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REVIEW

Emerging Role of Acquired Mutations and Clonal Hematopoiesis in Atherosclerosis

— Beyond Conventional Cardiovascular Risk Factors —

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Accumulating evidence suggests that conventional cardiovascular risk factors are incompletely predictive of cardiovascular disease, as a substantial risk remains even when these factors are apparently managed well. In this context, clonal hematopoiesis has emerged as a new and potent risk factor for atherosclerotic cardiovascular disease and other cardiometabolic conditions. Clonal hematopoiesis typically arises from somatic mutations that confer a competitive advantage to a mutant hematopoietic stem cell, leading to its clonal expansion in the stem cell population and its progeny of blood leukocytes. Human sequencing studies and experiments in mice suggest that clonal hematopoiesis, at least when driven by certain mutations, contributes to accelerated atherosclerosis development. However, the epidemiology, biology and clinical implications of this phenomenon remain incompletely understood. Here, we review the current understanding of the connection between clonal hematopoiesis and atherosclerosis, and highlight knowledge gaps in this area of research.

Key Words: Aging; Atherosclerosis; CHIP; Inflammation; TET2

Despite the efficacy of drugs that target traditional cardiovascular risk factors (e.g., cholesterol-lowering drugs), increasing evidence shows that a substantial risk of atherosclerotic cardiovascular disease (ASCVD) remains, even in individuals who achieve massive reductions in blood cholesterol and are apparently at low cardiovascular risk.[1–3] These observations have led to an increasing interest in the identification of new ASCVD risk factors, and mechanisms of atherosclerosis that are independent of conventional risk factors and susceptible to be targeted for improvements in the prediction, prevention and treatment of ASCVD. In this context, recent evidence supports an important role of acquired mutations in the hematopoietic system, typically associated with the development of leukemia, in atherosclerosis and related CVD.

Clonal Hematopoiesis: A Novel Risk Factor for ASCVD

Hematopoietic stem cells (HSC) are known to acquire random mutations constantly as an individual ages.[4–6] Although most of these mutations are neutral "passenger" mutations, a few of them affect a "driver" gene, providing a selective advantage to the mutant HSC that leads to the progressive expansion of the mutant cell over the years, a phenomenon that can be referred to as somatic mutation-driven clonal hematopoiesis (CH).[7,8] Several definitions have been used to describe CH and related hematological conditions, but the one that has achieved greater impact, particularly in the cardiology field, is that of CHIP or 'Clonal Hematopoiesis of Indeterminate Potential'. CHIP is specifically defined as the presence of somatic mutations in myeloid malignancy-associated genes in the blood or bone marrow (BM) that are present at ≥2% variant allele frequency (i.e., 4% mutant cells, if monoallelic mutations) in individuals without a diagnosed hematologic disorder.[9] CH is strongly linked to aging. Although CH-related mutations can be acquired randomly at any point in life, the likelihood of having acquired a driver mutation evidently increases as an individual ages. In this regard, CH can be estimated to be present in >20% of cancer-free individuals >60 years old,[10–15] although reported rates of CH depend on sequencing sensitivity, and it is expected that this number will increase as more sensitive sequencing strategies are used to study this phenomenon.

CH is strongly associated with increased risk of hematological malignancies and all-cause death.[10,11] Despite this, most individuals carrying a single somatic mutation capable of driving CH will never develop blood cancer, which typically arises as a result of the serial acquisition of multiple driver mutations in a HSC clone over time. In contrast, individuals with CH, most frequently with a single

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Table. Most Frequently Mutated Genes Identified as Candidate Drivers of Clonal Hematopoiesis

Driver gene	Description
DNMT3A	Epigenetic regulator of gene transcription that catalyzes de novo DNA methylation
TET2	Epigenetic regulator of gene transcription that modulates DNA methylation through catalytic (methylcytosine oxidation) and noncatalytic actions
ASXL1	Chromatin-binding protein and epigenetic regulator of gene expression
PPM1D	Protein phosphatase and negative regulator of the DNA damage response
JAK2	Nonreceptor tyrosine kinase and mediator of JAK/STAT signaling
SF3B1	RNA splicing factor
SRSF2	RNA splicing factor
TP53	Regulator of gene transcription and mediator of the DNA damage response
GNAS	Guanine nucleotide-binding protein, signaling regulator
GNB1	Guanine nucleotide-binding protein, signaling regulator

The relative frequency of mutations may vary across studies depending on sequencing sensitivity and age range.

