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Clinical relevance of clonal hematopoiesis in the oldest-old population

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Abstract:
Clonal hematopoiesis of indeterminate potential (CHIP) is associated with increased risk of cancers and inflammation-related diseases. This phenomenon becomes very common in oldest-old individuals, in whom the implications of CHIP are not well defined. We performed a mutational screening in 1794 oldest-old individuals enrolled in two population-based studies and investigate the relationships between CHIP and associated pathologies. Clonal mutations were observed in one third of oldest-old individuals and were associated with reduced survival. Mutations in *JAK2* and splicing genes, multiple mutations (*DNMT3A, TET2, ASXL1* with additional genetic lesions) and variant allele frequency {greater than or equal to}0.096 had positive predictive value for myeloid neoplasms. Combining mutation profiles with abnormalities in red blood cell indices improved the ability of myeloid neoplasm prediction. On this basis, we defined a predictive model that identifies 3 risk groups with different probabilities of developing myeloid neoplasms. Mutations in *DNMT3A, TET2, ASXL1* or *JAK2* (most occurring as single lesion) were associated with coronary heart disease and rheumatoid arthritis. Cytopenia was a common finding in oldest-old population, the underlying cause remaining unexplained in 30% of cases. Among individuals with unexplained cytopenia, the presence of highly-specific mutation patterns was associated with myelodysplastic-like phenotype and a probability of survival comparable to that of myeloid neoplasms. Accordingly, 7.5% of oldest-old subjects with cytopenia had presumptive evidence of myeloid neoplasm. In conclusion, specific mutational patterns define different risk of developing myeloid neoplasms *vs.*

inflammatory-associated diseases in oldest-old population. In individuals with unexplained cytopenia, mutational status may identify those subjects with presumptive evidence of myeloid neoplasms.

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Key Points

- In oldest-old population, specific mutational patterns define different risk of developing myeloid neoplasms *vs.* inflammatory-associated diseases.

- In oldest-old individuals with unexplained cytopenia, mutational status identifies subjects with presumptive evidence of myeloid neoplasms.

Abstract

Clonal hematopoiesis of indeterminate potential (CHIP) is associated with increased risk of cancers and inflammation-related diseases. This phenomenon becomes very common in oldest-old individuals, in whom the implications of CHIP are not well defined. We performed a mutational screening in 1794 oldest-old individuals enrolled in two population-based studies and investigate the relationships between CHIP and associated pathologies. Clonal mutations were observed in one third of oldest-old individuals and were associated with reduced survival. Mutations in *JAK2* and splicing genes, multiple mutations (*DNMT3A, TET2, ASXL1* with additional genetic lesions) and variant allele frequency ≥0.096 had positive predictive value for myeloid neoplasms. Combining mutation profiles with abnormalities in red blood cell indices improved the ability of myeloid neoplasm prediction. On this basis, we defined a predictive model that identifies 3 risk groups with different probabilities of developing myeloid neoplasms. Mutations in *DNMT3A, TET2, ASXL1* or *JAK2* (most occurring as single lesion) were associated with coronary heart disease and rheumatoid arthritis. Cytopenia was a common finding in oldest-old population, the underlying cause remaining unexplained in 30% of cases. Among individuals with unexplained cytopenia, the presence of highly-specific mutation patterns was associated with myelodysplastic-like phenotype and a probability of survival comparable to that of myeloid neoplasms. Accordingly, 7.5% of oldest-old subjects with cytopenia had presumptive evidence of myeloid neoplasm. In conclusion, specific mutational patterns define different risk of developing myeloid neoplasms *vs.* inflammatory-associated diseases in oldest-old population. In individuals with unexplained cytopenia, mutational status may identify those subjects with presumptive evidence of myeloid neoplasms.

Introduction

Exome sequencing studies have identified the frequent age-dependent clonal expansion of somatic mutations in the hematopoietic system.[1-5] Clonal hematopoiesis of indeterminate potential (CHIP) describes individuals with hematologic malignancy-associated mutations in blood or marrow, but without other diagnostic criteria for a hematologic malignancy[5] and is associated with increased risk of cancers (in particular myeloid neoplasms) and chronic inflammatory diseases (coronary heart disease).[1-6]

The phenomenon of CHIP becomes very common in oldest-old population (people aged 80+ years),[7-10] that represents the fastest growing age segment in developed countries.[9,11] In these individuals, clinical implications of CHIP are expected to be relevant but are not well defined.[7-8]

The incidence of solid cancers and myeloid neoplasms increases with age and mortality is higher after the age of 75.[12] The risk of several chronic inflammatory diseases is age-related as well, leading to large prevalence of frailty and disability among oldest-old people.[13] We hypothesized that the study of oldest-old population can contribute to define the relationship between specific mutational patterns in the hematopoietic system and the individual risk of developing cancers *vs.* other adverse events (chronic inflammatory diseases).

Anemia is a common finding in the elderly and is associated with worse cognitive and functional outcomes and increased mortality.[14-18] Underlying cause of anemia remained unexplained in 30% of cases, and a proportion of unexplained cytopenia may account for myeloid neoplasms.[15,18] We hypothesized that the study of CHIP may contribute to determine specific causes of anemia in elderly people and to define personalized treatment strategies to mitigate anemia-related negative sequela.

The primary objective of the present study was to evaluate the prevalence of CHIP and the relationships between CHIP and associated pathologies in oldest-old population. The secondary objective was to analyze clinical outcome of patients affected with unexplained cytopenia and to define the clinical effect of clonal abnormalities among these individuals. The definitions of Idiopathic Cytopenia of Unknown Significance (ICUS) and Clonal Cytopenia of Unknown Significance (CCUS) were applied to identify individuals with non-clonal *vs.* clonal unexplained cytopenia.[5,19]

Patients and methods

Study procedures are in accordance with the Declaration of Helsinki. Ethics Committees of Humanitas Research Hospital and Mario Negri Pharmacological Institute, Milan Italy approved the study. Written informed consent was obtained prior to blood sampling. (ClinicalTrials.gov number: NCT03907553)

Study population

Study procedures are described in *Supplementary_File_1*.

We analyze subjects included in the two population-based studies enriched in oldest-old individuals (80+ years), i.e., "Health_&_Anemia"[19,21] and "Monzino_80+"[24].

"Health_&_Anemia" is a prospective study (2003-2018) aimed to investigate clinical consequences of anemia in the elderly.[15,17] We studied 1059 oldest-old subjects (median age 83 years, range 80-105) in whom peripheral blood samples collected at study enrollment were available for mutational screening. At study enrollment (May, 2003) clinical history was collected and complete blood count was performed. When a hemoglobin concentration was below WHO reference criteria for anemia (<12g/dL in women and <13g/dL in men), further investigations were made to define specific causes of anemia. Follow-up was updated to December, 2018. A total of 344,565 laboratory tests were available during follow-up. Data on hospitalization and mortality were available for all subjects. Diagnosis of chronic inflammatory diseases and cancers were defined according to International Classification of Diseases for Oncology ninth edition CM (ICD-9-CM) codes and local cancer registry data. Diagnosis of myeloid neoplasms was based in addition on information provided by revision of bone marrow biopsy reports provided by Hematology Unit of Biella Hospital. Three investigators (MR, ER and MGDP) have reviewed independently all this information and a final diagnosis of myeloid neoplasms was provided by a consensus meeting.

