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Evaluation of Clonal Hematopoiesis and Mosaic Loss of Y Chromosome in Cardiovascular Risk: an 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 study, we aimed to ascertain the 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 an 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 Three-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, in MI(-) 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 study involving patients with a history of myocardial infarction, Fawaz et al. found no significant contribution of clonal hematopoiesis and mosaic loss of the Y chromosome to the incidence of myocardial infarction and atherosclerosis. Although the evidence provided by the study is **incomplete** due to its small sample size, the findings are **valuable** for guiding future larger studies that will further investigate this significant and controversial subject.

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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 (Berry et al., 2012).

Recently, clonal hematopoiesis of indeterminate potential (CHIP) has emerged as a potential new risk factor of cardiovascular diseases (Jaiswal et al., 2014). 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% (Steensma et al., 2015). 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 (Jaiswal et al., 2014). 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 (Jaiswal et al., 2017). 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 (Fuster et al., 2017; Jaiswal et al., 2017). 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, Kessler et al., 2022). But even with these criteria, no association between CHIP and atherothrombotic events was found in a validation cohort of 173 585 subjects (Kessler et al., 2022), which has also been suggested in another work studying several hundred thousand subjects (Kar et al., 2022). 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 (Bick et al., 2020; Vlasschaert et al., 2023a). However, the impact of 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 (Saiki et al., 2021). In particular, mosaic loss of chromosome Y (mLOY) has been shown to be frequent in male subjects without evidence of hematological malignancy (Wright et al., 2017; Zhou et al., 2016). mLOY was associated with cardiovascular diseases (Loftfield et al., 2018; Sano et al., 2022), and can be detected in a rather high proportion of subjects with CHIP (Ljungström et al., 2022; Zink et al., 2017). Finally, while *TET2* mutations promote inflammation and atherosclerosis in mouse models (Jaiswal et al., 2017), mLOY was shown to switch macrophages from a pro-inflammatory to a pro-fibrotic phenotype (Sano et al., 2022). 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 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 Three-City (3C) study cohort (MI(-) subjects, Supplementary Figure S1) (3C Study Group, 2003). 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 carotid atheroma volume was performed by trained physicians of the hospital University of Bordeaux, 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 (Lafitte et al., 2013). 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 (Supplementary 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 (Luque Paz et al., 2021) and reported in Supplementary Table S2. According to the definition of CHIP, only mutations with a VAF ≥ 2% were retained. Although we did not used error-corrected sequencing, the high depth (2111X in median) coupled with bioinformatic tools and manual curing allowed us to reliably detect variants with a VAF≥1%.

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. We determined the background noise of our technique by analyzing the DNA of control subjects (men under 40 years old with a normal karyotype, as assessed by conventional cytogenetic studies). We observed that only a signal corresponding to 9% of cells with mLOY could be considered different from background noise. By analyzing a dilution series of control DNA, we demonstrated the reliability of our ddPCR assay in estimating the proportion of cells with mLOY and its ability to detect as low as 10% of cells with mLOY. Considering the background noise, we established our threshold for confirming the presence of mLOY at 9% of cells with mLOY.

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 and Cox statistical models 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).

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**, Jaiswal et al., 2017, 2014; Zink et al., 2017) 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 a significant association between age and CHIP prevalence (p<0.001). Similarly, the prevalence of mLOY increased with age (p<0.001, **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**), when analyzing only *DNMT3A* and *TET2* mutations (data not shown), or when making further adjustment on CVRF.

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.

Table 1

Characteristics of the population at the time of inclusion

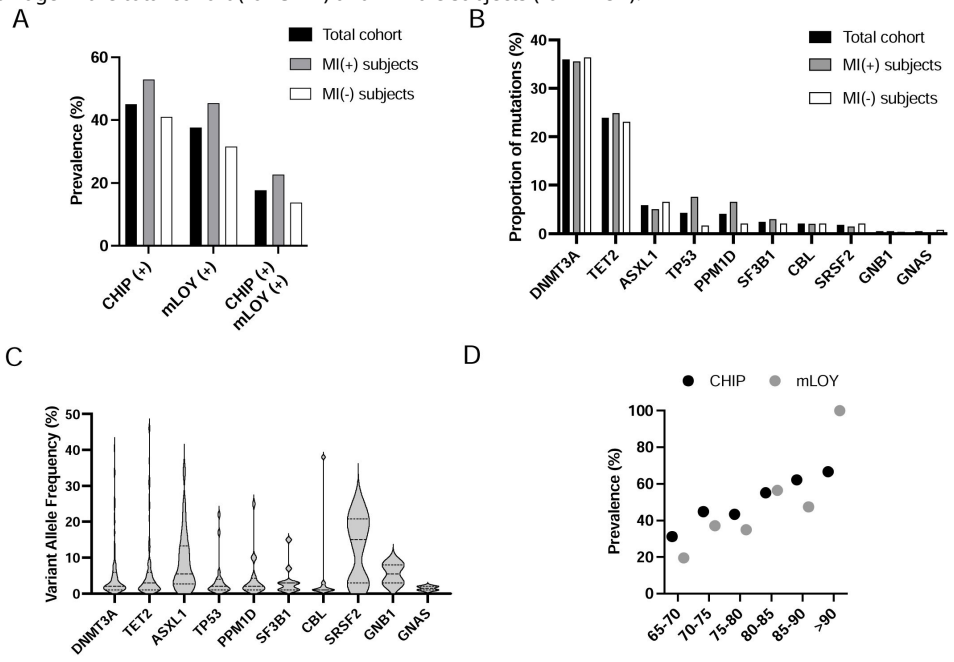
	All subjects n = 446	MI(+) subjects n=149	MI(-) subjects n=297	p-value
Male, n (%)	**257 (57.6)**	**98 (66)**	**159 (54)**	**0.015**
Median age, years (Q1;Q3)	**76.4 (71.9;80.9)**	**82.0 (78.0;86.0)**	**73.6 (70.6;77.8)**	**$P < 10^{-4}$**
Cardiovascular risk factors				
BMI, kg/m^2 (Q1;Q3)	25.5 (23.6;28.3)	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.

