A NINE-YEAR LONGITUDINAL STUDY IN THE BLOOD OF A SUPERCENTENARIAN REVEALS AN EXTENSIVE SUBCLONAL ARCHITECTURE AND ONGOING CLONAL DYNAMICS

RUNNING TITLE: ARCH IN SUPERCENTENARIAN

Erik B. van den Akker[1,2,3+#], Stavros Makrodimitris[3,5+], Marc Hulsman[3,4,5], Martijn H. Brugman[6], Tanja Nikolic[6], Ted Bradley[5,7], Quinten Waisfisz[5], Frank Baas[7], Marja E. Jakobs[7], Daphne de Jong[5], P. Eline Slagboom[2], Frank J.T. Staal[6], Marcel J.T. Reinders[1,3], and Henne Holstege[3,4,5#].

Affiliations

1. Leiden Computational Biology Center, Leiden University Medical Center, Leiden, 2300RC, The Netherlands
2. Section of Molecular Epidemiology, Leiden University Medical Center, Leiden, 2300RC, The Netherlands
3. Pattern Recognition & Bioinformatics, Delft University of Technology, Delft, 2628CD, The Netherlands
4. Alzheimer Center Amsterdam, Department of Neurology, Amsterdam Neuroscience, Vrije Universiteit Amsterdam, Amsterdam UMC, Amsterdam, The Netherlands
5. Department of Clinical Genetics, Vrije Universiteit Amsterdam, Amsterdam UMC, Amsterdam, The Netherlands
6. Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands
7. Department of Clinical Genetics, Amsterdam Medical Center, Amsterdam UMC, Amsterdam, The Netherlands

+Equally contributed

#Correspondence: Erik B. van den Akker, e.b.van_den_akker@lumc.nl and Henne Holstege, h.holstege@amsterdamumc.nl

Material & correspondence

Erik B. van den Akker, PhD; Leiden Computational Biology Center, Leiden University Medical Center, Leiden, The Netherlands; Einthovenweg 20, 2333 ZC, Leiden, The Netherlands; Tel: +31 (0)71 526 85 57; Fax: +31 (0)71 526 82 80; E-mail: e.b.van_den_akker@lumc.nl; website: http://www.lcbc.nl

Henne Holstege, PhD; Amsterdam Alzheimer Center & Clinical Genetics, Amsterdam UMC—location VUmc, de Boelelaan 1118, 1081 HZ Amsterdam, The Netherlands; Tel: +31 (0)20 444 08 16; E-mail: h.holstege@amsterdamumc.nl; website: www.100plus.nl

Key points

Deep sequencing of a longitudinal blood sample drawn from a healthy elderly individual at age 103, 110 and 111 years revealed that a single *DNMT3A*-mutated HSC and its clonal descendants produce over 75% of the peripheral blood.

A longitudinal analysis spanning a 9-year period reveals an extensive subclonal architecture with ongoing clonal dynamics reminiscent of myeloid dysplasia and malignancies.

While over 22% of the CD4 T-cells were derived from the mutated HSC, *in vitro* and *in vivo* evidence revealed an active and functional T-cell thymic output.

Abstract

Age Related Clonal Hematopoiesis (ARCH) arises when a hematopoietic stem cell (HSC) acquires a somatic mutation that confers a competitive growth advantage. ARCH mutations are associated with a tenfold increased risk of myeloid aberrancies, have been implicated in an increased risk for type II diabetes and cardiovascular disease, but are also frequently observed in apparently healthy elderly individuals. We longitudinally tracked the contributions of individual HSCs to peripheral blood and its sorted subsets in a healthy a supercentenarian at ages 103, 110 and 111 years. At age 110, up to ~75% of the peripheral blood cells were derived from a single HSC, carrying a splice-donor mutation in DNA (cytosine-5)-methyltransferase (*DNMT3A*). During the course of life, this HSC had undergone at least four additional clonal events, each giving rise to subclonal expansions, including a relatively recently evolved clonal lineage that quickly gained dominance within the timeframe of our sampling. The mutated HSC and its clonal descendants have clear myeloid over lymphoid preference, producing 87.4% of the granulocytes and 77.8% of the monocytes versus 10.6% of the T-cells and 7.4% of the B-cells. Within the lymphoid branch, we observed a 3-fold larger contribution to CD4 T-cells (22%) compared to CD8 T-cells (6.8%), as well as the presence of naïve T-cells that were capable of robust proliferation when tested *in vitro*. Together with surprisingly high Sj TREC content in sorted T cells this suggested a functional thymic output in this supercentenarian.

Introduction

Age-related Clonal hematopoiesis (ARCH) is an inevitable physiological consequence of ageing [Zink 2017], conferring a tenfold increased risk for the development of myeloid dysplasia or malignancies [Jaiswal 2014; Genovese 2014]. In addition to hematological disease, ARCH has also been associated with prevalent type 2 diabetes [Bonnefond 2013], prevalent chronic obstructive pulmonary disease [Zink 2017], incident cardiovascular disease [Jaiswal 2017], and vascular or all-cause mortality [Jaiswal 2014, Genovese 2014; Jaiswal 2017; Zink 2017]. While these associations suggest a potential role for ARCH in a broad spectrum of age-related low-grade inflammatory syndromes [Bowman 2018], its heterogeneity with respect to clinical outcomes has also been noted [Steensma 2015, Buscarlet 2017]. For instance, ARCH has been postulated to constitute an early phase of myelodysplasia and leukemia [Steensma 2015], yet the absolute risk to progress to one of these clinical entities remains overtly small. In addition, while ARCH becomes increasingly prevalent with age [Xie 2014, Jaiswal 2014, Genovese 2014] and can become very extreme in the exceptionally old [Holstege 2014], its association with all-cause mortality seems to wane in these oldest old [van den Akker 2016]. Collectively, these findings illustrate that we are far from understanding the molecular pathophysiological transitions that ARCH may initiate during ageing.

ARCH arises when an ageing hematopoietic stem cell (HSC) acquires a somatic mutation that confers a competitive growth advantage, leading to its gradual expansion [Busque 2012]. ARCH-associated mutations typically target genes previously linked with myeloid aberrancies, most prominently in genes encoding epigenetic regulators such as *DNMT3A*, *TET2* and *ASXL1* [Busque 2012; Jaiswal 2017; Genovese 2014]. In addition to ARCH caused by known candidate drivers (ARCH-CD), others have shown that a significant part of ARCH occurs in the absence of known drivers (ARCH-UCD) [Genovese 2014; Zink 2017]. These studies exploited the fact that HSCs accumulate somatic mutations over the course of life [Busque 2012], thus effectively tagging each individual HSC with a unique genetic 'barcode' [Zink 2017]. We previously applied this paradigm of clonal tracing to the blood of an apparently healthy 115-year-old woman, which revealed that up to 65% of her peripheral blood was derived from a single expanded HSC [Holstege 2014]. While this study exemplified that extreme ARCH does not preclude attaining an extreme age in good health, it also raises questions about the long-term temporal stability of extreme ARCH, its subclonal dynamics, its potential restriction to particular immune lineages, and its impact on overall immuno-competence.

We have applied various deep sequencing techniques on a longitudinal blood sample drawn from an apparently healthy elderly individual at age 103, 110 and 111 years to study extreme ARCH. Our results provide unique insights into the clonal architecture and evolution of extreme ARCH, its temporal stability, its lineage preferences and its putative impact on T-cell immunity during ageing. Most importantly, our data highlights the fact that clonal evolution may readily occur in healthy elderly individuals, even at highly advanced ages, and differentially contributes to the replenishment of functional immune subsets. Most strikingly, our data suggests that extreme ARCH and a functional CD4 T-cell immunity may readily co-occur in the healthy exceptionally old, and as such may comprise a hallmark of achieving exceptional age in good cognitive health.

Methods

Full details of the methods used are given in the **Supplemental Methods & Material**.