As a second cohort, we analyzed 735 individuals (median age 90 years, range 80-104) enrolled in the "Monzino_80+" prospective study (2002-2018) aimed at investigating relationships between age, cognitive decline and dementia.[20]

Due to the fact that data collection on myeloid neoplasms diagnosis was less accurate in "Monzino_80+" population with respect to "Health_&_Anemia" cohort (see *Supplementary_File_1*), we analyzed in addition 727 subjects aged ≥75<80 years from "Health_&_Anemia" study, to specifically validate the predictive value of clinical and mutational features on the risk of developing myeloid neoplasms.

Finally, to compare clinical features and outcome of oldest-old subjects with CHIP to those of patients with myeloid neoplasms, we analyzed a sex- and age-matched population of patients affected with myelodysplastic syndrome from the retrospective EuroMDS database. (2000-2018, ClinicalTrials.gov number: NCT04174547) Each subject with CHIP was matched with 5 patients with the same year of birth and sex; overall, 255 patients with myeloid neoplasms were included in this analysis.

Mutation screening

Using peripheral blood DNA we looked for mutations in 47 genes related to myeloid neoplasms. (Gene list is available in *Supplementary_Table_1*, sequencing procedures and variant calling are available in *Supplementary_File_2)*[5]

CHIP was defined as the presence of a clonal blood cell population associated with a hematologic malignancy-related mutation at a variant allele frequency (VAF)≥0.01. Median coverage was 3455×. Mutations with VAF<0.10 were re-sequenced on an independent platform. The effectiveness of re-sequencing in confirming genomic variants with VAF≥0.01 and <0.10 was of 96.5%, while DNA sequencing was significantly less performant and reproducible below the threshold of 0.01(P=0.02). The technique we used missed clonal skewing in the absence of mutations in putative myeloid neoplasms driver genes.[22]

Statistical analysis

Numerical variables were summarized by median and range; categorical variables were described with count and relative frequency (%) of subjects in each category.

Survival analyses were performed with Kaplan-Meier method and differences between groups were evaluated by log-rank test. Cox models were built to estimate hazard ratio (HR, with 95% CI) for probability of overall survival and risk of developing coronary heart disease and chronic inflammatory diseases (only incident cases were considered in the analyses).

The accuracy of mutational factors in predicting the risk of developing myeloid neoplasms was analyzed (only incident cases were considered in the analyses). The accuracy of categorical variables (presence *vs.* absence of mutations in a specific gene) was estimated by calculating time-dependent positive and negative predictive value (PPV, NPV).[23] For variables measured on a continuous scale (variant allele frequency, VAF), time-dependent ROC curve, the corresponding area under the ROC curve (AUC) and the optimal cutoff point were calculated by using online available *cenROC* R package.[24,25]

To define a risk score for developing myeloid neoplasms, HR from a multivariable Cox analysis on “Health_&_Anemia” population including age, sex, mutational status and non-mutational parameters as covariates were used. A diagnosis of myeloid neoplasm was considered as event; subjects were censored at the end of follow-up or at time of death. The goodness of concordance for the predictive score was measured by concordance index (C-index), internal 5-folds cross-validation and independent external validation.

Cumulative incidence of myeloid neoplasms was calculated by Kaplan-Meier method (death for any cause was considered as competing-event in the estimation of cumulative incidence function).[26] Left truncation was applied when calculating the cumulative incidence of myeloid neoplasms with age as the time scale.[27]

Data Sharing

Data are found under accession number PRJNA736552.

Results

Prevalence and clinical effect of CHIP in oldest-old population

We studied prevalence of CHIP and relationship between CHIP and probability of survival in oldest-old population. Analyses were performed on both "Health_&_Anemia" and "Monzino_80+" cohorts.

Mutations were observed in 32.6%[95%CI 29.4-35.5] and 26.0%[22.9-29.8] of subjects enrolled in "Health_&_Anemia" and "Monzino_80+" cohorts, respectively. The majority of variants in both cohorts occurred in three genes: *DNMT3A, TET2 a*nd *ASXL1.* (prevalence of mutated genes is reported in *Figure_1, Supplementary_Figure_1* and *Supplementary_Table_2)*

CHIP was more common in males *vs.* females (P=0.02 and P=0.005, respectively) and its prevalence increased with age (P=0.001 and P=0.03, respectively). Considering genes grouped according to functional patterns in both cohorts, we observed a significant increase of mutations with age in epigenetics and cohesin complex-related genes (P=0.01 and P=0.02, respectively). Considering single genes, we observed a significant increase in the prevalence of *TET2* and *ASXL1* mutations after the age of 90 (P=0.021 and P=0.032, respectively). After testing for gender bias, we observed two genes significantly more mutated in males than in females: ZRSR2 and U2AF1.

We focused on centenarians in both cohorts (n=44) stratified according to the presence of comorbidity (including heart disease, diabetes, stroke, cancer, osteoporosis, thyroid condition, Parkinson's disease and chronic obstructive pulmonary disease).[28] Individuals with chronic age-related illness before the age of 100 were 33 *vs.* 11 who remained disease-free at age 100. Prevalence of CHIP was higher in patients with *vs.* without comorbidity (62% *vs.* 20%,P=0.015).

Subjects with CHIP were older with respect to individuals without CHIP (median age of individuals without CHIP *vs.* those with 1 mutation *vs.* those with ≥2 mutations was 83y *vs.* 84y *vs.* 85y, respectively, in the "Health_&_Anemia" cohort, P=0.019; and 90y *vs.* 91y *vs.* 94y, respectively, in the "Monzino_80+" cohort, P<0.001). The presence of CHIP was associated with a lower probability of survival (P<0.001 in both cohorts) and prognosis was even poorer in subjects carrying ≥2 mutations. (P<0.001 in both cohorts, *Figure_2*). The independent association between CHIP and increased mortality was maintained in a multivariable analysis including age, sex and cytopenia as covariates (HR 1.28[1.1-1.9], P=0.009 and HR 1.37[1.2-1.71],P=0.006, in the "Health_&_Anemia" and "Monzino_80+" cohort, respectively) and when focusing on cancer-related death (HR 1.91[1.44-2.21],P=0.001 and HR 2.13[1.94-2.39],P=0.002) and non cancer-related mortality (HR 1.43[1.29-1.77],P=0.02 and 1.56[1.31-1.8],P=0.01) as separate outcomes.

Focusing on subjects carrying ≥2 mutations in both cohorts, the independent effect of carrying ≥2 mutations on mortality was maintained in a multivariate analysis including age, sex and cytopenia as covariates (HR 1.4[1.19-2.31,P=0.008]. Considering specific causes of death, a higher prevalence of cancer-related deaths was observed in individuals carrying ≥2 mutations *vs.* both subjects carrying 1 mutation and those without CHIP (P=0.028 and P=0.009, respectively)

CHIP and risk of developing myeloid neoplasms in oldest-old population

We investigated the relationship between CHIP and risk of developing myeloid neoplasms. Prevalent and incident cases were 16 and 25, respectively. We calculated the time-dependent PPV and NPV for developing myeloid neoplasms at the age of 95y for most relevant genomic features. (*Supplementary_Figure_2*) Absence of mutations had high NPV (0.89[0.86-0.92]), while the presence of CHIP *per se* had low PPV (0.11[0.09-0.16]). Among investigated genes, splicing genes (*SF3B1, SRSF2, U2AF1, ZRSR2*) and *JAK2* have the highest PPV (0.58[0.41; 0.63] and 0.70[0.41-0.98], respectively). To evaluate the impact of multiple mutations in the same individual, we focused on the three most commonly mutated genes (*DMNT3A, TET2, ASXL1*) comparing single mutations with co-mutation patterns. PPV of mutations in *TET2, DNMT3A* or *ASXL1* with co-mutation patterns was higher than PPV of single lesions (0.28[0.14; 0.41] vs. 0.08[0.02-0.23],P=0.001). (*Supplementary_Figure_2*) Overall, mutations in splicing genes, mutations in *JAK2* gene and co-mutation patterns involving *TET2, DNMT3A* and *ASXL1* accounted for 75% of myeloid neoplasms diagnosed in subjects with prior CHIP.

