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Telomere loss in Philadelphia-negative hematopoiesis after successful treatment of chronic myeloid leukemia: evidence for premature aging of the myeloid compartment

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ABSTRACT

Telomere shortening, a well-known marker of aging and cellular stress, occurs under several conditions in the hematopoietic compartment, including aplastic anemia and following iatrogenic noxae. We decided to verify whether pathological telomere erosion also arises in restored Philadelphia-negative (Ph-negative) hematopoiesis following successful treatment of chronic myeloid leukemia (CML). Eighty-one CML patients in complete cytogenetic remission were compared to 76 age-matched healthy subjects. Myeloid cells of CML patients had shorter telomeres than controls (6521 bp vs 7233 bp, $p < 0.001$). This difference was specific for the myeloid compartment, since it was not observed in lymphoid cells (6774 bp vs 6909 bp, $p = 0.620$). Acquired Ph-negative cytogenetic abnormalities ($p = 0.010$), lack of complete molecular remission ($p = 0.016$) and age ($p = 0.013$) were independent predictors of telomere shortening. Telomere dynamics were assessed over a median follow-up period of 22 months. We documented accelerated non-physiological ongoing telomere shortening in 17/59 CML patients (28%). Patients experiencing grade 2-4 hematological toxicity, during CML remission possessed significantly shorter telomeres compared to those lacking toxicity ($p = 0.005$ for any toxicity, $p = 0.007$ for anemia). CML patients suffer from significant and often ongoing telomere stress resulting in premature and selective aging of the myeloid compartment which might have long-term consequences on function and integrity of Ph-negative hematopoiesis.

Article keywords: telomere shortening, cytogenetic abnormalities, bone marrow failure, tyrosine kinase inhibitors, hematopoiesis

1. INTRODUCTION

Tyrosine kinase inhibitors (TKI) have dramatically changed the natural history of chronic myeloid leukemia (CML) (Baccarani et al, 2009; Cervantes and Mauro, 2011; Goldman, 2009). The vast majority of CML patients exhibit sustained cytogenetic and molecular responses, and the expected five-year overall survival rate exceeds 90% (Goldman, 2009; Kantarjian et al, 2009). Cytogenetic and molecular responses in CML result in hematopoietic reconstitution by Philadelphia-negative (Ph-negative) cells. Ph-negative hematopoiesis is usually polyclonal, apparently unrelated to the CML population, and probably sustained by non-neoplastic stem cells previously overwhelmed by the CML clone (Claxton et al, 1992; Bumm et al, 2003; 2010).

Although post-CML Ph-negative hematopoiesis allows patients to attain physiological or nearly physiological blood counts, it is unknown whether this hematopoiesis is genetically and functionally comparable to that observed in age-matched normal subjects. Indeed, a number of observations suggest that Ph-negative hematopoiesis following successful CML treatment actually bears a number of defects which are also observed in healthy elderly subjects. These defects include: a) clonal hematopoiesis in a minority of cases, a non-physiological condition also observed in cases of defective stem cell function (Bumm et al, 2003); b) additional cytogenetic abnormalities (CA), reminiscent of those observed in myelodysplastic syndromes occurring in 2-15% of patients in complete cytogenetic remission (CCyR) after treatment with TKI or other agents (Medina et al, 2003; Paquette et al, 2010; Jabbour et al, 2007; Deininger et al, 2007); c) hyporegenerative anemia and/or neutropenia (O'Brien et al, 2003; Quintàs- Cardama et al, 2009); d) a small but definite increase in the risk of developing neoplasms typical of elderly subjects such as myelodysplastic syndromes and/or secondary leukemias (Sharpless et al, 2007. Jabbour et al, 2007; Deininger et al, 2007; Kovitz et al, 2006; Navarro et al, 2007). Little information is available on the mechanisms responsible for these defects. Potential explanations, which are not mutually exclusive, include primitive or acquired defects of Ph-negative stem cells proliferative stress associated with bone marrow (BM) repopulation, depletion of the stem cell pool and treatment toxicity.

Telomeres consist of short tandem G-rich nucleotide repeats at the ends of eukaryotic chromosomes associated with a protein regulatory complex. The primary function of telomeres is ensuring effective capping of open DNA ends (de Lange, 2009; Martinez and Blasco, 2011; Blasco, 2007; Vaquero-Sedas and Vega-Palas, 2011). In most mammals with a prolonged lifespan, including humans, telomeres acquired the specific role of "sensors" of proliferative and oxidative damage. Telomeres undergo progressive shortening when

somatic non-neoplastic cells proliferate or suffer oxidative stress; excessive telomere shortening results in cell senescence and proliferative arrest (Collado et al, 2007; Vaziri et al, 1994; Hills et al, 2009). This mechanism prevents the malignant transformation of cells that have undergone excessive genotoxic stress or proliferation. A physiological consequence of telomere function is the progressive shortening of telomeres in the vast majority of somatic tissues during normal life (Aubert and Lansdorp, 2008; Brümmendorf and Balabanov, 2006). Several reports indicate that non-physiological stresses such as extensive proliferation or oxidative stress induce accelerated telomere shortening in both extra-hematopoietic and hematopoietic tissues (Brümmendorf and Balabanov, 2006; Passos et al, 2007; Guachalla and Rudolph, 2010) Moreover also the genetic background plays a major role in telomere dynamics as the presence of hypomorphic mutations of h-TERT and other genes have shown to be associated to accelerated telomere shortening (Hills and Lansdorp, 2009; Xin et al, 2009). In the hematopoietic compartment, accelerated telomere shortening possibly related to both environmental injury and constitutional background has been documented in aplastic anemia (Ball et al, 1998; Brümmendorf et al, 2001; Calado and Young, 2008) and after exogenous stresses, such as chemotherapy (Ricca et al, 2005) and BM transplantation (Wynn et al, 1998; Gadalla and Savagel, 2011; Rocci et al, 2007; Notaro et al, 1997; Drummond et al, 2007). Although the consequences of accelerated telomere shortening remain to be elucidated, this phenomenon has been correlated with the degree of stem cell damage (Notaro et al, 1997) and appears to persist over time (Rocci et al, 2007).

Based on these considerations, we speculated that Ph-negative hematopoiesis emerging after successful CML treatment could have undergone significant telomere shortening due to prolonged coexistence with the tumor clone, the proliferative effort associated with BM repopulation following successful clearance of Ph-positive hematopoiesis, and/or direct pharmacological inhibition of telomere preservation mechanisms due to TKI treatment (Uziel et al, 2005; Mor-Tzuntz et al, 2010). We wished to evaluate the extent of telomere damage in Ph-negative hematopoiesis after successful CML treatment. We associated this damage with clinical parameters with particular interest to patients developing additional CA. We also monitored telomere dynamics over time and sought associations between telomere shortening and hematological toxicity.

2. PATIENTS AND METHODS

2.1 Patients

This study was approved by the local Ethical Committee (Protocol Number 0052352). All subjects provided written informed consent for the research use of peripheral blood (PB) sample leftovers obtained during routine clinical examinations. All patients treated at the Hematology Divisions of the University of Turin and the Amedeo Avogadro University of Eastern Piedmont were included in this analysis if the patient had a history of Ph-positive CML and experienced CCyR lasting one year or more achieved by any treatment modality except allogeneic transplantation. Cytogenetic response was defined according to standard criteria based on the percentage of Ph-positive metaphases as previously described (Kantarjian et al, 2002). In order to enrich our panel for patients with acquired Ph-negative CA, three additional institutions (Hematology divisions of S. Eugenio, Tor Vergata University Hospital, Rome, Policlinico Mangiagalli e Regina Elena, Milan, A.O. SS. Antonio e Biagio, Alessandria, Italy) provided samples from patients meeting the selection criteria and showing evidence of CA. Age, sex, date of diagnosis, Sokal prognostic score (Sokal,1987) , and treatment schedule were recorded for all patients (Table 1). The times to achievement of hematological, cytogenetic, and molecular remission were also recorded.

Once CCyR was achieved, patients underwent cell blood count (CBC) monitoring and reverse-transcription polymerase chain reaction for the BCR-ABL fusion transcript at least every three months, and BM cytogenetics at least once every 24 months (Baccarani et al, 2009). Any post-CCyR hematological toxicity was evaluated and monitored according to NCI CTCAE v3.0 (Trotti et al, 2003). All hematological toxicities were considered for association with telomere length except for those having an obvious and reversible clinical explanation, such as iron deficiency or blood loss. Complete (CMoIR) and major molecular remission (MMoIR) were defined by real time quantitative and or nested PCR as the absence of a BCR-ABL amplification and a BCR-ABL/ABL ratio less than 0.1%, respectively, according to the standard criteria of European LeukemiaNet (Baccarani et al, 2009). All patients were on treatment at the time of first telomere length determination as detailed in Table 1. In particular, patients diagnosed before that date received different treatment modalities, generally consisting of interferon alpha (INF- α) or cytosine-arabioside (Ara-C) + INF- α (Table 1). However all patients were switched to imatinib when the drug became available, except patients already in CMoIR with INF- α alone (Table 1). All patients diagnosed from 2003 received imatinib 400 mg/day as the first line of therapy (Table 1).

In our population, the following treatment modification were recorded after first telomere length determination (table 1S); patients with molecular relapse were either escalated to imatinib 600 mg/day (three patients) or

switched to dasatinib 100 mg/day (three patients) according to the treating physician's decision (Table S1). Treatment reduction was considered in cases of grade 3 or more hematological or non-hematological toxicity (Table S1) (12 patients). In selected cases (five patients), patients with CMoIR lasting at least two years were considered for imatinib discontinuation according to Mahon et al, 2010 (Table S1); one patient discontinued treatment based on personal decision against treating physician advice. Whenever possible, CML patients underwent repeated telomere measurements (after at least 12 months from the first determination) in order to assess the dynamics of telomere erosion over time. Patients were excluded from this analysis if loss of CCyR was observed. Patients who discontinued treatment but were still in CCyR were considered.