Subject recruitment and phenotyping

Main subject of this study was W111, a Dutch female who lived for 111 years and 10 months with no dementia and whose blood showed no signs of hematological malignancies (**Supplemental Material**). W111 is a participant of the 100-plus Study, a study aimed to investigate Dutch citizens who have attained an age of 100 years or older with an exceptional good cognitive health [Holstege 2017]. Seven years prior to her participation in the 100-plus Study, W111 enrolled in the Leiden Longevity Study, a study aimed to investigate the determinants of familial longevity and healthy ageing [Schoenmaker]. The 100-plus Study and Leiden Longevity Study have been approved by their respective local Medical Ethical Committees and all participants gave informed consent. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Sequencing

Biomaterial obtained at various time points were included in our analyses, spanning a total time period of 8 years and 11 months. DNA was isolated from peripheral blood, its sorted subsets, a skin biopsy, or a *post-mortem* brain biopsy. Peripheral blood and a skin biopsy taken at age 110 years and 3 months were Whole Genome Sequenced (WGS) with SOLiD (Life Technologies) at 80x. Amplicon sequencing was performed on peripheral blood, its sorted subsets, and a brain biopsy using Ion Torrent Proton Ampliseq (Thermo Fisher Scientific) at 6000x. The TruSight Myeloid panel (Illumina) was used to resequence 54 genes known to be mutated in myeloid neoplasia in the peripheral sample blood drawn at 110 years and 3 months using a MiSeq instrument (Illumina).

Identification of mosaic somatic mutations

Whole genome sequencing data in peripheral blood and a skin biopsy taken at age 110 years and 3 months was used to identify somatic mutations, i.e. mutations that were detectable in blood, but not in the skin biopsy. To this end, Single Nucleotide Polymorphisms (SNPs) were called in both samples using GATK Haplotypecaller. Obtained calls were compared using in-house software, *Sommix*, to account for a potential contamination of the skin biopsy with blood cells, and to remove variant calls with strand biases or more than two alleles. Putative somatic mutations were validated by performing amplicon sequencing on peripheral blood taken at 110 years and 3 months and the *post-mortem* brain biopsy Ion Torrent Proton Ampliseq sequencing at 6000x. Variants were called using the Torrent Variant Caller (TVC) version 5.0.0 (Life Technologies), and mutations again called in peripheral blood, but not in the brain biopsy were considered to be confirmed somatic mutations.

Identification of candidate driver mutations

Whole genome sequencing data in peripheral blood drawn at age 110 years and 3 months was also analysed for the presence of candidate driver mutations in 16 genes previously reported to be recurrently mutated in the blood of apparently healthy elderly individuals: *DNMT3A*, *TET2*, *ASXL1*, *TP53*, *JAK2*, *SF3B1*, *GNB1*, *CBL*, *SRSF2*, *GNAS*, *BRCC3*, *CREBBP*, *NRAS*, *RAD21*, *U2AF1*, *PPM1D* [Xie 2014, Jaiswal 2014, Genovese 2014, van den Akker 2016]. For this purpose, SNPs and short indels called by GATK

Haplotypecaller were consecutively filtered according to the following five criteria. *i*) Positioned within or near, max 5 nucleotides, of the coding sequence of a transcript annotated to the aforementioned genes (hg19, UCSC refgene definitions). *ii*) A minimal read depth of the mutant allele of 3 and 6 for respectively SNVs and indels. *iii*) A protein altering predicted impact (UCSC Variant Annotation Integrator, NCBI Refseq curated subset). *iv*) Adhering to the gene specific mutational profiles, as previously compiled [Jaiswal *et al*. 2017]. *v*) Called in blood, but not in brain. A *DNMT3A* splice mutation was identified and confirmed by resequencing the peripheral sample blood drawn at 110 years and 3 months using the Illumina TruSight Myeloid panel and MiSeq instrument at 961x and visualized in Integrative Genome Viewer (IGV, Broad Institute, version 2.4.9).

Inference of subclonal architecture and subclonal lineage dynamics

Amplicon sequencing of peripheral blood collected at ages at age 102 years and 11 months, 110 years and 3 months, and 111 years and 10 months, a period of time spanning nearly 9 years, were used to infer the subclonal architecture. For this purpose, SciClone version 1.1.0 [Miller 2014] was run at default settings to cluster the panel of confirmed somatic mutations on Variant Allele Frequencies (VAFs) measured in peripheral blood at these three time points. Obtained clusters mark subclonal expansion events. Cluster annotations and VAFs were subsequently analysed with SCHISM version 1.1.2 [Niknafs 2015], at default settings, to infer a phylogenetic tree describing the most probable order in which these subclonal expansion events must have occurred. Each node of the obtained tree represents a subclonal lineage that arose by a consecutive acquisition of one or multiple clonal events, as represented by the structure of the phylogenetic tree. The percentage of cells carrying a particular subclonal expansion event was computed using the median VAF of the mutations assigned to its respective cluster. To obtain percentages of cells belonging to particular subclonal lineages, i.e. cells carrying particular combinations of events, each subclonal expansion was corrected for its immediate child events in the tree.

Assessing the dominance and preference of subclonal events in sorted subsets

Amplicon sequencing of sorted leukocyte subsets of peripheral blood drawn at age 110 years and 3 months and 111 years and 10 months were used to assess the percentages of cells that carry a particular clonal event in each sorted subset. For this purpose, median VAFs over mutations marking clonal events were computed using amplicon sequencing data created within each sorted subset. Apparent differences in the proportions of clonal events were further analysed on a per-mutation basis. For this purpose, the ratio of the VAF within a particular subset and within its corresponding peripheral blood sample was modelled as a function of the VAF in the peripheral blood sample, using linear models in R.

Immuno-phenotyping

The flow cytometric analysis, PBMC were incubated with a titrated cocktail of antibodies for 30 minutes on ice in the dark. Subsequently, cells were washed with FACS buffer (PBS supplemented with 2% FCS and Na-azide). Samples were acquired using FACS Fortessa-X20 (BD Biosciences). Antibody mix consisted of: FITC-anti-CD57 (clone HCD57); PE-anti-CD28 (clone CD28.2); APC-Cy7-anti-CD4 (clone RPA-T4); PE-Cy7-anti-CD197 (CCR7, clone 3D12); Pacific Blue-anti-CD27 (clone M-T271), BV650-anti-CD8 (clone SK1), all were from BD Pharmingen (USA). Alexa Fluor 700-anti-CD45RA (clone HI100); BV605-anti-CD31 (clone WM59) were from Biolegend (San Diego, USA). Phenotyping data were analysed using FlowJo software (TreeStar).

TRECs analyses

This assay is based on the rearrangement of the T-cell receptor occurring early during T-cell development [Dik 2005]. This rearrangement results in the formation of a coding joint (CJ) which

remains stably present in the genomic DNA, and a signal joint (SJ) on the corresponding excision circle (TREC). As with every cell division, SJ is diluted, while CJ is stably maintained in the genomic DNA, it is possible to derive the number of divisions the assayed cells have undergone from the difference between CJ and SJ [Zelm 2007]. TRECs analyses were essentially performed as previously described [van der Weerd 2013]. In short, DNA was extracted from peripheral blood or sorted T-cell subsets, after which two parallel real-time PCRs are performed that quantify the signal joint (SJ) or the coding joint (Cj) relative to an internal control of Albumin.

T-cell stimulation using a thymidine incorporation assay

Antigen-dependent T-cell proliferation capacity was determined using the mixed leukocyte reaction (MLR). For this, PBMC's from the test samples were cultured with irradiated HLA-mismatched PBMC (3000 rad). To test a general capacity of T cells to proliferate, PBMC were stimulated with Interleukin-2 (IL-2, 25 IU/ml). Cells were incubated in triplo in the 96-well round bottom plates in IMDM medium conditioned with Glutamine, Pen/Strep and 10% inactivated Human Serum (HS, Sanquin, Amsterdam) for 5 days. Radioactive thymidine (2 µCi/ml) was added in the last 18 hrs of the culture to enable incorporation in the DNA of proliferating cells and the radioactivity in DNA recovered from the cells was measured using a scintillation beta-counter. Obtained data (counts per minute, CPM) were used to calculate stimulation index by dividing the CPM values of the test samples by the CPM of test PBMC cultured in medium alone.

