Response to Reviewers

Reviewer #1: This study follows two Italian cohorts to examine the association of mild anemia and all-cause mortality. The topic is important, but here are some aspects to be considered.

We are grateful to the Reviewers for the several challenging issues they raised which gave us the opportunity to improve the quality of our manuscript.

1. “Objective of the present study was to prospectively investigate the long-term effect of mild anemia and mild anemia types on all-cause mortality in the young-old (65-84 years) and old-old (80+ years) from two population-based studies.”
   This is a causal question. The methods in this paper do not allow a clear causal interpretation (and it has little to do with this being a cohort study). I’d suggest that the use of the term “effect” is reconsidered.
   **Response**
   Following the Reviewer’s suggestion we have changed the term “effect” with the more appropriate “association”:
   “Objective of the present study was to prospectively investigate the long-term association of mild anemia and mild anemia types on all-cause mortality in the young-old (65-84 years) and old-old (80+ years) from two population-based studies.”

2. The setting up of the two cohorts is confusing. Seems that the distinguishing features of the final cohorts are cohort entry age. The 65-84 year olds comprise participants from H&A65-84, while 80+ comprise participants from both M80+ and H&A65-84. Some 80+ individuals, thus, are present in the 65-84 year olds cohort, and others in the 80+ cohort. What is the rationale? I feel that this muddles the interpretation of the effect estimates, obscuring the effect of both age and location. Why not, either have H&A and M80+ as the two distinct cohorts, or have 65-80 and 80+ as two distinct cohorts.
   **Response**
   We aimed to study the long-term association between mild anemia and mortality in both the young-old and the old-old. This latter age group is one on which very few data are available or almost absent if mild anemia instead of anemia of any grade is considered. The rationale behind our choice was consequently to investigate this association in two sufficiently large cohorts of young-old and old-old from the general population. The Monzino 80-plus (M80+) is a study specifically designed to study the oldest-old, even though the primary objective was not to explore anemia/mild anemia. The Health and Anemia (H&A) study was performed in two distinct stages well mirroring the above categorization of old age: as reported under “Study settings and participants”, “All registered individuals aged 65 to 84 years in 2003 were eligible (study years: 2003-2018). In 2007 the study was extended to all residents 85 years or older (study years: 2007-2018)”. Moreover, data on the association of mild-anemia with mortality in the young-old cohort (65-84 years) over the first 3.5 years have already been published (reference 16). Therefore, we thought it was sounder not to modify the composition of the original H&A 65-84 cohort by moving its 80-84 age group to the subsequent H&A 85+ cohort.
The old-old are variously defined as persons aged 80 or 85 years and older. In this regard we performed several sensitivity analyses (Supplementary Tables S1, S2, and S3) and all gave comparable results. We have now also analyzed the four cohorts the Reviewer suggested (i.e.: H&A65-79, [H&A80+ and M80+], H&A65+, and M80+) and the results are very similar to those reported in the original Table 2. We have now also reported the results of the suggested cohort analyses in the supplementary material.

Sentence added to sensitivity analyses:
“Results were akin also setting-up different cohorts: H&A65-79 (S4 Table), H&A80+ together with the M80+ (that is all individuals aged 80 years or older; S4 Table), H&A65+ (S5 Table), or M80+ (S5 Table).”

3. I think it might be useful to explicitly define cohort entry. To me it seems that participants entered the cohort upon providing blood samples, in 2003 or 2007 (H&A) or 2002 or 2009-2019 [2010?] (M80+). Is this correct? This of course results in the major problem of prevalent anemia, which is only partially accounted for in the incident anemia secondary analysis. This needs to be discussed as a major limitation.

Response
The Reviewer is correct: participants entered the cohorts analyzed in the present study upon providing blood samples (even though for the Monzino 80–plus study the date of the first main study visit preceded that of the blood draw [please see answer to comment 6]). We have now added your suggestion under the “Study design”:
“All participants entered the cohorts on the date of their blood draw”.

Mild anemia is a condition most elderly persons do not even realize that they have until it is unexpectedly identified in a complete blood count (likely the most common among the routine blood tests) and even in that eventuality it is often disregarded in everyday clinical practice. These prevalent cases are exactly those seen in general practice. Even though in our view prevalent anemia does not represent a major problem, the present study has investigated the association between anemia and mortality also in a prospective cohort of young-old and old-old without anemia. Investigating the association of incident anemia with mortality was not a secondary analysis (“to assess the effect of change in anemia status …”), rather one of the main and novel aims of the present study (all the available literature investigated this association in prevalent cases of anemia and only two among these studies also in incident cases, one in a selected population of young old [29] and one in a cohort of 85-years-olds [11]. Prevalent cases have actually had anemia for a longer period of time than that calculated from blood sampling. However, prevalent cases, being less susceptible to the exposure, represents the selected healthier sub-group surviving to the beginning of the study (selective survival) and the risk associated with mild anemia found in prevalent cases would thereby be underestimated. In accordance with this explanation, mortality rate over the first seven years of follow up was lower among prevalent cases of anemia than among incident cases. A result in agreement with that of the Leiden 85 study (mortality rates not reported): prevalent anemia (follow-up: 5 years): HR: 1.41 (1.13-1.76); incident anemia (follow-up: 7 years [< 5 years]): HR: 2.08 (1.60-2.07) [reference 11]). Consistently, elderly subjects with thalassemia trait have a lifelong coexistence with mild anemia and are not at risk of a shorter survival, thus tempering the difference between mild anemia and non-anemia with respect to mortality. In fact, if the 63...
participants with thalassemia trait were removed from the analysis of the H&A 65-84 cohort (the 7 thalassemic subjects in the H&A 85+ are too few for any further analysis), all HRs over 0-15, 0-7 or 8-15 years of follow-up would consistently increase with respect to those analyzing the complete cohort.

For the problem of incorrect classification or changing anemia status over time when hemoglobin is determined only once as in prevalent cases, please also see the answer to comment 8.

Following the Reviewer’s request we have discussed the point raised under the limitation part of the Discussion:
“Since prevalent cases have had the condition for some time before the study starts, analyzing prevalent cohorts, as almost exclusively done in the available literature, assumes that the individuals are allocated to the anemia group at the time of blood drawing, while only prevalent cases that are alive at that time are actually included in the analyses. These cases, being less susceptible to the exposure, represent the selected healthier sub-group surviving to the beginning of the study (selective survival). Not including the subset of prevalent cases at risk before baseline but that fails to survive until the sampling date leads to a study population biased toward favorable survival and the risk associated with mild anemia found in prevalent cases would thereby be underestimated. In accordance with this consideration, mortality rate over the first seven years of follow up was lower among prevalent cases of anemia than among incident cases. A result also in agreement with that of the Leiden 85 study [11]. Consistently, elderly participants with thalassemia trait have a lifelong coexistence with mild anemia and are not at risk of a shorter survival, thus tempering the difference between mild anemia and non-anemia with respect to mortality. Furthermore, the present together with two others [11,29] are the only studies that investigated the association between anemia and change in hemoglobin concentration with mortality also in non-anemic cohorts”.

Under sensitivity analysis:
“If the 63 participants with thalassemia trait (62 with mild and 1 with moderate anemia) were removed from the analysis of the H&A 65-84 cohort, hazards of death from mild anemia would consistently increase with respect to those analyzing the complete cohort: fully-adjusted HRs (95% CIs): 1.45 (1.22-1.72) over 0-15 years, 1.78 (1.41-2.25) over 0-7 years, and 1.21 (0.94-1.57) over 8-15 years”.

4. How hemoglobin was measured should be mentioned in methods.
Response
We have now added the methods used to measure hemoglobin at the beginning of the “Definitions of anemia and mild anemia” section:
“Hemoglobin concentration together with the other tests included in the complete blood count were determined on automated hematology analyzer instruments at the laboratory of Biella Hospital (H&A) and Laboratorio Milano, Milano (M80+)”.

5. How were the various types of anemia classified?
Response
As indicated at the end of the “Definitions of anemia and mild anemia” section, definitions used to classify the anemia types were reported under the Supplementary methods of the Supporting Information.

6. When exactly were the questionnaires administered and informed consent obtained: at cohort entry, before, after? I’d have thought that the history of hematological disorders is a critical covariate in this analysis, which is missing

Response
Informed consents were signed just before blood sampling in both studies. Questionnaires were administered after blood sampling in the H&A study. In the M80+ study, blood sampling was carried out in an ad hoc additional visit at the place of residence on average within 1.9 months of the scheduled study visit during which, after the administration of the questionnaire and tests, the subject's willingness to participate in the blood sub-study (which was not the primary objective of the study for which another specific informed consent was previously signed) was investigated.

Changes made to the original manuscript:
At the end of “Study setting and participants”: “Written informed consent was obtained from participants before blood sampling. Participants in the M80+ study had also previously signed the informed consent to participate in the main study. Written informed consent was also obtained from informants in both studies”.

