Hematopoietic alterations in chronic heart failure patients by somatic mutations leading to clonal hematopoiesis

Acquired somatic mutations in genes associated with myeloid neoplasms have been recently identified in blood cells of elderly individuals without any hematologic abnormalities, leading to clonal blood cell expansion.[1,2] Once the detected variant allele fraction (VAF) has exceeded 0.02 (2%), the entity is technically named clonal hematopoiesis of indeterminate potential (CHIP), and somatic mutations in epigenetic regulators such as *DNMT3A* and *TET2* are most common.[3,4] CHIP is increased with age and is associated with adverse overall survival caused by a higher incidence of cardiovascular diseases, chronic post-ischemic heart failure (CHF), and hematologic malignancies. However, the direct consequences on the cellularity and distribution of blood cell lineages and hematopoietic stem and progenitor cells (HSPC) in the bone marrow (BM) caused by distinct CHIP mutations are still unknown in humans. Therefore, we quantitatively assessed the peripheral blood (PB) and BM cell composition in patients carrying CHIP-driver mutations with a VAF of at least 0.02 compared to Non-CHIP carriers in a clinically well-characterized cohort of CHF patients. Patients with *TET2* mutations demonstrated increased numbers of leukocytes without a bias towards a specific blood cell lineage. Moreover, the CD34$^+$ HSPC compartment was significantly enlarged and CD133$^+$CD34$^+$ HSPC, which are particularly enriched on stem cells, were increased in numbers in patients with *TET2* mutations, thus indicating a net expansion of HSPC in patients with CHF carrying *TET2* CHIP-driver mutations. Surprisingly, patients with *DNMT3A* CHIP-driver mutations did not show an enlarged HSPC compartment, which stands in contrast to the excessive self-renewal of *Dnmt3a*-null hematopoietic stem cells in mouse models. Thus, somatic mutations in the CHIP-driver gene *TET2* are associated with

