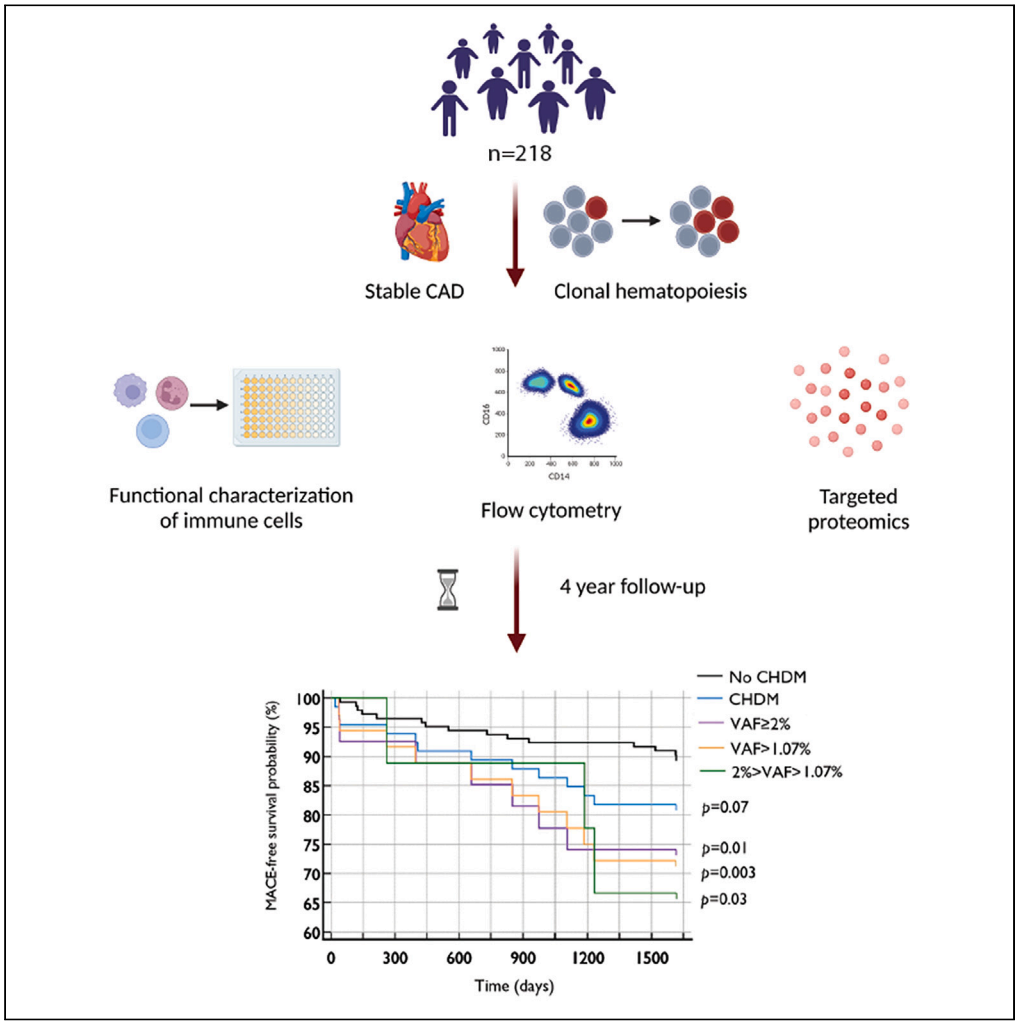
iScience

CellPress
OPEN ACCESS

Article

Clonal hematopoiesis is associated with cardiovascular events in patients with stable coronary artery disease



Mihaela I. Dregoesc, Helin Tercan, Adrian B. Ţigu, ..., Alexander Hoischen, Niels P. Riksen, Adrian C. Iancu

niels.riksen@radboudumc.nl (N.P.R.)
adrianiancu56@gmail.com (A.C.I.)

Highlights

In chronic CAD patients, CHIP is associated with cardiovascular events

This association is present for CHDMs with a VAF >1.07%

CHDMs are not associated with monocyte phenotype and function

CHDMs are associated with higher tissue factor, PAR-1, and matrilysin

Dregoesc et al., iScience 27, 109472
April 19, 2024
https://doi.org/10.1016/j.isci.2024.109472

iScience

CellPress
OPEN ACCESS

Article

Clonal hematopoiesis is associated with cardiovascular events in patients with stable coronary artery disease

Mihaela I. Dregoesc,[1,9] Helin Tercan,[2,9] Adrian B. Ţigu,[3] Siroon Bekkering,[2] Leo AB. Joosten,[2,4] Mihai G. Netea,[2,5] Rosanne C. van Deuren,[6] Alexander Hoischen,[2,6,7] Niels P. Riksen,[2,8,10,*] and Adrian C. Iancu[1,8,*]

SUMMARY

Clonal hematopoiesis (CH) is a risk factor for atherosclerotic cardiovascular disease, but the impact of smaller clones and the effect on inflammatory parameters is largely unknown. Using ultrasensitive single-molecule molecular inversion probe sequencing, we evaluated the association between CH and a first major adverse cardiovascular event (MACE) in patients with angiographically documented stable coronary artery disease (CAD) and no history of acute ischemic events. CH was associated with an increased rate of MACE at four years follow-up. The size of the clone predicted MACE at an optimal cut-off value of 1.07% variant allele frequency (VAF). Mutation carriers had no change in monocytes subsets or cytokine production capacity but had higher levels of circulating tissue factor, matrilysin, and proteinase-activated receptor-1. Our study identified CH driver mutations with a VAF as small as 1.07% as a residual cardiovascular risk factor and identified potential biomarkers and therapeutic targets for patients with stable CAD.

INTRODUCTION

Innate immune cells are critical for the development and destabilization of atherosclerotic plaques. Recently, clonal hematopoiesis (CH) was identified as risk factor for atherosclerotic coronary artery disease (CAD). CH is defined as the process in which somatic mutations in hematopoietic stem cells in the bone marrow lead to expansion of leukocyte clones.[1]

The most frequent clonal hematopoiesis driver mutations (CHDM) involve mutations in the genes *DNMT3A* (DNA methyltransferase 3a), *TET2* (tet methylcytosine dioxygenase 2), *ASXL1* (ASXL transcriptional regulator 1), and *JAK2* (Janus kinase 2).[2] These mutations provide a selective proliferation or survival advantage to the hematopoietic stem cells in which they occur, which then differentiate and contribute disproportionately to the population of mature blood cells.[3–5] While the presence of these mutations is associated with a 10-fold increased risk of hematological malignancies, they also confer a higher risk for cardiovascular disease (CVD).[4,5] Individuals carrying such mutations at a variant allele frequency (VAF) of at least 2%, and in the absence of hematologic disease, are considered as having clonal hematopoiesis of indeterminate potential (CHIP).[1,6]

Several population-based studies have shown that CHIP is associated with a higher incidence of CVD, including CAD and stroke.[2,7] Recent large biobank studies using exome sequencing to characterize CHIP status showed that most CHIP gene mutations were found in *DNMT3A*, and they confirmed the association with incident CVD,[8] including myocardial infarction, stroke, and peripheral arterial disease.[9] Kessler et al., however, did not find an association with *DNMT3A* mutations and CVD in a population based cohort.[8] One very recent large biobank study showed that CHIP was an independent predictor of recurrent adverse events and all-cause mortality in patients with established atherosclerotic CVD.[10] In smaller prospective studies using targeted sequencing of approximately 50 whole genes recurrently mutated in CH, the presence of CHDMs was also associated with prognosis in patients with established CVD. The presence of somatic *DNMT3A* and *TET2* mutations

[1]"Iuliu Hatieganu" University of Medicine and Pharmacy, Department of Cardiology –"Niculae Stăncioiu" Heart Institute, 19-21 Calea Moţilor, 400001 Cluj-Napoca, Romania
[2]Radboud University Medical Center, Department of Internal Medicine and Radboud Institute for Molecular Life Sciences, Geert Grooteplein Zuid 28, 6525 GA Nijmegen, the Netherlands
[3]MEDFUTURE Research Center for Advanced Medicine, Department of Translational Medicine, "Iuliu Haţieganu" University of Medicine and Pharmacy, 4-6 Louis Pasteur, 400349 Cluj-Napoca, Romania
[4]Department of Medical Genetics, Iuliu Hatieganu University of Medicine and Pharmacy, 4-6 Louis Pasteur, 400349 Cluj-Napoca, Romania
[5]Department of Immunology and Metabolism, Life and Medical Sciences Institute, University of Bonn, Carl-Troll-Str. 31, 53115 Bonn, Germany
[6]Radboud University Medical Center, Department of Human Genetics, Geert Grooteplein Zuid 855, 6525 GA Nijmegen, the Netherlands
[7]Radboud Expertise Center for Immunodeficiency and Autoinflammation and Radboud Center for Infectious Disease (RCI), Radboud University Medical Center, Geert Grooteplein-Zuid 10, 6525 GA Nijmegen, the Netherlands
[8]Senior author
[9]These authors contributed equally
[10]Lead contact
*Correspondence: niels.riksen@radboudumc.nl (N.P.R.), adrianiancu56@gmail.com (A.C.I.)
https://doi.org/10.1016/j.isci.2024.109472

was associated with an increased incidence of death and major adverse cardiovascular events (MACE) in acute myocardial infarction survivors.[11] Moreover, CHIP presence was associated with worse short-term outcomes in patients presenting with cardiogenic shock complicating acute myocardial infarction.[12] In patients who suffered an ischemic stroke, the presence of CHDMs with a VAF>1% was associated with an increased risk of recurrent stroke, myocardial infarction, and all-cause death.[13] However, whether CHDMs predict a first cardiovascular event in patients with stable CAD is unknown.

While most studies on CH and CVD are restricted to CHDMs with a VAF >2%,[2,11] or even 10%,[8] there is few evidence for an adverse cardiovascular effect of smaller clone sizes. In patients after stroke, CHDM with a VAF>1% are associated with recurrent MACE.[13] Also, for patients with chronic ischemic heart failure, clone sizes with a VAF <2% are associated with worse outcomes at follow-up.[14]

In this study, we aimed to investigate whether the presence of CHDMs predict a first MACE in patients with angiographically proven stable CAD. To this end, we used an ultrasensitive single molecule molecular inversion probe-based technique, that only captures a subset of known CHDM loci, but with an ultra-high sensitivity which detects very low VAFs.[15]

Unique to our study is that we also assessed innate immune cell phenotype using flow cytometry and cytokine production capacity in all subjects, in addition to targeted proteomics of plasma to explore potential underlying mechanisms of how CHDMs predispose to CVD.