Results

Study design and data collection in a super-centenarian

Subject of this study was W111, a Dutch female who lived for 111 years and 10 months in a good cognitive condition with no signs of hematological malignancies (**Supplementary**). W111 was included in the 100-plus Study [Holstege 2017] at age 110 and 2 months. Seven years prior to her participation in the 100-plus Study, W111 enrolled in the Leiden Longevity Study [Schoenmaker 2006] at age 102 and 11 months. DNA was isolated from peripheral blood and bio-banked. Various types of biomaterial were obtained at three consecutive time points, age 103, 110, and 111 respectively, spanning a period of 8 years and 11 months (**Figure 1**). Obtained materials included peripheral blood (PB) samples, its flow sorted subsets (granulocytes (G), monocytes (M), T-cells (T, T4, T8) and B-cells (B)), a skin biopsy (S) at age 110 and a post-mortem brain biopsy (C)).

Deep sequencing of peripheral blood reveals an advanced state of age-related clonal hematopoiesis

We conducted Whole Genome Sequencing (WGS, SOLiD, Life Technologies, 80x) in biomaterial obtained at age 110 to identify mutations that have accumulated in the hematopoietic stem cell compartment during W111's lifespan. To this end, DNA derived from peripheral blood was sequenced and compared with DNA derived from skin. Using an *in-house* bioinformatics pipeline (Sommix, **Methods**) we identified 650 putative single nucleotide variations (SNVs) of somatic origin (**Table S1**). We screened for candidate driver mutations amongst the 650 identified SNVs employing the definitions of Jaiswal *et al.* [Jaiswal 2017] (**Table S2**), which revealed a splice-donor site mutation in intron 11 of DNA (cytosine-5)-methyltransferase 3A (*DNMT3A,* NM_022552.4, chr2:25,469,028 C>T, c.1429+1 G>A). Amplicon sequencing (TruSight Myeloid Sequencing Panel, Illumina, MiSEQ sequencing, Illumina) confirmed this variant at 961x with an estimated variant allele frequency (VAF) of 0.378 (**Figure 2, Table S3**), which suggests that 75.6% of W111's peripheral blood cells were derived from a single clone carrying this *DNMT3A* splice-donor mutation. Moreover, custom targeted re-sequencing panels (Ion Torrent Proton Ampliseq, Thermo Fisher Scientific) were successfully designed for 474 out of 650 (72.9%) remaining mutations (**Table S4**) and 307 (64.8%) were confirmed. To summarize, the number of identified somatic mutations and the dominance of the candidate driver mutation indicate that the large majority of W111's peripheral blood production is derived from a single DNMT3A-mutated HSC, thus indicating a very extreme age-related clonal hematopoiesis.

Longitudinal analysis of somatic mutations during a 9-year period reveals a dynamic and extensive clonal architecture

The longitudinal design of our study provides a unique opportunity to investigate the long-term stability and subclonal dynamics of extreme ARCH. To this end, we subjected the peripheral blood samples taken at ages 103 and 111 years to the targeted re-sequencing panel comprising of the 307 confirmed somatic mutations (Ion Torrent Proton Ampliseq 2000x, Thermo Fisher Scientific). All 307 mutations identified at age 110 years were also observed at ages 103 and 111 (**Figure 3A**), indicating the long-term stability of extreme ARCH. While VAFs generally increased between ages 103 and 110 years, VAFs remained equal or lowered between ages 110 and 111 years. Nevertheless, VAFs were highly inter-correlated between timepoints (Pearson's $r_{103->110}$ = 0.983 and $r_{110->111}$ = 0.988, **Supplemental Figures**). Moreover, density distributions of VAFs obtained per time point showed similar profiles and were characterized by multiple peaks. As these observations are suggestive of an underlying subclonal architecture, we subjected the VAF data obtained from all three time points to a SciClone analysis [Miller 2014] (**Methods**). In accordance, this analysis assigned the 307 confirmed somatic mutations to five independent clonal events (A-E, **Figure 3A**). Subsequent modelling with SCHISM [Niknafs 2015] indicated that these five

clonal events most likely occurred consecutively within a single clonal lineage, that terminates into two independent child events D and E (**Figure 3B, Methods, Supplemental**). This implies the existence of two competing subclonal sister lineages, that are both derived from a shared ancestral HSC carrying events A-C, after which they have diversified by acquiring either event D or E. When accounting for the dependencies between clonal events within the same subclonal lineages, we observed that changes in dominance, i.e. changes in VAF, of clonal event A-C are largely explained by changes in dominance of event E, and not event D (Figure 3C, Methods). In fact, while clonal event D exhibits a near equal contribution of approximately 16.5% to peripheral blood at ages 103, 110 and 111 years, clonal event E quickly gained clonal dominance from approximately 6.1% at age 103 to 17.9% at ages 110 and 111. Collectively, these results indicate the existence of an extensive subclonal architecture in W111's hematopoietic stem cell compartment (Figure 3D), reminiscent of the subclonal evolution that must have occurred prior to this study. In addition, our results suggest that the competition between subclonal lineages seems to be ongoing, even at this extremely advanced age. Overall, our results indicate that W111's peripheral blood production has been governed by a single mutated stem cell and its subclonal lineages within a period spanning at least 9 years, underpinning the long-term stability of extreme ARCH.

The founding clone and its descendants are myeloid biased, yet also produce lymphoid progeny with a preference for CD4+ T-cells

Next, we investigated to what extent the founding clone (A) and its forthcoming descendants (B,C,D and E) contributed to various major cell subsets present in peripheral blood. To this end, peripheral blood drawn at age 110 was FACS-sorted into granulocytes, monocytes, T-cells and B-cells (**Figure 1**). DNA was isolated from these cells and subjected to targeted re-sequencing of the previously identified panel of 307 somatic mutations (Ion Torrent Proton Ampliseq 2000x, Thermo Fisher Scientific). Comparison of VAFs between different cell subsets (**Figure 4A**) indicated a significant higher clonal contribution of the mutated HSCs and its subclonal descendants to the myeloid branch producing 87.4% of the granulocytes ($G_{VAF,\ age=110}$ = 43.7%) and 77.8% of the monocytes ($M_{VAF,\ age=110}$ = 38.9%), compared to 67.0% of the total peripheral blood cells ($PB_{VAF,\ age=110}$ = 33.5%). Interestingly, somatic mutations were generally also observed in DNA derived from cell subsets of the lymphoid lineage, albeit at considerably lower VAFs. Nevertheless, approximately 10.6% and 7.4% of T- and B-cells ($T_{VAF,\ age=110}$ = 5.3%; $B_{VAF,\ age=110}$ = 3.7%) respectively were derived from the founding clone and its descendants, demonstrating its multipotency, yet also its myeloid bias. Flow-sorting and targeted sequencing within an additional blood sample drawn at age 111 did not only confirm both the myeloid bias and the multipotency (**Figure 4A**), but also pointed to a striking bias present within the lymphoid branch. Median VAFs were significantly higher in CD4 T-cells (22.2% of the cells, $T4_{VAF,\ age=111}$ = 11.1%),as compared to CD8 T-cells (6.4% of the cells, $T8_{VAF,\ age=111}$ = 3.2%, p<0.001, Wilcoxon) and B-cells (6.0% of the cells, $B_{VAF,\ age=111}$ = 3.0%, p<0.001, Wilcoxon). Hence, these results indicate that the contributions of the founding clone and its descendants are not restricted to the myeloid lineage, but also include the lymphoid lineage, and specifically CD4 T-cells.