We then explored the best cutoff value of VAF for developing myeloid neoplasms. VAF showed a significant accuracy as evaluated by time-dependent ROC curve (AUC was 0.88[0.81-0.94],P<0.001). At the age of 95y, a VAF of 0.096 was found as optimal cutoff value (sensitivity 0.84, specificity 0.83).

In a multivariable analysis including the number of mutations per subject and the most frequently mutated genes, having co-mutation patterns involving *TET2, DNMT3A* and *ASXL1* (HR 4.64[2.11-12.23],P<0.001), carrying splicing mutation (HR 10.63[5.23-17.68],P<0.001) or having VAF >0.096 (HR 2.35[1.22-5.48],P=0.021) were independent predictors for developing myeloid neoplasms.

Finally, we aimed to study whether non-mutational factors may improve the capability to capture individual risk of developing myeloid neoplasms. We focused on changes in red blood cell (RBC)-indices (mean corpuscular volume [MCV] and red blood cell distribution width [RDW]) that occur as early phenotypic abnormalities in subjects who later develop myeloid neoplasms.[4]

In “Health_&_Anemia” cohort, we found that high MCV (>98fl) and RDW (>14) values at study enrollment were associated with reduced survival. (P=0.01 and P=0.008, respectively, *Supplementary_Figure_3*) In a multivariable analysis including splicing mutations, co-mutation patterns involving *TET2, DNMT3A* and *ASXL1* and VAF as covariates, abnormal RBC-indices are associated with higher risk of developing myeloid neoplasms, independently from mutational features (HR 2.02[1.18-4.7],P<0.001).

Definition of a risk score for developing myeloid neoplasms according to mutational status and RBC-indices

We aimed to define a risk score for developing myeloid neoplasms according to mutational status and RBC-indices. Subjects from “Health_&_Anemia” cohort entered this analysis.

HR from multivariable Cox analysis including age, sex, mutational status and RBC-indices as covariates were used to define a risk score. (details are reported in *Figure_3*) A score of 1 was assigned for abnormalities in RBC-indices and VAF>0.096, a score of 2 was assigned for co-mutation patterns involving *TET2, DNMT3A* and *ASXL1,* and a score of 5 was assigned for splicing mutations. Risk groups were defined as low (score 0-1), intermediate (score 2-4) and high (score ≥5). A simplified risk classification was also provided. Cumulative incidence of myeloid neoplasms was significantly different among these three risk groups (P<0.001). In particular, in high risk individuals (3% of the whole oldest-old general population), cumulative incidence of myeloid neoplasm was 14%, 34% and 42% at the age of 85, 90 and 95 years, respectively. The accuracy of the predictive score was good (C-index 0.851) and was confirmed by internal 5-folds cross validation (mean C-index in test sets was 0.849).

We performed in addition an external validation on an independent cohort of 727 subjects aged ≥75<80y from “Health_&_Anemia” study. (*Figure_3* and *Supplementary_Figure_4*) The analyses performed on the validation cohort confirmed a high concordance of the model (C-index was 0.889), thus suggesting a high generalizability of the results.

Clonal evolution in old-oldest population with multiple samples available

We studied clonal evolution in 96 subjects from "Health_&_Anemia" cohort, in which multiple samples were available over a period of 4 years.

CHIP was found at baseline in 22 cases (23%): during follow up, 2 individuals acquired additional mutations, 10 displayed ≥0.05 VAF increase, while in 3 cases CHIP was lost. In 13/74 subjects without mutations at baseline, CHIP was acquired during follow-up (17.5%). We identified 2 subjects in whom clonal evolution preceded a diagnosis of a myeloid neoplasm (myelodysplastic syndrome in both cases). (*Figure_4* and *Supplementary_Table_3*)

Relationship between CHIP, coronary heart disease and chronic inflammatory diseases in oldest-old population

We aimed to define the relationship between CHIP and risk of developing chronic inflammatory diseases. We analyzed "Health_&_Anemia" cohort, in which the diagnosis of chronic inflammatory diseases was systematically recorded.

Coronary heart disease was defined as a history of myocardial infarction or coronary revascularization after the time of DNA collection. Prevalent and incident cases were 57 and 27, respectively. We defined *ASXL1, TET2, DNMT3A* and *JAK2* mutations as high-risk for vascular events.[6] Participants with CHIP had a significantly higher risk of coronary heart disease with respect to those without mutations (HR 1.61[1.28-3.21],P=0.02). When considering patients with high-risk mutations, HR increased to 2.21[1.45-4.01],P=0.006 and the effect was maintained when adjusting for sex, age smoking, hypertension and hyperlipidemia (not shown). Mutations in splicing genes were not associated with an increased risk of coronary heart disease (HR 0.91[0.79-1.65],P=0.84).

As a further step, we investigated the possible role of CHIP in other chronic inflammatory diseases (stroke, diabetes, arthritis and autoimmune diseases). We observed preliminary evidence of increased risk of developing rheumatoid arthritis (12 prevalent and 6 incident cases) in participants with *vs.* without high-risk mutations.[6] (HR 4.79[1.9-17.62],P=0.039) However, due to the low number of cases observed, this finding should be interpreted with caution.

Clinical relevance of CHIP in oldest-old individuals with unexplained cytopenia.

We aimed to specifically analyze clinical outcome of oldest-old patients affected with unexplained cytopenia and to define the clinical effect of clonal abnormalities among these individuals. The definitions of Idiopathic Cytopenia of Unknown Significance (ICUS) and Clonal Cytopenia of Unknown Significance (CCUS) were applied to identify individuals with non-clonal *vs.* clonal unexplained cytopenia.

Individuals from both "Health_&_Anemia" and "Monzino_80+" cohorts entered this analysis. The most common cytopenia reported at study enrollment was anemia (14.9% and 29.5% respectively).[15-18] The underlying cause of persistent (>6 months) cytopenia was unexplained in 29% and 34% of cases, respectively. (*Table_1*) Prevalence of CHIP was not significantly different in oldest old subjects with *vs.* without cytopenia (30.5% *vs.* 29.6%, respectively, P=0.24). Focusing on subjects affected with anemia stratified according to the underlying cause, no significant difference on the prevalence of CHIP was observed among different groups of patients. We noticed an enrichment of splicing gene mutations in patients with unexplained anemia with respect to subjects with anemia associated with specific underlying cause (P=0.031)

We considered subjects with unexplained cytopenia from both studies (n=133), stratified according to the presence of mutations as ICUS (n=82) *vs.* CCUS (n=51,38%).[19] Subjects with CCUS showed a significantly lower probability of survival compared with ICUS (P=0.002), while no significantly different probability of survival was noticed between ICUS and individual without cytopenia. (*Figure_5*)

As we observed a difference in survival among CCUS *vs*. ICUS, we tested the hypothesis that highly-specific mutation patterns for myeloid neoplasms may provide presumptive evidence of hematological malignancy in patients with unexplained cytopenia even in absence of definitive morphological criteria. According to our findings, highly-specific mutation patterns for myeloid neoplasms were defined as the presence of mutations of splicing factors, co-mutation patterns involving *TET2, ASXL1* or *DNMT3A* and/or mutations with VAF>0.096.