RESULTS

The cohort included 218 patients with a mean age of 64.9 years and a mean body mass index of 29.3 kg/m^2. Men represented 68.3% of the population. Arterial hypertension was diagnosed in 90.8% of patients, while diabetes mellitus was present in 35.8% of them. The mean baseline left ventricular ejection fraction was 53.2%. Statin therapy was administered in 92.2% of the cohort. Paroxysmal or persistent atrial fibrillation was present in 12.8% of the patients. The baseline characteristics of the population are presented in Table 1.

Clonal hematopoiesis driver mutations

CHDMs were present in 33% of the cohort (72 patients), and 13.3% of the cohort (29 patients) met the criteria for CHIP. The prevalence of CHDM was higher in women then in men (41.6% vs. 26.7%, p = 0.02). We identified CHDMs in 13 individual genes (Figure 1; Table S1). The most frequent mutations were in *DNMT3A* (56.9%), *TET2* (11.1%), and *JAK2* (6.9%) (Figure 1A). *DNMT3A* was the most frequently mutated gene in patients older than 55 years, in both males and females (Figures 1B and 1C).

The association between CHDM and major adverse cardiovascular events

The median follow-up time was 4 (3.7–4.1) years. A MACE was recorded in 12.4% of the cohort (27 patients). During follow-up, 3.2% of patients developed an acute myocardial infarction and 3.7% developed an acute ischemic stroke. A history of atrial fibrillation was present in only one of the patients who developed ischemic stroke. Cardiovascular mortality reached 7.3%, while the rate of non-cardiovascular death was 3.7%. During follow-up, 8.1% of patients underwent myocardial revascularization by either PCI or coronary artery bypass graft in the setting of an acute coronary syndrome, while 9.5% of them underwent elective, planned myocardial revascularization procedures.

In univariate Cox proportional-hazards regression analysis, the presence of CHIP was strongly associated with MACE (HR 3.19; 95%CI 1.27–8.01; p = 0.01). ROC curves analysis showed that in patients with CHDM, the VAF predicted MACE at an optimal cut-off value > 1.07% (AUC 0.701; 95%CI 0.575–0.807; p = 0.007) (Figure 2A). Also, the presence of a CHDM with VAF>1.07 was associated with an increased risk for MACE at follow up (HR 3.40; 95%CI 1.49–7.78; p = 0.003).

A gene-specific analysis showed that the presence of *DNMT3A* mutations was associated with a higher rate of MACE as compared to the group without CHDMs, but the result did not reach statistical significance (HR 1.79; 95%CI 0.68–4.72; p = 0.23). The presence of non-*DNMT3A* mutations was associated with a higher rate of ischemic events and cardiovascular mortality at follow-up (HR 2.46; 95%CI 0.93–6.49; p = 0.06). The survival curves are presented in the Supplementary material file (Figure S1).

There was also a significant association of smaller size clones (VAF between 1.07% and 2%) with ischemic events and cardiovascular mortality (HR 3.86; 95%CI 1.09–13.57; p = 0.03). The survival curves are presented in Figure 2B.

Multivariable Cox proportional-hazards models showed that both CHIP and CHDM with VAF>1.07 were associated with MACE independent of conventional cardiovascular risk factors (age, sex, body mass index, smoking, systolic blood pressure, diabetes mellitus, LDL-cholesterol, and BNP value) (Table S4).

Association of CHDM with baseline characteristics and cardiovascular risk factors

In our study cohort, there was no significant association between age and the presence of CHDM (p = 0.19), nor between age and VAF (p = 0.33). However, carriers of CHDMs with a VAF>1.07% were older as compared to patients without CHDMs (67.4 $\pm$ 7.6 vs. 64.4 $\pm$ 8.9 years, p = 0.05).

We further explored whether the presence of CHDM or CHIP was associated with other demographic characteristics or with classical cardiovascular risk factors (Table 2). Female sex (p = 0.02) and systolic blood pressure (p = 0.02) were associated with the presence of CHDM. In multivariable logistic regression analysis, the association of both parameters with CHDM was independent of age (p = 0.03 for each parameter). Elevated systolic blood pressure was also associated with CHIP (Table 2).

CHDM carriers had a higher clinical SYNTAX Score as compared to patients without mutations (p = 0.02).

Table 1. Baseline patient characteristics in the study cohort

PARAMETER	n = 218
Clinical data	
Age (years), mean ± SD	64.9 ± 8.7
Males, n (%)	149 (68.3)
Females, n (%)	69 (31.6)
Ethnicity: Eastern European, n (%)	218 (100)
BMI (kg/m2), mean ± SD	29.3 ± 3.5
Waist (cm), mean ± SD	101.9 ± 11.6
Smokers, n (%)	19 (8.7)
Heart rate (beats/min), median (Q1-Q3)	68 (60–75)
Hypertension, n (%)	198 (90.8)
SBP (mmHg), mean ± SD	137.9 ± 22.4
DBP (mmHg), mean ± SD	79.7 ± 10.9
Diabetes, n (%)	78 (35.7)
Laboratory data	
Glycemia (mg/dL), median (Q1-Q3)	109.1 (97.5–131.3)
Total cholesterol (mg/dL), mean ± SD	167.6 ± 46.6
LDL-cholesterol (mg/dL), mean ± SD	91.4 ± 33.8
HDL-cholesterol (mg/dL), mean ± SD	42.9 ± 12.7
Triglycerides (mg/dL), median (Q1-Q3)	151.2 (106.4–181.9)
Creatinine (mg/dL), median (Q1-Q3)	0.9 (0.8–1.1)
eGFR (ml/min/1.73m^2), median (Q1-Q3)	82.4 (68.1–100.7)
Echocardiographic data	
LVEF (%), mean ± SD	53.2 ± 9.1
Concomitant medication	
Aspirin, n (%)	181 (83)
P2Y12 inhibitor, n (%)	134 (61.4)
Anticoagulant (AVK/NOAC), n (%)	28 (12.8)
Betablocker, n (%)	181 (83)
ACE inhibitor/ARB, n (%)	168 (77)
Calcium channel blocker, n (%)	64 (29.3)
Nitrate, n (%)	72 (33)
Statin, n (%)	201 (92.2)
Fibrate, n (%)	14 (6.4)
Atherosclerosis severity	
SYNTAX Score I, median (Q1-Q3)	11 (7–21)
SYNTAX Score II – PCI, median (Q1-Q3)	29.5 (22.2–35.7)
SYNTAX Score II – CABG, median (Q1-Q3)	26.7 (20.3–33.4)
Revascularization	
Complete, n (%)	67 (31.3)

ACE = angiotensin converting enzyme; ARB = angiotensin receptor blocker; AVK = antivitamin K; BMI = body mass index; CABG = coronary artery bypass graft; DBP = diastolic blood pressure; eGFR = estimated glomerular filtration rate; HDL-cholesterol = high-density lipoprotein cholesterol; LDL-cholesterol = low density lipoprotein cholesterol; LVEF = left ventricular ejection fraction; n = number; NOAC = novel oral anticoagulant; PCI = percutaneous coronary intervention; Q1 = 1st quartile; Q3 = 3rd quartile; SBP – systolic blood pressure.

Exploring how CHDMs affect MACE: Association with leukocytes, and monocyte subsets and function

To explore potential mechanism of how the presence of CHDMs increases the risk for cardiovascular events, we assessed their correlation with circulating leukocyte numbers, and circulating monocyte subsets and function (cytokine production capacity).

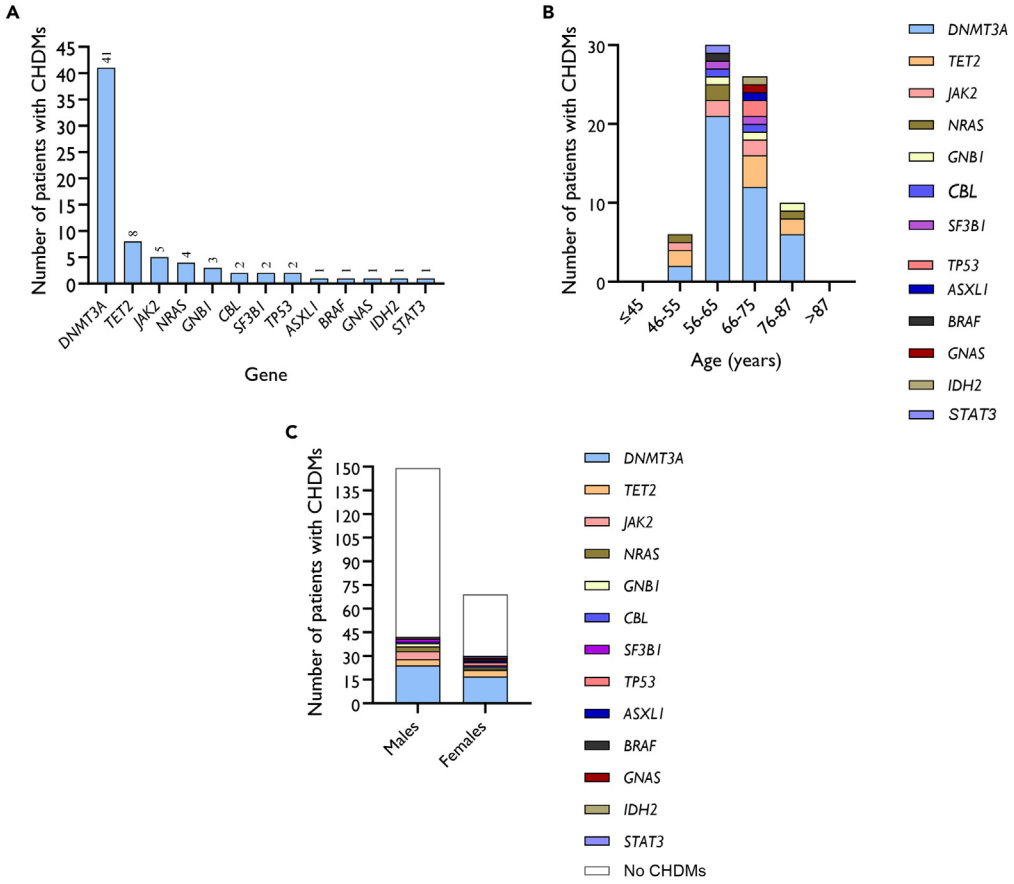


Figure 1. CHDMs in the study cohort

(A) The distribution of mutations in the cohort; (B) Number of individuals with CHDMs per age category; (C) Number of individuals with CHDMs per sex category. CHDM = clonal hematopoiesis driver mutations.