A panel of age-matched healthy subjects was used as control population for telomere length analysis. For these subjects age and sex were recorded. Samples from healthy subjects were obtained either directly or through local transfusion and geriatric outpatient services.

2.2 Cytogenetic assays

In order to monitor modifications in cytogenetic response and to identify the emergence of acquired CA, cytogenetic analyses were performed in all CML patients at diagnosis, every 3-4 months until CCyR achievement, and every 12-24 months during follow-up. Conventional cytogenetic analysis was performed in BM cells using the standard G-banding technique (Brothman et al, 2009). At least 20 metaphases per patient were analyzed and BM specimens were examined in direct or short-term (24-hour) cultures. Patients were defined as CA-positive if their Ph-negative cells displayed the same CA on at least two consecutive determinations, with a cut-off value of 10% of mitosis (at least 2/20), after achieving CCyR (Deininger et al, 2007).

2.3 Cell separation

For telomere restriction fragment length (TRF-L) analysis white blood cells from PB samples of patients and controls were separated by sedimentation on 33% Emagel (Medacta SA, Luxemburg, Belgium) to eliminate red blood cells. Granulocytes and mononuclear cells were then separated by density gradient (Ficoll-Paque, GE Healthcare, Buckinghamshire, UK). Mononuclear cells were further processed to separate monocytes from lymphocytes by phagocytosis with opsonized heat-inactivated yeast (Ferrero et al, 2001). After separation, pellets of polymorphonucleates (PMN) and monocyte-depleted peripheral blood mononuclear cells (MDPBMC) were stored at -80 °C for TRF-L analysis.

2.4 Telomere restriction fragment length (TRF-L) analysis

TRF-L was determined by Southern blot on PMN and in MD-PBMC as reported elsewhere (Ladetto et al, 2004). Briefly, 2 µg of genomic DNA were digested with HinfI and RsaI restriction enzymes (Roche Diagnostics, Mannheim, Germany). Telomere restriction fragments were separated by 0.8% agarose gel electrophoresis. Gels were transferred to a positively charged nylon membrane (Roche Diagnostics) and UV cross-linked. Hybridization and detection were performed using the TeloTAGGG Telomere Length Assay Kit (Roche Diagnostics) as previously described elsewhere (Ladetto et al, 2004). Membranes were scanned and peak TRF-L was measured with the KODAK DIGITAL SCIENCE 1D Software v3.0 (Scientific Imaging Systems, New Haven, CT, USA) elsewhere (Ladetto et al, 2004). The reproducibility of the method has been tested on 70 samples by performing the same experiment in two different experimental sessions by different technicians. In all cases, both "peak TRF-L" and "mean TRF-L" were calculated the former as described elsewhere (Boultwoold et al, 2000; Cottliar et al, 2003; Akiyama et al, 2000) the latter as described by Harley et al, 1990 and recommended by the producer (Roche Diagnostics, Mannheim, Germany).

2.5 Statistical methods

Patient characteristics were tested using the Pearson χ^2 test for discrete variables and the Mann-Whitney and Kruskal-Wallis tests for continuous variables. Telomere distribution was tested as a continuous dependent variable by various univariate and multivariate general linear models (Mardia et al, 1979), either for the entire study population or only CML patients. When the entire population was considered age, sex, and CML diagnosis were analyzed as independent predictors of telomere loss. When the analysis was restricted to only CML patients the following parameters were considered as independent predictors of telomere loss: age, sex, time to CCyR, status of MoIR, Sokal score, CA, type of treatment administration (imatinib alone vs combined therapy vs INF- α alone). All reported p-values were two-sided at the conventional 5% significance level. Data were analyzed with SPSS 18.0.1 (SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1 Patient features

Between February 2007 and January 2010, 73 consecutive CML patients from the University of Turin and the Amedeo Avogadro University of Eastern Piedmont meeting the previously mentioned criteria were enrolled in the present study. Median time from CCyR achievement to samples collection for telomere determination was 36 months (range: 4-191). Eight additional non-consecutive patients with CA were referred from other centers and added to the population under evaluation, resulting in a group of 81 CML patients (Table 1), including 15 patients with CA (Table 2). With the exception of CA, these eight additional patients did not substantially differ from the study population (data not shown). In particular patients with CA had a median age of 59 years (range:36-74) vs a median age of 64 years (range 23-88) among those without CA. Main biological and clinical features of CML patients at diagnosis and at study entry are reported in table 1. Subsequent clinical events including hematological toxicity, disease monitoring, and treatment changes are reported in Table S1. Table 2 describes the features of patients with CA. The control panel included 76 samples from age-matched healthy subjects with a male/female ratio of 42/34 and a median age of 59 years (range 40-102 years).

3.2 Telomere length analysis in myeloid and lymphoid compartments

Peak TRF-L was evaluated in PMN and MDPBMC in CML patients and controls. The telomere length of CML patients (median 6774 bp, range 4520-9985 bp) did not differ in the lymphoid compartment from control subjects (median 6909 bp, range 4494-11470 bp, $p = 0.620$, Fig. 1A). In contrast, PMNs of CML patients exhibited significant telomere shortening ($p < 0.001$, Fig. 1B) compared to controls; the median peak TRF-L of CML patients was 6521 bp (range 4301-8481 bp), while control subjects possessed a median peak TRF-L of 7233 bp (range 4834-12338 bp). Interestingly, in CML patients the telomeres of myeloid cells were shorter than lymphoid cells, as opposed to what was observed physiologically (data not shown) (Robertson et al, 2000). Given the heterogeneity of telomere length in human subjects, univariate and multivariate general linear models were applied to the entire population to define the relative impact of CML history, particularly with respect to age, that has a well established impact on telomere length of PMN. The model demonstrated that age and CML history were both independently associated with telomere shortening ($p < 0.001$ and $p < 0.001$, respectively; Fig. 1C). As expected, sex had no significant influence on TL of PMN ($p = 0.200$). Similar findings were observed when by using mean TRF-L instead of peak TRF-L with a small underestimation of mean-TRF-L vs peak TRF-L that was consistent among all subgroups examined (data not

shown)

3.3 Telomere length and cytogenetics

Fifteen patients at the time of sample collection for telomere analysis had evidence of CA in a concurrent or previous cytogenetic assay. To evaluate whether the presence of CA was associated with telomere length, we compared CML patients with and without CA and healthy controls. There was no difference in the time elapsed between CCyR and peak TRF-L analysis in the two populations ($p=0,6$). The presence of CA had no significant impact on the telomere length of MDPBMC (6690 bp with CA vs 6808 bp without CA, $p = 0.100$; data not shown) regardless of the percentage of abnormal mitoses. In contrast, PMN from patients with CA showed greater telomere shortening compared to patients without CA (6049 bp, range 4301-7518, and 6659 bp, range 4545-8481, respectively, $p = 0.020$; Fig. 2A). Notably, the difference between CML patients and healthy subjects remained statistically significant when patients with CA were excluded from the analysis ($p = 0.003$; Fig. 2B). While small patient numbers prevented detailed analysis of the impact of specific CA on telomere length, patients with CA involving the Y chromosome and 5q deletion had an inferior telomere shortening compared to other CA, particularly the chromosome 7 deletion and chromosome 8 trisomy (Table 2). The exclusion of patients with Y chromosome and 5q CA resulted in the identification of a patient subgroup with an even more severe telomere disruption compared with other CML subjects (5696 bp vs 6536 bp, $p = 0.006$, data not shown). Again, similar findings were observed when by using mean TRF-L instead of peak TRF-L (data not shown)

3.4 Telomere length and CML clinical features

To assess the relative impact of clinical variables and CA on telomere length, univariate and multivariate general linear models were applied to the CML population with respect to the following variables: age, sex, Sokal score, molecular remission, treatment schedule, and presence of CA. Sex, Sokal score, and treatment schedule had no significant impact on telomere length ($p = 0.814$, $p = 0.692$, and $p = 0.195$ respectively, data not shown). The highly significant multivariate linear model ($p = 0.001$) confirmed the association between CA ($p = 0.010$) and telomere shortening ($p = 0.013$) (Fig. 2C). Moreover the multivariate linear model demonstrated that age and the lack of CMoIR ($p = 0.016$, Fig. 2C) functioned as independent predictors of telomere shortening. Again we observed no differences in sampling time between cases with or without CMoIR ($p=0.3$).

3.5 Telomere length dynamics over time

Whenever possible, PMN from CML patients were prospectively studied at successive time points to verify telomere length L dynamics over time. At least two TRF-L determinations were available in 59 patients with a median follow-up of 22 months (range 12-34 months) between the first and last determination; three or more determinations were available for 23 of the 59 patients. The median peak TRF-L of PMN was 6378 bp at the first time point and 6157 bp at the last available time point ($p = 0.009$; Fig. 3A). As expected for the 23 patients with three or more time points, the median peak TRF-L of interim samples fell between the peak TRF-L at study entry and at final analysis: 6521 bp vs 6268 bp vs 6027 bp, (data not shown). A similar pattern was observed by using mean TRF-L instead of peak TRF-L (data not shown).

We next assessed telomere length kinetics patient by patient to verify whether CML patients were homogeneous or heterogeneous in their patterns of yearly telomere loss. Since time between the two telomere determinations was not uniform among patients (median 22 months, range 12-34), telomere loss of each patient was normalized by year. We were particularly interested in verifying whether telomere shortening in PMN occurred at a physiological rate or was accelerated at least in some subjects. To avoid overestimation of the number of patients with supraphysiologic telomeric loss stringent criteria were adopted which considered the results of our reproducibility analysis on 70 samples which never documented variability greater than 300 bp (median experimental variation of 129 bp, 25th and 75th percentile of 25 bp and 230 bp, respectively) and a physiological annual telomere loss of 50 bp (Hoffman et al, 2009). Based on these considerations patients were classified as follows: a) those exhibiting an annual TL increase greater than 300 bp were considered to have clear evidence of telomere recovery; b) those showing an annual telomere loss greater than 350 bp were considered as having a clear evidence of accelerated non-physiological telomere disruption. The remaining patients were considered as possessing telomere dynamics not clearly distinguishable from physiological aging. While no patient showed any evidence of telomere recovery, accelerated non-physiological telomere shortening was documented in 17/59 patients (28%). Other patients exhibited a pattern of telomere dynamics that could not be distinguished from physiological aging. Nevertheless, the median yearly telomere loss of 261 bp (range -1289 bp to +279 bp, Fig. 3B) recorded in the entire series revealed a level of telomere erosion greater than that usually observed with normal aging (50 bp year) (Hoffman et al, 2009; Ball et al, 1998).

