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Clonal hematopoiesis in clinical and experimental heart failure with preserved ejection fraction

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DISCLOSURES

None.

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Abstract

Background: Clonal hematopoiesis (CH), resulting from an array of nonmalignant driver gene mutations, can lead to altered immune cell function and chronic disease, and has been associated with worse outcomes in patients with heart failure (HF) with reduced ejection fraction (HFrEF). However, the role of CH in the prognosis of heart failure with preserved ejection fraction (HFpEF) has been understudied. Therefore, this study sought to characterize CH in patients with HFpEF and elucidate its causal role in a murine model.

Methods/Results: Using a panel of 20 candidate CH driver genes and a variant allele frequency (VAF) cutoff of 0.5%, ultra-deep error-corrected sequencing identified CH in a cohort of 81 patients with HFpEF (Age: 71 ± 6 years old, EF: 63% ± 5%) and 36 control individuals without a diagnosis of HFpEF (Age: 74 years old ±7 years old, EF: 61.5% ± 8%). Compared to control individuals, there was an enrichment of *TET2*-mediated CH in the HFpEF patient cohort (12% vs. 0%, respectively, p=0.02). Within the HFpEF cohort, patients with CH exhibited exacerbated diastolic dysfunction in terms of E/e' (14.9 vs. 11.7, respectively, p=0.0096) and E/A (1.69 vs. 0.89, respectively, p=0.0206) compared to those without CH. The association of CH with exacerbated diastolic dysfunction was corroborated in a validation cohort of 59 individuals with HFpEF. Accordingly, HFpEF patients with CH and an age ≥ 70 years old exhibited worse prognosis in terms of 5-year CV-related hospitalization rate (HR = 5.06, p= 0.042) compared to HFpEF patients without CH and an age ≥ 70 years old. To investigate the causal role of CH in HFpEF, non-conditioned mice underwent adoptive transfer with *Tet2*-wildtype or *Tet2*-deficient bone marrow and were subsequently subjected to a high fat diet/L-NAME combination treatment to induce features of HFpEF. This model of *Tet2*-CH exacerbated cardiac hypertrophy by heart weight to tibia length and cardiomyocyte size, diastolic dysfunction by E/e' and LV EDP, and cardiac fibrosis compared to the *Tet2*-wildtype condition.

Conclusions: CH is associated with worse heart function and prognosis in patients with HFpEF, and a murine experimental model of *Tet2*-mediated CH displays greater features of HFpEF.

Keywords

Clonal hematopoiesis; Heart failure; Prognosis; Biomarkers; Translational Studies

INTRODUCTION:

Heart failure (HF) is a clinical syndrome of breathlessness and/or fatigue caused by impaired cardiac function. Heart failure (HF) can be further categorized into heart failure with reduced ejection fraction (HFrEF) or heart failure with preserved ejection fraction (HFpEF), which are classically associated with a predominant systolic dysfunction and diastolic dysfunction, respectively.[1] Despite a similar prevalence of HFpEF and HFrEF,

the mortality rate of HFrEF has dropped significantly due to advancements in care while the mortality rate of HFpEF has remained largely uncurbed due to limited FDA-approved treatments for HFpEF.[2–4] The etiology of the HFpEF is poorly understood compared to that of HFrEF, yet recent findings suggest a pronounced role of inflammation, caused by diabetes, hyperlipidemia and hypertension, in the development of HFpEF.[5] However, these conditions alone are insufficient for the development of HFpEF, suggesting the presence of additional factors that contribute to the etiology. Thus, a better understanding of the mechanisms that contribute to HFpEF is required to address this prevalent yet underserved disease.

Clonal hematopoiesis (CH) is an emerging immunological phenomenon that has been implicated in different diseases.[6,7] In this process, hematopoietic stem cells (HSCs) incur somatic mutations as a consequence of aging, smoking, or other environmental/biological stresses. In some cases, these mutations occur in "driver" genes, such as *DNMT3A, TET2, TP53, ASXL1,* and *JAK2.*[8–13] When these driver genes are mutated, they provide a selective growth advantage to the HSCs. As a result, the mutant cell line outcompetes neighboring cells in the bone marrow niche and undergoes a clonal expansion. Furthermore, these mutations are maintained in the progeny cells of the HSC, and they can potentially affect the function of leukocytes and promote a chronic inflammatory state.[9,11–13] Recent studies have associated CH with worsened prognoses in HFrEF and in patients with an ischemic etiology of heart failure.[14–17] However, there is no current literature connecting CH to the prognosis of patients with HFpEF. In this study, we elucidated the significance of CH in populations of patients with HFpEF and control individuals without a diagnosis of HFpEF. Furthermore, we characterized the effects of *Tet2*-mediated CH in a murine model of HFpEF.

METHODS:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Clinical Data (Alberta HEART)

All participant data were sourced from the Alberta Heart Failure Analysis Research Team (HEART) cohort and described by Ezekowitz et. al.[18,19] Briefly, the cohort was recruited in Alberta, Canada, from 2010 to 2014 in an outpatient setting from a variety of clinics and the community at large. The cohort has been prospectively followed for clinical outcomes since inception in 2010 via annual administrative health data abstraction. Standard baseline demographics, laboratory, and other medical history were collected via medical record review and direct contact with the patient during study entry and following visits. Transthoracic echocardiography was performed with the subjects at rest in left lateral decubitus position using commercially available Phillips iE33 ultrasound imaging system (Philips Medical Systems) equipped with S5-phased or X5-phased array transducer. All images were digitally stored for offline analysis (Xcelera, Philips Medical System). Standard apical four- and two-chamber views were recorded with care taken to avoid foreshortening. LV volumes were measured from the apical four- and two-chamber views. Left ventricular end-systolic volume and end-diastolic volume were calculated using Simpson's biplane

method of discs. LVEF was subsequently derived and expressed as a percentage. Patients received standardized follow-up every three months, during which additional information was collected. Median follow-up was 1355 days (25th–75th percentile 854–1774). Patients were assigned to the control group or HFpEF group through an adjudication process conducted by team members with clinical experience and expertise. The adjudication process required two expert clinicians to review each case independently while blinded to each other's adjudication. The control population lacked a diagnosis of heart failure at any time during the study, were not risk of developing heart failure, and did not exhibit any symptomatology of heart failure at time of study entry. Given the diverse and ever-changing guidelines and criteria for HFpEF,[20] no strict criteria for HFpEF were employed. Instead, the two expert clinicians reviewed each case independently and assigned patients to HFpEF group based on medical history, echocardiography, other radiology testing, and laboratory information. The data for adjudication was sourced from past medical records and testing performed at study entry. Blood samples for ultra-deep, error-corrected sequencing were taken at time of study entry.

Data management and regulatory, ethical and administrative review for Alberta HEART was provided in-kind by the Canadian VIGOUR Centre (Edmonton, Alberta, Canada), and biologic samples were managed by the Canadian BioSample Repository (Edmonton, Alberta, Canada) at the University of Alberta, Canada. Written informed consent was obtained from all participants. The study is registered (clinicaltrials.gov NCT02052804). All analyses on laboratories and echocardiographic measurements were performed on baseline values, and patients with missing data for a given variable were excluded from analysis for that variable.

Validation Clinical Cohort (SCAN-MP)

Participant data was sourced from the ongoing clinical trial Screening for Cardiac Amyloidosis with Nuclear Imaging in Minority Populations (SCAN-MP) cohort as described by Ruberg et. al.[21] Briefly, subjects were recruited from Columbia University Irving Medical Center, Harlem Hospital, Yale University, and Boston Medical Center. All participants self-identified as Black or Hispanic of Caribbean origin. Heart failure was diagnosed by either the modified criteria utilized by Rich et al.[22] or a score ≥3 on the National Health and Nutrition Examination Survey (NHANES) for heart failure criteria. Modified criteria utilized by Rich et al. which include a history of acute pulmonary edema or the occurrence of at least two of the following that improved with diuretic therapy without another identifiable cause: dyspnea on exertion, paroxysmal nocturnal dyspnea, orthopnea, bilateral lower extremity edema or exertional fatigue. Additionally, patients with HFpEF had an ejection fraction >30%. Only HFpEF patients, who lacked a diagnosis of cardiac amyloidosis, were included in the present analysis. At time of enrollment, medical history, medication inventory, laboratories, and transthoracic echocardiography were obtained. At time of enrollment, blood samples were also collected for assessment of clonal hematopoiesis.