Under “Study design”: “Questionnaires were administered after blood sampling in the H&A study. In the M80+ study, blood sampling was carried out in an ad hoc additional visit at the place of residence on average within 1.9 months of the scheduled study visit during which, after the administration of the questionnaire and tests, the subject's willingness to participate in the blood sub-study was investigated. The questionnaire was administered by …”

Prevalence of hematological diseases other than anemia is low (about 2% in the present study) and not necessarily associated with mortality. Moreover, in the present study, about 65% of the hematological disorders other than anemia are represented by malignant neoplasms of the lymphoid and hematopoietic tissues which were already classified under “history of cancer”.

7. It is important to get a sense of the person time followed up, and the incidence rates of the events of interest, which have not been mentioned. What was the median follow-up? Were individuals who were not at risk at 8-end of follow-up excluded from the survival analysis from 8 to 15/11 years?

Response
Yes, individuals who were not at risk (i.e. those who died) by the end of the first period considered (0-7 years) were excluded from the analysis of the second period (8-15 or 8-11 years).

We have now specified under “Statistical analysis” that “To examine whether the effect of mild anemia was similar over time, two survival analyses were set up: from 0 to 7 years, and, in participants who survived the first seven years of follow up, from 8 to 15/11 years”.
We have also added the requested information on “person time followed up, and the incidence rates of the events of interest”. Please note that, in order to make the inserted parts intelligible, we report the whole beginning of the edited part:

“H&A65-84 cohort

During 15 years of follow-up after blood sampling, 230 anemic (66.9%; 205 mild anemic) and 1928 non-anemic (46.5%) individuals died (Fig 1). The median follow-up period was 14.0 years with 50,522 person-years of observation and a mortality rate of 4.3 per 100 person-years (4.1 in non-anemic and 7.1 in mild anemic participants). Over the 15-year follow-up, mortality risk was significantly increased in participants with anemia (fully-adjusted HR: 1.42; 95% CI, 1.22-1.65) and mild anemia (fully-adjusted HR: 1.35; 95% CI, 1.15-1.58). In the first seven years after blood collection (median follow-up: 7.0 years; person-years of observation: 28,607 years; incidence mortality rate: 2.9 per 100 person-years), compared with non-anemic … . From 8 to 15 years (3,574 participants; median follow-up: 7.4 years; person-years of observation: 21,915; incidence mortality rate: 6.1 per 100 person-years) the risk was still … .

H&A85+ and M80+ cohorts

During 11 years of follow-up, 526 anemic (94.1%; 449 mild anemic) and 1,137 non-anemic individuals (88.6%) died in the pooled 80+ cohort (Fig 1). The median follow-up period was 3.5 years with 7,871 person-years of observation and a mortality rate of 21.1 per 100 person-years (18.7 in non-anemic and 28.6 in mild anemic participants). Over the 11-year follow-up, mortality risk was significantly higher in participants with anemia (fully-adjusted HR: 1.32; 95% CI, 1.18-1.48) and mild anemia (fully-adjusted HR: 1.28; 95% CI, 1.14-1.44). In the first seven years after blood collection (median follow-up: 3.5 years; person-years of observation: 7,235 years; incidence mortality rate: 19.7 per 100 person-years), compared with non-anemic, … . From 8 to 15 years (287 participants; median follow-up: 2.3 years; person-years of observation: 637; mortality rate: 23.9 per 100 person-years) no significant difference … .

Risk of mortality associated with mild anemia in “healthy” elderly subjects

At baseline 1093 elderly persons (mean age 74.6, 64% women) from both H&A65+ and M80+ had no history of any of the diseases entered as confounders in multivariable analyses. The median follow-up period was 7 years with 7,043 person-years of observation and a mortality rate of 2.5 per 100 person-years (2.2 in non-anemic and 7.6 in mild anemic participants). Individuals with mild anemia (N = 74) showed an increased risk of mortality … .

Risk of mortality associated with anemia status assessed over time (under “Study design” or “Results”):

In H&A65-84+, a second blood sample was available for 692 subjects … . The mean (SD) time between samplings was 2.2 (0.1) years (median follow-up: 7.0 years; person-years of observation: 4,265; mortality rate: 3.8 per 100 person-years). …

Considering the 523 subjects without anemia at first sampling in H&A65-84+ (median follow-up: 7.0 years; person-years of observation: 3,286; incidence mortality rate: 3.3 per 100 person-years), the 27 incident cases (5.2%; mortality rate: 11.4 per 100 person-years) … . . . .
In M80+, a second blood sample was available for 366 subjects … . The mean (SD) time between samplings was 1.7 (1.0) years (median follow-up: 3.1 years; person-years of observation: 1,298; incidence mortality rate: 22.4 per 100 person-years). …

Considering the 279 subjects without anemia at first sampling in M80+ (median follow-up: 3.4 years; person-years of observation: 1,048; mortality rate: 20.5 per 100 person-years), the 65 incident cases at second sampling (23.3%; mortality rate: 31.8 per 100 person-years) showed an increased risk of mortality during the following 7 years compared to those who did not develop anemia (fully-adjusted HR: 1.47; 95% CI, 1.04-2.07). …

8. The assumption in the main analysis, where there is no censoring except for at event/administratively, is that once anemia is detected, it irreversibly affects the risk of death. How justified is this (particularly given that the authors conduct an analysis where they examined Anemia→No Anemia patients)? If it is not entirely justified, then this should be mentioned as a limitation.

Response

“In detecting and evaluating an anemia problem in a community, reference standards are necessary, even though they may be somewhat arbitrary” (WHO 1968). Several factors can influence the level of hemoglobin measured (for example, physiological fluctuations of plasma volume), especially in population-based studies. Since the diagnosis of anemia is defined as a concentration of hemoglobin of less than a conventional lower limit of normal, physiological fluctuations of hemoglobin concentrations can be incorrectly classified as either “anemia” (“false positive”) or “non-anemia” (“false negative”) when hemoglobin is determined only once. Moreover, among those diagnosed with an anemia type susceptible to treatment, a fraction can return over time to having a normal concentration of hemoglobin when successfully treated or the underlying causes have been eliminated. Whatever the assumption, considering how anemia is defined and the existence of anemia types susceptible of treatment, imply that anemia is not necessarily a chronic, everlasting condition. Moreover, please note that the present study has investigated the association between anemia and mortality also in prospective cohorts of young-old and old-old with two samplings and reported the risk associated precisely with change in anemia status (Table 4).

Please, see also the answer to comment 3.

Following the Reviewer’s request we have now added this point to the study limitations:

“A further potential limitation of studies assessing hemoglobin concentration on a single occasion, is that they cannot address within-individual changes in hemoglobin concentration over time that may potentially affect the results. However, the present study is one of only two attempts [11] to investigate also whether a change in anemia/non-anemia status would affect the risk of death. Generally, being anemic or non-anemic is a rather consistent status over time at old age in the general population: in the present study 84% of the study sample with two blood draws continued to be anemic or non-anemic on average over two years. However, since “false positives” (due to physiological fluctuations) and those successfully treated would decrease the mortality rate of the anemic group in which they were initially classified, whereas the
“false negatives” (due to physiological fluctuations) and incident cases would increase the mortality rate of the non-anemic group in which they were initially classified, the actual risk associated with the anemia status would tend to be underestimated in subjects with a single blood sample. Consistently, in both young-old and old-old cohorts, subjects with two hemoglobin determinations whose anemia “resolved” (whether they had been successfully treated after or “false positive” cases at first sampling) did not show a significantly increased risk of mortality compared to those who were consistently non-anemic, a result almost identical to that of the Leiden 85 study [11] and also of a retrospective study in a selected sample of patients with chronic heart failure (risk ratio 0.98 [0.73-1.36] [Tang et al. *J Am Coll Cardiol* 2008;51:569-576][41]. Whereas in those who became anemic at second sampling (whether they had been incident cases after or “false negatives” at first sampling) the risk was increased with respect to prevalent cases, again in agreement with the Leiden 85 study [11].

Considering fluctuations in concentrations over time, it should also be noted that when the upper limit of hemoglobin concentration for anemia and/or the lower limit for mild anemia adopted were higher (Table 2), the estimated risks for the association between anemia/mild anemia and mortality were very similar to those of the main analysis”.

Under the Results and in Table 4 we have now added also mortality risks associated with hemoglobin decline (per 1 g/dL decline) in the young-old and old-old with no anemia at baseline:

“In this cohort of young-old without anemia, also hemoglobin decline over the same period after the second sampling was associated with an increased risk of mortality: fully-adjusted (also for hemoglobin at first sampling) HR: 1.39 (95%CI, 1.10-1.76) per 1 g/dL decrease of hemoglobin concentration (Table 4). … In this cohort of old-old without anemia, also hemoglobin decline over the same period after the second sampling was associated with an increased risk of mortality (Table 4)”.

Under “Discussion”:

“…incident anemia and decline in hemoglobin concentration were significantly associated with ….”

