

Annual Review of Pathology: Mechanisms of Disease
**Cardiovascular Disease, Aging,
and Clonal Hematopoiesis**

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Annu. Rev. Pathol. Mech. Dis. 2020. 15:419–38

First published as a Review in Advance on
November 5, 2019

The *Annual Review of Pathology: Mechanisms of Disease*
is online at pathol.annualreviews.org

<https://doi.org/10.1146/annurev-pathmechdis-012419-032544>

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Keywords

somatic mutations, clonal hematopoiesis of indeterminate potential, CHIP, age-related clonal hematopoiesis, ARCH, *TET2*, *DNMT3A*, IL-1 β

Abstract

Traditional risk factors are incompletely predictive of cardiovascular disease development, a leading cause of death in the elderly. Recent epidemiological studies have shown that human aging is associated with an increased frequency of somatic mutations in the hematopoietic system, which provide a competitive advantage to a mutant cell, thus allowing for its clonal expansion, a phenomenon known as clonal hematopoiesis. Unexpectedly, these mutations have been associated with a higher incidence of cardiovascular disease, suggesting a previously unrecognized connection between somatic mutations in hematopoietic cells and cardiovascular disease. Here, we provide an up-to-date review of clonal hematopoiesis and its association with aging and cardiovascular disease. We also give a detailed report of the experimental studies that have been instrumental in understanding the relationship between clonal hematopoiesis and cardiovascular disease and have shed light on the mechanisms by which hematopoietic somatic mutations contribute to disease pathology.

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AGING AND CARDIOVASCULAR DISEASE: A MISSING PIECE OF THE PUZZLE

Cardiovascular disease is the most common cause of death worldwide, accounting for 31% of all deaths globally (1). In 2016, it was estimated that 17.9 million people died due to a cardiovascular cause, and of these deaths, 85% were attributed either to ischemic heart disease or stroke (1). In addition to death, cardiovascular disease poses an enormous financial burden and accounts for a substantial proportion of health-care spending and loss of productivity worldwide (2). Cardiovascular disease becomes much more prevalent with age, and, thus, with the world's population aging, the incidence of cardiovascular disease is expected to increase (3). Indeed, it has been predicted that by 2035 nearly one in four individuals will be 65 years or older (4). Despite strong epidemiological evidence linking cardiovascular disease with advancing age (3), we still have an incomplete understanding of how it drives disease progression. While the effect of aging on many cardiovascular diseases reflects, to a great extent, long-term exposure to traditional risk factors, such as hypertension, diabetes mellitus, hyperlipidemia, smoking, physical inactivity, and excessive alcohol consumption, these are still incompletely predictive of disease development in the elderly (5). Overall, this suggests that there are other unidentified age-related causal risk factors that drive the development and progression of cardiovascular disease. Thus, identifying and understanding these risk factors may uncover new ways to reduce the burden of cardiovascular disease.

SOMATIC MUTATIONS IN THE HEMATOPOIETIC SYSTEM AND AGING

In an effort to better understand the etiology of cardiovascular disease, research during the past two decades has focused on identifying common germline mutations that contribute to disease pathogenesis (6–8). These are mutations that are inherited from one's parents and are present in all tissues throughout the body. However, mutations may also arise spontaneously over the course of an individual's lifetime. These mutations are referred to as somatic or postzygotic mutations, and, in most cases, they result in no or little phenotypic consequences to the individual (9). In a few instances, however, a somatic mutation confers a competitive advantage to a cell, leading to progressive expansion of the mutant clone (10). As a result, the individual becomes a mosaic of cells with different genotypes, a phenomenon known as somatic mosaicism (9–11). Like germline mutations, somatic mutations can give rise to disease, and a well-known example of this is cancer (10, 12). However, with advances in sampling techniques and high-throughput sequencing technology, we are becoming increasingly aware that somatic mutations are also associated with the development numerous other diseases, including cardiovascular disease (13–17). Given the burden that cardiovascular disease imposes worldwide, there is a need to better understand the role that somatic mutations play in disease pathogenesis.

With advancing age, we accumulate mutations in our somatic cells and, therefore, it is not surprising that somatic mosaicism is a hallmark of aging in many tissues (18). Somatic mosaicism is more frequently detected in highly proliferative tissues, such as the hematopoietic system, which is responsible for producing more than 100 billion blood cells per day. In particular, it has been calculated that human hematopoietic stem and progenitor cells (HSPCs), the precursors to all mature blood cells, develop 0.13 ± 0.02 exonic mutations per year of life, and thus, it can be estimated that by age 50, an individual will accumulate an average of 5 coding gene mutations within each HSPC (19). If, by chance, one of these mutations confers a competitive advantage to the HSPC—such as by promoting its proliferation, self-renewal, survival, or some combination of these—this

may lead to expansion of the mutant clone at a disproportionate rate compared with other HSPCs (20). As a result, the HSPC-derived mutation will propagate through the hematopoietic system and into its immediate cellular progeny, giving rise to a genetically distinct population of mature blood cells.

Some of the earliest indications that clonal events occur within the aged hematopoietic system originated from studies examining the pattern of maternal-to-paternal X chromosome inactivation in the white blood cells of otherwise healthy females (21). The method used was originally devised to study the clonal origin of cancer, as early during female embryonic development each cell undergoes random inactivation of one of its X chromosomes, so a skewing of the predicted 1:1 ratio of the maternal:paternal X inactivation pattern can be suggestive of a clonal expansion. In these studies, it was observed that healthy females older than 60 years were more likely to exhibit significant skewing of one of their inactive X chromosomes when compared with younger females (21). A subsequent study undertaken more than a decade later identified that females who exhibited inactive X chromosome skewing in their blood were more likely to also possess a mutation in the epigenetic regulator and blood cancer driver gene ten eleven translocation 2 (*TET2*), suggesting that this particular genetic mutation was playing a role in driving the clonal expansion of blood cells within these women (22).