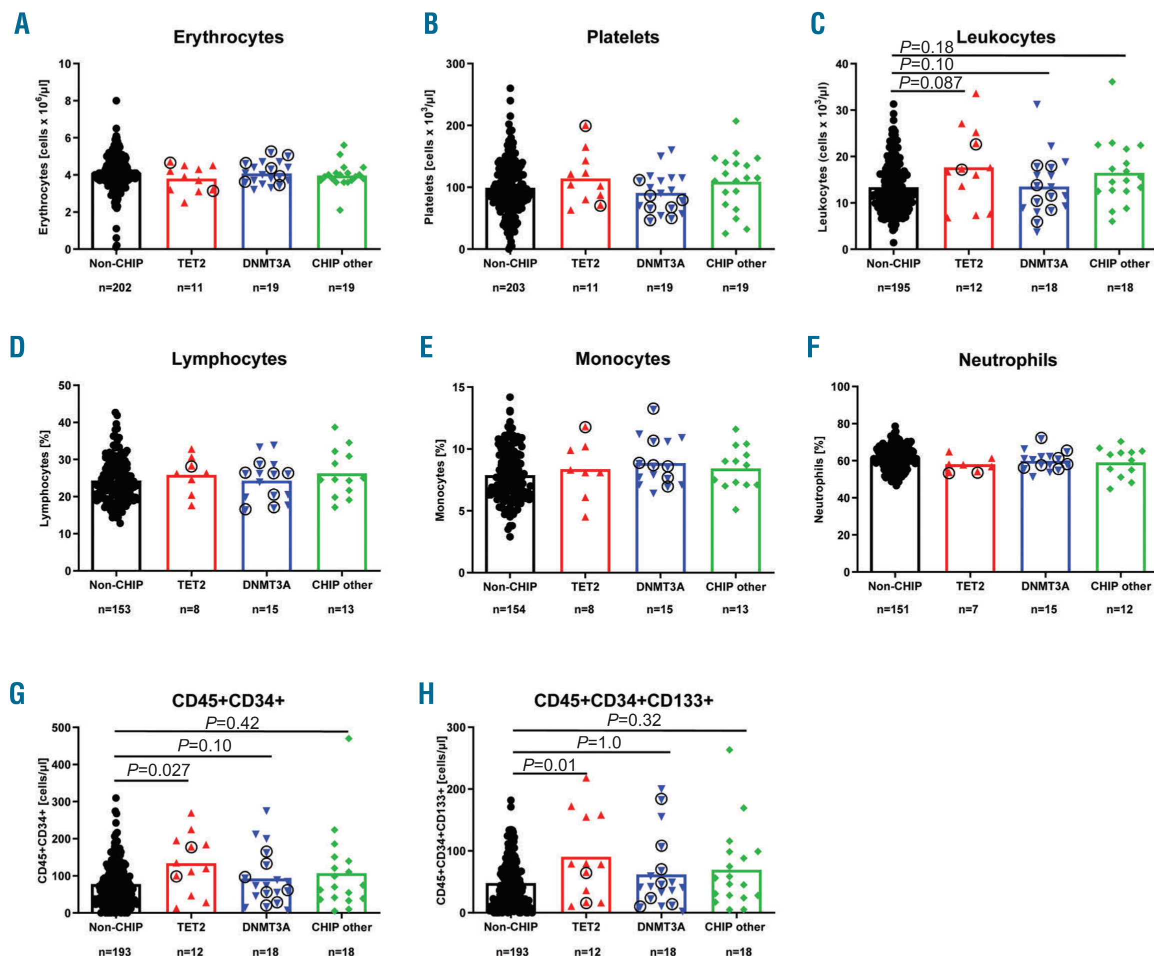

Figure 1. Bone marrow cell composition in chronic post-ischemic heart failure patients. Blood cell types were quantitatively determined *via* Sysmex measurements in Non-CHIP chronic post-ischemic heart failure (CHF) patients and CHIP-carriers. The CHF patients were further grouped according to the presence of a specific CHIP mutation. Absolute cell numbers of bone marrow (BM) erythrocytes (A), BM platelets (B), and BM leukocytes (C) are shown. The composition of BM leukocytes was further assessed for lymphocytes (D), monocytes (E) and neutrophils (F). (G and H) Hematopoietic stem and progenitor cells were quantitatively determined *via* flow cytometry using the Stem Cell Enumeration Kit (BD) in Non-CHIP CHF patients and CHIP-carriers. The CHF patients were further grouped according to the presence of a specific CHIP mutation. The numbers of CD34$^+$ stem and progenitor cells (G) and CD133$^+$CD34$^+$ stem cell-enriched cells (H) are shown here. Bars represent the mean. The number of included patients is shown below each group. Circles indicate data points from patients with more than one mutated CHIP-associated gene. Adjusted *P*-values between individual groups were determined by ANOVA testing and Bonferroni adjustment. Statistical analysis was performed with SPSS (Version 23.0, SPSS Inc.). CHIP: clonal hematopoiesis of indeterminate potential.

Table 1. Baseline characteristics of patient cohort according to their mutated CHIP- associated genes.

