Medicine

Comprehensive Evaluation of Clonal Hematopoiesis and Mosaic Loss of Y Chromosome in Cardiovascular Risk: A Thorough Analysis in prospective studies

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Abstract

Background

Clonal hematopoiesis of indeterminate potential (CHIP) was initially linked to a twofold increase in atherothrombotic events. However, recent investigations have revealed a more nuanced picture, suggesting that CHIP may confer only a modest rise in Myocardial Infarction (MI) risk. This observed lower risk might be influenced by yet unidentified factors that modulate the pathological effects of CHIP. Mosaic loss of Y chromosome (mLOY), a common marker of clonal hematopoiesis in men, has emerged as a potential candidate for modulating cardiovascular risk associated with CHIP. In this comprehensive study, we aimed to ascertain the precise risk linked to each somatic mutation or mLOY and explore whether mLOY could exert an influence on the cardiovascular risk associated with CHIP.

Methods

We conducted a meticulous examination for the presence of CHIP and mLOY using targeted high-throughput sequencing and digital PCR in a cohort of 446 individuals. Among them, 149 patients from the CHAth study had experienced a first myocardial infarction (MI) at the time of inclusion (MI(+) subjects), while 297 individuals from the 3-city cohort had no history of cardiovascular events (CVE) at the time of inclusion (MI(-) subjects). All subjects underwent thorough cardiovascular phenotyping, including a direct assessment of atherosclerotic burden. Our investigation aimed to determine whether mLOY could modulate inflammation, atherosclerosis burden, and atherothrombotic risk associated with CHIP.

Results

CHIP and mLOY were detected with a substantial prevalence (45.1% and 37.7%, respectively), and their occurrence was similar between MI(+) and MI(-) subjects. Notably, nearly 40% of CHIP(+) male subjects also exhibited mLOY. Interestingly, neither CHIP nor mLOY independently resulted in significant increases in plasma hsCRP levels, atherosclerotic burden, or MI incidence. Moreover, mLOY did not amplify or diminish inflammation, atherosclerosis, or MI incidence among CHIP(+) male subjects. Conversely, inMI(-) male subjects, CHIP heightened the risk of MI over a five-year period, particularly in those lacking mLOY.

Conclusion

Our study highlights the high prevalence of CHIP and mLOY in elderly individuals. Importantly, our results demonstrate that neither CHIP nor mLOY in isolation substantially contribute to inflammation, atherosclerosis, or MI incidence. Furthermore, we find that mLOY does not exert a significant influence on the modulation of inflammation, atherosclerosis burden, or atherothrombotic risk associated with CHIP. However, CHIP may accelerate the occurrence of MI, especially when unaccompanied by mLOY. These findings underscore the complexity of the interplay between CHIP, mLOY, and cardiovascular risk, suggesting that large-scale studies with thousands more patients may be necessary to elucidate subtle correlations.

eLife assessment

In this small-scale study, Fawaz et al. report a significant prevalence of somatic mutations such as Clonal Hematopoiesis of Indeterminate Potential (CHIP) and mosaic loss of Y chromosome (mLOY), but lack of their association with a history of myocardial infarction (MI). The study utilized sensitive techniques, including targeted high-throughput sequencing and digital PCR. The **valuable** findings by the authors present a contrast to earlier reports, yet they align somewhat with some studies that have demonstrated little or no link between clonal hematopoiesis and atherothrombotic events. Although the study offers **convincing** results, its limited sample size and brief follow-up period preclude drawing definitive conclusions.

Introduction

Atherothrombosis is the main cause of death worldwide. Traditional cardiovascular risk factors (CVRF), such as diabetes, smoking, dyslipidemia, hypertension, explain 70 to 75% of cardiovascular events suffered by patients. However, a significant part of these events remains unexplained given that 25 to 30% of people without any evident cause can present an atherosclerotic cardiovascular event (CVE), whereas not all high-risk subjects (according to traditional CVFR) experience such an event.[1]

Recently, clonal hematopoiesis of indeterminate potential (CHIP) has emerged as a potential new risk factor of cardiovascular diseases.[2] This condition results from the acquisition by a hematopoietic stem cell of somatic mutations in leukemia-driver genes, leading to clonal expansion of a population of hematopoietic cells without any clinical or biological sign of hematological malignancy. The definition of CHIP proposed to date, requires the detection of the mutation at a variant allele frequency (VAF) of more than 2%, representing a proportion of mutated cells of more than 4%.[3] The most commonly mutated genes in CHIP are *DNA Methyltransferase 3A* (*DNMT3A*) and *Ten Eleven Translocation 2* (*TET2*). In 2014, Jaiswal *et al* showed that CHIP was associated with a decreased survival mainly because of an increased atherothrombotic mortality. In particular, they observed a 2.0 fold-increased risk of myocardial infarction (MI) and a 2.6-fold increased risk of ischemic stroke.[2] In 2017, these data were confirmed, showing a 1.9-fold increased risk of coronary heart disease in the presence of CHIP, independently of traditional CVRF.[4] At the same time, a causative role of CHIP in inducing atherosclerosis has been demonstrated in animal models through the induction of a proinflammatory state.[4,5] More recently, Kessler *et al* showed in 454 803 subjects from the UK Biobank that the association of CHIP with atherothrombotic events was restricted to high VAF clones (*ie* ≥10%) with a much lower risk than initially demonstrated (HR=1.11).[6] But even with these criteria, no association between CHIP and atherothrombotic events was found in a validation cohort of 173 585 subjects,[6] which has also been suggested in another work studying several hundred thousand subjects.[7] Thus, the impact of CHIP on atherothrombosis in humans is not totally evident, possibly because of the existence of yet unidentified modulating factors that could potentiate or counteract the effect of CHIP. For example, the p.Asp358Ala variant of the IL6 receptor gene has been shown to decrease the atherothrombotic risk associated with CHIP.[8,9] However, the impact other genetic variants on the cardiovascular risk associated with CHIP remains unknown.

Gain or loss of chromosomes in hematopoietic cells appears to be as frequent as the acquisition of somatic mutations during aging.[10] In particular, mosaic loss of chromosome Y (mLOY) has been shown to be frequent in male subjects without evidence of hematological malignancy.[11,12] mLOY was associated with cardiovascular diseases,[13,14] and can be detected in a rather high proportion of subjects with CHIP.[15,16] Finally, while *TET2* mutations promote inflammation and atherosclerosis in mouse models,[4] mLOY was shown to switch macrophages from a pro-inflammatory to a pro-fibrotic phenotype.[14] Thus, because of their opposite effect on macrophages phenotype, mLOY could balance the effect of CHIP regarding the induction of inflammation and thus decrease the development of atherosclerosis and the resulting atherothrombotic risk. We thus hypothesized that mLOY could modulate the effect of CHIP in inducing inflammation, atherosclerosis and triggering atherothrombotic events.