SCAN-MP was approved by the Western Institutional Review Board (IRB) in a single IRB model (IRB#: 20183425). Written informed consent was obtained from all participants.

The study is registered (clinicaltrials.gov NCT03812172). All analyses on laboratories and echocardiographic measurements were performed on baseline values, and patients with missing data for a given variable were excluded from analysis for that variable.

Animal Studies/Mice

Wild-type mice (*Tet2*$^{+/+}$), *Tet2*-deficient mice (*Tet2*$^{-/-}$: B6(Cg)-*Tet2*$^{tm1.2Rao}$/J), and Pep Boy mice (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) were sourced from Jackson Laboratory (Stock #: 000664, 023359, 002014, respectively). All strains had a C57BL/6J background. *Tet2*$^{+/-}$ mice were used for breeding, and *Tet2*$^{-/-}$ and *Tet2*$^{+/+}$ male offspring were used for the test group and the control group, respectively. The animal protocols for these experiments were approved by the Institutional Animal Care and Use Committee of the University of Virginia.

Nonmyeloablative Bone Marrow Transplantation

Nonmyeloablative bone marrow transplantation was performed as previously published.[23] Briefly, 8- to 12-week-old Pep Boy mice were transplanted with suspensions of bone marrow cells from either Tet2$^{+/+}$ mice or Tet2$^{-/-}$ mice. Unfractionated bone marrow cells (5×10^6 cells/day) were injected via retro-orbital vein into non-irradiated recipients per day over 3 consecutive days (total: 1.5×10^7 cells).

HFpEF Model

After nonmyeloablative bone marrow transplantation, mice were randomized to receive either a control diet and water or high fat diet (S1850, Bio-serv) and N[w]-nitro-l-arginine methyl ester (L-NAME; 1.0 g/L, Sigma Aldrich, N5751) in the drinking water.[24] The drinking water was changed once per week. Throughout the length of the experiment, mice were maintained on a 12-hour light/dark cycle and had unrestricted access to food and water.

Statistical Analyses

Data are presented as mean and standard deviation unless otherwise noted. For continuous data with one variable, Shapiro Wilk test was performed to evaluate the data distribution. Non-normally distributed data was analyzed for statistical significance by Mann-Whitney U test while normally-distributed data was analyzed with Welch's *t* test and 1-way ANOVA with Dunnett multiple-comparison for data with one independent variable and 2 groups or >2 groups, respectively. For categorical variables, Fisher exact tests were performed to test statistical significance. For continuous data with two variables, a 2-way ANOVA with post hoc Sidak multiple comparison test was used to test for statistical significance. For multivariable analysis of continuous variables, a multivariable linear model was created adjusting for differences in backgrounds between groups. Data are reported as the coefficient ± standard error. For univariable and multivariable analysis of outcomes, a Cox proportional hazards model was employed. Data are reported as a hazard ratio and its corresponding 95% confidence interval.

RESULTS:

HFpEF patients with CH display worse cardiac diastolic function and outcomes

To investigate the significance of clonal hematopoiesis in HFpEF, peripheral blood samples were sourced from the Alberta HEART cohort.[19] Ultra-deep, error-corrected sequencing with a panel of 20 candidate driver genes was then employed to identify and quantify CH (Table S1). CH mutations only included frameshift, splicing, stop-gain, or nonsense variants, which had the potential to influence protein function. The sequencing methodology was sufficient to resolve mutations with variant allelic frequencies (VAFs) as low as 0.005. As such, we used this VAF as our threshold for diagnosing CH in patients. The cohort was composed of 81 patients with HFpEF and 36 control individuals without a diagnosis of HFpEF. As expected, the clinical parameters of the patients with HFpEF differed significantly from that of the control individuals with respect to hypertension, diabetes, smoking history, and a number of other comorbidities (Table S2). In this cohort, ultra-deep, error-corrected sequencing identified mutations in 12 known CH driver genes (Figure 1A). *DNMT3A* and *TET2* were the most commonly mutated genes. Of all the individuals with CH in the cohort, individuals with 1 mutation, 2 mutations, and 3+ mutations accounted for 77%, 19%, and 4%, respectively (Figure 1B). Consistent with previous reports,[7,16] CH prevalence increased with age (Figure 1C). Individuals between the ages of 60 to 69 years-old displayed a CH mutation prevalence of 24%, while 64% of individuals between 80 to 89 years-old displayed CH mutations. As shown in Figure 1D, patients with HFpEF possessed, on average, 4-fold larger CH clones than control individuals (VAF of 0.015 vs. 0.061, p=0.0197). Interestingly, there were no *TET2* mutations identified in control individuals, while 10 patients with HFpEF harbored a detectable *TET2* mutation (Figure 1E).

Further analysis characterized the functional significance of CH within the HFpEF population. The backgrounds of HFpEF patients with CH and HFpEF patients without CH were similar (Table 1). However, HFpEF patients with CH tended to be older (77 vs. 72 years, p = 0.001) and have a lower BMI (28 vs. 32 kg/m^2, p=0.008) and greater ACEi/ARB use (87% vs. 64%, p=0.01) than HFpEF patients without CH. HFpEF patients with CH had a worse baseline diastolic function compared to HFpEF patients without CH, as evidenced by an increase in both E/e' and E/A and decreased deceleration time (Figure 2A–2C). HFpEF patients with CH had elevated levels of the BNP and NT-proBNP biomarkers of HFpEF severity compared to HFpEF patients without CH at baseline (Figure 2D and 2E). Multivariate analysis was performed to adjust for age, BMI, and ACEi/ARB use (Table S3). After adjustment, E/e', E/A, and deceleration time remained statistically significant, and CH further became associated with increased left atrial volume index (LAVI). Additionally, many of these differences were maintained at different VAF cutoffs for identifying CH in both univariate and multivariate analyses, which corrected for corresponding differences in background characteristics (Tables S4–S5). Given the differences in heart function, we then evaluated whether this difference was maintained when only stratifying for the presence or absence of mutations in either of the two most common clonal hematopoiesis driver genes *DNMT3A* and *TET2* as has been done previously. [15,16,25] HFpEF patients with a *DNMT3A/TET2* mutation and HFpEF patients without *DNMT3A/TET2* mutation

had similar backgrounds (Table S6). However, HFpEF patients with a *DNMT3A/TET2* mutation tended to be female (70% vs. 46%, p=0.0339), have a lower BMI (28 vs. 31kg/m^2, p=0.0385), and have a lower prevalence of COPD (7% vs. 31%, p=0.0128). After adjusting for background differences, mutations in either *DNMT3A* or *TET2* were associated with worse diastolic function by E/A and deceleration time (Table S7). Furthermore, if using 0.01 or 0.02 as VAF cutoffs for diagnosing CH, mutations in either *DNMT3A* or *TET2* were associated with worse diastolic function by E/e'.

In a separate validation cohort, PBMCs were sourced from the ongoing SCAN-MP clinical trial,[21] which comprised HFpEF patients from minority populations. Ultra-deep, error-corrected sequencing was again performed to identify and quantify CH. HFpEF patients with CH and HFpEF patients without CH displayed no differences in background characteristics (Table S8). However, HFpEF patients with CH exhibited worse diastolic function by both E/e' (18.4 vs. 13.8, p=0.003) and e' alone (5.0 vs. 6.2, p=0.03) compared to HFpEF patients without CH (Figure S1A,B). Furthermore, this association held if HFpEF patients were stratified for the presence or absence of mutations in either *DNMT3A* or *TET2*. Despite no difference in background characteristics (Table S9), HFpEF patients with a *DNMT3A/TET2* mutation exhibited an increased E/e' (18.7 vs. 14.3, p=0.005) compared to HFpEF patients without a *DNMT3A/TET2* mutation (Figure S1C).