No obvious association between accelerated telomere loss and patient demographic or clinical features was observed (data not shown). Interestingly, the patient subgroup with accelerated telomere loss included also three patients (Fig. 3B, patients 1, 6, and 7) who discontinued treatment three, four, or six months

(respectively) after the first peak TRF-L determination. This patient subgroup also included two patients (Fig. 3B, patients 2 and 3) who lost molecular response while remaining in CCyR.

3.6 Association with CBC values

Associations between TL and post-CCyR hematological toxicity were analyzed (Table S1). We observed significantly shorter telomeres in patients experiencing hematological toxicity of any type or anemia ($p = 0.030$ and 0.010 , respectively; Fig. S1 A, B) compared to patients without hematological toxicity. This effect was not observed in patients with neutropenia or thrombocytopenia ($p = 0.210$ and 0.100 , respectively; Fig. S1 C, D). Even restricting the analysis to the smaller group of patients experiencing grade 2-4 toxicity, we found a significant association between TL and hematological toxicity of any type or anemia ($p = 0.005$ and 0.007 , respectively; Fig. 4A and B). Moreover also neutropenia resulted close to statistical significance ($p = 0.080$). Again, no significant difference was noted in timing of samples collection between patients with or without hematological toxicity ($p=0.4$ data not shown). The association between telomere length and post-CCyR hematological toxicity were observed also using mean TRF-L instead of peak TRF-L

4. DISCUSSION

In the current study we investigated the presence and extent of telomere shortening in Ph-negative hematopoiesis following successful treatment of CML. The myeloid population of CML patients displayed telomeres which are significantly shorter compared to those seen in age-matched healthy subjects, suggesting lineage-specific premature aging of the myeloid compartment as demonstrated by the presence of an undamaged lymphoid compartment. Telomere shortening was more pronounced in patients with CA or without CMoIR. We observed that telomeres from Ph-negative hematopoietic cells of CML patients displayed no recovery over time at least in presence of the standard ongoing treatment usually adopted in these cases; moreover, a pattern clearly suggesting accelerated non-physiological telomeric erosion occurs in at least one third of patients at follow-up. Finally, we identified an association between short telomeres and hematological toxicity, particularly anemia.

Critically shortened telomeres are commonly observed in a number of solid and hematological tumors, where they contribute to the establishment of the malignant phenotype (Hahn, 2003; Lin et al, 2010). Telomere erosion has been extensively investigated in Ph-positive CML cells as well (Keller et al, 2009). These cells also have shorter telomeres compared to healthy cells as frequently occur in human malignancies (Keller, et al 2009). Severe telomere shortening has additionally been associated with progression into blast crisis (Keller et al, 2009; Boulwood et al, 2000). Conversely, the achievement of CCyR after successful treatment has been associated with BM repopulation by Ph-negative hematopoietic cells characterized by longer telomeres compared to CML cells (Brümmendorf et al, 2003).

Telomere shortening is associated with physiological aging and a number of pathological conditions in non-neoplastic tissues. However, this shortening never reaches the level observed in cancer cells with the exception of subjects with severe deficiency of telomerase and telomerase related genes. This is mostly due to mechanisms such as senescence that limit proliferation and survival of cells undergoing excessive telomere erosion. In normal tissues, telomeres are effective sensors of genetic damage induced by multiple stressors; most notably proliferation and oxidative damage (Passos, 2007; Guachalla and Rudolph, 2010). The consequences of severe telomere shortening in non-neoplastic hematopoietic cells have yet to be fully elucidated. Several reports have shown that extensive telomere shortening occurs in aplastic anemia and following severe iatrogenic stress such as chemotherapy or transplantation (Calado and Young, 2008; Ricca et al, 2005; Wynn et al, 1998; Rocci et al, 2007; Notaro et al, 1997). Under these conditions, shortened telomeres may lead to functional exhaustion and genetic instability, the latter potentially implicated in the frequent late clonal complications of these conditions which are often highly reminiscent of those frequently

occurring during old age (Wagner et al, 2009; Wynn et al, 1998).

We wished to ascertain whether Ph-negative hematopoiesis following successful CML treatment showed evidence of telomere shortening similar to that observed after iatrogenic or immune-mediated noxae. Our report indicates that Ph-negative hematopoiesis undergoes severe telomere damage in the majority of CML patients. According to the general linear model, previous history of CML was associated with a telomere loss of 917 bp, a measurement not far from that reported in aplastic anemia and following BM transplantation (Ball et al, 1998; Wynn et al, 1998; Rocci et al, 2007). Substantial telomere shortening in all these conditions suggests a common physiopathological scenario characterized by a genetically and functionally impaired hematopoiesis, as demonstrated by the frequent occurrence of CA involving chromosome 8, 7, 5, and Y, and by the increased risk of myelodysplastic syndromes (Ball et al, 1998; Wynn et al, 1998; Rocci et al, 2007; Ricca et al, 2005; Notaro et al, 1997; Erdag et al, 2009; Maciejewski et al, 2002).

The link between shortened telomeres and impaired hematopoiesis is strengthened by the association of presence of CA and telomere shortening. This association has not yet been reported in other conditions, but we would not be surprised to find it also in aplastic anemia or autografted patients. Interestingly, short telomeres were observed both in cases with a high percentage of abnormal karyotypes as well as in those where CA were observed in 25% of cells or less, suggesting that telomere shortening is not a mere byproduct of the clonal abnormality.

The potential link between severe telomere erosion and hematopoietic damage is further strengthened by the association between telomere shortening and hematological toxicity. Our results therefore emphasize the value of telomere length as a broad sensor of cellular stress able to monitor the functional impairment of hematopoiesis as well as its tendency to give rise to pre-malignant clones harboring CA (Abruzzese et al, 2007).

Telomere shortening in Ph-negative hematopoiesis may result from several causes that are not mutually exclusive. An intrinsic defect of the hematopoietic stem cell which might precede the onset of Ph-positive hematopoiesis has been postulated in some studies. Such defective hematopoiesis might have undergone excessive telomeric stress due to proliferation or due to the presence of an intrinsically defective telomere-protection machinery as observed in other blood cancers. On the other hand the cohabitation with the Ph-positive clone may exert a deleterious effect on non-neoplastic Ph-negative stem cells, either directly or through disruption of the hematopoietic environment. These mechanisms might at least partly explain the

association between telomere shortening and lack of CMoIR. Telomere shortening may result from the proliferative effort required to repopulate the BM and sustain adequate hematopoiesis from a reduced stem cell compartment. At least theoretically, a negative effect of treatment (either TKI or other agents) on telomere dynamics may also result in telomere shortening, as imatinib may inhibit telomerase in hematopoietic cell lines (Uziel et al, 2005; Mor-Tzuntz et al, 2010). However, no evidence exists that this process occurs in normal cells; the low level of h-TERT expression in hematopoietic stem cells does not prevent age-related physiological telomere shortening (Zimmermann et al, 2004). Moreover, TKI-induced toxicity does not explain the lack of telomere shortening in the lymphoid compartment, which is characterized by significant expression of the *abl* gene (Brightbill and Schlissel, 2009) and is critically dependent on h-TERT activity during B- and T-cell immune responses (Lobetti-Bodoni et al, 2010). We observed major telomere shortening in the few patients who never received TKI, while ongoing non-physiological erosion was detectable in three patients who discontinued TKI due to persistent CMoIR. Taken together, these observations suggest that telomere disruption results from CML and not from a toxic effect of TKI.

We are intrigued by our novel though preliminary observation of accelerated, ongoing non-physiological telomere loss in a subset of patients. Although we were unable to conduct a case control analysis to formally prove this observation, our results suggest that several patients have an accelerated telomeric loss. The most likely cause of this phenomenon is related to over-stimulation of a compromised progenitor cell pool that has to undergo an increased number of proliferation rounds to ensure CBC similar to healthy subjects. We also possess preliminary evidence from a small patient series of 20 patients that both long-term and short-term progenitor cells are reduced in these patients (Lobetti-Bodoni C and Sia D, unpublished observation). An additional explanation, particularly in those cases with the most severe telomere annual loss, may be related to the presence of clonal hematopoiesis in a subset of patients. Finally some of these patients might bear hypomorphic variants of proteins associated to telomere elongation and or protection (Xin et al, 2009).

In the present study, Southern Blot (SB) had to be associated to a fairly complex separation procedure that nevertheless allowed to obtain excellent purity for both PMN and MD-PBMC. This choice appeared the most appropriate when the study was begun although Flow-FISH would probably appear more practical at the present time. However several experience including one from our laboratory showed excellent concordance when SB was compared to more modern techniques such as Flow-FISH cytometry indicating that the approach here employed despite its complication should not be considered as obsolete or imprecise

(Genuardi et al, 2011). Regardless of the method employed telomere length determination is simple, inexpensive, and suitable for large-scale applications, as demonstrated by a large bulk of studies on the most diverse pathological conditions (Fyhrquist et al, 2011; Yaffe et al, 2011; Jing et al, 2008).

Given the increased interest in defining risk patterns for late toxicity in CML and in other conditions associated with impaired hematopoietic function we believe that telomere length has potential of being validated in the future as a simple and effective marker of the genetic integrity of the myeloid compartment. However this require further extensive investigation, including careful analysis of temporal relationship between telomere shortening and occurrence of clinically relevant events such as CA or anemia.

