fragmented with increased numbers of pitted cells, echinocytes, spherocytes and other abnormally shaped erythrocytes. As cord blood haematological parameters are similar to that of new-borns, we noticed these deformities in our study too (**Figure 24 H**).

Next, we move on to accessing the osmotic fragility of the cell where we found that cord erythrocyte was less fragile. Decreased fragility may indicate higher integrity as well as elasticity in cord blood erythrocytes. Furthermore, decreased osmotic fragility also indicates increased pliability of the membrane (Saxena & Seshadri, 1983). Change in membrane pliability depends mainly on cholesterol phospholipid ratio (c/p ratio) and it is reasonably evident that cord blood or foetal blood having lower cholesterol and phospholipid content (Aletayeb et al., 2013).

We further proceed to overview the oxidative status of cord blood haemoglobin. In normal physiological conditions, haemoglobin oxidizes spontaneously to its met form along with the production of superoxide radicals, which in turn may augment the intracellular oxidative stress. The met-haemoglobin returns to its functional (reduced) form by met-haemoglobin reductase and other enzymatic systems to maintain a level within 3% (Faivre B, 1998). However, in the case of cord haemoglobin which mostly consists of foetal haemoglobin, having a higher affinity towards oxygen might show a disparate scenario. To confirm this, NBT-induced co-oxidation of purified haemoglobin was carried out, where we also noticed that the rate of met haemoglobin formation in cord blood is less as compared to normal adult haemoglobin. From this evidence, it is clear that the haemoglobin of cord blood is structurally different than the adult haemoglobin.

Next, our curiosity moved towards characterizing erythrocyte membrane proteins of cord blood, where we noticed a significant change in various paramount proteins, namely Spectrin, Band 3, Band 4.1 and Band 4.5 (GLUT1). Most remarkably we notice that the increased expression GLUT1 band appeared with reddish-violet coloration in Coomassie blue staining. We don't have a proper explanation for the reddish-violet coloration. However, it may be due to the acidic pH of cord blood. If pH is low which means that at birth, the baby had an elevated pCO_2 and/or an increased number of acidic by-products (mainly lactic acid) caused by anaerobic metabolism; or, there might be heam-GLUT1 adduct formation which needs to be

confirmed by further experiments. In **Figure 25 C**, band 4.5 (GLUT1) is not prominent in the adult erythrocyte membrane as compared to cord blood as GLUT1 expression or its copy number is less. The glucose transporter is significantly expressed in the foetal membrane because foetal cells exhibit rapid growth and differentiation, demanding an increased supply of energy-producing substrates. This is the reason for the prevalence of band 4.5 (GLUT1) in the foetal erythrocyte.

We also observed decreased expression of Band 3 in cord blood. Band 3 maintains the physical linkage to the cytoskeletal proteins as well as mediates HCO_3^- transport with an antiparallel influx of Cl^-, which may be less operative or functional in umbilical cord blood (Hsu, 2018). Moreover, the cytoskeletal proteins like Spectrin and Band 4.1, whose prime roles are to maintain the shape of erythrocyte, also showed lower expression in cord blood. The down regulations of such cytoskeletal proteins provide an explanation that either the erythrocyte membrane proteins of cord blood are not fully expressed or these are not in the effective functional states in maintaining the shape and integrity of the cell.

Besides portraying the membrane proteins, lipid peroxidation assay was also carried out to access the modulation by oxidants and other contexts. The most common method used is the estimation of aldehydic products by their ability to react with thiobarbituric acid (TBA) that yields 'thiobarbituric acid reactive substances' (TBARS). Lipid peroxidation is one of the valuable markers of oxidative stress and ROS production. We have found that there is a reduced level of lipid peroxidation in the cord erythrocyte membrane as compared to normal adults indicating that the cord erythrocyte might not be under the risk of oxidative cleavage of the protein backbone. So, we conclude that erythrocytes of cord blood are having a slightly different morphological layout and the oxidative milieu of haemoglobin, as well as erythrocyte, is at low risk of being damaged.

As aforementioned, some studies conclude, cord erythrocyte counts have higher values in term neonates compared to pre-terms and adults. In preterm babies the blood viscosity is lower compared to the term neonates which is due to low haematocrit values (Christensen et al., 2014), the higher viscosity of cord blood ensures more haemoglobin concentration and high RBC count, which is a direct result of increased erythropoietin production in cord blood. Our study showed the mean cellular count and MFI expression of CD235a Alexa 488 of CB RBC to be more than that of PB RBC. The estimated results were statistically significant and were in sync with established reports (Jacob, 2016). Our study tried to establish a connection

between the deeper dent formation and low osmotic fragility in cord erythrocytes, which may play a pivotal role in higher degrees of deformability than adults. A direct correlation between deeper indentation and higher deformability in cord erythrocyte may not be confirmed in any previous studies but we can assume more pliability is due to membrane stability, high HbF concentration, low lipid peroxidation, and deeper indentation in cord erythrocyte that in-turn favours a higher degree of deformability. CD235a marker study via flow cytometric analysis ensured a significantly high cord erythroid population than adults thus holding a clinical significance when blood transfusion in neonates takes place. This is probably an initial step taken towards the possible explanation of torus formation and deformability in erythrocytes supported by other biochemical parameters.

6.6. Conclusion

In our study, we primarily focussed on the morphological differences in red blood cells isolated from cord blood and adult peripheral blood using a high-resolution Scanning Electron Microscope (SEM). Studies regarding the SEM analysis of cord blood are limited and we succeeded in reporting some unique features of cord erythrocytes not recorded earlier. The reported morphological variations that focus on the difference in size, structure and membrane composition in cord erythrocytes ensure the different stages of haematological development in foetus, which is an interesting observation from the point of electron microscopy. To support our study on structural differences in cord and peripheral erythrocytes, we conducted some conventional biochemical analysis that also showed expected differences in cord RBC when compared to the adults. Another unique observation of membrane protein profile confirms a huge difference in cord and adult erythrocytes. Flow cytometry analysis yielded similar results with significant differences between cord and adult erythroid populations in both the scenarios that involved an antigen study with CD235a antibody and without any antigen profiling.

7. Comparative Analysis of Cord and Peripheral Blood Mononuclear Cells Using Scanning Electron Microscopy and Flow Cytometry

Chapter Summary

In this chapter, we tried to investigate the differences in cellular populations between umbilical cord blood (CB) and adult peripheral blood (PB), focusing on fresh and fixed samples using flow cytometry and scanning electron microscopy (SEM). We witnessed some unique difference in cord and peripheral blood erythrocyte population in our previous chapter, keeping the difference in our mind, we anticipated, if there are any obvious differences that can be recorded in the aforementioned blood types. Peripheral and cord blood were collected from individuals in Kolkata, and the analysis was performed without using surface antigens. Through flow cytomeric forward and side scattering, we witnessed significant differences in the cell counts of whole cord blood and isolated cord mononuclear cells (MNC) compared to adults, in both fresh and fixed states. SEM analysis highlighted similar morphological features between cord and adult leucocytes. However, a notable observation was the presence of reticulocytes in cord MNC which were absent in adult samples. The study was conducted, to understand the utility of flow cytometry for assessing cellular populations without antigen markers and record morphological insights through SEM, contributing to the understanding of CB and PB cell dynamics relevant to regenerative medicine.

7.1 Introduction

Human umbilical cord blood is rich in hematopoietic stem and progenitor cells. Its usage has gained interest in the medical and research field, after the discovery of its effective use in stem cell transplantation (Shearer et al., 2018; Tse & Laughlin, 2005) amongst adult and young recipients. There is an advancement in the rapid use of cord blood derivatives for the treatment of several hematopathological conditions and disorders like Fanconi's anaemia, (Gluckman et al., 2007) severe combined immune deficiency (SCID), (Fernandes et al., 2012) Krabbe's disease (Escolar et al., 2005) etc. Since cord blood (CB) is in use as an alternate source of blood transplantation (Broxmeyer et al., 1989) for almost more than two decades now, having a full picture of the cord blood cellular population is of utmost importance.

In the past much work has been done on the characterisation of cord blood nucleated cells using surface antigens (Chirumbolo et al., 2011; Hordyjewska et al., 2015) but the objective of our study was to establish differences in values of normal cord blood (CB) as well as adult

peripheral blood (PB) nucleated cells in fresh and fixed conditions using flow cytometry, without any use of antibody markers. Studying nucleated cell populations using forward and side scatter was done to ensure cost-effective cell analysis. Focus was given on population changes, cell count, and distribution pattern post-fixation. Flow cytometry measures and analyses single-cell suspensions' optical properties as they pass through a laser beam following hydrodynamic focussing. Forward scatter indicates cell size, while side scatter reflects cell complexity and granularity.

Previous studies revealed the ultrastructure of adult peripheral blood using high-resolution scanning electron microscopy (SEM). (Buys et al., 2013; Newell et al., 1976) SEM analysis was also done in case of diseased conditions like leukaemia and other erythrocyte disorders in peripheral blood. (Polliack, 1981) In 2002, a study was conducted to understand the ultrastructure of primary and cultured leucocytes obtained from cord blood, but the work focussed on the use of Transmission Electron Microscopy to analyse cord blood cells. (Mikami et al., 2002) Building on our previous study, which analysed the differences in erythrocyte populations between cord and adult peripheral blood, (Manna et al., 2021b) in this work, we intend to focus on examining the leukocyte populations in these blood sources. As little to no study exists on the three-dimensional structural analysis of cord mononuclear cells (MNC) using SEM, we aimed to draw a difference in the cell percentage and morphological structure of both cord blood and adult peripheral blood leucocytes using scanning electron microscopy.

In this study, we compared the cellular components of CB and PB in both fresh and fixed states, alongside a detailed morphological examination using high resolution image by scanning electron microscopy. Understanding the differences in cell percentages and their structural variations can be crucial for translational research. The purpose of the study was done to gain knowledge that may help us grasp the cellular dynamics and potential clinical uses of CB and PB, in order to develop better treatments and improving the effectiveness of regenerative medicine and hematopoietic therapies.

7.2 Materials and Methods:

7.2.1 Procurement of adult peripheral blood and cord blood

Peripheral blood was collected from consenting adult women, primarily postgraduate students or healthy individuals undergoing routine check-ups at Eden Hospital, Medical College, with an average age of 23.5 years. Approximately 10 ml of blood was drawn by venipuncture into

sterile EDTA vials. Umbilical cord blood was collected after consenting from pregnant mothers who delivered full-term new-borns (average maternal age: 26.1 years; gestation period: 38-40 weeks) at the same hospital. Around 30-35 ml of cord blood was collected in sterile heparinised collecting tubes immediately after delivery, details given in chapter 5 materials and methods. The study followed ethical guidelines and received approval from the Clinical Research Ethics Committee (CREC) of Calcutta School of Tropical Medicine and Medical College, Kolkata (**Ethical Approval Number: CREC-STM/52**). Participants with hypertension, diabetes, hepatitis, respiratory issues, blood infections, genitourinary diseases, eclampsia, or a history of miscarriages were excluded. Blood samples were promptly transported to the Department of Biochemistry and Medical Biotechnology Laboratory, School of Tropical Medicine, within 10-15 minutes in 4°-8°C ice containers. Written consent was obtained from the participants. (Manna et al., 2021a)

7.2.2 Isolation of mononuclear cells (MNC)

The isolation of mononuclear cells (MNC) from both peripheral and cord blood was done using density-gradient centrifugation within 30 minutes of procurement. The centrifugation of blood samples was conducted in a sterile environment within a Bio-Safety Cabinet. Blood samples stored at 4°C were diluted with an equal volume of cold, sterile 0.9% Sodium Chloride Solution (NaCl) or 1X Phosphate Buffered Saline Solution (PBS, pH 7.4). Each sample (5 ml) was layered over 3 ml of HiSep LSM 1077 solution (Himedia) at room temperature in a 15 ml centrifuge tube. Care was taken to avoid mixing the HiSep solution with the diluted blood. The tubes were then centrifuged at 500g for 25 minutes in a REMI (R8C) centrifuge machine. (Bieback, 2004)

Post centrifugation, the MNC layer was carefully isolated from the plasma and HiSep interface. The MNC layers from both peripheral and cord blood were washed twice with chilled 1X PBS by centrifugation at 200g for 5 minutes. The supernatants were discarded, and the MNC pellets were resuspended in FACS fluid (Becton and Dickinson) for further analysis.

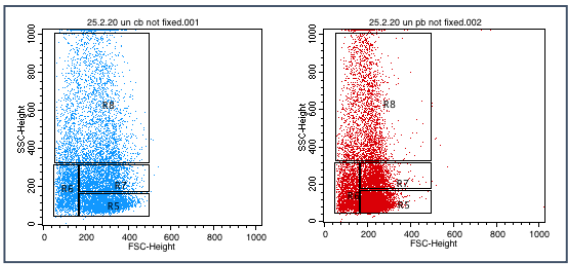
7.2.3 Flow cytometry analysis

Flow cytometric analysis was performed on fresh (Fr) and fixed (Fx) samples from peripheral blood (PB) and cord blood (CB). MNCs and whole blood (WB) were fixed using 1.5% paraformaldehyde (PFA) solution for 30 minutes in dark, washed with 0.9% NaCl, and resuspended in sheath fluid at $1x10^6$ cells/ml. Fresh samples were similarly prepared without

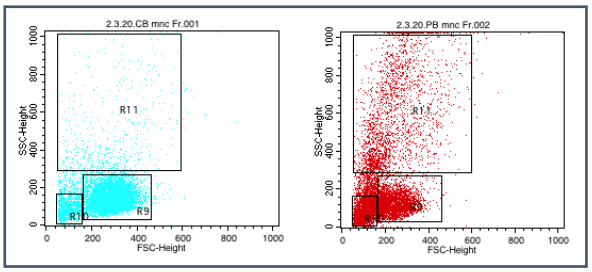
fixation. Forward scatter (FSC) and side scatter (SSC) of PB and CB samples were analysed using a Beckton and Dickenson FACS Calibur with Cell Quest Pro software.

Gating of blood cellular components was done to compare the differences in adult peripheral blood cells and umbilical cord blood cells in both fresh (Fr) and fixed (Fx) conditions. The gating strategies focused on grouping cellular components, highlighting the percentages of lymphocytes, monocytes, and granulocytes in corresponding positions on dot diagrams or plots for both CB and PB samples. The analysis considered the cell percentages, mean values, and variations in cellular distribution patterns when comparing different haematological parameters between fresh and fixed samples of PB and CB. The forward (FSC) and side (SSC) scatter settings were done with the logarithmic amplification scale for the dot plot analysis.

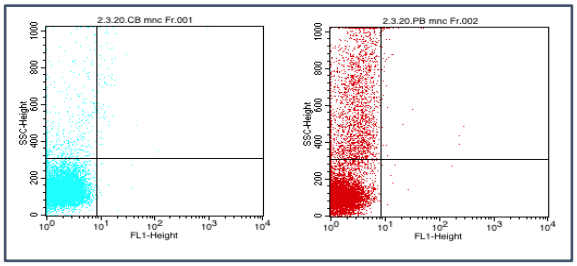
The dot plots were done for WCB (Fr) lymphocytes and WPB (Fr) lymphocytes; WCB (Fr) monocytes and WPB (Fr) monocytes (**Figure** 27 A) and **Table** 5 A; CB MNC (Fr) and PB MNC (Fr) (**Figure** 27 B) and **Table** 5 B; CB MNC (Fr) Quadrant and PB MNC (Fr) Quadrant (**Figure** 27 C) and **Table** 5 C; CB MNC (Fx) lymphocytes and PB MNC (Fx) lymphocytes; CB MNC (Fx) monocytes and PB MNC (Fx) monocytes (**Figure** 27 D) and **Table** 5 D; CB Buffy (Fr) lymphocytes and CB Buffy (Fx) lymphocytes; CB Buffy (Fr) monocytes and CB Buffy (Fx) monocytes (**Figure** 27 E) and **Table** 5 E; WCB (Fr) lymphocytes and WCB (Fx) lymphocyte; WCB (Fr) monocytes and WCB (Fx) monocytes (**Figure** 27 F) and **Table** 5 F and Quadrant of CB MNC (Fr) and CB MNC (Fx) (**Figure** 27 G) and **Table** 5 G.



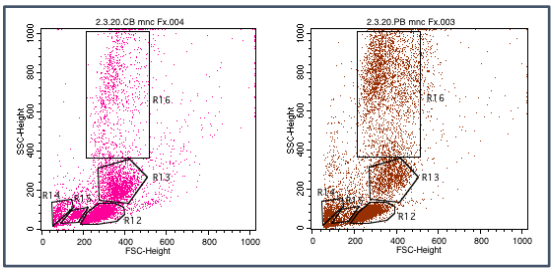
A. WCB (Fr) vs WPB (Fr)



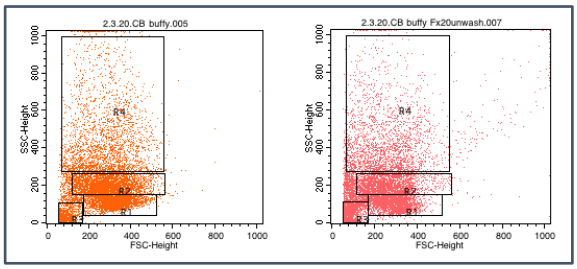
B. CB MNC (Fr) vs PB MNC (Fr)



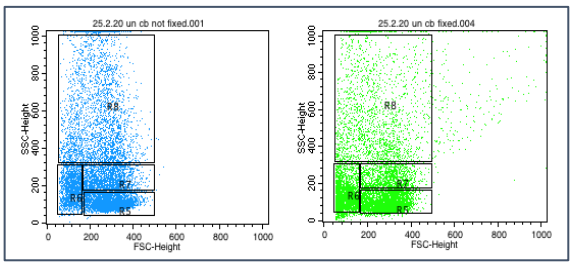
C. CB MNC (Fr) vs PB MNC (Fr) Quadrant



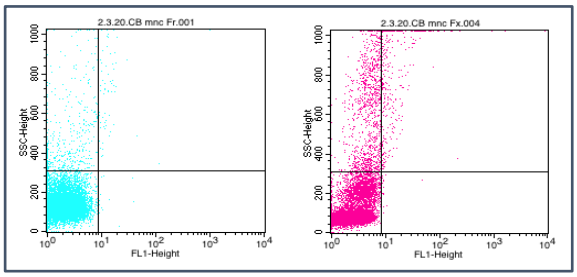
D. CB MNC (Fx) vs PB MNC (Fx)



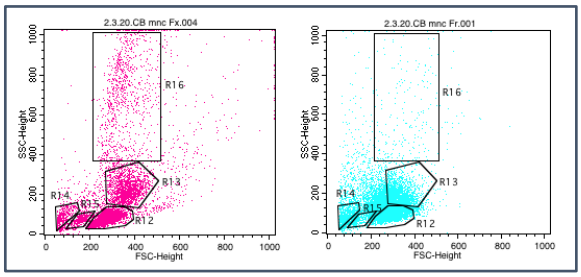
E. CB Buffy (Fr) vs CB Buffy (Fx)



F. WCB (Fr) vs WCB (Fx)



G. CB MNC (Fr) vs CB MNC (Fx) Quadrant



H. CB MNC (Fx) vs CB MNC (Fr)

Figure 27: Flowcytometric dot plot analysis of cord and adult peripheral blood cells in fresh and fixed states A. Forward scatter (FSC) and Side scatter (SSC) of fresh whole cord blood cells (WCB Fr) and fresh whole peripheral blood cells (WPB Fr); B. FSC and SSC of fresh cord blood mononuclear (MNC) cells (CB MNC Fr) and fresh peripheral blood MNC (PB MNC Fr); C. Quadrant graph of fresh cord blood mononuclear (MNC) cells (CB MNC Fr Q) and fresh peripheral whole blood MNC (PB MNC Fr Q); D. FSC and SSC of fixed cord blood MNC cells (CB MNC Fx) and fixed peripheral blood MNC (PB MNC Fx); E. FSC and SSC of cord blood buffy coat cells fresh (CB Buffy Fr) and fixed (CB Buffy Fx); F. FSC and SSC of fresh whole cord blood cells (WCB Fr) and fixed whole cord blood (WCB Fx); G. Quadrant graph of fresh cord blood MNC (CB MNC Fr) and fixed cord blood MNC (CB MNC Fx); H. FSC and SSC of fixed cord blood MNC (CB MNC Fx) and fresh cord blood MNC (CB MNC Fr);

Table 5: Percentage values of cord and peripheral blood WBC flow cytometric samples

Cell Population	Region/Gate	Gate %		Total events
		WCB (Fr)	WPB (Fr)	
Lymphocytes	R5	32.8	37.6	10000
Monocytes	R7	17.3	14.6	10000

A. Gated percentage of Lymphocytes and Monocytes of fresh whole cord blood (WCB Fr) and fresh whole peripheral blood (WPB Fr)

Cell Population	Region/Gate	Gate %		Total events
		CB MNC (Fr)	PB MNC (Fr)	
MNC	R9	79.7	35.2	10000

B. Gated percentage of Mononuclear Cells (MNC) of fresh cord blood MNC (CB MNC Fr) and fresh peripheral blood MNC (PB MNC Fr)

Cell Population	Quadrant	Gate %		Total events
		CB MNC (Fr)Q	PB MNC (Fr)Q	
MNC	Lower Left	95.2	78.2	10000

C. Quadrant of Mononuclear Cells (MNC) of fresh cord blood MNC (CB MNC Fr Q) and fresh peripheral blood MNC (PB MNC Fr Q)

Cell Population	Region/Gate	Gate %		Total events
		CB MNC (Fx)	PB MNC (Fx)	
Lymphocytes	R12	58.1	34.1	10000
Monocytes	R13	16.3	11.2	10000

D. Gated percentage of Lymphocytes and Monocytes of fixed cord blood MNC (CB MNC Fx) and fixed peripheral blood MNC (PB MNC Fx)

Cell Population	Region/Gate	Gate %		Total events
		CB Buffy (Fr)	CB Buffy (Fx)	
Lymphocytes	R12	27.8	25.7	10000
Monocytes	R13	32.2	24.1	10000

E. Gated percentage of Lymphocytes and Monocytes of cord blood buffy (Fr) and cord blood buffy (Fx)

Cell Population	Region/Gate	Gate %		Total events
		WCB (Fr)	WCB (Fx)	
Lymphocytes	R5	32.8	30.6	10000
Monocytes	R7	17.3	11.2	10000

F. Gated percentage of Lymphocytes and Monocytes of fresh whole cord blood (WCB Fr) and fixed whole cord blood (WCB Fx)

Cell Population	Quadrant	Gate %		Total events
		CB MNC (Fr)Q	CB MNC (Fx)Q	
MNC	Lower Left	95.2	86.5	10000

G. Quadrant of Mononuclear Cells (MNC) of fresh cord blood MNC (CB MNC Fr Q) and fixed cord blood MNC (CB MNC Fx Q)

7.2.4 Morphological studies of Mononuclear cells (MNC) under high resolution scanning electron microscopy (SEM)

Mononuclear cells isolated from adult and cord blood were fixed in 2.5% glutaraldehyde in PBS for 1 hour. The samples were then washed with 1X PBS three times each for 5 minutes, followed by sequential dehydration using 30%, 50%, 70%, 90%, and three times of 100% ethanol. After dehydration, the cells were dried, mounted and coated with platinum and observed under scanning electron microscope (Zeiss EVO18, Jena, Germany) with micrographs captured at an accelerating voltage of 15 kV.