Recent, large exome-sequencing and chromosomal mosaicism studies have expanded this association, detecting the presence of commonly occurring aberrant clonal expansions in hematopoietic cells from elderly individuals (11, 13, 15, 23–25). Specifically, two studies published in 2012 detected the presence of mosaic chromosomal alterations in the blood of otherwise healthy individuals, and the frequency of these alterations increased sharply with age (23, 24). In 2014, three independent studies used whole-exome sequencing of peripheral blood to examine the frequency of mutations within specific genes recurrently mutated in hematological malignancies (13, 15, 25). Although mutations in more than 100 candidate driver genes were associated with these clonal events within the hematopoietic system, most mutations occurred in just three genes that encode epigenetic regulators, specifically DNA methyltransferase 3 alpha (*DNMT3A*), *TET2*, and additional sex combs-like 1 (*ASXL1*) (13, 15, 25). Further, these studies showed that the frequency of these mutations increased with age, with approximately 10% of individuals older than 70 possessing at least one mutation in their white blood cells (13, 15, 25). While it was identified that individuals who harbored these mutations had an increased risk of developing a subsequent blood cancer, epidemiological studies showed that most will never develop a malignancy, as these conditions are relatively rare and generally require the acquisition of multiple oncogenic mutations, in accordance with the multistep leukemogenesis theory (13, 15, 26). Thus, based on these observations, this condition has been referred to as clonal hematopoiesis of indeterminate potential, or CHIP (27). In the literature, CHIP is defined by the presence of an expanded blood cell clone carrying a mutation in a known driver gene of hematological malignancy at a variant allele frequency (VAF) of at least 2% without meeting the standard diagnostic criteria for malignancy (i.e., cytopenia or abnormal blood cell counts, or both) (27). This distinction, while convenient, is largely based on the limits of mutation detection by sequencing methodology, rather than being based on epidemiological data or biological principles. Therefore, some researchers have labeled this definition as provisional and have suggested that it may be subject to change as we gain better insight into the impact that CHIP has on health and disease (27, 28). It is also important to note that clonal hematopoiesis can occur without the presence of a known driver mutation, perhaps by genetic drift in the HSPC pool during normal aging, by a mutation in an unidentified driver gene, or by a heritable epigenetic trait (29–31). In fact, studies estimate that mutations in unknown driver genes account for a substantial proportion of clonal events within the hematopoietic system (15, 31). Therefore, the

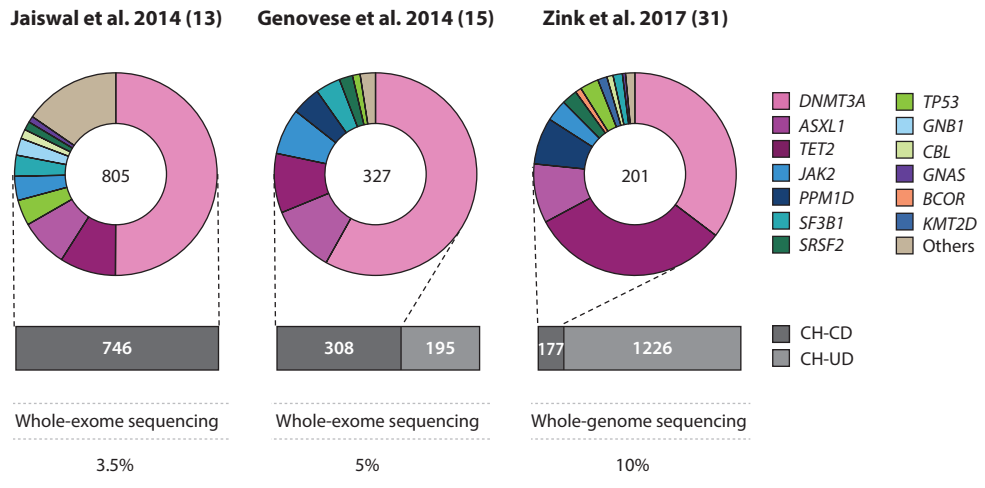


Figure 1

Summary of reported prevalence of clonal hematopoiesis. The bar graphs represent the total number of individuals with clonal hematopoiesis associated with (*dark gray*) and not associated with (*light gray*) known driver gene mutations reported in three independent studies (13, 15, 31). The donuts summarize the frequencies of somatic mutations in candidate driver genes; the numbers within the donuts indicate the total number of driver gene mutations detected. Abbreviations: CH-CD, clonal hematopoiesis with candidate driver mutations; CH-UD, clonal hematopoiesis with unknown driver mutations; SNV, single nucleotide variant; VAF, variant allele frequency.

term age-related clonal hematopoiesis (ARCH) has also been used to describe the presence of a clonal event observed in the hematopoietic system that is associated with advancing age (29).

Since the above-described epidemiological studies were reported, there has been increased attention paid to examining the prevalence of clonal hematopoiesis in other cohorts of individuals across the life span (31). Specifically, a study by Zink et al. (31) used nonbiased whole-genome sequencing to identify the presence of clonal hematopoiesis in the peripheral blood of 11,262 Icelanders. Unlike some of the earlier studies that used whole-exome sequencing (13, 25), this study did not select for candidate driver gene mutations and, thus, was able to detect clonal hematopoiesis at an even greater prevalence within the study population (31). Indeed, it was found that the overall frequency of mutations increased from 0.5% in individuals younger than 35 years to >50% in individuals older than 85 years, which is much higher than previous estimates. Interestingly, when the authors examined the frequency of mutations in candidate driver genes, it was found that they accounted for only a small proportion of the total number of clonal events, suggesting that there are other unidentified mutations that drive ARCH (Figure 1).

Related to this issue, advances in sequencing technology have allowed researchers to examine mutations at an even greater depth, and data arising from using this technology also suggest that clonal hematopoiesis is more prevalent than previously estimated, even when the analysis focuses only on candidate driver genes. For instance, ultradeep sequencing of mutational hot spots within select candidate driver genes has projected that the mean mutation frequency is >12% in individuals in their 60s, >19% in those in their 70s, >40% in those in their 80s, and >74% in those in their 90s (32). Similarly, a study using error-corrected sequencing, which allows for detection of clonal events at as low as 0.03% VAF, found that mutations in select candidate driver genes occurred in 95% of individuals aged 50–70 years, an age group for which previous studies estimated that only 5% of individuals had detectable clonal hematopoiesis (33). Overall, the findings

from these studies reveal that clonal hematopoiesis is almost ubiquitous by middle age, and the expansion of these clones occurs in a portion of individuals later in life. However, exactly what drives the clonal expansion of these mutations in some individuals and not others remains speculative. It is likely that genetics and environmental factors play roles in this process, perhaps by modulating the kinetics of clonal expansion or by altering the bone marrow niche to favor survival of the fittest HSPC clone (34). Certainly, the aging process itself has effects on hematopoiesis in terms of the output and function of HSPCs (35). However, longitudinal data on individuals with clonal hematopoiesis are limited. Current data suggest that mutant clones are relatively stable for more than a 10-year period (33, 36, 37). These findings suggest that their expansion is episodic rather than linear, perhaps indicating that variations in exposure to environmental stressors may alter the overall hematopoietic demand or lead to changes in the bone marrow niche per se. Nevertheless, it is apparent that further studies are warranted to better understand and identify the factors that drive the clonal expansion of mutant cells within the hematopoietic system.

