Supplementary Appendix

Supplement to: van Zeventer IA*, de Graaf AO*, Koorenhof-Scheele TN, van der Reijden BA, van der Klauw MM, Dinmohamed AG, Diepstra A, Schuringa JJ, Malcovati L, Huls G* and Jansen JH*. *Monocytosis and its association with clonal hematopoiesis in community-dwelling individuals: data from a prospective population-based cohort study.*

Supplementary Methods

Sample collection and biochemical measurements

Withdrawal of peripheral blood samples was done after an overnight fast. Thereafter, samples were directly processed for routine clinical chemistry assays at the University Medical Center Groningen. Complete and differential blood counts were determined on a XE2100-system (Sysmex, Japan). DNA was isolated from aliquots of peripheral blood specimens and stored in the Lifelines biobank for future use.

Definition of peripheral cytopenia or cytosis

The following cut-offs were used for definition of peripheral cytopenias: anemia, hemoglobin concentration <13.0 g/dL in men and < 12.0 g/dL in women; thrombocytopenia, platelet counts <150 x 10^9/L; and neutropenia, neutrophil counts <1.8 x 10^9/L. For cytosis, the following cut-offs were used: erythrocytosis, hemoglobin concentration >16.5 g/dL or hematocrit ≥48% in women or hemoglobin concentration >18.5 g/dL or hematocrit ≥52% in men; thrombocytosis, platelet count >400 x 109/L; leukocytosis, white blood cell count >10 x 10^9/L.

Design of targeted sequencing panel

The molecular inversion probe (MIP) design and protocol are based on previously described methods.[4] Modifications of this protocol are described below. Single molecule tagged molecular inversion probes (smMIPs) were designed for all target regions (Supplementary Table S1) using previously described procedures[5,6], in a tiling manner preferentially covering all target nucleotides with two smMIPs targeting both DNA strands independently. MIPs were designed to capture a target of 98 nucleotides and contain a single molecule tag consisting of a stretch of 10N nucleotides inserted between the backbone and ligation probe sequence. In case it was unavoidable to design smMIPs without common single nucleotide polymorphism variants in the respective capture arms, smMIPs were designed recognizing both alleles using degenerate nucleotides.

Library preparation and sequencing

Isolated DNA was sonicated using a Covaris R230 Focused-ultrasonicator (Covaris, Inc. MA, USA) with a standard protocol to obtain 400-bp fragments. Subsequently, library preparation and sequencing were performed as previously described[7] with the following adaptations. A volume of 7 µL containing 100 ng of genomic DNA was used as input per smMIP capture. Subsequently, 20 µL from the exonuclease-treated capture mixture was used for PCR in a total volume of 50 µL. The gDNA:smMIP ratio was set to 800:1 for high-quality blood-derived gDNA. The final pooled smMIP libraries were denaturated and diluted to a concentration of 1.1 nM. Sequencing was performed on a NovaSeq 6000 (Illumina, San Diego, CA) according to the manufacturer's protocol (300 cycles NovaSeq 6000 S1 reagent kit), resulting in 2 × 150 bp paired-end reads.

Data processing and variant calling

Bcl to fastq conversion and demultiplexing of barcoded reads was performed automatically and data was uploaded to a server running commercial analysis software (Sequence Pilot version 5.2.0, build 502 (JSI medical systems, Ettenheim, Germany). Single-molecule–directed assembly of duplicate reads to generate consensus reads after alignment was performed using the same software (Sequence Pilot, JSI medical systems, Ettenheim, Germany). Proper identification of sequence variants greatly relies on the settings used for variant calling, such as the minimal number of mutant unique smMIPs (>5 in our settings) and the minimal variant allele frequency (≥1%).

The following settings were used for variant calling using Sequence Pilot:

(i) Required Coverage/Min abs. cov., 20 combined
(ii) Mutations/Min abs. cov., 5 combined
(iii) (iii) Min % cov., 1% per dir

After variant calling using the commercial software, all variants were manually inspected and curated using a cut-off of 10 reads for minimal absolute coverage and in-house databases for recurrent artifacts, polymorphisms and mutations. Furthermore, Alamut Visual v2.10 (Interactive Biosoftware, 5 Rouen, France) and publicly available databases such as dbSNP, COSMIC, ClinVar, gnomAD, ESP were used to identify rare polymorphisms, which were excluded.

Isolation and sequencing of white blood cell subfractions

After blood withdrawal, cells were processed within 24 hours. Full blood was mixed 1:1 with FCS containing 20% DMSO. Samples were frozen in a controlled manner and stored in liquid nitrogen in the Lifelines biobank until further use. Cells were thawn with DNAseI in FSC containing Heparin and MgSO4. After washing twice with FACS buffer (PBS containing 1% FCS and 2mM EDTA), cells were stained in FACS buffer with CD45 - BUV395 (BD biosciences, 563792), CD15 - APCFire750 (Biolegend, 323041), CD14 – fitc (Biolegend, 325604), CD19 – ECD (Beckman Coulter, A07770), CD3 – PE (Beckman Coulter, A07747) and LIVE/DEAD fixable aqua dead cell stain (Invitrogen, L34965). Cells were measured on the cellsorter (BD FACSAria SORP) and viable Monocytes (CD14), Granulocytes (CD15) and T-lymphocytes (CD3) were selected. DNA was isolated from sorted and bulk (sample before sort) and amplified in duplo using the repli-G Midi kit (Qiagen, 150045).