7.3 Statistical Analysis

The comparison of flow cytometric values of fresh and fixed cord blood and adult peripheral samples was performed by using Independent Student's t-test with the normal distribution of different variables in SPSS version 16. Statistical significance was considered at $P < 0.05$.

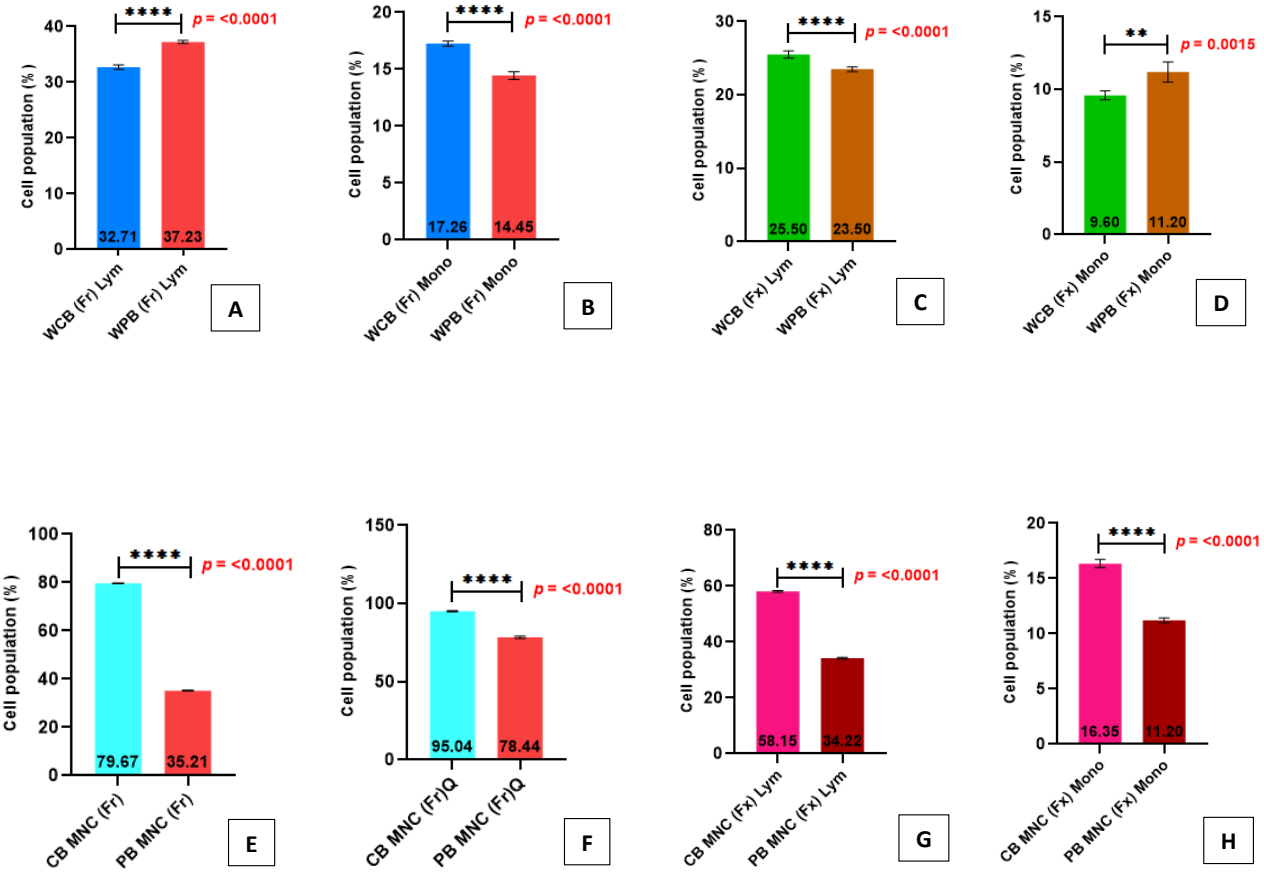
7.4 Results

7.4.1 Flow Cytometric Analysis

The analysis of cellular components from cord blood (CB) and peripheral blood (PB) revealed significant variations in their fresh (Fr) and fixed (Fx) states. In fresh whole cord blood (WCB (Fr)) the lymphocyte population (32.4±0.7) was 0.87-fold lower than fresh whole peripheral blood (WPB (Fr)) (37.2±0.2) (**Figure 28 A**), while in fixed samples, the lymphocyte value of WCB (Fx) (25.5±0.5) was 1.09-fold higher than WPB (Fx) (23.5±0.3) (**Figure 28 C**). Conversely, the monocyte population in WCB (Fr) (17.2±0.2) was 1.19-fold higher than in WPB (Fr) (14.4±0.3) (**Figure 28 B**), while the monocyte population in WCB (Fx) (9.6±0.3) was 0.86-fold lower than WPB (Fx) (11.2±0.7) (**Figure 28 D**).

The mean of CB mononuclear cells (MNC (Fr)) (79.6±0.1) was significantly higher at 2.26-fold compared to PB MNC (Fr) (35.2±0.1) (**Figure 28 E**), and the confirmation quadrant analysis showed similar results where the CB MNC (Fr) (95.0±0.4) to be 1.21 fold higher than PB MNC (Fr) (78.4±0.7) (**Figure 28 F**). In fixed states, CB MNC (Fx) lymphocyte (58.1±0.3) and monocyte (16.3±0.3) populations were 1.70-fold and 1.46-fold higher than PB MNC (Fx) lymphocyte (34.2±0.2) and monocyte (11.2±0.2) populations, respectively (**Figure 28 G, 28 H**).

Cord blood buffy coat analysis revealed CB Buffy (Fr) lymphocyte (27.8±0.1) and CB Buffy (Fr) monocyte (32.6±0.6) populations to be 1.09-fold and 1.35-fold higher than those in CB Buffy (Fx) lymphocyte (25.5±0.2) and CB Buffy (Fx) monocyte (24.2±0.3) populations, respectively (**Figure 28 I, 28 J**). We also compared fresh and fixed states of whole cord blood (WCB), where, the lymphocyte (32.4±0.6 vs. 30.4±0.8) and monocyte (17.2±0.8 vs. 11.2±1.0) values of WCB (Fr) were 1.07-fold and 1.54-fold of WCB (Fx), respectively (**Figure 28 K, 28 L**). The quadrant value of CB MNC (Fr) (95.2±0.6) was 1.10-fold higher than CB MNC (Fx) (86.3±0.6) (**Figure 28 M**). These observations highlight the distinctive cellular distribution and differences between cord and peripheral blood under different conditions.



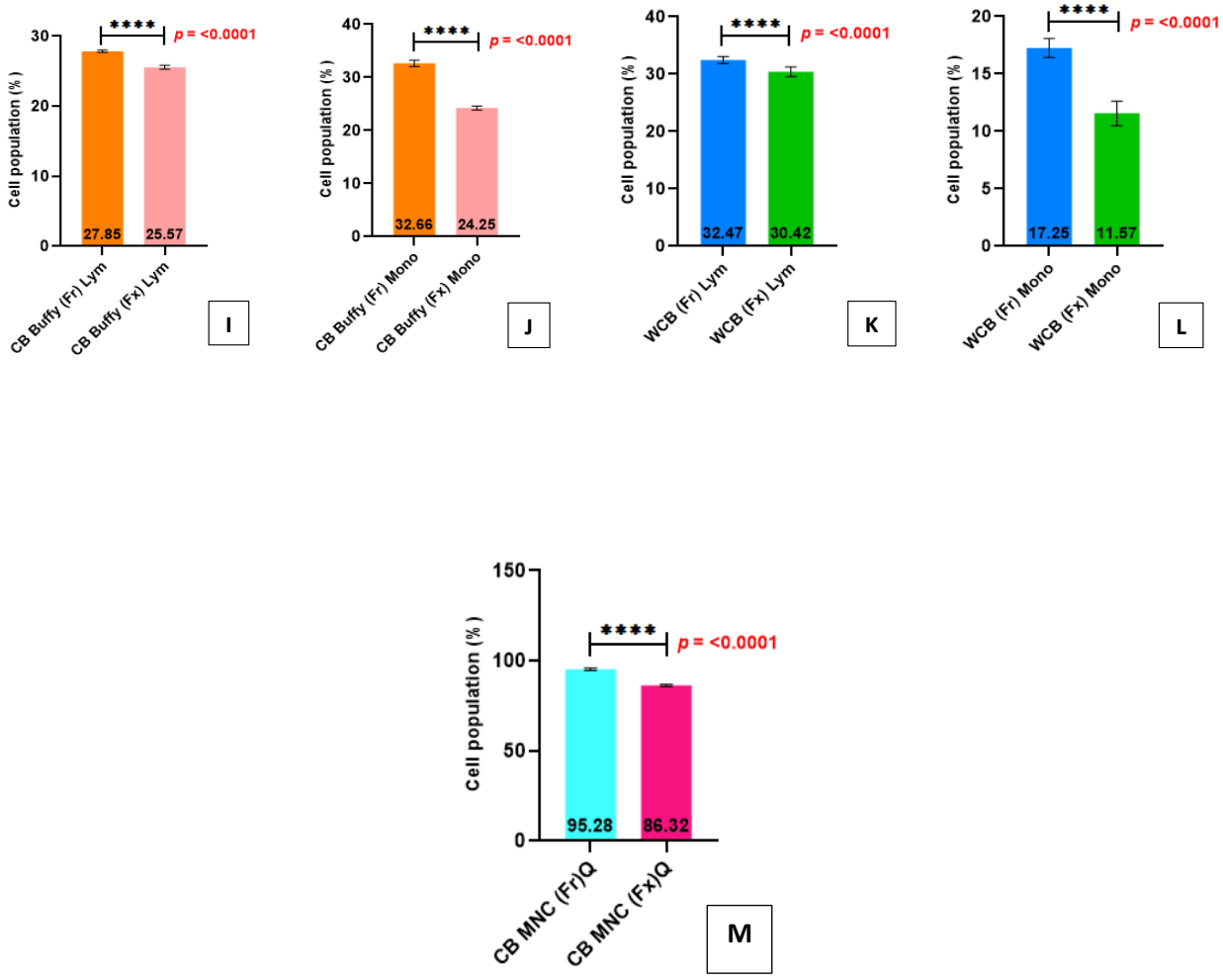
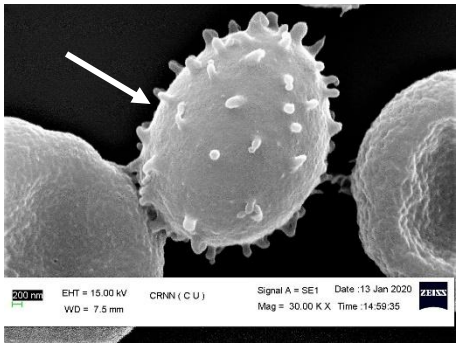


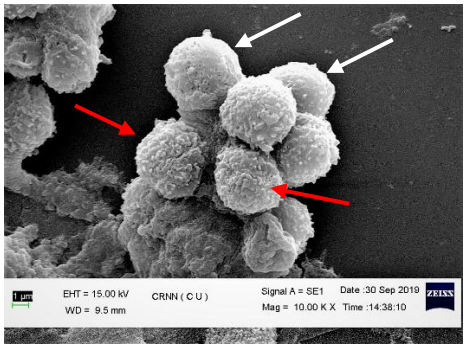
Figure 28: Graphical representations of cellular percentage differences from cord blood (CB) and peripheral blood (PB) samples in fresh and fixed states. **A**. Mean difference in Lymphocyte population of fresh whole CB and PB; **B**. Mean difference in Monocyte population of fresh whole CB and PB; **C**. Mean difference in Lymphocyte population of fixed whole CB and PB; **D**. Mean difference in Monocyte population of fresh whole CB and PB; **E**. Mean difference in mononuclear cell population of fresh CB (CB MNC Fr) and fresh PB (PB MNC Fr); **F**. Mean Quadrant difference in mononuclear cell population of fresh CB (CB MNC Fr) and fresh PB (PB MNC Fr); **G**. Mean difference in Lymphocyte cell population of fixed CB (CB MNC Fx) and fixed PB (PB MNC Fx); **H**. Mean difference in Monocyte cell population of fixed CB (CB MNC Fx) and fixed PB (PB MNC Fx); **I**. Mean difference in Lymphocyte cell population of fresh CB Buffy (Fr) and fixed CB Buffy (Fx); **J**. Mean difference in Monocyte cell population of fresh CB Buffy (Fr) and fixed CB Buffy (Fx); **K**. Mean difference in Lymphocyte cell population of fresh whole CB and fixed whole CB; **L**. Mean difference in Monocyte cell population of fixed whole CB and fixed whole CB; **M**: Mean Quadrant difference in mononuclear cell population of fresh CB (CB MNC Fr) and fixed CB (CB MNC Fx).

7.4.2 Scanning electron microscopy of cord and peripheral blood mononuclear cells

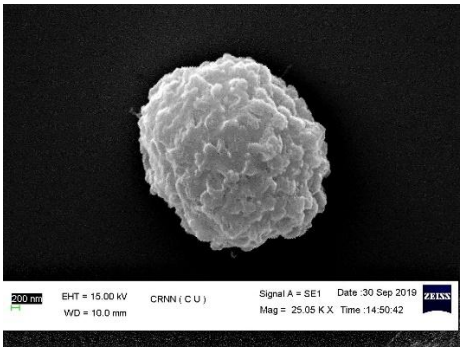
To study the morphological and cellular difference in CB and PB mononuclear cells (MNC), SEM analysis was carried out and three dimensional topographical aspects were noticed in cord leucocytes and adult peripheral blood. It was observed, the cord lymphocytes showed a mixed population of cells with pleomorphic microvilli. Some showed microvilli fewer in numbers, more spaced and shorter in length (stubby) (**Figure 29 A**), whereas most of them had short yet dense microvilli population on the surface of lymphocytes thus making them fully villous in appearance (**Figure 29 B**). The early myeloid cells both in cord and peripheral showed close similarity with short transverse ridges and undeveloped ruffles, so no clear differences in topographical structures were observed (**Figure 29 C and 29 D**). Both cord blood and peripheral blood monocytes have distinct transverse ridges and typical developed ruffled membranes (**Figure 29 E and 29 F**). There was no difference noticed in monocytes. SEM images of cord blood mononuclear cells showed reticulocytes whereas no reticulocytes were seen in peripheral mononuclear cells (**Figure 29 G and 29 H**).



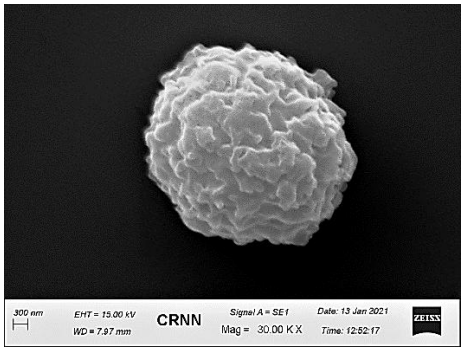
A. A single cord blood lymphocyte



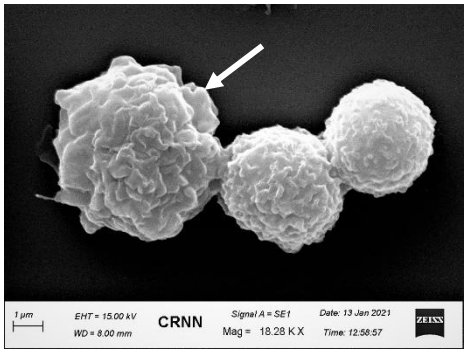
B. Clustered cord blood lymphocytes



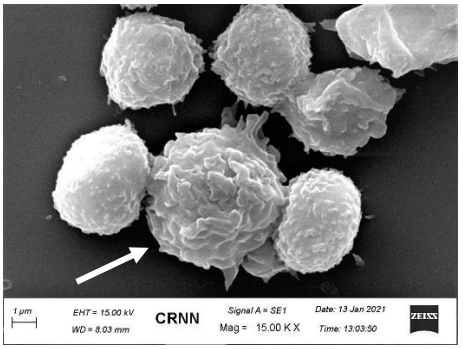
C. Cord blood early myeloid cell



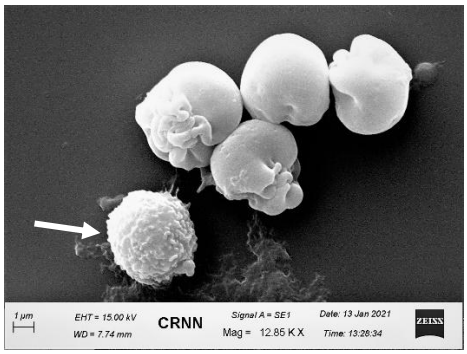
D. Peripheral blood early myeloid cell



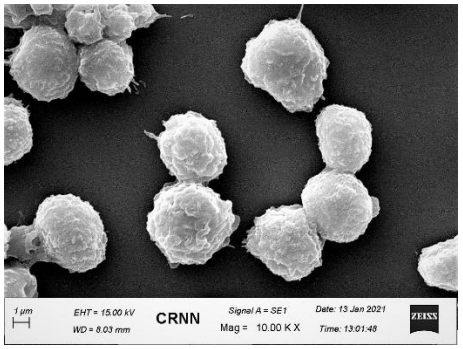
E. Cord blood mononuclear cells



F. Peripheral blood mononuclear cells



G. Cord blood reticulocytes



H. Peripheral blood lymphocytes with myeloid cells

Figure 29: Morphological study of cord and peripheral blood mononuclear cells under Scanning Electron Microscope (SEM). **A**. Cord blood lymphocyte showing fewer distantly placed, tiny microvilli (white arrow), Magnification in 30000X; **B**. Cord blood lymphocytes in cluster, showing dense, stubby microvilli giving fully villous appearance (red arrows), with few round early progenitor cells having few, scanty microvilli (white arrows), Magnification in 10000X; **C**. Cord blood early myeloid cells, Magnification in 25000X; D. Peripheral blood early myeloid cells, Magnification in 30000X; **E**. Cord blood monocyte (white arrow), Magnification in 18000X; **F**. Peripheral blood monocyte (white arrow), Magnification in 15000X; **G**. Cord blood lymphocyte (white arrow) and four reticulocytes, Magnification in 12000X; **H**. Peripheral blood cells showings lymphocytes and myeloid population, Magnification in 10000X.

7.5 Discussion

We have compared the cord and peripheral blood parameters by flow cytometric FSC and SSC analysis. The comparisons were drawn on the mean cell percentage obtained from the dot plot analysis of different hematopoietic cells in both fresh and fixed samples. The obtained data is expected to contribute in understanding the basic differences in cellular components of blood without any antigenic markers, when procured and studied from cord and peripheral sources. Though studying the differences of cell population on the basis of only FSC and SSC analysis is limited, we still tried to establish a simple procedure that would be cost effective and could give us an idea about the percentage of cells according to size, granularity, types and distribution in unfixed and fixed conditions.

The cellular component analysis in CB and PB revealed distinct differences in fresh and fixed states. Our study to investigate the difference in the cell population on the basis of fixation was inspired by the study (Pinto et al., 2005) conducted by Pinto et al, where the observation highlights 'fixation' of haematopoietic cells pre and post staining is a reliable method without compromising the cell percentage and MFI by flow cytometry. The observed result of FSC and SSC also gave us an idea that isolation and distribution of blood cellular components was better when the samples were fixed (**Figure 27 H**). The fresh samples did show isolations and the cell populations were merged in appearance, thus gating procedure while flow cytometric analysis caused minor discrepancy in some of the samples. In case of fixed samples the lymphocyte percentage was higher in WCB (Fx) than in WPB (Fx), this was in sync with previous reports, where undiluted whole cord blood's absolute lymphocyte mean value was more than the adult peripheral blood's lymphocyte count (BECK & LAM-PO-TANG, 1994; D'Arena et al., 1998; Katsares et al., 2009) but we cannot decipher why the mean lymphocyte of fresh WCB (Fr) was less than the WPB (Fr). In our study, the fresh CB samples showed higher monocyte counts compared to PB, indicating CB's richer monocyte content. This characteristic nature may enhance CB's therapeutic potential in regenerative medicine.