CLONAL HEMATOPOIESIS AND ALL-CAUSE MORTALITY: AN UNEXPECTED CONNECTION

To date, several independent reports have associated clonal hematopoiesis, either with or without candidate driver gene mutations, with an increase in all-cause mortality. The first and most prominent reports of this association originated from two large longitudinal studies published concurrently in the *New England Journal of Medicine* in 2014 (13, 15). These studies, which are also described above, used whole-exome sequencing of peripheral blood mononuclear cells from more than 29,000 individuals who were unselected for cancer or hematological malignancies (13, 15). It was found that clonal hematopoiesis linked to mutations in known driver genes of hematological malignancy or CHIP was associated with a 40–50% increase in all-cause mortality (13, 15). This association appeared to be correlated with age, as it was stronger in individuals who had their blood sequenced after the age of 70 (13). Further, the study by Genovese et al. (15) used nonbiased exome sequencing analysis, which allowed for the detection of clonal hematopoiesis with unknown driver mutations. It was documented that clonal hematopoiesis with mutations in unknown driver genes accounted for ~40% of all detectable clonal events; however, all-cause mortality was slightly lower for individuals bearing these types of mutations (15). Both of these studies observed that there was a marked increase in the frequency of hematological cancer in individuals with clonal hematopoiesis (13, 15), which is to be expected as mutations in driver genes represent an early step in the progression toward a hematological malignancy. However, hematological malignancies occur at a relatively low frequency in the general population, and, thus, could not fully explain the increase in all-cause mortality observed in these two studies (13, 15). In particular, the study by Jaiswal et al. (13) found that only one individual died due to a hematological neoplasm, and cause-specific analyses of 5,132 individuals confirmed a lack of association with cancer death. This lack of attributable cause in all-cause mortality led researchers to the question: What is the underlying cause of this increase in all-cause mortality in individuals who exhibit clonal hematopoiesis? In an unplanned secondary analysis, it was discovered that there was an association between clonal hematopoiesis and an increased incidence of coronary heart disease and ischemic stroke, suggesting that the increase in all-cause mortality could be explained by an increase in the incidence of these two conditions (13). Indeed, a cause-specific analysis confirmed the association between clonal hematopoiesis and death due to cardiovascular conditions (13). This surprising discovery has sparked increased interest in the relationship between somatic mutations in the hematopoietic system and cardiovascular disease, and the findings of the resultant studies are discussed in greater detail below.

Since these two studies were undertaken, additional epidemiological data have been published supporting an association between clonal hematopoiesis and an increase in all-cause mortality. The Icelandic study, also described above, which used barcoded, nonbiased whole-genome sequencing, reported an association between clonal hematopoiesis and all-cause mortality (31). In this particular study population, clonal hematopoiesis was detected at a high frequency, and it was found that the risk of mortality was similar for clonal hematopoiesis associated and not associated with candidate driver gene mutations, which differs somewhat from the findings of Genovese et al. (15). Most strikingly, however, it was found that individuals with detectable clonal hematopoiesis had a risk of all-cause mortality similar to those who had ever smoked (31), indicating that clonal hematopoiesis carries a similar risk to a well-established risk factor for all-cause mortality. Although this study did not examine cardiovascular outcomes, significant associations were detected between clonal hematopoiesis and smoking, smoking-related diseases, chronic pulmonary disease, and psychiatric disease (31). Expanding this association beyond specific gene mutations, a recent study by Loh et al. (30) reported an association between large clonal mosaic chromosomal alterations and all-cause mortality. Mutations involving large alterations of chromosomes have been an understudied area of clonal hematopoiesis, likely due to limitations in the methods of detection and the requirement for large study populations (11, 28). In the study by Loh et al. (30), 8,342 mosaic chromosomal alterations were detected within the blood of 151,202 participants in the UK Biobank using single nucleotide polymorphism-based computational techniques. Within the cohort, 71% of the detected alterations were classified as gains, deletions, or copy-number-neutral loss of heterozygosity, and the remaining 29% of events could not be inferred definitively (30). It was observed that the frequency of these types of mutations increased with age and was associated with a doubling in the risk of all-cause mortality (30). Similar to previous reports, this association could only partly be explained by cancer deaths after correcting for age, sex, and smoking status, and, thus, the authors speculated that some of these deaths might reflect the effects of these mutations on cardiovascular health (30). It should be noted that some of these aberrations likely affected genomic regions that include candidate driver genes, such as *DNMT3A* and *TET2* (30), which could possibly account for some of the increase in all-cause mortality observed in this study. Nevertheless, this study provides evidence that multiple DNA mutations beyond those that target known candidate driver genes can give rise to clonal hematopoiesis and have consequences for mortality.

CLONAL HEMATOPOIESIS AND CARDIOVASCULAR DISEASE: EVIDENCE FROM THE CLINIC

The discovery that cardiovascular disease could account for a substantial amount of the increase in all-cause mortality associated with clonal hematopoiesis (13) has led to further clinical investigations into the putative connection between the two phenomena (14, 38). As briefly discussed above, the study by Jaiswal et al. (13) in which this association was first detected, found that individuals with CHIP had a significantly increased risk of coronary heart disease (hazard ratio: 2.0) and ischemic stroke (hazard ratio: 2.6) after adjusting for age, sex, type 2 diabetes, systolic blood pressure, and body mass index. It was also noted that individuals with a VAF >10% had a higher risk of cardiovascular disease, suggesting that clone size may be correlated with risk (13). Prominently, the risks of coronary heart disease and ischemic stroke were higher in carriers of CHIP than in individuals with traditional risk factors, such as hypertension and high body mass index (13). However, the main aim of this study was to evaluate the frequency of hematopoietic somatic mutations in the general population rather than determine their association with cardiovascular disease. Therefore, a follow-up investigation was conducted to directly test the hypothesis that CHIP is associated with an increase in the incidence of cardiovascular disease (14). For this

analysis, whole-exome sequencing was performed on blood samples obtained from four case-control studies (two prospective and two retrospective) consisting of 4,726 individuals with coronary heart disease and 3,529 controls. In the prospective study cohorts, it was found that carriers of CHIP had an increased risk of coronary heart disease (combined hazard ratio: 1.9), which was comparable with what was found in the 2014 study (13, 14). Analogous to previous studies, most mutations were detected in just four genes, namely *DNMT3A*, *TET2*, *ASXL1*, and Janus kinase 2 (*JAK2*). To examine whether there were any gene mutation-specific differences in the risk of coronary heart disease, a meta-analysis was performed by combining the data from the prospective cohorts with the risks reported in previously published works (14). While there was no statistically significant difference in the risk of coronary heart disease for individuals carrying mutations in *DNMT3A* (hazard ratio: 1.7), *TET2* (hazard ratio: 1.9), or *ASXL1* (hazard ratio: 2.0), individuals who carried *JAK2* mutations displayed a marked increase in disease risk (hazard ratio: 12.0) (14). However, it should be noted that the sample of individuals who carried *JAK2* mutations was small, and this work should be viewed as preliminary. Further, an analysis of individuals within one of the prospective studies (known as BioImage) found that carriers of CHIP were more likely to have coronary artery calcification, and this appeared to be correlated with clone size (14). Analyses of the retrospective studies found there was also an association between carriers of CHIP and early-onset myocardial infarction (14). Overall, these findings add further weight to the initial report that CHIP is associated with an increased incidence of cardiovascular disease.

