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Letter to the Editor

Comparative analysis of clonal hematopoiesis of multipotent stem cells in healthy elderly in blood and bone marrow

To the Editor:

The identification of clonal hematopoiesis (CH) in healthy elderly individuals has added a significant new layer of understanding to the development of emerging myeloid neoplasms [1–3]. Likely due to limited sample availability, all major studies that initially described CH were exclusively performed using peripheral blood (PB) cells of healthy donors. Only one very recent study endeavored to study CH in bone marrow (BM) derived CD34 positive cells and found similar VAFs as compared to unfractionated peripheral blood [4]. Beyond this, little is known about the mutational burden of CH defining mutations in the bone marrow (BM) of healthy individuals. Furthermore, analysis of the BM of CH carrying individuals is necessary to elucidate the cells of origin and pathomechanisms leading to the preferential selection of these mutation carrying clones.

In this study we collected PB and the residual femoral head from n = 17 elderly patients undergoing hip replacement surgery (Table 1) at the University Hospital Mannheim, Germany and isolated mononuclear cells (MNC) from both PB and from the femoral head and expanded mesenchymal stromal cells (MSC) *in vitro* as previously published [5]. Subsequently, genomic DNA from BM, PB and MSCs was subjected to sequencing with the Illumina Myeloid Panel. Mutational analyses comprised of screening somatically acquired mutations in BM or PB using GATKs mutect2 and MSCs DNA as germline control. Mutations with VAF > 1% in either PB or BM were considered and annotated with annovar.

None of these patients had a previous medical history of a solid tumor, hematopoietic disorder or other severe past or present medical history. This cohort represented a typical elderly cohort with a median age of 73 years, normal blood counts and normal clinical chemistry parameters. We achieved a median coverage of 5 260× (95% > 1813×) across all amplicons. For validation of putative candidate mutations in PB or BM, independent PCR amplicons were amplified and sequenced after Nextera XT library construction, thereby achieving a median coverage of 10 191× (95% > 4 985×) for all amplicons. We detected clonal hematopoiesis in 6/17 (35%) of cases (Fig. 1A). The most frequently detected mutated gene was TET2 (3 cases) followed by DNMT3A in 2 cases. One patient carried a KIT p.S628N mutation. This CH frequency of 35% was higher than published in previous studies [2,6]. This discrepancy can possibly be explained by several factors: First, we used a targeted deep sequencing approach as opposed to exome-wide screening. This allowed for a deeper and thus more sensitive screening. Furthermore, we screened both PB and BM samples and found at least one case in which CH was not present in PB to a level of detection with 1%.

Correlation of clinical features associated with CH in our cohort (Table 2) revealed no statistically significant differences between both groups. Baseline blood parameters were equal between groups. Patients with CH tended to be older (70.4 vs 77.6 years, p = 0.16). In our cohort, the subgroup of < 70 year-olds, CH was present in 1 out of 76 (14%) cases, whereas clonal hematopoiesis was found in 5/10 (50%) cases among individuals with > 70 years of age. This is in line to a study that applied error-corrected sequencing to a healthy cohort [7] and found a CH frequency of up to 25% in the oldest group of 60–69 year olds.

We aimed to systematically correlate the clonal involvement in paired BM and PB samples and found that the clonal involvement was overall similar in BM and PB of the examined subjects and ranged from 1 to 34% VAF (r = 0.85 p = 0.08, Fig. 1B). In 83% of cases (5/6) the VAF was < 10% in both samples. One case (H16) harbored unexpected high VAFs displaying a TET2 mutation with a VAF of 21% in PB and 34% in BM. To determine which cell fractions carried the CH defining mutations, we FACS sorted various hematopoietic subfractions of BM and PB (Fig. 1C) including both stem- and progenitor compartments as well as terminally differentiated peripheral cell fractions from n = 3 patients with detectable CH (H07, H08, H012). H07 carried a TET2 p.R1404X mutation in BM samples with a VAF of 5% and only 1% in PB samples (Fig. 1D), which was at the lower level of detection in our conventional NGS approach. However, we were able to track this mutation in various progenitor cell fractions such as CD34+ and CD38+ cells. Of note, even in the most primitive hematopoietic stem cell gate (lin-CD34+CD38-CD90+), we found this mutation with a 5% VAF. This mutated CH clone was able to give rise to all compartments of differentiated hematopoiesis passing the mutational burden on into myeloid and erythroid progenitors with VAFs of 9% and 5% respectively. Also the CD14+ monocyte fraction in the PB showed positivity for the mutation with a VAF of 6%. Most importantly, B-lymphocytes