We further investigated the impact of CH on prognosis for patients with HFpEF, whose age ranged from 60 to 90 years old. HFpEF patients with CH and without CH displayed 5-year CV-related hospitalization rates of 43.5% and 26.9%, respectively (HR: 2.09, p=0.10, Figure S2A). We next examined prognosis by stratifying for an age ≥ 70 years old, at which many of these adverse events become more common. The backgrounds of HFpEF patients with CH and an age ≥ 70 years old and HFpEF patients without CH and an age ≥ 70 years old were similar (Table S10) except that HFpEF patients with CH tended to be older (80 vs. 77 years, p = 0.02) and have a lower BMI (28 vs. 31 kg/m^2, p=0.008). HFpEF patients with CH and an age ≥ 70 years old displayed a 5-year CV-related hospitalization rate of 51% while HFpEF patients without CH and an age ≥ 70 years old only had a 5-year CV-related hospitalization rate of 10% (HR=6.83, p=0.0041, Figure 3A–B). To eliminate the contribution of age and BMI, a Cox proportional hazards model for HFpEF patient outcomes was performed (Figure 3C). After adjusting for both age and BMI, HFpEF patients with CH and an age ≥ 70 years old maintained a statistically significant increase in 5-year CV-related hospitalization rate compared to HFpEF patients without CH and an age ≥ 70 years old, suggesting worse prognosis (HR=5.06, p=0.042). Furthermore, the difference in CV-related hospitalization rate is maintained at VAF thresholds for CH of ≥0.005, ≥0.01, and ≥0.02 in in both univariate and multivariate analyses, which corrected for corresponding differences in background characteristics (Tables S11–S12).

Given the difference in prognosis for patients with HFpEF and an age ≥ 70 years old, we then evaluated whether this difference was maintained at the age threshold when only stratifying for the presence or absence of mutations in either *DNMT3A* or *TET2*. Indeed, HFpEF patients with a *DNMT3A/TET2* mutation and an age ≥ 70 years old had a 5-year CV-related hospitalization rate of 64% while HFpEF patients without a *DNMT3A/TET2* mutation and an age ≥ 70 years old had a 5-year CV-related hospitalization rate

of 13% (HR =5.25, p=0.006, Figure 3A,3C). HFpEF patients with a *DNMT3A/TET2* mutation and an age ≥ 70 years old had a similar background to HFpEF patients without a *DNMT3A/TET2* mutation and an age ≥ 70 years old; however, they tended to be female (71% vs. 44%, p=0.04, Table S13). After adjusting for sex by Cox proportional hazards model, this difference in CV-related hospitalizations was maintained (HR: 4.40, p=0.017, Figure 3A,3C). Furthermore, this difference in CV-related hospitalization rate was maintained at VAF cutoffs of ≥0.005, ≥0.01, and ≥0.02 in a univariate analysis and maintained at VAF cutoffs of ≥0.005 and ≥0.01 in a multivariate analysis, which corrected for corresponding differences in background characteristics (Table S14). To understand how individual mutations in either *DNMT3A* or *TET2* modify prognosis, HFpEF patients were stratified for the presence or absence of either of these mutants. In multivariate analyses adjusting for differences in backgrounds of HFpEF patients with an age ≥ 70 years old (Table S15), *DNMT3A*-mediated CH was associated with a significant increase in CV-related hospitalization rate (HR: 5.39, p=0.018, Figure 3A,3D) while *TET2*-mediated CH was associated with a trending increase in CV-related hospitalization rate (HR; 2.92, p=0.076, Figure 3A,3E). In contrast to the findings with echocardiographic parameters and CV-related hospitalization data, analyses of all-cause mortality did not reveal associations with CH within the entire cohort (Figure S2B–C) or subgroups (Table S16), which may be due to the high degree of patient survival in the Alberta HEART cohort.

A murine model of HFpEF accelerates TET2-mediated hematopoietic cell expansion

Due to its enrichment in the HFpEF patient cohort, *TET2* was chosen for further mechanistic studies in mice. To model a more physiologically relevant state of CH, a small number of CD45.2 *Tet2*-WT (*Tet2*$^{+/+}$) or *Tet2*-deficient (*Tet2*$^{-/-}$) bone marrow cells were adoptively transferred to CD45.1 Pep Boy mice and allowed to expand for one month. Mice were then either continued on control diet and water or placed on a high-fat diet (HFD) and L-NAME drinking water combination treatment to induce features of HFpEF.[24] Serial measurements were performed at baseline, 5 weeks, and 12 weeks post-treatment induction (Figure 4A).

To evaluate clonal expansion in this model, flow cytometry of peripheral blood was performed. As previously published,[23] donor-derived *Tet2*$^{-/-}$ cells expanded more rapidly throughout all white blood cell lineages compared to donor-derived *Tet2*$^{+/+}$ cells, with a bias towards the myeloid and B cell populations (Figure 4B–4G). Interestingly, HFD/L-NAME enhanced *Tet2*-mediated cell expansion (Figure 4B, 4C, 4E, 4G). In accordance, flow cytometry of bone marrow revealed expansion of donor-derived *Tet2*$^{-/-}$ cells in the long-term and short-term hematopoietic stem cell pools (LT-HSC and ST-HSC, respectively) compared to donor-derived *Tet2*$^{+/+}$cells (Figure 5A–5F). Further, in parallel with peripheral blood chimerism, donor chimerisms of hematopoietic stem cell pools were significantly higher for *Tet2*$^{-/-}$ cells subjected to the HFD/L-NAME regimen compared to *Tet2*$^{-/-}$ cells exposed to the control conditions. HFD/L-NAME induced a leukocytosis by 3 months with increases in the absolute number of neutrophils, monocytes, and leukocytes for the *Tet2*$^{-/-}$ group (Figure S3). There were no differences in platelet counts or hemoglobin concentration throughout the conditions.

TET2-mediated CH exacerbates heart failure in a murine model of HFpEF

To induce HFpEF, a combination of an obesogenic diet and a hypertensive drug was employed. The HFD/L-NAME combination increased weight gain compared to respective mice on control diet and water (Figure 6A). Furthermore, *Tet2*$^{-/-}$ group exhibited increased weight gain compared to the *Tet2*$^{+/+}$ group on the HFD/L-NAME treatment. Additionally, the HFD/L-NAME combination treatment led to increased systolic blood pressure compared to the respective control group (Figure 6B).

To test whether *Tet2*-mediated CH modifies cardiac function in this model of HFpEF, serial echocardiographic analysis was performed on these mice. The HFD/L-NAME combination induced an increase in left ventricular filling pressures as estimated by an increase in the Doppler-derived E/e' ratio and measured directly via LV catheterization, collectively indicating worse diastolic heart function (Figure 6C–D). Moreover, the adoptive transplantation of *Tet2*$^{-/-}$ bone marrow exacerbated diastolic dysfunction compared to mice receiving *Tet2*$^{+/+}$ bone marrow. The *Tet2*$^{-/-}$ group on the HFD/L-NAME treatment also displayed worse cardiac hypertrophy and fibrosis compared with the *Tet2*$^{+/+}$ group of the same dietary treatment (Figure 6E–G).

DISCUSSION:

Clonal hematopoiesis is increasingly recognized as a key risk factor for cardiovascular disease. The accessibility of human peripheral blood samples and declining cost of ultra-deep error-corrected sequencing have streamlined the analysis of CH in clinical cohorts. In the last few years, numerous studies have been published on the associations between CH and CVD in patients[7] and its additive effect with traditional CVD risk factors.[26] Notably, CH is associated with increased risk of incident HF, and increased CVD morbidity and mortality in patients with HFrEF.[14–17] Despite these advances, past studies were either not stratified for etiology or only constrained to patients with HFrEF.[16] Thus, the present study has focused on the role of CH in HFpEF.

In this study, we investigated the significance of CH in control patients and HFpEF patients of the cardiometabolic phenogroup, as evidenced by the high BMI and diabetes prevalence. We found an enrichment of patients with *TET2* mutations in the HFpEF cohort versus control individuals. In an analysis of all 20 CH driver genes assayed, there was a significant increase in variant clone size in the patients with HFpEF versus the control group. However, due to the differences between the patients with HFpEF and control individuals, it is uncertain whether these findings are being driven specifically by the disease state of HFpEF or by one or more of its comorbidities/covariates. Thus, focusing on patients with HFpEF, analyses revealed that the presence of CH was associated with worse echocardiographic metrics of heart function. Specifically, the increased E/e' and E/A and decreased deceleration time collectively indicate worse diastolic heart function in patients with HFpEF and CH compared to those without CH. The increased left atrial volume index after multivariable adjustment suggests structural remodeling of the heart in HFpEF patients with CH compared to HFpEF patients without CH. Accordingly, these findings are associated with worse long-term prognosis as evidenced by increased 5-year CV-related hospitalization rate. Consistent with prior literature,[15,16,25] we also stratified for

the presence or absence of *DNMT3A/TET2* mutations and found similar results. Notably, the association between CH and worse prognosis appears to become more pronounced with age. Patients with HFpEF and an age ≥ 70 years old had 6-fold greater odds of experiencing a CVD-related hospitalization if they possessed a *DNMT3A* or *TET2* mutation. This age-dependent association of CH with CV-related hospitalization may be attributed to the greater prevalence of comorbidities and hospitalization in patients of advancing age. Furthermore, the significance of these findings is maintained when explicitly adjusting for covariates in a multivariable model (Table S17). In sum, the presence of CH appears to be an important determinant of the functional status and morbidity of HFpEF.

