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1 Telomere length is a critical determinant for survival in multiple  
2 myeloma

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16 **Running title:** Telomere based prognostication in Multiple Myeloma

17 **Keywords:** Multiple Myeloma, prognosis, telomere, genome instability

18

1 **Summary**

2 Patients with Multiple Myeloma (MM) exhibit variable clinical outcomes, which are  
3 incompletely defined by the current prognostication tools. We examined the clinical  
4 utility of high-resolution telomere length analysis as a prognostic marker in MM.  
5 Stratification of the cohort using a previously determined length threshold for  
6 telomere dysfunction revealed that patients with short telomeres had a significant  
7 shorter overall survival ( $P < 0.0001$ ; HR=3.4). Multivariate modelling using forward  
8 selection revealed that the most important prognostic factor was ISS, followed by  
9 age and telomere length. Importantly, each ISS prognostic subset could be further  
10 risk-stratified according to telomere length, supporting the inclusion of this  
11 parameter as a refinement of the ISS.

1 Despite the introduction of novel therapeutic modalities, patients with multiple  
2 myeloma (MM) display a heterogeneous clinical course, with survival ranging from a  
3 few months to over 10 years. Therefore, there is a requirement for reliable  
4 prognostic and predictive markers in this disease to allow for risk stratification and  
5 rational clinical decision-making. The most commonly used prognostic system in MM  
6 is the International Staging System (ISS) that is based on serum levels of both  $\beta_2$ -  
7 microglobulin and albumin (Greipp, *et al* 2005). Recently the ISS system has been  
8 improved upon by the inclusion of cytogenetic information to take into account the  
9 considerable genetic heterogeneity known to occur in this disease and the level of  
10 lactate dehydrogenase (Palumbo, *et al* 2015). Hyperdiploidy and the loss of whole  
11 chromosome arms is frequently detected in MM, this includes, amongst others,  
12 gains of 1q in 30% of cases and the loss of 17p in 7% of cases (Walker, *et al* 2010).

13  
14 Short dysfunctional telomeres are susceptible to DNA repair activities that can result  
15 in chromosomal fusion and the initiation of cycles of anaphase-bridging, breakage  
16 and fusion that can drive genomic instability and clonal evolution (Artandi, *et al*  
17 2000, Jones, *et al* 2014, Roger, *et al* 2013). Telomere dysfunction has been  
18 documented in numerous haematological malignancies (Jones, *et al* 2012), this is  
19 one putative mechanism that may lead to the genetic and clinical heterogeneity  
20 observed in MM (Wu, *et al* 2003) and may relate to changes in the 3D telomeric  
21 architecture that have been documented in MM cells (Klewes, *et al* 2013). Recently  
22 we have shown that high-resolution telomere analysis, combined with a functional  
23 definition of telomere length, can provide powerful prognostic information in several  
24 tumour types, including chronic lymphocytic leukaemia (CLL)(Lin, *et al* 2014),

1 myelodysplasia (Williams et al. in prep) and breast cancer (Simpson, *et al* 2015).

2 Here we sought to apply these technologies to examine the prognostic utility of

3 telomere length in MM.

4

5 Materials and Methods

6 Patients samples and cell separation

7 Patient samples were collected at diagnosis, prior to treatment, through treating

8 centres within the Heart of England NHS Foundation Trust and the Newcastle upon

9 Tyne NHS Foundation Trust between 1990 and 2005, with ethical approval from the

10 Newcastle Haematology Biobank (07/H0906/109+5) in accordance with the

11 declaration of Helsinki. Bone marrow samples from five MM patients were

12 fractionated into CD138<sup>+</sup> and CD138<sup>-</sup> cells using positive selection with CD138+

13 microbeads and the AutoMACS system (Miltenyi). Cytogenetics was not available on

14 these samples and thus the original ISS staging system was used for this study

15 (Greipp, *et al* 2005).

