# Landscape of Granulocyte Telomere Shortening in Cytopenia in India

**PARAMITA MANDAL** ( paramita.mandal2@gmail.com )
Nilratan Sircar Medical College and Hospital https://orcid.org/0000-0001-9971-342X
**Shyamali Dutta**
Nilratan Sircar Medical College and Hospital

**Research note**

**Keywords:** Telomere, cytopenia, FLOW-FISH, granulocyte, age

**Posted Date:** June 29th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-36887/v1

# Abstract

**Objectives:** Telomere length and telomerase activity have been implicated in the control of cell proliferation and genomic stability. We recently introduced a novel technique for measurement of the average telomere length in cells using fluorescence in situ hybridization (FISH) with peptide nucleic acid (PNA) probes and flow cytometry (flow-FISH).

***Results:*** Using this technique, we have analyzed the telomere length kinetics in subpopulations of unseparated peripheral blood leukocytes (PBLs) in a large population of healthy donors as well as patients with cytopenia. We only found significantly shortened telomeres from patient with cytopenia compared to age-adjusted controls. Strikingly, the telomere length in granulocytes from cytopenia patients differed significantly from controls. Furthermore, an inverse correlation between of granulocyte telomere length and age was found among healthy controls. However, surprisingly, telomere length in cytopenia patients with hypocellular marrow was significantly shorter from patients with non-hypocellular marrow. These results support the concept of accelerated granulocyte telomere shortening in cytopenia patients in India and suggest a potential use of telomere-length measurements as a prognostic tool in this group of disorders as well.


# Introduction:

Cytopenia is a reduction in the number of blood cells. It takes a number of forms viz. low red blood cell count resulting in anemia, low white blood cell count resulting in leukopenia or neutropenia, low platelet count resulting to thrombocytopenia. There is no epidemiological data on the true incidence of cytopenias from India and we have to depend on hospital based statistics.

Telomeres are the TTAGGG repeat sequences at the ends of chromosomes. They become progressively shorter at each cell division due to the end replication problem [1]. Telomeres protect genome from nucleolytic degradation, unnecessary recombination, repair, and interchromosomal fusion [2, 3]. As a normal cellular process, a small portion of telomeric DNA is lost with each cell division [4]. The average length of telomere repeats at the ends of chromosomes provides indirect information about their mitotic history [5, 6]. Telomere length may therefore serve as a biological clock to determine the lifespan of a cell and an organism [3, 7]. Human telomerase is a reverse transcriptase enzyme capable of counteracting telomere shortening by adding single-stranded telomeric DNA to the ends of chromosomes [8–10]. Telomerase is constitutively expressed in cells of the germ line and is abundant at variable levels [11–14]. A recent study [15] showed that telomerase gene therapy rescues the telomere length in mice with aplastic anemia.

Abnormal telomere shortening of Peripheral Blood leukocytes has been described in patients including aplastic Anemia, paroxysmal nocturnal hemoglobinuria (PNH) and myelodysplastic syndromes [3, 16, 17]. Telomerase activity was increased in lymphocytes from Immune thrombocytopenia (ITP) patients [18, 19]. The telomere length in nucleated peripheral blood (PB) cells indirectly reflects the mitotic history

of the hematopoietic stem cells (HSCs). As a normal cellular process, telomere length decreases with age [20, 21].

Earlier studies have provided an insight into the association of telomere shortening with different grades of cytopenia and act a predictor of response to immunosuppressive therapy [22, 23]. Our objective herein was to re-investigate the average leucocyte telomere length in different grades of cytopenias compared to healthy controls in Indian population. Particularly, we emphasized on determining the association of telomere length in subset population of leucocyte to identify the most representative leucocyte population for telomere length. We further explored the expression pattern of human telomerase gene (hTERT) among different grades of cytopenias and its association with telomere length. The secondary objective of the study was to establish the relationship of telomere length with bone marrow cellularity and establish the range of normal variation of telomere length in healthy subjects with normal blood counts.

# Methods:

### Ethics Statement

All samples were collected from the subjects with written informed consent approved by the institutional ethical committee for human experimentation of the NRS Medical College and Hospital, Kolkata, India.