Under “Abstract-Results”:

“In participants without anemia at baseline hemoglobin decline was also significantly associated with an increased mortality risk over seven years in both young-old and old-old”.

9. Another major assumption is that the covariates that predict death do not change between cohort entry and end of follow-up. This of course is not justified, and should be added to the limitations. On the issue of adjustment, the assertion that “Extensive adjustment may have led to underestimation of the association strength, since mild anemia could also be an effect of underlying pathological conditions,” is problematic given that the authors are interested in causal estimates. If after adjusting the estimates move towards the null, then that is the real effect. Had they been interested in mortality burden in a non-etiological sense, crude rates would have been important.

**Response**

Since the comorbidities considered as covariates are all chronic diseases or have chronic complications (e.g. stroke and myocardial infarction), the assumption is limited to the new occurrence (incidence) of these diseases after baseline. Please also note that this assumption as well is common to all the literature on the subject (and, incidentally, none
of the published studies has mentioned it among the limitations). However, we agree with the Reviewer.

In the Monzino 80-plus study, beyond baseline, another 8 follow-up assessments were available. Thus, limited to this study, it has been possible to further investigate the influence of time-varying covariates on the association between anemia and mortality in subjects with one (prevalent cases) as well as in subjects with two hemoglobin determinations (incident cases and hemoglobin decline). Moreover, in subjects with two hemoglobin determinations it was also possible to investigate how the association between baseline anemia and mortality was affected by change in anemia status (at second sampling) together with prevalent plus incident covariates over the following seven years of follow-up. We have now reported the results of these analyses in a new Table 5 (please, see the revised manuscript): all results confirmed those adjusted only for baseline covariates previously set out in Tables 2 and 4 and in the new Supplementary Table reporting only the results of the Monzino 80-plus study. Though slightly, all hazard ratios are consistently higher than those reported in the above mentioned Tables and Supplementary Table. Moreover, in the analysis where it has been possible to account for change in both anemia status and covariates, the risk associated with baseline anemia resulted moderately increased also with respect to the model further adjusted for change in covariates. This finding is in agreement with the observation that changes in anemia/non-anemia status over time would tend to underestimate the risk of mortality associated with anemia in subjects with a single blood sample (please see the answer to the previous comment).

Following the Reviewer’s request we have now added this point to the study limitations:

“Another possible limitation, common to all the literature on the subject, is that covariates potentially associated with death were assessed only at cohort entry, thus failing to account for the subsequent change over time of the covariates. However, restricted to the participants in the Monzino 80-plus study, the association between anemia and mortality could be further adjusted also for time-varying covariates and change in anemia status over time and the results confirmed those adjusted only for baseline covariates showing a slightly to moderately increased risk with respect to elderly subjects with a single blood sample and covariate assessment at entry, in agreement with the observation that changes in anemia/non-anemia status over time would tend to underestimate the risk assessed at baseline.”

Under “Statistical analysis” we have added:

“All covariates were assessed at baseline. Limited to the Monzino 80-plus study, beyond baseline, another 8 follow-up assessments were available. It was thus possible to further adjust the multivariable model also for the influence of time-varying covariates (age, habits, and intervening comorbidities) on the association between anemia and mortality in subjects with one (prevalent cases) as well as two hemoglobin determinations (incident cases and hemoglobin decline). Moreover, in subjects with two hemoglobin determinations it was also possible to investigate how the association between baseline anemia and mortality was affected by time-varying covariates together with anemia/non-anemia status at second sampling”.

Under “Results”, “H&A85+ and M80+ cohorts”:

“… was found (Table 2). Limited to the M80+ cohort, mortality risk associated with prevalent anemia and mild anemia was slightly increased when the model was further adjusted for time-varying covariates (anemia: HR 1.59 [95% CI: 1.39-1.82] over 11
years and HR 1.64 [95% CI: 1.42-1.89] over seven years; mild anemia: HR 1.57 [95% CI: 1.36-1.80] over 11 years and HR 1.60 [95% CI: 1.38-1.85] over seven years).

Under “Results”, “Risk of mortality associated with anemia status assessed over time”, at the end of the last paragraph:

“In all subjects with two hemoglobin determinations it was possible to control for both change in the anemia/non-anemia status together with time-varying covariates: the risk associated with baseline anemia resulted moderately increased also with respect to the model further adjusted only for time-varying covariates (Table 5). In non-anemic subjects with two hemoglobin determinations, the risk of mortality associated with incident anemia or change in hemoglobin concentration over the seven years following the second sampling was increased when also time-varying covariates were added to the “fully”-adjusted model (Table 5).”

Under “Discussion”:

“In the Monzino 80-plus study the risk associated with prevalent and incident anemia was even higher when the multivariable model could be further adjusted also for time-varying covariates and, limited to all old-old with two blood samplings, for change in anemia status at second sampling.”

Under “Discussion”, strength:

“No previous study has attempted to adjust also for the influence of change in anemia/non-anemia status and time-varying covariates”.

With regard to the discussion on the issue of the “extensive adjustment”, following the Reviewer’s observation we have removed the statement in the revised manuscript.

10. About 50% participated in each cohort. The authors report data on similarity in variable distribution between those who did and did not participate. However, to infer whether there was selection bias, one needs to know whether there were differences in variables that affect both anemia and mortality risk. As such, only differences in age, sex, and prevalence in dementia. But surely other variables may be associated with anemia and mortality risk that may be differentially distributed between participants and non-participants. For example, what about diabetes, htn, cancer, etc.

Response

Probably there must be a misunderstanding. We compared participants and non-participants only for age and sex simply because age and sex were/are the only two variables available in the municipality registry offices also for non-participants (i.e., eligible residents who refused or were untraceable). At any rate, age and sex are two of the main determinants of anemia and mortality and, at least for age, also of most chronic diseases.

Having a different study aim, the eligible for the blood sub-study of the M80+ were not all the eligible residents, rather the sub-population of those among all residents who accepted to participate in the main study (some 90% of all the eligible residents in any case). We also explored the distribution of dementia and cognitive function in participants and non-participants in the blood sub-study not because these variables are associated with mortality (a matter of fact) or anemia (under scrutiny; in the Monzino study for example anemia was not significantly associated with an increased risk of developing dementia [Lucca et al. Alzheimers Dement 2020; 16 (S10): abstract]), but, as reported in the manuscript, because these were the primary research targets (in other words, the traits under investigation), that is, those characteristics that could have
influenced the elderly subjects in the decision to take part or not in the study/sub-study and thus possibly determine a self-selection bias. Since the entire cohort of old-old was the result of the pooling of two cohorts (H&A85+ and M80+), the only available information for the H&A85+ cohort and consequently for the entire 80+ cohort was that regarding age and sex.

By the way, at initial visit in the M80+ study diabetes, hypertension, and cancer were more prevalent among participants with blood sampling than among those without, even though the difference was not statistically significant for diabetes (p = 0.203) and cancer (p = 0.239).

10[bis]. How was the stratified randomization for the second venous sampling conducted, on survivors or on all initial participants. How many of the initial participants died during this time?

Response

Under “Study settings and participants” section, we have now specified the process of stratified randomization:

“… recontacted during 2005-2006: initial participants were stratified at baseline in anemic and non-anemic strata, all eligible consenting anemic and a random sample of eligible consenting non-anemic participants were included in the study on the association of mild anemia with cognitive, functional, mood, and QoL outcomes [21].

“How many of the initial participants died during this time?”

We have now reported under the “Study settings and participants” section (“Risk of mortality associated with anemia status assessed over time”):

“In H&A65-84, a second blood sample …: … among the 344 consenting participants with baseline anemia, 29 died and 146 were not found or withdrew their consent to participate at the time of the second blood draw; among the 655 consenting participants without anemia at baseline, 20 died and 112 were non traceable or withdrew their consent to participate at the time of the second blood draw”.

For the M80+:

“Of the initial 1,115 participants, 366 accepted to donate a second blood sample (mean age: 89.7; men: 23.0%; with anemia: 23.8%; mean Hb: 13.1 g/dL); 667 did not or could not donate a second blood sample (mean age: 90.4; men: 26.5%; with anemia: 36.0%; mean Hb: 12.7 g/dL) and 82 died before the next visit (mean age: 92; men: 28.1%; with anemia: 57.3%; mean Hb: 11.8 g/dL). Of the 667 oldest-old with only one blood sample, 620 continued to participate in the Monzino study but refused a second blood sample; 30 could not be traced and 17 refused to continue to participate in the Monzino study)”.

11. The incidence rates and person time follow up should be mentioned for all analyses.

Response

Please, see answers to comment 7 and related changes.

12. What are the reference group in the analyses reported in table 4?

Response

We have now slightly changed footnote “c” of Table 4:

“Reference group: subjects consistently non-anemic at both samplings” (instead of: “with respect to subjects consistently non-anemic at both samplings”).
In the text it was already reported that “Considering the 523 subjects without anemia at first sampling, the 27 incident cases … compared to those who did not develop anemia … . When subjects were categorized according to anemia status at both samplings (Table 4), compared to subjects constantly non-anemic (n = 496), …” (under “Results”).