16 DNA extraction and single telomere length analysis

17 DNA was extracted from sorted cell populations using the Qiagen DNeasy blood and

18 tissue extraction kit, according to the manufacturers instructions. Single telomere

19 length analysis (STELA) at the XpYp telomere was performed as previously described

20 (Baird, *et al* 2003, Britt-Compton, *et al* 2006, Capper, *et al* 2007). A minimum of 6

21 PCR reactions per sample were carried out for each test DNA and DNA fragments

22 were resolved by agarose gel electrophoresis, and detected by Southern

23 hybridisations and phosphorimaging.

24 Statistical methods

1 Statistical analysis was carried out using Prism 6.0 (Graphpad) and SAS version 9.3  
2 software (SAS Institute). Univariate comparisons for overall survival (OS) were  
3 conducted with the log-rank test and displayed as Kaplan Meier curves.  $P < .05$  was  
4 considered significant. Analyses of time to event outcomes with respect to  
5 continuous variables or those with less than two categories, together with  
6 multivariable analyses, were performed using a Cox proportional hazard model with  
7 forward selection.  $P < 0.05$  was considered significant.

8

## 9 **Results and discussion**

10

11 We used Single Telomere Length Analysis (STELA) of the XpYp telomere to generate  
12 telomere length profiles from unsorted bone marrow samples derived from a cohort  
13 of 61 patients with MGUS and 134 patients with MM (Figure 1A). STELA is a high-  
14 resolution technology capable of detecting telomeres within the length ranges at  
15 which telomere fusion is known to occur (Letsolo, *et al* 2010, Lin, *et al* 2014). STELA  
16 also provides information on telomere length distributions, which relate to the  
17 clonality, replicative history and the cell purity of the tumour cell population  
18 analysed. At the individual patient level, it was apparent that the telomeres in both  
19 the MGUS and MM bone marrow samples displayed considerable length  
20 heterogeneity (Figure 1A), with an overall mean of the SDs obtained from these  
21 distributions in the MGUS cohort of 2.23 kb and 2.12 kb for the MM cohort. This was  
22 in contrast to a similar analysis in CLL (Lin, *et al* 2010) where purified tumour cells  
23 were analysed and individual telomere length distributions were significantly more  
24 homogeneous (mean SD = 1.28) compared to those observed in MM and MGUS  
25 patients ( $p < .0001$ ; Mann-Whitney). The telomere length heterogeneity in the MM

1 samples was consistent with the differing replicative histories of the numerous cell  
2 populations that composed the unsorted bone marrow samples analysed.

3

4 The majority of telomere length profiles derived from MM patient samples displayed  
5 multi-modal distributions with sub-populations of cells displaying more extensive  
6 telomere erosion (highlighted in red in Figure 1A). To examine the telomere length  
7 distributions of purified myeloma plasma cell populations, we undertook cell sorting  
8 in a subset of myeloma bone marrow samples (n = 5) and analysed the CD138<sup>+</sup> and  
9 CD138<sup>-</sup> cell fractions separately. Distinct differences in the telomere length  
10 distributions were apparent (Figure 1B), with CD138<sup>+</sup> plasma cells (mean = 2.40kb)  
11 displaying significant telomere erosion compared to the CD138<sup>-</sup> cells (mean = 5.26kb;  
12 p = .008). These data indicate that the shorter telomere length distribution observed  
13 in unsorted MM samples represent CD138<sup>+</sup> malignant plasma cells. Importantly  
14 these data are consistent with MM plasma cells exhibiting an extensive replicative  
15 history that is distinct to that of bone marrow CD138<sup>-</sup> cells in these patients.

16

17 Overall, telomere length was shorter in MM compared to MGUS patient samples (P =  
18 .017; Mann-Whitney) with 19% of MM samples within the fusogenic range (Figure  
19 1C); the telomere length threshold below which we detected telomere fusions in CLL  
20 (3.81kb)(Lin, *et al* 2014). Telomere length declined as a function of age in MM at  
21 rates consistent with the extensive literature on telomere dynamics in normal  
22 peripheral blood samples (-25 bp/year, p = .00056; Figure 1D)(Muezzinler, *et al*  
23 2013). However, there was no significant difference in telomere length between any  
24 of the International Staging System (ISS) subgroups (p = .27; Figure 1C) or between

1 sexes ( $p = .22$ ).