CCUS with highly-specific mutation patterns frequently showed macrocytic anemia and/or multilineage cytopenia (30 of 39 cases) consisting with a myelodysplastic syndrome phenotype. Two subjects died because of acute myeloid leukemia, while the most frequent cause of death was cardiac disease (25 subjects).

We then compared clinical features and outcomes of subjects with CCUS with those of an age- and sex-matched population affected with myeloid neoplasms (myelodysplastic syndromes) reported to retrospective EuroMDS database. (n=255, *Supplementary_Table_4*) No significant differences were observed in probability of survival between CCUS with highly-specific mutation patterns with respect to patients with myeloid neoplasms, while CCUS without highly-specific mutation patterns showed higher probability of survival with respect to CCUS with highly-specific mutation patterns. (HR 2.05[1.61-4.64],P=0.06 *Figure_5*).

Considering CCUS with highly-specific mutation patterns as “potential myeloid neoplasms”, 7.5% of oldest-old subjects with cytopenia had presumptive evidence of myeloid neoplasm.

Discussion

We observed that CHIP is a common finding in oldest-old population (30% of individuals) and that specific mutational profiles are associated with distinct clinical outcomes.

Mutations in splicing genes (mostly occurring as single genetic lesion)[29] showed the highest predictive value for myeloid neoplasms,[1-4,30] while they are not significantly associated with increased risk of developing chronic inflammatory diseases.

By contrast, the positive predictive value for myeloid neoplasms of isolated mutations in *TET2, DNMT3A* and *ASXL1* genes was lower, and additional genetic events are required to give rise to a myeloid neoplasm.[1-4,30] These mutations are mostly linked to increased risk of myocardial infarction and arthritis.[1-4,6,31]

Focusing on prediction of myeloid neoplasms, both number of mutations per subject and size of the mutant clone (VAF) had significant positive predictive value.[30] We observed in addition that abnormalities in RBC-indices significantly improve the capability of molecular features to capture individual risk of developing myeloid neoplasms.[4] By combining specific mutational patterns, size of mutant clone and abnormalities in RBC-indices, we defined three groups of individuals with different risk of developing myeloid neoplasms. In details, we identified a small population (3% of individuals aged 80 years or older) in which cumulative incidence of myeloid neoplasm was 14%, 34% and 42% at the age of 85, 90 and 95 years, respectively. Importantly, the predictive power of the score was confirmed by an external, independent validation.

Clonal evolution was a frequently observed event in oldest-old subjects with sequential samples available and in some cases preceded the occurrence of an overt myeloid neoplasm, suggesting that serial analysis may improve clinical monitoring.[30]

We had the opportunity to study CHIP in centenarian according to different morbidity profiles. CHIP is common in centenarians with age-associated diseases, while rarely observed those who attain their 100th birthday without comorbidity. The possible relationship between absence of CHIP and exceptional longevity should be addressed by specific investigations.[32]

Finally, we hypothesized that myeloid neoplasms could be underdiagnosed in oldest-old population, especially in cases with unexplained cytopenia.[14,32] We observed that among individual with unexplained cytopenia, the presence of a highly-specific mutation pattern for myeloid neoplasms[30,33] is associated with reduced survival. According to mutational features, 7.5% of oldest old subjects with cytopenia may have a presumptive evidence of myeloid neoplasm. The study of CHIP is therefore expected to contribute to determine specific causes of cytopenia in elderly people and to define personalized treatment strategies.

Since CHIP was described, caution is suggested against adopting mutational testing in clinical practice. In fact, the presence of mutations "*per se*" in a given individual has only limited predictive power as conversion to overt diseases is rare regardless of mutation status.[1-3,34] Our findings improve the capability to capture clinical information at individual patient level with respect to the presence of specific mutation patterns. These data support the rationale for prospective studies including CHIP as a part of conventional laboratory investigations to evaluate the health general status of elderly people and drive strategies to prevent adverse events.

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Data Availability Statement

According to Data Availability Guidelines for Blood Journal, in case of accepted manuscript we will provide access to DNA sequence obtained from peripheral blood samples of the present study, through the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) public repository

Authorship Contributions

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Writing the manuscript: all

Revision and approval of the manuscript: all

Disclosure of Conflicts of Interest

Marianna Rossi. Consulting or Advisory Role: Pfizer, Celgene, IQvia, Janssen

Manja Meggendorfer. Employment: MLL Munich Leukemia Laboratory

Francesco Passamonti. Speakers' Bureau: Novartis, AOP Orphan Pharmaceuticals

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Pierre Fenaux. Honoraria: Celgene. Research Funding: Celgene

Niccolò Bolli. Consulting or Advisory Role: Janssen. Speakers' Bureau: Celgene, Amgen

Wolfgang Kern. Employment: MLL Munich Leukemia Laboratory. Leadership: MLL Munich Leukemia Laboratory. Stock and Other Ownership Interests: MLL Munich Leukemia Laboratory

Torsten Haferlach. Employment: MLL Munich Leukemia Laboratory. Leadership: MLL Munich Leukemia Laboratory. Consulting or Advisory Role: Illumina

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Tables

Table 1. Prevalence and leading causes of anemia and other cytopenias at study enrollment in subjects aged 80y or older from "Health_&_Anemia" and "Monzino_80+" cohorts. The classification of anemia and other cytopenias (based on the hematologic findings) was supported by the clinical conditions and pharmacological therapies of the elderly (criteria are defined on *Supplementary_File_1*). Anemias and other cytopenias that could not be classified into any of the previously defined categories were considered to be of "unexplained origin". A panel of the three physicians reviewed, discussed and reached a final consensus for each case of cytopenia with discrepant classification.

	"Health_&_Anemia" study	**"Monzino_80+" study**	**P**
Number of subjects	1059	735	
Women (%)	697 (65.8)	546 (74.3)	<0.001
Men (%)	362 (34.2)	189 (25.7)	
Age y median (range)	82.8 (80-105)	90.5 (80.5-103.9)	<0.001
≥80<85 y (%)	725 (68.5)	128 (17.4)	<0.001
≥85<90 y (%)	235 (22.2)	215 (29.3)	
≥90<95 y (%)	71 (6.7)	247 (33.6)	
≥95<100 y (%)	24 (2.3)	105 (14.3)	
≥100 y (%)	4 (0.3)	40 (5.4)	
Death (%)	683/1059 (64.5)	695/735 (94.5)	<0.001
Blood count at study enrollment			
Hb g/dl median (range)	13.6 (7.3-18)	13.1 (6.7-16.8)	<0.001
HCT % median (range)	42.4 (22.9-55.5)	39.2 (22-50.6)	<0.001
MCV fL median (range)	95.9 (61.9-123.3)	92.2 (58.6-121.8)	<0.001
RDW fL median (range)	14.4 (12.3-21.6)	13.4 (11.3-22.2)	<0.001
WBC x10^9/L median (range)	6.4 (1.6-40.3)	6.5 (2.3-78.9)	0.664
ANC x10^9/L median (range)	3.8 (0.6-21.2)	3.8 (1.2-15.2)	0.059
PLT x10^9/L median (range)	222 (52-1117)	231 (25-915)	0.044
Anemia at study enrollment			
All (%)	158/1059 (14.9)	217/735 (29.5)	<0.001
Women (%)	94/697 (13.5)	142/546 (26)	<0.001
Men (%)	64/362 (17.7)	75/189 (39.7)	<0.001
Anemia by age (years)			