To assess the potential prognostic significance of CHIP in patients with cardiovascular disease, a more recent study by Dorsheimer et al. (38) examined the long-term prognosis of patients with CHIP and chronic heart failure (CHF) of ischemic origin. In a cohort of 200 CHF patients, the frequency of CHIP was assessed in bone marrow-derived mononuclear cells using deep, targeted amplicon sequencing of 56 genes previously associated with CHIP and myeloid malignancies (38). It was found that 18.5% of patients within this cohort were carriers of CHIP and had a VAF of at least 2%. Most mutations were detected in *DNMT3A* and *TET2*, which is similar to what has been reported in previous studies assessing clonal hematopoiesis in the general population. The authors also noted that there were smaller numbers of patients with mutations in lysine demethylase 6A (*KDM6A*) and BCL6 corepressor (*BCOR*). Consistent with earlier reports, carriers of CHIP were significantly older compared with noncarriers, although it was found that CHIP carriers did not differ with respect to clinical parameters of heart function and disease stage (38). However, in a subsequent analysis of patients harboring either *DNMT3A* or *TET2* mutations, it was found that patients with these gene-specific mutations had overall a worse long-term clinical outcome in terms of both death and death combined with rehospitalization for CHF compared with noncarriers, even after adjusting for age (38). To determine whether this association was correlated with clone size, the threshold of mutation calling was lowered to a VAF of 0.5% (38). Using this new threshold, it was identified that there were an additional 66 and 53 patients with *DNMT3A* and *TET2* mutations, respectively, with clone sizes between 0.5% and 2% VAF. When patients were grouped based on VAF (i.e., no mutation, VAF < 1%, VAF between $\geq 1\%$ and < 2%, or VAF > 2%), it was found that there was a dose-dependent relationship between clone size and clinical outcome, including clone sizes between 1% and 2% VAF, which is lower than the proposed threshold for CHIP designation (38). Regardless, this small clinical study supports the overarching hypothesis that somatic mutations in the hematopoietic system, and particularly those in the candidate driver genes *DNMT3A* and *TET2*, are associated with progression and poorer prognosis in patients with CHF. More recently, Mas-Peiro et al. (39) found an association between mutations in *DNMT3A* and *TET2* and poorer prognosis in 279 patients treated for aortic valve stenosis. However, further studies using larger cohorts will be required to validate and examine these associations more deeply.

In addition to the above-described studies, there has been a report that the presence of CHIP in patients undergoing autologous stem cell transplantation for lymphoma is associated with worse survival and death due to cardiovascular conditions (40). Interestingly, in this study, it was documented that there was a much higher frequency of mutations in protein phosphatase magnesium/manganese dependent 1D (*PPM1D*) and tumor protein 53 (*TP53*), both of which are classic tumor suppressor genes. In individuals undergoing cytotoxic therapy prior to stem cell transplantation, the hematopoietic system is likely under extreme stress, and it is thought that mutations in tumor suppressor genes, such as *TP53* and *PPM1D*, confer the mutated HSPCs with a survival advantage against genotoxic stress induced by chemotherapy, a phenomenon known as therapy-associated clonal hematopoiesis (41–44). Indeed, it was found that 30% of individuals undergoing autologous stem cell transplantation for lymphoma were carriers of CHIP (40). This finding is similar to those of other studies, which have reported enrichment of *PPM1D* mutations within the blood of patients who have been previously treated for solid-organ tumors (45–47). Thus, in general, this study may suggest that somatic mutations in genes such as *TP53* and *PPM1D* may also be associated with an increased incidence of cardiovascular disease, particularly in patients who have previously undergone chemotherapy (40). While of interest, further studies are required to directly assess this relationship, particularly as these genes are not as frequently mutated in the general population.

CLONAL HEMATOPOIESIS AND CARDIOVASCULAR DISEASE: EVIDENCE FROM THE LABORATORY

The descriptive nature of the aforementioned human studies does not allow us to determine whether a causal relationship exists between CHIP and an increased risk of cardiovascular disease, as the association between these two phenomena could be secondary to confounding factors or simply reflect a shared consequence of the aging process. Moreover, in clinical studies it is difficult to assess directionality and the study factors that may drive the clonal expansion of cells. This is important, as it has been suggested that chronic inflammation and other stresses associated with cardiovascular disease may promote somatic mutagenesis and the clonal expansion of mutant cells (48–50). Hence, animal models have recently proved to be powerful tools for gaining a better understanding of the relationship between clonal hematopoiesis and cardiovascular disease (14, 51–54) (**Figure 2**). The forthcoming sections describe recent experimental work examining the effects of clonal hematopoiesis associated with mutations in the key driver genes *TET2*, *DNMT3A*, and *JAK2* on cardiovascular disease and the wider implications of these findings for human cardiovascular health.

TET2

As indicated above, one of the most commonly mutated genes associated with an increased incidence of and death due to cardiovascular conditions is *TET2* (13, 14, 38). The protein encoded by *TET2* is an α -ketoglutarate and an Fe^{2+} -dependent enzyme that catalyzes DNA methylation by converting 5-methylcytosine into 5-hydroxymethylcytosine, thus promoting the transcriptional activation of genes (55, 56). Furthermore, depending on the cellular and molecular environment, *TET2* may also induce the repression of genes by recruiting histone deacetylases to gene promoters (51, 57). *TET2* was the first gene reported to exhibit acquired mutations in the blood cells of individuals without hematological malignancies (22), and, to date, more than 130 different *TET2* mutations have been identified in the blood cells of humans (13, 15, 22, 25, 31, 56, 58). Most of these mutations are small insertions or deletions, or nonsense or missense point mutations, often