2

3 The median telomere length of the MM cohort provided modest prognostic  
4 resolution (HR = 1.61 (1.04-2.53),  $p = .03$ ; data not shown). In contrast, use of the  
5 previously determined telomere dysfunction threshold (Lin, *et al* 2014) was highly  
6 prognostic for overall survival in MM (HR = 3.42 (3.67-15.81),  $P < .0001$ ; Figure 2A); a  
7 striking observation, given the variable contributions of plasma cells to the unsorted  
8 heterogeneous samples analysed.

9

10 Consistent with previous reports, the ISS provided strong prognostic information in  
11 our MM cohort (HR = 3.56 (2.92-9.13),  $p < .0001$ ; Figure 2B), that was similar to that  
12 derived using the telomere dysfunction threshold. In order to assess whether  
13 telomere length could add prognostic resolution to the ISS, we performed  
14 multivariate analysis on 113/131 (86.3%) MM samples on which we had all relevant  
15 clinical data. In a model which included the potential covariates of mean telomere  
16 length, gender, age, ISS sub-groups and the telomere dysfunction threshold (3.81kb),  
17 the most important prognostic factor was ISS, followed by age and telomere length  
18 below 3.81kb (Table 1). After adjustment for ISS and age, telomere length <3.81kb  
19 was associated with significantly shorter survival (HR = 2.23 (1.26-3.96),  $P = .006$ ;  
20 Figure 2C). Despite the prognostic independence of telomere length, there was  
21 evidence of an interaction between ISS and this parameter, with the effect of short  
22 telomeres less prominent in patients with high risk ISS score ( $P = .05$ ). However, in  
23 univariate analyses, stratified by ISS score, short telomeres still were associated with  
24 significantly worse survival in high risk ISS patients ( $P = .02$ ). Overall, patients with



1 good or standard risk ISS who manifested short telomeres, or high risk ISS patients  
2 with long telomeres had intermediate survival when compared to concordant groups  
3 consisting of good/standard risk ISS and long telomeres and high risk ISS and short  
4 telomeres (Figure 2D). These findings suggest that a refinement of the risk  
5 classification could be obtained by incorporating telomere length assessment into  
6 the ISS for MM. Given the obvious differences observed between malignant plasma  
7 cells and other bone marrow constituents, we speculate that purification of the MM  
8 tumour cells would further enhance the clinical utility of high-resolution telomere  
9 length analysis in this disease.

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4 **Conflict of Interest Statement:**

5 SH declares no conflict of interests; REJ declares no conflict of interests; NHH  
6 declares no conflict of interests; CF declares no conflict of interests other than co-  
7 authorship of a patent application based on some of this work; GHJ declares no  
8 conflict of interests; JMA declares no conflict of interests; GP declares no conflict of  
9 interests; CP declares no conflict of interests other than co-authorship of a patent  
10 application based on some of this work; DMB declares no conflict of interests other  
11 than co-authorship of a patent application based on some of this work.

12

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21

1 **Table 1. Multivariate modelling revealed three significant independent variables.**

2

Summary of Forward Selection					
Step	Effect entered	DF	Number In	Chi-Square test statistic	p-value
1	ISS 3	1	1	34.7751	<.0001
2	age	1	2	18.1900	<.0001
3	TL <3.81kb	1	3	7.7095	0.0055

3 ISS 3: Multiple myeloma International Scoring System stage 3

4 TL <3.81kb: mean telomere length of less than 3.81kb

5 Using a Cox proportional hazards model with forward selection, only three variables  
6 were deemed to hold independent prognostic significance ( $p \leq .05$ ).