Contributions of clones A-E within each of the sorted cell subsets of granulocytes, monocytes, and B cells are roughly proportional to that observed in peripheral blood of the corresponding time point, yet for T-cells some deviations were observed (**Figure 4B**). For all three sorted T-cell subsets we observe a relatively low contribution of clone E relative to its corresponding peripheral blood sample. When analyzing at the level of the individual somatic mutations, we observed that mutations assigned to smaller clones had a disproportionately lower VAF in the T-cell subsets as compared mutations assigned to larger clones (**Figure 4C-E**, **Supplementary**). No such trends were observed for B-cells (**Supplementary**), suggesting that the observed bias in clonal blood production relates to T-cells specifically and not to all lymphoid progeny.

W111 has immuno-competent naïve CD4-T cells

Ageing is associated with profound changes in immune subset composition, i.e. aged HSCs produce many more myeloid cells than B lymphocytes, and the thymus, responsible for *de novo* production of naive T cells, involutes [Mitchel 2010, Nasi 2016]. In accordance, flow cytometric analyses of peripheral blood at age 110 analyses revealed high myeloid to B lymphocyte ratios, significant numbers of senescent T cells and type 2 monocytes. Moreover, the majority of CD4 and CD8 T-cells were senescent, as apparent by the expression of CD57 (**Methods, Figure 5A-B**). However, ~5% of CD45RA+CCR7+ naïve T-cells expressed the marker CD31, suggesting that these are recent thymic emigrants [Kohler 2009]. In accordance, assessment of the replication history of T-cells by T-cell receptor excision circles (TRECs) assays indicated that both CD4 and CD8 T-cells had undergone a number of divisions comparable to that of middle-aged healthy controls (**Methods**, **Figure 5C, Supplemental**) with a TREC content of 3-6%, comparable to that of young adults [Nasi 2016]. Collectively, our results imply that the thymus of W111 was still active at age 110.

To validate that W111's T-cells are in fact capable of mounting an active immune response, we performed flow cytometric analysis of peripheral blood at age 111. Both the CD4 and CD8 T-cell subsets contained considerable fractions of CD25+CD69+ cells (**Figure 5D, Supplemental**), marking *in vivo*-activated cells. While considerable percentages of senescent T cells were also observed, the significant fraction of naive and *in vivo* activated T-cells suggested that a significant number of W111's T-cells are fully functional. Therefore, we performed two different types of *in vitro* proliferation assays (**Methods**). In both the IL2/TCR-dependent and the allogeneic assay, CD4 T-cells, on a per cell basis, were at least as capable of mounting an immune response as compared to T-cells obtained from healthy middle-aged controls (**Figure 5D, Supplemental**). Together, these results indicate that at least a part of the T-cell compartment was not immune senescent and still capable of mounting vigorous naive responses consistent with the presence of functional thymic activity in W111.

Discussion

Using a combination of whole genome and targeted deep sequencing we identified and validated somatic mutations in a longitudinal blood sample drawn from an apparently healthy woman at ages 103, 110, and 111 years. We identified an extensive clonal architecture within the hematopoietic system, with a *DNMT3A* splice-site mutation occurring early in clonal evolution, and at least four additional independent events that each gave rise to subsequent subclonal expansions. The latest subclonal expansion occurred within the timeframe of our study and marked the rise of a new subclonal lineage at the expense of an older sister lineage, indicating that clonal competition was still ongoing at this advanced age. Clonal tracing in sorted cell subsets revealed that all subclonal expansions were observed in all sampled blood subsets, irrespective of myeloid or lymphoid descendance. Although overall myeloid biased, we also observed a relative expansion within the lymphoid lineage of CD4+ T-cells compared to CD8+ T-cells or B-cells. Lastly, functional assays suggest that, in contrast to expectations at this highly advanced age [Nasi 2006], at least a part of the CD4+ T-cells of W111 were not immune senescent. Not only did TRECs analyses indicate that part of the CD4+ T-cells were descendant of recent thymic immigrants, also cultured T-cells were capable of mounting vigorous naive responses consistent with the presence of functional thymic activity. Collectively, our results comprise a unique account of an exceptionally long lived individual with a hematopoietic system characterized by extensive ARCH and an exceptional CD4 T-cell immune capacity.

We have identified and validated 308 unique somatic mutations in the blood from an exceptionally old, but apparently healthy individual at age 110. Identified somatic mutations included a splice-donor mutation in intron 11 of *DNMT3A* (NM_022552.4, chr2:25,469,028 C>T, c.1429+1 G>A), a somatic mutation that has recurrently been reported in patients with hematopoietic or lymphoid malignancies (COSM5945645) [Shin 2016, Wong 2018, Zehir 2017]. A clinical examination of peripheral blood, however, revealed neither cytopenias nor dysplastic morphologies (**Supplemental Material**). Collectively, these observations fit the postulated inclusion criteria for Clonal Hematopoiesis of indeterminate Potential (CHiP) [Steensma 2015], also referred to as Age Related Clonal Hematopoiesis (ARCH) [Schlush 2018], to describe mostly elderly individuals carrying a pre-leukemic mutation with unclear clinical implications. The extreme mutational burden of the identified candidate driver mutation (VAF = 37.8% at 961x), indicates that up to 75.6% of the cells in peripheral blood are derived from a single *DNMT3A*-mutated HSC. This is in accordance with our previous study in an apparently healthy 115-year-old, which revealed that up to 65.0% (VAF = 32.5%) of the cells in her peripheral blood were derived from a single mutated HSC [Holstege 2014]. Importantly, the current study not only confirms that ARCH can become very extreme at exceptionally advanced ages, it also critically extends this observation by underpinning its temporal stability, namely by showing its presence at three consecutive time-points spanning nearly nine years. While previous studies also investigated the temporal stability of ARCH, the crucial difference is that these were conducted in individuals with clonal hematopoiesis at an exceedingly low dominance [Young 2016] comprising ARCH cases with at best a questionable clinical relevance [Steensma 2015]. The temporal stability observed in our study suggests that at exceptional ages, extreme ARCH is neither a momentary flux of the hematopoietic system, nor a condition posing immediate and overt health risks.

In the current study, we have exploited clonal tracing to conduct very detailed studies into the underlying clonal architecture of clonal hematopoiesis and its temporal dynamics during a nine-year interval of aging to the highest ages. This study design has yielded several unique insights into the biology of the ageing human haematopoietic stem cell compartment. For instance, clonal tracing revealed an extensive clonal architecture in which at least five independent successive clonal events have emerged. Whereas the initiating clonal expansion could be attributed to a *DNMT3A* splice site mutation, no additional candidate drivers were detected amongst the identified somatic mutations, that

could explain an increase in replicatory fitness required for any of the successive subclonal expansions to occur. While this result might be explained by undetected or incomplete knowledge on driver mutations [Zink 2017], it could also point to non-genetic mechanisms, e.g. so-called 'epi-mutations' [Jost 2014]. Especially in view of the mutation in *DNMT3A*, a key epigenetic regulator active during myelopoiesis [Challen 2011] and responsible for maintenance of DNA methylation [Zhang 2016], it can be imagined that the successive events might be the result of a gradual loss of epigenetic control within the mutated HSC [Abdel-Wahab 2013]. Yet another possibility is that the *DNMT3A* mutation magnifies the alternating temporal activity of HSCs as occurring under normal conditions [Bystrykh 2012]. Each of the observed clonal expansion events would then constitute a temporary re-activation of the mutated HSC from a dormant state [Takizawa 2011]. To summarize, our findings not only warrant future research into the underlying mechanisms driving clonal dynamics during normative aging of the hematopoietic system, they also stress the importance of methods, such as clonal tracing [Genovese 2014; Zink 2017; Holstege 2014], that do not depend on the presence of known driver mutations.