In this study, we used sensitive technics to determine the prevalence of both CHIP and mLOY in 2 cohorts of subjects. We sought to determine whether CHIP and mLOY significantly increase the cardiovascular risk separately. We also searched to determine in humans whether mLOY could impact the effect of CHIP on inflammation, atherosclerosis burden or atherothrombotic risk.

Methods

Patients

For this study, we recruited 446 patients: 149 with a first MI and 297 without a MI or other CVE at inclusion. The 149 subjects with a MI were enrolled in the CHAth study between March 2019 and October 2021 (MI(+) subjects). The main eligibility criterion was suffering from a first MI of atherosclerosis origin after 75 years of age without evidence of hematological malignancy (Supplementary Figure S1). They were included between 4+/-2 months after the acute event, in order to assess their basal inflammatory state. In the presence of any clinical sign or factor

associated with inflammation, the appointment was reported in order not to skew biological and genetic data. Additionally, we ensured that the subjects had not been vaccinated against SARS-Cov2 within 15 days of enrollment. The study was approved by the institutional review board (IRDCB 2019-A02902-05), and registered (*https://www.clinicaltrials.gov*: NCT04581057). All participants gave written informed consent before inclusion in the study. Eligibility and exclusion criteria are more detailed in the Supplementary Methods.

As a control cohort, we selected subjects without any history of CVE at inclusion in the 3-city (3C) study cohort (MI(-) subjects, Supplementary Figure S1).[17] The 3C study is a prospective study that enrolled 9294 subjects of 65 years or more who were selected upon electoral lists. These subjects were followed for several years (up to 12 years) to detect the development of dementia from a vascular origin. As such, they benefitted from a stringent cardiovascular follow-up, with adjudication of all cardiovascular events (in particular occurrence of MI). Among these subjects, we selected the 297 subjects who did not present any CVE before inclusion. Seventy-nine of them presented a MI during follow-up. The remaining 218 subjects had no atherothrombotic event during follow up, and were matched on age, sex and CVRF with those who had a MI during follow up (Supplementary Figure S1).

Clinical and biological parameters measured at inclusion

In MI(+) subjects, a routine cardiovascular evaluation was performed at inclusion (between 2 and 7 month after MI occurrence). Subjects were asked about presence of dyspnea or angina, evaluated with NYHA and CCS scales. Information was obtained from medical records about a potential recurrence of a cardiovascular event since the index event (new MI, coronary revascularization, stroke, hospitalization for acute heart failure). Traditional cardiovascular risk factors were noted. Routine biological analyses comprising blood count, high-sensitive CRP (hsCRP), lipid profile, HbA1c were performed in the laboratory of the university hospital of Bordeaux on fresh samples.

For MI(-) subjects, data available at inclusion included hsCRP level, lipid profile and traditional CVRF. No blood count was available but none of the subjects developed cancer (including hematological malignancy) during follow-up suggesting that detectable somatic mutations were indicative of CHIP and not hematological malignancy.

Measurement of atherosclerosis burden

For MI(+) subjects, a transthoracic echocardiography was performed at inclusion by trained cardiologists and ejection fraction calculated. Supra-aortic trunks ultrasonography with 3D-measurement of atherosclerotic volume was performed by trained physicians, using a Philips iU22 probe equipped with a linear-3D volume convertor VL13-5 (Philips). Images were analyzed using the Vascular Plaque Quantification software on the QLAB 10.2 system (Philips). Carotid stenosis quantification was done with NASCET criteria. Functional ischemic testing was performed at the clinicians' discretion. For MI(-) subjects, atherosclerosis burden was assessed at the inclusion by ultrasound echography recording detection of atherosclerotic plaques, atherosclerotic plaque numbers and intima-media thickness.

Follow up of subjects

For MI(+) subjects, the follow-up was conducted with a standardized questionnaire previously validated in clinical trials.[18] Recurrence of MI as well as any significant cardiovascular event (cardiovascular death, acute coronary syndrome, stroke or transient ischemic attack, congestive heart failure, secondary coronary revascularization, or peripheral vascular surgery) occurring between the initial event and the year after inclusion in the study were recorded. All medical records of participants who died, or who reported on the questionnaire that they had experienced cardiovascular symptoms between baseline and follow-up evaluations, were reviewed by one of

the investigators, and the patient practitioners were contacted. For MI(-) subjects, a follow up was performed for up to 12 years. All cardiovascular events were recorded (including MI) with adjudication upon medical records by an expert committee (**Figure 1**).

Search for somatic mutations and mosaic loss of chromosome Y

For both MI(+) and MI(-) subjects, DNA was extracted from total leukocytes obtained at inclusion. The search for somatic mutations was carried out by the Laboratory of Hematology of the University Hospital of Bordeaux using the Next Generation Sequencing (NGS) panel designed for the diagnosis and follow-up of myeloid hematological malignancies. The genes tested are detailed in Supplementary Table S1.

Briefly, target sequences were captured using the SureSelect technology (Agilent). Sequencing was performed on a NextSeq 550Dx Instrument (Illumina technology) and analyzed using an in-house bioinformatics pipeline (see Supplementary Methods for more details). Variants interpretation was performed independently by two biologists according to criteria previously described[19] and reported in Supplementary Table S2. According to the definition of CHIP, only mutations with a VAF ≥ 2% were retained.

The search of mLOY was performed thanks to an in-house droplet digital PCR technic using the following primers and probes:

- - Primer-amel-Fwd: 5'-CCCCTGGGCACTGTAAAGAAT
- - Primer-amel-Rev: 5'-CCAAGCATCAGAGCTTAAACTG
- - Probe-amelX: 5'-HEX-CCAAATAAAGTGGTTTCTCAAGT-BHQ
- - Probe-amelY: 5'-FAM-CTTGAGAAACATCTGGGATAAAG-BHQ.

Briefly, 75 ng of DNA was mixed with ddPCR supermix for Probes (no dUTP, Biorad), primers (0.9µM each) and probes (0.25µM each). The emulsion was prepared with the QX-100 (Biorad). The amplification program was as follows: 10-minutes denaturation at 95°C, followed by 40 cycles of 30 seconds at 94°C, 1 minute at 55°C, and inactivation of 10 minutes at 98°C. The number of droplets positive for amelX and amelY was determined on the QX-200 droplet reader (Biorad) using the Quantasoft software version 1.5 (Biorad). At least 10,000 droplets were analyzed in each well. A cut off of 9% of cells with mLOY defined the detection of a mLOY based on the study of 30 men of less than 40 years who had a normal karyotype as assessed by conventional cytogenetic study.