	CHIP *TET2* (n=13)	CHIP *DNMT3A* (n=19)	Other CHIP (n=20)	Non-CHIP (n=216)	*P*
Age (years; n=268)	67 ± 5 65 (64; 71)	66 ± 12 67 (56; 73)	67 ± 9 69 (61; 75)	62 ± 11 62 (54; 71)	0.03
Sex male (%; n=268)	62	79	85	84	0.19
Hypertension (%; n=266)	92	84	95	74	0.07
Hyperlipidemia (%; n=264)	85	82	80	76	0.83
Diabetes mellitus (%; n=268)	39	32	45	33	0.95
Current or former smoking (%; n=265)	62	79	65	65	0.66
Family history of coronary artery disease (%; n=243)	55	59	36	56	0.52
NYHA class (n=268)	2.2 ± 0.8 2 (2; 3)	2.3 ± 0.8 2 (2; 3)	2.2 ± 0.7 2 (2; 3)	2.3 ± 0.8 2 (2; 3)	0.96
Seattle Heart Failure Score; (n=214)	0.4 ± 1.2 0.2 (-0.8; 1.5)	0.4 ± 0.6 0.3 (-0.1; 0.9)	0.3±0.9 0.1 (-0.3; 0.6)	0.3 ± 1.0 0.2 (-0.3; 0.9)	0.98
Left ventricular ejection fraction (LVEF; %; n=253)	33.3 ± 9.5 35 (23; 43)	29.4 ± 8.3 31 (20; 35)	38 ± 13 37 (29; 49)	32.0 ± 11.8 30 (24; 40)	0.09
NT-proBNP serum levels (pg/mL); (n=238)	1913 ± 1811 1121 (463; 2,888)	2217 ± 1829 1960 (775; 3,046)	2,039 ± 3,805 502 (324; 1,974)	1,929 ± 3,786 911 (354; 1,910)	0.99
High sensitivity C-reactive protein levels (mg/dL; n=264)	0.85 ± 1.80 0.27 (0.10; 0.84)	1.39 ± 2.65 0.50 (0.13; 0.99)	0.73 ± 0.91 0.29 (0.21; 1.23)	1.17 ± 5.12 0.27 (0.13; 0.72)	0.97
PB Hemoglobin (g/dL); (n=267)	13 ± 2 13 (12; 15)	14 ± 1 14 (13; 15)	14 ± 1 14 (13; 15)	14 ± 2 14 (13; 15)	0.32
PB Hematocrit (%); (n=266)	38 ± 4 38 (34; 41)	41 ± 4 41 (39; 44)	39 ± 4 40 (36; 42)	41 ± 5 41 (38; 44)	0.12
PB Thrombocytes (x10^3/μL); (n=204)	253 ± 76 248 (186; 279)	214 ± 86 197 (162; 222)	196 ± 54 184 (150; 248)	213 ± 61 210 (168; 253)	0.25
PB Leukocytes (x10^3/μL); (n=267)	7.5 ± 1.6 7.4 (6.3; 8.1)	8.4± 4.2 7.9 (6.7; 9.0)	7.8± 2.1 7.4 (6.2; 9.4)	7.8 ± 2.5 7.4 (6.1; 8.8)	0.80
PB MCV (fL); (n=198)	90.4 ± 4.8 91.4 (85.1; 93.3)	92.5 ± 5.1 92.6 (90.8; 96.2)	90.4 ± 3.9 90.9 (88.6; 93.6)	91.0 ± 4.9 91.0 (88.3; 93.8)	0.64
PB MCH (pg); (n=198)	30.2 ± 1.6 30.7 (29.0; 31.3)	31.5 ± 2.5 31.9 (30.6; 32.7)	30.5 ± 2.1 31.3 (29.9; 31.8)	30.8 ± 1.9 30.7 (29.8; 31.8)	0.44
PB MCHC (g/dL); (n=198)	33.5 ± 1.0 33.7 (32.5; 34.4)	34.0 ± 1.1 34.0 (33.4; 34.8)	33.7 ± 1.4 33.8 (33.0; 35.0)	33.8 ± 1.0 33.8 (33.3; 34.4)	0.69
BM Erythrocytes (x10^6/μL); (n=251)	3.8 ± 0.7 3.9 (3.2; 4.5)	4.1 ± 0.6 4.0 (3.6; 4.6)	4.0 ± 0.7 3.9 (3.7; 4.1)	4.1 ± 1.0 4.1 (3.6; 4.6)	0.70
BM Platelets (x10^3/μL); (n=252)	114 ± 42 104 (80; 143)	91 ± 33 90 (66; 115)	109 ± 46 115 (72; 140)	99 ± 43 95 (74; 125)	0.39
BM Leukocytes (x10^3/μL); (n=243)	17.6 ± 8.4 17.0 (9.1; 24.6)	13.5 ± 6.6 12.5 (8.8; 17.7)	16.5 ± 7.0 15.2 (12.5; 21.8)	13.4 ± 5.4 12.2 (9.5; 16.6)	0.02
BM Neutrophils (% of leukocytes); (n=185)	58.1± 3.9 57.8 (54.1; 61.3)	59.9 ± 5.3 60.5 (55.7; 62.3)	59 ± 8 62 (52; 65)	61.6 ± 6.5 62.0 (56.9; 66.5)	0.28
BM Lymphocytes (% of leukocytes); (n=189)	25.9 ± 5.0 26.3 (21.5; 30.0)	24.3 ± 5.6 26.0 (19.8; 28.6)	26.3 ± 6.4 24.6 (20.8; 31.6)	24.4 ± 6.0 23.6 (19.9; 28.6)	0.66
BM Monocytes (% of leukocytes); (n=190)	8.4 ± 2.3 8.2 (6.6; 10.1)	8.9 ± 2.0 8.5 (7.1; 10.6)	8.4 ± 1.8 8.7 (7.1; 9.9)	7.9 ± 2.1 7.6 (6.3; 9.5)	0.29
BM CD45$^+$/CD34$^+$ (% of CD45$^+$) (n=234)	0.71 ± 0.27 0.68 (0.60; 0.94)	0.66 ± 0.49 0.54 (0.40; 0.89)	0.58 ± 0.46 0.45 (0.30; 0.70)	0.57 ± 0.28 0.53 (0.38; 0.74)	0.31
BM CD45$^+$/CD34$^+$/CD133$^+$ (% of CD45$^+$/CD34$^+$) (n=232)	67.6 ± 20.6 73.3 (60.5; 80.6)	64.3 ± 20.2 68.2 (56.4; 74.4)	67.4 ± 14.1 71.8 (59.4; 76.1)	61.0 ± 19.9 66.0 (53.5; 75.7)	0.38