Clonal tracing over consecutive time points revealed intricate patterns of subclonal dynamics. By reconstructing clonal lineages and tracing them over time, we showed that a relatively recently evolved clonal lineage (E) quickly gained dominance in comparison to its competing sister lineage (D). While the mutations marking lineage E were sometimes at the border of detection at the start of our study (median VAF = 3.0%), they rapidly grew in dominance in ensuing years to produce up to 18% of the peripheral blood (VAF = 9.0%) at age 110. This aggressive expansion parallels patterns of clonal evolution otherwise observed in overt myeloid disease, i.e. myelodysplastic syndromes [Silvas-Coelho 2017, Makishima 2017], myeloproliferative neoplasms [Lundberg 2014] or relapsed acute myeloid leukemia [Ding 2012, Christen 2019], in which subclonal lineages may completely replaced. Whether these complete wipeouts occur within the healthy aged hematopoietic stem cell compartment remains to be determined. Nevertheless, our results indicate that the temporal plasticity and resulting subclonal architecture otherwise associated with dysplasia or malignancies readily occurs in the hematopoietic stem cell compartment of healthy elderly individuals, and should therefore be regarded as a process of normal ageing.

All clonal events were observed in all sampled blood subsets, irrespective of myeloid or lymphoid descendance. This is in agreement with recent findings [Arends 2018, Buscarlet 2018], independently indicating that *DNMT3A* mutations can be potent drivers of both the myeloid and lymphoid lineages, thereby giving rise to a so-called myeloid-lymphoid ARCH. Interestingly, while overall myeloid biased, we also observed a bias within the lymphoid lineage for CD4+ T-cells over CD8+ T-cells and B-cells. While only 6.8% of the CD8 T-cells and 6.0% of the B-cells were derived from the mutated clone, over 22% of the CD4 T-cells were descendants from the mutated clone. This could imply that a *DNMT3A*-instigated ARCH exhibits a skewed renewal not only towards the myeloid lineage, but also to particular cell subsets within the lymphoid lineage. However, a more detailed analysis of these lineage biases suggested that, similar to the myeloid-lymphoid bias, the CD4+ over CD8+ bias might in part be explained by the differences in renewal rates. Under the assumption that the median VAF of a mutational event can serve as a proxy of its relative age, we observed within all three sorted T-cell subsets a bias towards the older mutational events. This overall trend can be explained by the longevity of T-cells; changes in T-cell producing progenitors would only become slowly apparent in the total pool of T-cells. Interestingly, this trend is steeper for CD8+ T-cells than for CD4+ T-cells, indicating an even slower replacement rate for CD8+ T-cells as compared to CD4 T-cells. Collectively, our results suggest that the generally observed age-related skewing of the blood composition could in part be attributed to an ARCH-induced enhanced renewal of blood cells specifically contributing to particular immune subsets.

A recent report by Hashimoto *et al.* [Hashimoto 2019] corroborates our observation of a clonally expanded CD4 T-cell subset as a potential hallmark of the supercentenarian immune system. While

Hashimoto *et al.* establish the clonal expansion of CD4 T-cells with the use of T-cell receptor analyses, they conclude that it is most likely that the clonal expansion has been triggered by a sustained antigenic stimulation. However, they did not take into account that ARCH is practically unavoidable at an advanced age [Zink 2017], and as such may confer a competing explanation. Supportive evidence that ARCH-associated mutations may indeed contribute to the clonal expansion of functional lymphoid subsets was recently provided by Fraietta *et al.* [Fraietta 2018] in a paper elegantly reporting on an incidental finding in a study on CAR T-cell immunotherapy. In this study, CD8+ T-cells were genetically redirected to target tumor cells of patients with B-cell malignancies, so-called CAR T-cells. While transplantation should elicit a polyclonal T-cell response in the patient, clearing malignant B-cells, one patient showed a monoclonal outgrowth of CAR T-cells. Sequencing revealed that the procedure to genetically redirect the T-cell had also disrupted a gene associated with ARCH: *TET2*. Interestingly, *TET2*-deficient CAR T-cells were immuno-competent and the patient achieved full remission. In accordance with this study, our results *in vitro* and *in vivo* indicate that the CD4 thymic output was descended from recent thymic immigrants, and moreover, was capable of eliciting a fierce naive response. Collectively, this raises the possibility that the functional CD4 T-cell immunity, an immune state that is very exceptional for this highly advanced age, could be attributed to lymphoid descendants of the mutated stem cells.

This study also has limitations. Foremost, this study has been conducted with the material derived from only a single healthy supercentenarian. Hence, it is imperative that more of these highly unique individuals are investigated in order to generalize our findings to a broader context. Moreover, the nature of our study precludes any inferences on the causality between the two main observation done within this study, namely the co-occurrence between an extreme *DNMT3A*-instigated ARCH and an exceptionally functional CD4 T-cell immunity. Nevertheless, we believe that the nature of the presented findings warrants future research into the causes of a functional CD4 immunity that seems quintessential for achieving an exceptional age in good health.

To conclude, while a significant part of ARCH research is focused on the identification of new disease associations and determining minimal thresholds on clonal dominance to have adverse health effects, we shifted our focus to the other end of the spectrum, where extreme individuals of oldest old have been reported to exhibit extremely advanced states of ARCH, with yet unexplored consequences for immune functioning. We have applied various deep sequencing techniques on a longitudinal blood sample drawn from an apparently healthy elderly individual at ages 103, 110 and 111 years to study extreme ARCH in detail. Our results provide unique insights into the clonal architecture and evolution of extreme ARCH, its temporal stability, its lineage preferences and its putative impact on T-cell immunity during ageing. Most importantly, our data highlights the fact that extensive clonal evolution may readily occur in healthy elderly individuals, even at highly advanced ages, and may contribute to the replenishment of functional immune subsets that under normal conditions would have been depleted and seem to be essential for maintaining good health.

Acknowledgements

The 100-plus Study was supported by Stichting Alzheimer Nederland (WE09.2014-03), Stichting Diorapthe (VSM 14 04 14 02) and Stichting VUmc Fonds. The Leiden Longevity Study has received funding from the European Union's Seventh Framework Programme (FP7/2007-2011) under grant agreement number 259679. This study was supported by the Netherlands Consortium for Healthy Ageing (grant 050-060-810), in the framework of the Netherlands Genomics Initiative, Netherlands Organization for Scientific Research (NWO); by BBMRI-NL, a Research Infrastructure financed by the Dutch government (NWO 184.021.007).

Author contributions

Competing financial interests

None of the authors have competing interests to declare.