Table 1
Patient characteristics.

parameter	median (range)
Sex (m/f)	7/10
Age (years)	73 (54–96)
WBC (E9/L)	7.6 (5.5–9.7)
Hb (g/dL)	14.1 (13.2–15.1)
MCV (fl)	87.9 (82.2–94.5)
MCH (pg)	30 (27–32)
Plt (E9/L)	268 (152–353)
Creatinine (mg/dL)	0.95 (0.65–1.24)
BUN (mg/dL)	40.7 (21.3–59.1)
Albumin (g/L)	38.6 (34–41.6)
LDH (U/L)	244 (193–334)

WBC: white blood count, Hb: hemoglobin, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, Plt: Platelets, BUN: Blood urea nitrogen, LDH: Lactate dehydrogenase.

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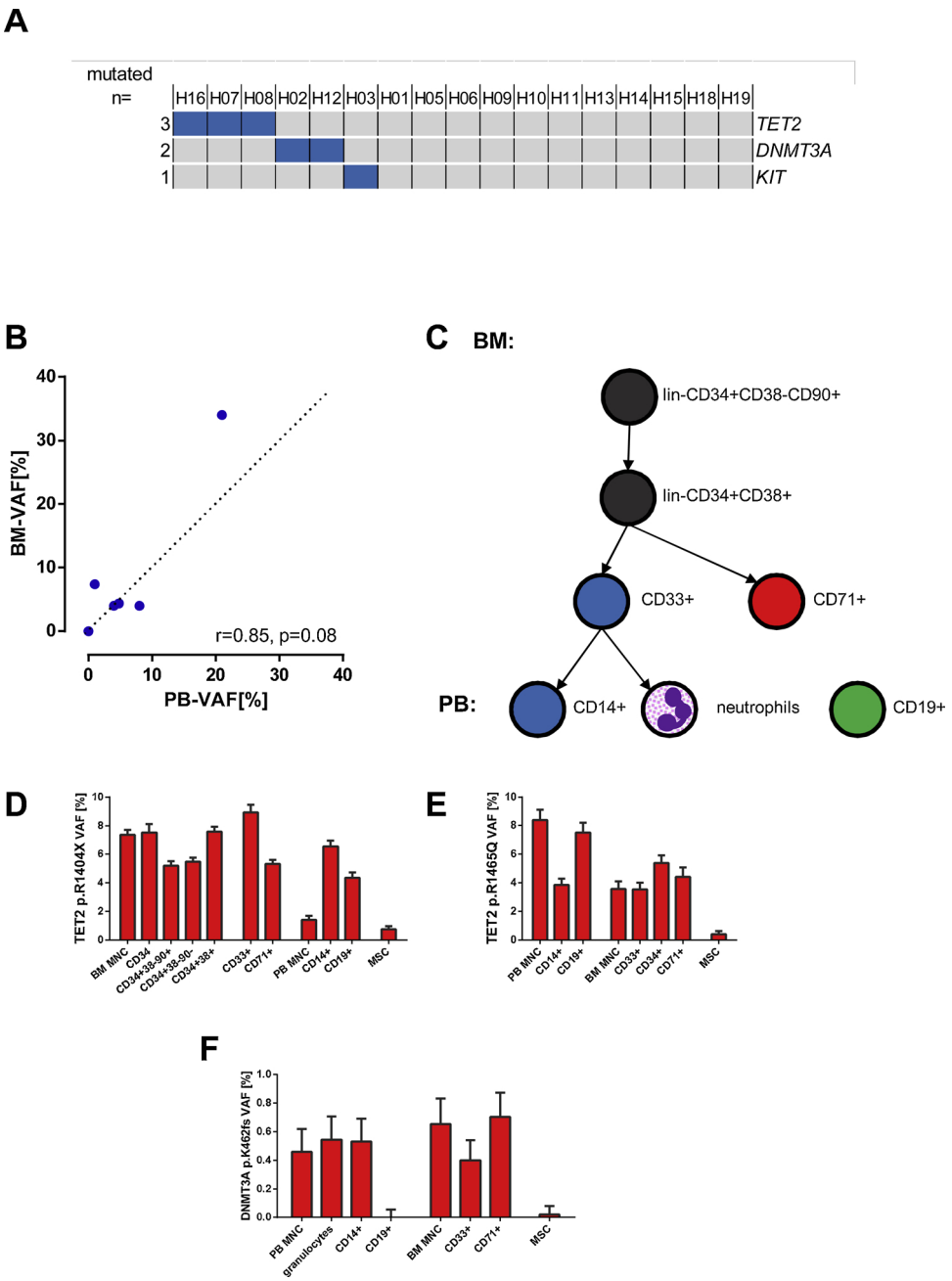

Fig. 1. (A) Summary of CH defining mutations detected within the cohort with validated VAF > 1% in either BM or PB. (B) Quantitative correlation of VAF in PB and BM. (C) Schematic overview of schema for FACS sorting of selected subfractions. VAF in various hematopoietic subfractions for patients (D) H07, (E) H08, (F) H12.