Our study extends upon previous clinical findings connecting CH and heart failure. Studies have reported that *DNMT3A*- and/or *TET2*-mediated clonal hematopoiesis is associated with worse prognosis in patients with chronic ischemic HF and following ST-segment elevation myocardial infarction.[15,25] It has also been reported that patients with *DNMT3A*- and/or *TET2*-mediated clonal hematopoiesis exhibit adverse HF progression irrespective of ischemic or nonischemic etiology.[16] Yu et al. found that CH, particularly mutations in the *ASXL1*, *TET2* and *JAK2* driver genes, was associated with incident heart failure in an analysis of 5 large biobanks.[17] More recently, the study by Shi et al.[27] focused specifically on the role of CH in incident HFpEF. This study reported that CH, using a VAF threshold of 2%, was associated with incident HFpEF in individuals younger than 65 years old, but this association was not found when the entire cohort was analyzed. Our data suggests that the association between CH and HFpEF can extend to patients older than 65 years old, and that VAFs as low as 0.5% may be predictive of this condition. The significance of this finding is bolstered by the fact that small clones become almost ubiquitous with advanced age.[28] We found that lower VAF cutoffs tended to better stratify patients in terms of prognosis as evidence by the higher hazards ratio with lower VAF cutoffs (Table S13). This may be due to the fact that these small clones can expand over the course of follow-up and consequently, exert a greater biological effect with time. Thus, traditional DNA sequencing approaches, which are limited by their sequencing depth and therefore, VAF detection limit, are not able to detect these clones, which appear to harbor notable prognostic significance. As a consequence, though previous work has documented a pronounced effect of CH on prognosis,[6,15,16,25,29,30] these data may be an underestimation of the true effect of CH.

To examine HFpEF in an experimental model, we utilized a combination of an obesogenic diet and the hypertensive drug L-NAME, as previously described.[24] This model was chosen as it reproduces aspects of the metabolic syndrome and consequent features of HFpEF that can be observed in these patients and further mirrors the cardiometabolic phenogroup largely investigated in our clinical cohort. Furthermore, it does not exploit genetic perturbations that are generally not observed in patients. As such, it was deemed the more physiologically realistic and applicable model for our murine studies. Additionally, the non-irradiated adoptive transfer model of clonal hematopoiesis was employed to model the spontaneous development and expansion of *Tet2*-deficient clones. This model avoids dynamic changes induced in both the hematopoietic niche and cardiovascular system, and appetite loss as a consequence of irradiation.[31–33] Together, we reasoned that this combined model more faithfully produces features of HFpEF and *TET2*-mediated CH for mechanistic studies.

As previously published in the adoptive transfer model,[23] *Tet2*-deficient hematopoietic cells were found to expand more rapidly than *Tet2*-sufficient clones. However, the HFD/L-NAME treatment accelerated expansion of *Tet2*-deficient donor cells in both peripheral blood and hematopoietic stem cell lineages. Analogously, these data mirrored the increased VAF of CH clones observed in patients with HFpEF compared to control individuals. This accelerated expansion may be partially attributed to increased hematopoiesis. Compared to the $Tet2^{-/-}$ group on control conditions, the $Tet2^{-/-}$ group on HFD/L-NAME treatment exhibited leukocytosis with elevation of several WBC lineages. Furthermore, hypertension has been previously demonstrated to increase hematopoiesis in murine and human models.[34] Thus, these differences may reconcile the lack of accelerated *Tet2*-deficient clonal expansion observed when mice are subjected to a high fat diet alone, atherogenic stimuli, or other models of heart failure.[10,35,36] Moreover, under the conditions of experimental HFpEF, the increased chimerism may further exacerbate the sequelae of *Tet2*-mediated CH.

In this murine model, *Tet2*-mediated clonal hematopoiesis exacerbates several features of HFpEF. The $Tet2^{-/-}$ group manifested increased E/e', LV EDP, HW/TL, and cardiac fibrosis compared to the $Tet2^{+/+}$ group on the HFD/L-NAME treatment, suggesting worse diastolic function, cardiac hypertrophy, and cardiac fibrosis. This again parallels our clinical data, which showed that patients with HFpEF and CH have significantly worse diastolic function. In summary, our data suggests that *Tet2*-mediated clonal hematopoiesis directly exacerbates heart failure in a murine model of HFpEF and reproduces many findings observed in a human cohort of HFpEF.

This study extends upon previous work in elucidating causal connection between *TET2*-mediated CH and cardiovascular disease.[7] Previously, our lab has shown that Tet2-deficiency in HSCs spontaneously leads to HFrEF in aging mice.[23] Additionally, Tet2-deficiency in HSCs exacerbates heart failure in ischemic models of HFrEF such as myocardial infarction and non-ischemic models of HFrEF such as pressure overload and angiotensin II infusion.[9,10] Collectively, *Tet2*-mediated CH exacerbates HFpEF and HFrEF in murine models of disease.

We acknowledge there are several limitations to the present study. First, for our clinical studies, the sample size is modest, and all patients were recruited from the same geographic location. However, in an analysis of a small cohort from the SCAN-MP study, we were able to corroborate that CH was associated with diastolic dysfunction. In the future, larger multicenter studies are required to further corroborate these findings and potentially reveal novel associations, whose effect sizes were too small to uncover in our analyses. Second, we acknowledge that HFpEF is a diverse disease state with myriad pheongroups.[37,38] Our cohort was composed predominately of the cardiometabolic phenogroup, in which patients are typically obese and diabetic. As such, the role of CH in other HFpEF phenotypes remains outstanding. Third, it will be informative to discern whether CH has an impact on CV-related mortality through the analysis of larger cohorts or a longer follow-up. Regarding this point, cohorts of Canadian patients with HFpEF, such as the one examined herein, may exhibit reduced morbidity and mortality due to the predominance of Caucasians enrolled in the study.[39,40] Fourth, the adoptive transfer of Tet2-deficient HSCs in experimental studies will generate larger homozygous clones rather than the heterozygous clones typically

observed in humans. However, this is common for almost all experimental models, which employ exaggerated stimuli to model disease in a temporally and fiscally feasible manner. Additionally, it has been previously demonstrated that similar to Tet2 deficiency, Tet2 heterozygosity promotes clonal expansion and cardiac dysfunction, albeit to a lesser extent.[10,23] Fifth, we acknowledge that we only employed one murine model of HFpEF. However, many other models of HFpEF don't reliably reproduce many of the clinical phenotypes of HFpEF.[41] Furthermore, models that resemble the clinical phenotype tend to utilize a combination of an obesogenic diet, hypertensive drug, and aging. Since the other robust models of HFpEF are largely redundant with our own, they weren't examined in our study. Finally, male mice were used exclusively for this study since females are more resistant to HFD-induced metabolic disturbances and inflammation.[42]

In closing, *TET2*-mediated clonal hematopoiesis is enriched among patients with HFpEF, and HFpEF patients with CH display worse heart function and prognosis, particularly in individuals 70 years and older. Furthermore, experimental models of HFpEF and *Tet2*-mediated CH suggest this relationship to be causal and may serve as a platform to further elucidate the pathophysiology and possible treatments for HFpEF exacerbated by CH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 4A was created with BioRender (BioRender.com).