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).



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 (Fuster et al., 2017). On the contrary, mLOY was shown to decrease the inflammatory phenotype of macrophages (Sano et al., 2022). Therefore, we searched to determine whether 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), or when adjusting further on CVRF. 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), or when adjusting further on CVRF.

Altogether, our results suggest that in both the context of a recent MI and in healthy individuals, CHIP is not associated with a systemic inflammation or an increased atherosclerotic burden. Additionally, mLOY does 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). Contrary to other studies, we did not observe an association between CHIP and an increased incidence of MI (HR 1.033 [0.657;1.625] after adjustment on age, sex and CVRF). In comparison, hypercholesterolemia and smoking tended to associate with stronger risk of incident MI (HR = 1.474 [0.758;2.866] and 1.866 [0.943;3.690], respectively). Similarly, neither mLOY nor the association between CHIP and mLOY were 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

Table 2

CHIP and mLOY are not associated with increased hsCRP level

	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 3

CHIP and mLOY are not associated with an increased atherosclerotic burden

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.

≥5%, to subjects with a proportion of cells with mLOY ≥50%, to CHIP associated with *DNMT3A* or *TET2* mutations (Supplementary Figure S3A-S3B, Supplementary Table S10), or when making further adjustment on CVRF. 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=0.014, **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 test, p=0.010, **Figure 2D**) and a significantly lower median time to MI compared with other subjects (Kruskall-Wallis test, p=0.007). CHIP(+)/mLOY(-) subjects also presented a higher 5-year incidence of MI using Cox models after adjustment on age and CVRF (HR 7.81, p=0.0388). 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 (Jaiswal et al., 2017, 2014). 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 (Fuster et al., 2017; Jaiswal et al., 2017). 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 (Bick et al., 2020; Busque et al., 2020; Kar et al., 2022; Kessler et al., 2022; Vlasschaert et al., 2023a). Moreover, there has been a scarcity of evidence linking CHIP to an increased burden of atherosclerosis in human subjects (Heimlich et al., 2024; Jaiswal et al., 2017; Wang et al., 2022; Zekavat et al., 2023). 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 an 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 3C 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,(Genovese et al., 2014; Jaiswal et al., 2014) likely

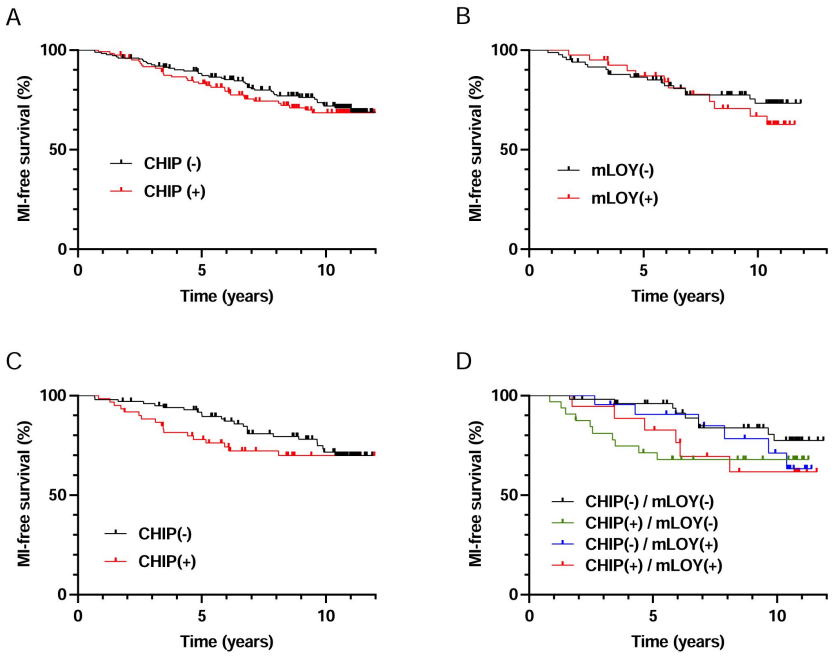


Figure 2

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.

attributable to the enhanced sensitivity of our sequencing technique. Importantly, our estimated prevalence aligns with studies employing similarly sensitive high-throughput sequencing techniques.(Guermouche et al., 2020; Mas-Peiro et al., 2020; Pascual-Figal et al., 2021; van Zeventer et al., 2021) 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.(Khoury et al., 2022) Notably, all our analyses also yielded identical results when comparing CHIP(+) patients to subjects without any detectable mutations or when comparing all patients with somatic mutations with a VAF≥1% to those without.