13. It was surprising to see such a strong HR among No anemia→Anemia. Again rates will help clarify this.

Response
We are not sure we have understood why “it was surprising to see such a strong [?] HR among “No anemia→Anemia”. If the surprise refers to the evidence of a stronger association in incident cases than in prevalent ones, please see answers to comments 3 and 8. As noted above, our results are in agreement with those of the Leiden 85 study: “We found that incident anemia in participants beyond the age of 85 years had an even stronger impact on mortality than prevalent anemia at age 85.” (reference 11, p. 156). Also Ishani et al. analyzing a selected sample of patients with heart failure participating in the SOLVD trial found that prevalent anemia was associated with a 44% increase in the hazard of all-cause mortality, whereas incident anemia with a 108% increase (J Am Coll Cardiol 2005;45:391-399).

14. “In these two large prospective population-based studies, elderly persons with mild anemia had an overall 40% increased risk of dying”: mentioning a single estimate without meta-analyzing is confusing.

Response
The results of the pooled analyses of the H&A and Monzino studies were reported in supplementary S2 Table (now S1), as pointed out in the “Results” section (line 257 of the original manuscript): “S2 Table summarizes the results in the pooled young-old and old-old cohorts”. We have now reported the main results of this pooled analysis also in the manuscript:

“S2 Table summarizes the results in the pooled young-old and old-old cohorts. Compared with non-anemic, mortality risk was significantly higher in elderly individuals with mild anemia in the first seven years after blood collection (fully-adjusted HR: 1.40; 95% CI, 1.26-1.57) as well as over the entire 11-year follow-up period (fully-adjusted HR: 1.29; 95% CI, 1.17-1.43). From 8 to 11 years no significant difference in mortality risk between anemic and non-anemic elderly persons was found”.

15. “The risk was somewhat higher in young-old (63%) than in old-old (38%), clearly due to a life expectancy twice as long at age 65 (18.5 years) than at age 80 years (8.5 years) (2003-5)”. Not very clear to me how this follows the life expectancy estimate. Do the authors mean that at an younger age there are fewer causes of death and that anemia is a greater contribution to mortality risk and so the risk is higher? If so, this is a good argument, but needs elaboration. Otherwise, please clarify.

Response
We agree with the Reviewer’s criticism of our interpretation and have decided to remove this sentence from the revised manuscript. Changes in the first sentence of the “Discussion” section: “… an overall 40% increased risk of dying, 60% in young-old and 38% in old-old”.
16. KM in 80+ separates immediately after cohort entry, indicating confounding/prevalent exposure related bias. Otherwise kindly justify mild anemia may lead to death within 2 months of follow-up

Response
Since not only mild anemia but multiple factors influence survival, by visualizing a Kaplan Meir curve it is not possible to estimate the effect of a factor. Being a cohort of very old, of course they start to die from the very beginning of the observed period, be they subjects with (mean age 91.2 years) or without (mean age 89.4 years) anemia: after one month 12 mild anemic and 14 non-anemic died, 21 and 22 respectively after two months, 30 and 32 after three months, and 46 and 44 after fourth months, when the difference between groups reached statistical significance at multivariable analysis. Therefore, our interpretation is that the rapid separation is mainly due to the strong background mortality, condition in which a hazard ratio can have a visible effect. With regard to “confounding” bias, it should also be noted that Cox proportional hazard models were adjusted for age, sex, education, smoking status, alcohol consumption, hypertension, diabetes, heart failure, myocardial infarction, chronic respiratory failure, chronic renal insufficiency, cancer, transient ischemic attack, stroke, parkinsonism, dementia, hospitalization during the previous year, and study. And with regard to “prevalent exposure related bias”, “KM in 80+ separate immediately after cohort entry” also when incident cases versus consistently non-anemic cases were analyzed (see Figure below). With regard to “confounding/prevalent exposure related bias”, please see also the answers to comments 3, 8, and 9, and Table 5.
17. “Cohort studies cannot determine causality”, “Only randomized controlled trials could finally establish whether restoring normal hemoglobin concentrations by treating the specific causes of mild anemia at older ages could safely revert or reduce the observed risks”: neither statement is true, consider changing.

Response
In compliance with the Reviewer’s observation, we have changed the two statements:
Under limitations:
“Although prospective cohort studies can help to assess a causal association, further experimental trials would contribute to establish true causality.”
Last sentence of the conclusions:
“Randomized controlled trials could aid in establishing whether restoring normal hemoglobin concentrations by treating the specific causes of mild anemia at older ages could safely revert or reduce the observed risks”.
Reviewer #2: Summary
This study examined whether mild anemia is associated with increased all-cause mortality in individuals aged 65-84 years and 80+ between 2002/2003 and 2017/2018. Two cohorts were constructed (H&A 65-84 and H&A 85+, M80+), with follow-up of 15, 11, and 15 years, respectively. During the study period, mild anemia was associated with a higher hazard of all-cause mortality, compared with those without anemia (HR: 1.42, 95% CI 1.22-1.65 in H&A 65-84 and HR: 1.32, 95% CI: 1.18-1.48 in H&A 85+ and M80+).

We are grateful to the Reviewers for the several challenging issues they raised which gave us the opportunity to improve the quality of our manuscript.

Abstract
- Please clarify the results section of the abstract as it provides results of the 0-7 years analysis although that analysis is not mentioned in the methods section of the abstract. Additionally, only the 0-7 years results are presented rather than the full results. Further, the title mentioning the 15-year mortality should be modified considering that one of the cohorts has a maximum follow-up of 11 years.

Response
According to the guidelines the abstract should not exceed 300 words. Since mild anemia, in both the young-old and the old-old, was not significantly associated with mortality over the second part of the time period investigated (8-15 and 8-11 years), we decided to report more conservatively the results of the first (0-7 years) instead of the entire time period considered.
Following the Reviewer’s remarks, we have now changed the abstract results: “…. mortality risk over 15/11 years was significantly higher in individuals with mild anemia compared with those without (young-old: fully-adjusted HR: 1.35, 95%CI, 1.15-1.58; old-old: fully-adjusted HR: 1.28, 95% CI, 1.14-1.44)”.

With regard to the title, we reported for conciseness only the 15-year mortality because for the old-old there was also available a (comparable) finding over a 15-year time period for the 895 participants aged 85+ years in the Monzino 80-plus study (see S1 Table: fully-adjusted HR: 1.38, 95% CI, 1.18-1.63) and now also for the entire Monzino cohort of 1.115 subjects aged 80+ years (fully-adjusted HR: 1.36, 95% CI, 1.18-1.57). In any case, in the Abstract it was already specified that “Objective of the study was to investigate the association of mild anemia (…) with all-cause mortality over 11-15 years”.
Following the Reviewer’s remarks, we have also changed the title: “Mild anemia and 11- to 15-year mortality in old-old and young-old: Results from two population-based cohort studies”.

Introduction
- Please add more information on anemia i.e., is this a transient vs long-lasting “exposure”? If transient, is anemia thought to have lasting effects?

Response
Is this a transient vs long-lasting “exposure”? Naturally anemia can be either a temporary (for example, due to a bleeding, peri-operative blood loss, chemotherapy, in general, any anemia that can be successfully treated or the underlying cause of which
can be eliminated) or a chronic disorder. The chronic is the most common condition among the elderly population. In fact, “the management of anemias in older individuals is a clinical challenge, especially when the etiology remains uncertain [one fourth to one third of anemias in the elderly remains unexplained] and/or (multiple) comorbidities are present” (Stauder et al. 2018). Besides, “in detecting and evaluating anemia problem in a community, reference standards are necessary, even though they may be somewhat arbitrary” (WHO 1968) and several factors can influence the level of hemoglobin measured (for example, physiologic fluctuations of plasma volume). Just because anemia can be a temporary condition, the present study has investigated the association between anemia and mortality also in a prospective cohort of young-old and old-old with two samplings.

“If transient, is anemia thought to have lasting effects?” As far as we know, the present study is the only one available investigating the association of mortality with change in anemia status in the general population of both young-old and old-old. Except for a study in a cohort of 85-year-olds (the Leiden 85 study, reference 11), we do not know of any other population-based study investigating whether a temporary (mild) anemia might have a lasting detrimental effect on health: the answer probably depends on how long that temporary lasts and on the cause underlying the mild anemia (for example, a congenital condition such as thalassemia trait would not seem to affect survival at all).

For the unexpressed implication of the question, please see the answer to the first comment under “Study design”…

Under introduction we have now added:

“Almost all studies assessed the relationship between anemia and mortality exclusively in prevalent cases, who, however, were already exposed to the condition at the time of blood sampling. Moreover, a single measurement of hemoglobin concentration cannot investigate the association of change in anemia/non-anemia status to subsequent mortality”.