### Selection of study subjects and controls

Study subjects were recruited from patients attending the outpatient department (OPD) of the hospital. A review of complete blood counts of patients, obtained from an automated hematology analyser was done. Controls were selected from persons with normal blood counts including voluntary blood donors and relatives of patients.

### Data Collection

Patients presenting with cytopenias were evaluated by detailed clinical history and physical examination and tests like liver function test and bone marrow biopsy and aspiration tests were performed. Peripheral blood samples were collected from patients and healthy controls by aseptic method. A total of 176 cases of cytopenias and 101 healthy control samples were collected for the study. Three categories of cytopenia patients were selected for the study like aplastic anaemia (n=101), thrombocytopenia (n=31) and anaemia (n=41). There were 31 cases of beta thalassaemia major and minor and E Beta thalassaemia in the cases with anaemia.

### Telomere length measurement by FLOW-FISH method

The average telomere length was determined by FLOW-FISH method according to Baerlocher et al. [24]. The subset populations of leucocytes were identified by CD45RA antibody and the average telomere length was recorded in each subset population. We have attempted to identify the average telomere length in 3 leucocyte populations such as (i) CD45RA positive lymphocytes or immature T cells, (ii)

CD45RA negative lymphocytes or mature T cells and (iii) granulocytes. Each day to day experiments were standardized by “Molecules of equivalent soluble fluorochrome” (MESF) Quantum$^{TM}$FITC-5 premix fluorescent beads (Bangs Laboratories) and was used to both calibrate the individual instrument and to establish a fluorochrome-based standard curve for the assay.

The compensation and threshold instrument setting of flow cytometer was done by standard procedure of manufacturer’s (Beckman Coulter) protocol. The median fluorescence value of FL1 channel was correlated with the MESF value from the standard curve. The difference of MESF values of the unstained” and ”stained with Telomere-PNA” was represented as the telomere length of each samples and absolute telomere length was calculated from the formula by Kapoor et al. [25].

### Expression of hTERT in lymphocytes by RT-PCR

This study of hTERT expression in leucocyte was undertaken to determine the hTERT expression. Total RNAs, from the blood samples collected in PAXgene blood RNA tubes was isolated, by Trizol RNA extraction method. cDNA will be prepared by using random hexamers, one microgram of DNase I treated total RNA from each sample was reverse transcribed using the primer in a 20 ml reaction mix. The hTERT mRNA expression will be determined by RT-PCR compared to endogenous GAPDH expression. For this assay, 100 ng of cDNA was used in a 10 µl reaction mixture with PCR Master Mix and 25 ng of both forward and reverse RT primers as mentioned in **Table S1**. The amplified products were analyzed in 2% agarose gel and the specific amplicon will be visualized under UV after ethidium bromide staining.

### Statistical analysis

Between groups (Case and controls) comparison of means and analysis of variance will be done by binary logistic regression analysis. Linear regression will be used for correlation of age with telomere length (TL). Chi-square test and Mann Whitney U test will be used to test the significance of bone marrow cellularity, clinical parameters and cytopenia with variations of TL distribution.

# Results:

### Association of telomere length with cytopenias

According to FLOW-FISH based measurement of telomere length, the telomere length was significantly (p=0.001) shorter in all kinds of cytopenias patients compared to controls. Particularly the telomere length was significantly (p=0.03) shorter in granulocyte subpopulation of leucocytes in the cytopenias patients. On the contrary, there was difficulty to distinguish the CD45 RA + and CD45 RA – leucocytes subset populations because the cellular morphology might be changed due to hybridization in high temperature. Similar trend of telomere shortening was observed in the ungated population of leucocytes in the aplastic anemia patients (p=0.002), immune thrombocytopenia patients (p=0.002) and anaemia cases (p=0.006) compared to controls (**Figure 1)**. The distribution of telomere length among control samples were divided into 5 quartiles and the proportion of individuals of controls and cytopenias as

were calculated in each quartiles. The proportion of control samples were comparatively higher in upper quartiles of telomere length compared to cytopenias and the proportion of cytopenia samples were comparatively higher in lower quartiles of telomere length compared to controls (**Figure 2**).