There was no difference in leukocytes number and differentiation between patients with or without CHDM, except for a slightly higher number of eosinophils in patients with CHDM (p = 0.01). In addition, there was no difference in monocyte subsets (classical, intermediate, non-classical), and no significant difference in CD11b expression (Table S5).

We also investigated cytokine production capacity by *ex vivo* stimulation of PBMCs with LPS, Pam3Cys and heat-killed *C. albicans*. Figure S2 shows that 24 h cytokine production was not significantly different between patients with or without CHDM or CHIP. Also, for the 72 h production of IL-17, IL-22, and IFN-gamma, there was no difference between the patients with and without a CHDM.

Exploring how CHDMs affect MACE: Association with circulating proteins

To explore whether circulating proteins are associated with the presence of CHDMs and with MACE, we used targeted proteomics and assessed relative cardiovascular and inflammatory protein levels between patients with and without CDHM. Table 3 shows proteins that were higher in patients with CHDMs: plasma renin (REN) (p = 0.002), leptin (LEP) (p = 0.003), tissue factor (TF) (p = 0.01), interleukin-18 (IL-18) (p = 0.01), matrilysin (matrix metalloproteinase-7, MMP-7) (p = 0.02), tyrosine-protein kinase Mer (MERTK) (p = 0.02), monocyte chemotactic protein 2 (MCP-2) (p = 0.02), thrombomodulin I (p = 0.03), and AMBP (p = 0.03). In patients with CHIP, only three proteins were significantly higher: IL-18 (p = 0.009), IL-12B (p = 0.03), and proteinase-activated receptor 1 (PAR-1) (p = 0.04). Of these proteins, TF (p = 0.006), MMP-7 (p = 0.007), and PAR-1 (p = 0.01) were also associated with MACE. Analysis of publicly available scRNA-seq data confirmed increased expression of the tissue factor gene (F3) in unstimulated classical monocytes from subjects with *DNTM3A* mutations compared to non-CHDM controls (relative fold 1.14%, *p*-adj = 1.45E-5).

DISCUSSION

The results of our study add several insights to the growing body of evidence that clonal hematopoiesis contributes to atherosclerotic CVD. First, we showed that an inexpensive smMIP-based sequencing technique for CHDMs can be used for the prediction of a first MACE in patients with symptomatic stable CAD and angiographically documented coronary atherosclerosis. Although this technique only covers a selection of known CHDM hotspots (including the entire *DNMT3A* gene), it can reliably detect CHDMs up to a VAF of >0.1%. Using this

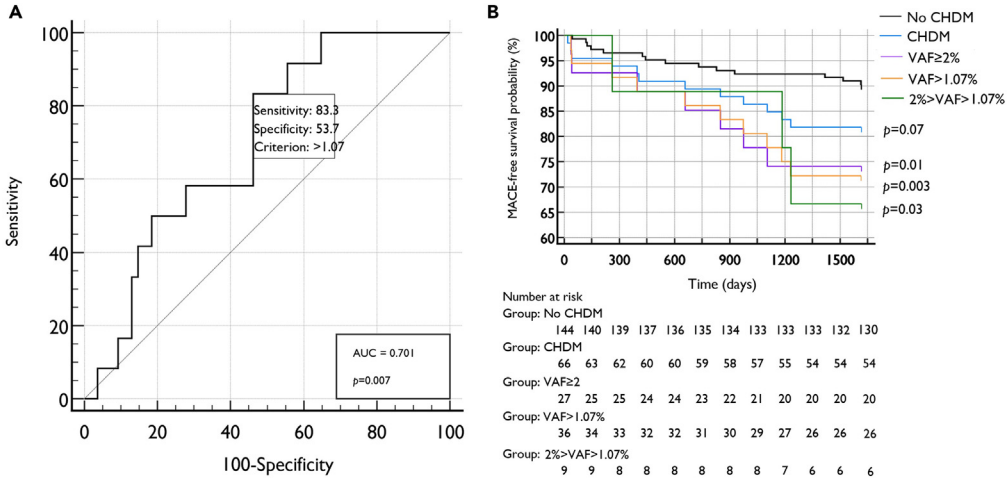


Figure 2. The association between CHDM and MACE

(A) ROC curve for the association between VAF and MACE at 4 years follow-up. VAF>1.07% predicts MACE with a Sensitivity of 83.3% (AUC 0.701; 95%CI 0.575–0.807; p = 0.007).

(B) MACE-free survival according to the presence of CHDMs and the size of the clone. All comparisons are made to the group without CHDM. Patients who suffered a non-cardiovascular death were excluded from the analysis. AUC = area under the curve; CHDM = clonal hematopoiesis driver mutations; MACE = major adverse cardiovascular events; ROC = receiver operating characteristic; VAF = variant allele frequency.

approach, we showed that the presence of CHDMs with a VAF >1.07% is associated with ischemic events and cardiovascular mortality. Secondly, we explored potential mechanisms that link CHDMs with CVD by assessing their association with monocyte phenotype and circulating inflammatory proteins, which is unique compared to previous studies. There was no difference in monocyte subsets in patients with or without CHDMs, nor was there a significant difference in the cytokine production capacity of PBMCs. The concentrations of several circulating proteins were significantly associated with CHDMs and CHIP, with TF, MMP-7 (matrilysin), and PAR-1 also being predictive of MACE.

While previous studies have linked CHIP to lower survival in patients with acute myocardial infarction, or ischemic heart failure,[11,16] or with recurrent adverse events and all-cause mortality in patients with established atherosclerotic CVD,[10] this study is the first to demonstrate that CHDMs are associated with a first ischemic event and cardiovascular mortality in patients with stable CAD. All patients received secondary preventive treatment with antithrombotic therapy and statins (92.2%). Hence, our study shows that CH contributes to the residual cardiovascular risk in these patients.

A VAF higher than 2% has been commonly used as cut-off to define CHIP,[6,11,16–18] and in this study we showed that CHIP was significantly associated with MACE independent of demographic and clinical factors. The cut-off of 2% was earlier chosen mainly based on limited sensitivity of whole genome sequencing techniques to detect smaller clone sizes. Only few studies showed that CHDMs with lower VAF can also have cardiovascular consequences. Arends et al. showed in patients after ischemic stroke, that the presence of CHDMs with a VAF ≥ 1% was associated with a higher risk for future cardiovascular events.[13] A recent report established an optimized cut-off value for *DNMT3A* and *TET2* mutations for prediction of all-cause death in patients with heart failure of 1.15% and 0.73%, respectively.[14] Also in our study, further ROC analysis revealed that CHDMs predict the occurrence of acute ischemic events and cardiovascular death when the VAF is >1.07%. Because of the relatively small sample size and of the subsequent low number of events per patient group, this result should be considered as hypothesis generating and should be further validated in larger cohorts.

The conventional strategy of CHDM identification is though whole-exome sequencing, which is expensive and can yield incidental findings. To circumvent this, we used an inexpensive smMIP-based method for targeted enrichment of CHDM hotspots that can identify CHDMs reliably up to a VAF of 0.1%. Using 300 probes, we covered CHIP/CH-related hotspots in 24 genes, including the entire *DNMT3A* gene since this is the gene with the most CHDMs. In the other genes, we only cover a minority of all CHDMs that have been discovered up to date. Despite this limited coverage, our approach strongly predicts the occurrence of ischemic events and cardiovascular mortality in our patient cohort.

The strength of our study is that we assessed in detail circulating immune cell phenotype and function as well as circulating inflammatory proteins in all 218 patients, which enabled us to explore potential mechanisms that link CHDM presence with MACE. Current insights are mainly derived from experimental animal studies focusing on CH-related mutations in specific genes. Mice with 10% *TET2*-deficient bone marrow cells develop accelerated atherosclerosis because *TET2*-deficient macrophages exhibit an increase in NLRP3 inflammasome-mediated interleukin-1β secretion.[19] In a macrophage cell line complete inactivation of *TET2* promoted gene expression of IL-1β and IL-6, whereas *DNMT3A* inactivation increased the expression of IL6, Cxcl1 and 2.[20] There are only few data in humans that explored these mechanisms. First, Abplanalp et al. used single cell RNA sequencing of PBMCs in six patients with heart failure harboring *DNMT3A* CHDMs and showed that the circulating monocytes and T cells of these patients demonstrated a significantly higher expression of inflammatory genes, while the authors did not investigate cytokine production.[21] Interestingly, the inflammatory transcriptional changes associated with *DNMT3A* mutations

Table 2. The association between baseline parameters, MACE, and the presence of CHDMs