ICD-O codes used to identify myeloid malignancies

The Netherlands Cancer Registry (NCR) codes disease morphology according to the International Classification of Diseases for Oncology (ICD-O).[8] The ICD-O first edition was used for case ascertainment until 1992, and from 1993-2000 the second edition (ICD-O-2) was used. The Lifelines study population was included starting in 2006. As a result, the third edition of the ICD-O (ICD-O-3, used in the NCR 2001 to 2011) and the updated ICD-O-3 (used in the NCR from 2012 onwards) were used to identify incident cases of hematological malignancies, including myeloid malignancies. We used ICD-O code 9945 to identify cases of chronic myelomonocytic leukemia.

R packages for analysis and visualization

Cox proportional hazards and competing risk regression analyses as well as visualization of survival were performed using R packages *survminer* (https://cran.r-project. org/web/packages/survminer/index.html), *survival* (http://cran.r-project. org/web/packages/survival/index.html), *cmprsk* (http://cran.r-project.org/web/packages/cmprsk/index.html) and *riskRegression* (https://cran.r-project.org/web/packages/riskRegression/index.html). The R package *compareGroups* (https://github.com/isubirana/compareGroups) was used for construction of tables with characteristics. All remaining figures were generated using *ggplot2* (https://github.com/tidyverse/ggplot2).

Supplementary Table 1. Overview of genes and regions in the sequencing panel.

Gene	Reference transcript	ENSEMBL reference transcript	Exon	Targeted codons/region
ASXL1	NM_015338	ENST00000375687	13 (partially)	exon 13
BRAF	NM_004333.4	ENST00000288602	15 (partially)	codon 600
CALR	NM_004343	ENST00000316448	9	exon 9
CBL	NM_005188	ENST00000264033	8-9	exon 8 and 9
CSF3R	NM_156039	ENST00000373103	14, 17	codon 618, 615 and exon 17
DNMT3A	NM_175629	ENST00000264709	2-23 (all coding exons)	all coding exons
ETNK1	NM_018638	ENST00000266517	3 (partially)	codon 243-244
EZH2	NM_004456	ENST00000320356	2-20 (all coding exons)	all coding exons
FLT3_835	NM_004119	ENST00000241453	20 (partially)	codon 835-842
IDH1	NM_005896	ENST00000415913	4 (partially)	codon 132
IDH2	NM_002168	ENST00000330062	4 (partially)	codon 140, 172
JAK2	NM_004972	ENST00000381652	12, 14 (partially)	codon 617 and exon 12
KIT	NM_000222	ENST00000288135	8 (partially), 17 (partially)	codon 816, 419
KRAS	NM_004985	ENST00000256078	2-3 (partially)	a.o. codon 12, 13, 61
MPL	NM_005373	ENST00000372470	10 (partially)	codon 515, 505
MYD88	NM_002468.4	ENST00000417037	4-5 (partially)	codon 265 and 232
NOTCH1	NM_017617.4	ENST00000277541	34 (partially)	codon 2514
NPM1	NM_002520	ENST00000517671	11 (partially)	codon 288-290
NRAS	NM_002524	ENST00000369535	2-3 (partially)	a.o. codon 12, 13, 61
RUNX1	NM_001754	ENST00000437180	2-9 (all coding exons)	all coding exons
SETBP1	NM_015559	ENST00000282030	4 (partially)	codon 850-910
SF3B1	NM_012433	ENST00000335508	13-16	codon 575-790
SRSF2	NM_003016	ENST00000392485	1 (partially)	codon 95, 96
TET2	NM_001127208	ENST00000380013	3-11 (all coding exons)	all coding exons
TP53	NM_000546	ENST00000269305	2-11 (all coding exons)	all coding exons
U2AF1	NM_006758	ENST00000291552	2, 6 (partially)	codon 34, 157
WT1	NM_024426	ENST00000332351	7, 9	exon 7 and 9

Supplementary Table 2. Characteristics and peripheral blood counts of cases with monocytosis (n=167) and controls (n=501), stratified by the presence of clonal hematopoiesis.

	Controls without CH	Controls with CH		Monocytosis without CH	Monocytosis with CH	
	N=323	N=178	P-value[1]	N=82	N=85	P-value[2]
Age (years)	67.0 [62.0;71.0]	69.0 [64.2;73.0]	0.001	66.0 [62.0;70.0]	70.0 [66.0;75.0]	0.001
Male sex	136 (76.4%)	233 (72.1%)	0.351	59 (72.0%)	64 (75.3%)	0.753
Monocyte count (10^9/L)	0.53 (0.16)	0.54 (0.14)	0.398	1.10 (0.11)	1.14 (0.12)	0.023
WBC count (10^9/L)	6.09 (1.59)	6.06 (1.50)	0.807	8.58 (1.65)	8.61 (1.80)	0.896
Neutrophil count (10^9/L)	3.33 (1.21)	3.44 (1.23)	0.350	4.91 (1.46)	4.85 (1.56)	0.803
Basophil count (10^9/L)	0.03 (0.02)	0.03 (0.02)	0.214	0.04 (0.02)	0.04 (0.03)	0.168
Eosinophil count (10^9/L)	0.21 (0.14)	0.20 (0.15)	0.502	0.23 (0.13)	0.27 (0.16)	0.093
Lymphocyte count (10^9/L)	1.99 (0.59)	1.85 (0.59)	0.009	2.30 (0.71)	2.30 (0.82)	0.937
Hemoglobin concentration (g/dL)	14.6 (1.25)	14.4 (1.25)	0.104	14.6 (1.11)	14.6 (1.53)	0.951
Erythrocyte count (10^9/L)	4.82 (0.38)	4.73 (0.41)	0.014	4.80 (0.37)	4.75 (0.49)	0.435
Hematocrit (L/L)	0.44 (0.03)	0.43 (0.03)	0.299	0.44 (0.03)	0.44 (0.04)	0.794
Platelet count (10^9/L)	232 (51.2)	224 (52.2)	0.097	257 (63.1)	251 (66.9)	0.541
MCV (fL)	90.4 (4.30)	91.5 (3.96)	0.003	91.6 (4.58)	92.3 (3.71)	0.281
Concurrent cytopenia*	39 (12.1%)	27 (15.2%)	0.40	5 (6.10%)	10 (11.8%)	0.313
Concurrent cytosis[$]	7 (2.2%)	6 (3.4%)	0.558	19 (23.2%)	19 (22.4%)	1.000

