Author's response to reviews

Title: The aberrant asynchronous replication -- characterizing PHA-stimulated lymphocytes of patients with hematological malignancies -- is erased following stem cell transplantation

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Author's response to reviews: see over
Dear Editor,

Re: MS: 3332547253061776

We addressed the reviewers' comments in the revised manuscript please see bellow a point-by-point response to the concerns. We also revised the title of the manuscript as requested by the editorial team.

I hope that you will find the revised form suitable for publication in BMC Cancer

Thanks for handling the matter

Lydia Avivi
Corresponding Author

Response to reviewers’ comments

Editorial Team Comments:

The title should be revised:
The title was revised to: The aberrant asynchronous replication — characterizing lymphocytes of cancer patients — is erased following stem cell transplantation (please see the title page of the revised manuscript).

Reviewer: Mathew J Thayer (#1)

Comment 1: The last sentence of the first full paragraph on page 18 needs to be clarified. What is alloCST? (alloSCT?),
The whole sentence was modified as requested (please see revised manuscript, page 19, lines 3-7).
Certainly, it should be alloSCT (page 19 line 4).

Comment 2: The implication that asynchronous replication of TP53 is functionally akin to LOH at the TP53 locus needs to be verified by expression data.
As far as we know there is no solid data showing directly that loss of synchrony at the TP53 locus is associated with loss of expression of a single allele at this locus. We accepted this comment and removed the issue of LOH from the Discussion section and the corresponding reference from the list of references (please see, revised manuscript, lines 3-5 in the paragraph starting on page 20 and terminating on page 21).
**Reviewer: Jose Rueff (#2)**

**Comment 1:** On what basis can one group together cancer patients highly differing in the etiology, status and prognosis of their malignant disease?

The group of cancer patients was divided into three subcategories: a subgroup not exposed to any treatment modality (ADI); a subgroup put through various therapeutic treatments, except transplantation (PRE); and a subgroup that underwent a successful transplantation (POST). The results speak for themselves and give a clear cut answer to the reviewer question. Briefly, all 20 patients from the ADI group and 14 from the PRE group revealed an aberrant replication pattern of all the three tested loci, expressed in high asynchronous values, similar to those normally characterizing an imprinted locus (Figs. 3 and 4). At the same time, the cancer-free (CON) group (20 cases) revealed the expected (normal) pattern of allelic replication; normal patterns of replication, synchronous replication (low SD values) for biallelically-expressed genes and asynchronous (high SD values) for imprinted and other monoallelically expressed genes. These are epigenetic characteristics established in early developmental stages and are independent of age (reviewed in the Introduction).

**Comment 2:** The large age variation also adds extra factors to hamper solid conclusions from the study.

Please see my response to Comment 1. In addition, note that the SD values for AML1 and TP53 obtained in cells of cancer-free elderly men studied previously (references 7 and 8) are low and similar to those obtained in normal embryonic cells (Amiel et al, *Eur J Hum Genet* 1998; 6:359 and Amiel et al, *Eur J Hum Genet* 1999; 7:223).

**Comment 3:** It is difficult to agree that loss of the aberrant asynchronous replication could serve as good biomarker of a successful treatment.

The facts that malignancy, in its broader sense, is accompanied by reversible epigenetic alterations and that asynchronous replication of biallelically-expressed genes is a reversible epigenetic aberration associated with cancer, allow one to suggest that allelic replication patterns offer a potential tool for monitoring the success of cancer treatments. The return of asynchronous replication can similarly be a sign of the return of malignancy. While we have only shown here that the epigenetic aberration associated with malignancy is reversible with in vitro 5-azacytidine, our previous work showed that, in this respect, the effect of a drug applied in vitro (G-CSF) is similar to that observed via in vivo application (please see reference 21).
Comment 4: The conclusion that loss of the aberrant asynchronous replication could be used for long-term follow-up of the patients and as marker of therapeutic success goes beyond the data presented at this stage. Please see my reaction to Comment 3.

Comment 5: The authors make use of the impressive and somehow imprecise concept of cancerous phenotype’ (page 20, line 3) which by all means needs clarification. To clarify the matter the whole sentence was rewritten (please see page 20, the two last lines and page 21, first line).

Comment 6: In what concerns persistence or recurrence of aneuploidy it is not clear if that effect might have been due to G-CSF treatment of the donors’ cells, and consequently not an effect of the malignancy. In general, increased levels of aneuploidy were described in lymphocytes of patients with a malignant disease who were not exposed to alloSCT or to any other therapeutic modality associated with G-CSF (please see Figs. 3 and 4; and references 8, 16, 18 and 21). Thus, it is not clear why one should question whether aneuploidy is associated with the malignant status or not.

Yet, since patients who underwent transplantation showed 100% of donor chimerism (see page 7, line 9), it is reasonable to assumed that their lymphocytes are, in fact, donors' cells and as such show the long term effect of G-CSF (normal replication patterns and increased levels of aneuploidy; see reference 21). To make it clear the lines dealing with this matter were rewritten (please see page 19, lines 3-7).

Minor Essential Revisions

The paper should supply data on hybridization efficiency and specificity. In our present study the percentage of aneuploidic cells observed in control sample (Figs. 3 and 4) expresses, in fact, hybridization efficiency and specificity of each probe in question. Clearly, efficiency correlates with the frequency of monosomic cells and specificity with the frequency of multisomic cells.

Reviewer: Yaqub Hanna (#3)

Comment 1: Average age values of the different groups of patients should be provided. We added the mean and median age values for each group of patient to the Materials and Methods section (please see lines 2-5 and 7 in the paragraph starting on page 7 and terminating on page 8).
Comment 2: Patients without hematological malignancy, but with some chronic inflammation should be provided as controls.

We added a group of 14 urologic patients with a common medical problem, matching in sex and age, referred to biopsy because of suspected cancer. Each was checked with the AML1 and the CEN17 probes. The results show that patients (4 cases), diagnosed, following biopsy, with cancer revealed high SD values, similar to those observed in patients with hematological malignancies. Yet, the rest (10 cases), who were found to be cancer-free, showed low SD values. It happened so that of the 10 cancer-free subjects 3 were diagnosed with chronic inflammation of the prostate gland. This may diminish the reviewer's concern that an infection is the cause leading to the epigenetic aberration described here. Please see: page 8, first full paragraph; page 16, second paragraph; Figure 6; and corresponding legend on page 30.

Drs. S. Cytron and M. Maschevich conceived, collected the samples and performed the medical and cytogenetic analyses of the above described, new added study. They both were added to the Authors' list of the revised manuscript.

Comment 3: The mechanistic implication of the demethylating drug 5-azacytidine, should be better discussed.

We added few lines in the discussion section to meet this comment and also two new references (please see, revised manuscript, lines 3-11 in the first full paragraph on page 21; and references #37 and #38 on page 28).