PARAMETER	No CHDM (n = 146)	CHDM (n = 72)	p value	VAF<2% (n = 43)	p value	VAF≥2% (n = 29)	p value
Clinical data							
Age (years)	64.4 ± 8.9	66.1 ± 8.3	0.19	65.8 ± 8.9	0.35	66.3 ± 7.2	0.28
Female sex (%)	26.7	41.6	0.02	41.8	0.07	41.3	0.11
BMI (kg/m^2)	29.1 ± 3.6	29.8 ± 3.2	0.15	30.1 ± 3.5	0.11	29.4 ± 2.8	0.50
Waist (cm)	101.7 ± 12.1	102.4 ± 10.7	0.66	102.6 ± 11.4	0.67	102.2 ± 9.7	0.81
Smokers (%)	10.3	5.5	0.24	4.6	0.25	6.9	0.57
Heart rate (beats/min), median (Q1 – Q3)	68 (60–75)	69 (60–74.2)	0.66	70 (60–75)	0.74	68 (61–70)	0.73
SBP (mmHg)	135.5 ± 19.3	142.6 ± 27.1	0.02	140.1 ± 28.7	0.22	146.1 ± 24.7	0.01
DBP (mmHg)	79.8 ± 11.2	79.7 ± 10.3	0.97	79.4 ± 10.6	0.85	80.2 ± 10.2	0.86
Hypertension, %	90.4	91.6	0.76	93.0	0.60	89.6	0.90
Diabetes, %	36.3	34.7	0.82	23.2	0.11	51.7	0.12
Laboratory data							
Total cholesterol (mg/dL)	165.2 ± 41.1	172.4 ± 56.0	0.56	173.4 ± 54.0	0.37	170.9 ± 59.8	0.52
LDL-cholesterol (mg/dL)	89.7 ± 28.7	94.9 ± 42.3	0.43	98.7 ± 46.5	0.16	89.2 ± 35.4	0.94
HDL-cholesterol (mg/dL)	43.2 ± 13.6	42.4 ± 10.6	0.68	43.1 ± 11.4	0.67	41.4 ± 9.5	0.51
Triglycerides (mg/dL), median (Q1 – Q3)	147.4 (105.4–172.2)	162.2 (107.6–204.7)	0.31	155.8 (110.8–200.7)	0.64	170.8 (101.4–233.4)	0.23
Glycemia (mg/dL)	122 ± 41.9	122.6 ± 41.0	0.68	118.1 ± 36.4	0.72	129.4 ± 46.8	0.39
Creatinine (mg/dL), median (Q1 – Q3)	0.9 (0.8–1.1)	0.90 (0.76–1.10)	0.98	0.9 (0.7–1.1)	0.85	0.8 (0.7–1.1)	0.92
eGFR (ml/min/1.73m^2), median (Q1 – Q3)	84.4 (69.5–100.5)	79.9 (62.0–102.6)	0.46	79.5 (61.9–97.6)	0.32	80.4 (59.0–106.7)	0.96
Echocardiography							
LVEF (%)	53.1	53.2	0.80	53.4	0.84	52.9	0.87
Medication, %							
Aspirin	80.8	87.5	0.25	88.3	0.25	86.2	0.49
P2Y12 inhibitor	57.5	69.4	0.10	67.4	0.24	72.4	0.13
Betablocker	82.8	83.3	0.93	79.0	0.57	89.6	0.36
ACE inhibitor/ARB	76.7	77.7	0.86	81.4	0.51	72.4	0.62
Statin	91.8	93.1	0.75	90.7	0.83	96.5	0.41
Atherosclerosis severity							
Three-vessel disease, %	28.7	33.8	0.44	34.8	0.43	32.1	0.71
SYNTAX Score I, median (Q1 – Q3)	11.5 (7–22)	11 (7–17)	0.84	12 (7–17)	0.87	9.5 (7.2–19.5)	0.88
SYNTAX Score II – PCI, median (Q1 – Q3)	27.9 (21.1–33.6)	32.3 (25.3–37.3)	0.02	33.3 (28.0–37.3)	0.01	30.5 (23.1–41.1)	0.32
Complete revascularization, %	30.7	32.3	0.81	30.2	0.94	35.7	0.60
MACE, n (%)	14 (9.6)	13 (18.1)	0.07	6 (13.9)	0.41	7 (24.1)	0.02

Values are presented as mean ± SD, unless otherwise stated.

ACE = angiotensin converting enzyme; ARB = angiotensin receptor blocker; BMI = body mass index; CHDM = clonal hematopoiesis driver mutations; DBP = diastolic blood pressure; eGFR = estimated glomerular filtration rate; HDL-cholesterol = high-density lipoprotein cholesterol; LDL-cholesterol = low density lipoprotein cholesterol; MACE = major adverse cardiovascular events; n = number; PCI = percutaneous coronary intervention; Q1 = 1st quartile; Q3 = 3rd quartile; SBP = systolic blood pressure.

Table 3. The association between proteomic biomarkers and the presence of clonal hematopoiesis driver mutations

PARAMETER	No CHDM (n = 146)	CHDM (n = 72)	p value	VAF<2% (n = 43)	p value	VAF≥2% (n = 29)	p value
MERTK	6.6 (6.3–6.8)	6.7 (6.4–6.9)	0.04	6.8 (6.5–7.0)	0.008	6.6 (6.3–6.8)	0.86
REN	6.9 (6.2–7.5)	7.2 (6.9–7.8)	0.005	7.3 (6.9–7.8)	0.003	7.1 (6.5–7.9)	0.21
AMBP	7.9 (7.8–8.0)	8.0 (7.8–8.1)	0.02	8.0 (7.8–8.1)	0.11	8.0 (7.8–8.2)	0.06
TF	6.1 (5.8–6.3)	6.2 (6.0–6.3)	0.02	6.1 (6.0–6.3)	0.06	6.2 (5.9–6.4)	0.10
LEP	6.7 (6.0–7.2)	7.0 (6.2–7.8)	0.004	7.0 (6.5–7.7)	0.01	7.0 (6.0–8.1)	0.07
TM	9.8 (9.6–10.1)	9.9 (9.7–10.1)	0.02	9.9 (9.7–10.2)	0.07	9.9 (9.7–10.1)	0.11
IL-18	8.9 (8.6–9.3)	9.1 (8.8–9.5)	0.01	9.1 (8.7–9.4)	0.19	9.2 (9.0–9.5)	0.007
IL-12B	6.0 (5.5–6.4)	6.2 (5.6–6.5)	0.16	5.9 (5.6–6.3)	0.85	6.3 (5.8–7.0)	0.02
PAR-1	8.8 (8.6–9.2)	9.0 (8.8–9.4)	0.03	9.0 (8.7–9.3)	0.28	9.1 (8.8–9.6)	0.02

Values are presented as median and interquartile range (Q1 – Q3).

All comparisons are made to the group without CHDM.

AMBP = protein AMBP; IL = interleukin; CHDM = clonal hematopoiesis driver mutations; LEP = leptin; MERTK = tyrosine-protein kinase Mer; PAR-1 = proteinase-activated receptor 1; Q1 = 1st quartile; Q3 = 3rd quartile; REN = renin; TF = tissue factor; TM = thrombomodulin; VAF – variant allele frequency.

occurred in a much larger population of cells than the population harboring the specific mutation. A potential explanation for this finding is that cells with CHDM indirectly modulate the function of non-CHDM-containing immune cells, either in bone marrow or in circulation. In addition, in individuals from the UK Biobank, genetically reduced IL-6 signaling abrogated the increased cardiovascular risk associated with CHIP, suggesting that increased IL-6 signaling mediates this increased risk.[22] While this finding was recently validated by the same group of authors in a larger sample of patients,[23] another study using the same whole exome sequencing data from the UK Biobank did not confirm the role of IL-6 inhibition in reducing the risk of CVD among CHIP carriers.[8] These discrepancies were attributed to the different filtering strategies applied for CHIP in the two studies,[23] the discordant results underlining the challenges of using whole exome sequencing data to identify true CH from artifacts in large population biobanks.[23] Importantly, in addition to experimental studies showing direct effects of experimental CH on atherosclerosis, there are also indications for reversed causality.[24] Heyde et al. used mathematical modeling and experimental studies in mice to illustrate that the presence of atherosclerosis itself accelerates CH by increased proliferation rates of bone marrow progenitor cells.[24] Exacerbated expansion of progenitor cells with somatic CHDM also occurs in the context of obesity-induced inflammation in mice.[25] Similarly, a large prospective cohort study in obese individuals showed that clone sizes increased with age in individuals with obesity, but not in those who underwent bariatric surgery.[26] This suggests that CH and atherosclerosis can reinforce each other in a vicious cycle.[27]

We and others have shown that isolated PBMCs,[28,29] and their bone marrow progenitors[30] from patients with established CAD are characterized by an increased cytokine production capacity. We hypothesized that in our cohort, PBMCs from patients with CHDM or with CHIP are characterized by a higher cytokine production capacity. Although point estimates of all pro-inflammatory cytokines were higher in patients with CHIP, mainly for IL-6 and IL-1β, this did not reach statistical significance. This suggests that in our cohort the adverse cardiovascular effect of CHDMs is not due to increased overall PBMC cytokine production. These findings do not exclude, however, that there is heterogeneity in cytokine production capacity of specific immune cell types (such as the monocytes), with a higher capacity in those cells containing the CHDM, which is not reflected in a higher overall PBMC cytokine production. In addition, our results do not exclude increased cytokine production capacity of monocytes with a *TET2* mutation since we only identified few *TET2* CHDMs. Future studies using single cell sequencing and stimulation assays of purified cell populations are necessary to explore this in more detail.

In addition, we showed that the concentrations of several circulating proteins were higher in patients with CHDM, three of these (TF, MMP-7, and PAR-1) being predictive of MACE. TF is a potent initiator of the coagulation cascade and is present in blood following plaque rupture.[31] During the early stages of atherosclerosis, cytokines induce the expression of TF in monocytes, while at later stages, TF is also detected in macrophages, endothelial cells, smooth muscle cells, as well as in the necrotic core of plaques.[32,33] TF is also found in the circulating monocytes of patients with acute myocardial infarction,[33] and it was associated with an adverse prognosis in patients with unstable angina.[34] Analysis of previously published scRNA-seq data from patients with *DNMT3A* CHDMs and control subjects revealed increased TF expression in classical monocytes,[21] suggesting that increased monocyte TF production could contribute to the higher circulating TF concentration in these patients. PAR family of proteins are expressed on platelets and contribute to inflammatory signaling by activation and cleavage through thrombin,[35] leading to increased circulating PAR-1 levels. Previous studies showed an increased expression of PAR-1 in the hearts of patients with ischemic and idiopathic dilated cardiomyopathy,[36] while animal studies have shown that its deficiency was associated with a reduced mRNA expression of proinflammatory and profibrotic markers.[37] PAR-1 is also expressed on monocytes and its expression increases after LPS stimulation.[38] In *ApoE−/−* mice, drugs targeting PAR-1 signaling attenuate atherosclerosis formation.[39] Therefore, a possible explanation for the development of acute ischemic events in patients with CHDM could be related to the occurrence of vulnerable plaques, or to rapidly progressive atherosclerosis on the background of chronic systemic inflammation. Finally, MMP-7 concentration was higher in patients with CHDM and was associated with an increased risk of ischemic events and cardiovascular mortality. Breakdown of the extracellular matrix of atherosclerotic plaques is considered an important event that triggers plaque destabilization, and circulating MMP-7 concentrations have been associated with cardiovascular event rate in patients with carotid stenosis.[40] In carotid plaques, MMP-7 was localized to macrophages,

and in primary monocytes *in vitro* MMP-7 expression could be increased by stimulation with TNF, in the presence of hypoxia and oxLDL.[41] The higher concentration of circulating TF, PAR-1, and MMP-7 in patients with CHDM and stable CAD highlights the potential role of these proteins as biomarkers or possible therapeutic targets. Future studies are warranted to confirm these findings and show causality. Another interesting observation is the higher systolic blood pressure in patients with CHDM, that was also previously reported.[14] The higher plasma renin concentration in these patients suggests involvement of the renin-angiotensin-aldosterone system, but additional studies are warranted to further explore the mechanisms and origin of TF, PAR-1, MMP-7, and renin upregulation in the context of CHDMs.