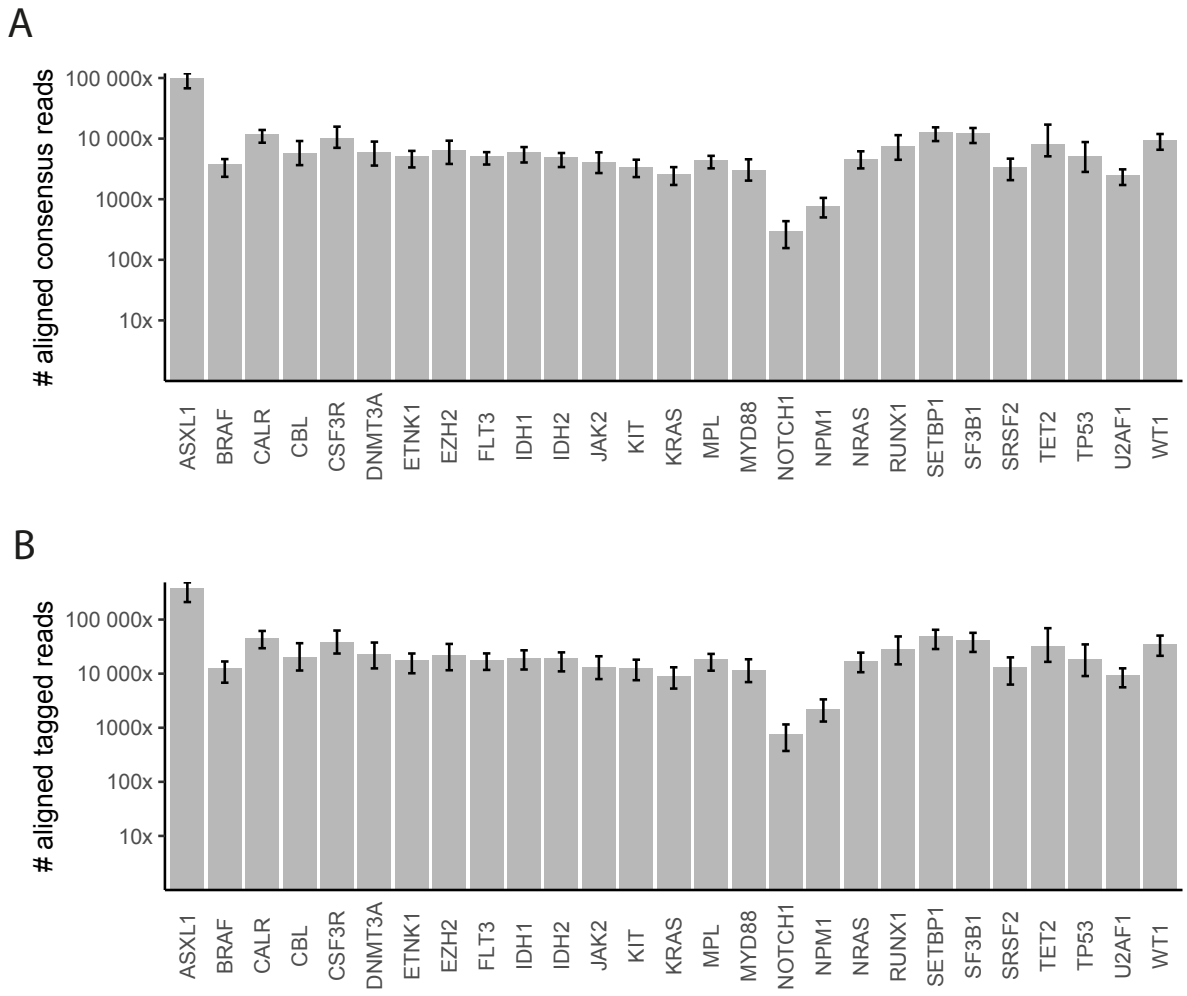
MCV, mean corpuscular volume; CH, clonal hematopoiesis; WBC, white blood cell. *A concurrent cytopenia was defined as follows: anemia, hemoglobin concentration <12.0 g/dL in women or <13.0 g/dL in men; thrombocytopenia, platelet count <150 x 10^9/L; neutropenia, absolute neutrophil count <1.8 x 10^9/L. [$]A concurrent cytosis was defined as follows: erythrocytosis, hemoglobin concentration >16.5 g/dL or hematocrit ≥48% in women or hemoglobin concentration >18.5 g/dL or hematocrit ≥52% in men; thrombocytosis, platelet count >400 x 10^9/L; leukocytosis, white blood cell count >10 x 10^9/L. [1]P-value for comparison of controls with and without CH. [2]P-value for the comparison of monocytosis cases with and without CH.