7

8

9

10

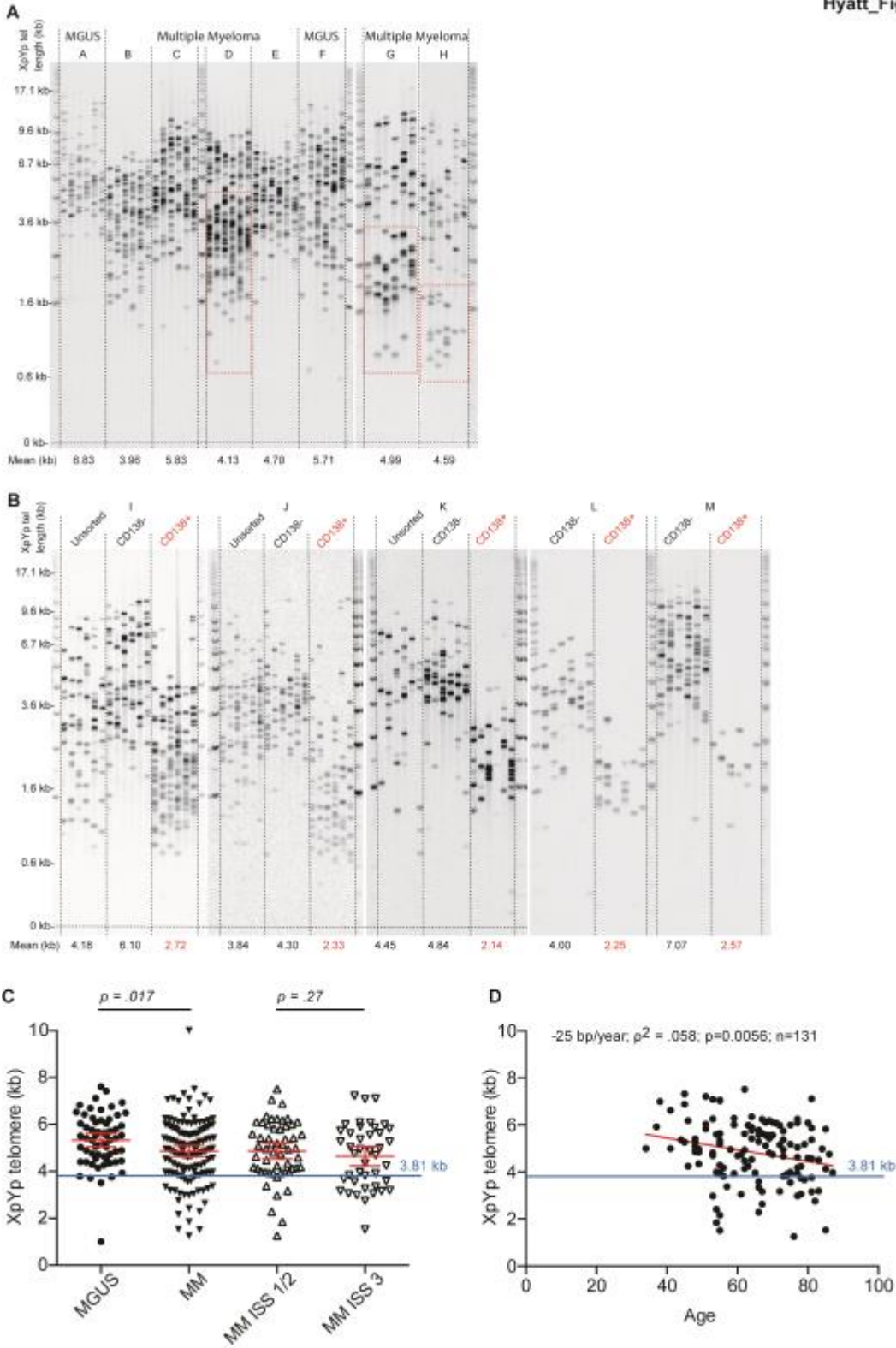
1 **Figure Legends**

2 **Figure 1. MM and MGUS exhibit heterogeneous telomere length profiles.** (A)