Our results suggest that telomere disruption may negatively impact cellular performance due to senescence-associated exhaustion of the stem cell pool and/or increased risk of transformation linked to critically shortened telomeres (Collado et al, 2007; Robertson et al, 2000; Hahn, 2003) leading to a physiopathological scenario which is similar to what occurs during old age (Von Figura et al, 2009) . The identification of a mechanistic link between telomere shortening and stem cell damage is clearly beyond the scope of this study. However, these mechanisms must be addressed to further clarify the patterns linking telomere damage and hematopoietic stem cell exhaustion and/or transformation in CML and other conditions associated with severe telomere damage. From a clinical perspective, additional data are required to verify whether telomere shortening correlates not only with CA and hematological toxicity, but also with the onset of myelodysplastic syndromes.

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Authorship and Conflict of interest disclosures

CLB and ML were the principal investigators and wrote the paper. CLB, EG performed the research and analyzed the data. CCS performed the in vitro experiments. RP was the statistical consultant. EB, LM, AR, DD, MZ, MB, DB co-coordinated the research. DF, DS, GG, EC, VG, GI, ML, EB, FR, MP, PP, GG and MB contributed to recruitment of the patients and samples.

The authors have no relevant conflicts of interest to disclose.

REFERENCES

- Abruzzese, E., Gozzetti, A., Galimberti, S., Trawinska, M. M., Caravita, T., Siniscalchi, A., Cervetti, G., Coletta A & De Fabritiis P., 2007. Characterization of Ph-negative abnormal clones emerging during imatinib therapy. *Cancer*, 109, 2466-2472.
- Akiyama, M., Asai, O., Kuraishi, Y., Urashima, M., Hoshi, Y., Sakamaki, H., Yabe, H., Furukawa, T., Yamada, O., Mizoguchi, H., & Yamada, H., 2000. Shortening of telomeres in recipients of both autologous and allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 25,441-447
- Aubert, G., & Lansdorp, P.M., 2008. Telomeres and aging. *Physiol Rev* 88,557-579.
- Baccarani, M., Cortes, J., Pane, F., Niederwieser, D., Saglio, G., Apperley, J., Cervantes, F., Deininger M., Gratwohl A., Guilhot F., Hochhaus A., Horowitz M., Hughes T., Kantarjian H., Larson R., Radich J., Simonsson B., Silver R.T., Goldman J., Hehlmann R. & European LeukemiaNet., 2009. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. *J. Clin. Oncol.* 27, 6041-6051.
- Ball, S. E., Gibson, F. M., Rizzo, S., Tooze, J. A., Marsh, J. C., & Gordon-Smith, E. C., 1998. Progressive telomere shortening in aplastic anemia. *Blood*, 91, 3582-3592.
- Blasco, María A., 2007. The epigenetic regulation of mammalian telomeres. *Nat. Rev.Gen.* 8, 299-309.
- Boulwood, J., Peniket, A., Watkins, F., Shepherd, P., McGale, P., Richards, S., Fidler, C., Littlewood T.J. & Wainscoat J.S., 2000. Telomere length shortening in chronic myelogenous leukemia is associated with reduced time to accelerated phase. *Blood*, 96, 358-361.
- Brightbill, H., & Schlissel, M. S., 2009. The effects of c-Abl mutation on developing B cell differentiation and survival. *Int. Immun.* 2, 575-585.
- Brothman, A. R., Persons, D. L., & Shaffer, L. G., 2009. Nomenclature evolution: Changes in the ISCN from the 2005 to the 2009 edition. *Cytogenet. Genome Res.* 127, 1-4.
- Brümmendorf, T H, Maciejewski, J. P., Mak, J., Young, N. S., & Lansdorp, P. M., 2001. Telomere length in leukocyte subpopulations of patients with aplastic anemia. *Blood* 97, 895-900.
- Brümmendorf, Tim H, Ersöz, I., Hartmann, U., Bartolovic, K., Balabanov, S., Wahl, A., Paschka, P., Kreil S., Lahaye T., Berger U., Gschaidmeier H., Bokemeyer C., Hehlmann R., Dietz K., Lansdorp P.M., Kanz L. & Hochhaus A., 2003. Telomere length in peripheral blood granulocytes reflects response to treatment with imatinib in patients with chronic myeloid leukemia. *Blood*, 101, 375-376.
- Brümmendorf, T H, & Balabanov, S., 2006. Telomere length dynamics in normal hematopoiesis and in disease states characterized by increased stem cell turnover. *Leukemia* 20, 1706-1716.
- Bumm, T.M., C., Al-Ali, H.-K., Krohn, K., Shepherd, P., Schmidt, E., Leiblein, S., Franke C., Hennig E., Friedrich T., Krahl R., Niederwieser D. & Deininger M.W., 2003. Emergence of clonal cytogenetic abnormalities in Ph- cells in some CML patients in cytogenetic remission to imatinib but restoration of polyclonal hematopoiesis in the majority. *Blood* 101, 1941-1949.
- Bumm, T, Deininger, J., Newell, A. H., Lawce, H., Olson, S., Mauro, M., Druker, B. & Deininger M., 2010. Clonal chromosomal abnormalities in CD34+/CD38- hematopoietic cells from cytogenetically

normal chronic myeloid leukemia patients with a complete cytogenetic response to tyrosine kinase inhibitors. *Leukemia* 24, 1525-1528.

- Calado, R. T., & Young, N. S., 2008. Telomere maintenance and human bone marrow failure. *Blood* 111, 4446-4455.
- Cervantes, F., & Mauro, M., 2011. Practical management of patients with chronic myeloid leukemia. *Cancer* 117, 4343-4354.
- Claxton, D., Deisseroth, A., Talpaz, M., Reading, C., Kantarjian, H., Trujillo, J., Stass, S., Gooch G. & Spitzer G., 1992. Polyclonal hematopoiesis in interferon-induced cytogenetic remissions of chronic myelogenous leukemia. *Blood* 79, 997-1002.
- Collado, M., Blasco, M. A., & Serrano, M., 2007. Cellular senescence in cancer and aging. *Cell* 130, 223-233.
- Cottliar, A, Pedrazzini, E., Corrado, C., Engelberger, M.I., Narbaitz, M, & Slavutsky I. 2003. Telomere shortening in patients with plasma cell disorders. *Eur J Haematol* 71, 334-340
- de Lange, T., 2009. How telomeres solve the end-protection problem. *Science* 326, 948-952.
- Deininger, M. W. N., Cortes, J., Paquette, R., Park, B., Hochhaus, A., Baccarani, M., Stone, R., Fischer T., Kantarjian H., Niederwieser D., Gambacorti-Passerini C., So C., Gathmann I., Goldman J.M., Smith D., Druker B.J. & Guilhot F., 2007. The prognosis for patients with chronic myeloid leukemia who have clonal cytogenetic abnormalities in philadelphia chromosome-negative cells. *Cancer* 110, 1509-1519.
- Drummond, M. W., Balabanov, S., Holyoake, T. L., & Brummendorf, T. H., 2007. Concise review: Telomere biology in normal and leukemic hematopoietic stem cells. *Stem Cells* 25, 1853-1861.
- Erdag, G., laMeck, J. M., Meloni-Ehrig, A., Matyakhina, L., Donohue, T., Srinivasan, R., Mowrey, P., Kelly J., Smith A. & Childs R., 2009. Long-term persistence of nonpathogenic clonal chromosome abnormalities in donor hematopoietic cells after allogeneic stem cell transplantation. *Cancer Genet. Cytogenet.* 190, 125-130.
- Ferrero, D, Foli, C., Giaretta, F., Argentino, C., Rus, C., & Pileri, A., 2001. Growth advantage of chronic myeloid leukemia CFU-GM in vitro: survival to growth factor deprivation, possibly related to autocrine stimulation, is a more common feature than hypersensitivity to GM-CSF/IL3 and is efficiently counteracted by retinoids +- alpha-interferon. *Leukemia* 15, 422-429.
- Fyhrquist, F., Silventoinen, K., Saijonmaa, O., Kontula, K., Devereux, R.B., De Faire, U., Os, I., Dahlöf, B., 2011. Telomere length and cardiovascular risk in hypertensive patients with left ventricular hypertrophy: The LIFE study. *J. Hum. Hypertens.* 25, 711-718.
- Gadalla, S. M., & Savage, S. A., 2011. Telomere biology in hematopoiesis and stem cell transplantation. *Blood Rev* 25, 261-269.
- Genuardi E., Ruggeri M., Drandi D., Caltagirone S., Mastrocola R., Bernocco E., Lobetti-Bodoni C., Boi M., Monitillo L., Mantoan B., Mantella E., Isaia G., Bergui L., Coscia M., Boccadoro M., Massaia M., Omedè P. & Ladetto M. (2011). Telomere length measurement in the clinically oriented research setting: a comparison between southern blot (SB), real time quantitative pcr (RQ-PCR) and flow- fish (FF). *Haematologica* 96(s3), 34(abstract #71).
- Goldman, J. M., 2009. Treatment strategies for CML. *Best Pract. Res. Clin. Haematol.* 22, 303-313.