≥80<85 (%)	81/725 (11.2)	21/128 (16.4)	0.142
≥85<90 (%)	41/235 (17.4)	49/215 (22.8)	0.249
≥90<95 (%)	25/71 (35.2)	87/247 (35.2)	0.930
≥95<100 (%)	9/24 (37.5)	40/105 (38.1)	0.971
≥100 (%)	2/4 (50)	20/40 (50)	1.000
Severity of anemia			
Mild (normal value to 10 g/dl) (%)	141/158 (89.3)	197/217 (90.7)	0.910
Moderate (9.9-8 g/dl) (%)	16/158 (10.1)	16/217 (7.3)	0.389
Severe (<8 g/dl) (%)	1/158 (0.6)	4/217 (2)	0.320
Causes of anemia			
Iron deficiency (%)	9/158 (5.7)	14/217 (6.4)	0.777
Beta thalassemia (%)	12/158 (7.6)	5/217 (2.3)	0.021
B12/folate deficiency (%)	18/158 (11.4)	41/217 (18.8)	0.091
Renal (%)	29/158 (18.4)	49/217 (22.6)	0.419
Chronic disease (%)	16/158 (10.1)	16/217 (7.3)	0.389
Multifactorial anemia (%)	23/158 (14.6)	18/217 (8.2)	0.087
Unexplained anemia (%)	41/158 (25.9)	74/217 (34.1)	0.216
Neutropenia at study enrollment			
All (%)	10/1059 (1)	26/735 (3.5)	<0.001
Unexplained (%)	4/10 (40)	11/26 (42.3)	0.936
Thrombocytopenia at study enrollment			
All (%)	51/1059 (4.8)	56/735 (7.6)	0.021
Unexplained (%)	9/51 (17.6)	16/56 (28.5)	0.294
Unexplained cytopenia	48/1059 (4.5)	85/735 (11.5)	<0.001

Figure Legends

Figure 1. Clonal hematopoiesis of indeterminate potential (CHIP) in oldest-old subjects from "Health_&_Anemia" and "Monzino_80+" cohorts. Panels ***A*** and ***C*** show the prevalence of most frequently mutated genes in the two cohorts (considering mutated and unmutated patients). Panels ***B*** and ***D*** show the number of persons with 1, 2, or more than 2 variants.

Figure 2. Panels ***A*** and ***C*** show cumulative probability of overall survival according to the presence of CHIP in oldest-old subjects from "Health_&_Anemia" and "Monzino_80+" cohorts, respectively. Panels ***B*** and ***C*** show cumulative probability of overall survival according to the number of variants (0 *vs.* 1 *vs.* 2 or more) in old oldest subjects from "Health_&_Anemia" and "Monzino_80+" cohorts, respectively.

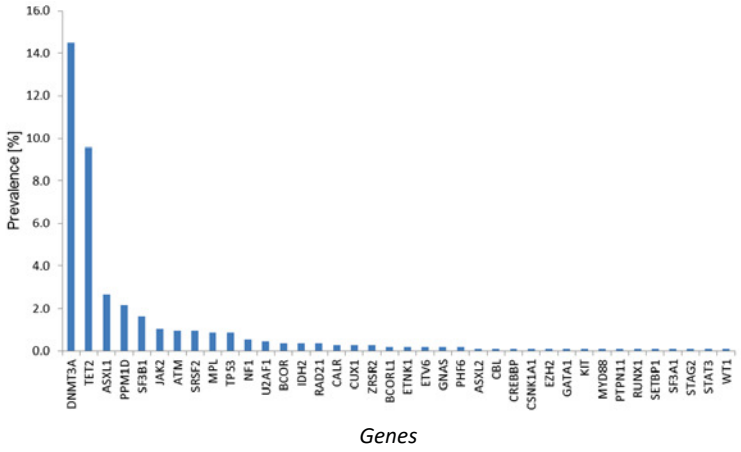
Figure 3. (***A***) Definition of a score based on specific mutational patterns (CHIP) and red blood cell (RBC)-indices to predict the risk of developing myeloid neoplasms. To define a risk score for developing myeloid neoplasms, we used HR from a multivariable Cox analysis on "Health_&_Anemia" cohort (learning cohort) adjusted for age and sex, including mutational status (splicing mutations, co-mutation patterns involving *TET2, DNMT3A* and *ASXL1*, and VAF>0.096) and non-mutational parameters (RBC-indices) as covariates. A diagnosis of myeloid neoplasm (including myelodysplastic syndrome and acute myeloid leukemia) was considered as event; subjects were censored at the end of follow-up or at time of death. (***B***) Cumulative incidence (CI) of myeloid neoplasms for oldest-old individuals stratified into 3 risk categories (learning cohort). Cumulative incidence was calculated by Kaplan-Meier method (death for any cause was considered as competing-event in the estimation of CI function). Left truncation was applied to calculate CI of myeloid neoplasms with age as the time scale. (***C***) Validation of the score on an independent cohort of 727 subjects aged ≥75<80y from "Health_&_Anemia" study.

Figure 4. (***A***) Clonal evolution in subjects from "Health_&_Anemia" cohort with multiple sample available (n=96); (**B**) Clonal evolution - Subject #269. This female subject was born on 1921. In 1999, she displayed normal blood count. In 2003 a first mutational screening was performed with evidence of a mutation in SF3B1 gene with 0.02 VAF. At this time, the subject showed normal hemoglobin level (12.1 g/dl), RDW (12) and MCV (87). Since 2003 this subject experienced increasing in RDW and MCV, and in 2007 a mild anemia was observed (10 g/dl). At this time, mutational screening showed increase in SF3B1 VAF (0.24). In 2008, a diagnosis of myelodysplastic syndrome (MDS) with ring sideroblasts was performed. (**C**) Clonal evolution -Subject #1145. This

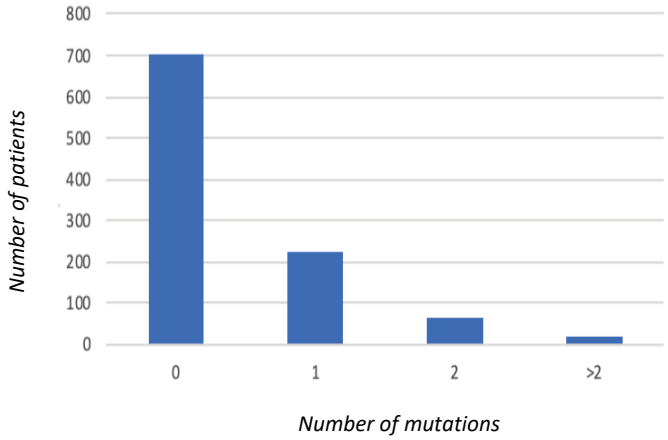
male subject was born on 1922. In 1999 blood count was normal. In 2003 a first mutational screening revealed a mutation in TET2 gene with 0.02 VAF. At this time, the subject showed a mild anemia (12.7 g/dl), with increased RDW (14.2) and normal MCV (89). In 2007 hemoglobin level decreased to 11.8 g/dl, and mutation analysis showed an increase in TET2 mutation VAF to 0.16. From 2007 to 2012 a further decrease in hemoglobin level together with increasing in RDW and MCV value was noticed. In 2013, a diagnosis of myelodysplastic syndrome (MDS) with unilineage dysplasia was performed.