Supplementary Table 2. Characteristics and peripheral blood counts of cases with persistent or corrected monocytosis or loss to follow-up.

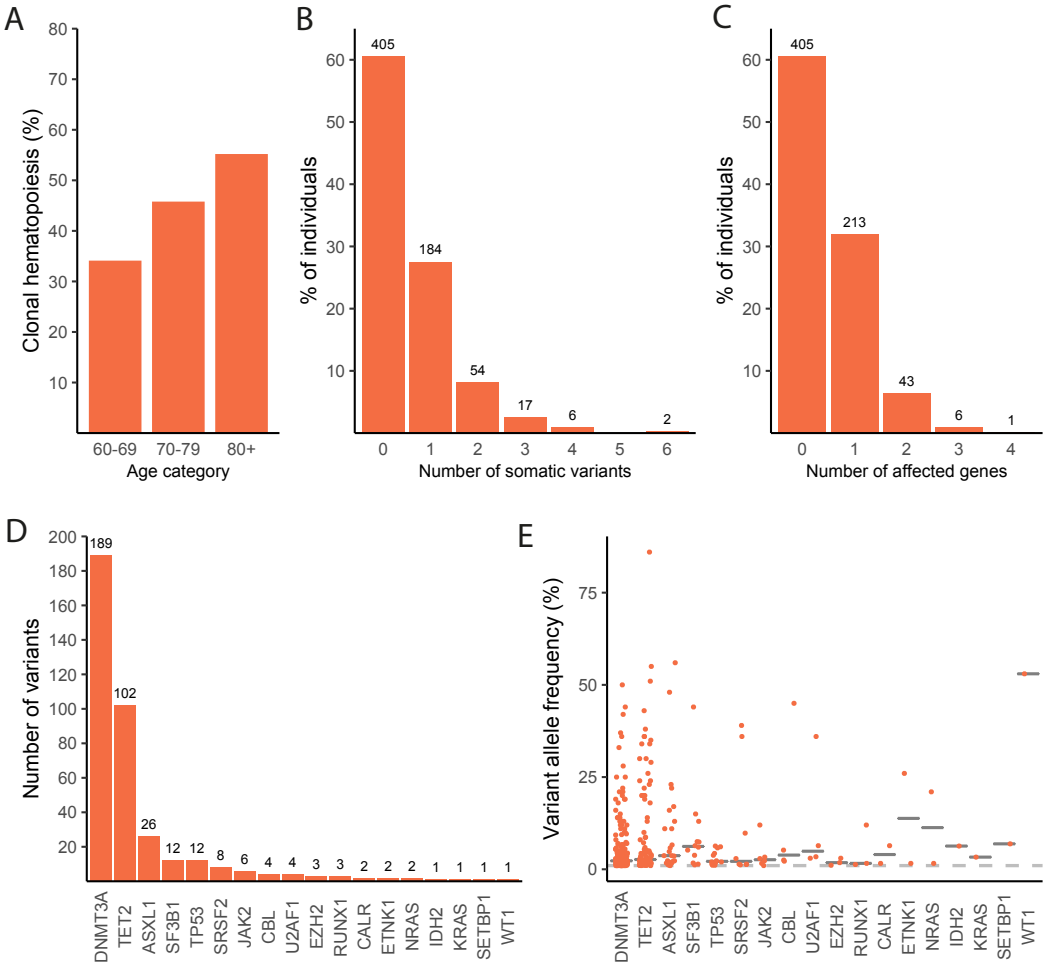
	Corrected	No follow-up	Persistent		
	N=72	N=65	N=30	P-value	N
Age (years)	66.5 [62.8;70.2]	70.0 [65.0;75.0]	67.0 [62.0;70.0]	<0.001	16644
Male sex	54 (75.0%)	45 (69.2%)	24 (80.0%)	<0.001	16644
Monocyte count (10^9/L)	1.10 (0.11)	1.13 (0.13)	1.14 (0.12)	0.000	16644
WBC count (10^9/L)	8.42 (1.78)	8.66 (1.60)	8.89 (1.83)	<0.001	16643
Neutrophil count (10^9/L)	4.68 (1.56)	5.04 (1.46)	5.01 (1.47)	<0.001	16644
Basophil count (10^9/L)	0.04 (0.03)	0.04 (0.03)	0.05 (0.03)	<0.001	16644
Eosinophil count (10^9/L)	0.24 (0.13)	0.25 (0.16)	0.29 (0.15)	<0.001	16644
Lymphocyte count (10^9/L)	2.36 (0.79)	2.19 (0.76)	2.41 (0.73)	<0.001	16644
Hemoglobin concentration (g/dL)	14.8 (1.15)	14.3 (1.59)	14.9 (1.06)	<0.001	16643
Erythrocyte count (10^9/L)	4.85 (0.42)	4.70 (0.48)	4.75 (0.33)	<0.001	16643
Hematocrit (L/L)	0.44 (0.03)	0.43 (0.04)	0.44 (0.03)	<0.001	16643
Platelet count (10^9/L)	250 (61.5)	256 (71.3)	261 (59.6)	<0.001	16629
MCV (fL)	91.7 (3.92)	91.7 (4.61)	93.3 (3.50)	<0.001	16643
Concurrent cytopenia*	3 (4.17%)	9 (13.8%)	3 (10.0%)	0.107	16630
Concurrent cytosis$^{\$}$	14 (19.4%)	17 (26.2%)	7 (23.3%)	<0.001	16629
hsCRP (mg/L)	2.90 [2.00;7.80]	4.15 [1.42;8.00]	2.45 [1.40;4.05]	<0.001	5599
Deceased	7 (9.72%)	25 (38.5%)	4 (13.3%)	<0.001	16644
Number of medications used$^{\#}$	3.00 [1.00;5.00]	4.00 [1.00;6.00]	3.00 [1.25;4.75]	<0.001	16644