driver mutation, have an increased ASCVD risk that far exceeds their risk of developing hematological disease. An analysis of archived whole-exome sequencing data from thousands of individuals demonstrated that CH is associated with a >2-fold increase in the incident risk of atherosclerotic conditions, such as coronary artery disease and ischemic stroke, independent of conventional CVD risk factors.[11] Candidate CH driver mutations in that study and most other CH-related sequencing efforts[10,13–15] occur primarily in genes that are commonly mutated in myeloid cancers, such as *DNMT3A*, *TET2*, *ASXL1*, *TP53* and *JAK2* (**Table**). In those with CH in that study,[11] the average size of the identified mutant clones was ~20% of peripheral blood cells. Subsequent studies based on similar analyses of whole-exome/genome sequencing datasets have further validated the strong association between CH and incident risk of developing ASCVD.[16–19] Furthermore, a gene-specific analysis strongly suggested associations between increased risk of ASCVD and CH driven by acquired mutations in *DNMT3A*, *TET2*, *ASXL1* and *JAK2*.[16] Additional studies with large cohorts will be required to corroborate these single gene associations and to examine whether mutations in other less frequently mutated genes are also associated with atherosclerotic conditions.

With the exception of some specific mutations with a strong hematological phenotype (e.g., $JAK2^{V617F}$, discussed below), CH is not typically associated with major alterations in blood cell counts, suggesting that the CH/ASCVD association may be related to "qualitative" changes in BM-derived cell phenotypes rather than to changes in cell quantity. Importantly, in a scenario of CH, the expansion of the mutant clone in the HSC pool has a reflection in its progeny, leading to a substantial fraction of immune cells that carry the CH mutation. Therefore, CH has the potential to affect inflammatory responses, which might account for its connection to accelerated atherosclerosis. Supporting this possibility, carriers of acquired mutations in some CH driver genes have been reported to exhibit heightened levels of specific circulating cytokines.[15,20] Furthermore, single-cell transcriptomics analyses have further corroborated increased inflammatory gene expression in blood cells of individuals carrying somatic mutations in *DNMT3A* and *TET2*,[21,22] and, importantly, inherited genetic variants that reduce pro-inflammatory interleukin (IL)-6 signaling have been reported to attenuate the increased ASCVD risk associated with CH driven by mutations in these genes.[17] Hence, CH is emerging as an important driver of inflammation in atherosclerosis.

Although human sequencing data strongly suggest that CH is a new risk factor for ASCVD and a direct contributor to inflammation and atherosclerosis, these data need to be interpreted cautiously, as they do not allow cause-effect relationships to be established. The CH/ASCVD association could simply reflect shared consequences of the normal aging process or be secondary to confounding factors. Furthermore, these sequencing studies provide limited information on directionality. In this context, a variety of experimental approaches are currently being used to expand our understanding of the connection between CH and ASCVD.

CH and Atherosclerosis: Insights From Experimental Studies in Mouse Models

The availability of mouse strains carrying mutations in orthologs of some of the most commonly mutated genes in CH has enabled the testing of causation in the CH/ASCVD association. Although much work lies ahead, *TET2* and *JAK2*, 2 of the most important CH driver genes, have already been thoroughly studied in the context of experimental atherosclerosis.

In 2017, we reported experiments in atherosclerosis-prone $Ldlr^{-/-}$ mice, an animal model of hypercholesterolemia-driven atherosclerosis, that support the causal contribution of TET2-mutant CH to atherosclerosis development.[23] *TET2* encodes for an epigenetic regulator of gene transcription that is able to catalyze the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), a process that facilitates subsequent DNA demethylation and transcriptional activation.[24–26] Conversely, TET2 can also mediate transcriptional repression through noncatalytic actions, for instance by recruiting histone deacetylases to gene promoters.[27] Human sequencing studies have found that inactivating *TET2* mutations are the second most common driver of CH[10,11,13–15] and frequent in myeloid malignancies.[28,29] Mouse studies have shown that TET2 loss of function results in increased HSC self-renewal and, in some settings, a bias towards differentiation into the myeloid lineage.[30–33] In our study,[23] competitive BM transplantation strategies with $Tet2^{-/-}$ and $Ldlr^{-/-}$ mice were used to mimic the human scenario of expansion of a small number of TET2-mutant HSCs. In brief, $Ldlr^{-/-}$ recipients were