Figure 5. (***A***) Overall survival of subjects with unexplained cytopenia form both "Health_&_Anemia" and "Monzino_80+" cohorts stratified according to the presence of mutations as idiopathic (non-clonal) cytopenia of undetermined significance (ICUS) *vs.* clonal cytopenia of undetermined significance (CCUS). Probability of survival of individuals without cytopenia was also reported; (***B***) Overall survival of subjects with CCUS with highly specific mutational patterns for myeloid neoplasms *vs.* CCUS without specific mutational patterns and *vs.* age- and sex-matched patients affected with myeloid neoplasms (myelodysplastic syndromes, from EuroMDS database).

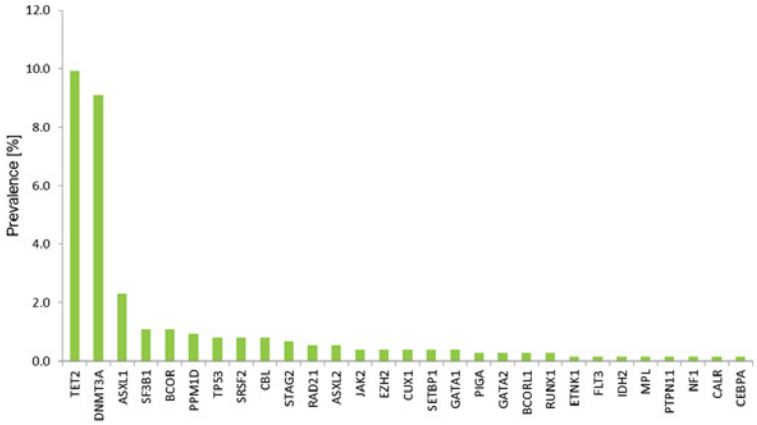
Fig.1 A) *Prevalence of most frequently mutated genes in the "Health_&_Anemia" cohort*



B) *Number of persons with 1, 2, or more than 2 variants in "Health_&_Anemia" cohort*



C) Prevalence of m*ost frequently mutated genes in the "Monzino_80+" cohort*



D) *Number of persons with 1, 2 or more than 2 variants in the "Monzino_80+" cohort*

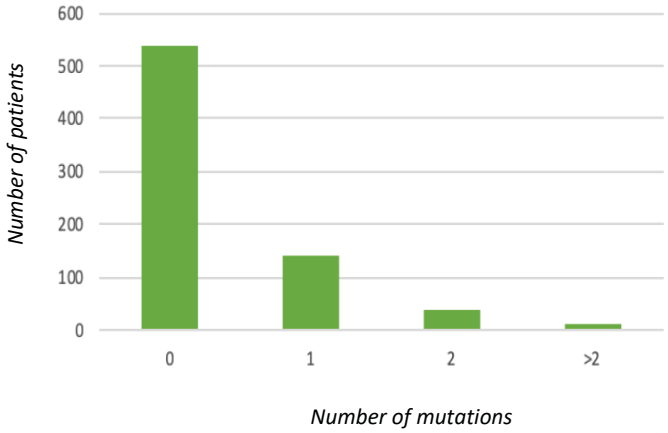
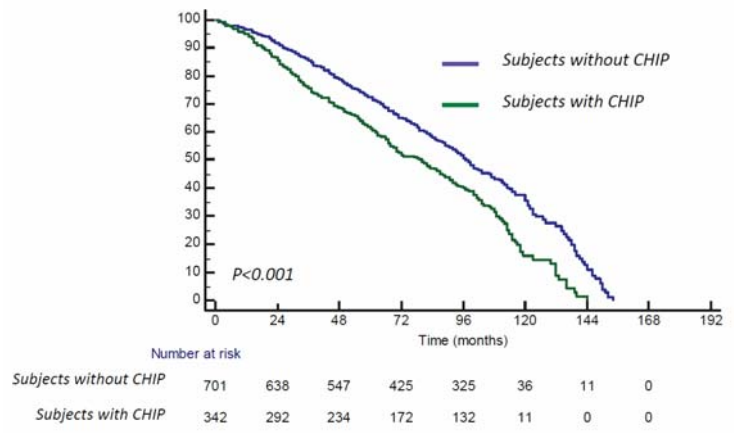
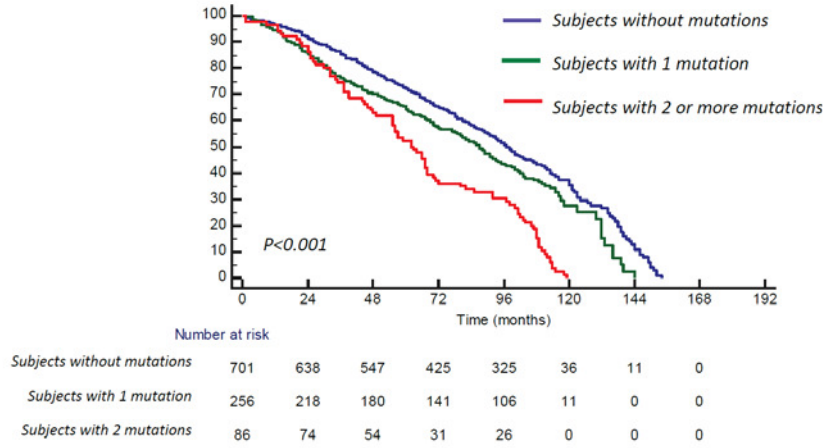


Fig.2

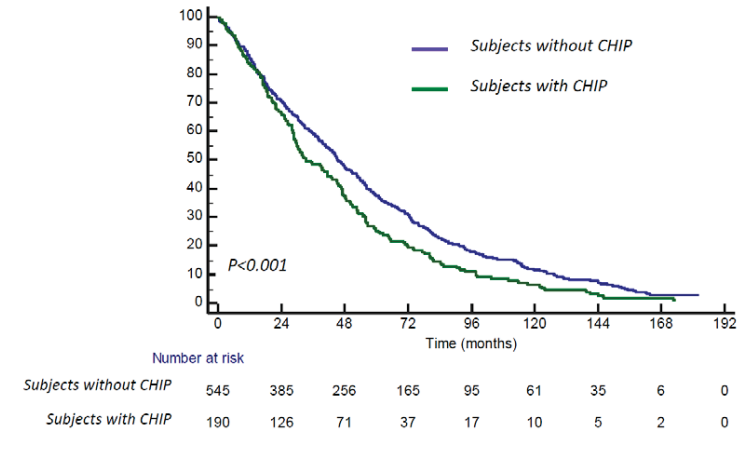
A) *Probability of survival according to the presence of CHIP in "Health_&_Anemia" cohort*



B) *Probability of survival according to the number of variants in "Health_&_Anemia" cohort*



C) *Probability of survival according to the presence of CHIP in "Monzino_80+" cohort*



D) *Probability of survival according to the number of variants in "Monzino_80+" cohort*