A recent study by Mas-Peiro et al. demonstrated an association between mLOY and increased post-transcatheter aortic valve replacement (TAVR) mortality (Mas-Peiro et al., 2023). 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 to allow a reliable mLOY detection and to distinguish it from the background noise signal. 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 (Forsberg et al., 2014) but aligns with recent findings from studies employing sensitive techniques.(Zink et al., 2017)

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,(Ljungström et al., 2022; Zink et al., 2017) 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 (Abplanalp et al., 2021). However, contradictory results have emerged when examining plasma markers of inflammation. Studies, including ours, have not consistently identified a significant increase in plasma hsCRP or proinflammatory cytokines in CHIP carriers (Cook et al., 2019; Pascual-Figal et al., 2021), whereas others have reported such associations (Bick et al., 2020; Busque et al., 2020). Importantly, our study did not show 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 (Arends et al., 2023; Böhme et al., 2022; Wang et al., 2022), 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 (Jaiswal et al., 2017), and Zekavat *et al.* suggested an increased atherosclerosis (Zekavat et al., 2023), these evaluations relied on self-reported atheroma and indirect parameters. In the context of coronary artery disease, only 2 studies addressed this question with conflicting results. Heimlich *et al* observed an increased prevalence of stenosis and obstructive stenosis in CHIP(+) subjects, particularly of the left main artery, while Wang *et al* did not notice any association between CHIP and the extent of coronary artery disease. (Heimlich et al., 2024; Wang et al., 2022) Our study stands as one of 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. (Mayerhofer et al., 2023)

Our study bears some limitations, the first of them being a relatively modest sample size of 449 subjects, which did not allow us to establish a direct association between CHIP, either alone or in conjunction with mLOY, and coronary heart disease. These results are in contradiction with previous studies based on cohorts composed of a high number of subjects (Jaiswal et al., 2014, 2017; Vlasschaert et al., 2023a). At the time of our study's initiation, the literature suggested a CHIP prevalence of 20% after 75 years and an increased MI risk associated with CHIP, with a hazard ratio of 1.7. Based on this data, a cohort of 112 cases would have been sufficient to demonstrate a more frequent presence of CHIP in MI(+) patients compared to MI(-) subjects with a power of 0.90 (more details are available in the Supplementary Methods). Although our study was not designed to demonstrate an association between CHIP and incident MI, we were able to confirm that the increased risk of MI associated with the presence of CHIP, if any, is lower 1.7, which is in accordance with more recent studies (Vlasschaert et al., 2023a; Zekavat et al., 2023; Zhao et al., 2024).

Different parameters could have also contributed to the discrepancy of our results with those of previous studies. First, the age of our subjects (≥75 years in the CHAth study, ≥65 years in the 3C study) is higher than the one of other cohorts. Jaiswal et al., 2017; Vlasschaert et al., 2023a; Zhao et al., 2024). Then our strategy to search for somatic variants was also different. In particular, we did not use the criteria defined by Vasschaert *et al* (Vlasschaert et al., 2023b) to cure variants that were called. This had a limited effect since 86.8% of the variants detected in our cohort were concordant with the criteria of Vlasschaert *et al* (Vlasschaert et al., 2023b), impacting the conclusion on the existence of a CHIP in only 15 patients. We also searched for a specific effect of *TET2* mutations on inflammation, atherosclerosis or risk of MI, as the literature suggests that they could be considered as "positive controls". However, we did not find any significant effect of *TET2* mutations. Finally, we were not able to reliably detect variants with a VAF<1% and could have missed the effect of low-VAF variants, as recently shown by Zhao *et al* (Zhao et al., 2024).

However, our results align with previous studies that reported either no difference in CHIP prevalence between individuals with MI and those without (Busque et al., 2020), or no significant association between CHIP and incident *de novo* or recurrent atherothrombotic events (Arends et al., 2023; David et al., 2022; Kar et al., 2022). Recently, Kessler et al. proposed that the increased risk of atherothrombotic events might be limited to CHIP with a VAF of 10% or higher (Kessler et al., 2022). However, even when considering this criterion, the association was not validated in a cohort of 173,585 subjects. Moreover, in our cohort the observed effect of CHIP on the risk of MI (HR = 1.033) was substantially lower than the ones observed for other established cardiovascular risk factors such as hypercholesterolemia (HR = 1.475) or smoking (HR = 1.865). This underscores the formidable challenges of identifying associations with low effect sizes, necessitating cohorts comprising hundreds of thousands of subjects to achieve statistical significance. Collectively, these findings suggest that any atherothrombotic risk associated with CHIP is limited in scope and cannot be used in clinical practice for the management of patients with a history of cardiovascular disease or a risk of atherothrombosis.