Statistical analyses

Univariate association analyses were conducted using Fisher exact or Chi-square test statistics for categorical variables. Analysis of variance was used for quantitative variables. Multivariate association analyses were performed using logistic or linear regression models as appropriate. Analyses were adjusted for age and sex (CHIP) or for age only (mLOY). We used raw values for all quantitative variables as they presented a normal distribution, except for CRP levels for which we analyzed log(CRP). Log-rank test statistics were employed to assess the association of clinical/biological variables with the incidence of future cardiovascular events.

All analyses were conducted using either the RStudio software (Posit team (2023). RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA. URL *http://www.posit.co/*.) or the PRISM software (GraphPad Prism version 9.5.1).

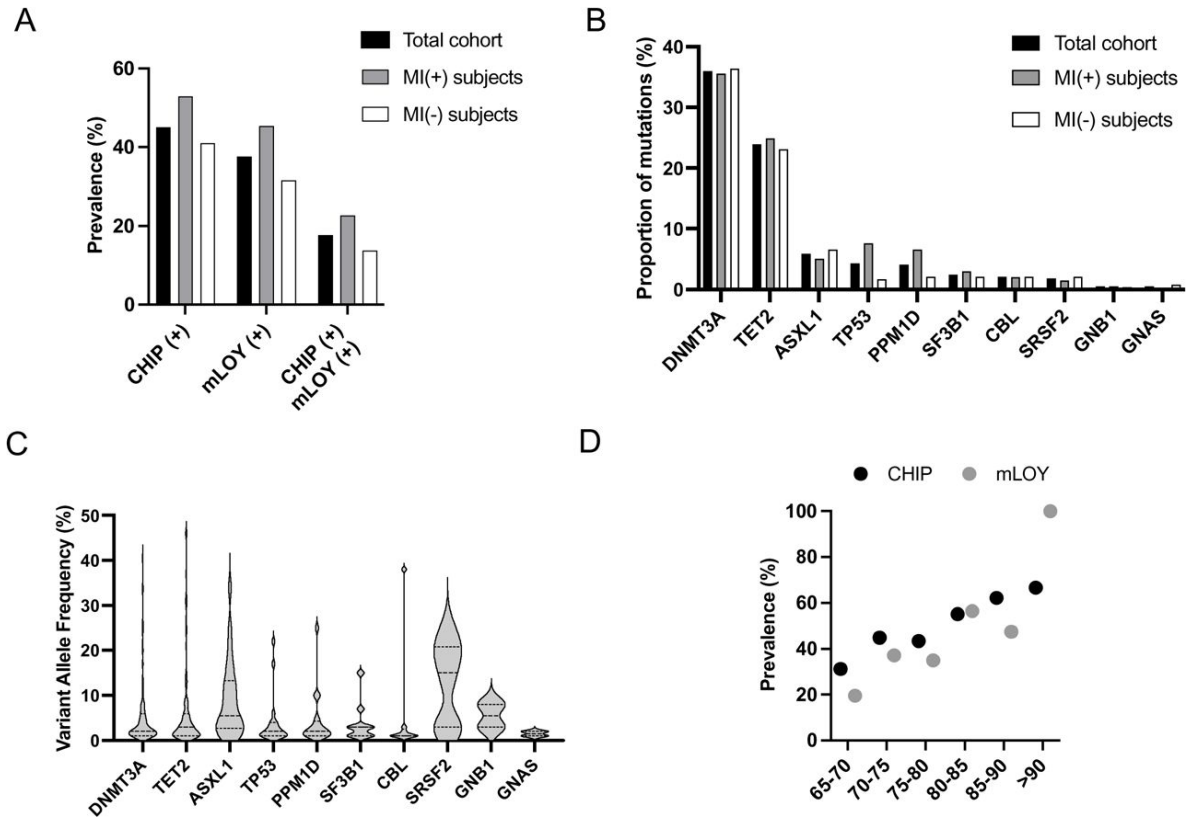

Figure 1

CHIP, mLOY and their combination are as frequent in MI(+) and MI(-) subjects

A: Prevalence of CHIP, mLOY and their combination in the total cohort of 449 subjects, in MI(+) as well as in MI(-) subjects. B: Mutational spectrum of CHIP expressed as the proportion of mutations detected in the indicated genes. C: VAF measured for the different mutations detected in the 449 subjects detected in the indicated genes. D: Prevalence of CHIP and mLOY depending on age in the total cohort (for CHIP) and in male subjects (for mLOY).

Results

Patient's characteristics

In this study, we aimed to decipher whether mLOY could alter the effect of CHIP in inducing a chronic inflammation that would favor the development of atherosclerosis and the incidence of atherothrombotic events. To answer this question, we analyzed 446 subjects from 2 prospective studies, 149 who presented a MI at inclusion and 297 who did not present any CVE before inclusion. The general characteristics of the 446 combined subjects are detailed in **Table 1**. Briefly, the median age was 76.4 years and 257 (57.6%) were males. Forty percent of them presented more than 2 CVRF. MI(+) subjects were older (due to the inclusion criteria), more frequently men, and presented a lower cardiovascular risk than MI(-) subjects (due to the initiation of treatment between the initial event and the inclusion).

CHIP and mLOY are detected as frequently in MI(+) and MI(-) subjects

Among the 446 subjects, at least one mutation with a VAF≥2% was detected in 201 persons (45.1%), defining CHIP(+) subjects (**Table 1**, **Figure 1A**). As previously described, *DNMT3A* and *TET2* were the 2 most frequently mutated genes. The other mutated genes were those previously described in CHIP (**Figure 1B**)[2,4,16] and the median VAF was 2% (**Figure 1C**). The mutational profile of CHIP(+) subjects is available in the Supplementary Tables S3 and S4 while the characteristics of CHIP(+) compared with CHIP(-) subjects are detailed in the Supplementary Tables S5 and S6. In this study, we considered subjects without any detectable mutation or with only mutations with a VAF below 2% as non-CHIP carriers (CHIP(-) subjects).

A mLOY was present in 83 (37.7%) male subjects (mLOY(+) subjects, **Table 1**, **Figure 1A**). The clinico-biological characteristics of mLOY(+) subjects compared with mLOY(-) subjects are detailed in the Supplementary Tables S5 and S6. The median proportion of cells with mLOY was 18% [12%;32%]. There was no significant association between CHIP and mLOY since 39 CHIP(+) subjects (39.8%) also carried a mLOY compared with 44 CHIP(-) subjects (36.1%, p=0.579). Finally, as previously demonstrated, CHIP(+) subjects were significantly older than CHIP(-) subjects (Supplementary Tables S5 and S6). We also observed an increasing prevalence of both CHIP and mLOY with age (**Figure 1D**).