Fixed samples demonstrated a notable decrease in cell counts, particularly in monocytes, underscoring the importance of sample handling. The less mean value in fixed samples could be because of the repeated washing of the isolated MNCs after fixation with 1.5% PFA solution that may lead to loss of some cells for the removal of residual PFA, unwanted RBC, debris and other granulocytes. Fresh CB mononuclear cells (MNCs) were significantly higher than those in PB, as confirmed by quadrant analysis. This suggests CB's robust cellular profile, making it

suitable for stem cell therapies and hematopoietic transplants. Our monocyte values in fresh samples were similar to the previous studies which showed high percentage of monocytes (classical) in CB when compared to APB. (Prabhu et al., 2016) Since our comparison did not involve neonates with related mothers but non-related volunteers, so there was a slight difference in the report with another paper, which showed less differences in lymphocytes population between neonates' CB and related mothers' PB.(Chirumbolo et al., 2011) The differences seen in fresh blood cellular counts with that of the fixed samples were probably because of less distribution and isolation of the subsets in FCS and SSC dot plots. This can be a new observation when fresh and fixed whole blood samples from cord and peripheral is compared. The buffy coat separated from the whole cord blood without using any density-gradient chemical also showed significantly high lymphocyte and monocyte values in fresh CB when compared to fixed CB similar to the whole cord blood. The estimated results were statistically significant and are reported in **Table** 6.

	t-value	**df**	**p-value**
WCB Mono (Fr)/WPB Mono (Fr)	16.795	10	< 0.01
CB MNC (Fr)/PB MNC (Fr)	548.001	10	< 0.01
CB MNC (Fr)Q/PB MNC (Fr)Q	47.13	10	< 0.01
CB MNC Lym (Fx)/PB MNC Lym (Fx)	134.243	10	< 0.01
CB MNC Mono (Fx)/PB MNC (Fx)	28.544	10	< 0.01
CB Buffy Lym/PB Buffy Lym	16.747	10	< 0.01
CB Buffy Mono/PB Buffy Mono	29.154	10	< 0.01
CB RBC (Fx)/PB RBC (Fx)	34.976	10	< 0.01
CB MNC Lym (Fr)/CB MNC Lym (Fx)	4.796	10	<0.01
CB MNC Mono (Fr)/CB MNC Mono (Fx)	10.25	10	<0.01
CB MNC (Fr)Q/CB MNC (Fx)Q	23.21	10	<0,01

Table 6: Statistical evaluations of cord and peripheral blood cells accessed using flow cytometry in fresh (Fr) and fixed (Fx) states

The major purpose of our study was to understand the morphology of cord blood leucocytes using scanning electron microscopy, as most of existing literature, highlights the morphology of adult peripheral leucocytes in both normal (Newell et al., 1976; Renau-Piqueras, 1978) and hematopathological conditions. (Polliack & de Harven, 1975; Soligo et al., 1994, 1995) Cord blood have higher number of primitive, immature hematopoietic cells compared to adult peripheral blood (Hordyjewska et al., 2015) so we intend to study the difference (if at all) is

observed in adult and cord whole blood and MNC population. The observed cord blood lymphocytes which are primitive in nature, (Harris et al., 1992; Veneri et al., 2009) exhibit similar morphology as the peripheral lymphocytes as studied by Hoffmann et al.. (Hoffmann et al., 1977) Majority of the cord blood lymphocytes have short or stubby, dense villous surface and few cells have distantly spaced, scanty microvilli.(Newell et al., 1976) These distantly spaced, less-numbered microvilli, round cells were probably the early progenitor cells, which are naïve and have high proliferative capacity (**Figure 29 B**). These almost featureless and scanty or distantly spaced microvilli cells are commonly observed in pre-B and T lymphoblastic leukemic bone marrow cells. (Soligo et al., 1995) The lengthening of microvilli projections occurs in the late developed stages or terminally differentiated stages in the haematopoietic lineage differentiation. No morphological differences in B and T lymphocytes were observed in cord or peripheral lymphocyte population, as both the cell populations from two groups showed villous appearances. There were no smooth surfaced cells observed as mentioned by Renau-Piqueras. (Renau-Piqueras, 1978) Early myeloid cells in both cord and peripheral showed short-transverse ridges and undeveloped ruffles unlike their matured monocytic stage which showed typical large flowy ruffles and distinct transverse ridges.(Polliack, 1981) Reticulocyte cells were noticed in cord blood mononuclear cells whereas little to no reticulocytes were observed in adult PB. The hypoxic environment in cord blood induces the production of erythropoietin, one of the hematopoietic growth factors, which favours the production of cord erythrocytes. As erythrocyte production is high in cord blood it is expected that there will be higher reticulocyte count in cord as compared to the adult peripheral blood for the production of red blood cells. (Jacob, 2016)

7.6 Conclusion

Our objective was to investigate the differences in leukocyte populations between cord blood and peripheral blood. Building on our previous findings of significant variations in erythrocyte populations, we anticipated notable differences in the white blood cell (WBC) study. This study was motivated by the premise that cord blood contains primarily naive, low immunogenic cells, while peripheral blood comprises more mature, highly immunopotent cells. In our study we focussed on the flow cytometry analysis of fresh and fixed cellular populations of whole blood cells and isolated mononuclear cells from both cord and adult peripheral blood samples. The mean cell counts of different cellular population showed significant differences and was in

sync with other studies which did not highlight flow cytometric analysis without any antigen markers. This simple yet cost effective advanced technique can be one of the procedures to study cellular differences in samples along with other sophisticated methods. We also emphasised on the three-dimensional topographical aspects in mononuclear cells isolated from cord and adult peripheral blood using Scanning Electron Microscope (SEM). For several decade focus was given in understanding the morphological differences in peripheral blood leucocyte types, bone marrow cell population in both normal and hematopathological conditions but SEM analysis considering cord blood mononuclear cells was hardly studied.

8. Therapeutic intervention using cord blood plasma factor (CBPF) in an experimental leukemic animal model

Chapter summary

The investigations of this chapter highlighted the therapeutic potential of cord blood plasma factors (CBPF) in an N-Ethyl-N-nitrosourea (ENU)-induced leukemic murine model. Onset of leukaemia in the model was confirmed through the significant presence of blast cells in peripheral blood when compared to control mice. When optimum level of blast percentages was recorded, the leukemic mice were treated intravenously with 100 µL cord blood plasma (CBP) every three days for 30 days. The effectiveness of CBPF was evaluated by assessing survivability, body weight, physical strength, bone marrow cytology, immune cell cytotoxicity, and histological changes in bone marrow and tissue architecture, both in pre- and post-treatment conditions. Further, transcriptional factor analysis using flow cytometry and leukemic marrow cellularity examination via scanning electron microscopy (SEM) were conducted to comprehend CBPF's therapeutic impact. Results disclosed a significant reduction in leukemic blasts in peripheral blood and bone marrow following CBPF treatment. A marked decrease in leukemic infiltration in extra-medullary tissues were also observed. Treatment with CBPF led to an increase in matured immune cells, enhancing their cytotoxic activity and indicating the possible blast differentiation. Significant transcriptional changes were observed, and marrow architecture showed noticeable improvements in treated mice compared to the control group. The findings suggest that CBPF effectively suppresses leukemic progression by reducing blast cell populations, enhancing immune response, reducing toxicity and restoring blast accumulation in tissue architecture. The study accentuates the potential of CBPF as a probable therapeutic agent against leukaemogenesis. These promising outcomes open avenues for further research to explore CBPF as a viable candidate in developing innovative treatment strategies for leukaemia along with the conventional treatment modalities.

8.1 Introduction

Haematological malignancies are heterogeneous group of neoplasms originate from genetically altered hematopoietic stem and progenitor cells (HSPCs) within the bone marrow (BM). Leukaemia is one such malignant condition which is characterised by neoplastic proliferation of haematopoietic blasts in BM which are either myeloid or lymphoid in origin. These malignant immature or partially matured proliferating white blood cells (WBC) accumulate in

the BM, eventually spills into the bloodstream, and often infiltrate various organs (Juliusson & Hough, 2016). Leukaemia is broadly classified into acute and chronic leukaemia, and each is further subdivided into lineage specific ones namely myeloid and lymphoid. Depending on the conditions and lineages, leukaemia can be acute myeloid leukaemia, acute lymphocytic leukaemia, chronic myeloid leukaemia, and chronic lymphocytic leukaemia (Y. C. Chang et al., 2012). The cause of such malignancies can be many and chemical mutagens are one of the triggering agents. Nitroso compounds (NOCs), like N-ethyl-N-nitrosourea (ENU), is one such potent leukaemogenic agent that induces leukaemia by affecting the bone marrow environment. Study reveals intraperitoneal injection (i.p.) of ENU induces leukaemia in mice through random gene mutations, one mutation per 1000 loci in the mouse genome (Nath & Maiti, 2022). Critical genes like TP53, NF-1, RUNX1, CEBPα, PU1 etc are significantly affected by mutations and lead to leukaemogenesis (Di Nardo & Cortes, 2016; Lindsley et al., 2015). Recently human umbilical cord blood has received prominent attention in regenerative medicine due to its therapeutic potential towards an array of diseases. Cord blood plasma contains a variety of growth factors, cytokines and other immunomodulatory factors that corroborate proliferation, differentiation and stimulate immune cells (Samarkanova et al., 2020). Cord blood transplantation (CBT) provides a potent graft-versus-leukaemia (GVL) effect, which has particularly confirmed its immunological efficacy in treating refractory leukaemia, while minimizing the risk of chronic graft-versus-host disease (GVHD) and relapse (Borrill et al., 2023).

This study aimed to explore the therapeutic potential of cord blood plasma factors (CBPF), rich in immunomodulatory and growth factors, on an ENU-induced leukemic murine model. We hypothesised that a biological concoction like CBPF may have the potential to exert anti-leukemic effects in in-vivo model without the standard chemical chemotherapy, partly by modulating immune-regulatory cells and by reducing the neoplastic blasts. The study reports CBPF-induced promising changes in hematopoietic profiles, bone marrow cellular morphology and tissue architecture, cytotoxic profiles, and transcription factors in the leukemic condition (**Figure 30**). The findings highlighted the promising potential of cord blood research, paving the way for innovative treatment modalities in leukaemia.

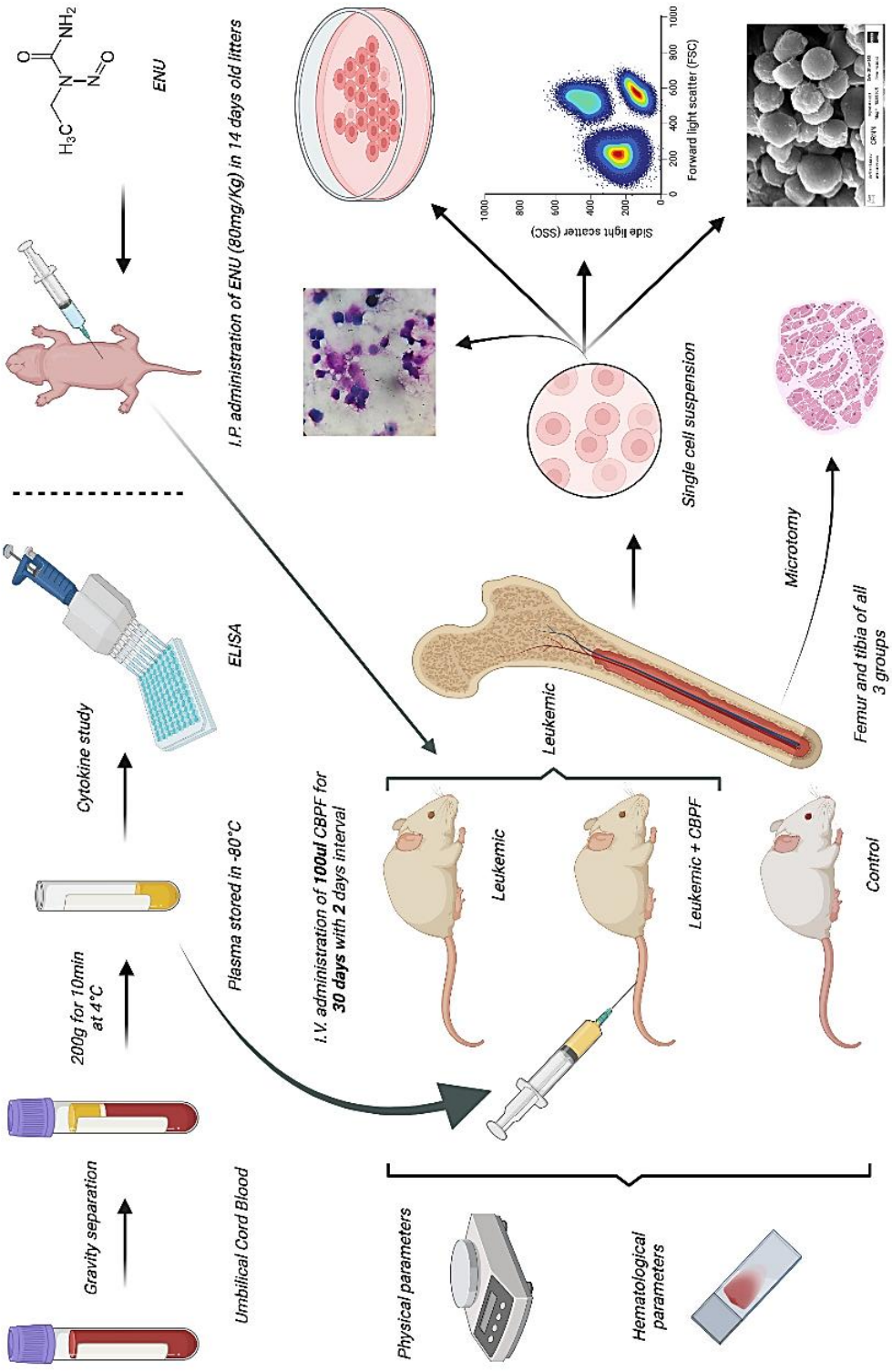


Figure 30: Schematic illustration of the experimental design to access the therapeutic potential of cord blood plasma, containing cord blood plasma factors (CBPF) in an ENU-induced leukemic mice model

8.2 Materials and methods

8.2.1 Procurement of cord blood

The postpartum umbilical cord blood was collected in sterile heparinised Falcon Tubes (Falcon, USA) (average sample volumes collected: 30-35 ml) from consenting mothers who delivered full-term new-borns (average age of mothers: 26.1 years and gestation period: 38-40 weeks) who underwent both vaginal and caesarean deliveries. In our study, the considered consenting mothers were non-smoking individuals with no history of recreational drug intake, hypertension, diabetes mellitus, jaundice, hepatitis, respiratory problems, blood infections like HPV and HIV and genitourinary diseases were not taken into consideration. Pregnant mothers having eclampsia and a history of miscarriage cases were also avoided at the time of sampling. The cord blood collection procedure and consenting is written in details in chapter 5 materials and methods.

8.2.2 Isolation of Cord Blood plasma (CBP)

For the isolation of cord blood plasma (CBP), the whole cord blood was centrifuged at 200g for 10 min at 4°C, the plasma was carefully pipetted out, pooled, and from the pooled CBP the aliquots of 200 µl were stored at -80°C till further use. The frozen CBP aliquots were thawed in a 37°C water bath before being used in the experiment. An individual lot of CBP is prepared from a single cord blood sample. Eight to ten different lots of CBP samples were pooled in as "pooled CBP". Analysis to determine the number of platelets in each sample was not performed; hence, the isolated and pooled plasma cannot be specifically characterised as "platelet rich" plasma (PRP) or "platelet-poor" plasma (PPP).

8.2.3 Determination of cord blood plasma factors (CBPF)

To quantify the cord blood plasma factor (growth factors and cytokines) levels, the stored plasma aliquots were thawed in a 37°C water bath and TGFβ, TNFα, IFNγ and IFNα2 were evaluated using the standard ELISA kits (RayBiotech, GA). The enzyme-linked immunosorbent assay (ELISA) tests were performed as per the manufacturer's instructions (RayBiotech). The plasma samples were analysed and the median value was taken for the analysis. The lower limits of detection for cytokines and growth factors were different for each experiment. Pooled adult peripheral blood (APB) plasma was used as a control to evaluate the cytokine levels of cord blood plasma.

8.2.4 Animal Maintenance

In-bred (immunocompetence) Swiss Albino mice (weight: 18-22 gm) and their pups were maintained under controlled room conditions (22 ± 2) °C in the research animal facility of the Calcutta School of Tropical Medicine (India). Animals had free access to a standard diet and water *ad libitum* under sterile conditions and were housed with a 12-hour light/dark cycle with constant temperature and humidity. All animal trials were performed in consent with the authorized guidelines of the Institutional Animal Ethical Committee (IAEC), and EU Directive 2010/63/EU, and their care was conducted in compliance with the Committee of Control and Supervision of Experiments on Animals, Government of India (Registration number: 681/GO/Re/S/02/CPCSEA).

8.2.5 Preparation and administration of ENU to induce leukaemia

The N-N'ethylnitrosourea (ENU) was procured from Sigma Aldrich (USA) and was stored at −20°C as instructed by the manufacturer (**Figure 31**). The recommended dose was reconstituted daily with 0.9% normal saline solution by measurement of the average body weight (kg) of each experimental mouse in correspondence to carcinogen weight (mg) across all groups except the control one. The Swiss Albino litter pups (weight: 4-5 gm) aging 10–14 days (**Figure 32**) of both the leukemic and treated groups were administered with a single intra-peritoneal (i.p.) dose of ENU (80 mg/kg) injection using a sterile 30-gauge needle to develop the leukemic condition in 6-8 months. The mice from the concerned groups were then monitored closely to determine the induction of the leukemic state and were confirmed by blood haemogram profiling as mentioned in previous studies documented (Basak et al., 2010; R. Chatterjee, Chattopadhyay, & Law, 2016a, 2016b; Chattopadhyay et al., 2019; Law et al., 2001, 2003).

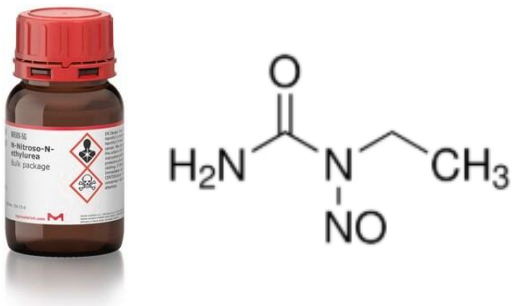


Figure 31: N-Ethyl-N-Nitrosourea, obtained from Sigma Aldrich and chemical structure

Figure 32: The Swiss Albino litter pups (weight: 4-5 gm) aging 10–14 days

8.2.6 Optimisation of cord blood plasma (CBPF) dose

Though few studies revealed the therapeutic potential of cord blood plasma administration in murine models, considering an optimal dose for the intravenous administration of CBP in our treated group was indeed challenging. Since little or no scientific research shed light on the therapeutic intervention of cord blood plasma factors concerning leukaemogenesis, we estimated an optimal dose of plasma administration for our experiment from the related research articles that utilised CBP in their work.

Castellano and their group administered 175µl of human plasma (derived from cord blood, young and aged individuals) intravenously (i.v.) 6-8 times for 2 weeks with 2-day intervals in young and aged mice to assess cord plasma's potential in neuronal activity (Castellano et al., 2017). In another study conducted by (Yoo et al., 2016), UCB plasma (1 mL/Kg) was administered intravenously in the tail vein of rats suffering from acute ischaemic stroke. CBP plasma treatment enhanced neurogenesis and reduced inflammation leading to significant post-stroke functional recovery. In an experiment, Cheng and their group subcutaneously injected 0.1ml (or 100µl) of UCB PRP three times in 3-day intervals in mice to promote hair follicle growth (Cheng et al., 2017). In an interesting experiment that did not involve hCBP, the plasma of young mice was delivered to old mice both intravenously (75µl) and intraperitoneally (i.p.) (150µl) per mouse per week for 16 months, so an average of 225µl/mouse/week or 900µl/mouse/month plasma was administered to assess the induction of rejuvenation in older mice (Shytikov et al., 2014). In a similar experiment isolated plasma from young mice that

exercised for 3 months was injected 100 µL of plasma into the tail vein of 12-month-old mice 10 times at 3-day intervals (T. W. Kim et al., 2020).

Since the published dose ranges vary greatly and acceptable dosages are highly dependent on animal size, type, model and the experimental situation, we searched for more concrete internationally accepted standard operating procedures (SOP) of different universities/institutes that have Animal Care Committee. As per Queen's University, for i.v. administration, not more than 10% of the rodent's total blood volume to be injected, the blood volume of a mouse is 72 mL/Kg. As per Boston University's Administration of Drugs and Experimental Compounds in Mice and Rats (IACUC) protocol, the maximum i.v. volume is 4 mL/Kg/hour. The Penn State IACUC guidelines recommend- the maximum volume for mouse tail vein i.v is 0.2ml or 200µl. The IACUC guidelines for the University of Colorado Denver were the same as the Penn State. As per the Animal Experimentation Ethics Committee (AEEC) of the Australian National University, the recommended i.v. dose in a mouse is <200 µl. Considering all the available options we decided to administer 100µl of CBP intravenously in the tail vein of our experimental murine model following the concept of 10% of the total blood volume as well as 5 mL/Kg (Diehl et al., 2001). If the average weight of our experimental mice is 20 gm and total blood volume (TBV) per mouse is approximately 72 ml/kg (recommended mean), which is 72 x 0.020 kg = 1.44 ml. Now 10% of the total volume of blood would be 0.144 ml or around 140 µl. Since it is the highest volume, we chose 100 µl for drug administration. As per the 5 mL/Kg concept, the optimum volume of injection will also be 100µl of induced drug. Moreover, a biological concoction like cord blood plasma has little to no toxic effect as an administered drug.

8.2.7 Experimental modelling and therapeutic administration of CBPF

The mice were divided into three (3) groups: A) control, B) leukemic and C) treated, each group having ten (10) mice. After constant invigilation over 6-8 months, when clinical signs of leukaemia became apparent in leukemic and treated groups (with more than 40% leukemic blasts in peripheral blood, rough fur, weakness, hunchback, or reduced motility) (**Figure 33**), the leukemic group was humanely sacrificed equally in all passages and the treated group was administered 100µl of CBPF intravenously for a month with an interval of 2 days. The control group consisting of healthy non-leukemic mice received an equal volume of saline solution in similar conditions.