References

1. Abdel-Wahab O, et al. Mutations in epigenetic modifiers in the pathogenesis and therapy of acute myeloid leukemia. Blood 2013;121(18):3563-3572.
   doi: https://doi.org/10.1182/blood-2013-01-451781
2. van den Akker EB, et al. Uncompromised 10-year survival of oldest old carrying somatic mutations in DNMT3A and TET2. Blood. 2016 Mar 17;127(11):1512-5.
   doi: https://doi.org/10.1182/blood-2015-12-685925
3. Arends CM, et al. Hematopoietic lineage distribution and evolutionary dynamics of clonal hematopoiesis. Leukemia. 2018 Sep;32(9):1908-1919.
   doi: https://doi.org/10.1038/s41375-018-0047-7
4. Bonnefond A, et al. Association between large detectable clonal mosaicism and type 2 diabetes with vascular complications. Nat Genet. 2013 Sep;45(9):1040-3.
   doi: https://doi.org/10.1038/ng.2700
5. Bowman RL, et al. Clonal Hematopoiesis and Evolution to Hematopoietic Malignancies. Cell Stem Cell. 2018 Feb 1;22(2):157-170.
   doi: https://doi.org/10.1016/j.stem.2018.01.011
6. Buscarlet M, et al. DNMT3A and TET2 dominate clonal hematopoiesis and demonstrate benign phenotypes and different genetic predispositions. Blood. 2017 Aug 10;130(6):753-762.
   doi: https://doi.org/10.1182/blood-2017-04-777029
7. Buscarlet M. et al, Lineage restriction analyses in CHIP indicate myeloid bias for TET2 and multipotent stem cell origin for DNMT3A. Blood. 2018 Jul 19;132(3):277-280.
   doi: https://doi.org/10.1182/blood-2018-01-829937.
8. Busque L, et al. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. Nat Genet. 2012 Nov;44(11):1179-81.
   doi: https://doi.org/10.1038/ng.2413
9. Bystrykh LV, et al. Counting stem cells: methodological constraints. Nat Methods. 2012 May 30;9(6):567-74.
   doi: https://doi.org/10.1038/nmeth.2043
10. Challen GA, et al. Dnmt3a is essential for hematopoietic stem cell differentiation. Nat Genet. 2011 Dec 4;44(1):23-31.
    doi: https://doi.org/10.1038/ng.1009
11. Christen F, et al. Genomic landscape and clonal evolution of acute myeloid leukemia with t(8;21): an international study on 331 patients. Blood. 2019 Mar 7;133(10):1140-1151.
    doi: https://doi.org/10.1182/blood-2018-05-852822
12. Dik WA, et al. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. J Exp Med. 2005 Jun 6;201(11):1715-23. doi: https://doi.org/10.1084/jem.20042524
13. Ding L, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature. 2012 Jan 11;481(7382):506-10.
    doi: https://doi.org/10.1038/nature10738
14. Fraietta JA, et al. Disruption of TET2 promotes the therapeutic efficacy of CD19-targeted T cells. Nature. 2018;558(7709):307–312.
    doi: https://doi.org/10.1038/s41586-018-0178-z

15. Genovese G, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med. 2014 Dec 25;371(26):2477-87.
doi: https://doi.org/10.1056/NEJMoa1409405
16. Hashimoto K, et al. Single-cell transcriptomics reveals expansion of cytotoxic CD4 T-cells in supercentenarians. bioRxiv 643528;
doi: https://doi.org/10.1101/643528
17. Holstege H, et al. Somatic mutations found in the healthy blood compartment of a 115-yr-old woman demonstrate oligoclonal hematopoiesis. Genome Res. 2014 May;24(5):733-42.
doi: https://doi.org/10.1101/gr.162131.113
18. Holstege H, et al. The 100-plus Study of cognitively healthy centenarians: rationale, design and cohort description. Eur J Epidemiol. 2018 Dec;33(12):1229-1249.
doi: https://doi.org/10.1007/s10654-018-0451-3
19. Jaiswal S, et al. Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med. 2014 Dec 25;371(26):2488-98.
doi: https://doi.org/10.1056/NEJMoa1408617
20. Jaiswal S, et al. Clonal Hematopoiesis and Risk of Atherosclerotic Cardiovascular Disease. N Engl J Med. 2017 Jul 13;377(2):111-121.
doi: https://doi.org/10.1056/NEJMoa1701719
21. Jost E, et al. Epimutations mimic genomic mutations of DNMT3A in acute myeloid leukemia. Leukemia. 2014 Jun;28(6):1227-34.
doi: https://doi.org/10.1038/leu.2013.362
22. Kohler S, et al. Life after the thymus: CD31+ and CD31- human naive CD4+ T-cell subsets. Blood. 2009 Jan 22;113(4):769-74.
doi: https://doi.org/10.1182/blood-2008-02-139154
23. Lundberg, P et al. Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. Blood. 2014 Apr 3;123(14):2220-8.
doi: https://doi.org/10.1182/blood-2013-11-537167
24. Miller MA, et al. SciClone: inferring clonal architecture and tracking the spatial and temporal patterns of tumor evolution. PLoS Comput Biol. 2014 Aug 7;10(8):e1003665.
doi: https://doi.org/10.1371/journal.pcbi.1003665
25. Mitchell WA, et al. Tracing thymic output in older individuals. Clin Exp Immunol. 2010 Sep;161(3):497-503.
doi: https://doi.org/10.1111/j.1365-2249.2010.04209.x
26. Nasi M, et al. Thymic output and functionality of the IL-7/IL-7 receptor system in centenarians: implications for the neolymphogenesis at the limit of human life. Aging Cell. 2006 Apr;5(2):167-75.
doi: https://doi.org/10.1111/j.1474-9726.2006.00204.x
27. Niknafs N, et al. SubClonal Hierarchy Inference from Somatic Mutations: Automatic Reconstruction of Cancer Evolutionary Trees from Multi-region Next Generation Sequencing. PLoS Comput Biol. 2015 Oct 5;11(10):e1004416.
doi: https://doi.org/10.1371/journal.pcbi.1004416
28. Makishima H, et al. Dynamics of clonal evolution in myelodysplastic syndromes. Nat Genet. 2017 Feb;49(2):204-212.
doi: https://doi.org/10.1038/ng.3742
29. Shapiro MB, et al. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. 1987 Sep 11;15(17):7155-74.
doi: https://doi.org/10.1093/nar/15.17.7155
30. Shlush LI. Age-related clonal hematopoiesis. Blood. 2018 Feb 1;131(5):496-504.

doi: https://doi.org/10.1182/blood-2017-07-746453

31. Schoenmaker M, et al. Evidence of genetic enrichment for exceptional survival using a family approach: the Leiden Longevity Study. Eur J Hum Genet. 2006 Jan;14(1):79-84. doi: https://doi.org/10.1038/sj.ejhg.5201508
32. Shin SY, et al. Mutation profiling of 19 candidate genes in acute myeloid leukemia suggests significance of DNMT3A mutations. Oncotarget. 2016 Aug 23;7(34):54825-54837. doi: https://doi.org/10.18632/oncotarget.10240
33. da Silva-Coelho P, et al. Clonal evolution in myelodysplastic syndromes. Nat Commun. 2017 Apr 21;8:15099. doi: https://doi.org/10.1038/ncomms15099
34. Steensma DP, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood. 2015 Jul 2;126(1):9-16. doi: https://doi.org/doi:10.1182/blood-2015-03-631747
35. Takizawa H, et al. Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. J Exp Med. 2011 Feb 14;208(2):273-84. doi: https://doi.org/10.1084/jem.20101643
36. van der Weerd K, et al. Combined TCRG and TCRA TREC analysis reveals increased peripheral T-lymphocyte but constant intra-thymic proliferative history upon ageing. Mol Immunol. 2013 Mar;53(3):302-12. doi: https://doi.org/10.1016/j.molimm.2012.08.019
37. Wong TN, et al. Cellular stressors contribute to the expansion of hematopoietic clones of varying leukemic potential. Nat Commun. 2018 Jan 31;9(1):455. doi: https://doi.org/10.1038/s41467-018-02858-0
38. Xie M, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. Nat Med. 2014 Dec;20(12):1472-8. doi: https://doi.org/10.1038/nm.3733
39. Zehir A, et al. Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. Nat Med. 2017 Jun;23(6):703-713. doi: https://doi.org/10.1038/nm.4333
40. van Zelm MC, et al. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med. 2007 Mar 19;204(3):645-55. doi: https://doi.org/10.1084/jem.20060964
41. Zhang X, et al. DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. Nat Genet. 2016 Sep;48(9):1014-23. doi: https://doi.org/10.1038/ng.3610
42. Zink F, et al. Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. Blood. 2017; blood-2017-02-769869; doi: https://doi.org/10.1182/blood-2017-02-769869

Figures and legends

Fig 1 Study design: At age 110 and 2 months, peripheral blood (PB) was drawn from W111 for DNA isolation prior and after cell sorting using FACS (granulocytes (G), CD3+ T-cells (T), CD19+ B cells (B)). In addition, DNA was isolated from a skin biopsy (S) performed two weeks after the initial blood draw. During follow-up at age 111 years and 10 months, one and a half year after inclusion, and three weeks prior to her death, peripheral blood (PB) was drawn and FACS sorted. DNA was isolated from peripheral blood (PB), granulocytes (G), CD3+CD4+ T-cells (T4), CD3+CD8+ T-cells (T8) and CD19+ B-cells (B). In addition, W111 gave consent for a post-mortem brain donation and DNA was isolated from the occipital cortex (C).