We observed a similar frequency of CHIP and mLOY in MI(+) and MI(-) subjects (**Figure 1A**, **Table 1**). Besides, the association of CHIP with mLOY was as frequent in MI(+) and MI(-) subjects (22.7% and 13.8%, p=0.797). Of note, MI(+) and MI(-) subjects presented similar proportions of mutated genes (**Figure 1B**) and VAF (Supplementary Figure 2). Similar results were also observed when considering CHIP associated with important clones (*i.e.* VAF≥5%, **Table 1**). or when analyzing only *DNMT3A* and *TET2* mutations (data not shown).

Altogether, these results suggest that CHIP and mLOY are very frequent but not associated with the existence of a history of MI, even when mLOY is associated with CHIP.

Neither CHIP, mLOY, nor their combination highly increase basal inflammation or atherosclerotic burden

It was demonstrated that *TET2* mutations were associated with the induction of a pro-inflammatory phenotype of macrophages.[5] On the contrary, mLOY was shown to decrease the inflammatory phenotype of macrophages.[14] Therefore, we searched to determine whether

	All subjects n = 446	MI(+) subjects n=149	MI(-) subjects n=297	p-value
Male, n (%)	**257 (57.6)**	**98 (66)**	**159 (54)**	**0.015**
Median age, years (Q1;Q3)	**76.4 (71.9;80.9)**	**82.0 (78.0;86.0)**	**73.6 (70.6;77.8)**	**$P < 10^{-4}$**
Cardiovascular risk factors				
BMI, kg/m^2 (Q1;Q3)	25.5 (23.6;28.3)	25.5 (23.6;28.5)	25.5 (23.6;28.1)	0.14
Diabetes, n (%)	**94 (21.2)**	**47 (32%)**	**47 (16%)**	**$p < 10^{-4}$**
Hypertension, n (%)	**362 (82.8)**	**107 (76.4)**	**255 (85.9)**	**0.020**
Total cholesterol, g/L (Q1;Q3)	**2.08 (1.72;2.38)**	**1.45 (1.25;1.72)**	**2.23 (2.00;2.47)**	**$p < 10^{-4}$**
LDL-c, g/L (Q1;Q3)	**1.25 (0.94;1.52)**	**0.77 (0.61;1.03)**	**1.39 (1.19;1.60)**	**$p < 10^{-4}$**
HDL-c, g/L (Q1;Q3)	**0.56 (0.46;0.66)**	**0.47 (0.39;0.57)**	**0.59 (0.50;0.68)**	**$p < 10^{-4}$**
Smoking, n (%)	32 (7.2)	6 (4.4)	26 (8.7)	0.117
Prevalence of CHIP and mLOY				
CHIP prevalence, n (%)	201 (45.1)	79 (53%)	122 (41%)	0.923
Prevalence of CHIP with VAF ≥5%	88 (19.7)	30 (20.1)	58 (19.5)	0.069
Subjects tested for mLOY, n	220	97	123	-
mLOY prevalence, n (%)	83 (37.7)	44 (45.4)	39 (31.7)	0.783
CHIP(+) / mLOY(+) prevalence, n (%)	39 (17.7)	22 (22.7)	17 (13.8)	0.797

Data are expressed as numbers and frequency or median, first and third quartiles. For quantitative values, comparisons were made by linear regression of log values adjusted for age and sex. For qualitative parameters, comparisons were made by the fisher test. For each variable, results are expressed among patients with available value.

Table 1

Characteristics of the population at the time of inclusion

mLOY could counter the inflammatory state that would be associated with CHIP. In the total cohort, CHIP(+) and CHIP(-) subjects presented similar levels of hsCRP, as did mLOY(+) and mLOY(-) subjects (**Table 2**). Similarly, hsCRP levels were not different in CHIP(-)/mLOY(-), CHIP(+)/mLOY(-), CHIP(-)/mLOY(+) and CHIP(+)/mLOY(+) subjects. The impact of CHIP and mLOY on hsCRP levels, either alone or in combination, was comparable in MI(+) or MI(-) subjects (**Table 2**). This was also true when restricting the analysis to *DNMT3A* and *TET2* mutated CHIP(+) subjects (Supplementary Table S7). Finally, subjects with important CHIP or mLOY clones did not present higher levels of hsCRP (Supplementary Table S8).

Very few data are available about atheroma burden associated with CHIP and/or mLOY in humans. Moreover, the modulation of the atherogenic effect of CHIP by mLOY remains unexplored. We thus asked whether CHIP alone or in association with mLOY was associated with an increased atherosclerotic burden. In MI(+) subjects we observed a similar proportion of multitroncular coronary lesions and carotid stenosis >50% in CHIP(+) and CHIP(-) subjects (**Table 3**). The global atheroma volume was explored by 3D ultrasound in 34 MI(+) patients, without difference between CHIP(+) and CHIP(-) subjects. Similar results were obtained when analyzing only CHIP(+) subjects carrying *TET2* and/or *DNMT3A* mutations (Supplementary Table S7), when comparing mLOY(+) and mLOY(-) subjects (**Table 3**), or when analyzing the association of CHIP with mLOY (Supplementary Table S9). Concordantly, in MI(-) subjects, all available atherosclerosis markers were similar between CHIP(+) and CHIP(-), as well as between mLOY(+) and mLOY(-) subjects (**Table 3**). This was also true when analyzing the effect of the different combinations of CHIP and mLOY (Supplementary Table S9). Once again, all these results were confirmed in subjects with a VAF ≥5% for CHIP or a mLOY≥50% (Supplementary Table S8).

Altogether, our results suggest that both in the context of a recent MI and in healthy individuals, CHIP were not associated with a systemic inflammation or an increased atherosclerotic burden. mLOY do not modulate inflammatory parameters or atherosclerosis, even in the presence of CHIP.

Neither CHIP, mLOY, nor their combination highly impact the incidence of MI

To decipher whether mLOY could impact the atherothrombotic risk associated with CHIP, we analyzed the incidence of MI during the follow-up of MI(-) subjects. Seventy-nine subjects developed a MI after inclusion in the study with a median delay of 5.00 years. Subjects with MI during follow-up did not differ significantly from those without MI in terms of demography or cardiovascular risk (Supplementary Table S10). CHIP, mLOY and their association were not significantly associated with an increased incidence of MI (**Figure 2A-B**, Supplementary Table S11). Concordantly, we did not observe any difference in the prevalence of CHIP, mLOY or their association between MI(-) subjects who suffered from a MI during follow up and those who did not (Supplementary Table S10). This was also the case when restricting the analysis to CHIP with a VAF ≥5%, a proportion of cells with mLOY ≥50% or to CHIP associated with *DNMT3A* or *TET2* mutations (Supplementary Figure S3A-S3B, Supplementary Table S10). These results suggest that the atherothrombotic risk associated with CHIP is moderate, and is not modulated by its association with mLOY. Moreover, neither CHIP, mLOY nor their combination were significantly associated with atherothrombotic recurrence (Supplementary Table S11).