Study settings and participants
-Although the authors provide references to previous publications for details of study population and design, this manuscript should include relevant details. For example, what are the data collection time points to assess changes in anemia status? In the study design section, the manuscript only specifies that “venous blood samples were collected at the place of residence” and that a random sample from the H&A 65-84 study were contacted in 2005/2006 to collect blood samples and consenting individuals in the M80+ cohort were asked “at following visits” to collect blood samples. This section should clearly specify the number of collections that occurred for the cohorts as well as the time frame, rather than only presenting the information in the Results section.

Response
Following the Reviewer’s requests we have specified the number of collections that occurred for the cohorts as well as the time frame by moving the information from the Result section to the “Study setting and participants” section:

“To assess change in anemia status, a stratified random sample of individuals from H&A65-84 was recontacted during 2005-2006 [1]. In H&A65-84, a second blood sample was available for 692 subjects (baseline: mean age 73.2 years, 55.4% women, mean [SD] hemoglobin concentration 13.6 [1.7] g/dL, 24.4% anemic). The mean (SD) time between samplings was 2.2 (0.1) years. … In M80+, participants who had
consented to donate a blood sample were asked at following visits whether they would agree to a further blood sampling [19]. In M80+, a second blood sample was available for 366 subjects (baseline: mean age 89.7 years, 77.1% women, mean [SD] hemoglobin concentration 13.1 [1.5] g/dL, 23.8% anemic). The mean (SD) time between samplings was 1.7 (1.0) years."

**Study design**
- The section mentions that venous blood samples were collected at the place of residence. Please clarify that this exposure was collected at the beginning of the study and thus marks the start of follow-up. Importantly, the limitation section should discuss how capturing prevalent cases of mild anemia (vs. incident) might impact the results.

**Response**
We have now added your suggestion under the “Study design” (second line):
“All participants entered the cohorts on the date of their blood draw”.

Mild anemia is a condition most elderly persons do not even realize that they have until it is unexpectedly identified in a complete blood count (likely the most common among the routine blood tests) and even in that eventuality it is often disregarded in everyday clinical practice. These prevalent cases are exactly those seen in general practice. Even though in our view prevalent anemia does not represent a major problem, the present study has investigated the association between anemia and mortality also in a prospective cohort of young-old and old-old without anemia. Investigating the association of incident anemia with mortality was not a secondary analysis (“to assess the effect of change in anemia status …”), rather one of the main and novel aims of the present study (all the available literature investigated this association in prevalent cases of anemia and only two among these studies also in incident cases, one in a selected population of young old [29] and one in a cohort of 85-years-olds [11].

Prevalent cases have actually had anemia for a longer period of time than that calculated from blood sampling. However, prevalent cases, being less susceptible to the exposure, represents the selected healthier sub-group surviving to the beginning of the study (selective survival) and the risk associated with mild anemia found in prevalent cases would thereby be underestimated. In accordance with this explanation, mortality rate over the first seven years of follow up was lower among prevalent cases of anemia than among incident cases. A result in agreement with that of the Leiden 85 study (mortality rates not reported): prevalent anemia (follow-up: 5 years): HR: 1.41 (1.13-1.76); incident anemia (follow-up: ? years [< 5 years]): HR: 2.08 (1.60-2.07) [reference 11]). Consistently, elderly subjects with thalassemia trait have a lifelong coexistence with mild anemia and are not at risk of a shorter survival, thus tempering the difference between mild anemia and non-anemia with respect to mortality. In fact, if the 63 participants with thalassemia trait were removed from the analysis of the H&A65-84 cohort (the 7 thalassemic subjects in the H&A 85+ are too few for any further analysis),
all HRs over 0-15, 0-7 or 8-15 years of follow-up would consistently increase with respect to those analyzing the complete cohort.

Following the Reviewer’s request we have discussed the point raised under the limitation part of the Discussion:
“Since prevalent cases have had the condition for some time before the study starts, analyzing prevalent cohorts, as almost exclusively done in the available literature, assumes that the individuals are allocated to the anemia group at the time of blood drawing, while only prevalent cases that are alive at that time are actually included in the analyses. These cases, being less susceptible to the exposure, represent the selected healthier sub-group surviving to the beginning of the study (selective survival). Not including the subset of prevalent cases at risk before baseline but that fails to survive until the sampling date leads to a study population biased toward favorable survival and the risk associated with mild anemia found in prevalent cases would thereby be underestimated. In accordance with this consideration, mortality rate over the first seven years of follow up was lower among prevalent cases of anemia than among incident cases. A result also in agreement with that of the Leiden 85 study [11]. Consistently, elderly participants with thalassemia trait have a lifelong coexistence with mild anemia and are not at risk of a shorter survival, thus tempering the difference between mild anemia and non-anemia with respect to mortality. Furthermore, the present together with two others [11,29] are the only studies that investigated the association between anemia and change in hemoglobin concentration with mortality also in non-anemic cohorts”.

Under sensitivity analysis:
“If the 63 participants with thalassemia trait (62 with mild and 1 with moderate anemia) were removed from the analysis of the H&A65-84 cohort, hazards of death from mild anemia would consistently increase with respect to those analyzing the complete cohort: fully-adjusted HRs (95% CIs): 1.45 (1.22-1.72) over 0-15 years, 1.78 (1.41-2.25) over 0-7 years, and 1.21 (0.94-1.57) over 8-15 years”.

- The questionnaires were administered by trained registered nurses in the H&A cohort and by psychologists in the M80+ cohort. Would the questionnaire responses expected to be different in the two cohorts based on the different assessors? Please also clarify the sentence on the agreement between interviewers on medical history queries being very high – were there more than one interviewer per individual?

Response
As extensively explained in the quoted reference 21, in the H&A65-84 study “on average, 46 days after the blood sample collection by the nurses, a thorough home interview was conducted by trained psychologists … . The information collected by the psychologists was blinded to that previously gathered by the nurses and the two interviews were used to control for the consistency of the medical histories reported by the participants. … Agreement between comparable items of the medical histories taken by the nurses and by the psychologists was very high (Cohen’s k between 0.84 and 0.93).” In the attempt to be concise our explanation was not clear. “Were there more than one interviewer per individual?” Only in the H&A65-84 study: a sample (more than seven hundred) of the initial participants in the H&A65-84 were included in the study on the association of mild anemia with cognitive, functional, mood and QoL
outcomes (reference 21) and, after the initial interview by the nurses, they were also interviewed by the psychologists. “Would the questionnaire responses expected to be different in the two cohorts based on the different assessors?” No, because the agreement between nurses and psychologists in the H&A was very high, and the psychologists employed the same questionnaire used in the M80+ study to collect the medical history in the H&A.

To be clearer, we have now added some of the key missing information:

“A questionnaire was administered by specifically trained registered nurses (in the H&A) and psychologists (in H&A85+, M80+, and the H&A65-84 sample entered in the incident study) to ascertain habits, present and past diseases, and hospital admissions. Agreement between nurses and psychologists on medical history queries on a large sample of participants in the H&A study was very high (Cohen’s k between 0.84 and 0.93) [21] and psychologists in the H&A employed the same questionnaire to collect the medical history used by the psychologists in the M80+ study”.

Statistical analysis
- It is unclear whether data from the H&A 85+ cohort is analyzed with the M80+. The previous sections do not mention the pooling of the older cohorts. It is only first mentioned in this section that the data for the H&A 85+ cohort and the M80+ cohort had been pooled. Please clarify specifically and earlier that pooling occurred and how, and discuss potential impacts of pooling these two cohorts given different data collection processes, different follow-up times etc.

Response
Under the objectives of the present study at the end of the Introduction, it is now specified that:

“Objective of the present study … . To examine the association of mild anemia with mortality in the old-old, the older cohorts from the H&A study (H&A85+) and from the Monzino 80-plus study (M80+), were pooled and followed-up over a period of 11 years (for the M80+, mortality data were available for a 15 year period)”.

At the beginning of the “Statistical analysis” we also added:

“To investigate the association of anemia/mild anemia with mortality over 11 years in the old-old, individual participant data from the H&A85+ and M80+ cohorts were pooled. The rationale behind this pooling was that both studies were prospective door-to-door population-based studies in the old-old with no exclusion criteria other than age; both had a long lasting follow-up and were conducted during more or less matching calendar years; life expectancy was very similar; mortality data on an ongoing basis from the Municipal Registry Offices was available for both cohorts; the modalities to collect health-related information were very similar or identical in both”.

“discuss potential impacts of pooling these two cohorts given different data collection processes” : actually data collection was identical in the two cohorts.