transplanted with suspensions of BM cells containing 10% *Tet2*$^{-/-}$ cells, and then fed a high cholesterol diet to induce hypercholesterolemia and atherosclerosis. The TET2-deficient cells expanded markedly in the BM, spleen and blood in this experimental setting, and this expansion led to accelerated atherosclerosis, with a ~60% increase in plaque size.[23] Furthermore, a similar, albeit milder effect was observed in transplantation experiments with *Tet2*$^{+/-}$ cells, which mimic the human scenario of CH better, given that somatic TET2 mutations are likely to be monoallelic in most individuals. An independent study also reported that TET2 inactivation in the entire hematopoietic system increases atherosclerotic plaque size in *Ldlr*$^{-/-}$ mice.[16] No quantitative differences in peripheral white blood cell counts were observed in these studies,[16,23] arguing against the possibility that leukocytosis is an important driver of accelerated atherosclerosis in TET2-mutant CH. Instead, mechanistic studies with myeloid-specific TET2-knockout mice and primary macrophages suggested that this accelerated atherosclerosis is mostly due to the pro-inflammatory properties of TET2-deficient macrophages,[16,23] predominantly to the overproduction of the pro-inflammatory cytokine IL-1β.[23,34,35] These studies identified TET2 as a novel regulator of IL-1β transcription and NLRP3 inflammasome-mediated IL-1β secretion.[23] Consistent with these findings, treatment with a pharmacological inhibitor of the NLRP3 inflammasome, which mediates IL-1β post-translational processing and secretion in atherosclerosis,[36] suppressed accelerated atherogenesis in mice carrying TET2-deficient hematopoietic cells.[23] Further supporting a strong connection between TET2-mutant CH and heightened IL-1β expression, human studies have found increased circulating levels of IL-1β in carriers of somatic TET2 mutations in blood cells, but not in carriers of other CH-related mutations.[15]

The JAK2^{V617F} gain-of-function mutation is another important driver of CH, which has been associated with myeloproliferative neoplasms[37,38] and an increased risk of atherothrombotic and vascular disease.[16,39] JAK2 is a non-receptor tyrosine kinase and a central mediator of JAK/STAT signaling. In marked contrast to somatic variants in *TET2* and most other known CH driver genes, the JAK2^{V617F} mutation is strongly associated with substantial hematological abnormalities in both mice and humans.[37,38,40–42] *Ldlr*$^{-/-}$ mice carrying JAK2^{V617F}-expressing hematopoietic cells exhibit a complex phenotype, with accelerated atherosclerosis development, as well as leukocytosis, erythrocytosis, thrombocytosis, neutrophilia, increased vascular inflammation and erythrophagocytosis.[43,44] Curiously, this acceleration of atherogenesis occurs despite the existence of lower blood cholesterol levels in the hematopoietic JAK2^{V617F} mutant mice. Humans carrying this mutation also exhibit lower circulating cholesterol levels,[45] although the mechanism underlying this observation remains unclear and warrants further investigation. Mice with myeloid-restricted expression of the JAK2^{V617F} mutation exhibit accelerated atherosclerosis development, in parallel with increased macrophage proliferation and expanded necrotic cores in atherosclerotic plaques.[44] Mechanistically, this phenotype appears related to overactivation of the double-stranded DNA-sensing inflammasome AIM2. Activation of AIM2 results in secretion of IL-1β and IL-18 and, accordingly, JAK2^{V617F} mice exhibit increased serum levels of IL-18,[44] which is consistent with observations in humans.[15] Increased JAK2^{V617F} macrophage proliferation is, however, dependent on IL-1 signaling. Although not changing overall plaque size, IL-1 antagonism with anakinra or IL-1β inhibition with a neutralization antibody reduced the JAK2^{V617F} macrophage burden and proliferation in atherosclerotic plaques.[44]

Beyond *TET2* and *JAK2*, ongoing studies are addressing whether mutations in other known CH driver genes affect experimental atherosclerosis development. The evaluation of the effects of *DNMT3A* mutations is particularly relevant, given that this gene is the most frequently mutated CH driver identified to date. *DNMT3A* encodes an enzyme that carries out de novo DNA methylation, although emerging evidence suggests that it can also facilitate DNA demethylation in some settings.[46] Most DNMT3A mutations associated with CH are predicted to cause loss of function. Yet, the effect of DNMT3A inactivation on atherosclerosis development is unclear and its effects on the function of mature immune cells are complex and poorly understood. In T cells, DNMT3A inactivation can increase the expression of the proatherogenic cytokine interferon-γ (IFNγ),[47,48] but also that of atheroprotective IL-13.[49] In macrophages, *DNMT3A* haploinsufficiency affects the expression of hundreds of genes; while it results in increased expression of some pro-atherogenic genes, such as several CXCL family members, it also decreases the expression of central drivers of plaque inflammation, such as TLR4.[46] Experimental studies with mouse models of DNMT3A-mutant CH will be required to examine whether somatic mutations in this gene affect atherosclerosis development and related CVD.