CHIP in the absence of mLOY may accelerate the occurrence of MI

In order to search for a combined effect of CHIP with mLOY on the risk of incidence of MI, we finally focused our analysis on MI(-) male subjects. In this population, we observed that MI occurred earlier in CHIP(+) subjects, with a significant increased 5-year incidence of MI (log-rank test, p=0.0142, **Figure 2C**). Such an effect was not observed in MI(-) female subjects (log-rank test, p=0.9402, Supplementary Figure S3C). Interestingly, this effect was more pronounced in CHIP(+)/mLOY(-) subjects who presented a significantly higher 5-year incidence of MI (log-rank

	hsCRP level All subjects	p value	hsCRP level MI(+) subjects	p value	hsCRP level MI(-) subjects	p value
CHIP(-)	1.64 (1.00;3.69)	0.652	1.40 (1.00;4.00)	0.600	1.71 (0.97;3.22)	0.141
CHIP(+)	2.00 (1.00:3.90)		2.20 (1.10;5.00)		1.63 (0.91;2.54)	
mLOY(-)	1.45 (0.99;2.75)	0.156	1.8 (1.0;4.8)	0.149	1.41 (0.74;2.16)	0.358
mLOY(+)	1.73 (1.01;4.00)		2.4 (1.03;4.5)		1.2 (0.99;2.99)	
CHIP (-) mLOY (-)	1.11 (0.76;2.19)	0.410	1.00 (0.77;2.72)	0.430	1.35 (0.79;2.14)	0.570
CHIP (+) mLOY (-)	1.87 (1.00;3.03)		2.30 (1.35;7.35)		1.43 (0.72;2.37)	
CHIP (-) mLOY (+)	2.20 (1.02;4.00)		2.50 (1.30;4.00)		1.37 (0.95;3.75)	
CHIP (+) mLOY (+)	1.23 (1.01;3.80)		2.00 (1.02;4.75)		1.17 (1.03;1.73)	

hsCRP Data are expressed as median, first and third quartiles. Comparisons were performed by linear regression of log values adjusted for age and sex. hsCRP levels are expressed in mg/L For each variable, results are expressed among patients with available value.

Table 2

CHIP and mLOY are not associated with increased hsCRP level

Atherosclerosis burden evaluation in MI(+) subjects							
	All patients (n = 149)	**CHIP (-)** (n = 70)	**CHIP (+)** (n = 79)	**p-value**	**mLOY (-)** (n = 53)	**mLOY (+)** (n = 44)	**p-value**
Multitroncular lesions, n (%)	68 (45.6)	29 (41.4)	39 (49.4)	0.484	25 (47.2)	20 (45.4)	0.717
Carotid stenosis ≥ 50%, n (%)	7 (4.7)	2 (2.8)	5 (6.3)	0.317	2 (3.8)	3 (6.8)	0.451
Global atheroma volume (mm^3), median (Q1;Q3)	499.5 (408.0;604.5)	455.0 (374.0;555.0)	520.0 (411.5;611.5)	0.333	601.0 (412.0;718.0)	492.0 (344.5;600.5)	0.707
Atherosclerosis burden evaluation in MI(-) subjects							
	All patients (n = 297)	**CHIP (-)** (n = 175)	**CHIP (+)** (n = 122)	**p-value**	**mLOY (-)** (n = 84)	**mLOY (+)** (n = 39)	**p-value**
Patients with atherosclerotic plaque, n (%)	135 (45.4)	81 (46.3)	54 (44.3)	0.997	34 (40.5)	19 (48.7)	0.537
Number of plaque, median (Q1;Q3)	1 (1;2)	2 (1;2)	1 (1;2)	0.258	2 (1;2)	2 (1;2)	0.863
Intima Media Thickness (mm), median (Q1;Q3)	0.68 (0.60;0.76)	0.67 (0.60;0.76)	0.68 (0.59;0.74)	0.897	0.67 (0.62;0.76)	0.72 (0.57;0.83)	0.706

Data are expressed as numbers and frequency or median, first and third quartiles. For quantitative values, comparisons were made by linear regression of log values adjusted for age and sex. For qualitative parameters, comparisons were made by the fisher test and logistic regression. For each variable, results are expressed among patients with available value.