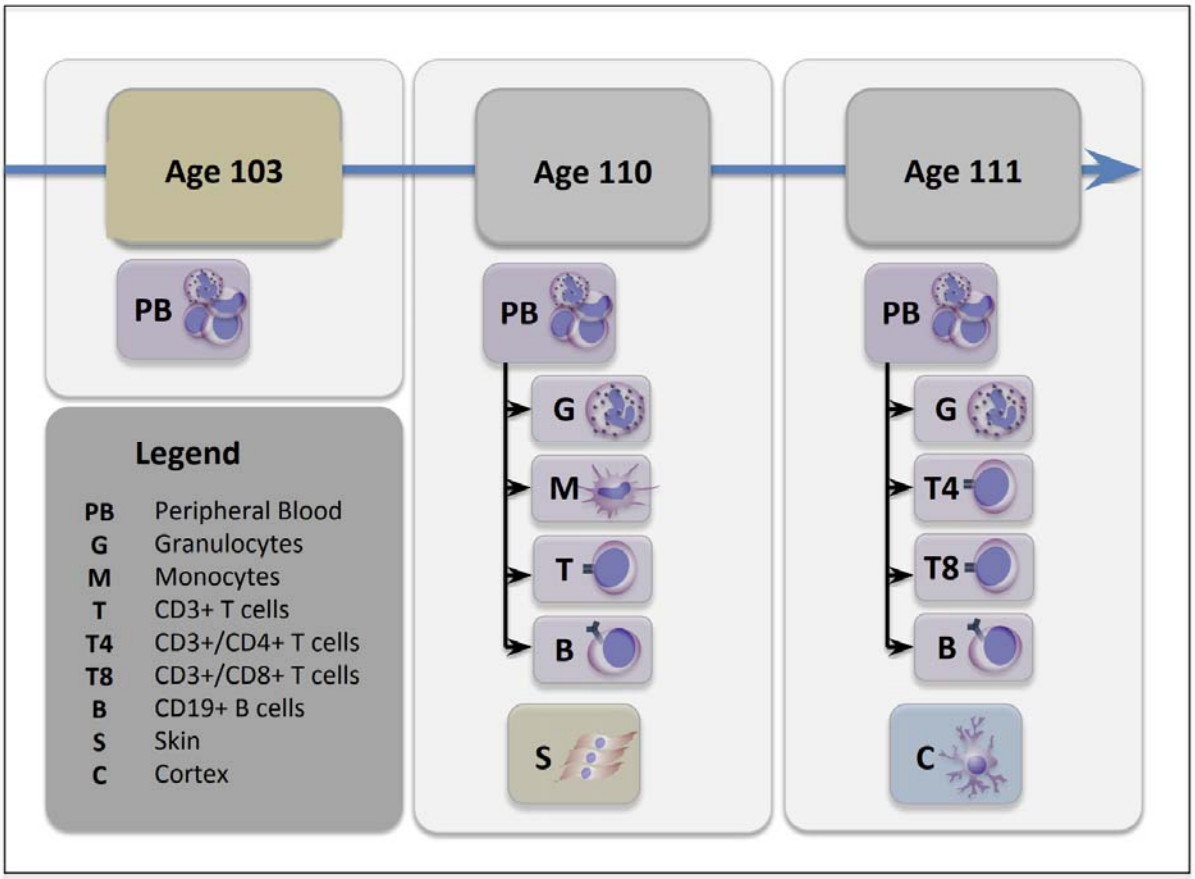

Figure 2: IGV plot of the splice-donor site mutation in *DNMT3A* detected in W111's peripheral blood drawn at age 110. The identified mutation (NM_022552.4, chr2:25,469,028 C>T, c.1429+1 G>A) alters the G nucleotide of the highly conserved GT intronic sequence [Shapiro 1987]. Amplicon sequencing (TruSight Myeloid Sequencing Panel, Illumina) was performed at 961x and indicated a VAF of 37.8% for the mutant allele.

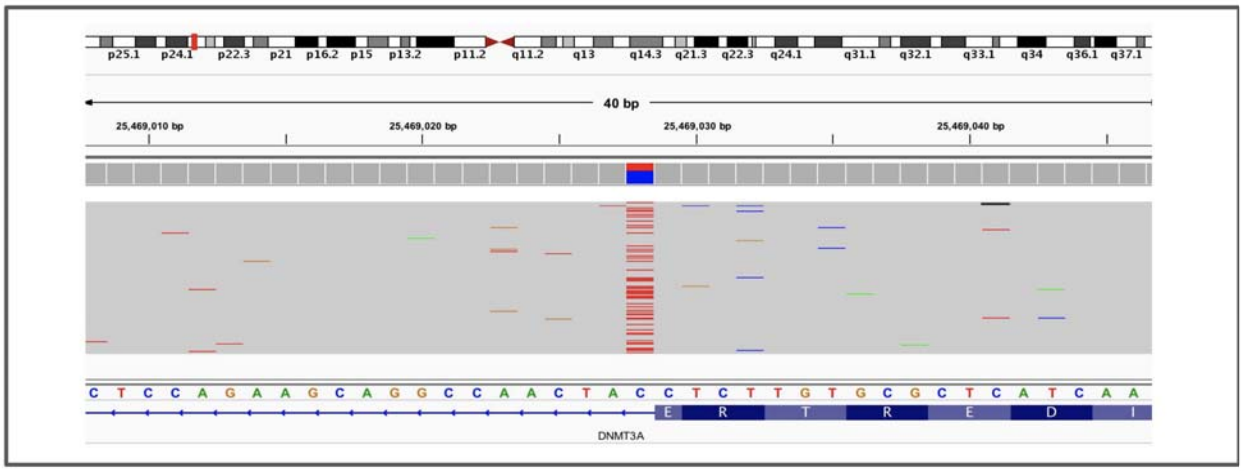

Figure 3: **Deep sequencing of longitudinal samples reveals the clonal architecture within the peripheral blood of elderly subject with age-related clonal hematopoiesis.** [**A**] Variant allele frequencies of the 307 confirmed somatic mutations at ages 103, 110 and 111. Lines connect the same mutations measured at different ages. Mutations were assigned to five independent clonal events (A-E) using SciClone [Miller 2014] and colored accordingly. [**B**] Subsequent analysis with SCHISM [Niknafs 2015] revealed the most likely underlying clonal architecture. The number of somatic mutations supporting each clonal event are listed next to the clones. [**C**] Median VAFs after subtraction of the median VAF of the descendant clonal event indicates that clone E displays the most variation. [**D**] Reconstruction of subclonal evolution. Time frames A-E correspond to the periods in which passenger mutations (crosses) were accumulated until a clonal event driving expansion (thunder) was encountered. Widths of the time frames are roughly proportional to the number of mutations detected for each event. The y-axis reflects the relative contribution of a HSC to overall peripheral blood production. 'WINDOW' refers to our window of observation ranging from age 103 to 111, a 9-year period characterized by the expansion of clonal event E.