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BM CD45+/CD34+ (cells/µL) (n=241)	134 ± 80 127 (60; 192)	93 ± 75 74 (41; 137)	107 ± 109 73 (38; 143)	78 ± 59 65 (34; 110)	0.01
BM CD45+/CD34+/CD133+ (cells/µL) (n=241)	90 ± 69 79 (21; 157)	62 ± 60 42 (20; 78)	70 ± 65 51 (27; 99)	48 ± 39 39 (15; 72)	<0.01

Continuous variables are shown as mean ± standard deviation; median (interquartile range), categorical variables are shown as frequency (%). MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration. The *P*-value for continuous variables were determined by ANOVA testing and for categorical variables by χ^2 test (two-sided). CHIP: clonal hematopoiesis of indeterminate potential.

an increased leukocyte production and an enlarged HSPC compartment including stem cells in the BM of patients with CHF.

DNMT3A encodes for a methyl transferase and *TET2* for a demethylase.[5] Mutations in these genes often occur in myelodysplastic syndrome, myeloproliferative neoplasms, and acute myeloid leukemia (AML).[5–7] *DNMT3A* and *TET2* mutations are mostly loss-of-function mutations.[5] CHIP has gained enormous medical interest since the prevalence of CHIP is age-dependent and associated with decreased overall survival,[2] which is only partly caused by a moderate risk to develop hematologic malignancies,[1,2] but rather caused by cardiovascular diseases.[8] Atherosclerosis is accelerated in patients harboring CHIP,[8] at least in those with *DNMT3A* and *TET2* mutations, as also supported by mouse models.[9,10] We recently identified a strong association of *TET2/DNMT3A* CHIP-driver mutations and adverse clinical outcome in patients with chronic post-ischemic heart failure.[11] Although the loss of *Tets2* and *Dnmt3a* have been studied in the context of hematopoiesis and hematologic disorders in mouse models,[12–14] the direct consequences on the cellularity and distribution of blood cell lineages and HSPC in the BM caused by distinct CHIP mutations are still unknown in humans.