Table 3

CHIP and mLOY are not associated with an increased atherosclerotic burden

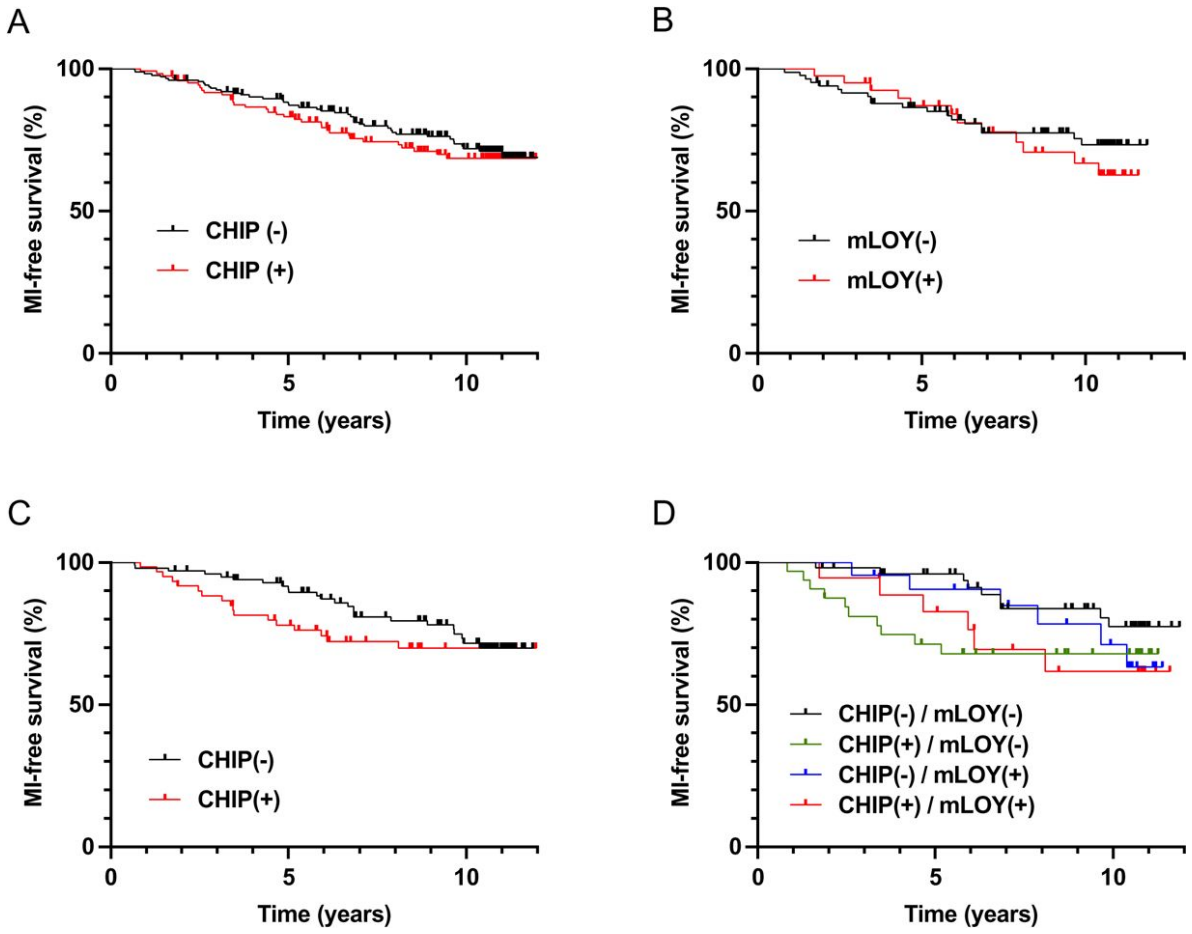

Figure 2

CHIP and mLOY do not increase significantly the risk of incident MI, but could accelerate it in male subjects.

Incidence of MI during follow up according to the presence of CHIP (A) or mLOY (B) in MI(-) subjects. Incidence of MI during follow up according to the presence of CHIP (C) or the combination of CHIP and mLOY (D) in male MI(-) subjects. Survival was compared between the different groups with log-rank tests.

test, p=0.0104, **Figure 2D**) and a significantly lower median time to MI compared with other subjects (Kruskall-Wallis test, p=0.007). Altogether, our results suggest that CHIP do not increase the risk of MI, but may accelerate its incidence, particularly in the absence of mLOY.

Discussion

Clonal hematopoiesis of indeterminate potential (CHIP) has previously been implicated in decreased overall survival, primarily due to its association with an increased incidence of cardiovascular diseases such as coronary artery disease (CAD) and stroke.[2,4] Experimental models have suggested that this association may arise from the pro-inflammatory phenotype of mutated monocytes/macrophages, contributing to the development of atherosclerotic plaques.[4,5] However, recent findings in the literature have presented a complex and sometimes contradictory picture. Studies have reported varying results regarding the relationship between CHIP, inflammation markers, and atherothrombotic events, challenging the initial notion of a high atherothrombotic risk associated with CHIP.[6,7,9,20,21] Moreover, there has been a scarcity of evidence linking CHIP to an increased burden of atherosclerosis in human subjects.[4,22] Additionally, limited data are available on the potential impact of chromosomal abnormalities, particularly mosaic loss of the Y chromosome (mLOY), on atherothrombosis in individuals with CHIP. In this study, we sought to investigate whether mLOY could modulate the effects of CHIP concerning systemic inflammation, atherosclerotic burden, and the risk of atherothrombotic events. To achieve this, we employed sensitive techniques, including targeted high-throughput sequencing and digital PCR, to analyze samples from two cohorts of meticulously phenotyped subjects.

In contrast to many previous studies, we conducted a comprehensive analysis involving two distinct cohorts. The “cases” were individuals recruited from the CHAth study within 2 to 7 months following their first myocardial infarction (MI) after the age of 75. We also established a “control cohort” comprising 297 subjects from the 3-city cohort, none of whom had experienced CVE before inclusion. This allowed us to assess the effects of CHIP and mLOY on inflammation and atherosclerosis independently of pre-existing cardiovascular disease.

Our analysis revealed a remarkably high frequency of CHIP, with an estimated prevalence of 45% among our 446 subjects, with a median age of 76.4 years. This prevalence exceeded initial reports of 15-20% determined by whole exome sequencing (WES) in individuals aged 70-80,[2,23] likely attributable to the enhanced sensitivity of our sequencing technique. Importantly, our estimated prevalence aligns with studies employing similarly sensitive high-throughput sequencing techniques.[24–27] Furthermore, our approach allowed us to reliably detect mutations with a variant allele frequency (VAF) as low as 1%. We chose to define subjects with detectable mutations at a VAF of 1% as CHIP(-), aligning with the WHO definition of CHIP.[28] Notably, all our analyses also yielded identical results when comparing CHIP(+) patients to subjects without detectable mutations.

A recent study by Mas-Peiro et al. demonstrated an association between mLOY and increased post-transcatheter aortic valve replacement (TAVR) mortality.[29] In their investigation, a mLOY ratio threshold of 17% was deemed the most relevant for discriminating patients’ mortality risk. In our study, we employed a mLOY threshold of 9% to identify mLOY, a value determined through DNA samples from patients for whom the absence of mLOY had been definitively established through conventional cytogenetic analysis and an age below 40 years. Using this threshold, we frequently detected mLOY in male subjects within our cohort, with an estimated prevalence of 37.7% among 220 subjects. This prevalence surpasses that reported by Forsberg et al [30] but aligns with recent findings from studies employing sensitive techniques.[16]

Surprisingly, our cohort did not reveal any significant association between the presence of CHIP and the detection of mLOY. This contrasts with the results of two recent studies,[15,16] possibly explained by the heightened sensitivity of our methodology in reliably detecting both somatic mutations and mLOY, which may exist in very small proportions of blood cells.

Mouse models of CHIP have recently suggested that somatic mutations are linked to a pro-inflammatory phenotype of mutated monocytes/macrophages, an observation supported by human samples using single-cell RNA sequencing.[31] However, contradictory results have emerged when examining plasmatic markers of inflammation. Studies, including ours, have not consistently identified a significant increase in plasma hsCRP or proinflammatory cytokines in CHIP carriers,[25,32] whereas others have reported such associations.[20,21] Importantly, our study did not observe any significant association between the detection of CHIP(+/-)mLOY and plasma levels of IL1ß and IL6 in MI(+) subjects, suggesting that CHIP's association with increased systemic inflammation may depend on specific stimulating factors. Notably, recent studies have reported elevated levels of plasma inflammatory markers in CHIP carriers during atherothrombotic events, such as MI or stroke,[33–35] indicating that CHIP may amplify systemic inflammation under specific conditions, but not necessarily in a basal state, as in our study.

