Supplementary Material to:

CLONAL HEMATOPOIESIS IN ELDERLY TWINS: CONCORDANCE, DISCORDANCE AND MORTALITY

Next-Generation Sequencing panel

We designed a TruSeq Custom Amplicon panel (Illumina, San Diego, CA, USA) with an amplicon length of 250 base pairs (bp) covering more than 95% of known mutations identified in publications on clonal hemaptopoiesis[1–6].Library preparation was performed according to the manufacturer's protocol, including pre-PCR addition of random unique molecular identifier (UMI) nucleotide strands. Genes and chromosol coordinates are listed below:

Gene	**Chr**	**hg19 coordinates (start stop)**	
ASXL1	20	(310 25234	310 21081)
ASXL2	2	(25964888	26101101)
DNMT3A	2	(25457124	25536945)
IDH1	2	(209113424	209112994)
IDH2	15	(90631736	90632009)
PPM1D	17	(58740350	58740919)
RAD21	8	(117859733	117866713)
SF3B1	2	(198266563	198267800)
SRSF2	17	(74732237	74733248)
TET2	4	(106155075	106197718)
TP53	17	(7572833	7579961)
JAK2	9	(5073674	5073808)
GNB1	1	Full coding sequence	
GNAS	20	(57415162	57415896)
	20	(57478578	57480535)
	20	(57484212	57485886)
ETV6	12	(11803056	12043986)
CREBBP	16	(3786120	3786810)
	16	(3781300	3781802)
NRAS	1	(115256421	115256599)
	1	(115258671	115258781)
KRAS	12	(25380168	25380346)
	12	(25398208	25398318)
CBL	11	Full coding sequence	
BRCC3	X	(154299797	154348431)
BCOR	X	(39911356	39937188)

Sequence analysis

Each read of the 150bp paired-end raw FASTQ included a 6bp UMI tag. Using the extract program in the UMI-tools software (v0.5.0)[7], the UMI 6mers were removed from the read sequence, combined with the corresponding mate-pair into a 12bp identifier and moved to the read name files of each mate. Corresponding FASTQ files where aligned to the reference hg19 genome with the bwa-mem implementation of the Burrows–Wheeler algorithm[8]. PCR duplicates were removed based on the UMI information using the dedup program in UMI-tools. QC and visual inspection of the alignments and amplicon coverage were assessed with the R package TarSeqQC[9]. Identification of the variants on the filtered BAM files was performed with two variant callers; Freebayes[10] and VarDict[11]. All the variants were annotated using snpEff (v4.2)[12] for gene name, amino acid change, rare variants (ExAC, dbSNP), cancer mutation records (COSMIC) and effect predictor (VEP).

Variant calling

Curation of mutations was performed blinded to clinical data. A manual assessment of all variants was performed, which included visualization in Integrated Genome Viewer (Broad Institute, Cambridge, MA). Exclusion criteria were:

- Coverage below 200x
- Alternative variant with read count below 5
- Variant allele frequency below 2%
- Area with excessive local noise
- Strand biased call explained by sequencing error
- Synonymous changes
- Known sequencing artefact based on >1000 samples analyzed by the same panel
- Known as a common polymorphism in the normal population (>1% frequency in ExAC, TOPMED or 1000G)
- Annotated as benign polymorphism in ClinVar
- Known rare variants and missenses mutations with a variant allele frequency of 40-60%, and not previously described as pathogenic. In MZ twins the presence of rare variants in both twins confirmed a germline origin.

Additional assessment criteria supporting inclusion of variant

- Variants identified in multiple UMI families[13]
- Known COSMIC reference

For each individual gene we used the following filtering:

Gene	Chromosome	Accession	Reported mutations used for variant calling
ASXL1	20	NM_015338	Frameshift/nonsense/splice-site in exon 11-12
ASXL2	2	NM_018263	Frameshift/nonsense/splice-site
DNMT3A	2	NM_022552	Frameshift/nonsense/splice-site; Missense in p.292-350 / p.482-614/ p.634-912
IDH1	2	NM_005896	Missense at R132
IDH2	15	NM_002168	Missense at R140 and R172