Table 2
Clinical parameters associated with CHIP.

parameter (mean +95% CI)	Non-CHIP (n = 11)	CHIP (n = 6)
Age (years)	70.4 (63.8–76.9)	77.6 (67.1–88.3)
sex (female:male)	6:5	4:2
Hb (g/dL)	14.2 (13.8–14.6)	13.8 (12.7–14.8)
MCV (fl)	86.9 (84.6–91.1)	86.5 (84.2–88.8)
MCH (pg)	29.9 (28.7–31.1)	29.3 (27.7–30.9)
WBC (e9/L)	7.12 (6.2–8.0)	8.2 (6.5–9.9)
Plt (e9/L)	243 (204–284)	275 (175–352)

were also positive for the TET2 mutation, albeit to a lower extent (VAF 4%). Similar multipotent involvement was seen for case H08 (Fig. 1E). In H12 we found a low-level 13bp deletion in DNMT3A causing a pre terminal stop codon with allele burden below 1% in PB and BM (Fig. 1F). Due to the nature of our sequencing approach, a valid quantitative assessment of this variant was not possible. Nevertheless, the mutation was qualitatively confirmed in all assessed hematopoietic subfractions except for CD19+ B-lymphocytes. In summary in all examined cases, the CH defining mutation was acquired at the level of a multipotent hematopoietic stem cell with clonal involvement in both myelo- and lymphopoiesis confirming a recent study by Arends et al. [4]. This has also been observed in acute myeloid leukemia [8] and myelodysplastic syndromes [9,10], affirming the hypothesis of common molecular roots of these diseases.

Since hip replacement surgery is a significant stress and inflammatory stimulus we were able to study aged human hematopoietic function in response to this severe artificial wounding scenario. As early