Mutations in DNA damage response genes are also common in individuals who exhibit CH, particularly mutations that affect the transcriptional regulator p53 and the p53-inhibitory phosphatase PPM1D/Wip1. Although mutations in these genes can be found in cancer-free individuals,[10,13–15] they are more frequent in individuals with a history of cancer and treatment with radiation or cytotoxic drugs.[50,51] Consistent with this observation, experimental studies in mice support that cytotoxic cancer therapies facilitate the clonal expansion of pre-existing mutant hematopoietic clones harboring mutations in these genes,[52–55] which has given rise to the concept of cancer therapy-related CH. This phenomenon may be relevant in the context of CVD, as cancer survivors exhibit a high risk of adverse CVD outcomes. Although a number of experimental studies in mice have evaluated the role of p53, *Ppm1d* and other DNA damage response genes in atherosclerosis,[56–63] whether carrying a fraction of cells that bear mutations in these genes is sufficient to affect atherosclerosis development remains unclear and warrants investigation.

Beyond Atherosclerosis, CH in Other Cardiometabolic Conditions

As discussed, mechanistic studies suggest that some CH mutations amplify pro-inflammatory pathways that are at the center of a variety of age-related conditions. Hence, it is likely that CH, at least when driven by certain mutations, is connected to other diseases of aging beyond cancer and ASCVD (**Figure 1**). In this context, a substantial burden of evidence links CH to accelerated progression of heart failure (HF), a major cause of morbidity and mortality in elderly individuals. Sequencing studies have found that CH is common among HF patients, affecting more than one-third of individuals who suffer this condition.[64–66] Importantly,

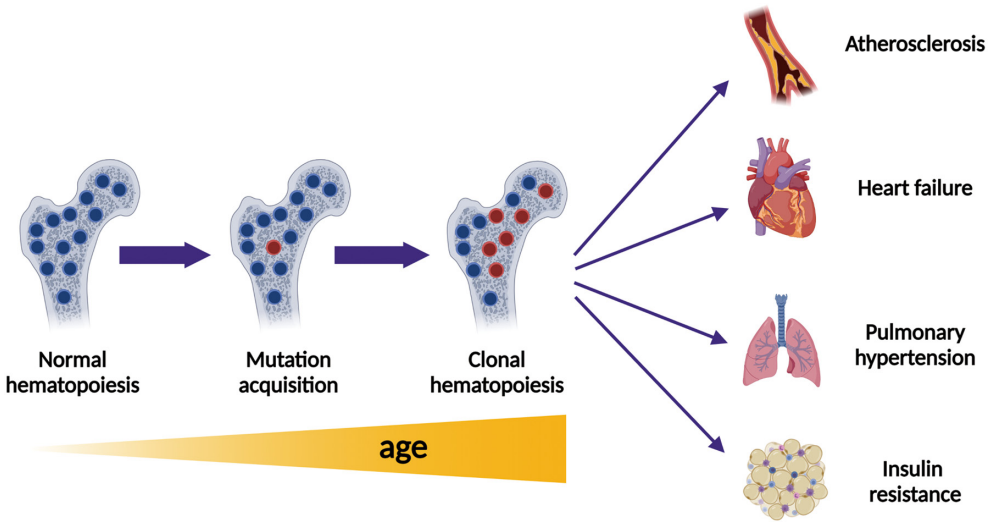

Figure 1. Clonal hematopoiesis (CH): an emerging risk factor for atherosclerotic cardiovascular disease and other age-related conditions. Hematopoietic stem cells accumulate mutations over the years and some of these mutations provide a competitive advantage to the mutant stem cell by promoting its self-renewal, proliferation or survival. This selective advantage eventually leads to the clonal expansion of the mutant stem cell in the bone marrow and, through its cellular progeny, in blood and inflamed tissues infiltrated by blood cells, such as atherosclerotic plaque. Sequencing studies in humans and experimental studies in mice suggest that certain CH-related mutations contribute to the development of atherosclerosis and other age-related conditions where dysfunctional inflammation plays a central role. (Figure created with BioRender.com.)