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NON-STANDARD ABBREVIATIONS AND ACRONYMS

CH	clonal hematopoiesis
HSC	hematopoietic stem cells

HfrEF	heart failure with reduced ejection fraction
HfpEF	heart failure with preserved ejection fraction
DNMT3A	DNA (cytosine-5)-methyltransferase 3A
TET2	Tet Methylcytosine Dioxygenase 2
TP53	Tumor protein P53
ASXL1	ASXL transcriptional regulator 1
SF3B1	Splicing factor 3b subunit 1
STAG2	Stromal antigen 2
RAD21	RAD21 cohesin complex component
U2AF1	U2 small nuclear RNA auxiliary factor 1
CBL	Cbl proto-oncogene
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
MPL	MPL proto-oncogene, thrombopoietin receptor
KRAS	KRAS proto-oncogene, GTPase
IDH1	Calreticulin (CALR)Isocitrate dehydrogenase 1
IDH2	Isocitrate dehydrogenase 2
JAK2	Janus kinase 2
NRAS	NRAS proto-oncogene, GTPase
PTPN11	Protein tyrosine phosphatase non-receptor type 11
RUNX1	RUNX family transcription factor 1
ZRSR2	Zinc finger CCCH-type, RNA binding motif and serine/arginine rich 2
Tet2$^{+/+}$	wild-type mice
Tet2$^{-/-}$	*Tet2*-deficient mice
HFD	high-fat diet
L-NAME	N[w]-nitro-l-arginine methyl ester
LT-HSC and ST-HSC	long-term and short-term hematopoietic stem cell pools
CANTOS	Canakinumab Antiinflammatory Thrombosis Study
ED	Emergency Department

CV	Cardiovascular
(MMP) cells	Multipotent progenitors
(LSK) cells	Lin-Sca1^{+}c-Kit^{+}
BMI	Body mass index
COPD	Chronic obstructive pulmonary disease
CKD	Chronic kidney disease
ACEi	Angiotensin-converting enzyme inhibitors
ARB	Angiotensin II receptor blocker
CCB	calcium channel blocker
LVEF	Left ventricular ejection fraction
BSA	body surface area
LAVI	left atrial volume index
LVPWd	left ventricular posterior wall at end diastole
LVMI	left ventricular mass index
LVEDV	left ventricular end-diastolic volume
LV EDP	Left ventricular end-diastolic pressure
BNP	brain natriuretic peptide
NT-proBNP	N-terminal pro b-type natriuretic peptide

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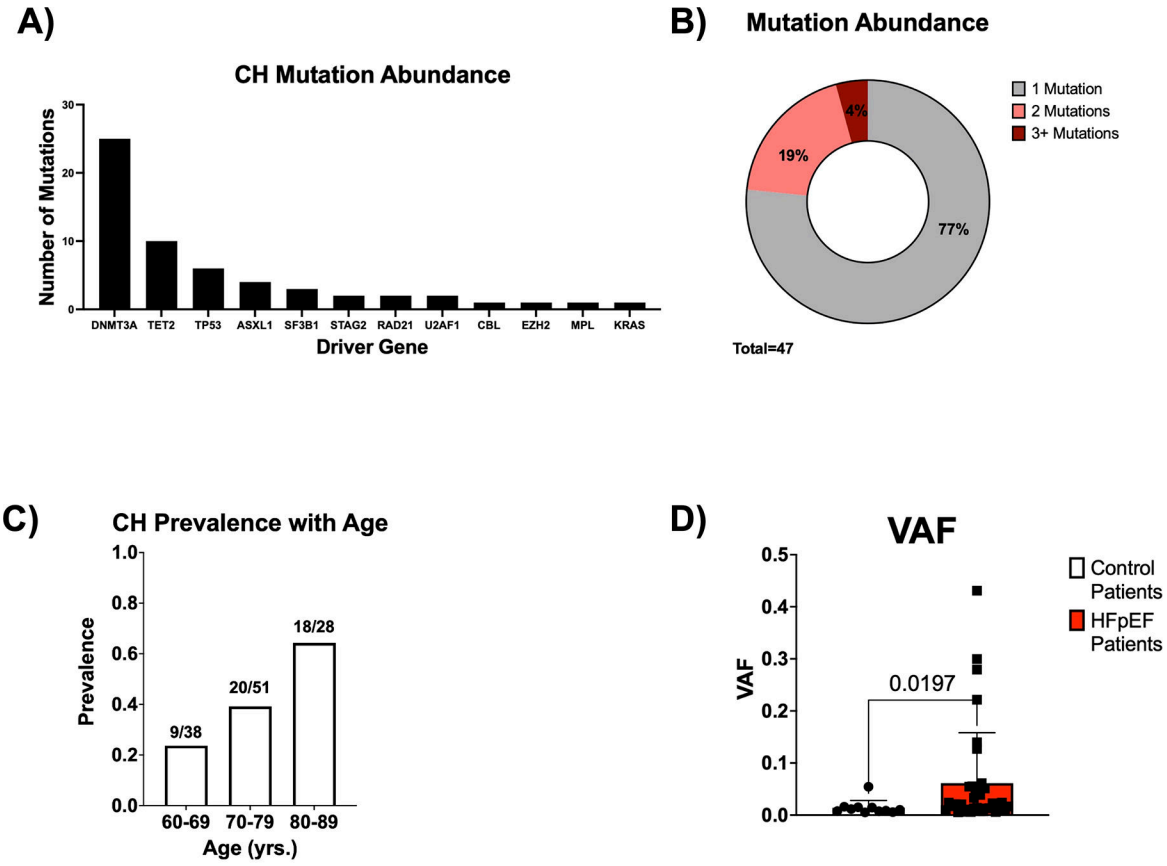
CLINICAL PERSPECTIVE

What is new?

- *TET2*-driven CH was enriched in a cohort of patients with HFpEF.
- In patients with HFpEF, CH was associated with worse diastolic heart function and outcome.
- In a murine model of HFpEF, *Tet2*-mediated CH led to higher echocardiographic E/e', greater left ventricular end-diastolic pressure, and greater cardiac fibrosis.

What are the clinical implications?

- *TET2*-driven CH may represent a novel pathophysiologic mechanism in HFpEF.
- Our findings establish a rationale for measuring CH in patients with HFpEF to predict future outcomes.
- Targeting *TET2*-mediated CH may be beneficial to prevent or treat HFpEF.



E)

CH Mutation Prevalence			
	Control individuals	HFpEF patients	p-value
Total Subjects	36	81	
CH	11(0.305)	36(0.444)	0.1125
DNMT3A	5(0.139)	20(0.247)	0.1413
TET2	0(0)	10(0.123)	0.0211
TP53	1(0.027)	5(0.062)	0.3987
ASXL1	2(0.056)	2(0.025)	0.3613
SF3B1	0(0)	3(0.037)	0.3280

Figure 1. Characterization of clonal hematopoiesis in the Alberta HEART patient cohort.

A. Abundance of the specified driver gene mutation in the patient cohort (n=58). **B.** Proportion of patients with the specified number of CH mutations (n=47). **C.** CH prevalence as a function of age in the patient cohort (n=117). **D.** VAF for the largest clone identified in patients with CH in control individuals and HFpEF patients. Statistical significance was determined by Mann-Whitney U test (n= 11 control individuals and 36 HFpEF patients). **E.** Prevalence of CH and driver gene mutations in control individuals and patients with HFpEF.

Statistical significance was determined by Fisher's Exact Test (n= 36 control individuals and 81 HFpEF patients).