In our study, guideline-based secondary prevention measures were implemented. Two-thirds of the participants had a history of elective myocardial revascularization, and more than 90% of them were on statin therapy. This comes to emphasize the role of immune cells in the residual risk in patients with stable CAD. Validation of these results in larger, prospective studies is needed to establish their value for the clinical practice.

In conclusion, we showed that smMIP-based detection of a limited set of CHDMs in patients with symptomatic stable CAD is a strong biomarker associated with an increased rate of a first ischemic event and cardiovascular mortality at follow-up. This could not be explained by overall differences in monocyte subsets or activation or overall PBMC cytokine production capacity. Mutation carriers had increased circulating concentrations of TF, MMP-7, and PAR-1 through a mechanism that needs further exploration. We provide evidence on the role of CH as a residual cardiovascular risk factor and identify potential biomarkers and therapeutic targets for patients with stable CAD.

Limitations of the study

The main limitation of our study is the relatively small sample size, which led to a limited statistical power when stratifying the cohort according to the size of the clones Similarly, the analysis restricted to the CHDMs in *DNMT3A* should be considered exploratory because of the smaller sample size. However, the selection of a well-defined, homogeneous cohort of patients with symptomatic stable CAD, angiographically documented coronary atherosclerosis, and without a history of acute cardiovascular events allowed us to obtain consistent results. In addition, the identification of CHDMs with a highly sensitive method allowed for the detection of relatively small clone sizes, and a higher sensitivity to detect known CHDMs/CHIP mutations compared to standard approaches including exome sequencing. A disadvantage of our targeted sequencing approach was that we only detected a part of the CHDMs in genes other than *DNMT3A*. When we initially designed our smMIPs, we specifically targeted loci containing the majority of CHDMs known at that time.[15] A comparison however of these loci with all currently reported CHDMs in the UK Biobank shows that this approach identifies less than 50% of all CHDMs in non-DNMT3A genes such as *TET2* and *ASXL1*. Therefore, our data preclude any strong conclusions with regard to specific non-*DNMT3A* CHDMs, and our conclusions are only robust for *DNMT3A* CHDMs, which are the most abundant CHDMs and which have recently been unequivocally demonstrated to cause atherosclerosis in experimental models.[42] Nonetheless, to eliminate false positives, we performed an independent identification of our candidate CHDMs in other publicly available datasets. This ensured the validity of candidate CHDMs identified in this study. Future studies using targeting smMIP sequencing should expand the loci to include at least the entire *TET2* gene.

Our study included patients with established CAD. Since we did not include a control group of healthy individuals, we cannot estimate whether the CHDMs prevalence in our group is higher. Comparisons with existing literature is troublesome because of differences in sequencing strategies. We recently used the similar smMIP sequencing approach to detect CHDMs in an otherwise healthy group of individuals with a BMI >27 kg/m^2.[43] The average age was 67 years. We detected a CHDM in 29% of all individuals, and CHIP in 11%. This is comparable to the frequencies detected in our current cohort. However, differences in BMI and geographical region (Romania vs. the Netherlands) preclude any firm conclusions about this comparison.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - Blood sampling
  - Identification of candidate clonal hematopoiesis driver mutations (CHDMs)
  - Peripheral blood mononuclear cells isolation and stimulation
  - Flow cytometry
  - Proteome analysis by proximity extension assays technology
  - Follow-up
  - Analysis of publicly available scRNAseq data
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109472.

ACKNOWLEDGMENTS

This work was supported by grants from.

HORIZON 2020 European Research Program – "REPROGRAM: Targeting epigenetic REPROGRamming of innate immune cells in Atherosclerosis Management and other chronic inflammatory diseases" (grant agreement no. 667837) to A.I., L.A.B.J., M.G.N., and N.P.R.

European Research Council (ERC) Advanced Grant (#833247) and a Spinoza grant of the Netherlands Organisation for Scientific Research to M.G.N.

CVON grant from the Dutch Cardiovascular Alliance (DCVA) and Dutch Heart Foundation (CVON2018-27, IN CONTROL II) to M.G.N., L.A.B.J., and N.P.R.

European Research Area – Cardiovascular Disease (ERA-CVD) Joint Transnational Call 2018 supported by the Dutch Heart Foundation (JTC2018, project MEMORY, 2018T093) to N.P.R. Competitiveness Operational Program grant of the Romanian Ministry of European Funds (HINT, P_37_762, MySMIS 103587) to L.A.B.J.

The Dutch Heart Foundation (2018T028) to S.B.

AUTHOR CONTRIBUTIONS

M.I.D. and H.T. contributed to the conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing of the original draft, reviewing and editing, visualization, supervision, project administration.

A.B.T., S.B., L.A.B.J., M.G.N., R.C.V.D., and A.H. contributed to the methodology, validation, formal analysis, resources, data curation, writing, reviewing, and editing.

N.P.R. and A.C.I contributed to the conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing of the original draft, reviewing, and editing, visualization, supervision, project administration, and funding acquisition.

M.I.D., H.T., N.P.R., and A.C.I. have directly accessed and verified the underlying data reported in the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: November 4, 2023
Revised: January 23, 2024
Accepted: February 29, 2024
Published: March 11, 2024

REFERENCES

1. Libby, P., Sidlow, R., Lin, A.E., Gupta, D., Jones, L.W., Moslehi, J., Zeiher, A., Jaiswal, S., Schulz, C., Blankstein, R., et al. (2019). Clonal Hematopoiesis: Crossroads of Aging, Cardiovascular Disease, and Cancer: JACC Review Topic of the Week. J. Am. Coll. Cardiol. *74*, 567–577. https://doi.org/10.1016/j.jacc.2019.06.007.
2. Jaiswal, S., Natarajan, P., Silver, A.J., Gibson, C.J., Bick, A.G., Shvartz, E., McConkey, M., Gupta, N., Gabriel, S., Ardissino, D., et al. (2017). Clonal Hematopoiesis and Risk of Atherosclerotic Cardiovascular Disease. N. Engl. J. Med. *377*, 111–121. https://doi.org/10.1056/NEJMoa1701719.
3. Challen, G.A., Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J.S., Bock, C., Vasanthakumar, A., Gu, H., Xi, Y., et al. (2011). DNMT3A is essential for hematopoietic stem cell differentiation. Nat. Genet. *44*, 23–31. https://doi.org/10.1038/ng.1009.
4. Moran-Crusio, K., Reavie, L., Shih, A., Abdel-Wahab, O., Ndiaye-Lobry, D., Lobry, C., Figueroa, M.E., Vasanthakumar, A., Patel, J., Zhao, X., et al. (2011). TET2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. Cancer Cell *20*, 11–24. https://doi.org/10.1016/j.ccr.2011.06.001.
5. Zink, F., Stacey, S.N., Norddahl, G.L., Frigge, M.L., Magnusson, O.T., Jonsdottir, I., Thorgeirsson, T.E., Sigurdsson, A., Gudjonsson, S.A., Gudmundsson, J., et al. (2017). Clonal hematopoiesis, with and without candidate driver mutations, is common in the elderly. Blood *130*, 742–752. https://doi.org/10.1182/blood-2017-02-769869.
6. Steensma, D.P., Bejar, R., Jaiswal, S., Lindsley, R.C., Sekeres, M.A., Hasserjian, R.P., and Ebert, B.L. (2015). Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood *126*, 9–16. https://doi.org/10.1182/blood-2015-03-631747.
7. Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P.V., Mar, B.G., Lindsley, R.C., Mermel, C.H., Burtt, N., Chavez, A., et al. (2014). Age-related clonal hematopoiesis associated with adverse outcomes. N. Engl. J. Med. *371*, 2488–2498. https://doi.org/10.1056/NEJMoa1408617.
8. Kessler, M.D., Damask, A., O'Keeffe, S., Banerjee, N., Li, D., Watanabe, K., Marketta, A., Van Meter, M., Semrau, S., Horowitz, J., et al. (2022). Common and rare variant associations with clonal haematopoiesis phenotypes. Nature *612*, 301–309. https://doi.org/10.1038/s41586-022-05448-9.
9. Zekavat, S.M., Viana-Huete, V., Matesanz, N., Jorshery, S.D., Zuriaga, M.A., Uddin, M.M., Trinder, M., Paruchuri, K., Zorita, V., Ferrer-Pérez, A., et al. (2023). TP53-mediated clonal hematopoiesis confers increased risk for incident atherosclerotic disease. Nat. Cardiovasc. Res. *2*, 144–158. https://doi.org/10.1038/s44161-022-00206-6.
10. Gumuser, E.D., Schuermans, A., Cho, S.M.J., Sporn, Z.A., Uddin, M.M., Paruchuri, K., Nakao, T., Yu, Z., Haidermota, S., Hornsby, W., et al. (2023). Clonal Hematopoiesis of Indeterminate Potential Predicts Adverse Outcomes in Patients With Atherosclerotic Cardiovascular Disease. J. Am. Coll. Cardiol. *81*, 1996–2009. https://doi.org/10.1016/j.jacc.2023.03.401.
11. Wang, S., Hu, S., Luo, X., Bao, X., Li, J., Liu, M., Lv, Y., Zhao, C., Zeng, M., Chen, X., et al. (2022). Prevalence and prognostic significance of DNMT3A- and TET2-clonal haematopoiesis-driver mutations in patients presenting with ST-segment elevation myocardial infarction. EBioMedicine *78*, 103964. https://doi.org/10.1016/j.ebiom.2022.103964.