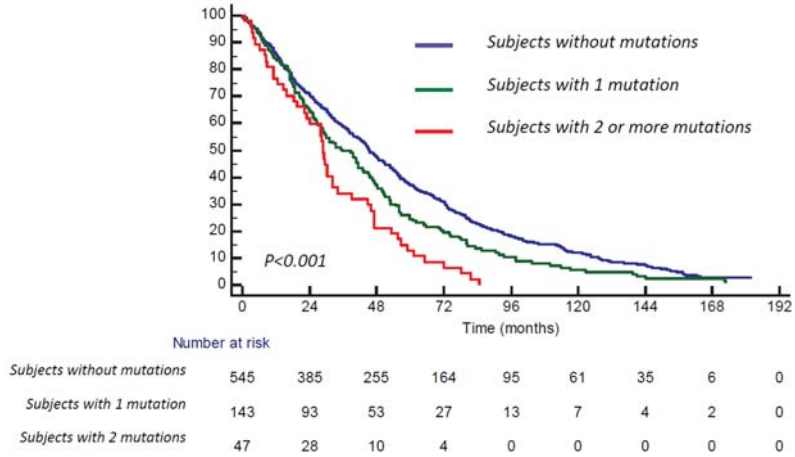


Fig.3

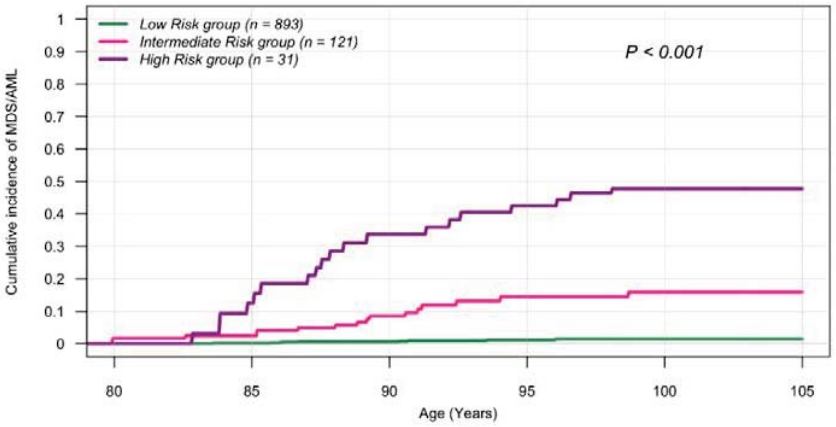
A) *Score to predict individual risk of developing myeloid neoplasms*

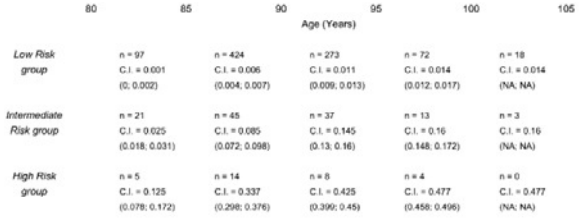
Parameter	HR (multivariable Cox regression analysis) [95%CI]	Score Value*
Presence of abnormal RBC-indices as defined as increased MCV (>98) and/or RDW (>14) value	2.61 [1.18-6.21]	1
Somatic mutation related to CHIP with VAF >0.096	3.24 [1.32-7.91]	1
Presence of TET2, DNMT3A, or ASXL1 mutations combined with other genetic lesions	5.02 [1.82-11.57]	2
Presence of spicing gene mutations	12.3 [6.78-23.85]	5

** Risk groups are defined as:*

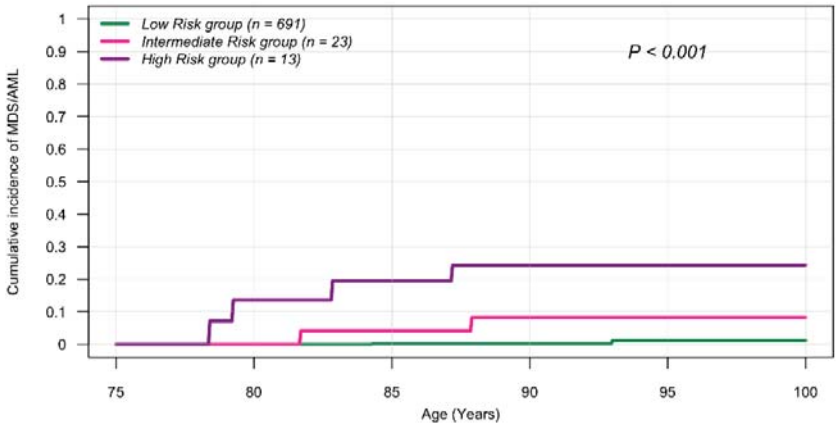
RISK GROUP	SCORE VALUE	RISK FACTORS
Low Risk	0-1	Absence of risk factors; Presence of one of the following risk factors: abnormal RBC-indices or CHIP with VAF >0.096
Intermediate Risk	2-4	≥2 of the following risk factors: abnormal RBC-indices; CHIP with VAF >0.096; TET2, DNMT3A, or ASXL1 mutations combined with other genetic lesions
High Risk	≥5	Spicing gene mutations with or without additional risk factors

B) *Cumulative incidence [95% CI] of myeloid neoplasms for oldest-old individuals stratified into three risk categories (learning cohort)*





C) *Validation of the score in an independent cohort of 727 subjects aged* ≥75<80 *years from "Health_&_Anemia" study.*



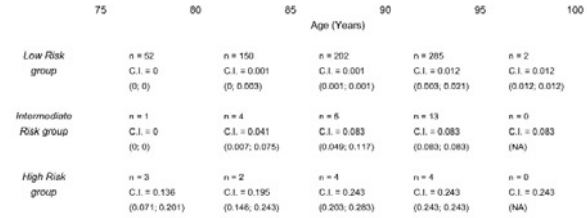
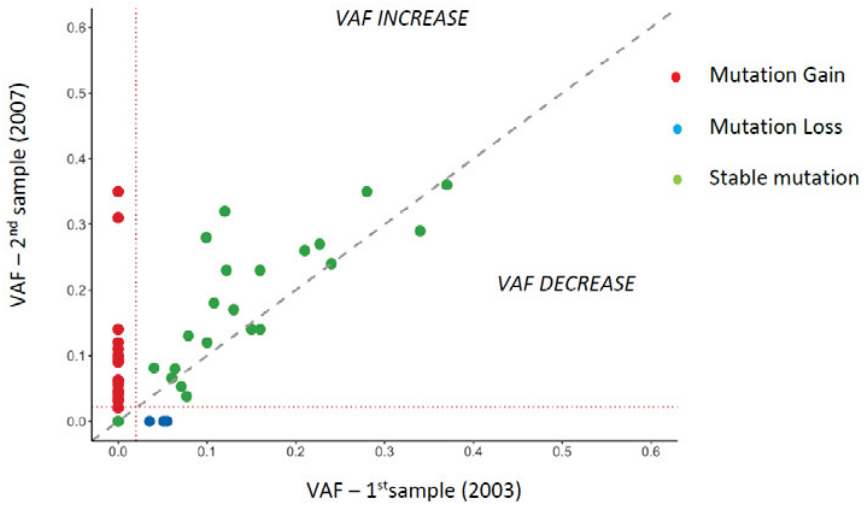
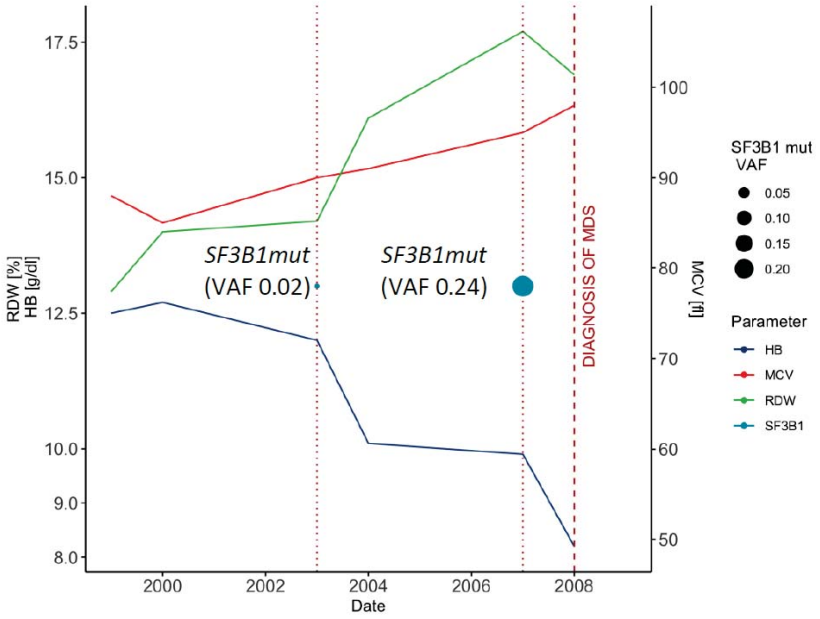