PPM1D	17	NM_003620	Frameshift/nonsense in exon 5/6
RAD21	8	NM_006265	Frameshift/nonsense/splice-site
SF3B1	2	NM_012433	Missense in terminal HEAT domains (p.529-1201)
SRSF2	17	NM_003016	Missense/deletion involving p.P95
TET2	4	NM_001127208	TET2 Frameshift/nonsense/splice-site; Missense in conserved domains (p.1104-1481 and p.1843-2002)
TP53	17	NM_001126112	Frameshift/nonsense/splice-site; Missense in DNA-binding domain (p.95-288); Missense at P72 / R337
JAK2	9	NM_004972	JAK2 V617F and Missense/indel in aa range p.536-547
GNB1	1	NM_002074	Missense at K57 / I80
GNAS	20	NM_016592	GNAS Missense at R201
ETV6	12	NM_001987	Frameshift/nonsense/splice-site
CREBBP	16	NM_004380	Frameshift/nonsense/splice-site
NRAS	1	NM_002524	Missense at G12 / G13 / Q61
KRAS	12	NM_033360	Missense at G12 / G13 / Q61 / A146
CBL	11	NM_005188	Missense in Linker/RING finger domains p.345-434
BRCC3	X	NM_024332	Frameshift/nonsense/splice-site
BCOR	X	NM_001123385	Frameshift/nonsense/splice-site

Hematological cancer and cytopenia ICD-10 codes.

All diagnosis from the cohort was identified and we created two groups of diagnoses

Hematological cancers including the following ICD-10 diagnosis.

DD45 - Polycythemia vera
DD46 - Myelodysplastic syndromes
DD47 - Mast cell neoplasms of uncertain behavior
DC81 - Hodgkin lymphoma
DC82 - Follicular lymphoma
DC83 - Non-follicular lymphoma
DC84 - Mature T/NK-cell lymphomas
DC85 - Other specified and unspecified types of non-Hodgkin lymphoma
DC86 - Other specified types of T/NK-cell lymphoma
DC88 - Malignant immunoproliferative diseases and certain other B-cell lymphomas
DC90 - Multiple myeloma and malignant plasma cell neoplasms
DC91 - Lymphoid leukemia
DC92 - Myeloid leukemia
DC93 - Monocytic leukemia
DC94 - Other leukemias of specified cell type
DC95 - Leukemia of unspecified cell type
DC96 - Other and unspecified malignant neoplasms of lymphoid, hematopoietic and related tissue

The group of unspecified cytopenia included the following diagnoses

DD619A – Pancytopenia, unspecified DD693 - Immune thrombocytopenic purpura DD696 - Thrombocytopenia, unspecified DD613 - Idiopathic aplastic anemia DD63 - Anemia in chronic diseases classified elsewhere DD618 - Other specified aplastic anemias and other bone marrow failure syndromes DD649 - Anemia, unspecified DD694 - Other primary thrombocytopenia DD70 – Neutropenia

Supplementary Table 1a and b Cox regression model including the VAF grouped as maximum VAF<10% vs >=10%.

A

Variable	HR	95% CI	P
VAF < 10%	1.16	0.94-1.43	0.115
VAF >=10%	1.120	0.93-1.54	0.205
Age (years)	1.11	1.08-1.13	<0.001
Sex (male)	1.15	0.91-1.45	0.252
Smoking (1st tertile)	1.02	0.81-1.29	0.852
Smoking (2nd tertile)	1.22	0.96-1.55	0.101
Smoking (3rd tertile)	1.70	1.23-2.35	0.001

***VAF = Variant allele frequency**

B

Variable	HR	95% CI	P
VAF < 10%	1.12	0.91-1.38	0.299
VAF >=10%	1.16	0.91-1.49	0.267
Sex (male)	1.14	0.90-1.44	0.267
Smoking (1st tertile)	1.03	0.82-1.29	0.787
Smoking (2nd tertile)	1.26	1.00-1.59	0.053
Smoking (3rd tertile)	1.73	1.26-2.37	0.001

***VAF = Variant allele frequency**

In table **A** time since blood sample is used as the underlaying time scale whereas age is used as the underlying the time scale in **B**, and almost similar results were found.

Supplementary Table 2

Risk of developing hematological cancer, competing risk analysis according to CHIP mutational status

Variable	HR	95% CI	P
Mutation (yes/no)	**1.80**	**0.87-3.75**	**0.115**
Age (years)	**0.94**	**0.86-1.04**	**0.234**
Sex (male)	**0.88**	**0.42-1.82**	**0.722**
Smoking (1st tertile)	**0.88**	**0.32-2.43**	**0.808**
Smoking (2nd tertile)	**0.123**	**0.016-0.95**	**0.045**
Smoking (3rd tertile)	**1.04**	**0.44-2.48**	**0.932**

The risk of developing a hematological cancer (as defined above), was not associated with clonal hematopoiesis after adjusting for age, sex and tobacco consumption. A total of 27 twins developed a hematological cancer during follow up. Of those 19 were classified as a lymphoid neoplasm and eight as a myeloid neoplasm.