“discuss potential impacts of pooling these two cohorts given … different follow-up times”. For both studies dates of death during the first 11 years after blood sampling were obtained on an ongoing basis from the Municipal Registry Offices. Since for the M80+ mortality data were available also for a further four years, we decided to truncate the follow-up time to the shortest period (thus obtaining the same duration in both studies) for the main analyses. We clarified this in the Methods: “To investigate the
association of anemia/mild anemia with mortality over 11 years in the old-old, individual participant data from the H&A85+ and M80+ cohorts were pooled”. With regard to the potential impact of pooling these two cohorts, the inspection of results showed in supplementary S2 Table (previous S1) leaves little doubt. For the M80+ study, we have also added a new supplementary S6 Table where the results of the 0-11 and 0-15 periods are placed side by side for inspection.

- For the analysis of the effect of mild anemia over time, how was person-time handled in the analysis? Were individuals contributing to the 0-7 years group up until they reached past 7 years of follow-up?

Response
Individuals who were not at risk (i.e. those who died) by the end of the first period considered (0-7 years) were excluded from the analysis of the second period (8-15 or 8-11 years).

We have now specified under “Statistical analysis” that “To examine whether the effect of mild anemia was similar over time, two survival analyses were set up: from 0 to 7 years, and, in participants who survived the first seven years of follow up, from 8 to 15/11 years”.

- For the analysis re-defining the criteria for mild anemia, please indicate the year that the changes in lower limits and WHO criteria were proposed.

Response
There are no reference/established criteria for “mild anemia”. Actually WHO criteria (2011) are recent and were published after those commonly used and considered in the present study (Dallman, 1984; Groopman and Itri, 1999; Wilson et al., [2004]). Current debate is about the definition of the lower limit of normal hemoglobin concentration for the diagnosis of anemia. WHO’s most commonly used definition of anemia (1968) has been questioned by Beutler and Waalen (2006) who proposed different lower limits of normal hemoglobin concentration for men and women according to different age groups (20-59 and 60+ years).

We have included also this information in the manuscript under “Definitions of anemia and mild anemia”: “Anemia was defined according to the most commonly used WHO criteria (1968) as a hemoglobin concentration lower than 12 g/dL in women and 13 g/dL in men [22]. Along with most grading systems [23-25], mild anemia was defined by Dallman (1984), Groopman and Itri (1999), Wilson et al. (2004) as a hemoglobin concentration between 10.0 and 11.9 g/dL in women and 10.0 and 12.9 g/dL in men.” Under “statistical analysis: “To investigate whether WHO criteria (1968) may have affected the estimated effect of mild anemia on mortality, we re-evaluated this association using slightly higher lower limits of normal hemoglobin concentration to define anemia in white adults proposed by Beutler and Waalen in 2006 (lower than 12.2 g/dL in women and lower than 13.2 g/dL in men) [26]. We further tested this association also using recent WHO criteria (2011) for mild anemia (lower limit of hemoglobin concentration: 11 g/dL) [27].”

- Please indicate in the flow chart how many individuals had further blood collection.

Response
Following the Reviewer’s suggestion we have added to the flow chart the number of participants with a second blood sampling in the H&A65-84 and M80+ cohorts.
Were the individuals who refused blood collection at baseline different than those who did not, aside from age and sex (e.g., comorbidities)?

**Response**

Probably there must be a misunderstanding. We compared participants and non-participants only for age and sex simply because age and sex were/are the only two variables available in the municipality registry offices also for non-participants (i.e., eligible residents who refused or were untraceable). At any rate, age and sex are two of the main determinants of anemia and mortality and, at least for age, also of most chronic diseases.

Having a different study aim, the eligible for the blood sub-study of the M80+ were not all the eligible residents, rather the sub-population of those among all residents who accepted to participate in the main study (some 90% of all the eligible residents in any case). We also explored the distribution of dementia and cognitive function in participants and non-participants in the blood sub-study not because these variables are associated with mortality (a matter of fact) or anemia (under scrutiny; in the Monzino study for example anemia was not significantly associated with an increased risk of developing dementia [Lucca et al. *Alzheimers Dement* 2020; 16 (S10): abstract]), but, as reported in the manuscript, because these were the primary research targets (in other words, the traits under investigation), that is, those characteristics that could have influenced the elderly subjects in the decision to take part or not in the study/sub-study and thus possibly determine a self-selection bias.

Since the entire cohort of old-old was the result of the pooling of two cohorts (H&A85+ and M80+), the only available information for the H&A85+ cohort and consequently for the entire 80+ cohort was that regarding age and sex.

By the way, at initial visit in the M80+ study diabetes, hypertension, and cancer were more prevalent among participants with blood sampling than among those without, even though the difference was not statistically significant for diabetes (p = 0.203) and cancer (p = 0.239).

**Results**

- It appears that no individuals in the M80+ cohort were excluded due to death/not found as in the H&A cohort. If the 2,039 individuals were deemed eligible because they were alive at first interview, please add this detail to the first box of the flow chart.

**Response**

The Reviewer is right: the 2,039 are the individuals alive at first interview. As explained above (previous comment), the eligible for the blood sub-study were not all the eligible residents, but rather the sub-population of those among all residents who accepted to participate in the main study.

Following the Reviewer’s suggestion we have added this detail to the first box of the flow chart.

- This section mentions that the primary research targets were dementia and cognitive function. This is the first time the reader comes across this information. If those were the primary aims of the original cohorts, please specify briefly under “Study settings and participants”.

**Response**
Following the Reviewer’s suggestion we have specified the aim of the M80+ study under “Study settings and participants”:

“The M80+ is a prospective, door-to-door population-based study among oldest-old registered residents in the province of Varese, Italy (study years: 2002-2017) aimed at investigating cognitive decline and dementia.”

- For this study, the analyses are based on the exposure of mild anemia vs non-anemia. Table 1 should then present the baseline characteristics of individuals for each of these exposure groups.

**Response**
We have changed Table 1 as requested by the Reviewer. The amount of information to report has required splitting the Table in two: Table 1A for the H&A65-84 and Table 1B for the [H&A85+ and M80+].

- If exposure was assessed only at baseline, it is possible that the study captured both prevalent and incident cases of anemia. Capturing prevalent cases can be problematic especially when assessing the association between an exposure and mortality. For example, some individuals might have had mild anemia for several years prior to the baseline data collection. As mentioned before, these limitations must be discussed.

**Response**
Please see the answer to the same previous question: “Importantly, the limitation section should discuss how capturing prevalent cases of mild anemia (vs. incident) might impact the results”.

- It is unclear what the time axis is for the cohort. In the text, it appears that it is duration of follow-up in years, although the KM curves show time to death. What were the censoring events?

**Response**
Sorry, the legend of the x-axis in Figure 1 was wrong. We have changed it: “Years of observation since blood sampling (study start)”. We have also changed the Figure legend: “Fig.1 Survival by mild anemia status in the …”.

- What was the covariate assessment window?

**Response**
We have added this piece of information under the “Statistical analysis”: “… cancer, transient ischemic attack, stroke, parkinsonism, dementia. All covariates were assessed at baseline and for participants with two blood samples also at second sampling.

- What was the mean follow-up time?

**Response**
We have added the requested information under the “Results” section::

**H&A65-84 cohort**
During 15 years of follow-up after blood sampling, 230 anemic (66.9%; 205 mild anemic) and 1928 non-anemic (46.5%) individuals died (Fig 1). The median follow-up period was 14.0 years with 50,522 person-years of observation … .

**H&A85+ and M80+ cohorts**
During 11 years of follow-up, 526 anemic (94.1%; 449 mild anemic) and 1,137 non-anemic individuals (88.6%) died in the pooled 80+ cohort (Fig 1). The median follow-up period was 3.5 years with 7,871 person-years of observation ...

... Risk of mortality associated with mild anemia in “healthy” elderly subjects
At baseline 1093 elderly persons (mean age 74.6, 64% women) from both H&A65+ and M80+ had no history of any of the diseases entered as confounders in multivariable analyses. The median follow-up period was 7 years with 7,043 person-years of observation ...

Risk of mortality associated with anemia status assessed over time (under “Study design” or “Results”):
In H&A65-84+, a second blood sample was available for 692 subjects .... The mean (SD) time between samplings was 2.2 (0.1) years (median follow-up: 7.0 years; person-years of observation: 4,265; incidence mortality rate: 3.8 per 100 person-years) ...

Considering the 523 subjects without anemia at first sampling in H&A65-84+ (median follow-up: 7.0 years; person-years of observation: 3.286; incidence mortality rate: 3.3 per 100 person-years), the 27 incident cases (5.2%; mortality rate: 11.4 per 100 person-years) ...

In M80+, a second blood sample was available for 366 subjects .... The mean (SD) time between samplings was 1.7 (1.0) years (median follow-up: 3.1 years; person-years of observation: 1,298; incidence mortality rate: 22.4 per 100 person-years) ...

Considering the 279 subjects without anemia at first sampling in M80+ (median follow-up: 3.4 years; person-years of observation: 1,048; incidence mortality rate: 20.5 per 100 person-years), the 65 incident cases at second sampling (23.3%; mortality rate: 31.8 per 100 person-years) showed an increased risk of mortality during the following 7 years compared to those who did not develop anemia (fully-adjusted HR: 1.47; 95% CI, 1.04-2.07).