MCV, mean corpuscular volume; CH, clonal hematopoiesis; hsCRP, high sensitive CRP; WBC, white blood cell. *A concurrent cytopenia was defined as follows: anemia, hemoglobin concentration <12.0 g/dL in women or <13.0 g/dL in men; thrombocytopenia, platelet count <150 x 10^9/L; neutropenia, absolute neutrophil count <1.8 x 10^9/L. $^{\$}$A concurrent cytosis was defined as follows: erythrocytosis, hemoglobin concentration >16.5 g/dL or hematocrit ≥48% in women or hemoglobin concentration >18.5 g/dL or hematocrit ≥52% in men; thrombocytosis, platelet count >400 x 10^9/L; leukocytosis, white blood cell count >10 x 10^9/L. $^{\#}$As a proxy for comorbidity.

Supplementary Figure 1. Coverage across all included samples. Graphs below show the number of aligned consensus reads (A) and the number of aligned reads (B) for all genes included in the panel, for the entire sequenced cohort (n=668). Columns and error bars indicate median and interquartile range.

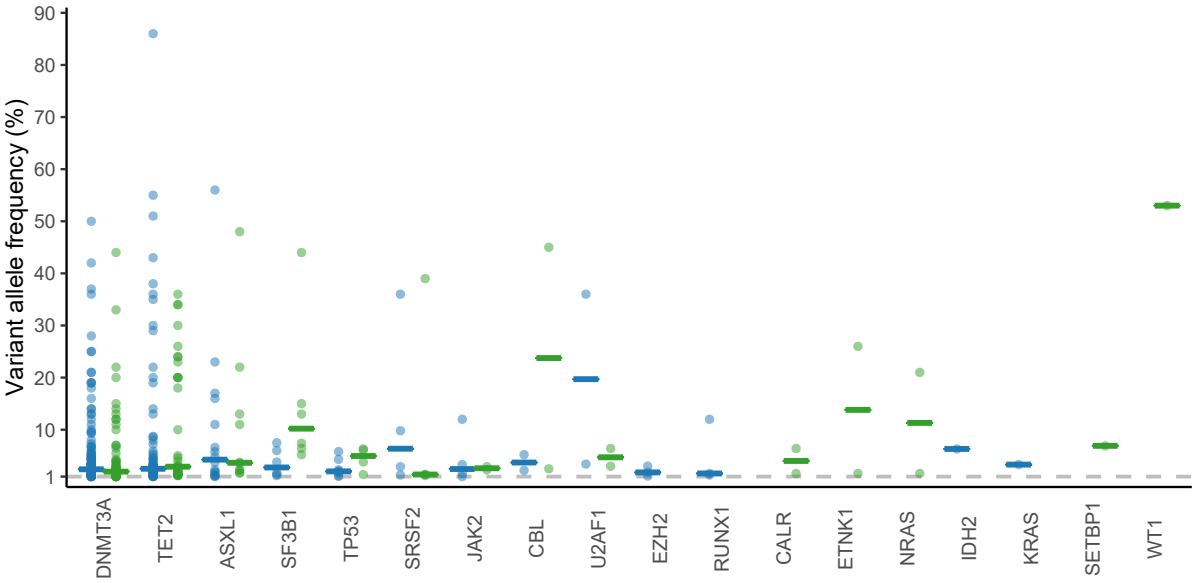


Supplementary Figure 2. Mutational spectra for the entire cohort. The graphs below show (A) the age-related emergence of clonal hematopoiesis, (B) the number of detected somatic variants and (C) the number of affected genes in the entire cohort with next-generation sequencing data (n=668). In addition, (D) the frequency of all somatic variants detected and (E) the distribution in variant allele frequency for all detected somatic variants are shown.