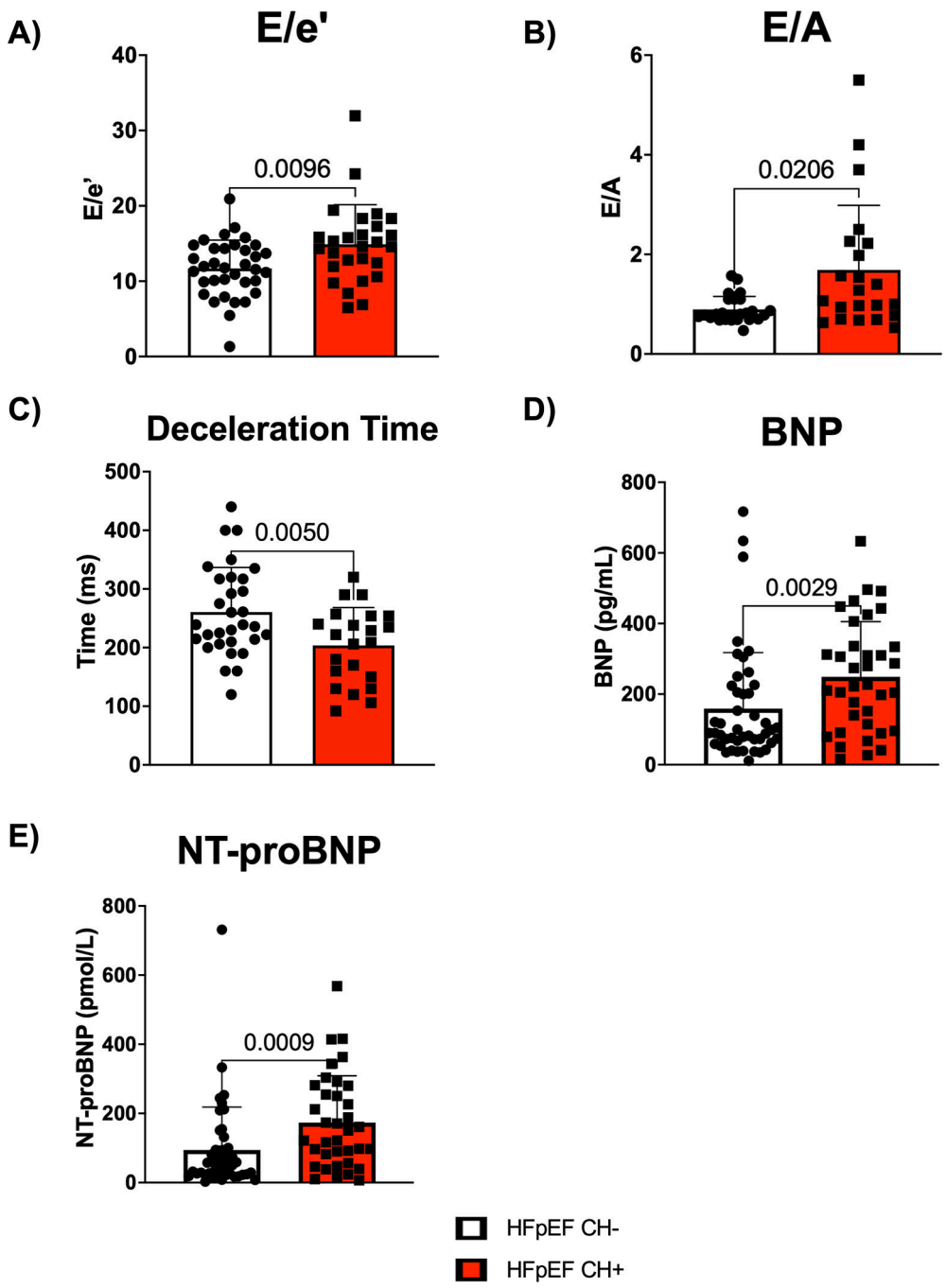


Figure 2. HFpEF patients with CH exhibit worse diastolic dysfunction and possess elevated levels of heart failure biomarkers at baseline.

A. Echocardiographic analysis of E/e' in HFpEF patients with and without CH. Statistical significance was determined by Mann-Whitney U test (n= 35 HFpEF CH− patients and 27 HFpEF CH+ patients). **B.** Echocardiographic analysis of E/A in HFpEF patients with and without CH. Statistical significance was determined by Mann-Whitney U test (n= 27 HFpEF CH− patients and 22 HFpEF CH+ patients). **C.** Echocardiographic analysis of deceleration time in HFpEF patients with and without CH. Statistical significance was determined by

Welch's *t* test (n= 31 HFpEF CH− patients and 22 HFpEF CH+ patients). **D.** Plasma brain natriuretic peptide (BNP) levels for HFpEF patients with and without CH. Statistical significance was determined by Mann-Whitney U test (n= 45 HFpEF CH− patients and 36 HFpEF CH+ patients). **E.** Plasma N-terminal pro b-type natriuretic peptide (NT-proBNP) levels for HFpEF patients with and without CH. Statistical significance was determined by Mann-Whitney U test (n= 45 HFpEF CH− patients and 36 HFpEF CH+ patients).

A)

Correlation of different CH mutations with patient outcomes		
	CV-related Hospitalization	
	HR (Univariable)	HR (Multivariable)
CH	6.83 (1.50-31.05, p=0.013)	5.06 (1.06-24.15, p=0.042)
DNMT3A/TET2	5.25 (1.60-17.25, p=0.006)	4.40 (1.30-14.94, p=0.017)
DNMT3A	3.55 (1.19-10.59, p=0.023)	5.39 (1.34-21.71, p=0.018)
TET2	2.92 (0.90-9.54, p=0.076)	2.92 (0.90-9.54, p=0.076)

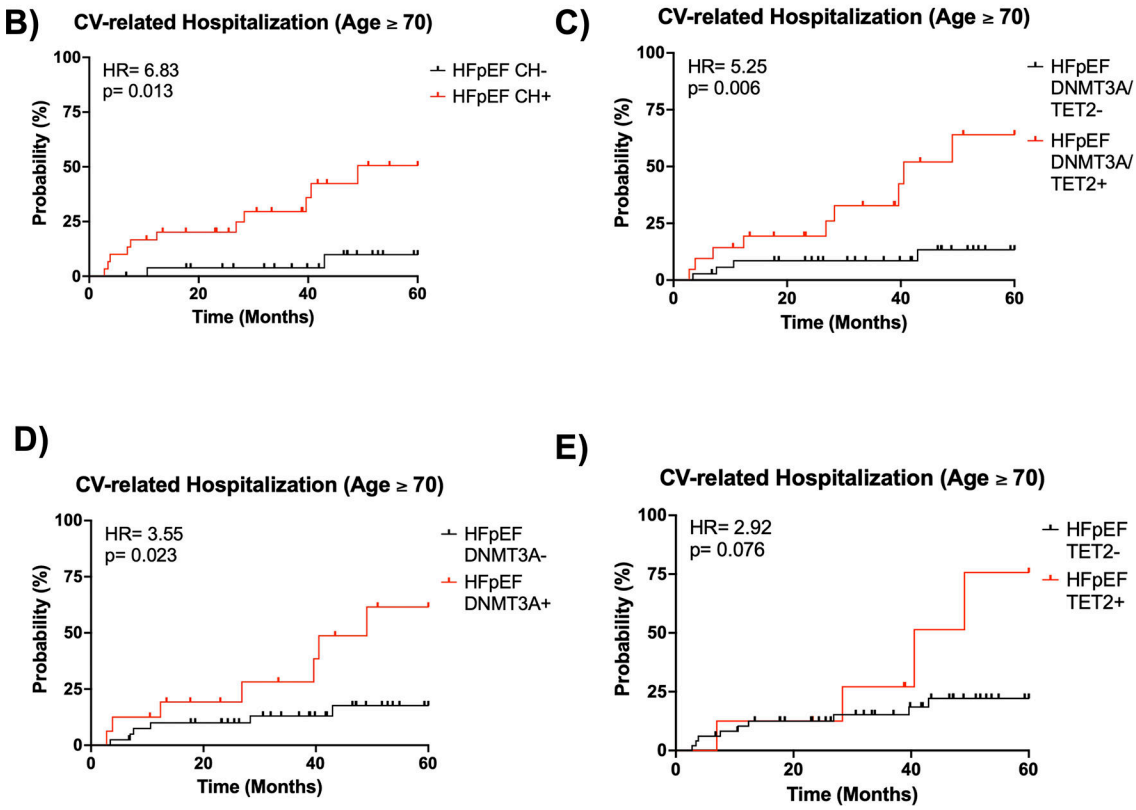


Figure 3. HFpEF patients with mutations in CH driver genes exhibit worse long-term prognosis.