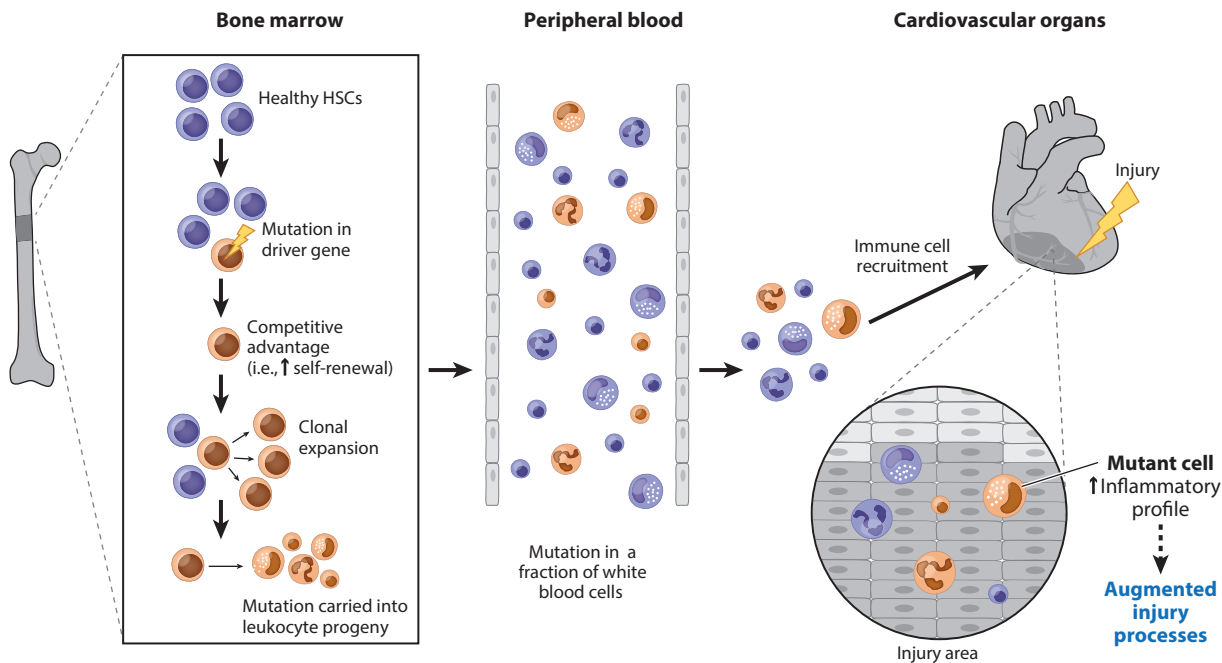


Figure 2

Central overview of age-related clonal hematopoiesis and how it drives cardiovascular disease progression. During the aging process, healthy hematopoietic stem cells (HSCs) may acquire a mutation in a driver gene. This mutation provides the mutant HSC with a competitive advantage, allowing for its expansion at a disproportionate rate compared with other HSCs within the bone marrow niche (clonal expansion). As a result, the HSC-derived mutation propagates through the hematopoietic system and into its immediate leukocyte progeny, giving rise to a genetically distinct population of mature white blood cells. During cardiovascular disease, inflammatory cells migrate to the injury site and are involved in promoting disease pathogenesis through a variety of mechanisms. In the setting of clonal hematopoiesis, HSC-derived mutations may result in changes to the inflammatory profile of the leukocyte progeny (i.e., elevated cytokine production). As a result, this altered inflammatory profile of leukocytes can exacerbate cardiovascular injury processes, driving disease progression.

resulting in changes to the catalytic site and, hence, a loss of protein function (13, 15, 56). It has also been reported that large alterations in chromosome 4q may lead to *TET2* loss of function in some instances (59). Since the discovery of *Tet2* in 2009, studies in rodents have shed light on its function within the hematopoietic system and have indicated that it plays an important role in regulating the self-renewal of HSPCs as well as myeloid cell differentiation (60–62). In particular, *Tet2* deficiency in mice leads to aberrant patterns of hematopoiesis, and *Tet2*-deficient HSPCs display increased self-renewal and proliferative capacity in competition assays compared with their wild-type counterparts (62, 63). Further, animals deficient in *Tet2* exhibit myeloid lineage skewing as well as extramedullary hematopoiesis, characterized by an increase in monocytes and neutrophils within the spleen (62, 64). Notably, studies have observed that not all *Tet2*-deficient mice will develop a hematological malignancy, consistent with the stepwise concept of blood cancer development (49).

To determine whether a causal relationship existed between *TET2* clonal hematopoiesis and cardiovascular disease, we recently assessed the impact of *Tet2* deficiency in HSPCs in several murine models of cardiovascular disease (51–53). For our initial assessment, a mouse model of

atherosclerosis was chosen, as atherosclerosis represents a root cause of both coronary heart disease and ischemic stroke, the two conditions in which an association was initially reported with clonal hematopoiesis (13). To model clonal hematopoiesis, competitive bone marrow transplantation (BMT) experiments were performed to introduce a small number of *Tet2*-deficient hematopoietic cells into atherosclerosis-prone *Ldlr*^{-/-} mice (51). This experimental setting mimics the human scenario of individuals carrying a *TET2* somatic mutation, since these mutations are initially carried by a small number of HSPCs, which will gradually expand over time. Consistent with this concept, it was found that following transplantation, *Tet2*-deficient cells expanded progressively in the bone marrow, spleen, and blood, and they exhibited a slight myeloid bias, with preferential expansion into the Ly6C^{high} monocyte population (51). Importantly, there was no effect of HSPC *Tet2* deficiency on the total number of white blood cells, which is analogous to what is observed in cancer-free individuals who exhibit clonal hematopoiesis associated with a *TET2* mutation (13, 15, 25). Notably, the expansion of *Tet2*-deficient cells in this manner accelerated atherosclerosis, leading to a marked increase in plaque size (51). To more closely model the human scenario, whereby most individuals bear a mutation in only one allele, equivalent experiments were performed using cells that were heterozygous for *Tet2*. In these experiments, heterozygosity of *Tet2* was sufficient to accelerate atherosclerosis, although the phenotype was milder than what was observed following the transplantation of homozygous cells (51). It was also noticed that the kinetics of clonal expansion were slower for heterozygous cells, indicating a gene dose-dependent effect. Further, myeloid-specific ablation of *Tet2* was sufficient to promote atherosclerosis, indicating that cells of myeloid origin and likely macrophages as well, play major roles in accelerating atherosclerosis associated with the clonal expansion of *Tet2*-deficient HSPCs (51). Credence was given to these findings when an independent group also reported similar outcomes in a mouse model of atherosclerosis (14). In that study, a conventional BMT approach was taken, whereby all transplanted hematopoietic cells were deficient in *Tet2* (14). In contrast to the approach taken by Fuster et al. (51), the approach in the study reporting similar outcomes led to the widespread presence of xanthomas, enlargement of the spleen, and macrophage infiltration into various tissues (14). Also, total hematopoietic deletion of *Tet2* resulted in a substantial increase in plaque size in this model of atherosclerosis (14). This study also found that myeloid-specific deletion of *Tet2* led to an augmented atherosclerotic lesion, providing additional evidence that the effects of hematopoietic *Tet2* loss of function are mediated through myeloid cells (14).

To corroborate and extend these studies, we recently examined the effect of *Tet2* clonal hematopoiesis on experimental heart failure (52, 53). In the initial studies, two distinct models of heart failure were employed, specifically the left anterior descending coronary artery ligation model of myocardial infarction and the transverse aortic constriction model of pressure overload-induced cardiac hypertrophy. It was found that hematopoietic *Tet2* inactivation, achieved by competitive BMT, led to more pronounced pathological cardiac remodeling in these models, as indicated by poorer cardiac function and greater myocardial hypertrophy, fibrosis, and inflammation (53). Further, it was found that myeloid-specific deletion of *Tet2* also led to accelerated heart failure, indicating that myeloid cell populations are likely involved in accelerating the pathology (53). An ensuing study strengthened these findings using an angiotensin II-induced pressure overload model of heart failure (52). In this study, *Tet2* inactivation was achieved using a lentivirus-mediated CRISPR-Cas9 system to create *Tet2* mutations in a small number of lineage-negative hematopoietic cells ex vivo. Following lentiviral transduction, BMT experiments were performed, similar to our earlier studies using competitive BMT (52). Importantly, the CRISPR-edited cells expanded in the bone marrow and blood, and the expansion characteristics were similar to what was observed in prior studies using cells from *Tet2*-deficient mice. Further, mice transplanted with CRISPR-edited cells displayed exacerbated pathological cardiac remodeling following angiotensin II

infusion, characterized by worse cardiac function and greater levels of fibrosis and inflammation (52). Notably, the profile of cardiac remodeling following angiotensin II infusion was similar between our CRISPR-mediated and conventional competitive BMT approaches, indicating that the CRISPR-Cas9 system is an efficient method to study clonal hematopoiesis associated with candidate driver mutations (52). Overall, the findings from these two studies in experimental heart failure indicate that clonal hematopoiesis associated with *Tet2* mutations accelerates adverse cardiac remodeling leading to heart failure.