However, we believe that our study has also some strength. 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 evaluated 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. Our findings that CHIP might expedite the onset of MI, particularly in the absence of mLOY warrant further investigation in larger subject cohorts.

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, GM 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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Editors

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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. The null findings regarding CHIP are partly in conflict with several larger studies in the literature. However, it must be noted that the authors did find trends to an association between CHIP and a higher incidence of MI during follow-up among those without a history of MI at baseline, which is more consistent with previous research work. The association with incident MI reached statistical significance in men, particularly in those not showing LOY, suggesting potential interactions between different clonally-expanded somatic mutations.

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. Reporting null findings is definitely relevant in an emerging field such as the role of somatic mutations in cardiovascular disease.

Weaknesses:

The study suffers from important limitations, which cast some doubts onto 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 previous reports (a hazard ratio of 1.7, mainly based on the findings by Jaiswal et al, NEJM 2014,2017). However, this analysis is simply based on the predicted prevalence of CHIP in MI(+) and MI(-) patients, and it does not consider the complex relationship between age CHIP and atherosclerotic disease. More advanced approaches to calculate statistical power may have provided a more accurate estimation. It must also be noted that recent work in much larger populations suggest that the overall effect of CHIP on atherosclerotic CVD is smaller than 1.7, 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 even 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 underpowered from a statistical point of view, so the authors' findings need to be interpreted with caution.

(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, which likely reflects the limited statistical power of the study to assess accurately the effects of CHIP mutations on atherosclerotic disease.

(3) One of the most essential features of CHIP is the tight correlation with age. In this study, the effect of age on CHIP (e.g. Supp. Tables S5, S6) is statistically significant, but substantially milder than in previous studies. Given the relatively modest effect size of age on CHIP 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. It must be considered, however, that the advanced age of the population may have confounded the analysis of these relationships, as acknowledged by the authors.

(4) CHIP represents just one type of clonal hematopoiesis (e.g. see https://doi.org/10.1182/blood.2023022222). In this context, it must be noted that the mutated genes included in the definition of "CHIP" here are markedly different than 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, DDX41... that are not prototypical CHIP genes. This is unlikely to have major impact on the main results, as the vast majority of mutations detected are indeed in bona fide CHIP genes, but it needs to be considered when interpreting the authors' findings. Furthermore, the strategy used here for CHIP variant calling and curation is substantially different than that used in previous studies. This is important, because such differences in the definition of CHIP and the curation of variants are at the basis of most conflicting findings in the literature regarding the effects of this condition. The authors estimate that the effect of these discrepancies on the definition of CHIP is limited, but small differences can have substantial impact in a study with limited sample size.

(5) A major 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. A larger sample size in these longitudinal analyses would provide deeper insights into the relationship between CHIP and MI.

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Author response:

The following is the authors' response to the original reviews.

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).

We thank the reviewer for his/her positive and open comment. We acknowledge that we did not use error-corrected sequencing method for our study. However, we do not fully agree with the statement that our NGS sequencing technique is “shallow”.

Considering our entire sequencing panel, we achieve a sequencing depth ≥100X and ≥300X for 100% [99%;100%] and 99% [99%;100%] of the targeted regions respectively. This corresponds to a median depth of 2111X [1578;2574] for all regions sequenced. When considering “CHIP genes”, the median depth is 2694X [1875;3785] for patients from the CHAth study and 3455X [2266;4885] for patients from the 3C study. More specifically, for *DNMT3A* and *TET2* genes, the median depths of sequencing are 2531X [1818;3313] and 3710X [2444;4901] for patients from the CHAth and 3C study respectively. These values are far much higher than the 300X recommended for NGS sequencing by capture technology by the French National Institute of Cancer. Coupling this high depth of sequencing with our bioinformatic pipeline that uses 3 different variant callers, a manual curing for all variants by trained hematobiologists and a bioinformatic tool to estimate the background noise allow us to detect somatic mutation with a VAF of 1% with a high accuracy. Noteworthy, our accuracy in detecting mutations in leukemia-associated genes is tested twice a year as part of our quality control program organized by the French Group of Molecular Biologists in Hematology (GBMHM). We added the information about the depth of sequencing in the Supplementary Methods section.

> *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.*

We thank the reviewer for his/her interest in this result. We also agree that it would be interesting to focus specifically on demonstrating the impact of mLOY in countering the cardiovascular risk associated with CHIP. We performed additional analysis to demonstrate that this effect was independent of age and cardiovascular risk factors and included this information in the results section.

However, we believe that it is also of interest to show negative results that, although probably due to limitation in sample size, suggest that the cardiovascular risk associated with CHIP is not as strong and clinically pertinent as initially suggested. Of note, if CHIP really increase the risk of Myocardial Infarction in a significant manner, they would be more frequently detected in subjects who suffered from a MI compared to those who did not, which was not observed in our cohort. Moreover, we were able to determine that if CHIP increases the risk of MI, they do it to a much lesser extent (HR = 1.03 for CHIP) -than other established cardiovascular risk factors such as hypercholesterolemia or tobacco use HR = 1.47 and HR = 1.86 respectively in our cohort), which questions the pertinence of considering for CHIP in the management of patients with atherothrombosis. These data have been added in the Results and Discussion sections.