To date, only a limited number of studies have successfully linked the presence of CHIP to an increased atherosclerotic burden. While Jaiswal *et al.* reported a higher calcic score in subjects with CHIP[4], and Zekavat *et al.* suggested an increased atherosclerosis,[22] these evaluations relied on self-reported atheroma and indirect parameters. Our study stands as the first to utilize direct markers of atherosclerosis, including global atheroma volume, in CHIP(+) subjects within the context of coronary artery disease. Strikingly, we did not detect a clear increase in atherosclerosis among CHIP or mLOY carriers, either individually or in combination. Conversely, increased atherosclerotic burden associated with CHIP has been observed in patients with stroke.[36]

Our cohort comprising 449 subjects was unable to establish a direct association between CHIP, either alone or in conjunction with mLOY, and coronary heart disease. These results align with previous studies that reported either no difference in CHIP prevalence between individuals with MI and those without,[21] or no significant association between CHIP and incident de novo or recurrent atherothrombotic events.[7,34,37] Recently, Kessler et al. proposed that the increased risk of atherothrombotic events might be limited to CHIP with a VAF of 10% or higher.[6] However, even when considering this criterion, the association was not validated in a cohort of 173,585 subjects. Notably, at the time of our study's initiation, the literature suggested an increased MI risk associated with CHIP, with a hazard ratio of 1.9-2.0. Based on this data, a cohort of 112 cases would have been sufficient to demonstrate a more frequent detection of CHIP in MI(+) patients compared to MI(-) subjects with a power of 0.90. This underscores the formidable challenges of identifying associations with low effect sizes, necessitating cohorts comprising hundreds of thousands of subjects to achieve statistical significance. Regarding specific mutations in *DNMT3A*, *TET2* or other genes, our study did not reveal differing effects on inflammation, atherosclerosis, or MI incidence. Collectively, these findings suggest that any atherothrombotic risk associated with CHIP is limited in scope.

In conclusion, our study does not provide substantive evidence supporting a significant association between CHIP and substantial systemic inflammation, a pronounced increase in atherosclerotic burden, or a markedly elevated atherothrombotic risk. Additionally, mLOY did not appear to significantly modulate inflammation or atherosclerosis in the presence of CHIP. However, our data suggests that CHIP might expedite the onset of MI, particularly in the absence of mLOY. These findings warrant further investigation in larger subject cohorts.

While our study bears some limitations, including a relatively modest sample size of 449 subjects and recruitment of subjects within 2 to 7 months post-MI, it possesses strengths. Notably, we employed highly sensitive techniques for the reliable detection of both CHIP and mLOY, surpassing the capabilities of large cohort studies relying on whole exome sequencing and SNP arrays. Furthermore, we meticulously assessed atherosclerotic burden using sensitive parameters and conducted a thorough evaluation of two cohorts—one comprising MI(+) subjects and the other MI(-) subjects—with precise cardiovascular phenotyping at inclusion and rigorous follow-up, including direct evaluation and adjudication of all CVE.

In summary, our findings provide a nuanced perspective on the relationship between CHIP, mLOY, and cardiovascular outcomes, highlighting the need for larger, more comprehensive studies to elucidate potential associations further.

Data Availability

All data produced in the present work are contained in the manuscript.

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Authorship

TC, OM and CJ designed the study. SF, YP, TC, AG and JB enrolled the subjects in the CHAth study and performed all cardiovascular evaluation. AS, CT and SD set up the 3 City Study Cohort. AB, SM, OM analyzed the NGS data. MD and DAT realized the statistical analysis. SF, SM, MD, DAT, CJ, OM and TC wrote the paper. All authors read and agreed to the final version of the manuscript.

Author approval

All authors have seen and approved the manuscript.

Disclosures

The authors report no conflict of interest.

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Registration

URL: *https://clinicaltrials.gov*; Unique identifier: NCT04581057.

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Reviewer #1 (Public Review):

This manuscript examines the individual and dual effects of CHIP and LOY in MI employing a cohort of ~460 individuals. CHIP is assessed by NGS and LOY is assessed by PCR. The threshold for CHIP is set at 2% (an arbitrary cutoff that is often used) and LOY at 9% (according to the Discussion text - this reviewer may have missed the section that describes why this threshold was employed). The investigation assessed whether LOY could modulate inflammation, atherosclerotic burden, or MI risk associated with CHIP. Neither CHIP nor LOY independently affected hsCRP, atherosclerotic burden, or MI incidence, nor did LOY presence diminish these outcomes in CHIP+ male subjects.

This study represents the first dual analysis of CHIP and LOY on CVD outcomes. The results are largely negative, contradictory to other studies (many with much larger sample sizes). I would attribute the limitation of sample size as a major contributor to the negative data. While the negative data are suspect, the "positive" finding that LOY abolishes the prognostic significance of CHIP on MI is of interest (and consistent with what is understood from mechanistic studies).

Overall, I enjoyed reading the paper, and it is of interest to the research community. However, I disagree with some of the authors' interpretations of the data. Generally, many conclusions on CHIP interpretation are based on the comparison of findings from very large datasets that have been evaluated by shallow NGS DNA sequencing. These studies lack sensitivity and accuracy, but this is counterbalanced by their very large sample sizes. Thus, they draw conclusions from the sickest individuals (ICD codes) with the largest clones (explaining the 10% VAF threshold). Here, the study has a well-phenotyped cohort, but as far as this reviewer can tell, the DNA sequencing is "shallow" NGS. Typically, to assess smaller datasets, investigators employ an error-correction method (DNA barcodes, duplex sequencing, etc.) for the sensitivity and accuracy of calling variants. Thus, the current study appears to suffer from this limitation (small sample sizes combined with NGS).

While the "negative" data from this study are inconclusive, the positive data (i.e. CHIP being prognostic for MI in the absence but not presence of MI) is of interest. Thus, the investigators may want to consider a shorter report that largely focuses on this finding.

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Reviewer #2 (Public Review):

Summary:

The preprint by Fawaz et al. presents the findings of a study that aimed to assess the relationship between somatic mutations associated with clonal hematopoiesis (CHIP) and the prevalence of myocardial infarction (MI). The authors conducted targeted DNA sequencing analyses on samples from 149 MI patients and 297 non-MI controls from a separate cohort. Additionally, they investigated the impact of the loss of the Y chromosome (LOY), another somatic mutation frequently observed in clonally expanded blood cells. The results of the study primarily demonstrate no significant associations, as neither CHIP nor LOY were found to be correlated with an increased prevalence of MI. Of note, the null findings regarding CHIP are in conflict with several larger studies in the literature.

Strengths:

Overall, this is a useful research work on an emerging risk factor for cardiovascular disease (CVD). The use of a targeted sequencing approach is a strength, as it offers higher sensitivity than the whole exome sequencing approaches used in many previous studies.