Figure 33: Physical deformities in pre-treatment leukemic animals- exhibiting weak stature, rough and thinning fur, visible ulcers (marked with red circles) and hunched back posture

During the drug (CBPF) administration, the non-anaesthetised mouse from the treated group was restrained in a restrainer (Turner, Pekow, et al., 2011) and the tail was thoroughly yet gently cleaned with 70% ethanol. Prior to injection, the tail veins of the animal were dilated by dipping it in lukewarm water for 5-10 minutes. Post dilation the tail was again cleaned with 70% ethanol and held with a slight tension between the thumb and index finger of the non-dominant hand. A 30 gauge 1ml syringe was prepped with no air bubble having 100µl of the CBPF. With the dominant hand, the needle of the syringe was placed almost parallel to the tail vein and the needle was smoothly and carefully inserted about 2-3 mm inside the vein (Turner, Brabb, et al., 2011). The initial puncture was within the caudal 1/3 of the tail. The drug was carefully injected with steady hands for 1-2 minutes and post-injection, the needle was carefully removed and gentle compression was applied at the injecting site to stop any bleeding for 2-3 minutes, before releasing the animal in its cage (**Figure 34**).

Figure 34: Intravenous (i.v) administration of CBPF in the caudal veins of leukemic mice from the treated group. 100µl of thawed CBPF was administered by a 30 gauge 1 ml syringe at 2-day interval for 30 days

8.2.8 Survivability, body weight and muscle strength

To evaluate the therapeutic potential of cord blood plasma factors (CBPF) on the survivability of mice with leukemia, the lifespan of the treated group was observed during the treatment period and compared to both the untreated leukemic and the control groups. Additionally, throughout the course of CBPF treatment, the weights of all three groups were monitored daily, starting from the initiation of the treatment. This comprehensive approach allowed for an assessment of CBPF impact on survivability and body weight changes across the experimental groups.

Cachexia is one of the frequent conditions encountered by leukemic patients. Cachexia is characterized by the loss of adipose cells and muscle atrophy leading to weight loss and a decline in the overall quality of life eventually resulting in reduced survival expectancy (Bindels et al., 2012). In this study, we wanted to estimate the effect of CBPF on the improvement of muscle strength and thus used the wire-hanging test to evaluate muscle strength in experimental mice. A wire cage lid, reinforced with duct tape along the edges, was utilized to prevent the mouse from moving beyond the perimeter. The mouse was initially positioned on top of the lid and gently prompted to grip the wire surface through a subtle shaking action. The lid was then slowly inverted within the cage, maintaining an elevation of 0.5 to 0.6 meters above a cushioned surface to prevent immediate descent but ensure safety. The latency (in seconds) to fall from the wire cage to the cushioned surface inside the cage was measured. The experiment records the time until the mouse falls (**Figure 35**). The trial was conducted three times for each mouse from all the groups and the longest duration value was used for the evaluation of the study. The resting pause between consecutive attempts was maintained for at least 3 minutes (Deacon, 2013; Jansone et al., 2016).

Figure 35: A representative picture of the wire-cage lid experiment: To study the grip strength of leukemic mice pre and post CBPF treatment

8.2.9 Haematopathological profiling pre and post CBPF administration

To administer the haematopathological profile of experimental mice, peripheral blood was obtained from every mouse of all the groups both pre and post treatment time-point by tail-vein puncture and total leukocyte count (TC), differential leukocyte count (DC), blast percentage, total red blood cell count (RBC) and haemoglobin contains were estimated.

Total Leukocyte count (TC):

For total leukocyte count, 10 µl of tail vein punctured heparinised peripheral blood was diluted with 190 µl of Tuerk fluid and incubated for 10 min at room temperature (RT). After incubation 150 µl of the solution mixer was charged in haemocytometer and total leukocytes were counted in the WBC chambers

Leishman Stain for differential count (DC):

Tail vein bleeds from the experimental groups was collected to study the peripheral haematological parameters. Peripheral blood was smeared onto clean, grease-free glass slides and was stained with Leishman (Sigma, USA) stain to examine the cytological profile and differential counts of blood cells. The smears were flooded with Leishman stain and were incubated for 3 minutes by covering the slides, thereafter the smears were flooded with distilled water for 7 minutes and finally washed with tap water and air dried. Blast percentage was estimated during Leishman staining.

Total red blood cell count:

For total red blood cell count, 10 µl of tail vein punctured heparinised peripheral blood was diluted with 1990 µl of Dacie's fluid and incubated for 10 min at RT. After incubation 150 µl of the solution mixer was charged in haemocytometer and total red blood cells were counted in the RBC chambers.

Haemoglobin estimation:

The peripheral blood haemoglobin estimation was performed colorimetrically by diluting 10µl of heparinised peripheral blood with 2.5ml of Drabkin's solution. The reading was taken at 540nm after 10 mins of incubation at RT.

8.2.10 Bone marrow isolation and preparation of single cell suspension

The animals from respective groups were humanely sacrificed and their hind limbs were surgically extracted. With sterile procedures, the femur and tibia bones were dissected, and bone marrow cells were aseptically flushed into RPMI-1640 media from Sigma, USA, using a sterile 26-gauge needle. These cells were then subjected to multiple rounds of pipetting and filtered through a 70 µm cell strainer. After straining, the cells underwent a triple wash with chilled RPMI-1640 media at 300g for 5 minutes at room temperature, aimed at effectively eliminating debris and achieving a single-cell suspension (R. Chatterjee, Chattopadhyay, & Law, 2016b). Bone marrow explants with >95% viability were studied using Trypan blue staining (Merck, India).

8.2.11 Bone marrow morphology pre and post CBPF administration

Bone marrow cellular studies were performed by staining bone marrow smears with Leishman (Sigma, USA), Sudan black B (SBB) and Periodic acid-Schiff (PAS) stains (Sigma, USA).

Leishman Stain:

Bone marrow from the experimental groups was collected to study the cell morphology and haematopathological status. The bone marrow of animals was smeared onto clean, grease-free glass slides and was stained with Leishman (Sigma, USA) stain to examine the cytological profile and disease condition pre and post treatment. The smears were flooded with Leishman stain and were incubated for 3 minutes by covering the slides, thereafter the smears were flooded with distilled water for 7 minutes and finally washed with tap water and air dried.

Sudan Black B staining:

Bone marrow smears from the experimental groups were first subjected to fixation in formaldehyde vapour for 15 minutes. Subsequently, they were immersed in a working solution of Sudan Black B and phenol buffer for one hour. Following this immersion, the slides underwent a series of rinses involving three changes in 70% alcohol, culminating in a final wash with tap water. The slides were then counter-stained using Leishman stain, mounted, and observed under an oil immersion objective of a light microscope (Olympus Ch20i, Japan).

Periodic Acid-Schiff staining:

Bone marrow smears from all three groups of animals were initially fixed in a solution containing formaldehyde and ethanol, the smeared slides then underwent washing under

running tap water, and were subsequently immersed in a periodic acid solution for 5 minutes, followed by multiple rinses using distilled water. Next, the slides were dipped into Schiff's reagent for 10 minutes and then subjected to another round of washing under running tap water. The slides were allowed to air dry, mounted, and were observed under an oil immersion objective using a light microscope (Olympus Ch20i, Japan).

8.2.12 Bone marrow immune functional efficacy pre and post CBPF administration

Cytotoxicity Assay:

In brief, effector cells (bone marrow cells), from the experimental groups were co-cultured with target cells (Sarcoma-180 cells) at a ratio of 10:1 for 18 hours at 37°C within a 4% CO2 humidified environment. Following this 18-hour incubation period, an equal volume of cell suspension was mixed with an equal volume of 0.4% trypan blue (Merck, India) dye solution, and the percentage of dead target cells was quantified using a haemocytometer chamber. This assessment was conducted to ascertain the effectiveness of the marrow cells in inducing the death of the target cells (R. Chatterjee, Chattopadhyay, Sanyal, et al., 2016; S. Chatterjee et al., 2010).

8.2.13 Bone marrow culture study pre and post CBPF administration

Short term culture:

Bone marrow cells (1×10^6 cells/ml) were cultured in 35 mm culture plates (Corning, USA) containing 3 ml of RPMI-1640 media (Sigma, USA) supplemented with 20% FBS (Fetal Bovine Serum, Lonza, USA). The cultures were maintained in a 5% CO2 humidified chamber at 37°C. Cellular observations were conducted at 0 h, 24 h, 48 h, and 72 h using an inverted microscope (Dewinter) at 40X magnification (Daw et al., 2019).

MTT Assay:

The NADP(H)-dependent cellular oxidoreductase enzyme serves as an indicator of cell viability by catalysing the reduction of the tetrazolium dye MTT (3,4,5-dimethylthiazol-2-yl)-2,5-dipenyl tetrazolium bromide) into its insoluble formazan derivative, which exhibits a purple coloration. The bone marrow cells ($1x10^6$ cells/ml) from the three experimental groups were seeded in 12 well plates with RPMI-1640 media (Sigma, USA) and 20% FBS supplementation, without the phenol red colour indicator (Thermo Fisher Scientific) and incubated at 37°C. Bone marrow cells were collected at time points 0 hours, 24 hours, 48 hours and 72 hours and were assessed for cell viability using the MTT assay. The formation of

formazan (purple colouration) within the cells was dissolved in isopropanol and subsequently, the optical density (OD) was measured at 540 nm by a colorimeter (EESDIGI-8F, India) (Daw et al., 2019)

8.2.14 Tissue architectural study of pre and post CBPF administration

Haematoxylin and Eosin (H&E) staining was performed to visualise and analyse the changes of the haematopathological scenario within the structural patterns of bone marrow, liver, kidney, and spleen sections of the experimental groups.

Histology of bone marrow:

The long bones, comprising the femur and tibia, were aseptically excised from the experimental groups and subsequently preserved in 10% neutral buffered formalin for a duration of 24 hours. Following the fixation process, decalcification was achieved by immersing the bones in a solution of 10% formic acid. Small sections were then meticulously obtained from various regions of the bones. These sections underwent a systematic progression through ascending alcohol concentrations and were subsequently embedded in paraffin. After paraffin blocks were prepared, 5-micrometer sections were precisely cut using a microtome machine and affixed onto slides that had been coated with albumin.

The fixed paraffin slides containing sections of bone marrow were subjected to a methodical deparaffinization process involving xylene treatment, followed by rehydration through a series of descending alcohol gradients (100%, 90%, 75%, 50%, and 30%) and distilled water. Subsequently, these slides were subjected to haematoxylin staining for a duration of 1 minute, followed by rinsing in tap water and immersion in an acid alcohol solution. Finally, the sections were counterstained with eosin for 30 seconds. The slides were then subjected to dehydration using escalating concentrations of ethanol (70%, 95%, 100%; 10 seconds each), mounted, and meticulously examined under an oil immersion objective on a light microscope, specifically utilizing the (Olympus Ch20i, Japan) (R. Chatterjee, Chattopadhyay, & Law, 2016b).

Histology of extra medullary organs:

Haematoxylin and Eosin (H&E) staining was conducted as per the previously described protocol to examine the structural patterns of bone marrow. Liver and spleen sections were also studied in all three experimental groups.

8.2.15 Flow cytometry

Bone marrow cells were obtained from the control animals, ENU-induced leukemic animals and ENU-induced leukemic animals administered with CBPF for treatment. These cells were washed twice with 1X PBS and subsequently fixed using 1.5% paraformaldehyde (PFA). The PFA-fixed bone marrow cells from the experimental groups were permeabilised by adding 90% chilled methanol and rigorous vortexing. The perforated bone marrow cells were then suspended in sheath fluid (BD Bioscience, USA) and divided into polystyrene FACS tubes (BD Bioscience, USA) at a concentration of $1x10^6$ cells/ml, followed by staining with primary monoclonal antibodies (Cell Signaling Technologies, USA), including anti-RUNX1, anti-CEBPα, anti-AhR, Oct-4, anti-GFI1, and anti-PU1 (raised in rabbit reactive against mouse proteins) for studying the expression of transcription factors. The primary antibody-stained cells were incubated at 37 °C for 30 minutes and subsequently, counter-stained with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, USA) or PE (Santa Cruz Biotechnology, USA) (goat anti-rabbit IgG) and incubated again in the dark at 37 °C for 30 minutes. The cells were then washed in PBS and subjected to analysis using a BD FACS Caliber flow cytometer (Becton Dickinson, USA) with Cell Quest Pro software (v9.1, Becton Dickinson), further analyses were done using FlowJo_v10.8.1 (trial version). Data obtained from the flow cytometry analysis included the determination of the mean fluorescence intensity (MFI) of the target proteins through the application of virtual gating.

8.2.16 Scanning Electron Microscopy

Small sections of bone marrow from respective experimental groups were fixed with 2.5% glutaraldehyde in PBS overnight. The marrow pieces were then subjected to serial dehydration technique, with 30%, 50%, 70%, 90%, and finally 100% (absolute) ethanol. The marrow pieces were then dried, mounted, and coated with platinum, and observed under a high-resolution scanning electron microscope (Zeiss EVO18 special edition SEM, Jena, Germany).

8.3 Statistical Analysis

The statistical analysis was performed using unpaired Student's t-test and One-way ANOVA, followed by post-hoc testing using the Tukey's Test. The quantitative data of the paper were presented as Mean ± Standard Deviation (SD). The level of significance, with $P \leq 0.05$ was considered statistically significant for all comparisons. Every experiment in this study was repeated three times to ensure reliability and consistency of the results.

8.4 Results

8.4.1 Analysis of Cord Blood Plasma Factors

Cord blood plasma holds distinctive properties and has significant therapeutic potential not only in regenerative medicine but also in the treatment of various haematological, oncological, and immune-related conditions. CBP is rich in cytokines and growth factors (Ehrhart et al., 2018b; Romanov et al., 2019) required for the growth and survival of haematopoietic stem cells (A. C. Lam et al., 2001; Pranke et al., 2001b). The variability in cord blood plasma component concentrations, influenced by donor-specific traits and delivery methods (Romanov et al., 2019), can impact the expression of analysed proteins, which was also observed in our study. The concentrations of CBP and APB plasma cytokines and growth factors are already shown in (**Figure 23 A to 23 D**). The ELISA kits had a lower and a higher detection limit of 18 pg/ml-4000 pg/ml for TGFβ; 30 pg/ml-6000 pg/ml for TNF-α; 1 ng/ml-400 ng/ml for IFNγ and 5 ng/L-1000 ng/L for IFNα2. The concentration of TGFβ (pg/ml) in CBP (3445±168.1) was significantly higher than adult (838.9±112.8), with p = <0.0001. The expression of TNF-α (pg/ml), IFNγ (ng/ml) and IFNα2 (ng/L) in CBP (249±26.57; 33.49±2.2; 38.71±1.47) showed significantly lower expression than their equivalent adult peripheral blood plasma (756.2±10.25; 81.57±8.57 and 92.35±1.91), with p values <0.0001; 0.0015 and <0.0001.

8.4.2 Effect of CBPF on the survivability, body weight and muscle strength of ENU induced leukemic mice:

The treated group exhibited a significant increase in the Kaplan Meier survivability curve when compared to the leukemic group with a p = < 0.0001, suggesting initial indications of the CBPF's potential in leukemic treatment (**Figure 36 A**).

The ENU-induced leukemic mice exhibited pronounced body weight (15.3±0.71 gm; p = <0.0001) and muscle mass loss compared to the control group (23.8±0.9 gm), suggesting significant adverse effect of the potent mutagen in the experimental cohort. Contrarily, there was a significant increase in the body weight (18.1±1.25 gm; p = 0.014) and improved physical appearance observed in the treated group when compared to the leukemic group following the cord blood plasma factors (CBPF) treatment (**Figure 36 B**).

The ENU-induced leukemic mice performed poorly in the wire-hanging test compared to the healthy control group. This sub-par performance, attributed to the muscle weakness and reduced fitness in leukemic mice, thus resulting in a significantly shorter hanging time from the wire-lid when compared to the control group. However, following the CBPF treatment, the performance quality of the treated cohort significantly improved, thus displaying a notable difference in hanging time compared to the leukemic group (**Figure 36 C**).

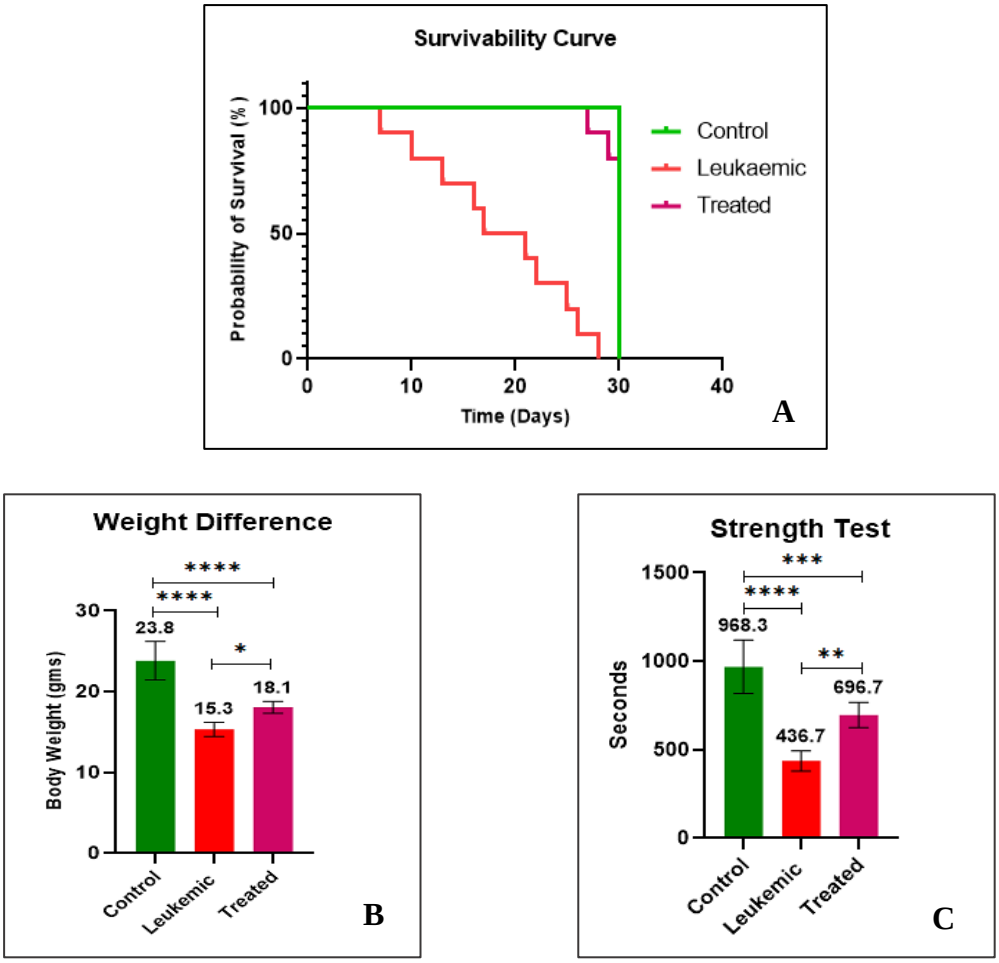


Figure 36: Effect of cord blood plasma factor (CBPF) treatment on experimental mice's physiological parameters. A. Effect of CBPF on the survivability of experimental animals, depicted using Kaplan Meier curve; B. CBPF effect on weight differences; C. CBPF effect on strength differences

8.4.3 Effect of CBPF administration on the haematopathological profile of ENU induced leukemic mice:

The blood hemogram profile revealed a significant increase in total leukocyte count in the leukemic cohort ($32.05 \pm 2.5 \times 10^3$cells/mm^3) with $p = < 0.0001$, when compared to the control group ($8.05 \pm 1.9 \times 10^3$ cells/mm^3). Furthermore, a notable decrease in the white blood cell count (WBC) was observed in the treated group ($27.52 \pm 2.1 \times 10^3$ cells/mm^3) with $p = 0.002$ compared to the leukemic mice, indicating a potential effect of CBPF on WBC scenario in leukemic condition (**Figure 37 A**). The neutrophil percentage (%) in leukemic group (49.87 ± 2.8) was significantly higher, resulting in neutrophilia than the control mice (33.67 ± 2.2), whereas the treated group (43.77 ± 2.6) showed significantly lesser than the leukemic group (**Figure 37 B**). Conversely, an inverse trend was observed in the lymphocyte percentage (%) of the leukemic group, where lymphocyte levels (15.12 ± 1.0) were significantly lower than those of the control (61.27 ± 2.1). In contrast, in the treated scenario, the lymphocyte percentage

(30.55 ± 1.8) improved significantly when compared to the leukemic condition (**Figure 37 C**). The administration of CBPF notably impacted the blast crisis in leukemic haematopathological profile, observed with a significant decrease in blast percentage (%) in the treated group (22.25 ± 4.5; p = <0.0001) when compared to the leukemic group (39.35 ± 3.6) (**Figure 37 D**). This accentuates the promising potential of CBPF treatment in mitigating blast crisis. The total red blood cell (RBC) count was decreased in the leukemic group (4.27 ± 1.2 × 10^6 cells/mm^3) compared to the control group (5.01 ± 0.7 × 10^6 cells/mm^3) and in the treated group (6.9 ± 0.9 × 10^6 cells/mm^3). The haemoglobin content was also decreased in leukaemogenic mice (14.72 ± 0.45 g/dl) compared to control (15.56 ± 1.52 g/dl) and treated group (17.21 ± 1.38 g/dl). Both the total RBC count and haemoglobin level showed no significant changes when compared across the groups (**Table 7**).