**Association of telomere length with age:**

We investigated the effect of age on telomere length among the cytopenia patients and control. Interestingly the granulocyte telomere length was negatively and significantly (p=0.031) correlated with the age of the control groups (**Figure 3)** which justified the natural phenomenon. But there was no correlation of telomere length and age of the cytopenias patients**.**

**Association of telomere length with bone marrow cellularity:**

Overall, cases with hypocellular marrows had significantly shorter telomere length compared to those with normal marrow cellularity (**Figure 3**).

**Association of telomere length with hTERT expression**

Telomerase expression by RT-PCR was observed in 32/44(72.7%) of cases and in 24/35(68.6%) of controls.

**Association of telomere length with severity of cytopenias in cases of aplastic anaemia**

There was no significant association of telomere length with different grades of anaemia, thrombocytopenia and neutropenia in cases of aplastic anaemia.

# Discussion:

We undertook the present study to investigate telomere length in various cytopenias in the Indian population. We also measured telomere length in healthy control subjects in age range 0–76 yrs. We focused on the two aspects of telomere shortening, i.e. association of telomere shortening with age in cases and controls and sought to identify the most representative leucocyte subset population for measurement telomere length by FLOW FISH (**Figure S1**). We have established that telomere shortening is a normal phenomenon associated with age in healthy subjects and that the correlation with age is seen in granulocytes.

In our study we noted that telomere length in both total leucocyte population and granulocytes was considerably shorter in all kinds of cytopenias patients compared to controls. In comparison, Brummendorf et al. [6] found that absolute telomere fluorescence was significantly associated aplastic anaemia compared to controls in only the granulocyte population and not in lymphocytes. This is similar to our finding of a lack of association of telomere length and age in various types of cytopenias including aplastic anaemia.

Qi et al. [19] depicted that the relative telomere length of peripheral blood mono nuclear cells (PBMC) in immune thrombocytopenia (ITP) patients was significantly shorter than in healthy controls. In our experimental study it has been noticed that significant granulocytes telomere length shortening occurs in aplastic anemia, immune thrombocytopenia, anemia. There have been no previous reports in the literature of telomere length variations in anaemia and thalassaemia.

AA patients in our study were among those with the shortest telomere compared and other cytopenias (Fig. 2). This led us to hypothesize that accelerated senescence of a reduced population of hematopoietic cells could contribute to shortening of telomere length in the pathogenesis of aplastic anaemia. Ball et al [26] demonstrate that significant telomeric shortening in AA, affecting both granulocyte and mononuclear cell fractions, probably occurs at the level of the hematopoietic stem cell.

We studied the telomere length variation in relation to marrow cellularity and found that telomere lengths when measured in all leucocytes were significantly shorter in cases with cytopenia and hypocellular bone marrow compared to those cases with cytopenia and normocellular bone marrow. There is no previous report in the literature regarding bone marrow cellularity and telomere length. In this study, we also observed that there was no correlation of hTERT expression and telomere length among cytopenias. We have visualized the hTERT expression even in cases with short telomeres.

## Conclusions:

We evaluated the telomere lengths of cytopenia patients at a single-cell level by quantitative FLOW-FISH method. Patients with cytopenia especially aplastic anaemia, thrombocytopenia, anaemia showed accelerated telomere shortening compared to the healthy control with a significant negative correlation with bone marrow cellularity. In our experiment, we observed a negative correlation of granulocyte telomere length with age only among controls but not among cases. Telomere length when measured in all leucocytes as well as granulocyte subpopulation correlated with the presence of cytopenia and telomere lengths in both groups were shortest in aplastic anaemia compared to other cytopenias. In aplastic anaemia telomere length in all leucocytes and granulocytes did not correlate significantly with the grades of cytopenia.

# Limitations:

Limited sample size in all categories of cytopenia patients might contribute the results of the study. Further work will be needed in large sample set and gene expression analyses as well as mutation analysis of the genes involved in telomere maintenance pathway elucidate the complete story of telomere biology and to explore efficient telomere targeting therapies in different grades of cytopenias.