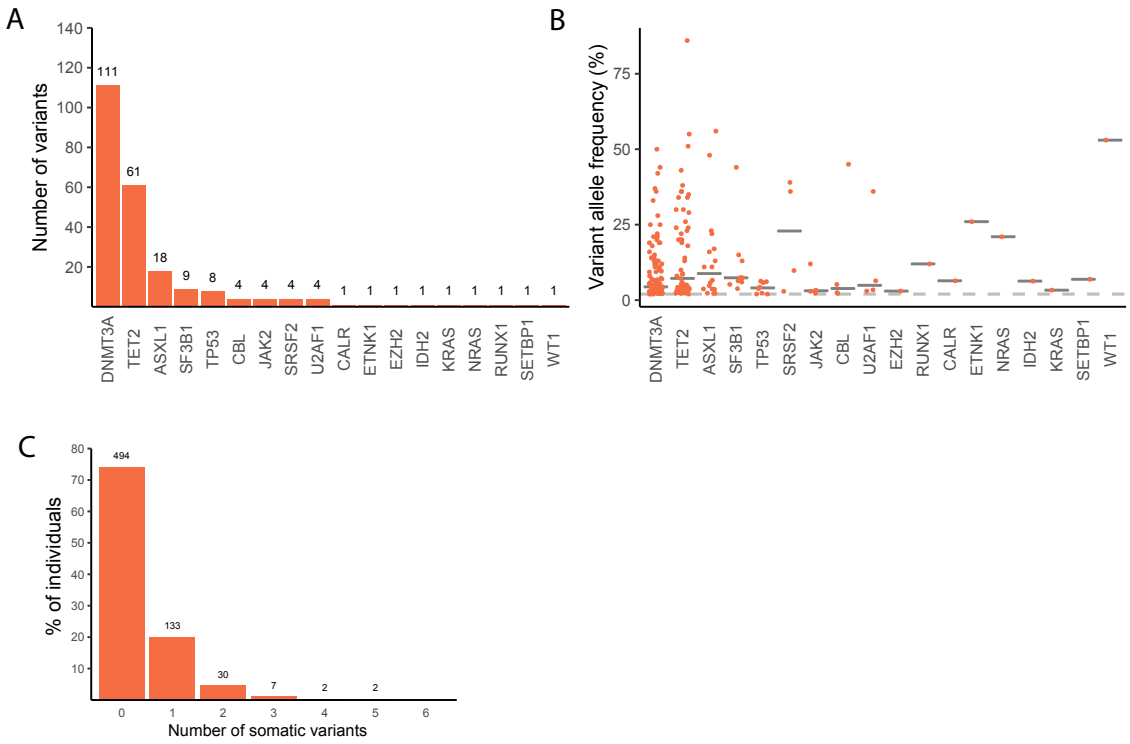


Supplementary Figure 3. Variant allele frequencies for detected variants in the monocytosis and control cohort.

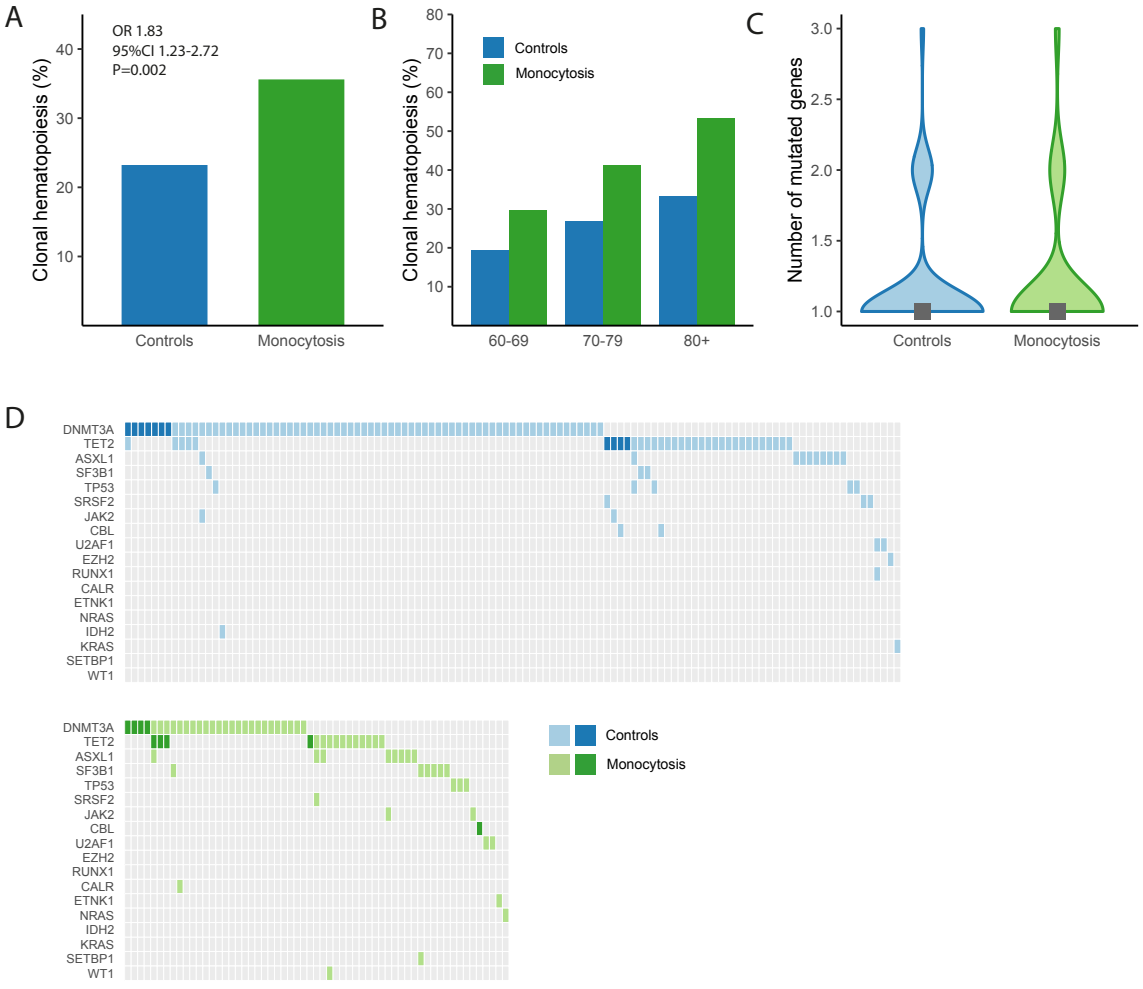
Individual data points correspond to somatic variants detected in the monocytosis (green, n=167) and control (blue, n=501) cohort. The median variant allele frequency for the variants detected in each gene is indicated.