3 Examples of STELA of the XpYp telomere in 8 MM and MGUS samples. The mean of  
4 the telomere length profiles are indicated in black below each sample. Red boxes  
5 indicate the shorter modal telomere length profiles in samples that display a  
6 multimodal telomere length distributions. (B) Comparison of cell sorted CD138<sup>+</sup> and  
7 CD138<sup>-</sup> sub-populations revealed distinctly shorter telomere length profiles in  
8 CD138<sup>+</sup> cells. (C) Scatter plot depicting mean telomere length measurements for  
9 cohorts of MGUS and MM patients, and MM ISS 1/2 and ISS 3 sub-groups. The  
10 upper limit of telomere dysfunction (3.81kb) is shown as a blue horizontal line.  
11 Statistical comparisons were undertaken using non-parametric Mann-Whitney  
12 tests. (D) Plotting mean telomere length as a function of age shown in years. P value  
13 was determined using Spearman correlation.

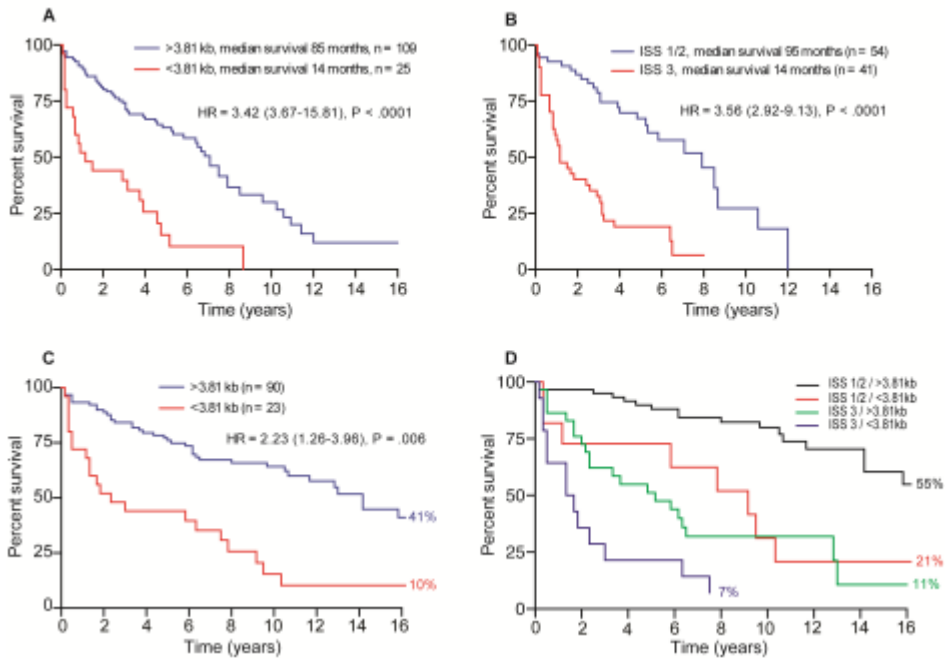
14 **Figure 2. Telomere length is highly prognostic in MM.** Kaplan Meier survival analysis

15 was performed on the MM cohort using the log-rank test (A) The telomere  
16 dysfunction threshold (<3.81kb) identified a subset of MM patients with inferior  
17 survival. (B) In keeping with previous reports, the ISS 3 sub-group also showed  
18 significantly inferior survival compared to the combined ISS 1/2 sub-group. (C) Even  
19 after adjustment for ISS and age, MM patients with telomere length <3.81kb had  
20 significantly shorter survival. (D) The combination of ISS and the telomere  
21 dysfunction threshold provided a refinement of their prognostic information. ISS 1/2  
22 patients with short telomeres, or ISS 3 patients with long telomeres had  
23 intermediate survival when compared to concordant groups consisting of ISS 1/2 and  
24 long telomeres and ISS 3 and short telomeres.



1  
2

Hyatt\_Figure 2



1