12. Böhme, M., Desch, S., Rosolowski, M., Scholz, M., Krohn, K., Büttner, P., Cross, M., Kirchberg, J., Rommel, K.P., Pöss, J., et al. (2022). Impact of Clonal Hematopoiesis in Patients With Cardiogenic Shock Complicating Acute Myocardial Infarction. J. Am. Coll. Cardiol. *80*, 1545–1556. https://doi.org/10.1016/j.jacc.2022.08.740.
13. Arends, C.M., Liman, T.G., Strzelecka, P.M., Kufner, A., Löwe, P., Huo, S., Stein, C.M., Piper, S.K., Tilgner, M., Sperber, P.S., et al. (2023). Associations of clonal hematopoiesis with recurrent vascular events and death in patients with incident ischemic stroke. Blood *141*, 787–799. https://doi.org/10.1182/blood.2022017661.
14. Assmus, B., Cremer, S., Kirschbaum, K., Culmann, D., Kiefer, K., Dorsheimer, L., Rasper, T., Abou-El-Ardat, K., Herrmann, E., Berkowitsch, A., et al. (2021). Clonal haematopoiesis in chronic ischaemic heart failure: prognostic role of clone size for DNMT3A- and TET2-driver gene mutations. Eur. Heart J. *42*, 257–265. https://doi.org/10.1093/eurheartj/ehaa845.
15. Acuna-Hidalgo, R., Sengul, H., Steehouwer, M., van de Vorst, M., Vermeulen, S.H., Kiemeney, L.A.L.M., Veltman, J.A., Gilissen, C., and Hoischen, A. (2017). Ultra-sensitive Sequencing Identifies High Prevalence of Clonal Hematopoiesis-Associated Mutations throughout Adult Life. Am. J. Hum. Genet. *101*, 50–64. https://doi.org/10.1016/j.ajhg.2017.05.013.
16. Dorsheimer, L., Assmus, B., Rasper, T., Ortmann, C.A., Ecke, A., Abou-El-Ardat, K., Schmid, T., Brüne, B., Wagner, S., Serve, H., et al. (2019). Association of Mutations Contributing to Clonal Hematopoiesis With Prognosis in Chronic Ischemic Heart Failure. JAMA Cardiol. *4*, 25–33. https://doi.org/10.1001/jamacardio.2018.3965.
17. Pascual-Figal, D.A., Bayes-Genis, A., Díez-Díez, M., Hernández-Vicente, Á., Vázquez-Andrés, D., de la Barrera, J., Vazquez, E., Quintas, A., Zuriaga, M.A., Asensio-López, M.C., et al. (2021). Clonal Hematopoiesis and Risk of Progression of Heart Failure With Reduced Left Ventricular Ejection Fraction. J. Am. Coll. Cardiol. *77*, 1747–1759. https://doi.org/10.1016/j.jacc.2021.02.028.
18. Mas-Peiro, S., Hoffmann, J., Fichtlscherer, S., Dorsheimer, L., Rieger, M.A., Dimmeler, S., Vasa-Nicotera, M., and Zeiher, A.M. (2020). Clonal haematopoiesis in patients with degenerative aortic valve stenosis undergoing transcatheter aortic valve implantation. Eur. Heart J. *41*, 933–939. https://doi.org/10.1093/eurheartj/ehz591.
19. Fuster, J.J., MacLauchlan, S., Zuriaga, M.A., Polackal, M.N., Ostriker, A.C., Chakraborty, R., Wu, C.L., Sano, S., Muralidharan, S., Rius, C., et al. (2017). Clonal hematopoiesis associated with TET2 deficiency accelerates atherosclerosis development in mice. Science *355*, 842–847. https://doi.org/10.1126/science.aag1381.
20. Sano, S., Oshima, K., Wang, Y., Katanasaka, Y., Sano, M., and Walsh, K. (2018). CRISPR-Mediated Gene Editing to Assess the Roles of Tet2 and Dnmt3a in Clonal Hematopoiesis and Cardiovascular Disease. Circ. Res. *123*, 335–341. https://doi.org/10.1161/CIRCRESAHA.118.313225.
21. Abplanalp, W.T., Cremer, S., John, D., Hoffmann, J., Schuhmacher, B., Merten, M., Rieger, M.A., Vasa-Nicotera, M., Zeiher, A.M., and Dimmeler, S. (2021). Clonal Hematopoiesis-Driver DNMT3A Mutations Alter Immune Cells in Heart Failure. Circ. Res. *128*, 216–228. https://doi.org/10.1161/CIRCRESAHA.120.317104.
22. Bick, A.G., Pirruccello, J.P., Griffin, G.K., Gupta, N., Gabriel, S., Saleheen, D., Libby, P., Kathiresan, S., and Natarajan, P. (2020). Genetic Interleukin 6 Signaling Deficiency Attenuates Cardiovascular Risk in Clonal Hematopoiesis. Circulation *141*, 124–131. https://doi.org/10.1161/CIRCULATIONAHA.119.044362.
23. Vlasschaert, C., Heimlich, J.B., Rauh, M.J., Natarajan, P., and Bick, A.G. (2023). Interleukin-6 Receptor Polymorphism Attenuates Clonal Hematopoiesis-Mediated Coronary Artery Disease Risk Among 451 180 Individuals in the UK Biobank. Circulation *147*, 358–360. https://doi.org/10.1161/CIRCULATIONAHA.122.062126.
24. Heyde, A., Rohde, D., McAlpine, C.S., Zhang, S., Hoyer, F.F., Gerold, J.M., Cheek, D., Iwamoto, Y., Schloss, M.J., Vandoorne, K., et al. (2021). Increased stem cell proliferation in atherosclerosis accelerates clonal hematopoiesis. Cell *184*, 1348–1361.e22. https://doi.org/10.1016/j.cell.2021.01.049.
25. Pasupuleti, S.K., Ramdas, B., Burns, S.S., Palam, L.R., Kanumuri, R., Kumar, R., Pandhiri, T.R., Dave, U.P., Yellapu, N.K., Zhou, X., et al. (2023). Obesity induced inflammation exacerbates clonal hematopoiesis. J. Clin. Invest. *133*, e163968. https://doi.org/10.1172/JCI163968.
26. Andersson-Assarsson, J.C., van Deuren, R.C., Kristensson, F.M., Steehouwer, M., Sjöholm, K., Svensson, P.A., Pieterse, M., Gilissen, C., Taube, M., Jacobson, P., et al. (2023). Evolution of age-related mutation-driven clonal hematopoiesis over 20 years is associated with metabolic dysfunction in obesity. EBioMedicine *92*, 104621. https://doi.org/10.1016/j.ebiom.2023.104621.
27. Stein, A., Metzeler, K., Kubasch, A.S., Rommel, K.P., Desch, S., Buettner, P., Rosolowski, M., Cross, M., Platzbecker, U., and Thiele, H. (2022). Clonal hematopoiesis and cardiovascular disease: deciphering interconnections. Basic Res. Cardiol. *117*, 55. https://doi.org/10.1007/s00395-022-00969-w.
28. Bekkering, S., van den Munckhof, I., Nielen, T., Lamfers, E., Dinarello, C., Rutten, J., de Graaf, J., Joosten, L.A.B., Netea, M.G., Gomes, M.E.R., and Riksen, N.P. (2016). Innate immune cell activation and epigenetic remodeling in symptomatic and asymptomatic atherosclerosis in humans *in vivo*. Atherosclerosis *254*, 228–236. https://doi.org/10.1016/j.atherosclerosis.2016.10.019.
29. Shirai, T., Nazarewicz, R.R., Wallis, B.B., Yanes, R.E., Watanabe, R., Hilhorst, M., Tian, L., Harrison, D.G., Giacomini, J.C., Assimes, T.L., et al. (2016). The glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease. J. Exp. Med. *213*, 337–354. https://doi.org/10.1084/jem.20150900.
30. Noz, M.P., Bekkering, S., Groh, L., Nielen, T.M., Lamfers, E.J., Schlitzer, A., El Messaoudi, S., van Royen, N., Huys, E.H., Preijers, F.W., et al. (2020). Reprogramming of bone marrow myeloid progenitor cells in patients with severe coronary artery disease. Elife *9*, e60939. https://doi.org/10.7554/eLife.60939.
31. Moons, A.H.M., Levi, M., and Peters, R.J.G. (2002). Tissue factor and coronary artery disease. Cardiovasc. Res. *53*, 313–325. https://doi.org/10.1016/s0008-6363(01)00452-7.
32. Steffel, J., Lüscher, T.F., and Tanner, F.C. (2006). Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications. Circulation *113*, 722–731. https://doi.org/10.1161/CIRCULATIONAHA.105.567297.
33. Suefuji, H., Ogawa, H., Yasue, H., Kaikita, K., Soejima, H., Motoyama, T., Mizuno, Y., Oshima, S., Saito, T., Tsuji, I., et al. (1997). Increased plasma tissue factor levels in acute myocardial infarction. Am. Heart J. *134*, 253–259. https://doi.org/10.1016/s0002-8703(97)70132-7.
34. Soejima, H., Ogawa, H., Yasue, H., Kaikita, K., Nishiyama, K., Misumi, K., Takazoe, K., Miyao, Y., Yoshimura, M., Kugiyama, K., et al. (1999). Heightened tissue factor associated with tissue factor pathway inhibitor and prognosis in patients with unstable angina. Circulation *99*, 2908–2913. https://doi.org/10.1161/01.cir.99.22.2908.
35. Lordan, R., Tsoupras, A., and Zabetakis, I. (2021). Platelet activation and prothrombotic mediators at the nexus of inflammation and atherosclerosis: Potential role of antiplatelet agents. Blood Rev. *45*, 100694. https://doi.org/10.1016/j.blre.2020.100694.
36. Moshal, K.S., Tyagi, N., Moss, V., Henderson, B., Steed, M., Ovechkin, A., Aru, G.M., and Tyagi, S.C. (2005). Early induction of matrix metalloproteinase-9 transduces signaling in human heart end stage failure. J. Cell Mol. Med. *9*, 704–713. https://doi.org/10.1111/j.1582-4934.2005.tb00501.x.
37. Antoniak, S., Cardenas, J.C., Buczek, L.J., Church, F.C., Mackman, N., and Pawlinski, R. (2017). Protease-Activated Receptor 1 Contributes to Angiotensin II-Induced Cardiovascular Remodeling and Inflammation. Cardiology *136*, 258–268. https://doi.org/10.1159/000452269.
38. Thaler, B., Hohensinner, P.J., Baumgartner, J., Haider, P., Krychtiuk, K.A., Schörgenhofer, C., Jilma, B., Hell, L., Fischer, M.B., Huber, K., et al. (2019). Protease-Activated Receptors 1 and 3 are Differentially Expressed on Human Monocyte Subsets and are Upregulated by Lipopolysaccharide Ex Vivo and *In Vivo*. Thromb. Haemostasis *119*, 1394–1402. https://doi.org/10.1055/s-0039-1692219.
39. Rana, R., Huang, T., Koukos, G., Fletcher, E.K., Turner, S.E., Shearer, A., Gurbel, P.A., Rade, J.J., Kimmelstiel, C.D., Bliden, K.P., et al. (2018). Noncanonical Matrix Metalloprotease 1-Protease-Activated Receptor 1 Signaling Drives Progression of Atherosclerosis. Arterioscler. Thromb. Vasc. Biol. *38*, 1368–1380. https://doi.org/10.1161/ATVBAHA.118.310967.
40. Moreno-Ajona, D., Irimia, P., Rodríguez, J.A., García-Velloso, M.J., López-Fidalgo, J., Fernández-Alonso, L., Grochowitz, L., Muñoz, R., Domínguez, P., Gállego-Culleré, J., and Martínez-Vila, E. (2020). Elevated circulating metalloproteinase 7 predicts recurrent cardiovascular events in patients with carotid stenosis: a prospective cohort study. BMC Cardiovasc. Disord. *20*, 93. https://doi.org/10.1186/s12872-020-01387-3.
41. Abbas, A., Aukrust, P., Russell, D., Krohg-Sørensen, K., Almås, T., Bundgaard, D., Bjerkeli, V., Sagen, E.L., Michelsen, A.E., Dahl, T.B., et al. (2014). Matrix metalloproteinase 7 is associated with symptomatic lesions and adverse events in patients with carotid atherosclerosis. PLoS One *9*, e84935. https://doi.org/10.1371/journal.pone.0084935.