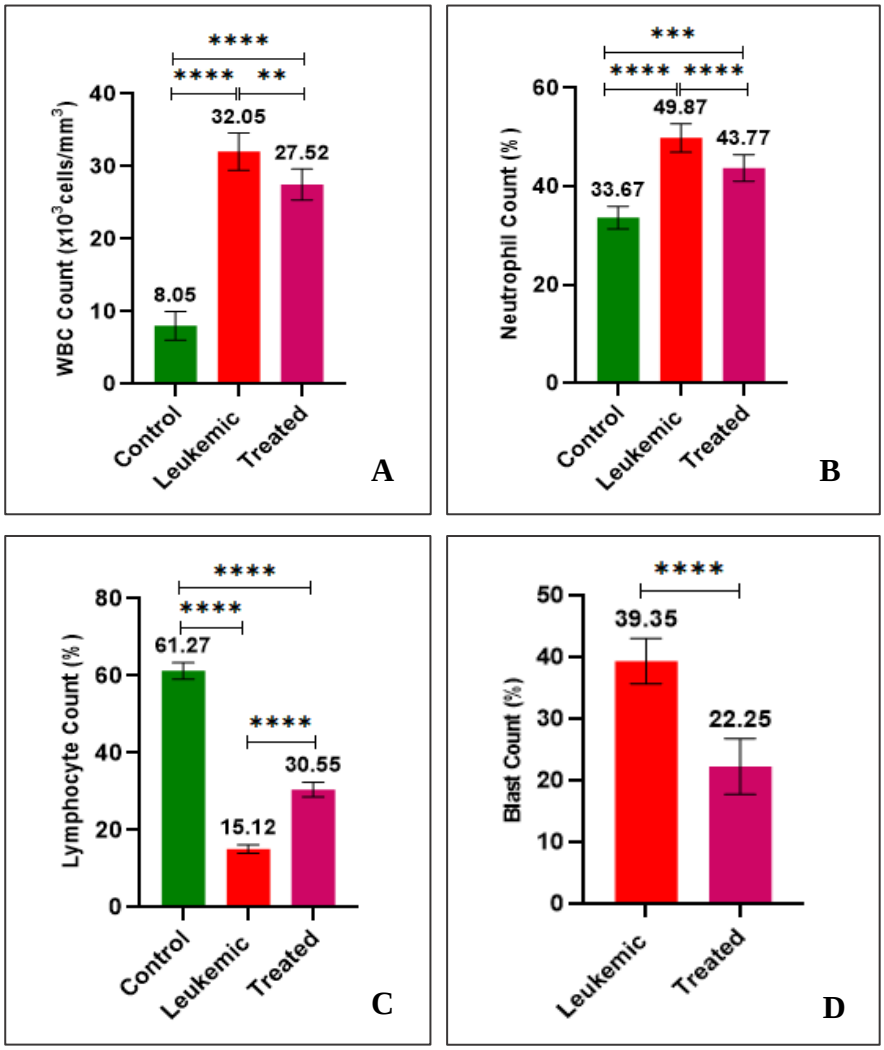


Figure 37: Effect of cord blood plasma factor (CBPF) treatment on experimental mice's haematological parameters: **A.** CBPF effect on white blood cell (WBC) count (10^3 cells/mm^3) differences; **B.** CBPF effect on neutrophil percentage differences; **C**. CBPF effect on lymphocyte percentage differences; **D**. Differences in blast percentages pre and post CBPF treatment

Haematological Parameters	*Control*	*Leukemic*	*Treated*
WBC Count (10^3 cell/mm^3)	**8.05 ± 1.9**	**32.05 ± 2.5**	**27.52 ± 2.1**
Neutrophil (%)	**33.67 ± 2.2**	**49.87 ± 2.8**	**43.77 ± 2.6**
Lymphocyte (%)	**61.27 ± 2.1**	**15.12 ± 1.0**	**30.55 ± 1.8**
Blast (%)	_	**39.35 ± 3.6**	**22.25 ± 4.5**
RBC (10^6 cells/mm^3)	**5.01 ± 0.7**	**4.27 ± 1.2**	**6.9 ± 0.9**
Haemoglobin (g/dl)	**15.56 ± 1.52**	**14.72 ± 0.45**	**17.21 ± 1.38**

Table 7: Haematological profile of peripheral blood smear in experimental groups

Haematological analysis of peripheral blood smears using Leishman staining revealed the presence of abnormal blasts from both myeloid and lymphoid lineages, along with the infiltration of dysplastic neutrophils and hypo segmented ring neutrophils (**Figure 38 A to 38 E**). These findings are indicative of the onset of leukaemia. Peripheral blood count served as the initial predictive measure to evaluate leukemic progression following treatment with CBPF.

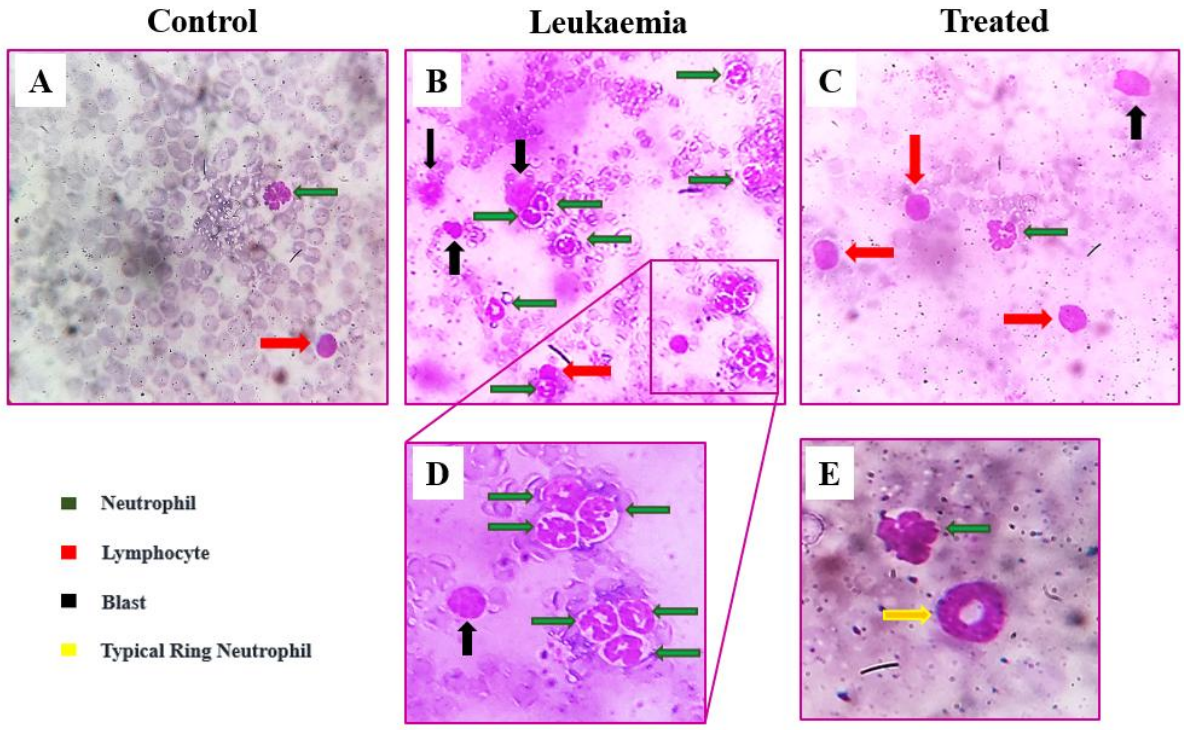


Figure 38: A representative comparison of Leishman stained peripheral blood from experimental groups: A. Peripheral blood smear of control animal showing neutrophil (green arrow) and matured lymphocyte (red arrow); B. Peripheral blood of a leukemic animal showing a significant increase in leucocytosis with increased infiltration of dysplastic neutrophils (green arrows), blast cells (black arrows) and a single matured lymphocyte (red arrow); C. Peripheral blood smear of a treated animal showing decreased blast cells (black arrow) and a hyper-lobed neutrophil (green arrow) with increased matured lymphocytes (red arrows). All the images A to C are captured in 40X magnification; D. Zoomed image (100X magnification) of leukemic dysplastic neutrophils (green arrows) and blast cell (black arrow); E. Zoomed image of a hyper-segmented abnormal neutrophil (green arrow) and a typical ringed neutrophil (yellow arrow) in leukemic condition

8.4.4 Effect of CBPF administration on the bone marrow morphology of ENU induced leukemic mice

ENU-induced leukemic bone marrow smears revealed a prominent disruption in the normal hematopoietic scenario. Cytopathological examination by Leishman staining confirmed the observational neoplastic proliferation of immature blast cells in bone marrow resulting in increased blast burden in leukemic condition (**Figure 39 B**). Furthermore, bone marrow smears from leukemic animals exhibited abnormal blast cells with no matured lymphocytes compared to controls (**Figure 39 A**). Notably, bone marrow smears from treated group displayed substantial decrease in blast cells with increased lymphocytic population ascertaining the therapeutic effect of CBPF (**Figure 39 C**).

Sudan Black B (SBB) is a fat-soluble dye with a strong affinity for neutral fats and lipids. It is used to distinguish between Acute Myeloid Leukaemia (AML) and Acute Lymphoid Leukaemia (ALL). The leukemic bone marrow smears exhibited strong positive staining which confirmed the presence of myeloblast cells (**Figure 39 F**) when compared to the control group (**Figure 39 E**). The bone marrow smears from the treated group showed prominent reduction in the SBB positive stained cells (**Figure 39 G**) thus confirming the effect of CBPF on leukemic condition. Studies revealed SBB positive cells in 3% of blasts typically indicates acute non-lymphoblastic leukaemia (Bennett et al., 1976). However, SBB positivity has been also documented in 1-2% of conventional Acute Lymphoblastic Leukaemia (ALL) cases (Mandal et al., 2019).

Periodic acid Schiff (PAS) stain is used to identify Acute Lymphoid Leukaemia blasts, where the glycogen in cytoplasm is oxidized to produce dialdehydes, which when react with Schiff's reagent, yield a purple-magenta colour. Acute lymphoblastic leukaemia and certain erythroleukaemia cases show strong PAS positivity due to glycogen accumulation, altered membrane composition and presence of vacuoles. The leukemic bone marrow smears exhibited strong block positivity (Gamal et al., 2018) which confirmed the presence of lymphoid blasts (**Figure 39 J**) when compared to the control group (**Figure 39 I**). The bone marrow smears from the treated group showed noticeable reduction in PAS positive cells (**Figure 39 K**), confirming the effect of CBPF on leukemic condition.

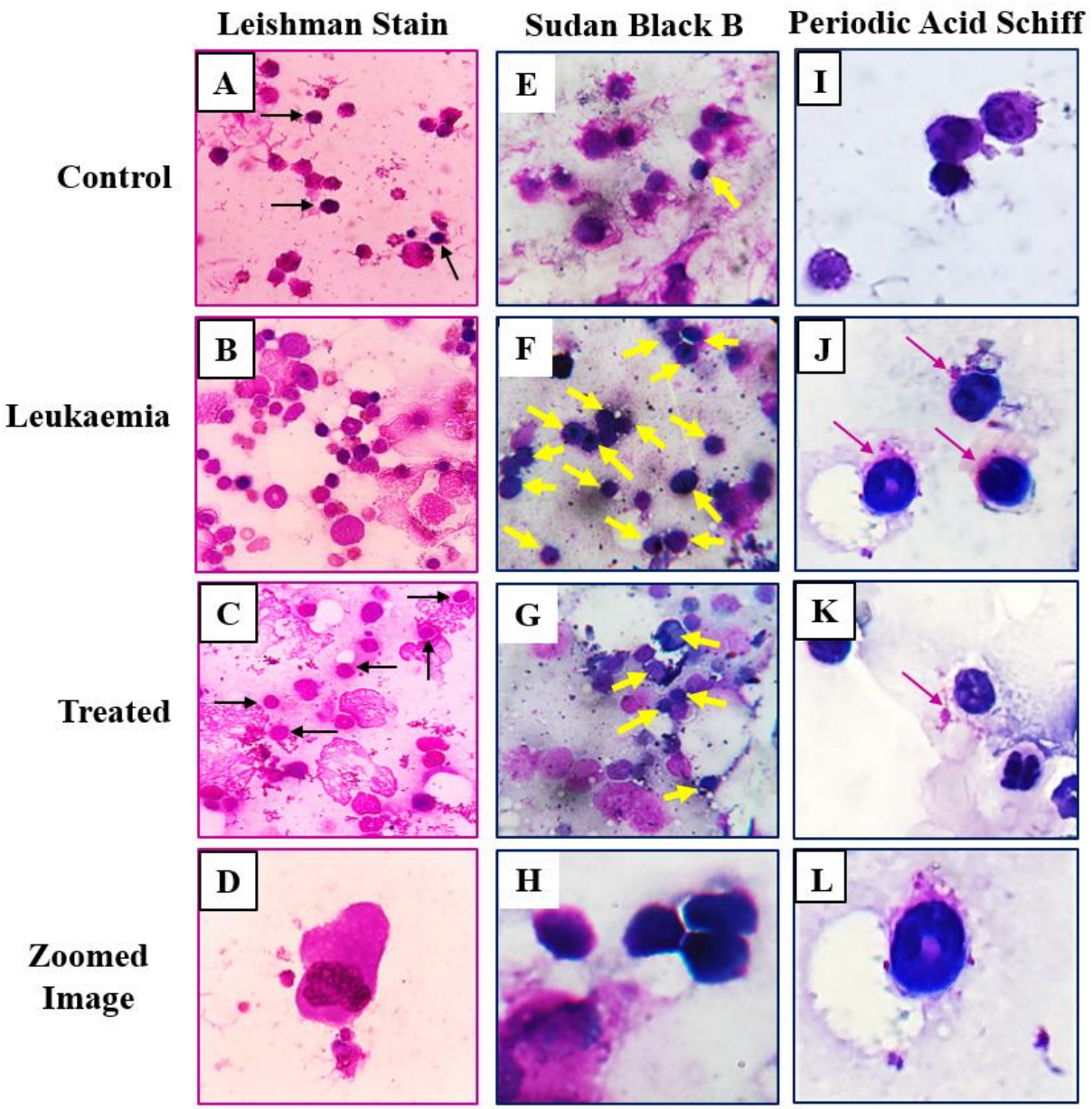


Figure 39: Bone marrow morphological analysis using cytological staining post-CBPF treatment: **A**. Leishman stained control bone marrow smear depicting matured lymphocytes (black arrows); **B**. Leishman stain depicting an increased abnormal blast over-crowding with no matured lymphocytes; **C**. Leishman stained treated bone marrow smear showing comparatively visible matured lymphocytes (black arrows); **D**. Representative (100X) image of a large Leishman stained megakaryoblast typically seen in leukemic condition; **E**. Dark blue Sudan Black B (SSB) stained (yellow arrow) myeloid blasts in control bone marrow smear; **F**. Bone marrow smear of leukemic group shows an increased number of myeloid blasts (yellow arrows) using SSB staining; **G**. Reduction of SSB stained myeloid blasts (yellow arrows) in treated bone marrow smear; **H**. Representative (100X) image of dark blue SSB stained azurophilic myeloid blast cells; **I**. Control bone marrow smear stained with Periodic Acid Schiff (PAS) stain; **J**. Leukemic bone marrow smear depicting increased PAS positive lymphoid blasts with magenta-coloured cytoplasmic granules (magenta arrows); **K**. Treated bone marrow smear depicting decreased PAS positive lymphoid blasts (magenta arrow); **L**. Representative (100X) image of a large PAS positive stained lymphoblastic cell with magenta-coloured cytoplasmic granules in cytoplasm.

8.4.5 Effect of CBPF administration on bone marrow immune functional efficacy

The cytotoxic efficacy was determined based on the percentage of dead target cells when co-cultured with the bone marrow derived cells. The cytotoxic efficacy percentage mean of the bone marrow derived cells from ENU-induced experimental group was highly reduced (1.66 ± 0.56, p = <0.0001) when compared to the control (8.66 ± 0.57). The cytotoxic efficacy of bone marrow cells, derived from post CBPF administered treated group showed significant recovery (3.33 ± 0.57, p = 0.028) when compared to the leukemic animals (**Figure 40**).

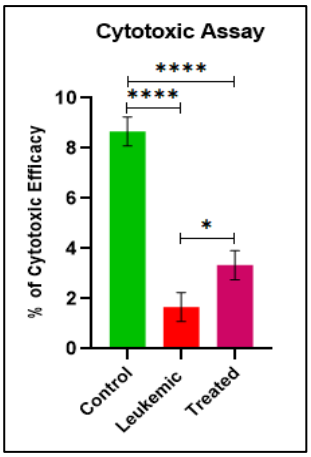
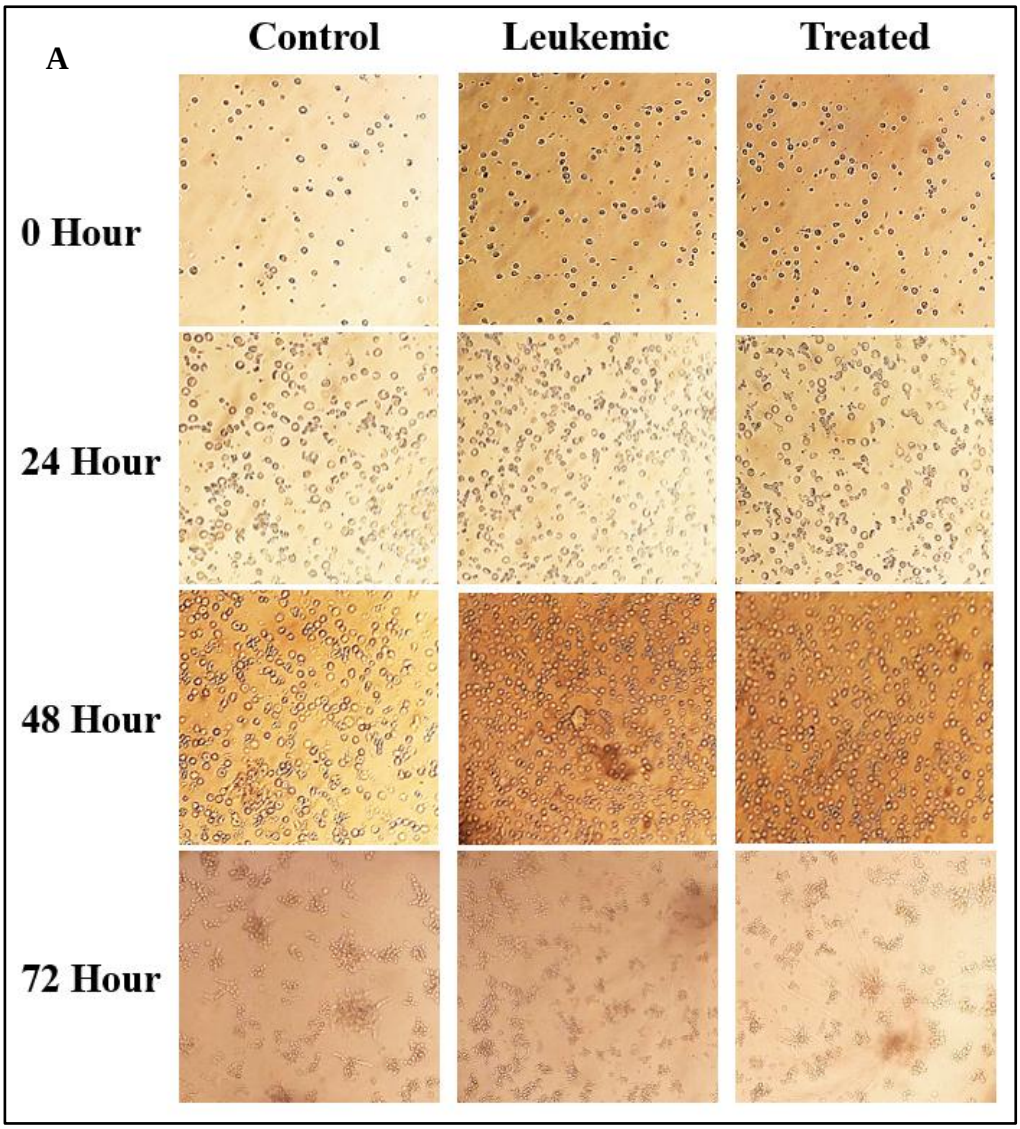


Figure 40: Effect of CBPF on cytotoxic efficacy of matured immune cells pre and post-CBPF treatment in leukemic and treated groups compared to the control

8.4.6 Effect of CBPF administration on bone marrow culture study

In short-term monoculture of bone-marrow cells, the leukemic cell population showed cellular proliferation at 24 hrs and aggressive growth at 48 hrs compared to the normal and treated group. It was noticed by 72 hrs the cell growth declined and cell death was prominent with clustering of dead cells (**Figure 41 A**).

The proliferation rate of bone marrow (BM) cells from the experimental groups was assessed using the MTT assay. The growth kinetics of cultured cells were monitored by measuring the optical density (OD) values at 0, 24, 48, and 72 hours of culture. The leukemic group exhibited a prominent increase in the proliferation rate compared to the control (p = <0.001), resulting in a darker purple colour indication of enhanced formazan formation at 48 hr culture. Cells from the post-CBPF treatment group did not show any significant difference in proliferative tendency when compared to the leukemic scenario for 24 hr and 48 hr culture but at 72 hrs of incubation significant reduction (p = 0.0143) in cell proliferation was observed when compared to the diseased condition (**Figure 41 B**).