We also believe that our study has the merit to assess directly the impact of CHIP on atheroma burden, which has been performed in only a limited number of studies in the context of coronary artery disease. This could not be possible by analyzing only male subjects in our cohort because it would further decrease the statistical power of our analyses.

> ***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.*

We thank the reviewer for his/her pertinent and constructive comments. We totally agree that our study presents a substantially smaller sample size as compared to the studies of Zekavat *et al*, Vlasschaert *et al* or Zhao *et al*.

The CHAth study was designed as a prospective study (which is not frequent in CHIP reports) to demonstrate that, if CHIP increase the risk of MI, they would be detected more frequently in patients who suffered from a MI compared to those who did not. To achieve this, we defined eligibility criteria to have a rather high prevalence of CHIP and optimize the statistical power of a study based on a limited number of patients. We thus enrolled patients who suffered from a first MI after the age of 75 years. These patients had to be compared with subjects from the Three-City study who had 65 years or more at inclusion and did not present any cardiovascular event before inclusion.

To determine the number of patients necessary to achieve our objective, we considered a CHIP prevalence of 20% in the general population after the age of 75 years, as estimated when we set up our study (Genovese *et al*, NEJM 2014, Jaiswal *et al*, NEJM 2014, Jaiswal *et al*, NEJM 2017). At this time the relative risk of MI associated with CHIP was shown to be 1.7, leading to an expected prevalence of CHIP of 37% in subjects who presented a MI. Based on these hypotheses, the recruitment of 112 patients in the CHAth would have been sufficient to detect a significant higher prevalence of CHIP in MI(+) patients compared to MI(-) subjects with a power of 0.90 at a type I error rate of 5%. These calculations were performed by the Research Methodology Support Unit of the University Hospital of Bordeaux. These data were added in the Supplementary Methods section to expose more clearly the design and objectives of the CHAth study.

Finally, we recruited 149 patients in the CHAth study and compared them to 297 control subjects. Although recruiting more patients than initially needed, we observed a similar prevalence of CHIP between our 2 cohorts, suggesting that the cardiovascular risk associated with CHIP is lower than the 1.7 increased risk claimed in most publications related to CHIP in the cardiovascular field. We have to notice that our study was not designed to demonstrate the impact of CHIP on the occurrence of MI during follow-up, which could explain our negative results due to a limited number of patients as stated by the reviewers. This statement has been added in the Supplementary Methods section. However, performing such analysis allowed us to confirm that the risk of MI associated with CHIP was lower than 1.7 and lower than the one associated with hypercholesterolemia or smoking.

We would like also to notice that the eligibility criteria for both CHAth and the Three-City study can have led to a selection bias, possibly contributing to the contradiction of our results with other studies. As stated before, in the CHAth study, only patients who experience a first MI after the age of 75 were enrolled. In the Three-City study, all subjects had 65 years or more at inclusion. On the contrary, most of the cohorts showing an association between CHIP and cardiovascular events were composed of younger subjects:

- Bioimage : median age 70 years (55-80 years)

- MDC : median age 60 years

- ATVB : subjects with a MI before 45 years

- PROMIS : subjects between 30 and 80 years

- UK Biobank : between 40 and 70 years at inclusion, median age of 58 years in the study of Vlasschaert et al.

- Zhao et al : median age of 53.83 years (45.35-62.39 years).

This last information was added in the Discussion section (lines 452-454).

> *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).*

We thank the reviewer for insisting on the fact that the initial HR of 2.0 observed by Jaiswal *et al* was shown to be smaller in more recent studies. This corresponds to what we wrote in the introduction (lines 103-109) and discussion (lines 365-370, 465-471).

> *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.*

We agree with the reviewer that the statistical power of our study is lower than the one of other studies, in particular those based on several hundred thousand patients. Whenever possible, we analyzed our data by combining MI(+) and MI(-) subjects. However, for some aspects such as atherosclerosis, we did not have the same parameters available for these 2 groups and had to analyze them separately, leading to a more limited statistical power. We also have to acknowledge that our study was not designed to demonstrate an effect of CHIP on incident MI (as stated before), limiting our statistical power to demonstrate an effect of CHIP +/- mLOY on the incident risk of coronary artery disease.

However, when designing our prospective study (CHAth study), we aimed to address the limitations of a small cohort and obtain rapid, significant results regarding the impact of CHIP. We hypothesized that if CHIP really increases the risk of myocardial infarction (MI), it would be detected more frequently in patients who have experienced a MI compared to those who have not. This study design would demonstrate the importance of CHIP in MI pathophysiology without requiring thousands of patients. However, we did not observe such an association questioning the relevance of detecting CHIP for the management of patients in the field of Cardiology. This was confirmed by the fact that in our cohort, the cardiovascular risk associated with CHIP appears to be low (HR = 1.03 [0.657;1.625] after adjustment on sex, age and cardiovascular risk factors) compared to hypercholesterolemia (HR = 1.474 [0.758;2.866]) or smoking (HR = 1.865 [0.943;3.690]). These data have been added in the Results and Discussion sections.