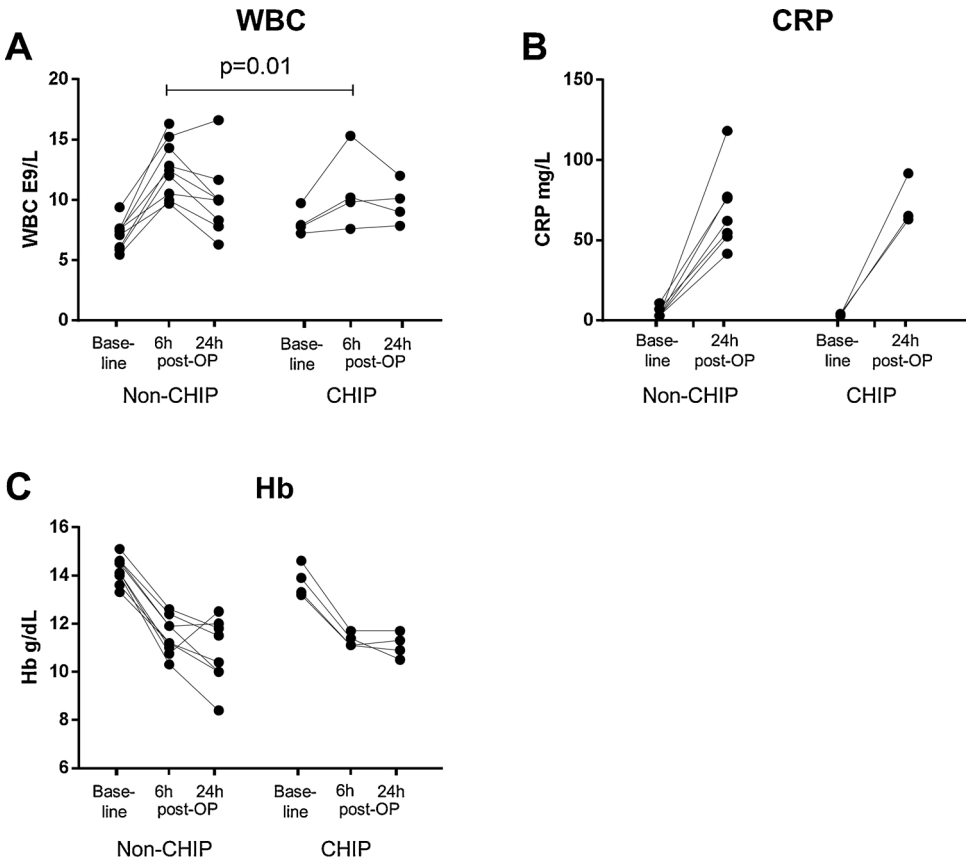

Fig. 2. Dynamic parameters during hip replacement surgery for patients with CH or non-CH. Hematopoietic parameter (A and C) at baseline, 6 h, 24 h. c-reactive protein (B) at baseline and 24 h.

as 4–6 h upon surgery a reactive rise in peripheral WBC (deltaWBC determined as first post-operative WBC – baseline WBC) could be observed in the routine blood counts taken to monitor perioperative Hb loss (Fig. 2A). Importantly the amplitude of this stress leukocytosis was reduced by 0.49 fold (mean increase in WBC 5.5E9/L, absolute 12.6E9/L in the CH vs. 2.5E9/L, absolute 10 E9/L in non-CH cohort, p = 0.01). In univariate Pearson's analysis, no other factor (age, sex, Hb, MCV, MCH, post CRP, Plt, Creatine, deltaHb) correlated with the rate of WBC cell increase. Importantly the effect of the presence of CH on the WBC amplitude retained statistical significance (p = 0.01) in a multivariate linear regression model (deltaWBC = CH + age + Hb + sex + CRP_d1 + Plt + Creatine, accounting for multiple testing). 24 h after surgery, this transient leukocytosis levelled back in both groups. To our knowledge, this is the first study that shows an impact of CH on *in vivo* functional parameters of healthy hematopoietic response in humans. This anergic leukocyte reaction upon the inflammatory stimulus of hip replacement surgery in CH patients is in line with previous reports [11] of poor mobilization of peripheral blood stem cells for patients undergoing autologous bone marrow transplantation. There patients with CH also displayed poorer mobilization after chemotherapy and G-CSF treatment resulting in increased collection days and an increase rate of failed mobilization. While our observation is limited by the retrospective nature of this data and relatively small cohort size, the difference nevertheless retained significance in a multivariate analysis. Other non-hematopoietic factors that indicate systemic inflammation or postoperative infection, such as C reactive protein showed a similar response 24 h after surgery in both groups (mean post-OP CRP 69 mg/L in non-CH vs 73 mg/L in the CH cohort, p = 0.79, Fig. 2B). Blood loss (as monitored by Hb decrease) and perioperative platelet counts were similar in both groups (Fig. 2C). Since the detected clone sizes were rather small in most samples, this relatively over proportionate anergic response in CH patients could possibly be explained by additional alterations in the bone marrow niche induced by the presence of CH clones in these patients or by other (e.g. epigenetic) effects.

In conclusion, our study supports the emergence of CH clones at the level of the hematopoietic stem cell compartment. Analysis of bone marrow specimens can pick up cases of CH, which would have been missed when analyzing only peripheral blood. We present first evidence that the presence of CH clones in the bone marrow of healthy individuals as defined by normal peripheral blood counts may have impact on the reactive capacity of the hematopoietic system upon response to exogenous stressors. Our study represents an additional layer of evidence for common roots of myeloid malignancies and shows that in few cases, CH clones emerge in the bone marrow that were not readily detectable with standard sequencing approaches.

Conflict of interest

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Johann-Christoph Jann, Florian Nolte, Maximilian Mossner, Johanna Flach, Eva Altrock, Nanni Schmitt
Department of Hematology and Oncology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

Henning Röhl
Department of Orthopedics and Traumatology, Diakonissenkrankenhaus, Mannheim, Germany

Ahmed Jawhar
Department of Orthopedics and Traumatology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

Uwe Neumann
Department of Surgery, Community Hospital, Reichenbach, Germany

Verena Nowak, Justine Danner, Julia Obländer, Iris Palme, Wolf-Karsten Hofmann, Daniel Nowak*
Department of Hematology and Oncology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany
E-mail address: daniel.nowak@medma.uni-heidelberg.de (D. Nowak).

* Corresponding author at: Medical Faculty Mannheim, Heidelberg University, Department of Hematology and Oncology, Pettenkofer Strasse 22, DE-68169, Mannheim, Germany.