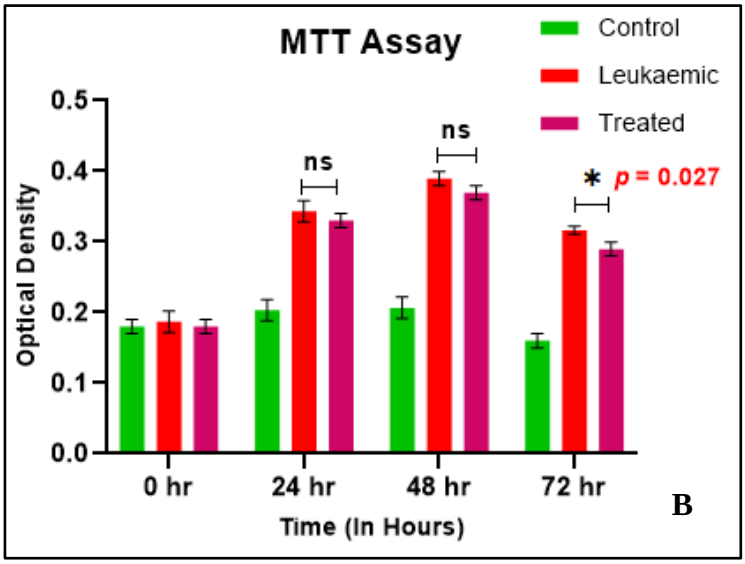


Figure 41: Effect of CBPF on cell culture and proliferation of bone marrow cells from experimental animals: A. Short-term bone marrow cell culture at 0, 24, 48 and 72 hours; B. MTT assay depicting the cell proliferation rate of control, leukemic and treated groups at 0, 24, 48 and 72 hours

8.4.7 Effect of CBPF administration on tissue architectural study

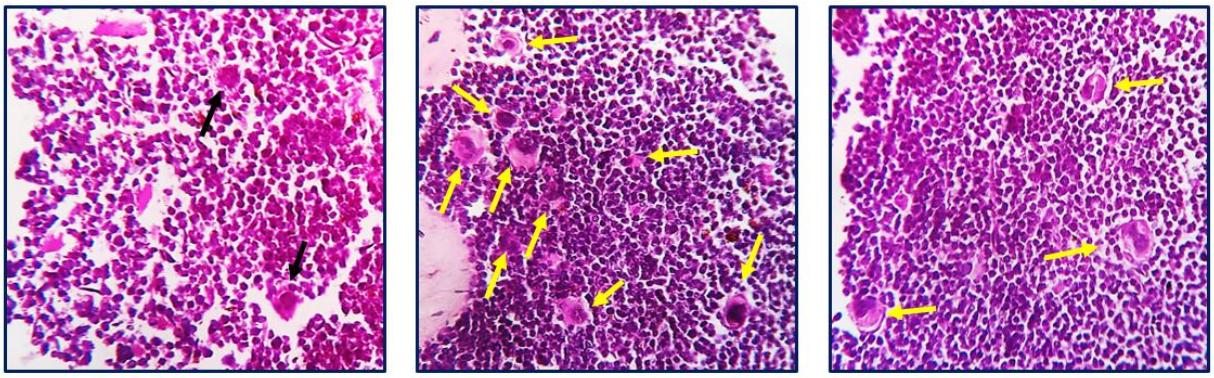
The ENU-induced assault on the bone marrow architecture, cellular morphology and microenvironment indicates a clear disruption of normal haematopoiesis in the leukemic condition. The histopathology of monomorphic blast burden in leukemic bone-marrow section depicts a diseased condition with neoplastic proliferation of atypical cells having high nuclear-cytoplasmic ratio. The marrow-architecture showed abnormality with aggressive growth and abnormal cellular clustering of myeloblast like cells (**Figure 42 B**). The control group exhibited polymorphic distribution of cells ensuring the different stages of blasts with normal cellular morphology and tissue architecture (**Figure 42 A**). The cellular morphology of CBPF-treated group showed some improvements with lesser aggregation of blast cells but no visible improvement in bone marrow architecture was observed (**Figure 42 C**).

When extra-medullary organs like liver and spleen were analysed, notable enlargement of these vital organs were observed in leukemic mice when compared to the control. Hepatomegaly (**Figure 42 D**) and splenomegaly (**Figure 42 H**) is evident in diseased group. Conversely, in the treated group, reduction in the size of liver and spleen was noticed, signifying the prominent effect of CBPF on leukemic mice.

In the leukemic group, liver tissue architecture exhibited notable infiltration of blast cells within the sinusoids (**Figure 42 F**), resulting in hyper cellularity compared to the control (**Figure 42 E**). On the contrary, blast infiltration in the treated group was significantly reduced (**Figure 42 G**), and histological sections of the CBPF-treated group displayed similar tissue morphology that of the control.

In the control group, histological sections of the spleen exhibited clear demarcation between the white and red pulp (**Figure 42 I**). However, in the ENU-induced leukemic group, the red pulp is diffused. The tissue architecture was distorted and infiltration of leukemic blasts was evident (**Figure 42 J**). Though no significant improvement in overall tissue architecture was observed in the CBPF-treated group but a notable reduction in infiltrated blasts was evident (**Figure 42 K**).

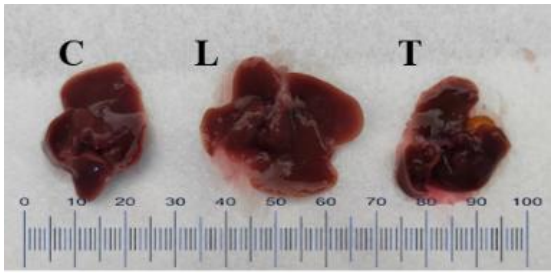
Bone Marrow Histology



Control **Leukemic** **Treated**

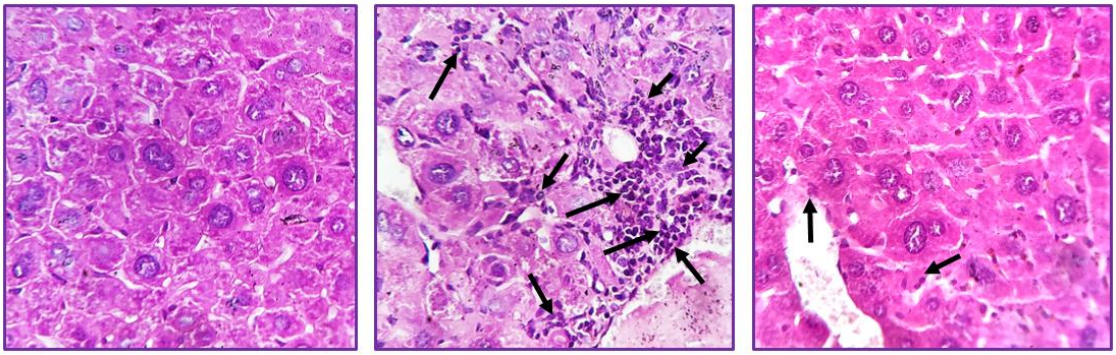
A B C

Difference in liver sizes



D

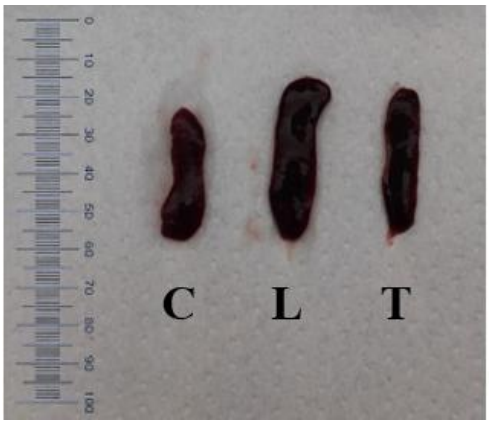
Liver Histology



Control **Leukemic** **Treated**

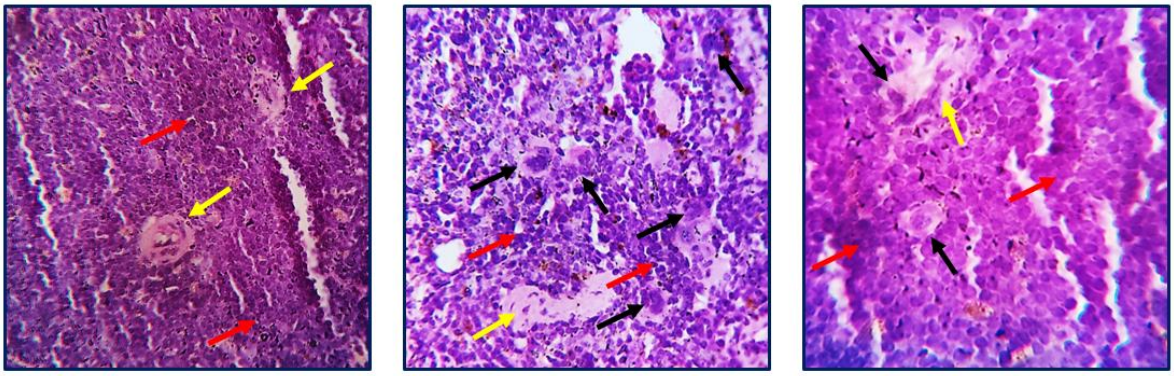
E F G

Difference in spleen sizes



H

Spleen Histology



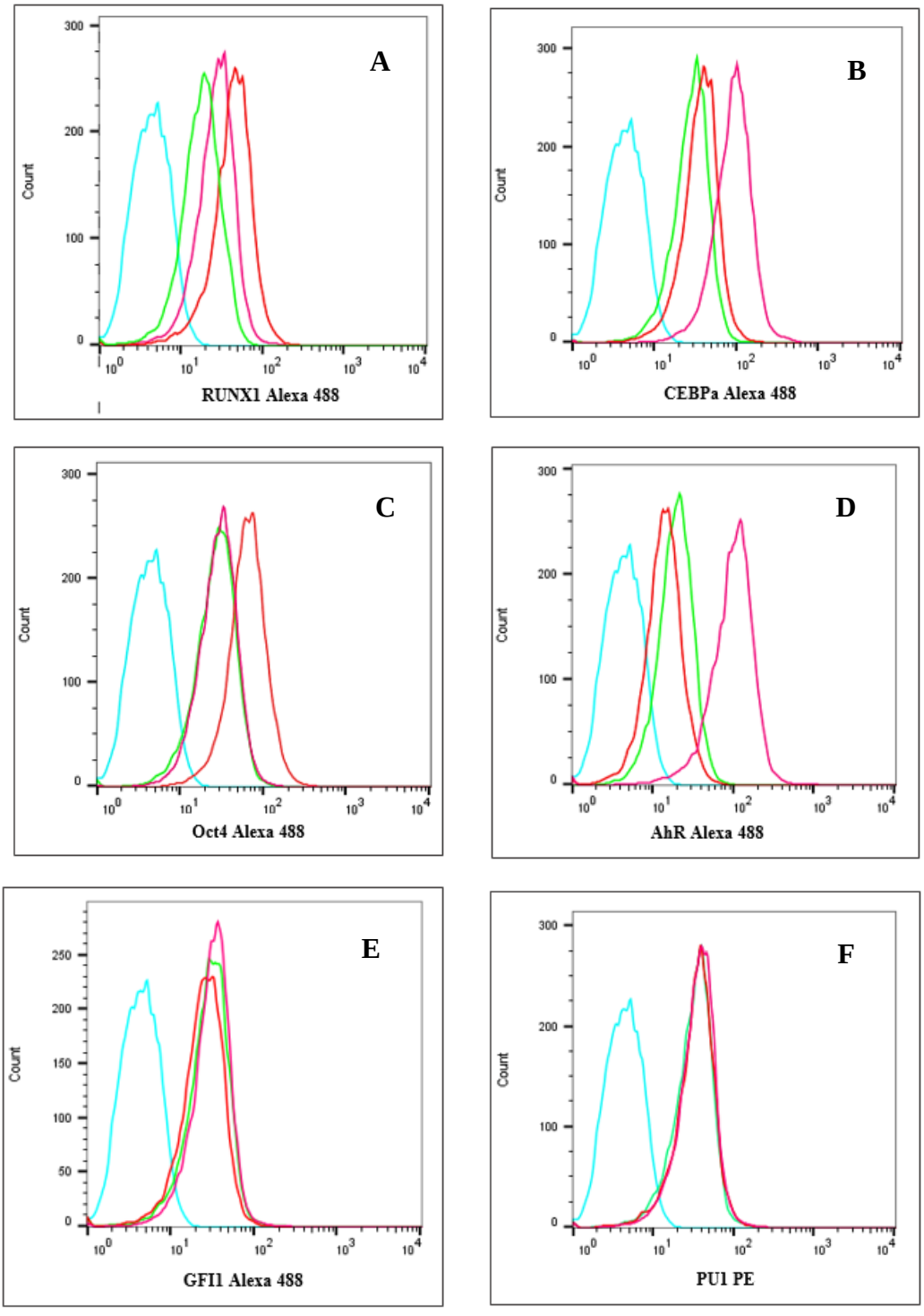
Control **Leukemic** **Treated**

I J K

Figure 42: Histolopathology status of bone marrow and other extra-medullary organs in pre and post-CBPF treatment using Haematoxylin and Eosin (H&E) staining: A. Bone marrow section from the control group showing normal cellular architecture with healthy polymorphic cells (black arrows); B. Aggressive monomorphic leukemic blast accumulation (black arrows) with clear disruption of tissue architecture in leukaemia group; C. Decreased in blast cells (black arrows) but with no major improvement in tissue architecture in treated condition; D. Effect of CBPF on size of livers in experimental mice with hepatomegaly seen in leukemic (L) animal; E. Healthy sinusoidal architecture in control liver; F. Notable blast infiltration (black arrows) in leukemic liver sinusoids with hyper-cellularity in tissue architecture; G. Comparatively healthy liver architecture with decreased in blast infiltration (black arrow) in treated group; H. Effect of CBPF on size of spleen in experimental mice with splenomegaly seen in leukemic (L) animal; I. Healthy tissue architecture with distinct red (red arrow) and white pulp (yellow arrow) in control spleen; J. Distorted tissue architecture with diffused red pulp (red arrow) and increased blast infiltration (black arrows) in leukemic spleen; K. Decreased in blast cells (black arrows) but with no major improvement in tissue architecture, red pulp (red arrow) and white pulp (yellow arrow) of spleen in treated condition

8.4.8 Effect of CBPF administration on transcription factors

The flow cytometry histogram overlays of the transcription factors from respective groups are represented in the (**Figure 43 A** to **43 F**). Flow cytometry analysis revealed significant upregulation of RUNX (**Figure 43 G**) in the hematopoietic population of leukemic mice compared to the control group (MFI; 49.27 ± 0.64 vs. 20.37 ± 1.51, respectively; $p < 0.0001$), indicating a 2.42-fold increase in expression. Post-treatment with CBPF led to a significant decline in RUNX expression compared to the leukemic group (MFI; 30.4 ± 1.51; $p = 0.0007$), representing a 1.62-fold decrease. Similarly, the expression of CEBPα (**Figure 43 H**) was significantly higher in leukemic bone marrow compared to the control group (MFI; 40.17 ± 1.56 vs. 31.50 ± 1.25, respectively; $p = 0.003$), with a 1.27-fold increase. However, in our study, a drastic increase in CEBPα expression was observed in the treated group compared to both the control and leukemic mice (MFI; 100.2 ± 2.65; $p < 0.0001$), indicating a 3.17-fold and 2.49-fold increase, respectively. Additionally, a significant increase in Oct4 expression (**Figure 43 I**) was observed in leukemic marrow compared to the control group (MFI; 71.50 ± 0.88 vs. 31.20 ± 1.05, respectively; $p = 0.0003$), representing a 2.29-fold increase. A marked decrease was observed post-treatment with CBPF (MFI; 33.6 ± 1.10; $p = 0.0005$), indicating a 2.13-fold decrease. Moreover, downregulation of AhR and GFI1 expression (**Figure 43 J and Figure 43 K**) was observed in leukemic marrow compared to the control group (MFI; 16.8 ± 1.45 and MFI; 26.83 ± 1.25, respectively, vs. MFI; 21.97 ± 0.7 and MFI; 31.13 ± 1.2, respectively; $p = 0.012$ and $p = 0.001$), representing a 1.32-fold and 1.75-fold decrease, respectively. However, a significant increase in AhR expression was observed in the treated group compared to the leukemic group (MFI; 103.7 ± 8.14; $p = 0.007$), indicating a 6.18-fold increase. Additionally, a notable increase in GFI1 expression was observed compared to the diseased group (MFI; 35 ± 0.7; $p = 0.019$), representing a 1.3-fold increase. The expression of PU1 (**Figure 43 L**) showed no significant difference between the leukemic group and the control group (MFI: 37.2 ± 0.25 vs 35.58 ± 0.58, respectively; $p = 0.05$). However, in the treated group, a significant increase in the PU1 expression (MFI: 41.03 ± 0.9; $p = 0.001$) was observed when compared to the leukemic group. Mean MFI data is represented in **Table 8**.


A
Count
RUNX1 Alexa 488
B
Count
CEBPa Alexa 488
C
Count
Oct4 Alexa 488
D
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AhR Alexa 488
E
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PU1 PE

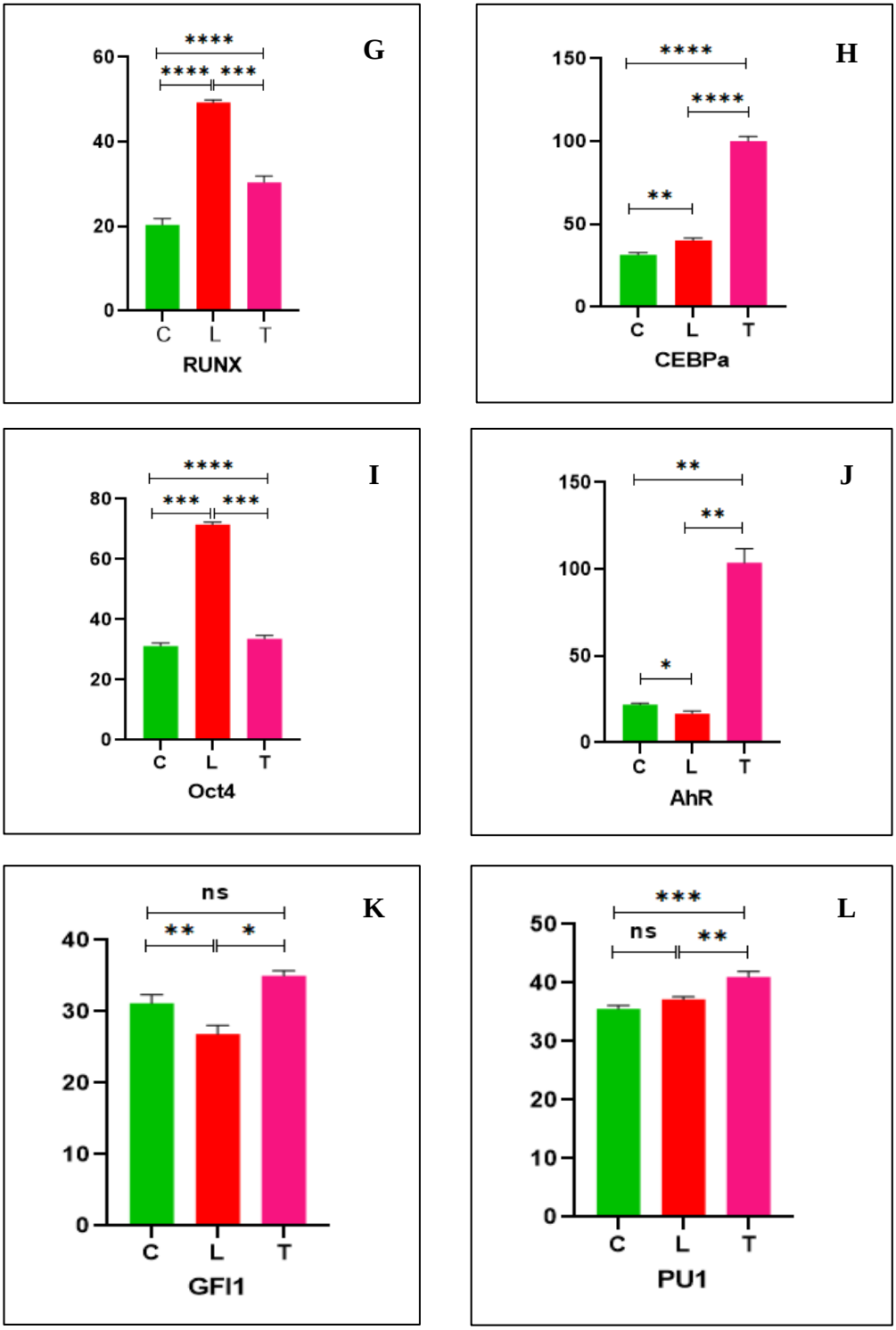

Figure 43: Flow cytometric analysis of transcription factors in pre and post-CBPF treatment: Histogram overlay of transcription factors in experimental mice A to F (Cyan: Isotype-untreated unstained, Green: Control, Red: Leukemic and Pink: Treated) and graphical representation of mean fluorescence intensity (MFI) differences G to L of transcription factors in pre and post-CBPF treatment groups

Transcription Factors	*MFI of Control*	*MFI of Leukemic*	*MFI of Treated*
RUNX1	**20.37 ± 1.51**	**49.27 ± 0.64**	**30.4 ± 1.51**
CEBPα	**31.50 ± 1.25**	**40.17 ± 1.56**	**100.2 ± 2.65**
4-Oct	**31.20 ± 1.05**	**71.50 ± 0.88**	**33.6 ± 1.10**
AhR	**21.97 ± 0.7**	**16.8 ± 1.45**	**103.7 ± 8.14**
GFI1	**31.13 ± 1.2**	**26.83 ± 1.25**	**35 ± 0.7**
PU1	**35.58 ± 0.58**	**37.2 ± 0.25**	**41.03 ± 0.9**

Table 8: Mean Fluorescence Intensity (MFI) expression of the transcription factors in experimental groups

8.4.9 Bone marrow ultrastructure analysis by Scanning Electron Microscopy

The scanning electron microscopy (SEM) ultrastructural analysis of ENU-induced bone marrow cells from the leukemic group revealed large irregular monomorphic cells with relatively smooth yet occasionally undulating surfaces and scattered tiny round vesicle like structures (**Figure 44 B**). These leukemic cells exhibited a sticky, extensive membrane processes to form mesh-like network, resulting in a inter-cellular clumping condition. In contrast, bone marrow morphology in the control group (**Figure 44 A**) showed relatively uniform-sized spherical polymorphic cells (indicative of different stages of cell development) with varied surface morphology, including short microvilli or transverse ruffles covering the entire surface. Compared to leukemic bone marrow cells, cells in the control group displayed more defined structures with distinct surface topography, resembling mature lymphoid and granulocytic origins. In the treated condition (**Figure 44 C**), large smooth irregular cells were observed, along with cells exhibiting a ruffled surface and microvilli. However, the number of smooth cells in the treated condition was lower, and cells were not visibly clumped, with no mesh-like network observed as seen in the leukemic group.