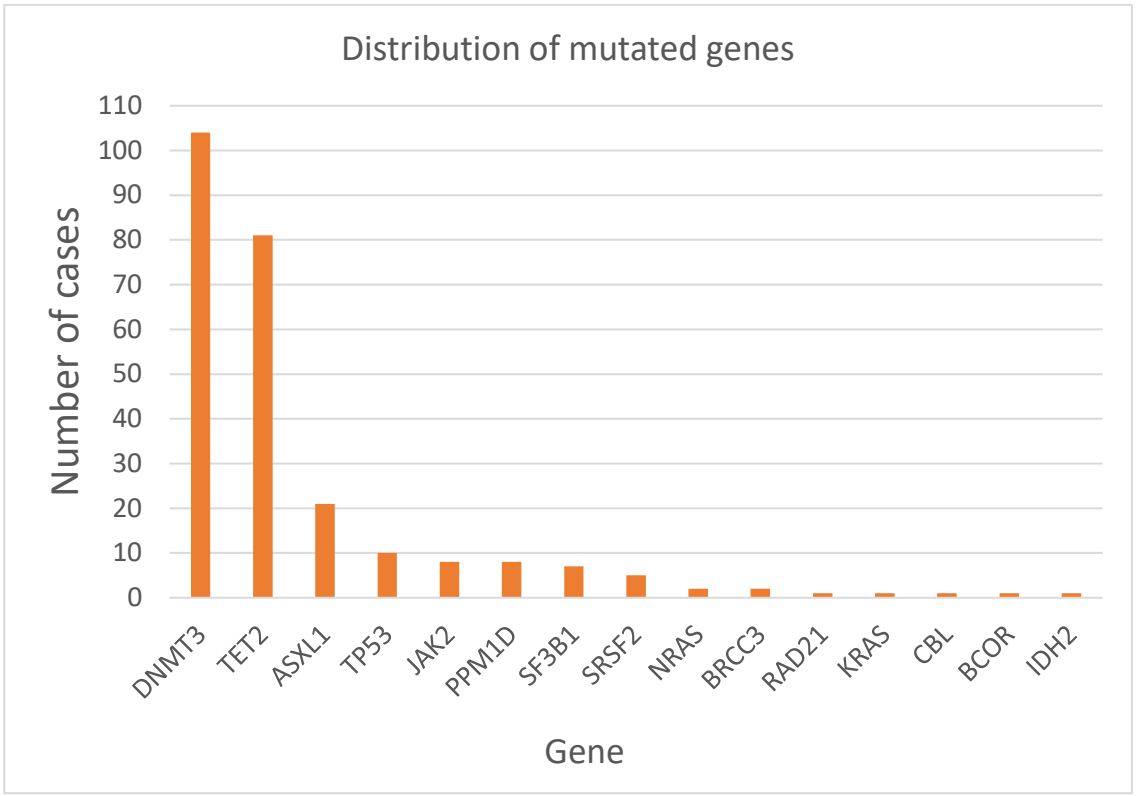
Supplementary Table 3

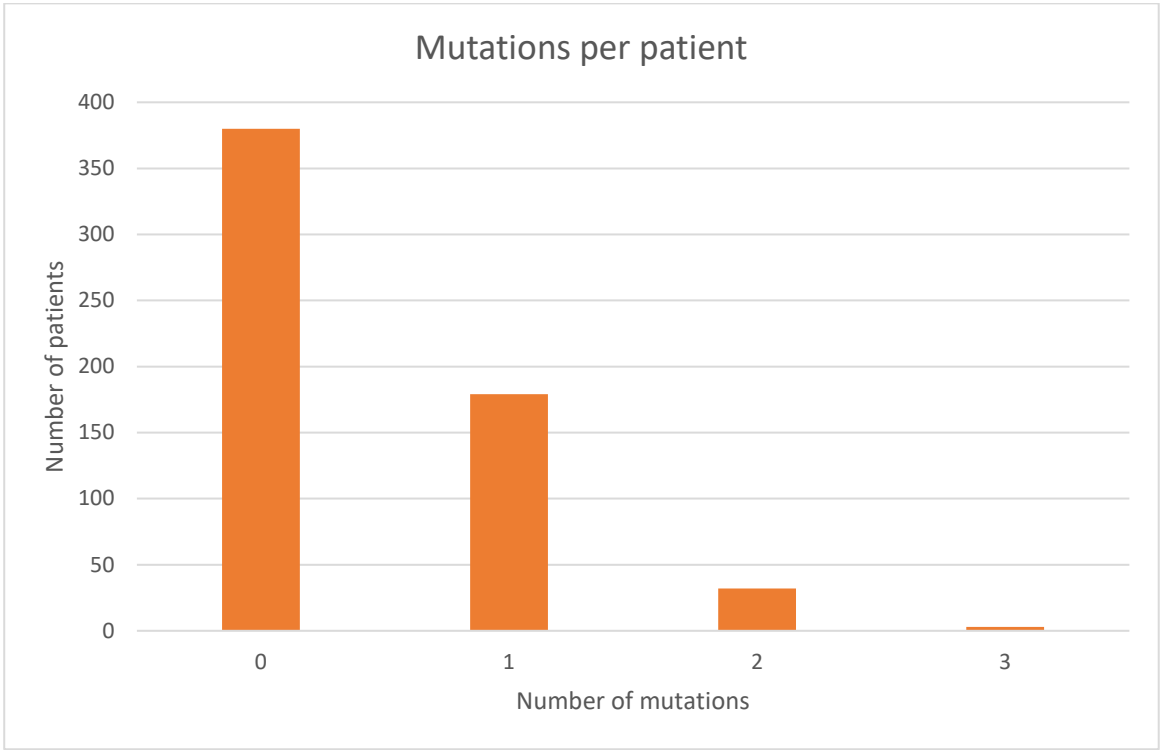
Risk of developing unspecified cytopenia, competing risk analysis according CHIP mutational status

Variable	HR	95% CI	P
Mutation (yes/no)	**1.59**	**0.99-2.54**	**0.052**
Age (years)	**0.96**	**0.91-1.02**	**0.158**
Sex (male)	**1.24**	**0.75-2.04**	**0.403**
Smoking (1st tertile)	**0.92**	**0.48-1.77**	**0.802**
Smoking (2nd tertile)	**0.63**	**0.31-1.30**	**0.211**
Smoking (3rd tertile)	**1.01**	**0.54-1.90**	**0.980**

A total of 74 twins developed an unspecified cytopenia during follow up, and as the only variable clonal hematopoiesis had a borderline significant association with the development of unspecified cytopenia after adjusting for age, sex and tobacco consumption.