... Was the type of anemia (i.e., anemia due to specific conditions) ascertained at baseline? If so, please indicate in the methods section. How can we be sure that the increased risk seen for anemia type is not due to the underlying condition, and what is the impact of adjusting for some of these comorbidities if they are also included in the exposure definition for anemia type?

Response
Yes, anemia type was ascertained at baseline. We have added it under “Definitions of anemia and mild anemia”:
“Anemia types were ascertained at baseline and their definitions are reported in S1 Methods”.

The underlying condition of most anemia types are not per se associated with a risk of mortality and in a good one fourth to one third of anemias in the elderly population the underlying causes are even still unknown (unexplained anemia). Numerous covariates were entered in multivariable analyses because, possibly being associated with mortality, they could explain whether the effect of mild anemia seen at univariate analysis was independent of other possible causes of death. With regard to “the impact of adjusting for some of these comorbidities if they [very few] are also included in the
exposure definition for anemia type” we reported among the limitations that “Extensive adjustment may have led to underestimation of the association strength, since mild anemia could also be an effect of underlying pathological conditions”. However, according to Reviewer 1 this assertion “is problematic given that the authors are interested in causal estimates. If after adjusting the estimates move towards the null, then that is the real effect”. Based on this comment we have decided to remove this sentence.

- With several criteria used in this study to define mild anemia, please provide the rationale in the methods section for the choice of cut-off used to report the primary results.

**Response**

As clarified above, there are no reference/established criteria for “mild anemia”: WHO criteria (2011) are recent and were published after those commonly used and considered in the present study (Dallman [1984], Groopman and Itri [1999], Wilson et al. [2004]). Given that WHO (1968) is the most commonly used definition of anemia both in clinic and research (all the 16 studies on the association of anemia and mortality used WHO criteria for the diagnosis of anemia), actually there were only two definitions of mild anemia: ≥ 10 g/dL of hemoglobin (Dallman [1984], Groopman and Itri [1999], Wilson et al. [2004]) and ≥ 11 g/dL of hemoglobin (WHO [2011]). In addition to its own merits, we chose to use the ≥ 10 g/dL cut-off level for reporting the primary results because we had already adopted it in the previous paper on the association between anemia and mortality over the first 3 years of follow-up in the H&A 65-84 cohort (Riva et al. 2009, reference 16), when WHO criteria (2011) were not yet published. In any case, by crossing the two criteria of anemia with the two of mild anemia, the reader has the possibility to see that whatever the range of hemoglobin considered to define mild anemia, the results found would have been more or less the same. Under “Definitions of anemia” we have now added:

“Anemia was defined according to the most commonly used WHO criteria (1968), as … Along with most grading system, mild anemia was defined by Dallman (1984), Groopman and Itri (1999) and Wilson et al. (2004) as …”.

Under “Statistical analysis”:

“To investigate whether WHO criteria (1968) may have affected the estimated effect of mild anemia on mortality, we re-evaluated this association using slightly higher lower limits of normal hemoglobin concentration to define anemia in white adults proposed by Beutler and Waalen in 2006 (lower than 12.2 g/dL in women and lower than 13.2 g/dL in men) [26]. We further tested this association also using recent WHO criteria (2011) for mild anemia (lower limit of hemoglobin concentration: 11 g/dL) [27].”

- This section mentions pooling the oldest-old cohorts because the hazard ratios were similar. This seems like an ad-hoc decision, which should not have been based on the HRs. Please provide a clearer rationale as to why the two cohorts were pooled in the first place, considering that they have different follow-up times and data collection methods.

**Response**

The Reviewer is perfectly right. The rationale for pooling the results of the two cohort studies was: both were prospective population-based studies in the old-old with no exclusion criteria other than age; both had a long lasting follow-up and were conducted
during more or less the same years; life expectancy was very similar; for both there was access to mortality data on an ongoing basis from the Municipal Registry Offices; the modalities to collect health-related information were very similar or identical in both. The inspection of Table S1 actually has only confirmed post hoc that the pooled results reported in Table 2 there were very similar results in both studies.

We have now modified the sentence in question under the “Results” section:

“H&A85+ and M80+ cohorts

Risk of mortality associated with anemia and mild anemia in subjects 85 years and older in both studies separately were quite similar (S1 Table), therefore H&A85+ and M80+ cohorts were pooled.”

We have added the rationale under the “Statistical analysis”:

“To investigate the association of anemia/mild anemia with mortality over 11 years in the old-old, individual participant data from the H&A85+ and M80+ cohorts were pooled. The rationale behind this pooling was that both studies were prospective door-to-door population-based studies in the old-old with no exclusion criteria other than age; both had a long lasting follow-up and were conducted during more or less matching calendar years; life expectancy was very similar; mortality data on an ongoing basis from the Municipal Registry Offices was available for both cohorts; the modalities to collect health-related information were very similar or identical in both”.

- A second sample was only available for 15.4% of subjects in the H&A 65-84 cohort. Were the individuals with a second blood sample different from those who did not have a second blood sample?

Response

“A second sample was only available for 15.4% of subjects in the H&A 65-84 cohort”: as we wrote under study design, “to assess change in anemia status, a stratified random sample of individuals from H&A65-84 was recontacted during 2005-2006 [1]”. Participants were stratified at baseline in anemic and non-anemic strata. We included all eligible consenting anemic and a random sample of eligible non-anemic participants. Considering that 2,306 subjects were not randomized, a second sample was available for 31.6% of participants in the H&A 65-84. Besides, because only subjects who accepted to be interviewed and tested (not to donate a second blood sample) entered into the randomization, a further 1,018 subjects who refused or were not found (and who, in any case for the most part would not be randomized because of the limited economic resources of the study), could be removed from the denominator, as well as 161 subjects who accepted to participate but at the time of the second blood sampling were deceased, untraceable or withdrew their consent to participate, a second sample was available for 68.6% of participants with one blood sample.

“Were the individuals with a second blood sample different from those who did not have a second blood sample?” Of the 4,494 subjects of the H&A 65-84 cohort, 3,802 had one blood sample and 692 two (15.4%):

| H&A 65-84         | 1 sample | 2 samples | All     |
|-------------------|----------|-----------|---------|
| Participants, No. | 3,802    | 692       | 4,494   |
| Female sex, No. (%) | 2,323 (61.1) | 383 (55.3) | 2,706 (60.2) |
| Age, mean (SD), years | 73.6 (5.2) | 73.2 (5.2) | 73.5 (5.2) |
| Education, mean (SD), years | 7.6 (3.9) | 8.1 (3.8) | 7.7 (3.8) |
Anemia and cancer cannot be matched because of the inclusion criteria of the study on the association of mild anemia with cognitive, functional, mood, and QoL outcomes (all participants with anemia or cancer consenting to be interviewed and tested were included).

Key characteristics of the entire group of participants with two blood samples are already reported in the first sentence of the “Results” section. Under “Study design” we have now added:

“Baseline characteristics of participants with one and two samplings were for the most comparable”.

- For the analysis capturing the second blood samples, were patient characteristics and comorbidities measured at baseline or at the time of the second blood sample?

**Response**

Patients characteristics and comorbidities were assessed both at baseline and at the time of the second blood sample. For investigating the association of incident cases and change in anemia status (Table 4), variables entered in the multivariable analyses were measured at the time of second follow-up.

Under “Statistical analysis” we have added:

“… cancer, transient ischemic attack, stroke, parkinsonism, dementia. All covariates were assessed at baseline and also at second sampling for participants with two blood samples.”

Under Table 4:

“…; F-A: "fully"-adjusted for age, sex, education, smoking, alcohol, hypertension, diabetes, heart failure, myocardial infarction, chronic respiratory failure, chronic renal insufficiency, TIA, stroke, cancer, dementia, hospitalization during the previous year. All covariates were assessed at second sampling”.

- It is worrisome that anemia was measured only at baseline (a time point not defined by any specific event), which included both prevalent and incident cases, and that the relationship between this exposure on mortality was assessed over up to 15 years later. Please discuss the impact of choosing an exposure definition that only captures the baseline status without accounting for changes in the exposure status or comorbidities over time, and includes prevalent and incident cases. Individuals included in the mild anemia group might not have been anemic for a large portion of the follow-up time, and vice versa.