- Guachalla, L. M., & Rudolph, K. L., 2010. ROS induced DNA damage and checkpoint responses: influences on aging? *Cell Cycle*, 9, 4058-4060.
- Hahn, W. C., 2003. Role of telomeres and telomerase in the pathogenesis of human cancer. *J. Clin. Oncol.* 21, 2034-2043.
- Harley, C.B., Futcher, A.B. & Greider, C.W., 1990. Telomere shorten during ageing of human fibroblast. *Nature* 341, 458-460.
- Hills, M., & Lansdorp, P. M., 2009. Short telomeres resulting from heritable mutations in the telomerase reverse transcriptase gene predispose for a variety of malignancies. *Ann. N.Y. Acad. Sci.* 1176, 178-190.
- Hills, M., Lücke, K., Chavez, E. A., Eaves, C. J., & Lansdorp, P. M., 2009. Probing the mitotic history and developmental stage of hematopoietic cells using single telomere length analysis (STELA). *Blood* 113, 5765-5775.
- Hoffman, J., Erben, Y., Zeiher, A.M., Dimmeler, S., & Spyridopoulos, I., 2009. Telomere length-heterogeneity among myeloid cells is a predictor for chronological ageing. *Exp Gerontol* 44, 363-366.
- Jabbour, E., Kantarjian, H. M., Abruzzo, L. V., O'Brien, S., Garcia-Manero, G., Verstovsek, S., Shan, J., Rios M.B., & Cortes J., 2007. Chromosomal abnormalities in Philadelphia chromosome negative metaphases appearing during imatinib mesylate therapy in patients with newly diagnosed chronic myeloid leukemia in chronic phase. *Blood* 110, 2991-2995.
- Jing, Z.G., Maeda, T., Sugano, M., Oyama, J.-I., Higuchi, Y., Suzuki, T., Makino, N., 2008. A percentage analysis of the telomere length in Parkinson's disease patients. *J. Geront. A Biol. Sci. Med. Sci.* 63, 467-473.
- Kantarjian, H., Sawyers, C., Hochhaus, A., Guilhot, F., Schiffer, C., Gambacorti-Passerini, C., Niederwieser, D., Resta D., Capdeville R., Zoellner U., Talpaz M., Druker B., Goldman J., O'Brien S.G., Russell N., Fischer T., Ottmann O., Cony-Makhoul P., Facon T., Stone R., Miller C., Tallman M., Brown R., Schuster M., Loughran T., Gratwohl A., Mandelli F., Saglio G., Lazzarino M., Russo D., Baccarani M., Morra E. & International STI571 CML Study Group, 2002. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N. Engl. J. Med.* 346, 645-652.
- Kantarjian, H. M., Larson, R. A., Guilhot, F., O'Brien, S. G., Mone, M., Rudoltz, M., Krahnke, T., Cortes J., Druker B.J. & International Randomized Study of Interferon and STI571 (IRIS) Investigators., 2009. Efficacy of imatinib dose escalation in patients with chronic myeloid leukemia in chronic phase. *Cancer* 115, 551-560.
- Keller, G., Brassat, U., Braig, M., Heim, D., Wege, H., & Brümmendorf, T. H., 2009. Telomeres and telomerase in chronic myeloid leukaemia: impact for pathogenesis, disease progression and targeted therapy. *Hematol. Oncol.* 27, 123-129.
- Kovitz, C., Kantarjian, H., Garcia-Manero, G., Abruzzo, L. V., & Cortes, J., 2006. Myelodysplastic syndromes and acute leukemia developing after imatinib mesylate therapy for chronic myeloid leukemia. *Blood* 108, 2811-2813.
- Ladetto, M., Compagno, M., Ricca, I., Pagano, M., Rocci, A., Astolfi, M., Drandi, D., di Celle P.F., Dell'Aquila M., Mantoan B., Vallet S., Pagliano G., De Marco F., Francese R., Santo L., Cuttica A., Marinone C., Boccadoro M. & Tarella C., 2004. Telomere length correlates with histopathogenesis

- according to the germinal center in mature B-cell lymphoproliferative disorders. *Blood* 103, 4644-4649.
- Lin, T. T., Letsolo, B. T., Jones, R. E., Rowson, J., Pratt, G., Hewamana, S., Fegan, C., Pepper C. & Baird D.M., 2010. Telomere dysfunction and fusion during the progression of chronic lymphocytic leukemia: evidence for a telomere crisis. *Blood* 116, 1899-1907.
 - Lobetti-Bodoni, C., Bernocco, E., Genuardi, E., Boccadoro, M., & Ladetto, M., 2010. Telomeres and telomerase in normal and malignant B-cells. *Hematol. Oncol.* 28, 157-167.
 - Maciejewski, H., Jaroslaw, P., Risitano, A., Sloand, E. M., Nunez, O., & Young, N. S., 2002. Distinct clinical outcomes for cytogenetic abnormalities evolving from aplastic anemia. *Blood*, 99, 3129-3135.
 - Mahon, F.X., Rea, D., Guilhot, J., Guilhot, F., Huguette, F., Nicolini, F., Legros, L., Charbonnier, A., Guerci, A., Varet, B., Etienne, G., Reiffers, J., & Rousselot, P., on behalf of the Intergroupe Francais des Leucémies Myéloïdes Chroniques (FILMC), 2010. Discontinuation of imatinib in patients with chronic myeloid leukemia who have maintained complete molecular remission for at least 2 years: the prospective multicentre Stop Imatinib (STIM) trial. *The Lancet* 11, 1029-1035.
 - Mardia K.V., Kent J.T.& Bibby J.M. Multivariate analysis. Academic Press, London, 1979.
 - Martínez, P., & Blasco, M. A., 2011. Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nature Reviews. Cancer* 11, 161-176.
 - Medina, J., Kantarjian, H., Talpaz, M., O'Brien, S., Garcia-Manero, G., Giles, F., Rios, M. B., Hayes K. & Cortes J., 2003. Chromosomal abnormalities in Philadelphia chromosome-negative metaphases appearing during imatinib mesylate therapy in patients with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase. *Cancer* 98, 1905-1911.
 - Mor-Tzuntz, R., Uziel, O., Shpilberg, O., Lahav, J., Raanani, P., Bakhanashvili, M., Rabizadeh, E., Zimra Y., Lahav M. & Granot G., 2010. Effect of imatinib on the signal transduction cascade regulating telomerase activity in K562 (BCR-ABL-positive) cells sensitive and resistant to imatinib. *Exp. Hematol.* 38, 27-37.
 - Navarro, J.-T., Feliu, E., Grau, J., Espinet, B., Colomer, D., Ribera, J.-M., Oriol, A., Granada I., Juncà J. & Millá F., 2007. Monosomy 7 with severe myelodysplasia developing during imatinib treatment of Philadelphia-positive chronic myeloid leukemia: two cases with a different outcome. *Am. J. Hematol* 82, 849-851.
 - Notaro, R., Cimmino, A., Tabarini, D., Rotoli, B., & Luzzatto, L., 1997. In vivo telomere dynamics of human hematopoietic stem cells. *Proc. Nat. Acad. Sci. USA* 94, 13782-13785.
 - O'Brien, S. G., Guilhot, F., Larson, R. A., Gathmann, I., Baccarani, M., Cervantes, F., Cornelissen, J. J., Fischer T., Hochhaus A., Hughes T., Lechner K., Nielsen J.L., Rousselot P., Reiffers J., Saglio G., Shepherd J., Simonsson B., Gratwohl A., Goldman J.M., Kantarjian H., Taylor K., Verhoef G., Bolton A.E., Capdeville R., Druker B.J. & IRIS Investigators, 2003. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N. Engl. J. Med* 348, 994-1004.
 - Paquette, R. L., Nicoll, J., Chalukya, M., Gondek, L., Jasek, M., Sawyers, C. L., Shah, N. P. & Maciejewski J., 2010. Clonal hematopoiesis in Philadelphia chromosome-negative bone marrow cells of chronic myeloid leukemia patients receiving dasatinib. *Leuk. Res.* 34, 708-713.
 - Passos, J. F., Saretzki, G., & von Zglinicki, T., 2007. DNA damage in telomeres and mitochondria

during cellular senescence: is there a connection? *Nucleic Acids Res.* 35, 7505-7513.

- Quintás-Cardama, A., De Souza Santos, F. P., Kantarjian, H., O'Brien, S., Faderl, S., Awais, A., Borthakur, G. & Cortes J., 2009. Dynamics and management of cytopenias associated with dasatinib therapy in patients with chronic myeloid leukemia in chronic phase after imatinib failure. *Cancer* 115, 3935-3943.
- Ricca, I, Compagno, M., Ladetto, M., Rocci, A., Dell'Aquila, M., Omedè, P., De Marco, F., D'Antico S., Caracciolo D., Ferrero D., Carlo-Stella C. & Tarella C., 2005. Marked telomere shortening in mobilized peripheral blood progenitor cells (PBPC) following two tightly spaced high-dose chemotherapy courses with G-CSF. *Leukemia* 19, 644-651.
- Robertson, J. D., Gale, R. E., Wynn, R. F., Dougal, M., Linch, D. C., Testa, N. G., & Chopra, R., 2000. Dynamics of telomere shortening in neutrophils and T lymphocytes during ageing and the relationship to skewed X chromosome inactivation patterns. *Br. J. Haematol.* 109, 272-279.
- Rocci, Alberto, Ricca, I., Dellacasa, C., Longoni, P., Compagno, M., Francese, R., Lobetti Bodoni, C., Manzini P., Caracciolo D., Boccadoro M., Ferrero D., Ladetto M., Carlo-Stella C. & Tarella C., 2007. Long-term lymphoma survivors following high-dose chemotherapy and autograft: evidence of permanent telomere shortening in myeloid cells, associated with marked reduction of bone marrow hematopoietic stem cell reservoir. *Exp. Hematol* 35, 673-681.
- Rossi, D., Lobetti Bodoni, C., Genuardi, E., Monitillo, L., Drandi, D., Cerri, M., Deambrogi, C., Ricca I., Rocci A., Ferrero S., Bernocco E., Capello D., De Paoli L., Bergui L., Boi M., Omedè P., Massaia M., Tarella C., Passera R., Boccadoro M., Gaidano G. & Ladetto M., 2009. Telomere length is an independent predictor of survival, treatment requirement and Richter's syndrome transformation in chronic lymphocytic leukemia. *Leukemia* 23, 1062-1072.
- Sharpless, N. E., Depinho, R. A., 2007. How stem cells age and why this makes us grow old. *Nat Rev Mol Cell Biol.*8,703-13.
- Sokal, J. E., 1987. Prognosis in chronic myeloid leukaemia: biology of the disease vs. treatment. *Baillière's Clin. Haematol.* 1, 907-929.
- Trotti, A., Colevas, A. D., Setser, A., Rusch, V., Jaques, D., Budach, V., Langer, C., Murphy B., Cumberlin R., Coleman C.N. & Rubin P., 2003. CTCAE v3.0: development of a comprehensive grading system for the adverse effects of cancer treatment. *Seminars Radiat. Oncol.* 13, 176-181.
- Uziel, O, Fenig, E., Nordenberg, J., Beery, E., Reshef, H., Sandbank, J., Birenbaum, M., Bakhanashvili M., Yerushalmi R., Luria D. & Lahav M., 2005. Imatinib mesylate (Gleevec) downregulates telomerase activity and inhibits proliferation in telomerase-expressing cell lines. *Br. J. Cancer* 92, 1881-1891.
- Vaquero-Sedas, M. I., & Vega-Palas, M. A., 2011. On the chromatin structure of eukaryotic telomeres. *Epigenetics*, 6. Epub ahead of print
- Vaziri, H., Dragowska, W., Allsopp, R. C., Thomas, T. E., Harley, C. B., & Lansdorp, P. M., 1994. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. *Proc. Nat. Acad. Sci. USA* 91, 9857-9860.
- von Figura G., Hartmann D., Song Z., Rudolph K.L., 2009. Role of telomere dysfunction in aging and its detection by biomarkers. *Mol Med* 87,1165-71.
- Wagner W., Bork S., Horn P., Krunic D., Walenda T., Diehlmann A., Benes V., Blake J., Huber F.X.,