## Abbreviations

1. FISH: Fluorescence in situ hybridization

2. PNA: Peptide nucleic acid
3. PBLs: Peripheral blood leukocytes
4. PNH: Paroxysmal nocturnal hemoglobinuria
5. ITP: Immune thrombocytopenia
6. PB: Peripheral blood
7. HSCs: Hematopoietic stem cells
8. hTERT: Human telomerase
9. OPD: Outpatient department
10. MESF: Molecules of equivalent soluble fluorochrome
11. TL: Telomere length
12. PBMC: Peripheral blood mono nuclear cells
13. AA: Aplastic anaemia

## Declarations

# • Ethics approval and consent to participate

All samples were collected from the subjects with written informed consent approved by the institutional ethical committee for human experimentation of the NRS Medical College and Hospital, Kolkata, India.

**• Consent for publication**

Consent for publication was taken from participants involved in the study.

**• Availability of data and material**

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

**• Competing interests**

The authors declare that they have no competing interests.

# • Funding

The financial support for the study was provided by Indian Council of Medical Research, New Delhi, India (Grant id: 53/1/2014-BMS dated. 20.03.2015) for the entire experiments of the study.

# • Authors' contributions

PM and SD: Performed the research, PM and SD: designed the study, PM and SD: analyzed the data, PM and SD: wrote the paper. All authors read and approved the final manuscript.

# Acknowledgements

We thank all the clinicians, researchers and laboratory technicians of Department of Haematology, NRS Medical College and Hospital for providing support in sample collection. We also acknowledge Principal of NRS Medical College and Hospital and Head of the Department of Haematology for providing the opportunity to do the study and Indian Council of Medical Research ((53/1/2014-BMS, date: 20.3.2015) for funding support. Last but not the least; we are thankful to the cytopenia patients as well as healthy blood donors who voluntarily percolated in the study.