Fig.4 **A)** *Clonal evolution in subjects from "Health_&_Anemia" cohort with multiple sample available*



B) *Clonal evolution - Subject #269*



C) *Clonal evolution -Subject #1145*

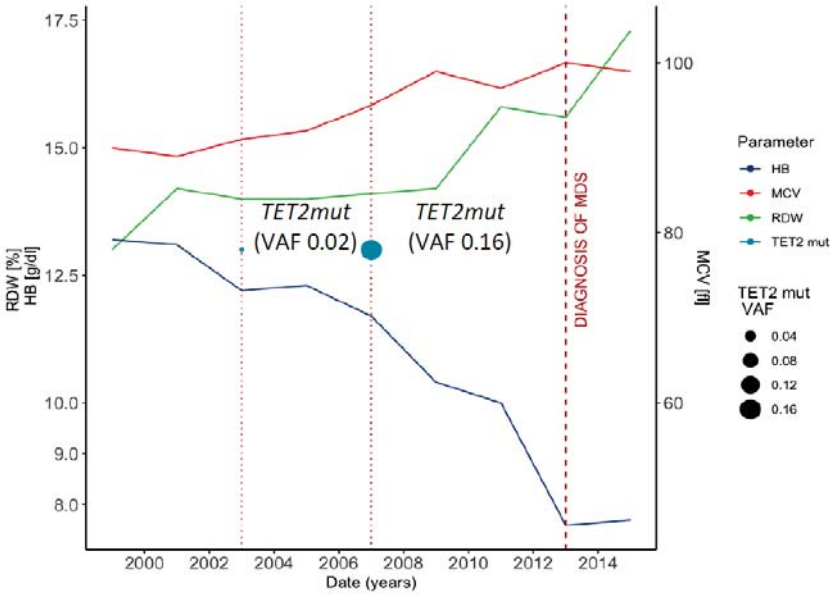
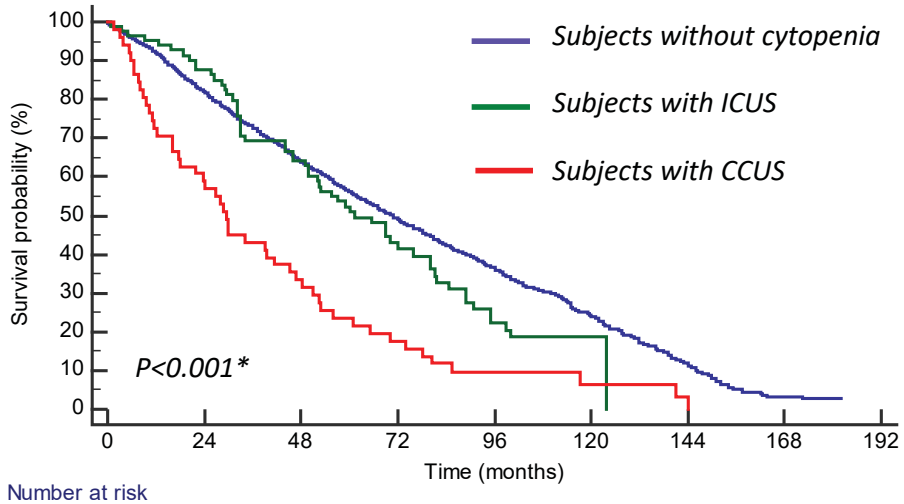

17.5
15.0
12.5
10.0
9.0
8.0
RDW [%]
HB [g/dl]
100
80
60
MCV [fl]
2000
2002
2004
2006
2008
2010
2012
2014
Date (years)
TET2mut
(VAF 0.02)
TET2mut
(VAF 0.16)
DIAGNOSIS OF MDS
Parameter
HB
MCV
RDW
TET2 mut
TET2 mut
VAF
0.04
0.08
0.12
0.16

Fig. 5

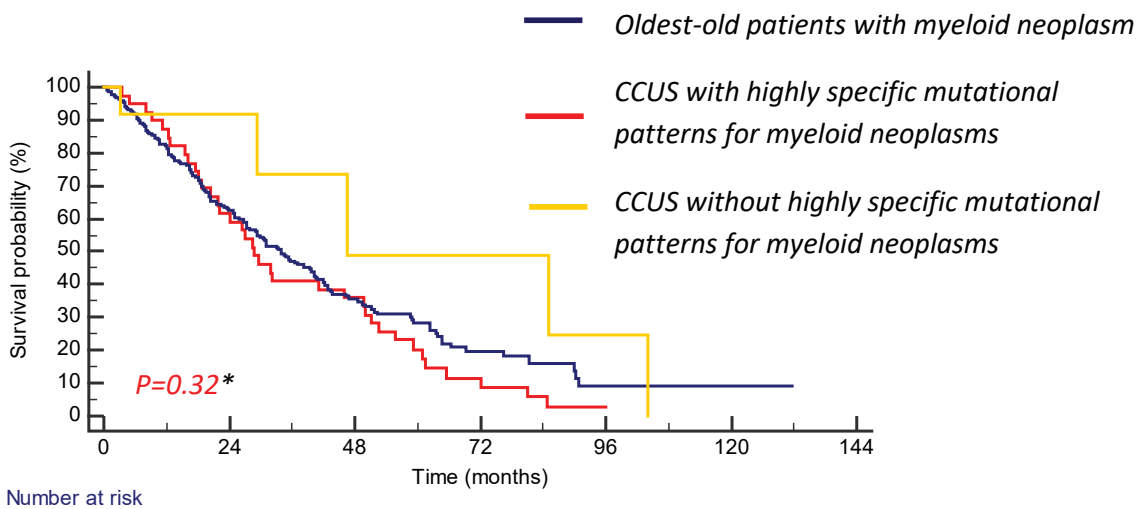
A) *Overall survival of subjects with unexplained cytopenia stratified according to the presence of mutations*



Number at risk									
Subjects without cytopenia	1661	1357	1055	777	561	117	52	8	0
ICUS	82	69	49	25	12	1	0	0	0
CCUS	51	30	17	9	4	2	1	0	0

* ICUS *vs*. subjects without cytopenia HR 0.81[0.75-0.97], P=0.35; CCUS *vs.* subjects without cytopenia HR 2.34 [1.98-3.04], P<0.001; CCUS *vs.* ICUS HR 2.06 [1.77-2.78], P=0.002

B) *Overall survival of CCUS with and without highly specific mutational pattern for myeloid neoplasms vs. oldest-old patients with myeloid neoplasms (myelodysplastic syndromes)*



Number at risk							
CCUS with specific mutations	39	24	14	4	0	0	0
MDS	255	120	45	17	2	1	0
CCUS without specific mutations	12	5	2	2	1	0	0

* CCUS with specific mutations *vs.* myeloid neoplasms HR 0.84 [0.75-0.97], P=0.29; CCUS with specific mutations *vs*. CCUS without specific mutations HR 2.05 [1.61-4.64], P=0.06)