In addition, we would like to mention that despite the limited number of subjects studied, we do not have only negative results. When studying only men subjects, we were able to show that CHIP accelerate the occurrence of MI, particularly in the absence of mLOY (Figure 2D). This effect was independent of age and cardiovascular risk factors (diabetes, cholesterol and high blood pressure). We added this last information in the results section of the manuscript, although we acknowledge that this has to be confirmed in future work.

> *(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.*

We thank the reviewer for this pertinent remark. It has indeed been shown that depending on the somatic mutation, the impact of CHIP on inflammation, atherosclerosis and cardiovascular risk is different. The studies cited by the reviewer suggest that *DNMT3A* mutations have a low impact on atherosclerosis/atherothrombosis while other “non-*DNMT3A*” mutations, including *TET2* mutations, have a greater impact. In particular, Zekavat *et al* suggested that *TP53*, *PPM1D*, *ASXL1* and spliceosome mutations have a similar impact on atherosclerosis/atherothrombosis to *TET2*.

To answer to the reviewer in our cohort, we did not find a clear association between the detection of *TET2* mutation with a VAF≥2% and:

- A history of MI at inclusion (p=0.5339)

- Inflammation (p=0.440)

- Atherosclerosis burden :

- In the CHAth study:

- p=0.031 for stenosis≥50%

- p=0.442 fir multitruncular lesions

- p=0.241 for atheroma volume

- in the 3C study :

- p=0.792 for the presence of atheroma

- p=0.3966 for the number of plaques

- p=0.876 for intima-media thickness

- Incidence of MI (p=0.5993)

Similarly we did not find any association between the detection of *TET2* mutations with a VAF≥1% and:

- A history of MI at inclusion (p=0.5339)

- Inflammation (p=0.802)

- Atherosclerosis burden :

- In the CHAth study :

- p=0.104 for stenosis≥50%

- p=0.617 fir multitruncular lesions

- p=0.391 for atheroma volume

- in the 3c study:

- p=0.3291 for the presence of atheroma

- p=0.2060 for the number of plaques

- p=0.2300 for intima-media thickness

- Incidence of MI (p=0.195)

However, analyzing the specific effect of *TET2* mutations reduces the cohort of CHIP(+) subjects to 61 individuals. In these conditions, considering a prevalence of "*TET2*-CHIP" of 13.5% (in our cohort) and a hazard ratio of 1.3 (Vlasschaert *et al*), the statistical power to show an increased risk of MI is only 16%.

> *(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.*

We thank the reviewer for highlighting this point. Although the difference of median age between subjects with or without a CHIP is not very important in our cohort, we did observe a significant association of CHIP with age:

- The differences in age were statistically significant both in the CHAth and 3C study (Supplementary Tables S5 and S6)

- We observed a significant association between age and CHIP prevalence (p<0.001 for the total cohort, p=0.0197 for the CHAth study, and p=0.0394 for the 3C cohort after adjustment on sex). This association was already shown in the figure 1. We added the significant association between age and CHIP prevalence in the Results section (line 279).

As stated before, we have to remind the reviewer that we enrolled only subjects of ≥75 years and ≥65 years in the CHAth and 3C studies respectively. This led to a median age in our cohort that was substantially higher than in other cohorts (in particular the UK Biobank and the different cohorts studied by Jaiswal et al). This could have contributed to an apparent milder effect of age on CHIP, even if this association was still observed.

> *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?*

The reviewer correctly pointed out that sex has been associated with various aspects of CHIP. While Zekavat *et al* reported that CHIP carriers were more frequently males, Kar *et al* (Nature *Genetics 2022)*, and Kamphuis *et al* (Hemasphere 2023) did not observe a difference in the prevalence of CHIP between males and females, but rather a difference in the mutational spectrum. Male presented more frequently *SRSF2*, *ASXL1*, *SF3B1*, *U2AF1*, *JAK2*, *TP53* and *PPM1D* mutations while females had more frequently *DNMT3A, CBL* and *GNB1* mutations.

In our study, the association between CHIP prevalence and age was indeed significant even after adjustment on sex (p<0.001 for the total cohort, p=0.0197 for the CHAth study and p=0.0394 for the 3C).

> *(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.*

We agree with the reviewer that our gene panel includes genes that are not considered prototypical CHIP genes. This acknowledgment has been added in the Supplementary Methods section. To perform this study, we did not design a specific targeted sequencing panel. We used the one that is used for the diagnosis of myeloid malignancies at the University Hospital of Bordeaux. *ANKRD26* and *DDX41* are genes that, when mutated, predispose to the development of hematological malignancies. *CALR* mutations are frequently detected in Myeloproliferative Neoplasms while *CCND2* mutation can be detected in acute myeloid leukemia among other diseases. As usually performed in our routine practice, we analyzed all the genes in the panel. However, as stated by the reviewer, most of the mutations we detected involved *bona fide* CHIP genes.

> *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).*

We agree with the reviewer, our strategy for CHIP variant calling and curation was substantially different from what has been used in other studies. We decided to apply the criteria we used in previous studies for the analysis of somatic mutation in myeloid malignancies. Because CHIP are defined by the detection of “somatic mutations in leukemia driver genes”, this appeared to follow the definition of CHIP.