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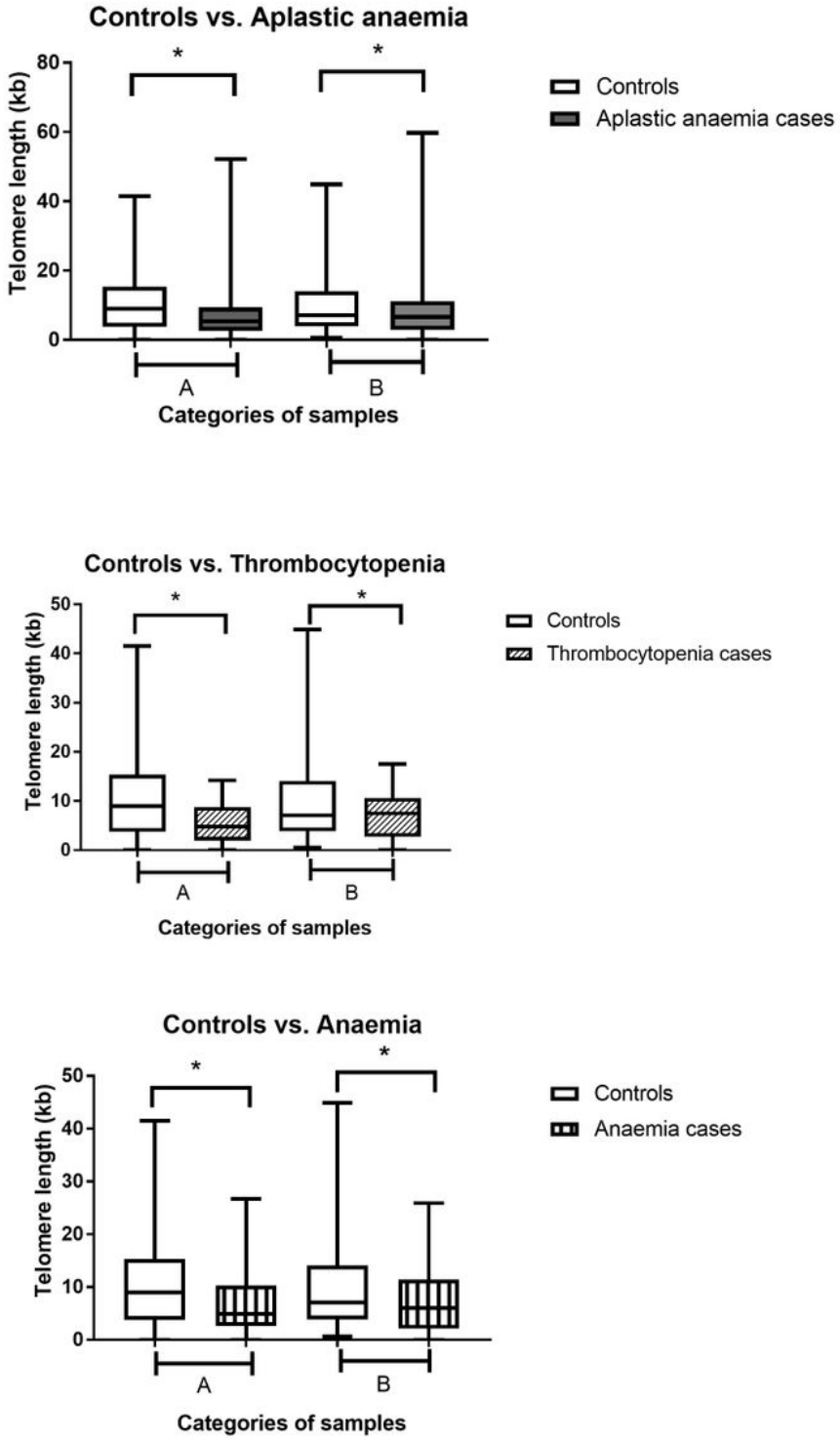
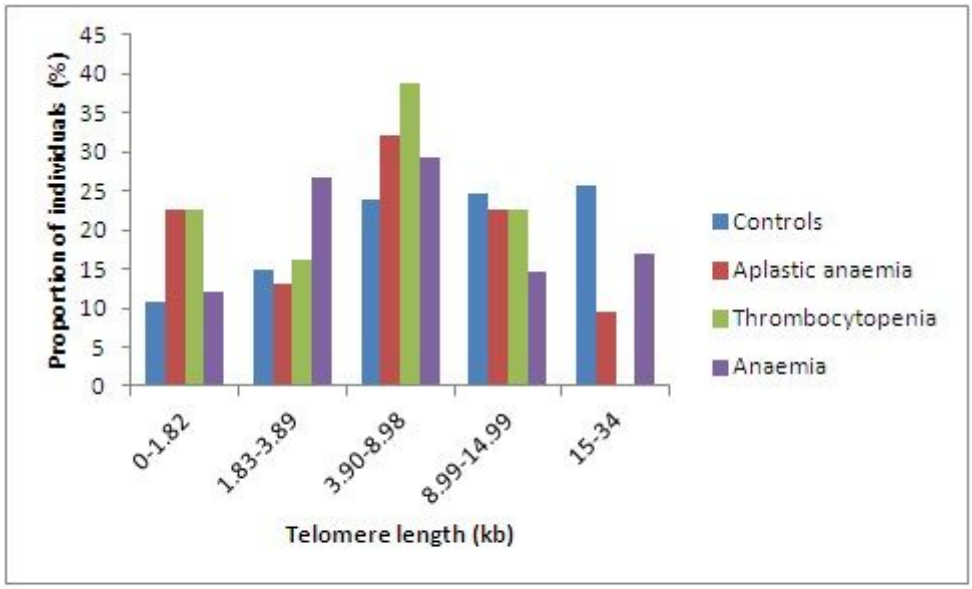
# Figures




## Figure 1

Box plot represents association of telomere length in cytopenias compared to healthy controls. (i) Aplastic anaemia compared to controls, (ii) thrombocytopenia compared to controls, (iii) anaemia compared to controls. A represents ungated leucocyte population and B represents granulocyte population. * denotes p value<0.05 and # denotes non significant p value.




## Figure 2

Distribution of telomere length across the individuals of controls and cytopenias.