Weaknesses:

Reporting null findings is definitely relevant in an emerging field such as the role of somatic mutations in cardiovascular disease. Nevertheless, the study suffers from severe limitations, which casts doubts on the authors' conclusions, as detailed below:

(1) The small sample size of the study population is a critical limitation, particularly when reporting null findings that conflict (partly) with positive findings in much larger studies, totaling hundreds of thousands of individuals (e.g. Zekavat et al, Nature CVR 2023, Vlasschaert et al, Circulation 2023; Zhao et al, JAMA Cardio 2024). The authors claim that they have 90% power to detect an effect size of CHIP on MI comparable to that in a previous report (Jaiswal et al, NEJM 2017). However, the methodology used to estimate statistical power is not described. Furthermore, the work by Jaiswal et al (NEJM 2017) showed a hazard ratio of approx. 2.0, but more recent work in much larger populations suggests that the overall effect of CHIP on atherosclerotic CVD is smaller, most likely due to the heterogeneity of effects of different mutated genes (e.g. Zekavat et al, Nature CVR 2023, Vlasschaert et al, Circulation 2023; Zhao et al, JAMA Cardio 2024). In addition, several analyses in the current manuscript are conducted separately in MI(+) (n= 149) and MI(-) (N=297) individuals, further limiting statistical power. Power is still lower in the investigation of the effects of LOY and its interaction with CHIP, as only men are included in these analyses. Overall, I believe the study is severely underpowered, which calls into question the validity of the reported null findings.

(2) Related to the above, it is widely accepted that the effects of CHIP on CVD are highly heterogeneous, as some mutated genes appear to have a strong impact on atherosclerosis, whereas the effect of others is negligible (e.g. Zekavat et al, Nature CVR 2023, Vlasschaert et al, Circulation 2023, among others). TET2 mutations are frequently considered a "positive control", given the multiple lines of evidence suggesting that these mutations confer a higher risk of atherosclerotic disease. However, no association with MI or related variables was found for TET2 mutations in the current work. Reporting the statistical power specifically for assessing the effect of TET2 mutations would enhance the interpretation of these results.

(3) One of the most essential features of CHIP is the tight correlation with age. In this study, the effect of age on CHIP (Supplementary Tables S5, S6) seems substantially milder than in previous studies. Given the relatively weak association with age here, it is not surprising that no association with MI or atherosclerotic disease was found, considering that this association would have a much smaller effect size. In addition, there are previous reports of sex-related differences in the prevalence of CHIP, is there an association between CHIP and age after adjusting for sex?

(4) The mutated genes included in the definition of "CHIP" here are markedly different than those in most previous studies, particularly when considering specifically the studies that demonstrated an association between CHIP and atherosclerotic CVD. For instance, the definition of CHIP in this manuscript includes genes such as ANKRD26, CALR, CCND2, and DDX41... that are not prototypical CHIP genes. This is unlikely to have a major impact on the main results, as the vast majority of mutations detected are indeed in bona fide CHIP genes, but it should be at least acknowledged. Furthermore, the strategy used here for the CHIP variant calling and curation seems substantially different than that used in previous studies, which precludes a direct comparison. This is important because such differences in the definition of CHIP and the curation of variants are the basis of most conflicting findings in the literature regarding the effects of this condition. Ideally, the authors should conduct sensitivity analyses restricted to prototypical CHIP genes, using the criteria that have been previously established in the field (e.g. Vlasschaert et al, Blood 2023).

(5) An important limitation of the current study is the cross-sectional design of most of the analyses. For instance, it is not surprising that no association is found between CHIP and

prevalent atherosclerosis burden by ultrasound imaging, considering that many individuals may have developed atherosclerosis years or decades before the expansion of the mutant clones, limiting the possible effect of CHIP on atherosclerosis burden. Similarly, the analysis of the relationship between CHIP and a history of MI may be confounded by the potential effects of MI on the expansion of mutant clones. In this context, it is noteworthy that the only positive results here are found in the analysis of the relationship between CHIP at baseline and incident MI development over follow-up. Increasing the sample size for these longitudinal analyses would provide deeper insights into the relationship between CHIP and MI.

(6) The description of some analyses lacks detail, but it seems that statistical analyses were exclusively adjusted for age or age and sex. The lack of adjustment for conventional cardiovascular risk factors in statistical analyses may confound results, particularly given the marked differences in several variables observed between groups.

(7) The variant allele fraction (VAF) threshold for identifying clinically relevant clonal hematopoiesis is still a subject of debate. The authors state that subjects without any detectable mutation or with mutations with a VAF below 2% were considered non-CHIP carriers. While this approach is frequent in the field, it likely misses many impactful mutations with lower VAFs. Such false negatives could contribute to the null findings reported here. Ideally, the authors should determine the lower detection limit of their sequencing approach (either computationally or through serial dilution experiments) and identify the threshold of VAF that can be detected reliably with their sequencing assay. The association between CHIP and MI should then be evaluated considering all mutations above this VAF threshold, in addition to sensitivity analyses with other thresholds frequent in the literature, such as 1% VAF, 2% VAF, and 10% VAF.

(8) The authors should justify the use of 3D vascular ultrasound imaging exclusively in the supra-aortic trunk. I am not familiar with this technique, but it seems to be most typically used to evaluate atherosclerosis burden in superficial vascular beds such as carotids or femorals. I am concerned about the potential impact of tissue depth on the accurate quantification of atherosclerosis burden in the current study (e.g. https://doi.org/10.1016/j.atherosclerosis.2016.03.002). It is unclear whether the carotids or femorals were imaged in the study population.

(9) The specific criteria used to define LOY need to be justified. LOY is stated to be defined based on a "A cut off of 9% of cells with mLOY defined the detection of a mLOY based on the study of 30 men of less than 40 years who had a normal karyotype as assessed by conventional cytogenetic study." As acknowledged by the authors, this definition of LOY is substantially different than that used in recent studies employing the same technique to detect LOY (Mas-Peiro et al, EHJ 2023). In addition, it seems essential to provide more detailed information on the ddPCR assay used to determine LOY, including the operating range and, more importantly, the lower limit of detection (%LOY) of the assay. A dilution series of a control DNA with no LOY would be helpful in this context.

(10) Our understanding of the relationship between CHIP and CVD is evolving fast, and the manuscript should be considered in the context of recent literature in the field. For instance, the recent work by Zhao et al (JAMA Cardio 2024, doi:10.1001/jamacardio.2023.5095) should be considered, as it used a similar targeted DNA sequencing approach as the one used here, but found a clear association between CHIP and coronary heart disease (in a population of 6181 individuals).

(11) The use of subjective terms like "comprehensive" or "thorough" in the title of the manuscript does not align with the objective nature of scientific reporting.

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