Data suggested that macrophages were playing a key role in the mechanism by which *Tet2* loss of function exacerbated cardiovascular pathology (51–53). However, *Tet2* deficiency did not affect macrophage proliferation, apoptosis, oxidized low-density lipoprotein uptake, or the expression of regulators that control cholesterol uptake (51). Thus, we hypothesized that *Tet2*-deficient hematopoietic cells may accelerate atherosclerosis by generating a pool of macrophages with enhanced proinflammatory actions. In support of this notion, isolated *Tet2*-deficient macrophages were found to display augmented inflammatory gene expression profiles and to produce greater amounts of the proinflammatory cytokine interleukin 1 β (IL-1 β) when treated with lipopolysaccharide (LPS) (51). Increased transcript and protein levels of IL-1 β were also detected in the plaques of atherosclerotic mice, possibly suggesting a causative role for IL-1 β (**Figure 3**). To

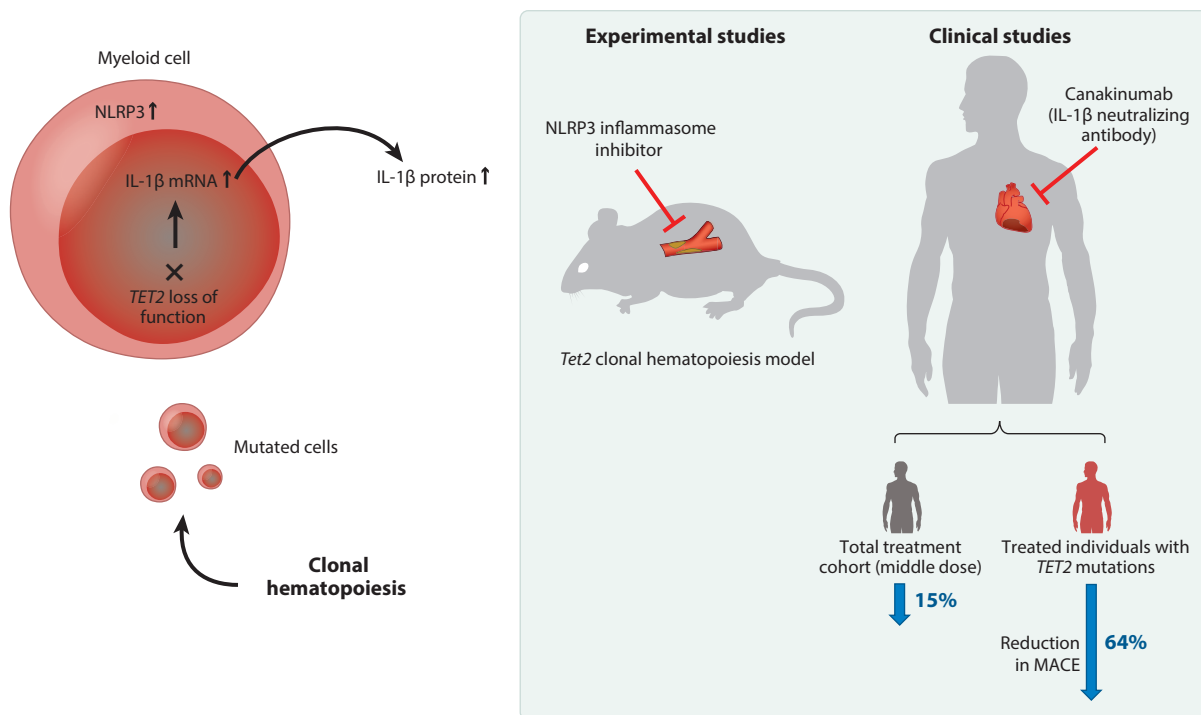


Figure 3

TET2-mediated clonal hematopoiesis accelerates cardiovascular disease through aberrant IL-1 β signaling. Somatic mutations in *TET2* within hematopoietic stem and progenitor cells lead to their clonal expansion, giving rise to myeloid cells that promote cardiovascular disease via aberrant activation of the NLRP3/IL-1 β pathway (51–53). Blockade of the NLRP3 inflammasome complex has been shown to reduce disease pathology associated with *Tet2* clonal hematopoiesis in mouse models of atherosclerosis and heart failure (51, 53). Moreover, a subanalysis of the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial indicated that individuals with somatic mutations in *TET2* responded more favorably to the IL-1 β neutralizing antibody canakinumab than did individuals without *TET2* mutations (66–68). Abbreviations: IL, interleukin; MACE, major adverse cardiovascular event.

better understand the connection between *Tet2* and IL-1 β , additional experiments were performed in isolated macrophages to interrogate how *Tet2* regulates IL-1 β production. These experiments revealed that *Tet2* plays a role in inhibiting the transcription of IL-1 β via histone deacetylase-mediated histone deacetylation rather than by promoting DNA methylation (51). This finding is analogous to a previous report that *Tet2* represses transcriptional activation of specific inflammatory genes in macrophages by recruiting histone deacetylase 2 to the gene promotor (57).

It is well established that IL-1 β is synthesized as a precursor protein (pro-IL-1 β) and requires cleavage for activity, often by an immune complex called the NLRP3 inflammasome (65). Studies in the atherosclerosis model found that *Tet2* also regulates components of the NLRP3 inflammasome complex and that selective inhibition of this complex with a small molecule inhibitor reduced lesion burden specifically within animals transplanted with *Tet2*-deficient bone marrow cells, thus confirming a role for IL-1 β and the NLRP3 inflammasome in the augmented pathology (51). In terms of the downstream actions of IL-1 β , findings suggested that IL-1 β released from *Tet2*-deficient plaque macrophages was perpetuating inflammation by promoting endothelial cell activation and P-selectin expression (51). In turn, this led to an increase in the recruitment of monocytes to the lesion site, regardless of the *Tet2* genotype, thereby augmenting vascular inflammation. Therefore, it can be speculated that a few *Tet2*-mutant cells in the plaque act in a catalytic manner by amplifying and perpetuating vascular inflammation and, thus, drive atherosclerotic plaque progression. Similarly, Jaiswal et al. (14) also reported that macrophages from *Tet2*-deficient animals were more proinflammatory and exhibited increased levels of various cytokines and chemokines, including IL-1 β , following stimulation with LPS. Moreover, in that study, atherosclerotic animals, which had transplants of *Tet2*-deficient bone marrow, appeared to have enhanced levels of circulating cytokines and chemokines compared with animals that had transplants of wild-type cells. The authors speculated that *Tet2* loss of function was accelerating atherosclerosis by promoting monocyte recruitment to the lesion site via enhanced CXCR2 signaling, although this concept was not examined directly. In subsequent studies using mouse models of heart failure, it was found that IL-1 β was involved in the augmented pathology associated with *Tet2* clonal hematopoiesis (52, 53). Specifically, increased transcript and protein levels of IL-1 β were observed in the hearts of animals transplanted with *Tet2*-deficient cells (52, 53). Similarly, inhibition of the NLRP3 inflammasome complex led to reduced cardiac remodeling and heart failure in the left anterior descending and transverse aortic constriction models (53). The mechanistic findings from these studies indicate that *TET2* regulates IL-1 β production at multiple levels, and, thus, loss of function of *Tet2* results in aberrant IL-1 β signaling, which, in turn, accelerates cardiovascular disease (51–53).