Control (10000x mag.) **Leukaemic (10000x mag.)** **Treated (10000x mag.)**

A **B** **C**

Figure 44: Morphological study of bone marrow cells using scanning electron microscopy (SEM) (10000X magnification) in pre and post-CBPF treatment: A. Control; B. Leukemic and C. Treated

8.5 Discussion

Leukaemia, a form of blood cancer in bone marrow is characterised by an uncontrolled clonal proliferation and accumulation of immature white blood cells, also known as the blast cells (Weeda et al., 2022). These blasts overcrowd the healthy matured cells refer as ‘cytes’ in blood and organs, are poorly differentiated and abnormal in nature. These immature leukocytes, having genetic abnormalities (De Kouchkovsky & Abdul-Hay, 2016), fail to perform their normal immune functions resulting in the increased susceptibility to infection (Aliyu et al., 2020; Kayser & Levis, 2022). Aliyu et al reported in their study how potent contributing factors like alkylating drugs, ionising agents and chemicals are responsible to induce chromosomal abnormalities resulting in DNA damage and eventually leading to leukaemia (Aliyu et al., 2019). Nitrosoureas, such as N-ethyl-N-nitrosourea (ENU), were reported to induce leukaemia by alkylating nucleobases, disrupting DNA integrity, which eventually lead to bone marrow suppression and the subsequent proliferation of leukemic cells (Aliyu et al., 2019). Therapeutic potential of cord blood was extensively discussed by studies who reported enhance neurogenesis and reduced inflammation in acute ischaemic stroke rats (Yoo et al., 2016), also in hippocampal function and memory restoration by plasticity enhancing proteins in aged mice treated with CBP (Castellano et al., 2017), Cord blood serum injection showed beneficial functional improvement in Alzheimer’s mouse (Habib et al., 2018), Umbilical cord serum’s efficacy in the treatment of corneal defects (Vajpayee et al., 2003; Yoon et al., 2011) and neurotropic keratitis in humans (Yoon et al., 2007).

Few studies have investigated the impact of cord blood plasma factors (CBPF) on in-vivo hematopoietic differentiation in leukemic models. Our study aimed to explore the therapeutic potential of a biological concoction like cord blood plasma factors (CBPF) in ENU-induced leukemic mice, without the use of standard chemotherapeutic drugs. However, the efficacy of such drugs are immensely effective and have prolonged the patient survival, they are often associated with severe side effects, including aplastic anaemia, bone marrow failure, chemotherapeutic resistance, immunosuppression, and cellular toxicity (Bhattacharyya & Law, 2022). We simply wanted to assess the efficacy of CBPF on leukaemia, and not trying to establish it as a ‘safer therapeutic alternative’.

In our ENU-induced leukemic model, mice displayed low body weights, probably due affected metabolism accompanied by increased leucocytosis in peripheral blood. The elevated percentage of leukemic blasts in circulation indicated the onset of leukaemia, which was further

substantiated by an increase in abnormal neutrophils and a decrease in haematological parameters like RBC and haemoglobin. Leukaemogenesis was further validated by cytochemical staining (SSB and PAS) of bone marrow smears for myeloid and lymphoid lineages. The presence of both myeloid and lymphoid blasts in marrow smears suggested a mixed leukemic condition without clear lineage specificity. Detection of abnormal increase of blasts and severely reduced matured cells were determined by Leishman staining. Treatment with cord blood plasma factor (CBPF) yielded promising results, with initial signs of recovery marked by increase body weights and strength along with a significant reduction in both abnormal neutrophils and blasts in peripheral blood. The treatment also revealed a notable decrease in observational myeloblasts and lymphoid blasts in the treated group. The efficacy of CBPF as a treatment in leukemic mice showed a significant increase in mature immune cells, such as lymphocytes, in the bone marrow, indicating a possible haematological differentiation and suggesting potential cytotoxic effects when co-cultured with sarcoma cells compared to the leukemic state. An observational decrease in the size of liver and spleen in CBPF treated group gives an insight of its potential efficacy which was further validated by histopathological examination using H&E staining. Reduction in blast cells in post-treatment marrow architecture, compared to the leukemic state and decreased infiltration of blasts in tissue architecture of treated condition ensures some therapeutic efficacy of the biological concoction.

RUNX1, a member of the Runt-related transcription factor family, is located on chromosome 21 and plays a crucial role in normal haematopoiesis. (Kurokawa, 2006; Link et al., 2010). It is responsible for the differentiation of myeloid and lymphoid lineage (K. Lam & Zhang, 2012). RUNX family proteins have been associated to several diseases and their aberrant expressions (Ito et al., 2015) are frequently seen in various cancers (Ge et al., 2014; Krishnan, 2023; Sun et al., 2019). The expression of the RUNX family proteins and their prognosis values greatly differ in leukemic conditions and several studies confirmed depending on their expression, they can act either as a tumour promoter or as a tumour suppressor in haematological malignancies (Osato, 2004; Sood et al., 2017; Speck & Gilliland, 2002). The loss of RUNX1 can lead to leukaemogenesis (Asou, 2003; K. Lam & Zhang, 2012), it was also documented ALL patients tend to have significantly higher RUNX1 gene expression level compared with controls (Sun et al., 2019). Such observation was also true for risk group stratification where high-risk (HR) patients were presented with higher levels of RUNX1 that correlates with greater leucocytosis (Szmajda-Krygier et al., 2022) and also in normal cytogenetics AML patients with poor outcomes (Fu et al., 2016). Our significant overexpression of RUNX1 in leukemic group

compared to the control reflected similar results and interestingly, a significant reduction of RUNX1 in CBPF treated group was observed when compared to the diseased indicating potential effect of CBPF on leukemic blast cells.

CCAAT Enhancer Binding Protein Alpha (CEBPα) is an intron less gene located on human chromosome 19q13 (Timchenko et al., 1995). It encodes a transcription factor (protein) that belongs to the family containing a basic leucine zipper (bZIP) motif (Pabst & Mueller, 2009). CEBPα plays a critical role in modulating gene expressions involved in cell cycle regulation (Q. F. Wang et al., 2003), body weight homeostasis, immune cell density and differentiation (Porse et al., 2001) and myeloid differentiation during haematopoiesis (Friedman, 2002; Pabst & Mueller, 2009; Zhang et al., 2004). Study revealed, the CEBPα gene expression is up-regulated during granulocytic differentiation and rapidly down-regulated during the alternative monocytic pathway (Radomska et al., 1998). The prognosis of Acute Myeloid leukaemia (AML) patients linked with CEBPα mRNA expression levels were studied (Akin et al., 2018; Su et al., 2022; van Waalwijk van Doorn-Khosrovani et al., 2003; Wouters et al., 2009) along with its expression in ALL patients (Chapiro et al., 2006; Szmajda et al., 2019). The expression of CEBPα in leukemic patients is debatable as research articles have reported contradictory expressions of the protein. Low expression of CEBPα was reported by (van Waalwijk van Doorn-Khosrovani et al., 2003) and (Grossmann et al., 2012) in leukemic cohort. On the other hand, (Hassan et al., 2021) have reported significant upregulation of CEBPα, which was associated with bone marrow hyper cellularity, high leukocyte count, increased peripheral blast percentage and poor progression-free-survival. (Gholami et al., 2019) in their study also reported a significant overall up-regulation of CEBPα in AML patients as compared to the healthy cohort. Fuchs reported the fine balance of CEBPα expression which determines the lineage oriented leukaemia, where overexpression of CEBPα in certain translocated mutation leads to BCP-ALL and a decreased expression can lead to AML (Fuchs, 2007). In our study a significant overexpression of CEBPα was observed in leukaemogenic mice when compared to the control group, suggesting a possible mutation in CEBPα genetic profile due to the potent mutagen, ENU. This increased expression may have led to the increased blast and neutrophil population. The concerning part was when an enhanced expression of CEBPα was also observed in the treated group which is not yet clear. Surprisingly in the treated group, the blast percentage was significantly decreased and the observed neutrophils were not showing any abnormal morphology which were evident in leukemic mice. So argumentatively, CBPF treatment increased CEBPα level that might have induced proper myeloid differentiation into

matured neutrophils by upregulation of lineage specific gene products simultaneously arrest cell proliferation (Fuchs, 2007).

The transcription factor, Oct4, belong to the Pit-Oct-Unc (POU) family of DNA binding proteins is present on chromosome 6 in the region of MHC complex (X. Wang & Dai, 2010). Oct4 protein is expressed in both embryonic and adult stem cells and usually associated with proliferation, pluripotency and self-renewal characteristics (Nichols et al., 1998; Pan et al., 2002), adult cells with Oct4 expression 'lacks differentiation' and could be a potential target for carcinogenesis (Tai et al., 2005). Several researchers have shown overexpression of Oct4 in germ cell tumours and somatic cancers (Atlasi et al., 2007; El Deeb & Abdelzaher, 2014; Gidekel et al., 2003). (Yin et al., 2015) in their study discussed frequent high expression of Oct4 could be a potential biomarker in AML patients when compared to the control group. (Picot et al., 2017) revealed that alteration in the expression of certain genes (including Oct4), which are responsible to maintain stem-ness, in AML cell lines they could contribute the transformation of normal cells to malignant cells with tumour forming capacities. (Aref et al., 2024) reported significant higher expression of Oct4 in both ALL and AML cohort as compared to the control. Expression of Oct4 declined at remission and elevated in relapse cases, suggesting its potential as a biomarker to improve risk stratification in acute leukaemia cases. In our study, the leukemic group exhibited significant overexpression of Oct4 compared to the control and treated groups, resulting in an increased burden of undifferentiated blasts. Administration of CBPF in the treated group led to a significant reduction in Oct4 expression, resulting in significant decrease in undifferentiated blast proliferation and subsequent increase in differentiated matured leucocytes.

The aryl hydrocarbon receptor (AhR) belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family. It is a ligand activated ubiquitous transcription factor having multiple critical cellular functions involving cell division, cell quiescence and inflammatory responses in diversified species (Hahn, 2002; McMillan & Bradfield, 2007; Rothhammer & Quintana, 2019). Like several transcription factors, AhR's paradoxical role as either an oncogene or a tumour suppressor depends on its expression in various cancers (Kolluri et al., 2017; Murray et al., 2014; Paris et al., 2021). Here we specifically focussed on the low expression of Ahr and its effect on cancer progression. (Ly et al., 2019) reported AhR signalling is suppressed in human AML blasts and is particularly downregulated in leukemic stem cell (LSC) enriched populations within leukaemia thus promoting self-renewal of leukemic blasts. (Gentil et al., 2018) in their study revealed significant reduction of AhR expression in chronic myeloid

leukaemia patients and UT-7 cell line as compared to the controls. Treatment with AhR agonist, FICZ significantly reduces leukemic clonogenicity in cell lines. We found a similar result in our study, where the expression of AhR was significantly reduced in leukemic group as compared to the control. This signifies the carcinogenic effect of ENU on the haematopoietic cells thus leading to the arrest of differentiation and promotion of cell proliferation. On the contrary, the CBPF treatment induced reduction of blasts cells in treated group and significant increase of matured lymphocytes. Another interesting study conducted by (Bunaciu & Yen, n.d.) reported treatment with retinoic acid, upregulated the AhR expression in undifferentiated promyelocytic HL60 leukemic cells, which simultaneously decreased the Oct4 expression resulting in the reduction of leukemic cell proliferation and differentiation of myeloid lineage cells. This cross-talk between Ahr and Oct4 transcription factors was also evidently expressed in our study, where with the increase of AhR expression in treated group, the Oct4 decreased leading to the reduction in blast burden and promoting haematopoietic differentiation.

Growth factor independent 1(GFI1) is a zinc-finger transcription factor (Phelan et al., 2010) that represses target gene transcription by recruiting histone deacetylase, histone de-methylases or histone mythyltransferases (Duan et al., 2005; McGhee et al., 2003; Zweidler-Mckay et al., 1996). It plays a critical role in haematopoiesis (Fraszczak & Moroy, 2017; Hock & Orkin, 2006) especially in the early development of lymphoid and myeloid cells (Möröy et al., 2008; Möröy & Khandanpour, 2011) and haematopoietic stem cells (Hock et al., 2004; Zeng et al., 2004). Studies revealed absence of GFI1 in mice and disabling mutations in human leads to severe neutropenia and accumulation of monocytic precursors (Fraszczak et al., 2019; Hock et al., 2003; Person et al., 2003; Zarebski et al., 2008) reported low GFI1 expression accelerates AML development and leads to the predisposition of myeloproliferative disorders. Reduced expression of GFI1 gene lead to genome-wide epigenetic changes involving in the pathogenesis of AML. The altered expression correlates to the patient survival and treatment, influencing the degree of disease progression (Möröy & Khandanpour, 2019). (Hönes et al., 2017) reported upregulation of GFI1 expression inhibits the leukemic expansion and colony formation *in vitro.* In humanised *in-vivo* AML mouse model, increased GFI1 drives myeloid differentiation. In our study we reported similar results where the ENU induced leukaemia expressed low expression of GFI1 leading to accumulation of blasts when compared to the control. In the treated condition, the GFI1 expression was significantly increased enabling the differentiation of haematopoietic population and reduction in leukemic proliferation signifying the possible therapeutic role of CBPF in leukemic mice.

PU1 is an ETS family transcription factor encoded by Spi1 gene. It plays multiple roles in haematopoiesis by directly regulating many genes of the myeloid, dendritic cell and B cell lineages. PU1 expression is highest in macrophages/monocytes and low to moderate in B-cells. Its action is important for the generation of haematopoietic precursors that would lead to lymphoid development (Dakic et al., 2005; Friedman, 2007; Houston et al., 2007; Iwasaki et al., 2005). Homozygous PU1 deletion leads to complete block in myeloid differentiation and heterozygous mutation leads to AML. In AML-ETO translocation fusion with PU1, the translocation downregulates the PU1 expression, this inactivation of the myeloid differentiating gene results in the proliferation of bone marrow cells in mice and overexpression of PU1 restores normal differentiation (Vangala et al., 2003). In our study, we observed a slight upregulation of PU1 expression in leukemic group as compared to the normal. Though the overexpression was not significant yet the observation was concerning and no rational could be drawn from the existing literature review. In the treated group, the CBPF treatment significantly enhanced the expression of PU1 as compared to the leukemic mice, which blocked the leukemic blast proliferation and restored the normal haematopoietic differentiation.

The scanning electron microscopy (SEM) analysis provided valuable insights into the ultrastructural characteristics of bone marrow cells in both ENU-induced leukemic and CBPF administered treated groups. In former studies it was revealed both immature lymphoid and myeloid cells exhibited smooth surfaces with sparse and flat micro-ridges (Soligo et al., 1987), leukemic cells from ‘immature’ non-lymphoid origin displayed smooth surface morphology with underdeveloped microprojections or ridge like processes, the leukemic cells from lymphoid origin also displayed similar features- smooth or undulating surfaces with little microprojections (Polliack et al., 1981), similar pattern was observed in myeloblastic and undifferentiated leukaemia that featured large smooth blasts (Polliack et al., 1975). Stages of differentiation and maturation of lymphocytes was discussed in (Polliack et al., 1981) where the presence of fewer microvilli in less matured cells were observed. In our study, the SEM images revealed the presence of large irregular monomorphic cells with relatively ‘smooth surfaces’ and scattered vesicles or blebs, indicative of immature cell morphology associated with leukaemia. Additionally, these leukemic cells exhibited abnormal structures with sticky, extensive cytoplasmic processes that form mesh-like network (Paraguassú-Braga et al., 2003), resulting in cell clumping, which may reflect altered cellular interactions and adhesion properties within the bone marrow microenvironment. Interestingly on treatment with CBPF, changes in the morphology of bone marrow cells were observed. While large irregular cells

were still present, indicating persistence of leukemic features, there was a reduction in the number of smooth cells and a notable absence of cell clumping and mesh-like networks, resembling characteristics observed in the leukemic group. Additionally, some cells in treated condition exhibited a ruffled surface and microvilli, resembling the mature lymphoid and granulocytic origin as observed in the control, suggesting potential cellular response to treatment indicating healthy hematopoietic function. However, SEM findings support the notion that treatment with CBPF may influence the ultrastructural features of bone marrow cells in leukemic mice. Further investigation is warranted to elucidate the underlying mechanisms driving these observed changes and their implications for leukaemia progression and therapeutic response.

8.6 Conclusion

In conclusion, the study investigated the potential therapeutic efficacy of cord blood plasma factors in ENU-induced leukemic mice. CBPF demonstrated possible haematological differentiation by increasing the matured immune cell population and significantly reducing the blast percentage in peripheral blood and bone marrow eventually promoting cytotoxic efficacy by the differentiated mature cells. Furthermore, effect of CBPF on leukemic condition was validated with the reduction in size of the extra-medullary organs like liver and spleen along with evident reduction of blast infiltration in tissue architecture. Effect of CBPF as a treatment with significant changes in the expression of transcription factors between pre- and post-treatment conditions, in ENU assaulted mice was promising. The scanning electron microscopy indicated the reduction of blast burden in post CBPF treated mice and exhibited healthy marrow architecture. Results of the present study, thus, indicate that CBPF has the potential to be used as an effective therapeutic agent in hematopathological conditions and should be further studied for its efficacy of cytokine profile both alone and in combination with standardized chemotherapeutic drugs.

9. General Discussion

In this study, we aimed to characterise the cellular components of fractionated human cord blood (UCB) and investigate the potential therapeutic properties of cytokine enriched UCB plasma, a biological concoction, on the haematopathological profile of an ENU-induced in-vivo leukemic mice model. Despite having an array of immunomodulatory factors, cord blood is usually considered as a biological waste and is often discarded post neonatal delivery leading to an immense loss of valuable samples that could be harnessed for research and therapeutic purposes. Although cord blood intervention has yielded remarkable results in the field of translational medicine since 1989 (Gluckman & Rocha, 2005), primarily in the treatment of patients with haematological and non-haematological diseases (Roura et al., 2015), its standalone use as a primary therapy in treatment of leukaemia is not extensively documented. The clinically approved, widely accepted and well documented treatment protocols for leukaemia involves the utilisation of chemotherapeutic agents. These clinically trialled drugs, although effective in inducing remission, are often associated with significant adverse effects that take a toll on the patients' physiological condition. Our investigation prioritised the possible treatment opportunity using a biological derived cord blood plasma fraction containing factors (CBPF) without the detrimental side effect in leukemic animals as commonly observed with standardised chemotherapeutic regime. The aim of the study was to evaluate the efficacy of CBPF in ameliorating the leukemic condition while minimising side effects, without claiming it as a 'safer therapeutic alternative.' We focussed solely on assessing CBPF's potential benefits and not advocating it as a replacement for current treatments.

In the current global scenario, increased anthropogenic activities and rampant industrialisation have led to a rise in environmental pollutants, including various carcinogenic compounds. N-nitroso compounds (NOCs) are one such recognised carcinogenic agents which are found in drinking water and foods, such as nitrate-cured meats and smoked fish, thus associated with several human cancers (Eichholzer & Gutzwiller, 1998; Hotchkiss, 1988; Mirvish, 1975; Ruiz-Saavedra et al., 2024) documented in the past couple of decades. Beyond food sources, these toxic, carcinogenic and mutagenic NOCs are also known to be present in the natural water bodies (D. Jain et al., 2020; Van Breda et al., 2019), tobacco smoke and emissions from rubber, pesticide, fertilizer, and tire industries (Descatha et al., 2005; Haorah et al., 2001). Studies have revealed the detrimental effect of NOCs in the development of various cancers in humans, (Loh et al., 2011; Straif et al., 2000; Zheng et al., 2019). ENU, a monofunctional alkylating NOC

agent is classified as a genotoxicant that can effectively bring in point mutations by targeting the oncogenes. It is extensively used as a positive control substance for genotoxicity study in animal models and also to bring in newer mutations in several organisms (Chen et al., 2002). ENU on entering a biological system, readily penetrates the intracellular environment and exerts harmful effects on the genomic and biochemical profile through DNA alkylation, carbamoylation of amino acids leading to protein structural modifications, and the induces cellular oxidative stress (Nazmeen & Maiti, 2018).

Cord blood plasma (CBP), typically procured from human umbilical cord blood (hUCB) by centrifugation is consideration as a biological waste product and is often discarded post neonatal delivery. However, studies over past decades have shown that CBP holds valuable properties to induce trophic effects when used for therapeutic purposes. CBP has been used as a substitute for standard serum in the growth and expansion of various human stem cells, like-hUCB-derived mesenchymal stem cells (MSC), human dental stem cells, hUCB-derived T-lymphocytes, and human endothelial colony-forming cells (ECF). Moreover, CBP has also shown therapeutic potential in animal models specifically in rodents and mice. Studies revealed, CBP when administered to rats with acute ischemic stroke was found to enhance neurogenesis and effectively reduce inflammation, leading to a significant recovery after the stroke. On having a plasticity-enhancing protein in CBP namely the tissue inhibitor of metalloproteinases 2, restoration of hippocampal function and improvement of memory in 18-month-old mice was observed. Cord blood's therapeutic effect was reported in an Alzheimer's disease mouse model, where a specific fraction from cord blood serum showed beneficial effects compared to adult blood serum. In humans, umbilical cord serum has been effectively used to treat corneal defects and neurotrophic keratitis. Additionally, cord blood is extensively used in the treatment of haematopathological conditions. Keeping these in mind, we sought to assess the cord blood plasma factor (CBPF) role in an in-vivo leukemic model.