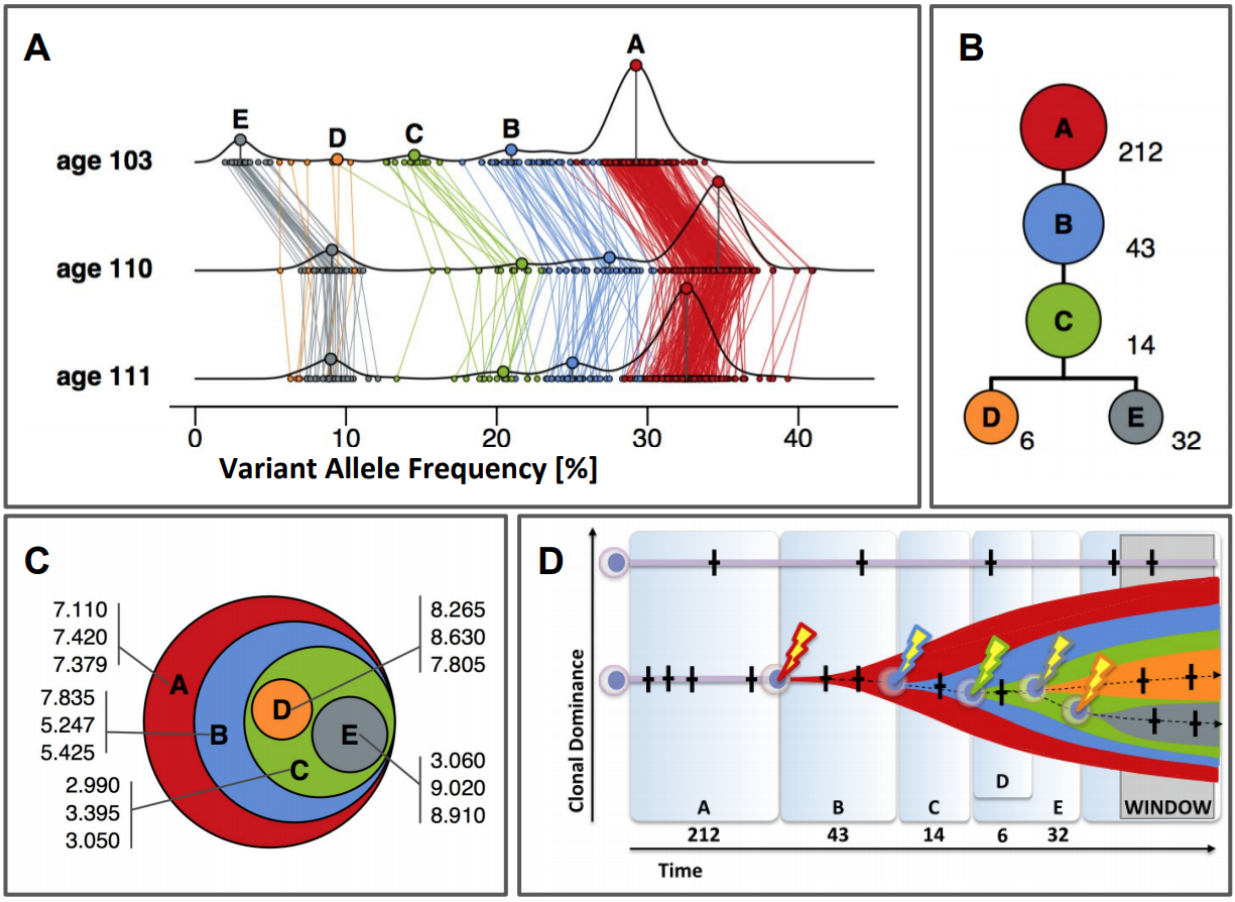

Figure 4: **Contributions of clonal events to the major subsets in peripheral blood. [A]** Violin plots of VAFs [%] in peripheral blood and its sorted subsets. M: Monocytes; G: Granulocytes; PB: Peripheral Blood; B: B-cells; T: T-cells; T4: CD4 T-cells; T8: CD8 T-cells. Numbers signify time points: 0: age 103; 1: age 110; 2: age 111. **[B]** Fractions of mutated cells per sorted cell subset derived from each clonal event. Barplots per subset add up to 100%. **[C-E]** For respectively T-cells, CD4 T-cells and CD8 T-cells, the ratio of VAFs for mutations within the sorted subset and the corresponding peripheral blood sample (y-axis) as a function of the VAFs in peripheral blood (x-axis). For all three T-cell subsets, clonal events with higher dominance have a disproportionately larger contribution to the production of the respective subsets.

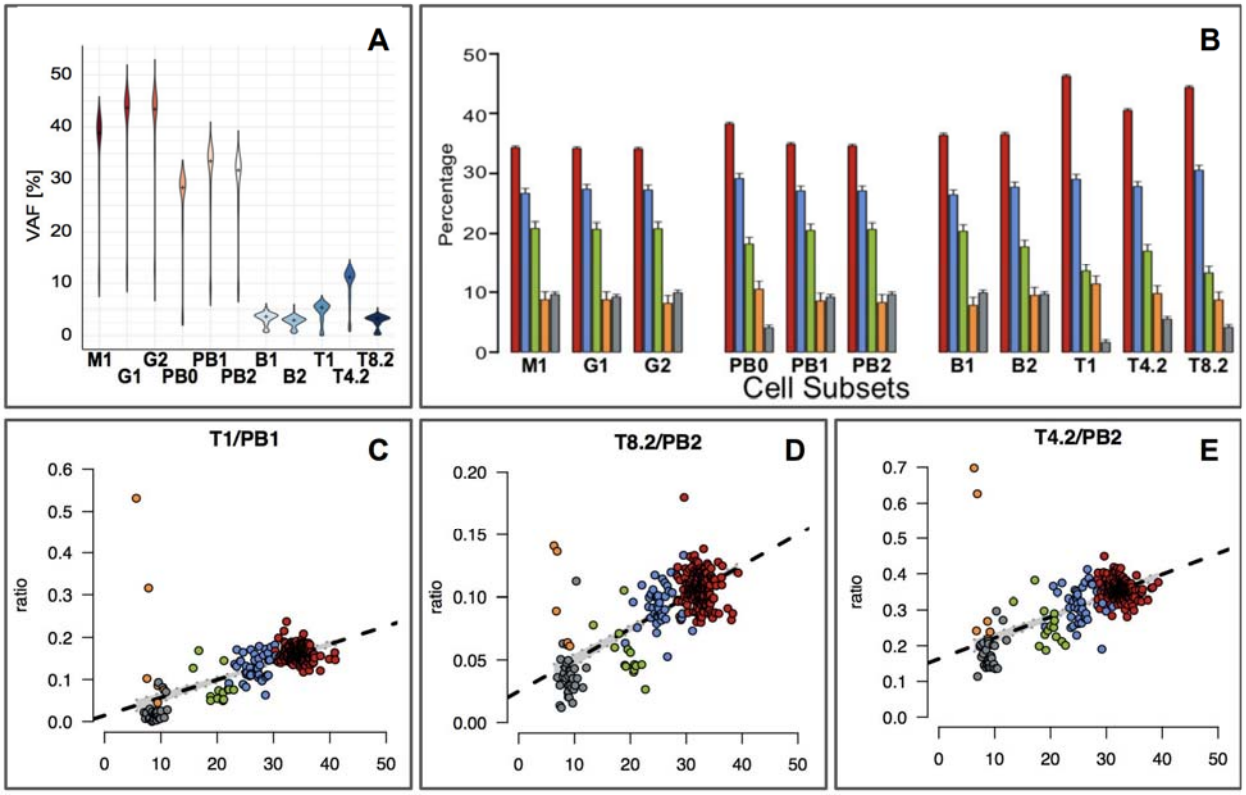

Figure 5: **Immune characterization of W111's T-cells drawn at age 110: [A]** Gating of live cells and CD4 and CD8 T-cells. **[B]** Subsequent gating of CD4+ T-cells; top left: CCR7-CD45RA+ (CD4+TEMRA) to top right: CD31+ (recent thymic immigrants); bottom left: CD27+CD28+ to bottom right: CD57+ (senescent) **[C]** Percentage of TREC positive cells in material derived from W111 (blue) or a middle-aged female control (grey). PB: Peripheral Blood cells; T: T-cells; T4: CD4 T-cells; T8: CD8 T-cells. Numbers signify time points: 0: age 103; 1: age 110; 2: age 111. **[D]** Stimulation Indices computed for IL2/TCR-dependent (IL2) and the allogenic (Allo) assay of cultured CD4 T-cells (dark grey) versus two middle-aged females used as control (lighter shades of grey).