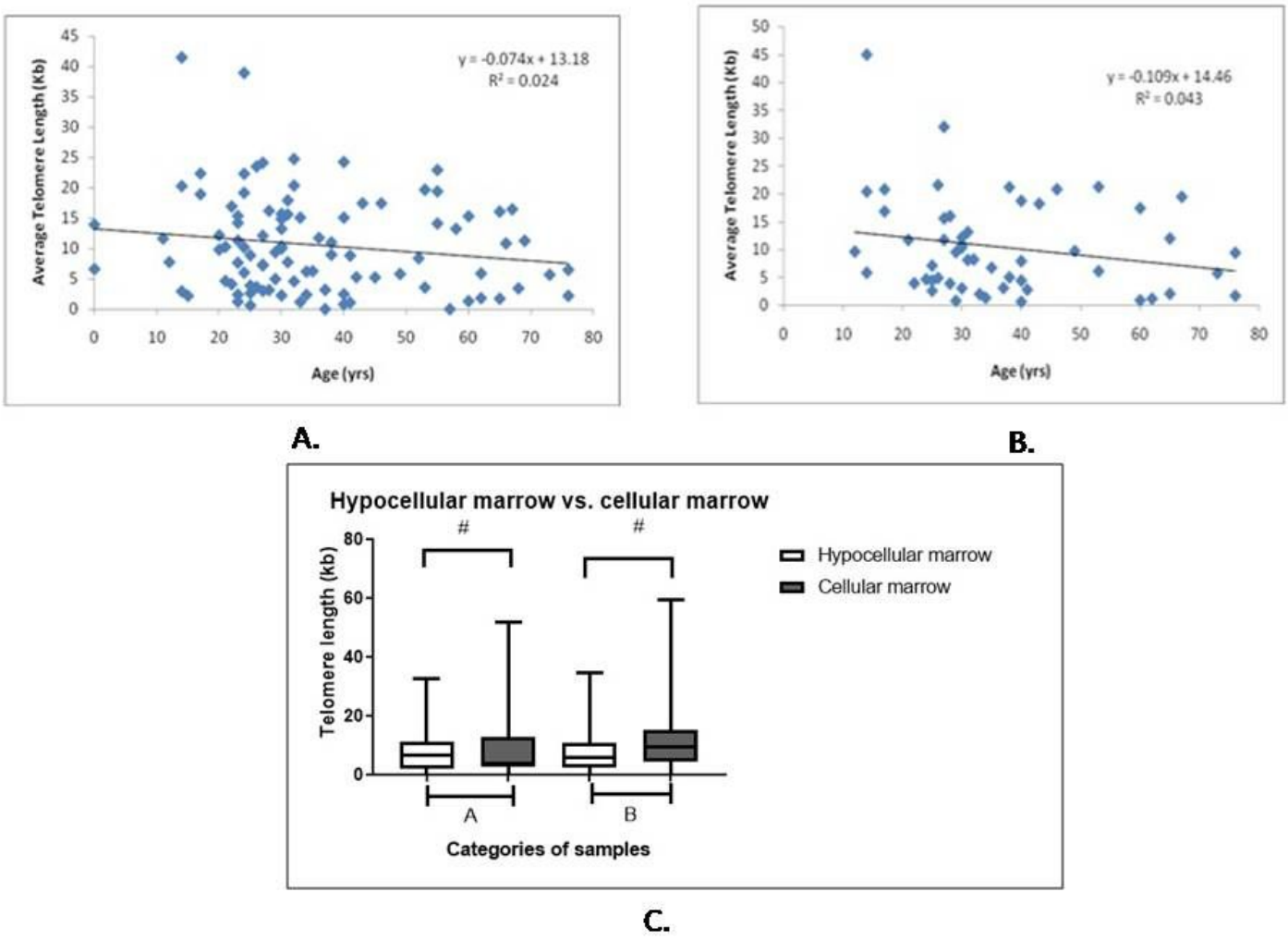



**Figure 3**

Linear regression analysis represents association of telomere length with age among healthy controls and association of marrow cellularity with telomere length among cytopenia patients. A. Average telomere length among ungated leucocyte population with age (p=0.188), B. Average telomere length among granulocyte population with age (p=0.031), C. Box plot represents association of telomere length in cytopenias with hypocellular marrow compared to cytopenias with cellular marrow. * denotes p value<0.05 and # denotes non significant p value.

# Supplementary Files

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- Supplementarydata.doc