Implications for the CANTOS Trial

These findings on *TET2*-mediated clonal hematopoiesis may have implications for understanding the outcomes of the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial. CANTOS was a large, placebo-controlled clinical trial that examined the effects of escalating doses of canakinumab, a neutralizing IL-1 β antibody, in patients who were post-myocardial infarction and had sustained levels of high-sensitivity C-reactive protein (hsCRP), a measure of inflammation. In this high-risk group, targeting IL-1 β at the middle dose (150 mg every 3 months) led to a significantly lower frequency of major adverse cardiovascular events (MACE) compared with the placebo group (66). In a prespecified secondary analysis of the reduction in CRP relative to events in CANTOS, it was found that MACE and all-cause mortality were highly dependent on the degree of CRP reduction (67). For canakinumab-treated patients who exhibited a reduction in hsCRP to <2 mg/L there were also reductions of 25%, 31%, and 31% in, respectively, MACE, cardiovascular mortality, and all-cause mortality (67). In contrast, the nonresponders (i.e., those

who on treatment had hsCRP levels of ≥ 2 mg/L) displayed no reductions in these end points. It had previously been hypothesized that individuals with *TET2*-mediated clonal hematopoiesis would have a better response to therapies that target the IL-1 β /NLRP3 inflammasome pathway (51). Support for this hypothesis was provided by a recent exploratory analysis of nearly 4,000 individuals enrolled in the CANTOS trial (68) that found patients with mutations in *TET2* displayed a 64% reduction in MACE in response to canakinumab therapy (**Figure 3**).

DNMT3A

DNMT3A belongs to a family of cytosine methylases and plays a key role in epigenetic regulation by catalyzing the addition of methylation marks to genomic DNA (69). *DNMT3A* is the most frequently mutated candidate driver gene associated with clonal hematopoiesis in the elderly (13, 15, 25, 31), and, as indicated above, it is associated with an increased risk of cardiovascular disease (13, 14, 39). Experimental studies examining the role of *Dnmt3a* within the hematopoietic system indicate it is involved in regulating HSPC self-renewal, and the loss of function of this enzyme has been reported to impair HSPC differentiation (70, 71). Moreover, mice that have *Dnmt3a* selectively deleted from their hematopoietic cells exhibit hallmarks of primitive hematopoiesis, including an upregulation of fetal hematopoietic gene expression and preferential myelopoiesis, as well as expansion of myeloid cells within the liver (72). Thus, it has been suggested that *Dnmt3a* regulates genes involved in the early development of the hematopoietic system.

To better understand the connection between clonal hematopoiesis associated with *DNMT3A* mutations and cardiovascular disease, our laboratory recently assessed the effect of hematopoietic *Dnmt3a* deficiency on experimental heart failure induced by angiotensin II infusion (52). *Dnmt3a* loss of function was achieved by genetic editing of lineage-negative hematopoietic cells using the same CRISPR-Cas9 approach that was used to produce *Tet2* loss of function, described above. Following lentiviral transduction, BMT experiments were performed to introduce a small number of *Dnmt3a*-mutated cells into mice. Unlike mutations in *Tet2*, *Dnmt3a* loss of function did not lead to increased chimerism of the edited cells during the 4-month course of this study (52). This finding is analogous to previous reports showing that *Dnmt3a*-deficient HSPCs expand only in aged animals or after sequential BMT (70, 73, 74), raising potential concerns regarding the use of murine models to study clonal hematopoiesis associated with mutations in *DNMT3A*. Nevertheless, in mice with *Dnmt3a*-mutated cell chimerism, between 5% and 10% exhibited worse cardiac function following angiotensin II infusion and greater cardiac remodeling, as indicated by increased cardiac fibrosis and myocyte cross-sectional area (52).

From a mechanistic point of view, *DNMT3A* is thought to play a role in regulating inflammatory pathways. For example, it has been reported that *DNMT3A* can restrain inflammatory pathways in mast cells (75) as well as regulate T helper cell polarization (76–78) and macrophage functions (78, 79). Consistent with these previous reports, our in vitro and in vivo data suggested that hematopoietic *Dnmt3a* loss of function was potentially promoting heart failure by augmenting inflammatory pathways (52). It was observed that mice transplanted with *Dnmt3a*-mutated cells had greater macrophage accumulation in the heart and increased expression of immune cell markers following angiotensin II infusion. Moreover, a *Dnmt3a*-deficient macrophage cell line displayed increased expression of the proinflammatory molecules Il-6, Cxcl1, Cxcl2, and Ccl5 following stimulation with LPS. Interestingly, this inflammatory profile differed from that observed for *Tet2*-deficient macrophages, which showed increases in Il-1 β , Il-6, and Ccl5, but not Cxcl1 or Cxcl2, following LPS stimulation (52). Although further experiments using primary cells from mice and humans are required, these findings highlight the concept that mutations in different driver genes may result in clones with divergent functions, which may ultimately drive disease

progression through different mechanisms. This could conceivably have implications for the design of future therapies, particularly as it has been observed in a subanalysis of the CANTOS trial that individuals with hematopoietic *TET2* mutations respond more favorably to canakinumab than do individuals without mutations in this gene (68). Overall, the findings from this study indicate that *Dnmt3a* loss of function within hematopoietic cells promotes inflammatory processes and heart failure (52). However, given the complex immunomodulatory properties of *DNMT3A* in multiple immune cell types (75–79), additional experimental studies are required to better understand how *DNMT3A* affects the pathogenesis of cardiovascular disease.