42. Rauch, P.J., Gopakumar, J., Silver, A.J., Nachun, D., Ahmad, H., McConkey, M., Nakao, T., Bosse, M., Rentz, T., Vivanco Gonzalez, N., et al. (2023). Loss-of-function mutations in Dnmt3a and Tet2 lead to accelerated atherosclerosis and concordant macrophage phenotypes. Nat. Cardiovasc. Res. *2*, 805–818. https://doi.org/10.1038/s44161-023-00326-7.

43. Tercan, H., Cossins, B.C., van Deuren, R.C., Rutten, J.H.W., Joosten, L.A.B., Netea, M.G., Hoischen, A., Bekkering, S., and Riksen, N.P. (2024). Association Between Clonal Hematopoiesis Driver Mutations, Immune Cell Function, and the Vasculometabolic Complications of Obesity. J. Am. Heart Assoc. *13*, e031665. https://doi.org/10.1161/JAHA.123.031665.

44. Knuuti, J., Wijns, W., Saraste, A., Capodanno, D., Barbato, E., Funck-Brentano, C., Prescott, E., Storey, R.F., Deaton, C., Cuisset, T., et al. (2020). ESC Guidelines for the diagnosis and management of chronic coronary syndromes. Eur. Heart J. *41*, 407–477. https://doi.org/10.1093/eurheartj/ehz425.

45. Toth, G.G., De Bruyne, B., Kala, P., Ribichini, F.L., Casselman, F., Ramos, R., Piroth, Z., Fournier, S., Piccoli, A., Van Mieghem, C., et al. (2019). Graft patency after FFR-guided versus angiography-guided coronary artery bypass grafting: the GRAFFITI trial. EuroIntervention *15*, e999–e1005. https://doi.org/10.4244/EIJ-D-19-00463.

46. van der Heijden, W.A., van Deuren, R.C., van de Wijer, L., van den Munckhof, I.C.L., Steehouwer, M., Riksen, N.P., Netea, M.G., de Mast, Q., Vandekerckhove, L., de Voer, R.M., et al. (2022). Clonal Hematopoiesis Is Associated With Low CD4 Nadir and Increased Residual HIV Transcriptional Activity in Virally Suppressed Individuals With HIV. J. Infect. Dis. *225*, 1339–1347. https://doi.org/10.1093/infdis/jiab419.

47. van Deuren, R.C., Andersson-Assarsson, J.C., Kristensson, F.M., Steehouwer, M., Sjöholm, K., Svensson, P.A., Pieterse, M., Gilissen, C., Taube, M., Jacobson, P., et al. (2021). Expansion of mutation-driven haematopoietic clones is associated with insulin resistance and low HDL-cholesterol in individuals with obesity. Preprint at bioRxiv. https://doi.org/10.1101/2021.05.12.443095.

48. van Deuren, R.C. (2021). CHMIP-RsCh-PIPELINE (GitHub). https://github.com/RosanneVanDeuren/CHMIP-RsCh-PIPELINE.

49. Bekkering, S., Blok, B.A., Joosten, L.A.B., Riksen, N.P., van Crevel, R., and Netea, M.G. (2016). *In Vitro* Experimental Model of Trained Innate Immunity in Human Primary Monocytes. Clin. Vaccine Immunol. *23*, 926–933. https://doi.org/10.1128/CVI.00349-16.

50. Weber, C., Shantsila, E., Hristov, M., Caligiuri, G., Guzik, T., Heine, G.H., Hoefer, I.E., Monaco, C., Peter, K., Rainger, E., et al. (2016). Role and analysis of monocyte subsets in cardiovascular disease. Joint consensus document of the European Society of Cardiology (ESC) Working Groups "Atherosclerosis & Vascular Biology" and "Thrombosis". Thromb. Haemostasis *116*, 626–637. https://doi.org/10.1160/TH16-02-0091.

51. Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D.N., Leenen, P.J.M., Liu, Y.J., MacPherson, G., Randolph, G.J., et al. (2010). Nomenclature of monocytes and dendritic cells in blood. Blood *116*, e74–e80. https://doi.org/10.1182/blood-2010-02-258558.

52. Assarsson, E., Lundberg, M., Holmquist, G., Björkesten, J., Thorsen, S.B., Ekman, D., Eriksson, A., Rennel Dickens, E., Ohlsson, S., Edfeldt, G., et al. (2014). Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. PLoS One *9*, e95192. https://doi.org/10.1371/journal.pone.0095192.

53. Dregoesc, M.I., Ţigu, A.B., Bekkering, S., van der Heijden, C.D.C.C., Bolboacă, S.D., Joosten, L.A.B., Visseren, F.L.J., Netea, M.G., Riksen, N.P., and Iancu, A.C. (2022). Relation Between Plasma Proteomics Analysis and Major Adverse Cardiovascular Events in Patients With Stable Coronary Artery Disease. Front. Cardiovasc. Med. *9*, 731325. https://doi.org/10.3389/fcvm.2022.731325.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD14-phycoerythrin, clone REA599	Miltenyi Biotec, Bergisch Gladbach, Germany	Cat#130-110-519; RRID: AB_2655052
CD16-fluorescein isothiocyanate, clone REA423	Miltenyi Biotec, Bergisch Gladbach, Germany	Cat# 130-113-392; RRID: AB_2726428
CD11b-allophycocyanin, clone REA713	Miltenyi Biotec, Bergisch Gladbach, Germany	Cat#: 130-110-554; RRID: AB_2654667,
Biological samples		
Peripheral blood through venous puncture, mean age 64.9 year old males and females	Homo sapiens	Medical ethics committee of "Iuliu Hatieganu" University of Medicine and Pharmacy, approval number 385/2017
Cell wash solution (BD Phosflow™ Perm/Wash Buffer I)	BD, Franklin Lakes, USA	Cat# 557885
Glutamine, 2 mmol/L in RPMI	Gibco, Dublin, Ireland	Cat# 25030081
Gentamicin, 10 mg/ml in RPMI	Merck, Darmstadt, Germany	Cat# 1405-41-0
Pyruvate, 1 mM/L in RPMI	Gibco, Dublin, Ireland	Cat# 11360070
Lipopolysaccharide from *Escherichia coli* Serotype 055:B5, *1 ng/mL*	Sigma-Aldrich, St. Louis, MO, USA	Cat# L2880
Pam3CysK4	EMC Microcollections Tubingen, Germany	Cat# L2000
Heat-killed *Candida albicans* conidia (UC820 strain)	American Type Culture Collection (ATCC), USA	Cat# MYA-3573
Ficoll-Paque	GE Healthcare, Chicago, USA	Cat# 17144003
Roswell Park Memorial Institute (RPMI) 1640	Lonza, Basel, Switzerland	12-115F BE12-115F
Critical commercial assays		
Human Duoset IL-6 ELISA	R&D systems	Cat# DY206; RRID:AB_2814717
Human Duoset TNF ELISA	R&D systems	Cat# DY210; RRID:AB_2848160
Human Duoset IL-1b ELISA	R&D systems	Cat# DY201; RRID:AB_2848158
Human Duoset IL-10 ELISA	R&D systems	Cat# DY217B; RRID:AB_2927688
Human Duoset IL-17	R&D systems	Cat# DY317; RRID:AB_2928042
Human Duoset IL-22	R&D systems	Cat# DY782; RRID:AB_2928043
IFN-γ PeliKine Compact	Sanquin, Amsterdam, the Netherlands	Cat#: M1933; RRID:AB_2935684
Target 96 Inflammation and Cardiovascular II panels Proximity Extension Assay	Olink Proteomics, Uppsala, Sweden	NA
Deposited data		
Gene regions sequenced, CHDM and smMIP probe characteristics	This paper, GitHub	Tables S1, S2, and S3
scRNAseq data	Publicly available dataset from Abplanalp et al.	https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.120.317104
Proteomics data	PeptideAtlas	accession no: PASS01721
Code (CHMIP-RsCh-PIPELINE)	GitHub	https://github.com/RosanneVanDeuren/CHMIP-RsCh-PIPELINE

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FlowJo software v10.8	BD, Franklin Lakes, USA	https://www.flowjo.com/solutions/flowjo/downloads
GraphPad Prism version 9.3.1 for Windows	GraphPad Software, CA, USA	https://www.graphpad.com/features
MedCalc v 20.019	MedCalc Software, Ostend, Belgium	https://www.medcalc.org/
Other		
Inturis	Inturis, Philips Medical Systems, Eindhoven, The Netherlands	https://www.philips.nl/healthcare/middelen/support-documentation/dicom-web-viewing
SYNTAX Score II calculator	Cardialysis, Boston Scientific	http://syntaxscore.org/calculator/syntaxscore/frameset.htm
Roche cobas c501 chemistry analyser	Roche Diagnostics, Basel, Switzerland	Cat# 04745914001
Sysmex-XN 1000 haematology analyser	Sysmex, Hamburg, Germany	NA
EVE automatic cell counter	VWR, West Chester, USA	NA
BD FACS Canto II Flow cytometer	BD, Franklin Lakes, NJ, USA	NA

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Professor Niels P. Riksen (niels.riksen@radboudumc.nl).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data of gene regions sequenced, identified CHDMs and the probes used in this study is available in Tables S1, S2, and S3.
- The proteomics results have been publicly deposited at PeptideAtlas (see key resources table).
- Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.
- This manuscript used previously developed CHMIP-RsCh-PIPELINE pipeline to identify CHDMs. The code used in the pipeline is publicly available on GitHub (https://github.com/RosanneVanDeuren/CHMIP-RsCh-PIPELINE/tree/main).

