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## Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Age-specific incidence rates of patients with chronic lymphocytic leukaemia in the Netherlands according to age, 1989–2016. Incidence rates are presented per 100 000 person-years and shown according to the following sexes: (A) males and females together, (B) males alone, and (C) females alone.

**Figure S2.** Age-specific incidence rates of patients with chronic lymphocytic leukaemia in the Netherlands per quinquennial years of age, 2003–2016. Incidence rates are presented per 100 000 person-years and shown according to sex. The period of 2003–2016 was chosen, as the incidence of CLL in the Netherlands remained comparatively steady as from 2003.

**Table SI.** Patient characteristics.

**Data S1.** Supplemental methods.

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# Response to: "Cytoplasmic dislocation of NPM1 and PU.1 in NPM1-mutated leukemia is obscured by paraformaldehyde fixation"

To the Editor:

Although we recognise that paraformaldehyde may influence the detection of NPM1 and PU.1 in immunofluorescence, the main goal of our study (Pianigiani *et al.*, 2020) was to test whether PU.1 localisation could be used to diagnose *NPM1*-mutated AML through

immunohistochemistry in B5-fixed bone marrow biopsies. B5 fixation is routinely used in our laboratory to detect cytoplasmic localisation of NPM1 in acute myeloid leukaemia, based on Falini *et al.* (2005). It is unlikely that B5 fixation allows for correct detection of NPM1 but not that of PU.1.

The data provided by Gu *et al.* in this Letter and in previous work (2018) support the hypothesis that NPM1 directly binds PU.1, leading to PU.1 relocation from the nucleus to the cytoplasm. Given the heterozygous nature of *NPM1* mutations, a significant proportion of PU.1 would be expected to still be localised in the nuclei of *NPM1*-mutated cells (even accounting for the small amount of wild-type NPM1 dragged to the cytoplasm by the mutant protein). However, no PU.1 is detected in the nuclei of *NPM1*-mutated untreated cells in the vast majority of immunofluorescence and western blot experiments reported by Gu *et al.*, arguing for an overestimation of the actual amount of PU.1 in the cytoplasm. Also, all the experiments by Gu *et al.* have been performed using a polyclonal anti-PU.1 antibody whose production has been discontinued years ago, making it difficult to reproduce the data.

Concerning the nuclear/cytoplasmic fractionation, it is possible that in our experiments a proportion of PU.1 was not separated from the nuclear fraction. Considering the correct localisation of control proteins in our blot, and assuming, as claimed by Gu *et al.* (2018), that almost all PU.1 should be in the cytoplasm of *NPM1*-mutated cells, one would expect to find a visible proportion of PU.1 in the cytoplasm, even after an incomplete separation. However, we did not detect PU.1 at all in any of the cytoplasmic fractions, even after applying longer exposure times.

We would like to emphasize that we do not rule out that a proportion of PU.1 may be found in the cytoplasm of AML cells. However, our data indicate that PU.1 localisation studied by immunohistochemistry should not be used to diagnose *NPM1*-mutated AML. More experiments are necessary to establish the exact proportion of PU.1 localised to the cytoplasm of leukemic cells, and to define the contribution of cytoplasmic PU.1 to the development and maintenance of *NPM1*-mutated AML.

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# Cytoplasmic dislocation of NPM1 and PU.1 in *NPM1*-mutated leukaemia is obscured by paraformaldehyde fixation

To the Editor:

Nucleophosmin (NPM1) is the most recurrently mutated gene in *de novo* acute myeloid leukaemia (AML), producing mutant-NPM1 that aberrantly accumulates in cytoplasm instead of nuclei. Why this should transform myeloid precursors was unknown. We discovered, using unbiased proteomic analyses, that NPM1 is a cofactor for the master transcription factor driver of granulo-monocytic lineage-fates PU.1, and that, crucially, mutant-NPM1 dislocates PU.1 into cytoplasm with it (Gu *et al.*, 2018). We showed that disruption of the granulo-monocytic master transcription factor hub in this way decouples exponential proliferation of myeloid progenitors from forward-differentiation to produce exponential self-replication (a transforming action) (Gu *et al.*, 2018). In a letter to the *British Journal of Haematology*, Pianigiani *et al.* (2020) contradicted our report by describing PU.1 location in nuclei, not cytoplasm, of *NPM1*-mutated AML cells. Here, we show why Pianigiani *et al.* incorrectly found the bulk of NPM1 and PU.1 in nuclei instead of cytoplasm of *NPM1*-mutated AML cells, and confirm again dislocation of both into cytoplasm.

Detection of intra-cellular proteins by immune-histochemistry or immune-fluorescence requires cell fixation/permeabilization. Paraformaldehyde, a commonly used fixative, is known to affect apparent subcellular locations of proteins and