- Eckstein V., Boukamp .P, Ho A.D., 2009. Aging and replicative senescence have related effects on human stem and progenitors cells. *PLoS One*. 9, e5846
- Wynn, R. F., Cross, M. A., Hatton, C., Will, A. M., Lashford, L. S., Dexter, T. M., & Testa, N. G., 1998. Accelerated telomere shortening in young recipients of allogeneic bone-marrow transplants. *Lancet* 351, 178-181.
 - Xin, Z.-T., Beauchamp, A. D., Calado, R. T., Bradford, J. W., Regal, J. A., Shenoy, A., Liang, Y., Lansdorp P.M., Young N.S. & Ly H., 2007. Functional characterization of natural telomerase mutations found in patients with hematologic disorders. *Blood* 109, 524-532.
 - Yaffe, K., Lindquist, K., Kluse, M., Cawthon, R., Harris, T., Hsueh, W.-C., Simonsick, E.M., Cummings, S.R., 2011. Telomere length and cognitive function in community-dwelling elders: Findings from the Health ABC Study. *Neurobiol. Aging* 32, 2055-2060.
 - Zimmermann, S., Glaser, S., Ketteler, R., Waller, C. F., Klingmüller, U., & Martens, U. M., 2004. Effects of telomerase modulation in human hematopoietic progenitor cells. *Stem Cells* 22, 741-749.

LEGEND TO FIGURES

Figure 1. Telomere length comparison of CCyR CML patients and age-matched healthy subjects.

A: TRF-L analysis in MD-PBMC. B: TRF-L analysis in PMN. C: the equation from multivariate analysis by general linear model of the entire population (healthy subjects and CML patients) and its graphic resolution in the absence or presence of significant predictors of telomere attrition in PMN. The impact of aging was calculated at the median age of the entire population (61 years).

Figure 2. Relation between telomere length loss and the presence of CA.

A: Telomere length in CML patients with normal karyotype versus CML patients with acquired CA. B: Telomere length in healthy subjects and in CML patients without CA. C: Equation of multivariate analysis on the CML population only, and its graphic resolution in the absence or presence of significant predictors of telomere shortening. The impact of age was calculated at the median age of the CML population (62 years).

Figure 3. Prospective evaluation of telomere loss over time.

A: Global TRF-L analysis at two time points of PMN with a median interval of 22 months. B: individual telomere loss in PMN calculated on a yearly basis. A variation of 300 bp was accepted as a reflection of the maximal inter-assay variability. An additional 50 bp loss was accepted as the maximal annual physiological loss; telomere shortening more than 350 bp was considered abnormal (in black columns).

Figure 4. Association between telomere loss and grade 2-4 hematological toxicity.

Relation between telomere length and G2-4 hematological toxicity (A), G2-4 anemia (B), G2-4 neutropenia (C), and G2-4 thrombocytopenia (D). Hematological toxicity was evaluated according to NCI CTCAE v3.0, recording the lowest levels of hemoglobin, granulocytes, and platelets displayed by patients were recorded between CCyR and the most recent follow-up.

Figure S1. Association between telomere loss and hematological toxicity of any grade.

Telomere length and any G 1-4 hematological toxicity (A), G 1-4 anemia (B), G 1-4 neutropenia (C), G1-4 thrombocytopenia (D). Hematological toxicity was evaluated according to NCI CTCAE v3.0, recording the lowest levels of hemoglobin, granulocytes, and platelets displayed by patients from CCyR to the most recent follow-up.

TABLES

Table 1: Patients demographic and clinical features at study entry

AGE (years)	
Median	61
Range	23-88
SEX	
Male	40/81
Female	41/81
SOKAL SCORE at diagnosis	
High	18/81
Intermediate	28/81
Low	35/81
TIME FROM DIAGNOSIS (years)	
Median	4
Range	1-18
TIME FROM TKI START (years)	
(when applicable)	2
	1-7
TIME FROM CCyR (years)	
Median	3
Range	1-12
MOLECULAR REMISSION	
CMoIR	20/81
MMoIR	45/81
No MoIR	16/81
CA*	15/81
THERAPY	
TKI inhibitors**	48/81
IFN- α alone	6/81
TKI after previous treatments***	27/81

*The consecutive series was enriched of eight patients with CA.

** Imatinib in 44 patients, dasatinib in four

*** INF- α followed by imatinib (16/27); ARA-C followed by imatinib (2/27); INF- α + ARA-C followed by imatinib (9/27)

Abbreviations:

TKI: tyrosine kinase inhibitors, CCyR: Complete Cytogenetic Remission, CMoIR: Complete Molecular Remission, MMoIR: Major Molecular Remission, No MoIR: No Molecular Remission, CA: acquired cytogenetic abnormalities, IFN- α : Interferon alpha

Table 2: Clinical features of patients with acquired cytogenetic abnormalities at study entry

*at least 2 cells with CA on 20 analyzed metaphases

Abbreviations: CA: Acquired Cytogenetic Abnormalities, CCyR: Complete Cytogenetic Remission, CMoIR: Complete Molecular Remission, MMoIR: Major Molecular Remission, No MoIR: No Molecular Remission, INF α : interferon alpha, ARA-C: cytosine arabinoside, TRF-L: Telomere Restriction Fragment Length, PMN: Polymorphonucleates

	CA	Mytosis* (%)	TRF-L PMN (bp)	Sex	Age (years)	Time from diagnosis (years)	Time from CCyR (years)	Molecular Response	Sokal Score	Treatment Schedule
CA 1	Del Y	50	6532	M	68	1	1	CMoIR	INT	imatinib
CA 2	47 XYY	25	6536	M	51	6	5	No MoIR	LOW	IFN- α , imatinib
CA 3	Del 5q13	70	6595	F	40	6	5	No MoIR	INT	imatinib
CA 4	Del 5q	100	7004	M	36	4	3	No MoIR	HIGH	IFN- α , imatinib
CA 5	Del 10	50	6510	F	45	8	3	CMoIR	HIGH	IFN- α , ARA-C, imatinib
CA 6	Del 20q	25	5018	M	73	7	1	No MoIR	INT	imatinib
CA 7	Trisomy 8	25	7518	M	64	2	1	No MoIR	INT	imatinib
CA 8	Trisomy 8	11	5683	F	58	9	3	MMoIR	HIGH	IFN- α , ARA-C, imatinib
CA 9	Trisomy 8,9	20	6012	F	59	11	7	CMoIR	LOW	dasatinib
CA 10	Trisomy 8	25	4836	F	74	10	9	MMoIR	HIGH	IFN- α , ARA-C, imatinib
CA 11	Trisomy 8	50	4301	F	57	12	2	MMoIR	INT	IFN- α , imatinib
CA 12	Del 7	50	6086	F	67	2	1	CMoIR	INT	imatinib
CA 13	Del 7	50	5700	M	71	8	3	MMoIR	INT	imatinib
CA 14	Del 7	70	5609	F	51	12	6	MMoIR	LOW	IFN- α , ARA-C + imatinib
CA 15	Del 7	60	5687	M	69	14	3	MMoIR	INT	IFN- α , ARA-C, imatinib