A. Univariate and multivariate analysis of CV-related hospitalization for HFpEF patients and an age ≥ 70 years old based on CH status, *DNMT3A/TET2*-driven CH status, *DNMT3A*-driven CH status, and *TET2*-driven CH status. Statistical significance and hazards ratio were determined by Cox proportional hazards model. **B.** 5-year CV-related hospitalization based on clonal hematopoiesis status for HFpEF patients with an age ≥ 70 years old. Statistical significance and hazards ratio were determined by Cox proportional hazards model (n= 27 HFpEF CH− patients and 30 HFpEF CH+ patients). **C.** 5-year CV-related hospitalization

for HFpEF patients with and without *DNMT3A/TET2*-driven CH and an age ≥ 70 years old. Statistical significance and hazards ratio were determined by Cox proportional hazards model (n= 36 HFpEF DNMT3A/TET2- patients and 21 HFpEF DNMT3A/TET2+ patients). **D.** 5-year CV-related hospitalization for HFpEF patients with and without *DNMT3A*-driven CH and an age ≥ 70 years old. Statistical significance and hazards ratio were determined by Cox proportional hazards model (n= 41 HFpEF DNMT3A- patients and 16 HFpEF DNMT3A+ patients). **E.** 5-year CV-related hospitalization for HFpEF patients with and without *TET2*-driven CH and an age ≥ 70 years old. Statistical significance and hazards ratio were determined by Cox proportional hazards model (n= 49 HFpEF TET2- patients and 8 HFpEF TET2+ patients).

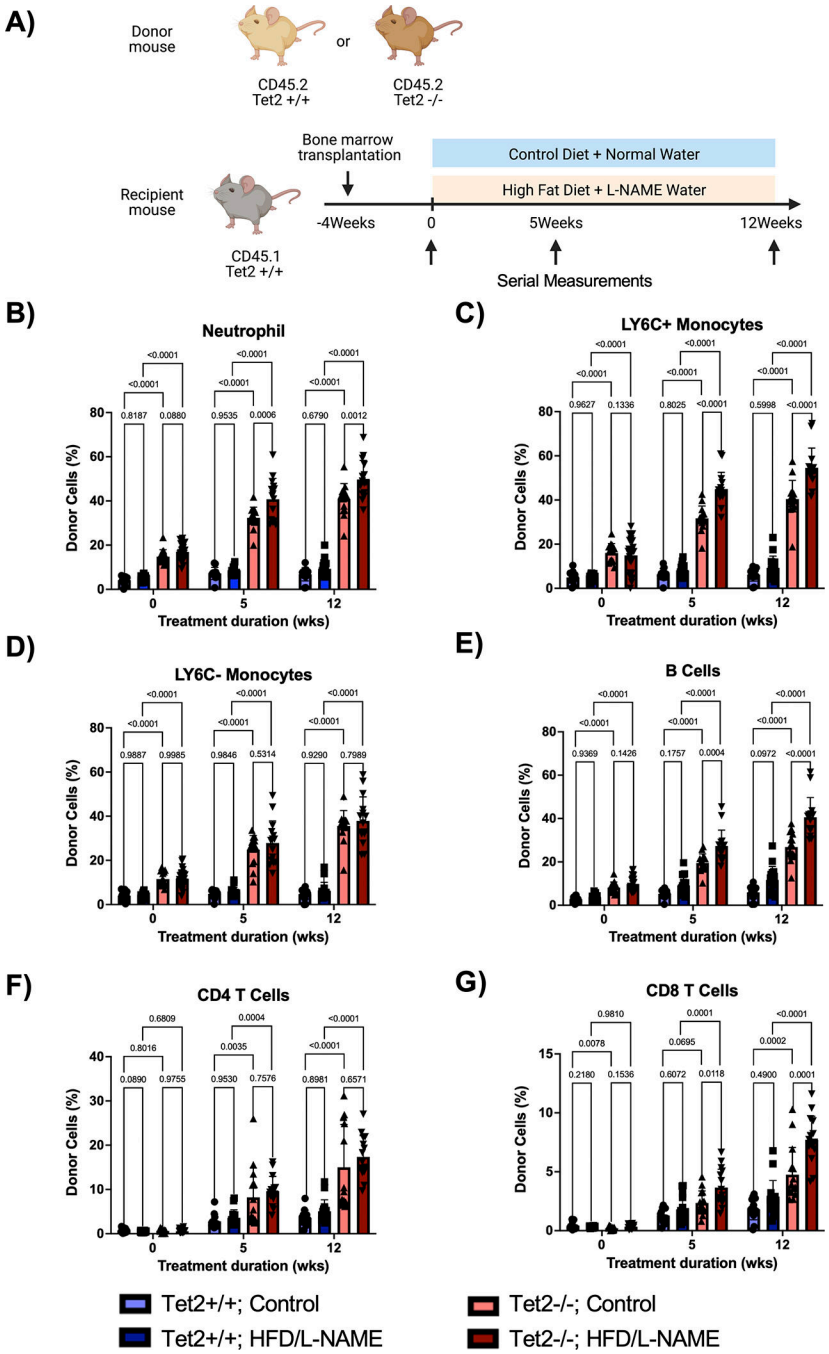


Figure 4. HFD/L-NAME treatment accelerates expansion of *Tet2*-deficient cells in peripheral blood.

A. Schematic of the experimental design. CD45.2 Tet2-sufficient or Tet2-deficient bone marrow was adoptively transferred to CD45.1 Pep Boy mice. One month after bone marrow transplantation, mice were started on either HFD/L-NAME combination treatment or continued on control diet and water. Serial measurements were taken at baseline, 5 weeks, and 12 weeks. **B-G.** Flow cytometric quantification of donor cell chimerism for neutrophils, Ly6C+ monocytes, Ly6C− monocytes, B cells, CD4 T cells, and CD8 T cells

in the peripheral blood at baseline and after 5 weeks and 12 weeks of treatment. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n= 13–26 per group).

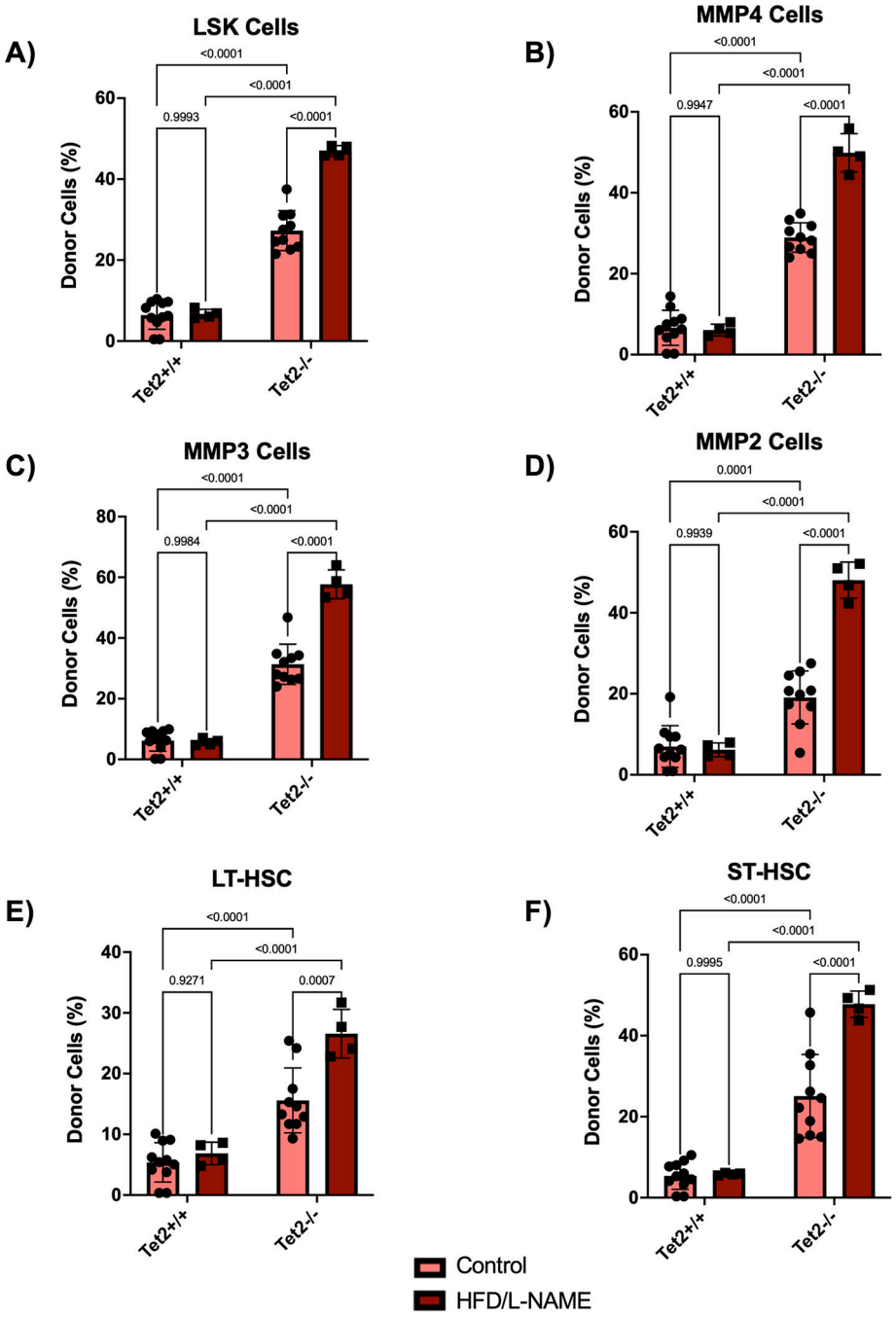


Figure 5. HFD/L-NAME treatment promotes expansion of *Tet2*-deficient cells in the hematopoietic stem and progenitor cells.