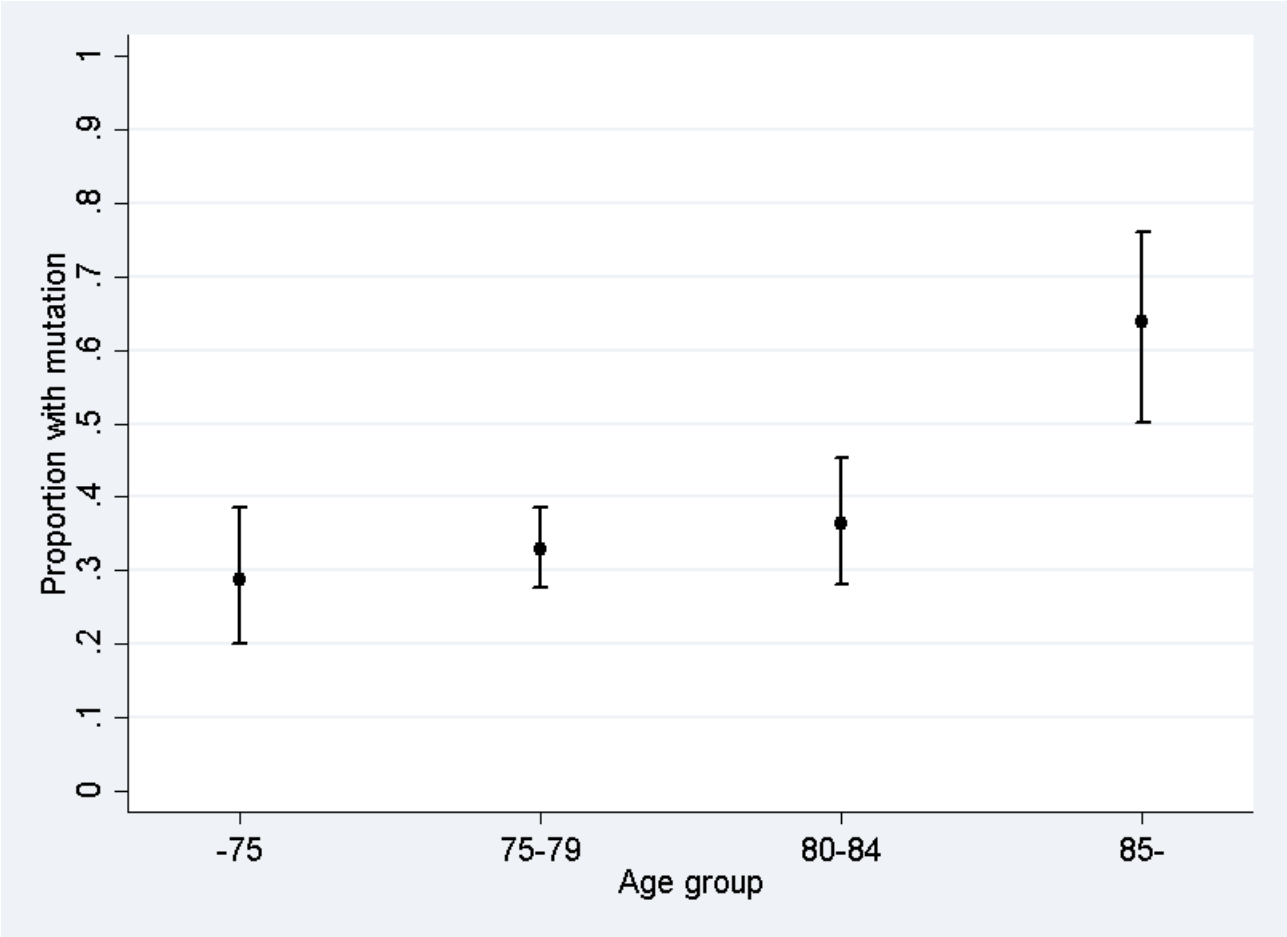
Supplementary Figure 1a and b





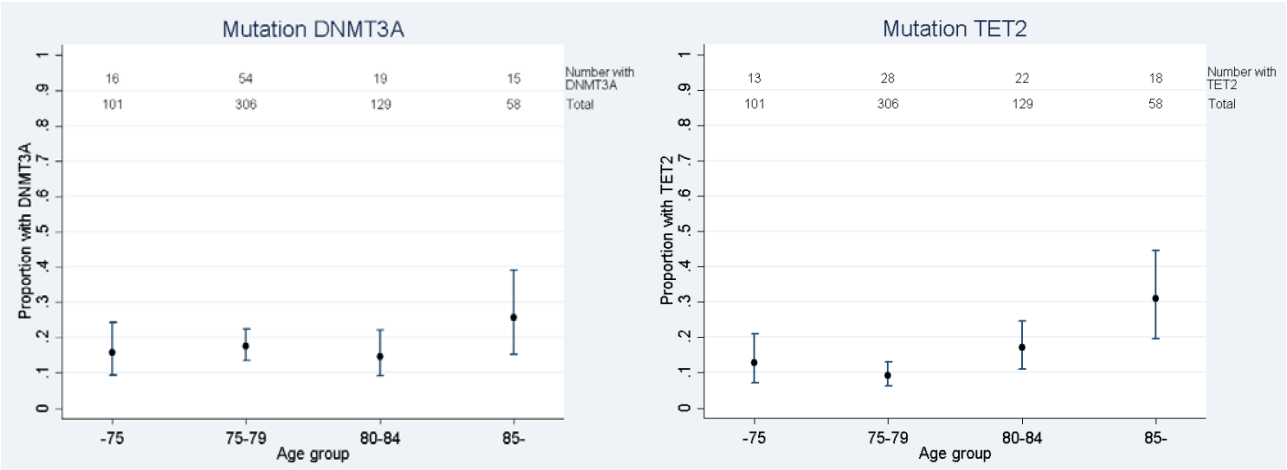
1a cases with a mutation in the affected gene and 1b number of different genes mutated per twin.