Figure 1

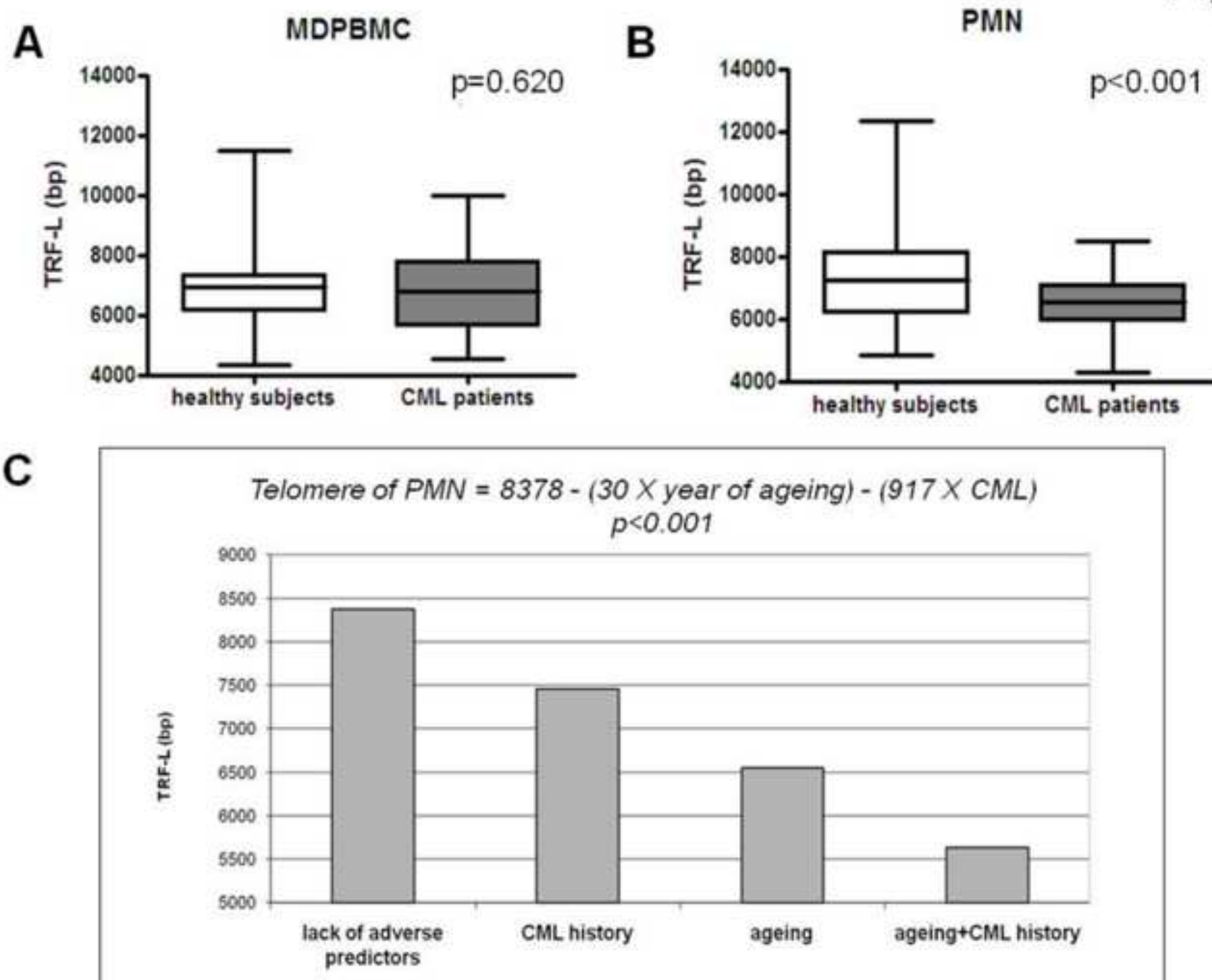


Figure 2
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Figure 2

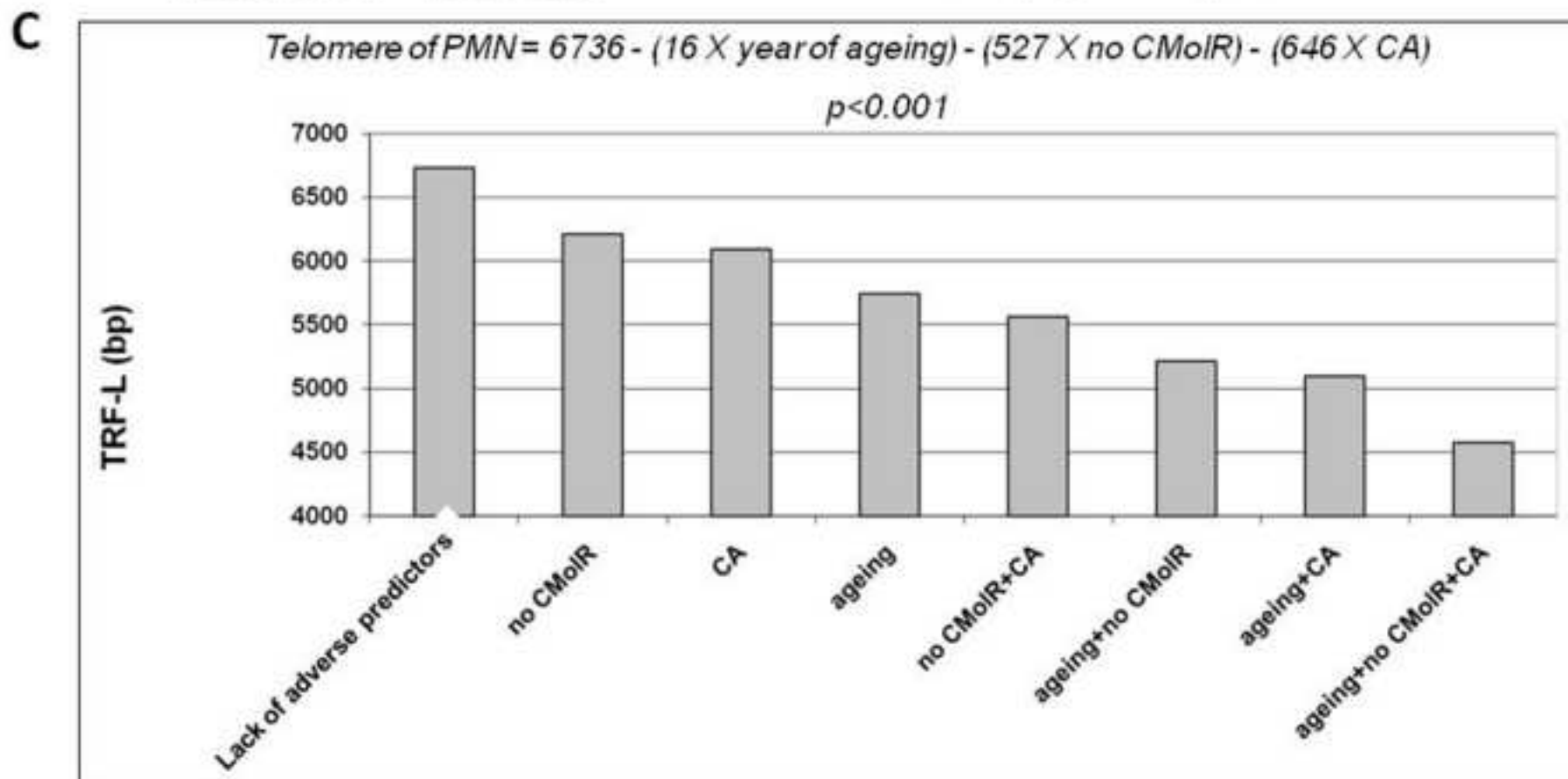
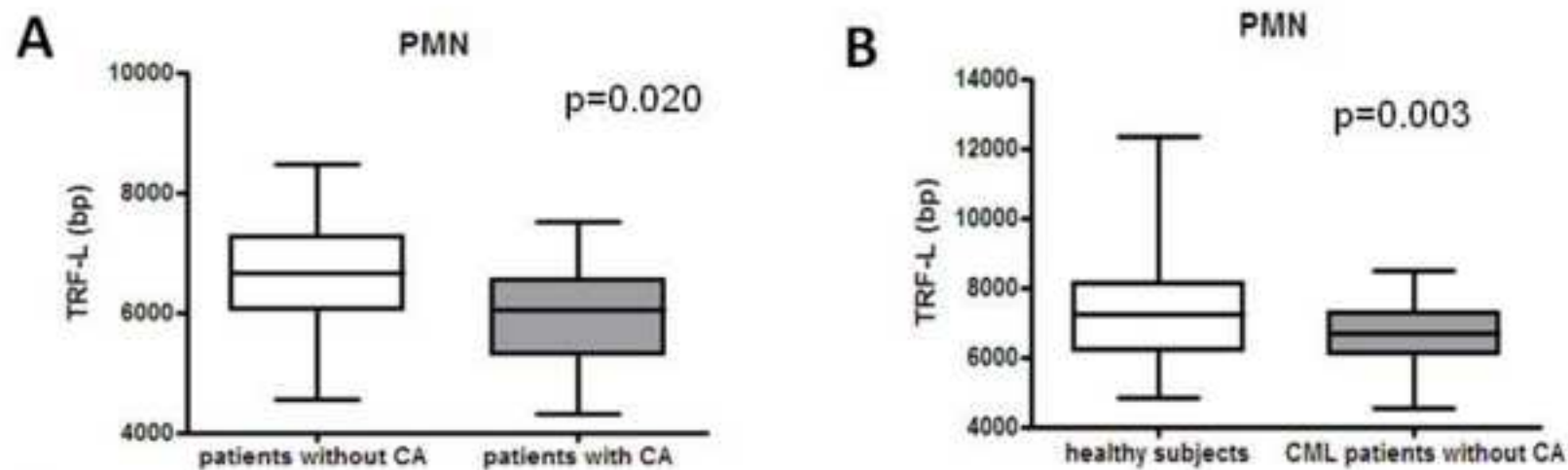