To examine whether distinct somatic mutations encoding for CHIP result in cellular blood alterations, we analyzed the PB and BM in a cohort of 268 CHF patients who participated in different trials examining the effects of intracoronary administration of autologous blood mononuclear cells (BMC) between June 2005 and July 2017 at the University Hospital of the Goethe University (Frankfurt/Main, Germany) according to their mutated genes.[11] All patients provided written informed consent and the ethics review board of the Goethe University (Frankfurt, Germany) approved the protocols. The study complies with the Declaration of Helsinki. These patients had a median age of 63 years, NYHA class 2 and a left ventricular ejection fraction of 32% due to a previous myocardial infarction. The BM and PB were analyzed at the same time point with a median time of 5 years since the last infarction.[11] Due to the retrospective nature of our analyses, not all clinical parameters were available for all patients at the time of sample analysis. BM aspirate (50 mL) was obtained from the iliac crest under local anesthesia. BMC were isolated by Ficoll density-gradient centrifugation, as previously reported.[11]

The determination of mutations in 56 genes associated with CHIP in BMC was performed *via* error-corrected deep targeted amplicon sequencing (TruSeq Custom Amplicon Low Input Kit, Illumina) with a median coverage across all samples of 4,290x before unique molecular identifier (UMI) family clustering and 638x with inclusion of UMI. 52 of 268 CHF patients did harbor CHIP-driver mutations with a VAF ≥0.02 at the time of analysis, which affected 63 different somatic mutations in 19 genes (*Online Supplementary Figure S1*). Mutations in *DNMT3A* and *TET2* were most prevalent affecting 32 patients (19 with *DNMT3A* and 13 with *TET2* mutations) in our CHF patient cohort. Other mutations were in *KDM6A* (4), *BCOR* (3), *ASXL* (3), *SF3B1* (3), *CBLC, TP53* and *ZRSR2* (two cases each) and 10 other genes (*Online Supplementary Figure S1* and *Online Supplementary Tables S1-2*), with an average VAF of 0.084 (range: 0.02-0.42).

In PB, hemoglobin, hematocrit, leukocyte numbers and the number of thrombocytes did not differ between carriers of CHIP-driver mutations and Non-CHIP patients (*Online Supplementary Table S3*), in agreement with previous studies in elderly people.[3] Likewise, blood cell composition in the BM of CHF patients did not differ with respect to the number of erythrocytes and platelets, or the percentage of neutrophils, lymphocytes and monocytes between carriers of CHIP-driver mutations and Non-CHIP patients. Interestingly, the leukocyte numbers were increased in patients carrying a CHIP-associated mutation (*Online Supplementary Table S3*).

Since *TET2* and *DNMT3A* mutations account for the vast majority of CHIP-driver mutations and were experimentally related to an increased inflammatory activity,[10,15] we focused subsequent analyses on the two most prevalent genes in our cohort, *DNMT3A* and *TET2*. The average age of patients carrying either *DNMT3A* or *TET2* mutations was similar (66±12 years and 67±5 years, respectively), but significantly higher compared to non-CHIP carriers (62±11 years, *P*=0.03). Sex distribution and smoking history was not different between CHIP and non-CHIP patient groups (Table 1). CHF patients carrying a *TET2-CHIP*-driver mutation had a trend towards lower hematocrit (Table 1) and increased leukocyte numbers (Figure 1C) without any significant bias in the distribution of neutrophils, lymphocytes and monocytes, and no change in platelet numbers (Figure 1A-F). The mean corpuscular volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC) were both similar between the groups. In contrast, CHF patients carrying a *DNMT3A* CHIP-driver mutation did neither show any changes in the PB hematocrit and hemoglobin level nor altered blood cell lineages in the BM (Figure 1A-F).