Supplementary Figure 2



Proportion of individuals with a mutation according to age group. There were 101 twins between 73-75 years, 306 between 75-79 years, 129 between 80-85 years and 58 were 85 years or above at the time of inclusion in the study in 1997.

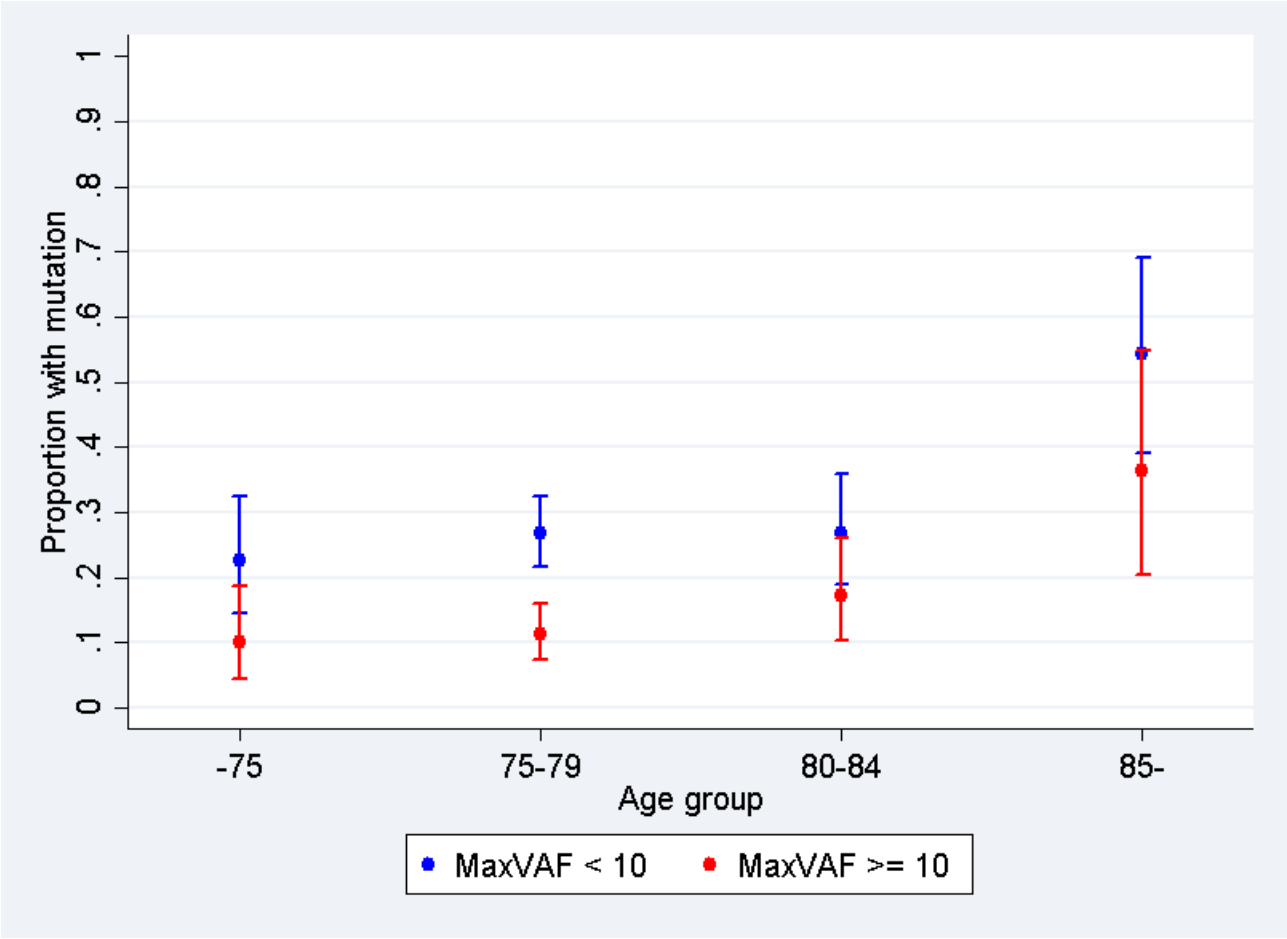
Supplementary Figure 3a and b.



A B

The distribution of mutations in (A) *DNMT3A* and (B) *TET2* according to age group. The total number and number affected in each age group can be seen above the barplot.

Supplementary figure 4



The proportion of samples grouped according to after the allele frequency of the mutations with the highest VAF within each case.

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