A-F. Flow cytometric quantification of donor cell chimerism in LSK cells, MMP4 cells, MMP3 cells, MMP2 cells, long-term hematopoietic stem cells (LT-HSC), and short-term hematopoietic stem cells (ST-HSC) of the bone marrow after 12 weeks of treatment. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n=4–11 per group).

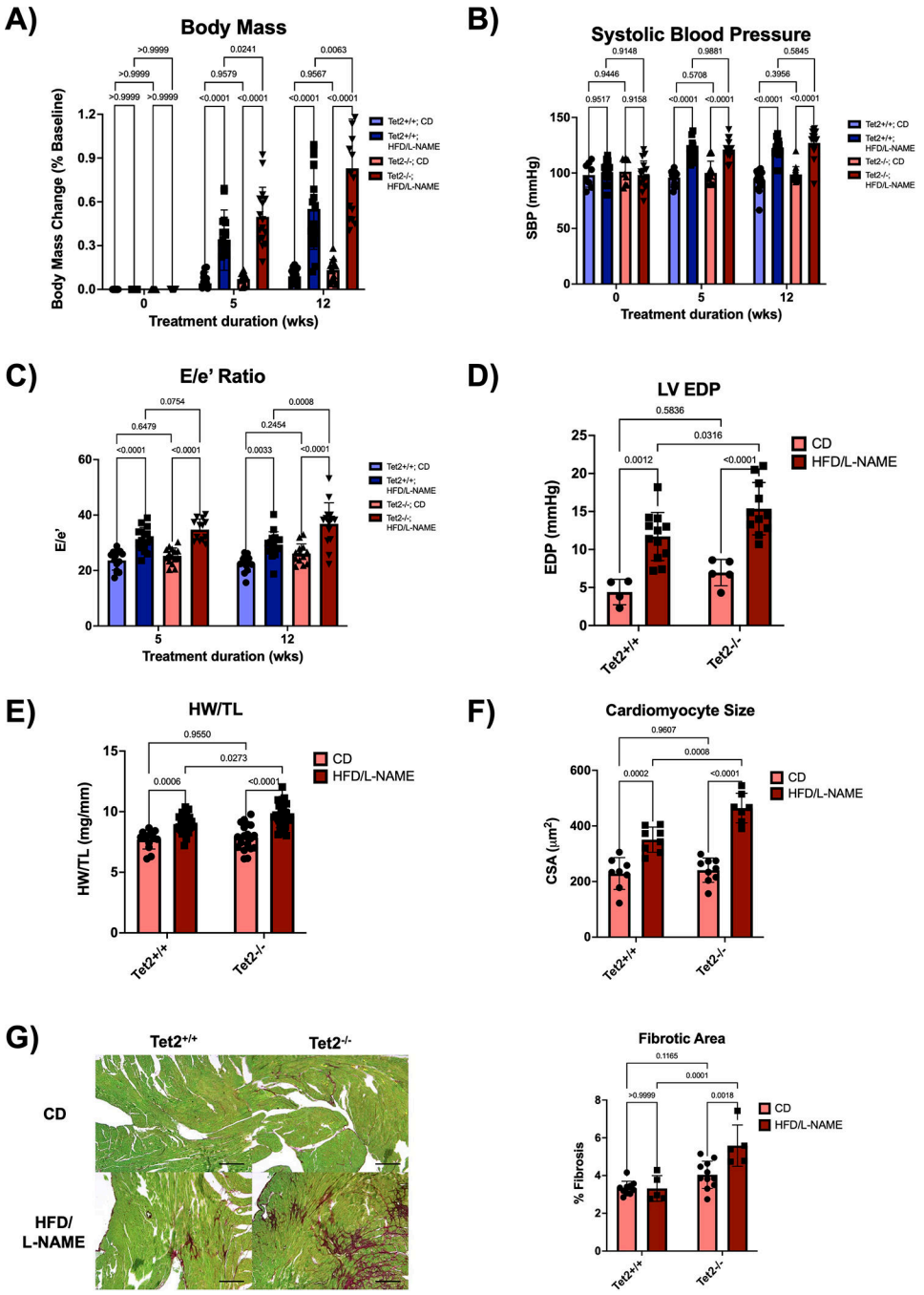


Figure 6. *Tet2*-mediated clonal hematopoiesis exacerbates cardiomyopathy in a model of HFpEF.

A. Normalized change in body mass relative to baseline body mass measured at the initiation of treatment. Measurements were performed at baseline and after 5 and 12 weeks of treatment. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n=14–17 per group). **B.** Systolic blood pressure measured by tail-cuff plethysmography at baseline and after 5 and 12 weeks of treatment. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n=8–17 per group). **C.** Serial echocardiographic analysis after 5 and 12 weeks of

treatment. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n= 13–16 per group). **D.** Left ventricular end diastolic pressure was obtained via LV catheterization after 12 weeks of treatment. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n=4–12 per group). **E.** Heart weight normalized to tibia length after 12 weeks of treatment. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n=13–28 per group). **F.** Quantification of cardiomyocyte cross-sectional area from heat germ agglutinin (WGA) stained hearts after 12 weeks of treatment. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n=7–9 per group). **G.** Representative images and analysis of Picrosirius Red/Fast Green staining of the hearts after 12 weeks of treatment. Scale bars are included in the lower right corner and correspond to 200 μm. Statistical significance was determined by 2-way ANOVA with post hoc Sidak multiple comparison test (n=5–11 per group).

Table 1.

Clinical characteristics for the HFpEF patients with and without clonal hematopoiesis.

HFpEF CH− vs HFpEF CH+ Patient Characteristics			
Measurement	**HFpEF CH−**	**HFpEF CH+**	**p-value**
Total	45	36	
Age (yrs)	72(7)	77(7)	0.0012
Female	21(0.47)	23(0.64)	0.0929
BMI (kg/m^2)	32(6)	28(5)	0.0083
Hypertensive	38(0.84)	33(0.92)	0.2635
Diabetes	19(0.42)	11(0.31)	0.1983
Hyperlipidemia	28(0.62)	23(0.64)	0.5316
COPD	14(0.31)	5(0.14)	0.0585
CKD	7(0.16)	8(0.22)	0.3144
Prior history of cancer	0(0)	0(0)	1.0000
History of smoking	24(0.53)	17(0.47)	0.3734
Prior myocardial infarction	11(0.24)	6(0.17)	0.2830
Prior coronary revascularization	0(0)	0(0)	1.0000
Atrial fibrillation or flutter	22(0.49)	23(0.64)	0.1302
Cerebrovascular Disease	4(0.09)	8(0.22)	0.0866
Peripheral Vascular Disease	1(0.02)	2(0.06)	0.4160
ACE inhibitor or ARB	39(0.87)	23(0.64)	0.0161
Beta-blocker	36(0.8)	25(0.69)	0.2015
Diuretic	36(0.8)	31(0.86)	0.3375
CCB	21(0.47)	13(0.36)	0.2331
Digoxin	3(0.07)	4(0.11)	0.3753
Antiarrhythmic	6(0.13)	7(0.19)	0.3283
Anticoagulation	21(0.47)	21(0.59)	0.2061
Antiplatelet	4(0.09)	1(0.03)	0.2570

Continuous variables are expressed as mean and standard deviation while categorical variables are described by absolute count and frequency. BMI: body mass index, COPD: chronic obstructive pulmonary disease, CKD: chronic kidney disease, ARB: angiotensin II receptor blocker, CCB: calcium channel blocker.