**Response**

With regard to the “prevalent cases” issue, please see the answer to the same previous question: “Importantly, the “limitations” section should discuss how capturing prevalent cases of mild anemia (vs. incident) might impact the results”.

| Characteristics                      | No. (%), Study 1 | No. (%), Study 2 | No. (%), Study 3 |
|--------------------------------------|------------------|------------------|------------------|
| Current smokers, No. (%)             | 562 (14.8)       | 94 (13.6)        | 656 (14.6)       |
| Former smokers, No. (%)              | 1,183 (31.2)     | 245 (35.5)       | 1,428 (31.9)     |
| Current alcohol use, No. (%)         | 2,731 (72.5)     | 513 (74.1)       | 3,244 (72.7)     |
| Former alcohol use, No. (%)          | 132 (3.5)        | 29 (4.2)         | 161 (3.6)        |
| Body mass index, mean (SD)           | 25.0 (4.1)       | 24.9 (3.9)       | 25.0 (4.1)       |
| Diabetes, No. (%)                    | 358 (9.5)        | 63 (9.2)         | 421 (9.4)        |
| Hypertension, No. (%)                | 2,007 (53.7)     | 388 (56.6)       | 2,395 (54.1)     |
With regard to the failure to account for changes in the exposure status over time in the prevalent cohort, ours is one of only two attempts [11] to investigate also whether a change in anemia/non-anemia status would affect the risk of death in the general population. We report below the same answer to a very similar point raised by the other Reviewer.

In detecting and evaluating an anemia problem in a community, reference standards are necessary, even though they may be somewhat arbitrary” (WHO 1968). Several factors can influence the level of hemoglobin measured (for example, physiological fluctuations of plasma volume), especially in population-based studies. Since the diagnosis of anemia is defined as a concentration of hemoglobin of less than a conventional lower limit of normal, physiological fluctuations of hemoglobin concentrations can be incorrectly classified as either “anemia” (“false positive”) or “non-anemia” (“false negative”) when hemoglobin is determined only once. Moreover, among those diagnosed with an anemia type susceptible to treatment, a fraction can return over time to having a normal concentration of hemoglobin when successfully treated or the underlying causes have been eliminated. Whatever the assumption, considering how anemia is defined and the existence of anemia types susceptible of treatment, imply that anemia is not necessarily a chronic, everlasting condition.

Moreover, please note that the present study has investigated the association between anemia and mortality also in prospective cohorts of young-old and old-old with two samplings and reported the risk associated precisely with change in anemia status (Table 4).

Following the Reviewer’s request we have now added this point to the study limitations:

“A further potential limitation of studies assessing hemoglobin concentration on a single occasion, is that they cannot address within-individual changes in hemoglobin concentration over time that may potentially affect the results. However, the present study is one of only two attempts [11] to investigate also whether a change in anemia/non-anemia status would affect the risk of death. Generally, being anemic or non-anemic is a rather consistent status over time at old age in the general population: in the present study 84% of the study sample with two blood draws continued to be anemic or non-anemic on average over two years. However, since “false positives” (due to physiological fluctuations) and those successfully treated would decrease the mortality rate of the anemic group in which they were classified, whereas the “false negatives” (due to physiological fluctuations) and incident cases would increase the mortality rate of the non-anemic group in which they were classified, the actual risk associated with the anemia status would tend to be underestimated in subjects with a single blood sample. Consistently, in both young-old and old-old cohorts, subjects with two hemoglobin determinations whose anemia “resolved” (had they been successfully treated after or “false positive” cases at first sampling) did not show a significantly increased risk of mortality compared to those who were consistently non-anemic, a result almost identical to that of the cited Leiden 85 study [11]) and also to that of a retrospective study in a selected sample of patients with chronic heart failure (risk ratio 0.98 [0.73-1.36] [Tang et al. J Am Coll Cardiol 2008;51:569-576][41]). Whereas in those who became anemic at second sampling (had they been incident cases after or “false negatives” at first sampling) the risk was increased with respect to prevalent
cases, again in agreement with the Leiden 85 study [11]. Considering fluctuations in concentrations over time, it should also be noted that when the upper limit of hemoglobin concentration for anemia and/or the lower limit for mild anemia adopted were higher (Table 2), the estimated risks for the association between anemia/mild anemia and mortality were very similar to those of the main analysis”.

Under the Results and in Table 4 we have now added also mortality risks associated with hemoglobin decline (per 1 g/dL decline) in the young-old and old-old without anemia at baseline:

“In this cohort of young-old without anemia, also hemoglobin decline between samplings was associated with a subsequent increased risk of mortality over seven years (Table 4). … In this cohort of old-old without anemia, also hemoglobin decline between samplings was associated with a subsequent increased risk of mortality over seven years (Table 4)”.

Under “Discussion”:
“Consistent with two studies in a selected population of young-old [28] and 85 years old [11], incident anemia and decline in hemoglobin concentration were significantly associated with increased risk of mortality in both age cohorts”.

Under “Abstract-Results”:
“In participants without anemia at baseline also hemoglobin decline was significantly associated with an increased mortality risk over seven years in both young-old and old-old”.

With regard to the failure to account for changes in comorbidities over time, we report below the same response to a very similar point raised by the other Reviewer. Since the comorbidities considered as covariates are all chronic diseases or have chronic complications (e.g. stroke and myocardial infarction), the assumption is limited to the new occurrence (incidence) of these diseases after baseline. Please also note that this assumption as well is common to all the literature on the subject (and, incidentally, none of the published studies has mentioned it among the limitations). However, we agree with the Reviewer.

In the Monzino 80-plus study, beyond baseline, another 8 follow-up assessments were available. Thus, limited to this study, it has been possible to further investigate the influence of time-varying covariates on the association between anemia and mortality in subjects with one (prevalent cases) as well as in subjects with two hemoglobin determinations (incident cases and hemoglobin decline). Moreover, in subjects with two hemoglobin determinations it was also possible to investigate how the association between baseline anemia and mortality was affected by change in anemia status (at second sampling) together with prevalent plus incident covariates over the following seven years of follow-up. We have now reported the results of these analyses in a new Table 5 (please, see the revised manuscript): all results confirmed those adjusted only for baseline covariates previously set out in Tables 2 and 4 and in the new Supplementary Table reporting only the results of the Monzino 80-plus study. Though slightly, all hazard ratios are consistently higher than those reported in the above mentioned Tables and Supplementary Table. Moreover, in the analysis where it has been possible to account for change in both anemia status and covariates, the risk associated with baseline anemia resulted moderately increased also with respect to the model further adjusted for change in covariates. This finding is in agreement with the observation that changes in anemia/non-anemia status over time would tend to
underestimate the risk of mortality associated with anemia in subjects with a single blood sample (please see the answer to the previous comment).

Following the Reviewer’s request we have now added this point to the study limitations:

“Another limitation, common to all the literature on the subject, is that covariates potentially associated with death were assessed only at cohort entry, thus failing to account for the subsequent change over time of the covariates. However, restricted to the old-old participants in the Monzino 80-plus study, the association between anemia and mortality could be further adjusted also for time-varying covariates and change in anemia status over time: the results confirmed those adjusted only for baseline covariates and showed a slightly to moderately increased risk with respect to elderly subjects with a single blood sample and covariate assessment at entry, in agreement with the observation that changes in anemia/non-anemia status over time would tend to underestimate the risk assessed at baseline.”

Under “Statistical analysis” we have added:

“All covariates were assessed at baseline. Limited to the Monzino 80-plus study, beyond baseline, another 8 follow-up assessments were available. It was thus possible to further adjust the multivariable model also for the influence of time-varying covariates (age, habits, and intervening comorbidities) on the association between anemia and mortality in subjects with one (prevalent cases) as well as two hemoglobin determinations (incident cases and hemoglobin decline). Moreover, in subjects with two hemoglobin determinations it was also possible to investigate how the association between baseline anemia and mortality was affected by time-varying covariates together with anemia/non-anemia status at second sampling”.

Under “Results”, “H&A85+ and M80+ cohorts”:

“… was found (Table 2). Limited to the M80+ cohort, mortality risk associated with prevalent anemia and mild anemia was slightly increased when the model was further adjusted for time-varying covariates (anemia: HR 1.59 [95% CI: 1.39-1.82] over 11 years and HR 1.64 [95% CI: 1.42-1.89] over seven years; mild anemia: HR 1.57 [95% CI: 1.36-1.80] over 11 years and HR 1.60 [95% CI: 1.38-1.85] over seven years)”.

Under “Discussion”:

“In the Monzino 80-plus study the risk associated with prevalent and incident anemia was even higher when the multivariable model could be further adjusted also for time-varying covariates and, limited to all old-old with two blood samplings, for change in anemia status at second sampling.”.

Under “Discussion”, strength:

“No previous study has attempted to adjust also for the influence of change in anemia/non-anemia status and time-varying covariates”.
Discussion
- The “dose-response relationships” are only stated in the abstract and discussion. This definition of the analysis should be explicitly stated in the methods (…to assess whether a dose-response relationship exist…”) and results. Further, it is unclear whether this dose relationship refers to the different cut-off criteria or the comparison of HRs between anemia and mild anemia (which, in the case of anemia, is based on very few individuals with non-mild anemia).

Response
In order to clarify that we refer to the results reported in Figure 2 we have now added the following sentence under the “Statistical analysis”:
“To assess whether a dose-response relationship existed, hemoglobin concentrations were divided into categories of 1 mg/dL and p for trend analyses carried out.”