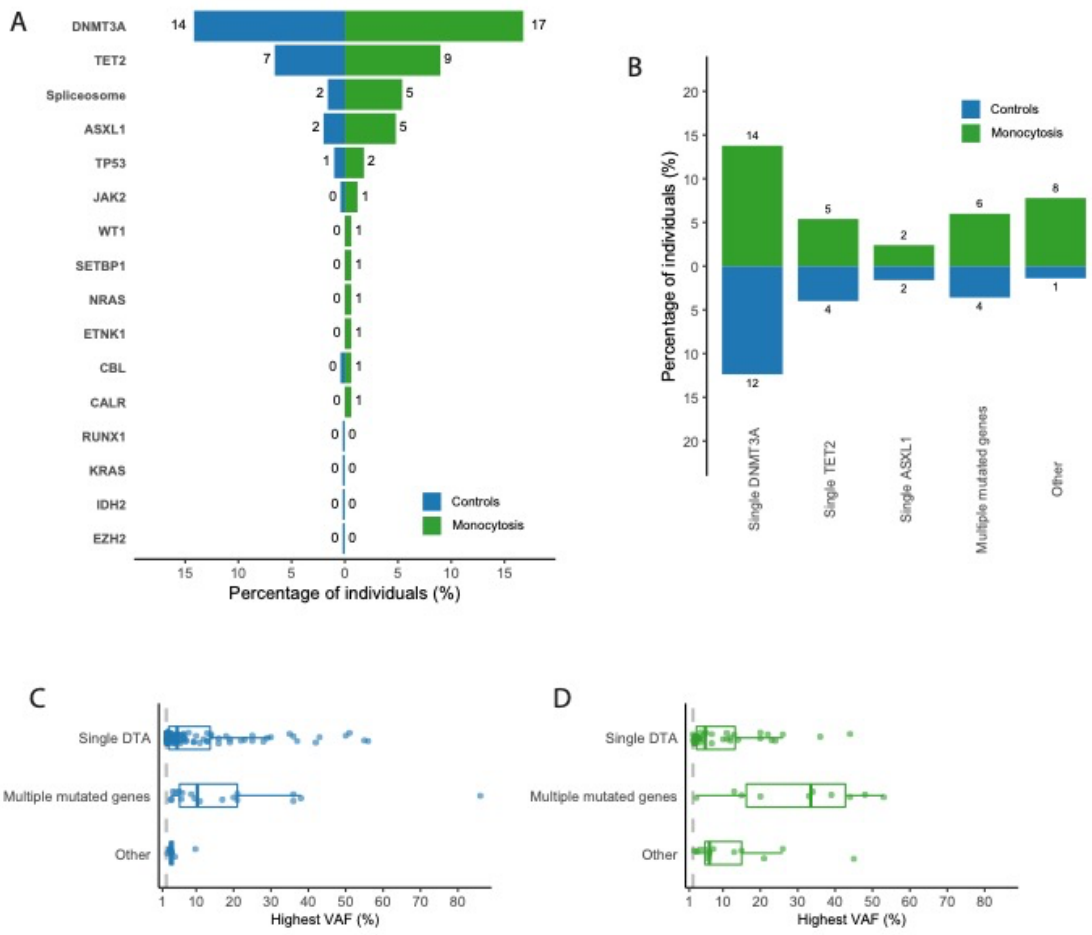
Supplementary Figure 4. Mutational spectrum restricted to "CHIP" [9]. Shown below is the mutational spectrum detected in the entire cohort with next-generation sequencing data (n=668) when restricted to n=232 variants detected at ≥2% variant allele frequency (VAF). The graphs below show (A) the frequency of all somatic variants detected and (B) the distribution in variant allele frequency for all detected somatic variants as well as (C) the number of detected somatic variants per individual.



Supplementary Figure 5. Case-control comparison of mutational spectrum restricted to "CHIP" [9] (I). Shown below are analyses for the comparison of mutational spectrum between monocytosis cases and controls, restricted to variants detected ≥2% VAF. (A) Prevalence of CH among all individuals with monocytosis (n=167) as compared to 1:3 matched controls (n=501). (B) Prevalence of CH according to age for individuals with monocytosis and controls. (C) Violin plot showing the distribution in the number of mutated genes for individuals with CH in the monocytosis (green) and control (blue) cohort. Gray rectangles indicate the median number. (D) Mutational landscape for the control (blue, top) and monocytosis (green, bottom) cohort. A darker shade indicates multiple mutations in the same gene.