Next, we investigated the HSPC compartment *via* flow cytometry using the quantitative Stem Cell Enumeration Kit (BD Biosciences) according to the supplier´s instructions.[16] First, we determined the percentage and absolute cell number of CD34+CD45+ HSPC. There was a significant increase of CD34+CD45+ HSPC in patients carrying a *TET2* CHIP-driver mutation (Figure 1G). Further restriction of our analyses on the immature HSPC com-

partment by addition of the anti-CD133 antibody (clone AC133, Miltenyi Biotech, Germany) to the Stem Cell Enumeration Kit revealed a significant increase in the number of $CD133^+CD34^+CD45^+$ HSPC, suggestive of a further enrichment for hematopoietic stem cells in carriers of the *TET2* CHIP-driver mutation (Figure 1H). Importantly, CHF patients harboring DNMT3A mutations did neither show an increase of HSPC nor an increase in the $CD34^+CD45^+$ compartment, which contrasts with experimental knockout mouse models using *Dnmt3a*-null hematopoietic stem cells, that demonstrate a strong self-renewal expansion.[12] Since two CHF patients with *TET2* mutations and seven patients with DNMT3A mutations harbored an additional mutation in another CHIP-associated gene with a VAF ≥0.02 (*Online Supplementary Table S1*), we excluded these patients from our BM analyses to avoid confounding effects of the second mutation. Again, the increase in BM leukocytes (P=0.04), $CD34^+CD45^+$ (P=0.02) and $CD133^+$ $CD34^+$ $CD45^+$ HSPC (P=0.001) remained significant only in the *TET2*-mutated CHF patient group (n=10), and not in the *DNMT3A*- or other CHIP-driver mutation groups (n=11 and 18, respectively), in comparison to non-CHIP CHF patients (n=195).

Finally, we determined the stem and progenitor cell distribution in CHF patients with *TET2*-driven clonal hematopoiesis with VAF between 0.005 and 0.01 and between 0.01 and 0.02. Only *TET2*-mutated patients with a VAF ≥0.02 harbored increased $CD34^+CD133^+CD45^+$ stem and progenitor cells (12 patients, 90±69 cells/μL), in comparison to patients with a VAF between 0.01 and 0.02 (7 patients, 43±39 cells/μL), a VAF between 0.005 and 0.01 (41 patients, 54±54 cells/μL), and patients without clonal hematopoiesis (153 patients, 50±41 cells/μL, ANOVA P=0.026), suggesting that an accelerated load of *TET2*-mutated cells is required to promote stem and progenitor cell expansion.

In summary, distinct somatic mutations leading to CHIP have a different outcome on blood cell composition and production. In our cohort of CHF patients, *TET2* mutations are associated with leukocytosis in the BM, while PB hematocrit is slightly reduced. Since the composition of the different leukocyte lineages is not largely altered, *TET2* mutations may directly impact on early stem/progenitor cells. Indeed, the absolute numbers of HSPC are significantly increased in CHF patients carrying a *TET2* CHIP-driver mutation. These results are the first to show that, in patients with CHF, *TET2* mutations are associated with a net increase of HSPC in humans, which is supported by mouse models with conditional *Tet2* deficiency.[14,17] However, mouse models simulating human CHIP with a subfraction of *Tet2*-deleted blood cell clones by transplantation into wild-type recipients have so far not reported on changes in HSPC cellularity,[10,15] which may be explained by a different responsiveness to altered extrinsic factors such as cytokines in mice and humans. In contrast, *DNMT3A* mutations in CHF patients did not significantly alter the numbers and distributions of PB and BM blood cells, nor did they impact on the cellularity of HSPC. This result is unexpected given the self-renewal promoting phenotype of murine *Dnmt3a*-null HSC.[12]

Clonal hematopoiesis may arise from a competition of mutated and non-mutated HSPC, or from successive expansion of mutated HSPC. In the latter, the HSPC compartment expands. As we show here, *TET2*-mutated CHF patients demonstrate a net increase of HSPC cellularity in their BM. *TET2*-mutated HSPC may become independent from the BM niches, thereby promoting their expansion, which may also lead to extramedullary hematopoiesis as shown in *Tet2* heterozygote mice.[14] Whether non-mutated HSPC are also affected in individuals with CHIP caused by *TET2* mutations in a paracrine, cell-extrinsic fashion, caused by an inflammatory milieu due to altered cytokine production, requires further investigation.

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