We also acknowledge that this discrepancy with the criteria defined by Vlasschaert *et al* could contribute to our findings that differ from those of other studies. We thus checked whether the variants detected were in accordance or not with the criteria defined by Vlasschaert *et al*. Pooling the 2 cohorts, we detected 439 variants, 381 of which were in accordance with the criteria established by Vlasschaert *et al*, representing a concordance rate of 86.8%. Moreover, the variants “wrongly” retained according to these criteria had an impact on the conclusion on the detection of CHIP in only 15 patients (because these variants were associated with a mutation in a *bona fide* CHIP gene and/or because its VAF was below 2%). Thus, the impact of CHIP variant calling and curation had only a limited impact on our results. This has been added in the discussion (lines 455-459).

However, we would like to discuss the criteria that have been defined by Vlasschaert *et al* which are probably too restrictive. For some genes, such as *ZRSR2*, in addition to frameshift and non-sens mutations that are expected to be associated with a loss of function, only some single nucleotide variations were retained (probably those detected by this group). In our patient 20785, we detected a c.524A>G, p.(Tyr175Cys) mutation that was not reported in the list published by Vlasscheart *et al*. However, this variant presents a VAF presumptive of a somatic origin (3%), affects the Zn finger domain of the protein and is observed in a male subject. Thus, it presents several criteria to consider it as associated with a loss of function. Similarly, the *CBL* variant c.1139T>C, p.(Leu380Pro) observed in our patient 21536, although not affecting the residues 381-421 of the protein (the criteria defined by Vlasschaert *et al*), has

been reported in 29 cases of hematological malignancies. It is thus likely to have a significant impact on the behavior of hematopoietic cells. Moreover, in the same patient, a *TET2* c.4534G>A, p.(Ala1512Thr) variant was detected. Although not affecting directly the CD1 domain, it has been reported in a case of AML with a VAF suggestive of a somatic origin (Papaemmanuil *et al*, NEJM 2016). The *SH2B3* gene is not considered by Vlasschaert *et al* as a *bona fide* CHIP gene, contrary to other genes involved in cell signaling such as *JAK2*, *GNAS*, *GNB1*, *CBL*. However, inactivating mutations in *SH2B3* can be detected in myeloid malignancies and were recently shown to drive the phenotype in some patients with a MPN (Zhang *et al*, American Journal of Hematology 2024). We could thus expect that this also happens in our patients 22591 and 21998 who harbor mutations of *SH2B3* (a SNV in the PH domain and a frameshift mutation respectively).

Regarding *BCOR*, *STAG2*, *SMC3* and *RAD21* genes, although frameshift mutations are the most prevalent, there are several reports on the existence of SNV in the context of hematological malignancies (COSMIC, Blood (2021) 138 (24): 2455–2468, Blood Cancer Journal (2023)13:18 ; https://doi.org/10.1038/s41408-023-00790-1).

We can also add that although Vlasschaert *et al* did not consider *CSF3R* and *CALR* as CHIP-genes, Kessler *et al* did. Because CHIP are an emerging field, it should be considered that the concepts that define it are expected to evolve, as demonstrated by the recent study of the Jyoti Nangalia's group (Bernstein et al, Nature Genetics 2024) who showed that 17 additional genes (including *SH2B3*) should be considered as driver of clonal hematopoiesis.

> *(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.*

We agree with the reviewer that increasing the sample size for longitudinal analyses would provide deeper insights into the relationship between CHIP and MI. Unfortunately, for the moment, we do not have access to additional samples of the 3C study and are not able to perform these additional analyses.

> *(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.*

The reviewer is right when saying that we adjusted our analyses on age and/or sex. This was done because as stated before, our results did not show a lot of significant differences. However, we reanalyzed our data, adjusting further the tests for conventional cardiovascular risk factors, and observed similar results. These data have been added in the results section (lines 286-287, 303, 319, 331-332, 341).

> *(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.*

We agree with the reviewer that the VAF threshold for identifying clinically relevant CH is still debated. As stated in the manuscript and by the reviewer, we used the conventional threshold of 2%. Considering that different studies have shown that the cardiovascular risk is increased in a more important manner for CHIP with a high VAF (Jaiswal et al, NEJM 2017, Kessler et al Nature 2022, Vlasschaert et al, Circulation 2023), it is not sure that considering variant with a very low VAF (below 2%) would help us in finding an impact of CHIP on inflammation, atherosclerosis or atherothrombotic risk.

However, as mentioned by the reviewer, variants with a low VAF could have a clinical impact as recently reported by Zhao *et al*. In France, the use of biological analysis for medical purposes imposes to demonstrate that all its aspects are mastered, including their performances. In that context, we determined that our NGS strategy allowed us to reliably detect mutation with a VAF down to 1% (data not shown). As stated in the discussion, we also analyzed our results considering variants with a VAF of 1% and found similar results (lines 394-395). The sensitivity analyses were already mentioned in the manuscript, as we also searched for an effect of CHIP with a high VAF (≥5%) and found no effect neither. We did not have a sufficient number of subjects carrying variants with a VAF≥10% to perform analysis with this threshold.