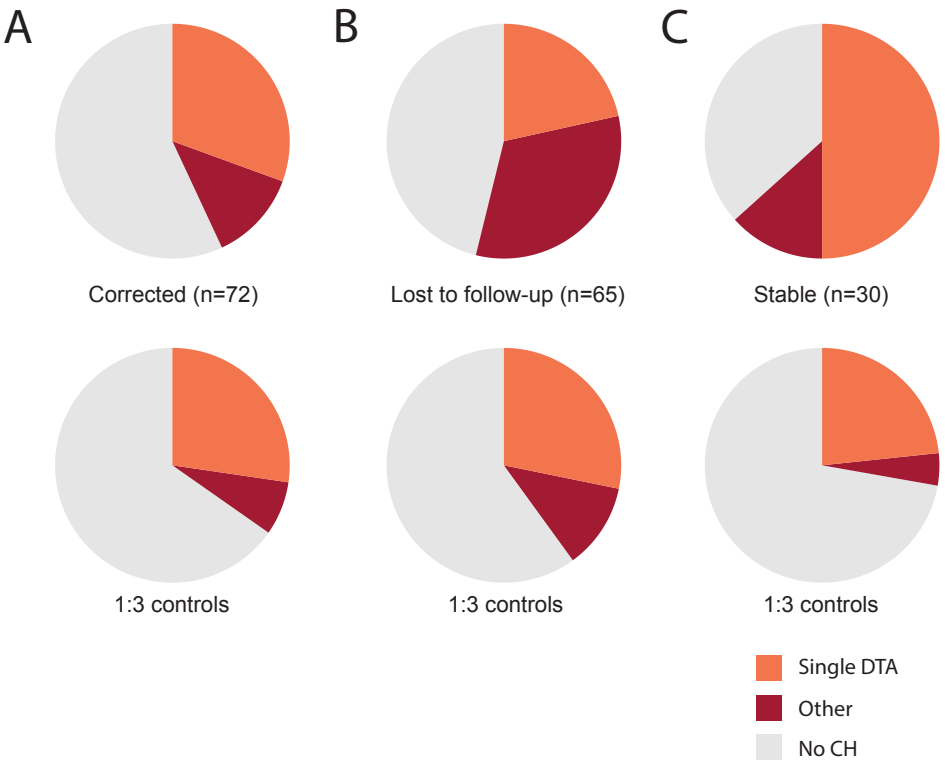


Supplementary Figure 6. Case-control comparison of mutational spectrum restricted to "CHIP" [9] (II). Shown below are analyses for the comparison of mutational spectrum between monocytosis cases and controls, restricted to variants detected ≥2% VAF. (A) Pyramid plot indicating the proportion of individuals with detected gene mutations within the monocytosis (green) and control (blue) cohort. The category of spliceosome mutations includes SF3B1, SRSF2 and U2AF1. The proportion of individuals carrying the gene mutation is given. (B) Bar plot showing the proportion of monocytosis cases (green, top) and controls (blue, bottom) with mutational spectra confined to mutated DNMT3A, TET2 or ASXL1, or multiple mutated genes. The category 'other' denotes isolated gene mutations other than DNMT3A, TET2 or ASXL1. The proportion of individuals for each category is given. (C) Highest detected VAF according to mutational spectrum for monocytosis cases (green) and control (blue). Individuals were classified as carrying CH confined to mutated DNMT3A, TET2 or ASXL1 (isolated DTA), CH involving multiple mutated genes and other isolated gene mutations (other). Boxes represent median, first and third quartiles. DTA, DNMT3A, TET2 or ASXL1; VAF, variant allele frequency.



Supplementary Figure 7. Mutational spectra for individuals according to the stability of monocytosis over time.

Subgroups of individuals with corrected monocytosis (n=72), without follow-up (n=65), and with stable monocytosis (n=30) are compared to their respective 1:3 matched controls. The proportion of individuals without clonal hematopoiesis (CH), with CH restricted to mutated DNMT3A, TET2 or ASXL1 (DTA) and with other mutational spectra is shown.



References

1. Sediq R, van der Schans J, Dotinga A, et al. Concordance assessment of self-reported medication use in the Netherlands three-generation Lifelines Cohort study with the pharmacy database IADB.nl: The PharmLines Initiative. Clin Epidemiol. 2018;10:981-989.
2. Slagter SN, van Vliet-Ostaptchouk JV, van Beek AP, et al. Health-Related Quality of Life in Relation to Obesity Grade, Type 2 Diabetes, Metabolic Syndrome and Inflammation. PLoS One 2015;10:e0140599.
3. Alberti KGMM, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009;120:1640-5.
4. O'Roak BJ, Vives L, Fu W, et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science 2012;338:1619-1622.
5. Boyle EA, O'Roak BJ, Martin BK, Kumar A, Shendure J. MIPgen: optimized modeling and design of molecular inversion probes for targeted resequencing. Bioinformatics 2014;30:2670-2672.
6. Eijkelenboom A, Kamping EJ, Kastner-van Raaij AW, et al. Reliable Next-Generation Sequencing of Formalin-Fixed, Paraffin-Embedded Tissue Using Single Molecule Tags. J Mol Diagn 2016;18:851-863.
7. Neveling K, Mensenkamp AR, Derks R, et al. BRCA Testing by Single-Molecule Molecular Inversion Probes. Clin Chem 2017;63:503-512.
8. Fritz AG, Percy C, Jack A, et al. International classification of diseases for oncology 3rd edition. World Health Organization Geneva 2000.
9. Steensma DP, Bejar R, Jaiswal S, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood 2015;126:9-16.