Figure 3

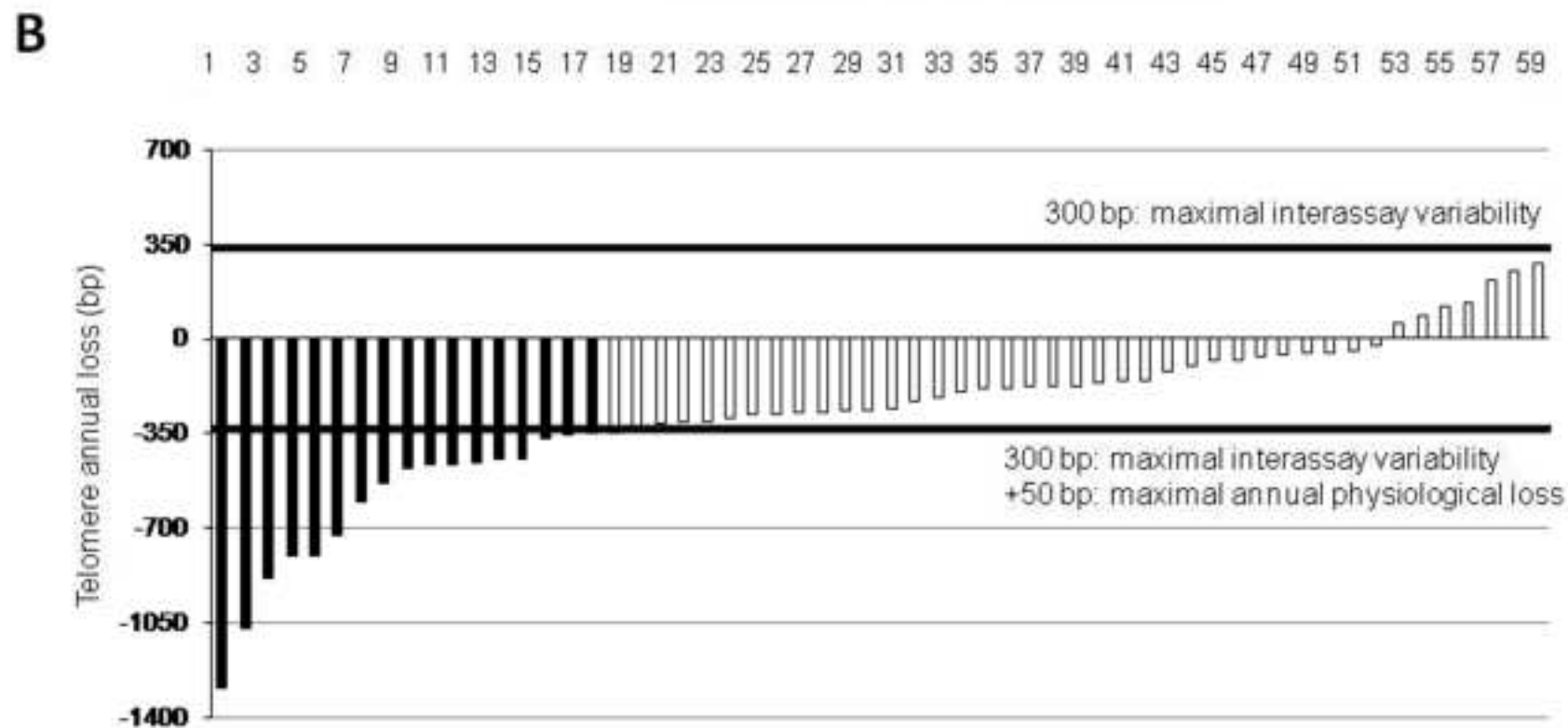
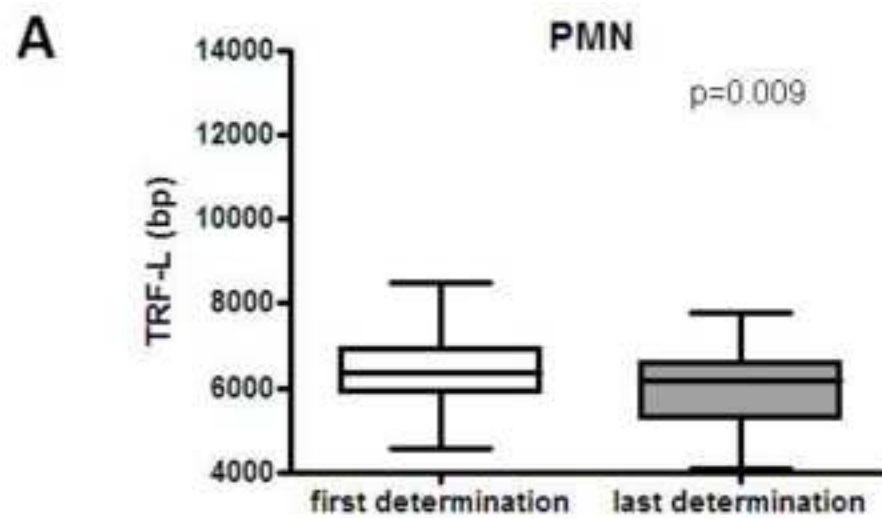
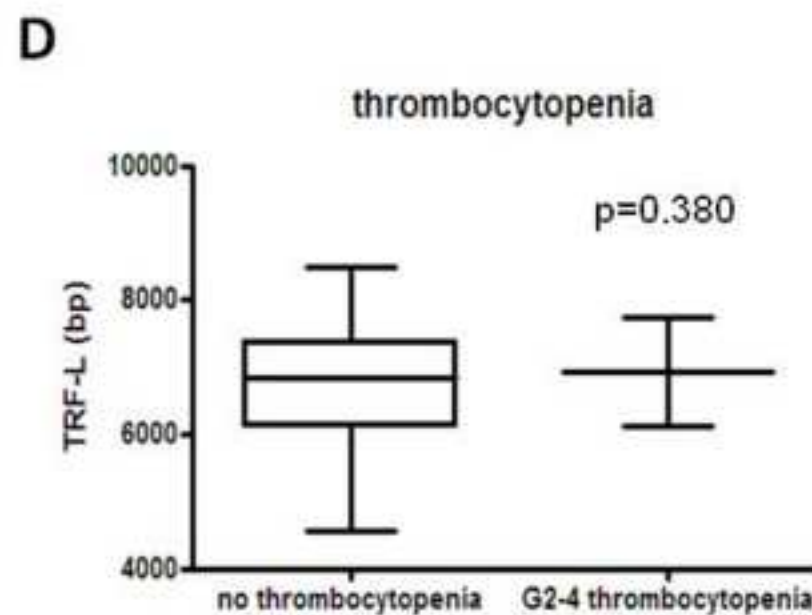
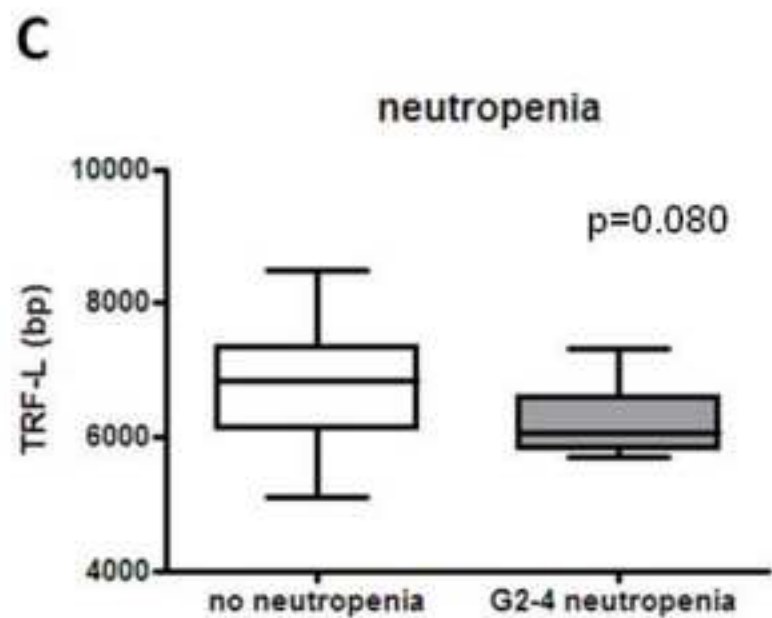
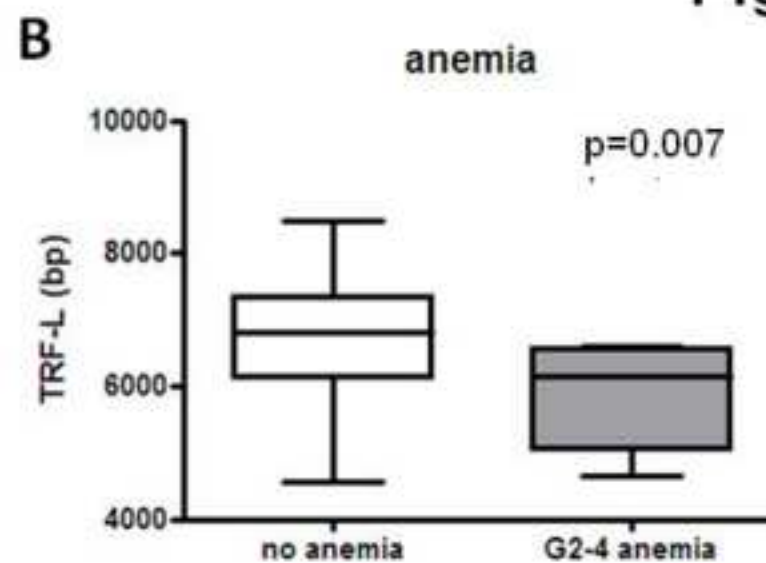
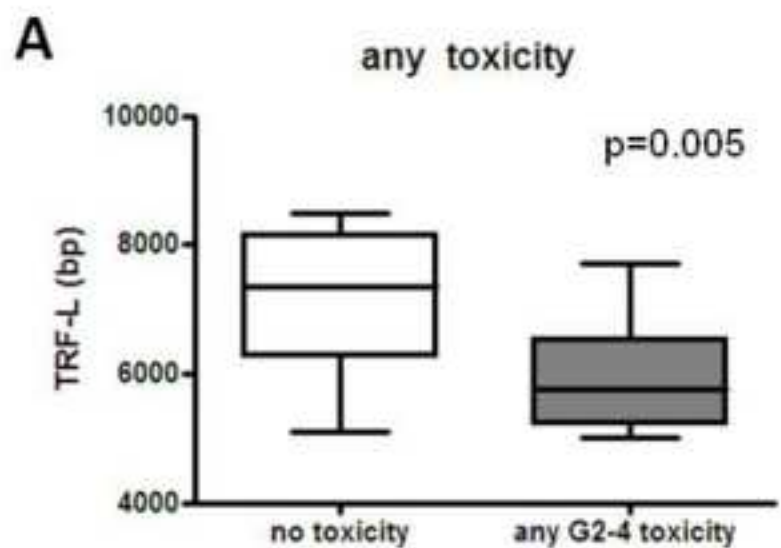


Figure 4



Supplementary Material

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Supplementary Material

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