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This was a prospective, observational, single-centre cohort study. Between May 2017 and September 2018, 1020 consecutive patients with symptomatic stable CAD were screened for inclusion. The study included men and women of Eastern European descent. The inclusion criteria were the detection of inducible myocardial ischemia at the treadmill or imaging stress testing and the presence of at least one coronary atherosclerotic stenosis on angiography. Coronary stenosis evaluation was performed by quantitative coronary angiography (Inturis, Philips Medical Systems, Eindhoven, The Netherlands) by two independent senior interventional cardiologists. All lesions with more than 90% diameter stenosis were considered significant.[44] An intermediate coronary lesion was defined as a luminal narrowing with a diameter stenosis $\geq$30% but $\leq$90%.[45]

The exclusion criteria consisted of any documented history of acute cardiovascular events.

The research protocol was approved by the Institution's Ethics Committee on research on humans (approval number 385/2017), and all patients gave written informed consent. All the procedures followed were in accordance with institutional guidelines. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

METHOD DETAILS

The classic cardiovascular risk factors were recorded in all patients.

To evaluate the extent of coronary atherosclerosis, SYNTAX Score I was calculated by a senior interventional cardiologist based on the most recent coronary angiography. SYNTAX Score II was calculated by combining the anatomic and clinical prognosis variables (http://syntaxscore.org/calculator/syntaxscore/frameset.htm).

Blood sampling

Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were measured in fasting plasma using a Roche cobas c501 chemistry analyser. Low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula. Total blood cell counts were determined with an automated Sysmex-XN 1000 haematology analyser (Sysmex, Hamburg, Germany).

Identification of candidate clonal hematopoiesis driver mutations (CHDMs)

CHDMs were identified in DNA obtained from whole blood by ultrasensitive single-molecule molecular inversion probe (smMIP) sequencing.[15,46,47] In brief, a total of 300 MIP probes were designed to cover the majority of well-known CHIP/CH-related hotspots in 24 genes, including *ASXL1*, *TET2*, and *DNMT3A*. The probes were designed in 2017, at the beginning of patient enrolment, based on the existing literature data at that time, and completely cover the *DNMT3A* gene (Supplementary material file – Tables S1, S2, and S3). For each sample two technical Polymerase Chain Reaction (PCR) replicates were sequenced, after which two independent data processing strategies were applied to identify CHDMs. Variants were further filtered by a targeted quality control workflow, in which final variant allele frequencies were calculated using samtools mpileup.[48] The average coverage for all individuals over the entire panel was 2,986x. Identified candidate CHDMs were validated in publicly available data sets. In the case of patients with multiple CHDMs, the CHDM with the highest VAF was included in the analysis.

Individuals with CHDMs were then divided into categories VAF<2% and VAF≥2% based on the arbitrary clinical cut-off for CHIP in accordance with the current literature.

Peripheral blood mononuclear cells isolation and stimulation

Non-fasting blood was collected in EDTA vacutainer tubes. Sample processing occurred within 2 hours. Plasma and serum were stored at -80°C until further use.

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (GE Healthcare, Chicago, USA) density gradient centrifugation for half an hour. PBMCs were washed by centrifugation with cold phosphate saline buffer and concentrated in Roswell Park Memorial Institute-1640 (RPMI) cell culture medium (Lonza, Basel, Switzerland) supplemented with 2 mmol/L glutamine (Gibco, Dublin, Ireland), 1 mmol/L pyruvate (Gibco, Dublin, Ireland) and 50 ug/mL gentamicin (Merck, Darmstadt, Germany).

Cells were counted using an EVE automatic cell counter (VWR, West Chester, USA). At least 20 million PBMCs were stored in freezing medium at -80°C and used for flow cytometry analysis.

To evaluate the cytokine production capacity, $1x10^5$ PBMCs per well were seeded in round-bottom 96-well plates (Eppendorf, Hamburg, Germany), and incubated with supplemented RPMI cell culture medium. Subsequently, cells were stimulated for 24h with 1 ng/mL lipopolysaccharide (LPS) (from Escherichia coli serotype 055:B5 (Sigma-Aldrich, St. Louis, MO, USA)), or 10 μg/mL Pam3Cys (P3C) (L2000, EMC microcollections, Tubingen, Germany).[49] To measure adaptive immune response, PBMCs were stimulated for 7 days in RPMI, with $1x10^6$/mL Heat-killed (HK) Candida albicans conidia (UC820 strain) supplemented with 10% human pooled serum (Sigma Aldrich, Saint Louis, USA).

After the 24h and 7 days incubation period, supernatants were collected after plate centrifugation and stored at -80°C until cytokine measurements with commercial enzyme-linked immunosorbent assay kits (TNF, IL-6, IL-1β, IL-17 and IL-22 (Duoset, R&D Systems, MN, USA); IFN-γ (PeliKine Compact, Sanquin, Amsterdam, the Netherlands)), following the instructions of the manufacturer.

Flow cytometry

Stored PBMCs were first washed with phosphate saline buffer. Cells were then washed two times with cell wash solution (BD, Franklin Lakes, USA) and centrifuged at 500xG at room temperature for 5 minutes. Over the remaining cell pellet, 10 μL of each CD14-phycoerythrin, CD16-fluorescein isothiocyanate and CD11b-allophycocyanin anti-human/mouse antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) were added and mixed with 250 μL of cell wash solution to avoid cell clumping. After a 30 minutes incubation at room temperature, one washing step with cell wash solution was performed and the pellet was re-suspended in 500 μL of cell wash buffer. The samples were analysed using a BD FACS Canto II Flow cytometer (BD, Franklin Lakes, NJ, USA). Monocytes were selected based on FSC/SSC and CD14/CD16 expression and monocyte subsets were identified in the CD14/CD16 plot as percentage of gated according to current guidelines.[50,51] Each subtype or the total monocyte population were then analysed for the presence of CD11b marker (Percentage positive and Median Fluorescent Intensity on positive cells). Data were analysed with FlowJo software v10.8 (Becton Dickinson). Single stains and unstained samples were used for compensation.

Proteome analysis by proximity extension assays technology

All EDTA plasma samples were shipped to Olink Proteomics AB (Uppsala, Sweden) for analysis. Using proximity extension assays (PEA) technology,[52] the levels of 177 inflammatory proteins from the Olink Cardiovascular II and Inflammatory panels were measured (Table S6).[53] Oligonucleotide-labelled antibody probe pairs bind to their targeted protein in each sample. When the two probes are brought in close proximity, the oligonucleotides will hybridize in a pair-wise manner.[53] To adjust for intra- and inter-run variation, data is quality controlled and normalized using an internal extension control and an inter-plate control (https://www.olink.com/resources-support/document-download-center/; accessed August 2020).[53] The final data is presented in Normalized Protein eXpression (NPX) values, which is an arbitrary unit on a log2-scale.[53] A high NPX value corresponds to a higher protein concentration.[53]

Follow-up

Patient follow-up was performed after four years. The endpoint consisted of the first major adverse cardiovascular event (MACE) and was the composite of cardiovascular death, acute myocardial infarction, or acute ischemic stroke. Information regarding outcomes was obtained from interviews with the patients or their relatives, from patient charts and discharge documents, from the hospital's electronic database, or from primary care physician records. Data regarding vital status was available in all patients from the electronic records of the national insurance company.

Analysis of publicly available scRNAseq data

Publicly available single cell RNAsequencing data from patients with heart failure with or without *DNMT3A* driver mutations were used.[21] Data from classical, intermediate, and non-classical monocytes were overlaid with findings from our own proteomics and prediction models to confirm gene expression differences in patients with *DNMT3A* CHDMs.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data distribution was assessed using Kolmogorov–Smirnov and D'Agostino tests. Quantitative continuous data were summarized as mean ± standard deviation (SD) whenever data followed the normal distribution; otherwise, median, and interquartile range (Q1–Q3, where Q1 = first quartile and Q3 = third quartile) were used. Groups were compared with Student t-test or Mann-Whitney U-test, as appropriate. Categorical data were presented as counts and proportions and compared with Chi-square test, or Fisher's exact test, as appropriate.

Univariable Cox proportional-hazards regression was used to evaluate the association between variables of interest and MACE. To evaluate MACE-free survival, patients who suffered a non-cardiovascular death were excluded from the analysis. The hazards ratio (HR), along with 95% confidence intervals (Cis) and p-values, were computed for each regression. For proteomic data, HR per 1 SD increase in each protein levels were computed. Multivariable Cox proportional-hazards regression analysis was used to identify variables independently associated with outcomes. The parameters that were statistically significant in the univariate analysis, namely sex and systolic blood pressure, together with age, were included in the multivariable models.

Receiver-operating characteristic (ROC) curves were constructed to evaluate the accuracy of VAF in predicting MACE. The area under the ROC curves (AUC) was determined as a scalar measure of performance. The Youden index was used to identify the ideal cut-off values from the ROC curves.

Kaplan-Meier curves were constructed for survival analysis. The log rank test was applied for comparison of event-free survival analysis.

Adjustment for multiple comparisons was performed. A Benjamini-Hochberg adjusted significance level was used, at a controlled false discovery rate (FDR) of 0.25 (p-value<0.042).

Statistical analysis was performed with GraphPad Prism version 9.3.1 for Windows (GraphPad Software, CA, USA) and MedCalc (v 20.019, MedCalc Software, Ostend, Belgium). All reported *p*-values are two-sided.