JAK2

Another relatively common gene that is mutated within the aged hematopoietic system is *JAK2* (1–3, 80). *JAK2* is a member of the Janus family of cytoplasmic non-receptor tyrosine kinases and plays an important role in immune signaling processes (81). In particular, it is involved in many cytokine signal transduction pathways, such as those of erythropoietin and thrombopoietin (81). The most frequent type of *JAK2* mutation associated with both clonal hematopoiesis and myeloproliferative disorders, such as polycythemia vera, is the V617F mutation, which is often denoted as *JAK2*^{V617F} (1–3, 80, 82). This mutation is a guanine-to-thymidine substitution, resulting in an amino acid substitution of valine for phenylalanine at codon 617 of *JAK2* (83). The mutation is thought to cause changes to the autoinhibitory site of JAK2 kinase, resulting in a constitutively active protein; thus, this type of mutation is considered a gain-of-function mutation (84). It has been suggested that *JAK2* mutations may occur in lineage-restricted stem cells, such as myeloid-restricted stem cells (85–87); however, studies have challenged this and have observed that individuals who harbor the *JAK2*^{V617F} mutation possess it in all blood cell compartments (88, 89). Moreover, it has been found that some common germline variants have a higher risk of developing mutations in *JAK2* (80, 90); however, the mechanisms by which these germline variants predispose individuals to developing mutations in *JAK2* remain unknown. A recent study examining the prevalence of the *JAK2*^{V617F} mutation in the peripheral blood of 19,958 adult Danish citizens found that 3.1% of individuals harbor this mutation (91). Of these *JAK2*^{V617F}-positive individuals, only 2.3% were diagnosed with a myeloproliferative neoplasm (15). While previous studies have documented individuals who possess the *JAK2*^{V617F} allele in their blood cells and do not exhibit overt changes in the levels of erythrocytes, platelets, or leukocytes, this study found that 42% of mutation-positive individuals had a more hyperproliferative blood cell count (15), which challenges the current definition of CHIP.

Recently, our laboratory examined the relationship between clonal hematopoiesis associated with *JAK2* gain of function and cardiovascular disease (54). Using a transgenic mouse strain that expressed human *JAK2*^{V617F}, competitive BMT experiments were performed to characterize the expansion characteristics of *JAK2* mutant cells. Interestingly, *JAK2*^{V617F} mutants expanded exclusively within the myeloid cell population, and this expansion was accelerated following myocardial infarction (54). However, at 16 weeks following competitive BMT, all mice showed signs of myeloproliferative disease, with elevated numbers of platelets and red blood cells; thus, this model was not ideal for studying CHIP (54). To avoid such complications, a lentiviral approach was developed, whereby exogenous *JAK2* expression was under the control of a myeloid-specific promoter and enhancer. Lineage-negative bone marrow cells were then transduced with the lentivirus encoding *JAK2*^{WT} (control) or *JAK2*^{V617F} (mutant) and transplanted into lethally irradiated mice. Importantly, mice transplanted with *JAK2*^{V617F} cells did not display alterations in blood cells or platelets, suggesting this was a superior model for studying clonal hematopoiesis in the setting of cardiovascular disease (54). Using the left anterior descending artery (or LAD) ligation model of

myocardial infarction and the transverse aortic constriction (or TAC) model of pressure overload, mice transplanted with a small number of $JAK2^{V617F}$ cells showed augmented indices of heart failure compared with animals transplanted with $JAK2^{WT}$ cells (54). Moreover, it was noted that there were elevated levels of proinflammatory mediators within the hearts of animals that received $JAK2^{V617F}$ mutant cells (54). Overall, this indicates that clonal hematopoiesis associated with the $JAK2^{V617F}$ mutation can accelerate heart failure, likely through the overactivation of inflammatory pathways. It has also been reported that total hematopoietic $JAK2$ gain of function accelerates experimental atherosclerosis (92). Specifically, it has been documented that the transplantation of $JAK2^{VF}$ bone marrow to $Ldlr^{-/-}$ atherosclerosis-prone mice results in larger and more complex atherosclerotic lesions following a high-fat, high-cholesterol diet (92). However, caution should be taken when interpreting these findings as the entire bone marrow was reconstituted with $JAK2$ mutant cells, and mice exhibited a myeloproliferative disease phenotype that is not representative of CHIP.

In terms of the potential underlying mechanism by which hematopoietic $JAK2$ gain-of-function mutations may promote cardiovascular disease, it has been documented that the mutation results in an increase in extracellular trap formation by neutrophils (NETs). More specifically it has been found that $JAK2^{V617F}$ human and mouse neutrophils have increased NET formation in response to ionomycin treatment in vitro (93). Moreover, it has been observed that $JAK2^{V617F}$ mutant cells from humans had increased expression of PAD4, an enzyme involved in the formation of NETs (93). Indeed, studies have reported that NETs can promote cardiac dysfunction in the setting of myocardial infarction (94, 95) and pressure overload (96); thus, increased NET formation may represent a potential mechanism by which hematopoietic $JAK2$ gain-of-function mutations promote cardiovascular disease. Furthermore, it is known that NET formation is involved in thrombosis, and it has been reported that mice heterozygous for the $JAK2^{V617F}$ allele in their hematopoietic cells show an increased propensity for developing spontaneous pulmonary thrombosis (93). Consistent with this, a case-control study has documented an association between JAK^{V617F} -positive CHIP carriers and major venous thrombotic events (93). Thus, an increase in thrombosis may account for the increase in the incidence of cardiovascular disease (i.e., myocardial infarction and stroke) observed in JAK^{V617F} -positive carriers.

PERSPECTIVES AND CONCLUDING REMARKS

Clonal hematopoiesis is an inevitable consequence of normal human aging, and recent clinical studies indicate that this condition is associated with an increased incidence of mortality and cardiovascular conditions (13–15, 31, 38–40). Recent experimental work has provided evidence that this relationship is causal and has shed light on the mechanisms through which mutations in *Tet2* and *Dnmt3a*, and $JAK2^{V617F}$ in hematopoietic cells contribute to pathology in models of atherosclerosis and heart failure (14, 51–54). Despite this progress, this area of research is very much in its infancy. To date, only a small number of candidate driver genes have been evaluated in models of cardiovascular disease. Furthermore, clonal hematopoiesis not attributed to known drivers has also been associated with an increase in all-cause mortality, and conceivably some of these deaths could be due to cardiovascular conditions (15, 30, 31). Thus, identifying these mutations and their relationship with cardiovascular disease will be an important step forward. Due to mechanistic differences, different genetic mutations will likely confer divergent functions on hematopoietic cells. Therefore, some mutations may be highly pathological, whereas others may be benign or perhaps even cardioprotective. It is also important to note that the same mutation may affect components of the cardiovascular system differently, and, thus, future experimental studies should examine the effects of clonal hematopoiesis on cardiovascular diseases beyond

atherosclerosis and heart failure. It also remains to be determined how clonal hematopoiesis can affect other age-related diseases, particularly those that are driven by inflammation. Continued research into this area could provide a rationale for the prognostic evaluation of somatic mutations in the hematopoietic system, as it could add to the predictive capabilities of traditional risk factors for cardiovascular disease, which were deduced ~50 years ago. Moreover, understanding how different mutations affect pathology may ultimately uncover novel approaches to treat cardiovascular disease.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health grants to K.W. (HL138014, HL1238014, HL126141, and HL132564) and grants to S.S. from the American Heart Association Postdoctoral Fellowship program (17POST33670076) and the Kanae Foundation for the Promotion of Medical Science.

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