Our research endeavour in the fifth chapter began with an aim to establish and standardise cord blood parameter reference range for the West Bengal population, while investigating potential differences in cord blood haematological profile between urban and rural populations. We know accurate reference values are critical for the assessment of neonatal health, and a standardised reference is essential to avoid ineffective clinical management during prognosis. Since previous studies predominantly reported cord blood reference ranges for Caucasian neonates and not much on South Asian groups, through this research we sought to fill this gap, focussing on the cord haematological profile of neonates in West Bengal specifically.

Our findings established notable differences in cord blood parameters based on delivery modes i.e. vaginal and caesarean delivery and regional differences i.e. urban-rural inhabitants. Neonates delivered in a reputed public hospital via normal vaginal birth showed significantly higher neutrophil and monocyte counts compared to the caesarean groups in the same hospital, whereas elevated lymphocyte counts were observed in both the scenarios. These observations align with prior studies; however, a notable finding was the increase of total white blood cell (WBC) counts in West Bengal cohort. This result differs from the existing reference ranges documented in global populations, including Caucasian, South East Asian, and African groups, as well as other Indian cities, suggesting possible environmental or regional influence on the haematological profile. Our study also revealed significant differences in neutrophil and lymphocyte enumeration between urban and rural populations. Neutrophil counts were statistically higher in rural areas, while lymphocyte counts were elevated in urban populations. These findings emphasise the need for population-specific reference ranges, as regional disparities can impact haematological assessments. Furthermore, red blood cell (RBC) values in the West Bengal population were within the accepted global reference ranges, while parameters like packed cell volume (PCV), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular haemoglobin concentration (MCHC) were consistent with South East Asian and European standards as anticipated. This, as significant differences were observed when comparing West Bengal values to those from other populations.

This study provides the first comprehensive analysis of cord blood parameter differences between urban and rural populations in West Bengal, while highlighting the need for regionally tailored reference intervals. Further exploration would unearth the underlying causes of these haematological differences and refine clinical practices based on population-specific data.

In our subsequent, sixth chapter, we aimed to investigate the differences in the morphological and biochemical properties of erythrocytes procured from UCB and adult peripheral blood (APB). UCB harbouring haematopoietic stem and progenitor cells (HSPCs) is increasingly used in therapies of genetic disorders, blood malignancies, and immune deficiencies. Based on our studies of morphology, osmotic fragility, oxidative status, and membrane protein expression, we tried to indicate some features that make UCB erythrocytes different from adult erythrocytes and show their possible advantages.

Scanning electron microscopy (SEM) revealed some unique features, where it was observed that the indentations in cord erythrocytes were deeper, while the dents in the adult cells were more uniform. These deeper indentations may be due to lower shear stress in cord blood, reduced by increased blood viscosity, low pH, and temperature elevations. In addition, the high concentration of foetal haemoglobin (HbF) in cord blood (Pritišanac et al., 2021) may increases the chance of cell deformability. Our SEM also showed an increased number of reticulocytes and significant erythrocyte deformities in cord blood, which are usually absent in adult blood.

The osmotic fragility of cord erythrocytes indicated their less fragile nature than adult cells. This reduced osmotic fragility infers greater physical integrity and elasticity in the cord erythrocyte membrane and could be attributed to lower cholesterol/phospholipid ratios. Further investigation into the oxidative status in cord blood haemoglobin revealed that the predominant HbF exhibited lower formation of met-haemoglobin, hence low oxidative stress, as compared to adult haemoglobin. Erythrocyte membrane protein analysis has shown striking differences in the expression level of Spectrin, Band 3, Band 4.1, and Band 4.5 between cord and adult blood, where cord blood expressed higher levels of GLUT1, probably due to the high demand for energy by rapidly growing foetal cells and low expression of Band 3 reflecting its reduced functional involvement in HCO3- transport. The lipid peroxidation assays demonstrated that the membranes of cord erythrocytes contain lower oxidative damage compared to adult erythrocytes, and hence, reduced risk of protein backbone cleavage. Additionally, our flow cytometric analysis using CD235a marker highlighted significantly higher cord erythrocyte population when compared to the adult peripheral blood.

Therefore, cord erythrocytes possess several unique morphological and biochemical features such as deeper indentations, lower osmotic fragility, and reduced oxidative stress that correlate with superior deformability and clinical significance for neonatal transfusion and other therapeutic uses.

Following the cord erythrocyte characterisation, in our seventh chapter, we tried to investigate a comparison between cord blood (CB) and adult peripheral blood (PB) white blood cells (WBC) using flow cytometric and scanning electron microscopic analysis. Another aspect of the study focused on understanding the effects of cell fixatives like 1.5% paraformaldehyde (PFA) solution on hematopoietic cells when preserved for future analysis, and whether the classical cellular distribution, based on size and granularity, is affected.

The flow comparison was based on the mean cell percentages of different haematopoietic cells using dot plot analysis in fresh and fixed samples, aimed to understand fundamental differences in cellular components without using antigenic markers. Although studying cell population differences through forward and side scatter (FSC and SSC) alone is limited, we attempted to develop a cost-effective procedure to estimate cell percentages based on size, granularity, type, and distribution in unfixed and fixed conditions. The results revealed notable differences in the cellular components of CB and PB between fresh and fixed samples. We observed the fixed samples provided better isolation and distribution of cellular components compared to fresh samples. We also reported, lymphocyte percentages were higher in fixed whole cord blood (WCB) compared to peripheral blood (WPB) and fresh CB samples also showed higher monocyte counts than PB, indicating a richer monocyte content in CB, which may enhance its therapeutic potential in regenerative medicine, this was documented in our population based study aligned with previous studies showing higher classical monocyte percentages in CB compared to PB. Quadrant analysis confirmed significantly higher mononuclear cell (MNC) counts in fresh CB compared to PB, suggesting CB's robust cellular profile, making it suitable for stem cell therapies and transplants.

Cord blood leucocytes contain more primitive hematopoietic cells than those in PB, as demonstrated by scanning electron microscopy. Previous investigations have shown that the majority of CB lymphocytes displayed short, dense villi and sparse microvilli. Adult PB did not have reticulocytes, while CB MNCs did. The hypoxic environment in cord blood, which promotes erythropoiesis, is probably the cause of the occurrence of reticulocytes. These results demonstrate CB's distinct morphological characteristics and point to its therapeutic benefits.

Before delving into the eight chapter of this thesis, we sought to explore and unravel the complexities of cord blood, an extraordinarily valuable resource that was once considered as a biological waste. Today, its immense potential in the field of clinical research and translational medicine is widely recognised and the graph of its utilisation is on the positive direction. For decades, cord blood had a profound impact on the treatment of non-communicative diseases, serving as a bridge between fundamental science and therapeutic advancement. Standing at the dawn of scientific evolution, cord blood is paving the way for its further advancement and beneficial utilisation. The standardisation of cord blood haematological profiling and its contribution to the global database, opens up several opportunities for this valuable biological product to be utilised and get valued for its altruistic property.

Leukaemia is characterised by the neoplastic proliferation of haematopoietic blasts and immature white blood cells in the bone marrow, out numbering the healthy matured cells and eventually rendering the immune system ineffective. The goal of the eight chapter was to explore, for the first time, the therapeutic benefits of human CBPF in a leukemic mouse model, induced by a potent carcinogenic chemical, N-Ethyl-N-nitrosourea (ENU). As studies have revealed the detrimental effect of most standard chemotherapeutic drugs on the treated patients like bone marrow suppression and immunosuppression, CBPF was chosen for its therapeutic property to assess its potential in providing the minimal side effects when used for leukemic amelioration. In our leukemic model, the ENU treated mice showed symptoms such as reduced body weight, crouched stature, weakness with increased leucocytosis, abnormal neutrophil and blast count, and reduced other haematological parameters like RBC and haemoglobin. Bone marrow analysis through Sudan Black B (SSB) and Periodic Acid-Schiff (PAS) cytochemical staining demonstrated myeloid and lymphoid blasts, revealing a mixed leukemic condition not particularly indicating a specific lineage. CBPF treatment showed promising therapeutic potential, evidenced by increased body weight and a significant decrease in abnormal neutrophils and blast cells in peripheral blood circulation in treated group. Additionally, CBPF-treated mice showed increased mature immune cells like lymphocyte, thus suggesting a possible haematopoietic differentiation.

Histopathological analysis using H&E staining showed a reduction in the size of liver and spleen tissues and a significant reduction in leukemic cell infiltration in the CBPF-treated group. The therapeutic effect was further confirmed in bone marrow samples, showing a decrease in blast cells and the restoration of normal marrow architecture. Scanning electron microscopy analysis of bone marrow post-CBPF treatment reveals reduced blast cells and restoration of healthy microenvironment architecture, highlighting CBPF's therapeutic impact. These valuable observations reflect the efficacy of CBPF treatment in ENU-induced leukemic mice.

This chapter also focused on key transcription factors involved in leukemogenesis and were analysed using flow cytometery. RUNX1, a critical regulator of haematopoiesis got significantly overexpressed in the leukemic group, while its expression in CBPF treated group showed marked reduction in its expression, suggesting CBPF may interfere with leukemic blast proliferation by modulating RUNX1. CEBPα, an essential regulator of myeloid differentiation also overexpressed in the leukemic condition. Despite the continued high expression of CEBPα post CBPF treatment, blast counts decreased, and neutrophils displayed normal morphology,

indicating that CBPF may have the potential to promote proper granulocytic differentiation while arresting abnormal cell proliferation. Oct4, a stem cell marker is associated with pluripotency and blast proliferation, was significantly elevated in our leukemic group but there was a notably decrease in its expression following the CBPF treatment, correlating with a reduction in undifferentiated blasts and an increase in mature immune cells. The aryl hydrocarbon receptor (AhR) was downregulated in leukaemia and was restored in the CBPF-treated group thus suggesting that CBPF may promote differentiation by enhancing AhR signalling. Furthermore, the upregulation of GFI1 in the treated group supports the role of CBPF in promoting haematopoietic differentiation thus reducing leukemic proliferation. Though PU1 didn't show convincing results but the overall findings indicate that CBPF modulates key transcription factors involved in leukemogenesis, leading to decreased blast proliferation, improved differentiation, and a potential therapeutic effect in leukaemia.

Further investigation into the molecular mechanisms of CBPF's therapeutic effects could have given us a more holistic picture but we had to accept the limitations of this study. The comprehensive analysis as elucidated in our study is expected to significantly contribute to the existing knowledge base and the insights gained from this research might lay the foundation for further exploration of cord blood use amongst the upcoming future generation.

10. General Conclusion

The study helped us understand the multifaceted potential of cord blood as a valuable resource in research, which can be utilised in clinical practices for patient-related treatments. The findings highlighted the importance of standardizing reference values of cord blood specific to the Indian context, focussing on the West Bengal population as extensively studied in Caucasian cohort. These reference ranges ensure more accurate clinical assessments and improved patient outcomes and the establishment of a local database of cord blood parameters can enhance diagnostic precision and develop therapeutic strategies personalised to the Indian population having diverse genetic and environmental backgrounds. The biochemical and morphological investigations of cord blood revealed the unique structural characteristics and features that might have a clinical benefit in the treatment of neonates when blood transfusion is considered as one of the treatment procedures. Further involvement of advanced techniques such as Scanning Electron Microscopy (SEM) and flow cytometry in our research, unveiled some distinct features that not only helped in understanding the fundamental differences between neonatal and adult immune systems but also opened several avenues for exploring cord blood's therapeutic applications, particularly in the field of regenerative medicine and immunotherapy. Our investigation of cord blood plasma factors (CBPF) in an ENU-induced leukemic mouse model endeavours its promising therapeutic efficacy- though its utilisation is not advocated as an alternative to the chemotherapeutic intervention. In our study, CBPF administration resulted in increased populations of matured immune cells and a significant reduction in the percentage of leukemic blasts in peripheral blood and bone marrow, suggesting that CBPF may have a dual role, one facilitating haematological differentiation while another promoting cytotoxic effects against leukemic blasts. Furthermore, the size reduction of extra-medullary organs like the liver and spleen, along with the improved bone marrow architecture in post-CBPF treatment mice, validated the potential of this biological concoction as an effective therapeutic agent in leukemic conditions. The implications of this research extend beyond immediate clinical applications as clinical intervention could be a prospect but not an effective replacement to standardised clinical practices. By further studying the unique properties of cord blood and its components, we can develop novel therapeutic strategies aimed at combating various haematological and non-haematological conditions along with the already existing effective drugs. As the understanding of cord blood's capabilities continues to evolve to enrich our knowledge, it is crucial for the medical community to recognise its importance not only as a source of naive stem and progenitor cells but also as a reservoir of bioactive

factors that may significantly contribute to patient care. Human cord blood holds immense prospects in modern medicine that are yet to be unearthed. The establishment of standardised reference values, coupled with ongoing research into its therapeutic applications, can transform cord blood from a biological waste product into a vital tool in fighting various health challenges. This research is just a tiny contribution to the continued exploration of cord blood and its derivatives in innovative treatments, ultimately paving way to improve health outcomes and enriching the landscape of translational medicine.

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List of Publications

1. Cord Blood Haematological Parameters Reference Range Difference in Urban and Rural Populations of West Bengal as Compared to Global Scenario
   *Sayak Manna, Indranil Dhar, Tapan Kumar Naskar, Sujata Law**
   https://doi.org/10.37871/jbres

2. Cord and peripheral blood erythrocyte analysis by scanning electron microscopy and flow cytometry
   *Sayak Manna, Payel Biswas, Rajen Haldar, Tapan Kumar Naskar, Sujata Law**
   https://doi.org/10.1111/ijlh.13810

3. Cord and peripheral blood mononuclear cell analysis by scanning electron microscopy and flow cytometry
   *Sayak Manna, Tapan Kumar Naskar, Sujata Law**
   https://doi.org/10.55184/ijpas.v76i03.266

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RESEARCH ARTICLE

Cord Blood Haematological Parameters Reference Range Difference in Urban and Rural Population of West Bengal as Compared to Global Scenario

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ABSTRACT

Background: Human umbilical cord blood is often discarded as a biomedical waste. We aimed to standardise a local cord blood reference range for the West Bengal population. The cord blood haematological values differ depending on ethnic, regional and genealogical groups, so we primarily focussed our study on the international and intra-national differences of cord blood parameters. Comparison between the cord blood values of urban and the rural population along with normal and caesarean deliveries was not recorded before.

Methods: Umbilical cord blood was procured from consenting mothers (average age: 26.1 and 38-40 weeks gestation period), post-delivery at Medical College, Kolkata. Cord blood parameters were divided into two major groups: urban and rural population, each group was further divided into two sub-groups depending on the mode of delivery: normal and caesarean delivery. Comparison was also drawn on the basis of other international groups having different ethnic backgrounds and inter-state individuals having similar ethnic background.

Results: Our results showed West Bengal's cord blood WBC value was higher and monocyte count was much less when compared globally. The neutrophil value was higher in rural as compared to urban and both the lymphocyte and platelet values of urban were recorded more than rural. Slight differences were recorded among urban-normal, urban-caesarean, rural normal and rural caesarean groups.

Conclusion: A new angle in terms of urban and rural population study is introduced in cord blood analysis. The standardisation of cord blood reference range for the West Bengal population is a new step for neonatal studies.

INTRODUCTION

Umbilical cord blood is rich in hematopoietic stem/progenitor cells [1] and an effective mode of transplantation [2] for its reduced or depressed immunological responses [3] and high availability. The cells with their immense ability of self-renewal and differentiation into multi hematopoietic lineages [4] can lead to the opening of various avenues including researches in translational medicine and other therapeutic measures [5,6]. The advantages of procurement of cord blood for hematopoietic cells include easy and harmless collection procedure, which is safe for both the mother and the new born, reduced graft-versus-host reactivity, low viral contamination [7] and a painless procedure [8]. When cord blood is procured through proper guidelines availability of sample is high and risk of transmissible infections is low [9]. Studies revealed umbilical cord blood can also be considered as an excellent alternative to neonatal blood for the evaluation of sepsis in infants [10,11].

Studies revealed extensive research work is already done to standardize

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ORIGINAL ARTICLE

Cord and peripheral blood erythrocyte analysis by scanning electron microscopy and flow cytometry

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Abstract

Introduction: Human umbilical cord blood is rich in hematopoietic cells. We aimed to focus on the morphological, biochemical, membrane protein profile and surface protein expression differences of erythrocytes, isolated from cord and adult peripheral blood using techniques such as high-resolution scanning electron microscopy (SEM), gel electrophoresis (SDS-PAGE) and flow cytometry.

Methods: Adult peripheral blood was collected from consenting adults, and umbilical cord blood was procured from consenting mothers, post-delivery at Medical College, Kolkata. We emphasized on cord and adult peripheral blood erythrocytes' morphological variations using SEM images and protein expression by flow cytometric analysis. Some conventional biochemical analyses such as osmotic fragility of the cell membrane, haemoglobin co-oxidation study and lipid peroxidation assay were done for supporting evidence along with membrane protein content using gel electrophoresis.

Results: Our SEM images indicated clear morphological variations in cord erythrocyte with a higher degree of cellular deformities and difference in membrane texture. Flow cytometric analysis of cord erythrocyte showed a significant difference in CD235a expression than adults. We observed an overexpression of GLUT1 and decreased expression of Band 3 in cord erythrocyte membrane. Our results also showed cord erythrocytes have low osmotic fragility, a slower rate of co-oxidation of cord haemoglobin and a lesser lipid peroxidation level than that of adults.

Conclusion: Cord blood erythrocytes have deeper indentations leading to higher flexibility, more oxygen-carrying capacity and less osmotic fragility in comparison with adult erythrocytes. The expression of CD235a and Band 4.5 (GLUT 1) was significantly higher in cord erythrocytes than peripheral adult erythrocytes.

KEYWORDS
cord blood, erythrocyte, flow cytometry, gel electrophoresis, peripheral blood, scanning electron microscopy

1 | INTRODUCTION

Umbilical cord blood is a biological waste, and it is an excellent source of low immunogenic hematopoietic stem and progenitor cells.[1] Studies revealed cord blood parameters are similar to that of the neonate or foetus venous blood[2] enabling it to be a safer and painless alternative source for various haematological studies,[3,4] blood transfusion in case of hematopathological conditions and therapies. During the procurement of stem cells from cord blood, RBCs of neonates are usually discarded and less importance is given

Sayak Manna and Payel Biswas: Contributed equally.

RESEARCH ARTICLE

Cord and peripheral blood mononuclear cell analysis by scanning electron microscopy and flow cytometry

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ABSTRACT

Background: Umbilical cord blood is a rich source of hematopoietic cells, which vary in phenotypic expressions from that of adult peripheral blood. Our study focused on differences in fresh and fixed cell populations isolated from umbilical cord and adult peripheral blood using flow cytometric analysis and high-resolution scanning electron microscopy (SEM). *Methods:* Adult peripheral blood (PB) and cord blood (CB) was collected from individuals from Kolkata. Our study emphasised on the differences in fresh and fixed cord and peripheral blood samples through forward and side scattering of flow cytometer and without any surface antigens. We also studied the morphological variations of both the cord and peripheral MNC using SEM images. *Results:* The flow cytometric analysis of whole cord blood and isolated cord MNC showed significant differences in counts when compared to adults in both fresh and fixed samples. Our SEM images indicated similar morphological features in cord leucocytes when compared to adult leucocytes. It was also observed presence of reticulocytes in SEM analysis only in cord MNC as compared to adult. *Conclusion:* Our study demonstrates the effectiveness of flow cytometry for analysing cellular populations in cord and adult peripheral blood, without requiring antigen markers. Additionally, our SEM analysis provides some insights on the morphology of mononuclear cells from CB and PB sources, enhancing our understanding of normal cellular dynamics in the regenerative medicine domain.

Keywords: Cord blood, Peripheral blood, Mononuclear cells, Scanning electron microscopy, Flow cytometry.

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INTRODUCTION

Human umbilical cord blood is rich in hematopoietic cells. Its usage has gained interest in the medical and research field, after the discovery of its effective use in stem cell transplantation[1,2] amongst adult and young recipients. There is an advancement in the rapid use of cord blood for the treatment of several hematopathological conditions and disorders like Fanconi's anaemia,[3] severe combined immune deficiency (SCID),[4] Krabbe's disease,[5] etc. Since cord blood (CB) has been in use as an alternate source of blood transplantation[6] for almost more than two decades now, having a full picture of the cord blood cellular population is of utmost importance.

In the past much work has been done on the characterisation of cord blood nucleated cells using surface antigens[7,8] but the purpose of our study was to establish differences in values of normal cord blood as well as adult peripheral blood nucleated cells in fresh and fixed conditions using flow cytometry, without any use of antibody markers. Studying nucleated cell populations using forward and side scatter was done to ensure cost-effective cell analysis. Focus was given on population changes, cell count, and distribution pattern post-fixation. Flow cytometry measures and analyses single-cell suspensions' optical properties as they pass through a laser beam following hydrodynamic focussing. Forward scatter indicates cell size, while side scatter reflects cell complexity and granularity.

Previous studies revealed the ultrastructure of adult peripheral blood using high-resolution scanning electron microscopy (SEM).[9,10] SEM analysis was also done in case of diseased conditions like leukaemia and other erythrocyte disorders in peripheral blood.[11] In 2002, a study was conducted to understand the ultrastructure of primary and cultured leucocytes obtained from cord blood, but the work focussed on the use of transmission electron microscopy (TEM) to analyse cord blood cells.[12] Building on our previous study, which analysed the differences in erythrocyte populations between cord blood (CB) and adult peripheral blood (PB),[13] we now tried to focus on examining the leukocyte populations in these blood sources. As little to no study exists on the three-dimensional structural analysis of cord mononuclear cells (MNC) using SEM, we aimed to draw a difference in the cell morphological structure of both cord blood and adult peripheral blood leucocytes using scanning electron microscopy.

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