CH is strongly associated with adverse clinical progression in patients with either ischemic[64,65,67] or nonischemic etiology of HF.[64] The observation of a significant association regardless of the presence of ischemic heart disease is relevant, because it argues against the possibility that these associations simply result from the strong connection between CH and atherosclerosis or the effects of cardiac ischemia on the hematopoietic system. Further supporting a link between CH and HF pathophysiology, studies with mice that are genetically engineered to lack TET2 suggest an important effect of inactivating mutations in this gene on cardiac dysfunction and inflammation.[35,68] Mouse studies also support a link between TET2-mutant CH and pulmonary hypertension, as mice with hematopoietic TET2 inactivation spontaneously develop evidence of this condition.[69] Furthermore, human data suggest an association between carrying a *TET2* mutation, either inherited or acquired, and the development of pulmonary hypertension.[69] Additional research is needed to verify the association between somatic *TET2* mutations that drive CH and this condition.

Beyond CVD, some sequencing studies suggest an association between CH and type 2 diabetes.[11] Although the nature of this connection and the underlying mechanisms remain scarcely investigated, experiments in mice show that the expansion of TET2-deficient hematopoietic cells aggravates age- and obesity-related insulin resistance (IR) by promoting IL-1β-driven inflammation in white adipose tissue.[34] Impaired glucose homeostasis and adipose tissue inflammation facilitate the development of CVD[70] and, therefore, these experimental studies suggest an additional mechanism by which TET2-mutant cells may contribute to CVD beyond their direct effects on the vascular wall and the myocardium. In addition, given that IR plays a key pathogenic role in diabetes, these findings provide experimental evidence supporting that some CH mutations may contribute to the development of diabetes. However, evaluating this possibility will require additional sequencing and mechanistic studies.

Nonmutational Regulators of CH

Ultrasensitive sequencing techniques that allow for the detection of a very small fraction of mutant blood cells (e.g., <1/1,000) suggest that the presence of low levels of hematopoietic cells with somatic mutations able to drive CH is almost ubiquitous by middle age.[71] However, in only a fraction of individuals the mutant clone undergoes marked expansion, reaching the currently most widely accepted 2% variant allelic fraction threshold that identifies pathophysiologically relevant CH. Indeed, sequencing studies of serially collected samples have shown that the dynamics of clonal expansion are highly heterogeneous: some individuals carry clones that remain stable in size for years, whereas others show marked growth.[11,71] These observations suggest the existence of additional factors that regulate the clonal expansion of mutant HSCs. Because mutant clone size is associated with the risk for ASCVD,[11,16–18] understanding the factors that modulate CH dynamics will be crucial to developing strategies for the management of CVD risk in individuals who carry CH-related mutations.

Inflammation driven by microbial products may be an important promoter of CH. In this context, treatment with bacterial lipopolysaccharide has been shown to enhance the competitive advantage of TET2-deficient HSCs in mice, related to hyperactive IL-6 signaling and improved cell survival.[72] Consistent with these results, dysfunction of the small intestinal barrier leading to bacterial translocation