> *(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.*

We apologize for the lack of precision in the Methods section. As stated by the reviewer, we evaluated the atherosclerosis burden in superficial vascular beds. We measured atheroma volume at the site of the common carotid (as described by B Lopez-Melgar, in Atheroslerosis, 2016). We did not analyze femoral arteries in this study. The sentence is now corrected in the Methods (lines 176-179).

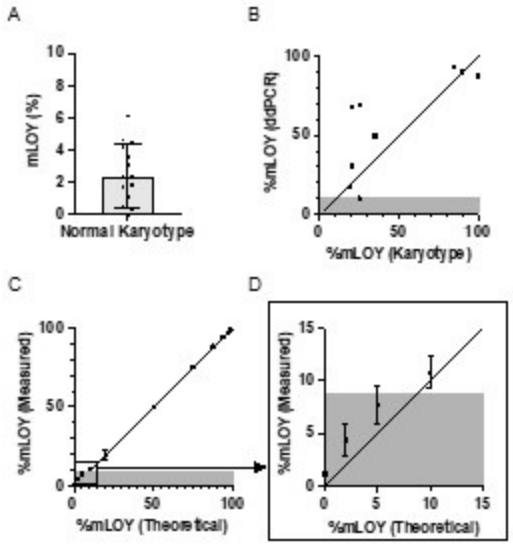
> *(9) The specific criteria used to define LOY need to be justified. LOY is stated to be defined based on a "A cut off of 9% of cells with mLOY defined the detection of a mLOY based on the study of 30 men of less than 40 years who had a normal karyotype as assessed by conventional cytogenetic study." 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.*

We apologize if the definition of the threshold for detecting mLOY was unclear. To test the performance of our ddPCR technique, we first determined the background noise by testing DNA obtained from total leukocytes in 30 men of ≤40 years who presented a normal

karyotype as assessed by conventional cytogenetic technics. In this control population supposed not to carry mLOY, we detected of proportion of cells with mLOY of 2,34+/-1,98 (see Author response image 1, panel A). We thus considered a threshold above 9% as being different from background noise (mean + 3 times the standard deviation).

We then compared the proportion of cells with mLOY measured by ddPCR and conventional karyotype and observed a rather good correlation between the 2 technics (R2=0.6430, p=0.0053, see Author response image 1, panel B). Finally, we tested the reliability of our ddPCR assay in detecting different levels of mLOY using a dilution series of control DNA (from an equivalent of 2% of cell with mLOY to 98% of cells with mLOY). We observed a very nice correlation between the theoretical and measured proportions of cells with mLOY (R2=0.9989, p<0.001, see Author response image 1, panel C). Of note, the proportion of mLOY measured for values ≤10% were concordant with theoretical values. However, considering the background noise determined with control DNA, we were unable to confirm that this "signal" was different from the background noise. Therefore, we set a threshold of 9% to define the detection of mLOY by ddPCR. It is also noteworthy that the 10% cell population with mLOY was consistently detected by the ddPCR technique. This has been added in the Methods section (lines 228-235).

Author response image 1.



> *(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).*

We thank the reviewer for this pertinent reference. We did not include it in the first version of our manuscript because it was not published yet when we submitted our work. We included this reference in the discussion (lines 451, 455, 464). We also included the recent study of Heimlich *et al* (Circ Gen Pre Med 2024, lines 464-468) who studied the association of CHIP with atherosclerosis burden.

> *(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.*

We removed the terms “comprehensive” and “thorough” from the title and the text.

Recommendations for the authors:

> ***Reviewing Editor:***
>
> *The Editors believe that in light of the small study the word Comprehensive has to be removed (including from the title and abstract).*

We agree and removed the term comprehensive from the title and the text.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Other comments:*
>
> *It has long been recognized that hsCRP does not adequately address the inflammation associated with CHIP. For example, see Bick et al Nature 2020; 586:763. Through an assessment of a large dataset, the regulation of multiple inflammatory mediators was associated with CHIP but not with CRP.*

We agree that hsCRP is probably not the most sensitive marker for inflammatory state associated with CHIP. However, it is the most commonly used one in medical practise. However, as indicated in the discussion (lines 418-420), we did not observe any association between CHIP and the plasmatic level of different cytokines (IL1ß, IL6, IL18 and TNFα) in patients enrolled in the CHAth study.

> *Many of the citations lack journal names, volumes, page numbers, etc.*

We apologize for this and corrected the citations.

> *Please provide more details on the methodology (i.e. is CHIP assessed only through NGS with no error correction?). Specify the rationale for why the 9% LOY threshold was employed. Provide this information in the Methods section.*

We added more details on the methodology as demanded in the results section (lines 212-214 and 228-235).

> *Supplementary Table S3 lacks headings. What are the designations for columns 6-8?*

We apologize for this and corrected the Table. Columns 6-8 correspond to the VAF, coverage of the variants and depth of sequencing, as for Table S4.

https://doi.org/10.7554/eLife.96150.2.sa0