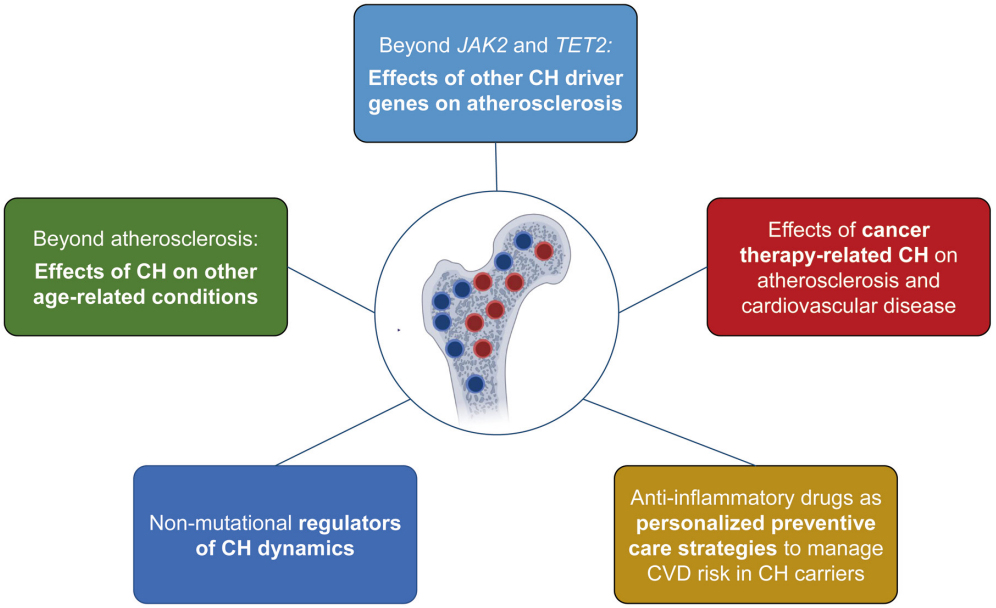

Figure 2. Relevant knowledge gaps in the connection between clonal hematopoiesis (CH) and atherosclerosis. Addressing these gaps in knowledge will require experimental studies in innovative animal models, longitudinal, highly sensitive sequencing studies in human cohorts deeply phenotyped at the cardiovascular level, and, ultimately, clinical trials that enroll carriers of CH-related mutations. CVD, cardiovascular disease.

into the blood and increased IL-6 production have been reported to accelerate the development of hematological phenotypes in TET2-deficient mice.[73] Evidence is also emerging that supports a role for infections and pro-inflammatory cytokines in DNMT3A-mutant CH. In mice, chronic mycobacterial infection accelerates the expansion of DNMT3A-deficient HSCs, which better retain their self-renewal capacity when exposed to chronic IFNγ-induced inflammatory stress.[74]

Beyond infections, metabolic stress-induced inflammation may also contribute to mutant cell expansion in some settings, because both hyperglycemia and obesity affect HSC biology.[75,76] In this context, hyperglycemic stress has been shown to exacerbate hematological phenotypes associated with TET2 inactivation in mice.[77] Furthermore, mathematical modeling supported by human and mouse data suggests that the atherosclerosis trait complex (i.e., the interplay of chronic inflammation, hyperlipidemia, and arterial plaque formation) may accelerate CH by increasing HSC proliferation.[78] This possibility has important implications because it suggests the existence of a pernicious cycle in which atherosclerosis enhances CH which, in turn, accelerates atherosclerosis development. In addition, this mathematical modeling raises the possibility of the existence of reverse causation in the CH/ASCVD association; that is, this association may reflect the effects of atherosclerosis on CH dynamics, rather than the effects of CH on atherosclerosis. Although this is an intriguing hypothesis, it is inconsistent with the results of experimental studies in mice and sequencing studies in humans that show that pharmacologic or genetic inhibition of specific inflammatory pathways blunts the effects of some CH-related mutations on atherosclerosis, without affecting mutant clone size or expansion rates.[17,23,44,79] Hence, further research is needed to determine conclusively the directionality of the CH/ASCVD association, which is of great clinical relevance, as there are ongoing efforts to develop clinical trials for CVD prevention with anti-inflammatory drugs aimed at targeting the effects of specific CH mutations.

Concluding Remarks and Future Directions

Collectively, sequencing studies in humans and experimental studies in mice have provided strong evidence supporting that somatic mutation-driven CH represents a previously unrecognized major risk factor for ASCVD and, potentially, other age-related conditions. However, many relevant questions related to the effects of CH on atherosclerosis remain unanswered and are the object of intensive research efforts (**Figure 2**). The pathophysiological and biological significance of CH will likely depend on the specific driver mutation. Although some CH-related genes, such as *TET2* and *JAK2*, have been thoroughly investigated in both humans and mouse models of atherosclerosis, further research is needed to understand the role of other common drivers of CH in this condition. In addition, it will be critical to investigate the factors that modulate CH dynamics and determine whether a mutant hematopoietic clone remains indolent or expands to dominate hematopoiesis and affect atherosclerosis development. Although recent studies have suggested several potential regulators of mutant HSC expansion, including atherosclerosis itself, their relevance in human CH remains speculative in most cases. Ultimately, dissecting the regulation of CH dynamics will only be achieved through the combination of carefully designed experimental studies in animal models of

CH, and longitudinal deep sequencing studies in deeply phenotyped human cohorts that allow the tracking of mutant clones over years. Such studies might provide the basis for the development of novel strategies for the management of CVD risk in CH mutation carriers.

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Disclosures

The authors declare no competing interests.

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