SUPPLEMENTAL MATERIAL

Genetic IL-6 signaling deficiency attenuates cardiovascular disease risk in clonal hematopoiesis

Supplemental Methods

Whole exome sequencing

UK Biobank

Genomic DNA from blood samples for the UKBiobank was sequenced at the Regeneron Genetics Center from the UK Biobank. Briefly, multiplexed DNA libraries were created and IDT's xGen probe library was used for exome target capture. The multiplexed samples were pooled and sequenced using 75 base pair paired-end reads on the Illumina Novaseq 6000 platform using the S2 flow cells. Sample-specific FASTQ files, representing all the reads generated for that sample, were then aligned to the GRCh38 genome reference with BWA-mem2. The resultant binary alignment file (BAM) for each sample contained the mapped reads' genomic coordinates, quality information, and the degree to which a particular read differed from the reference at its mapped location. Aligned reads in the BAM file were then evaluated to identify and flag duplicate reads with the Picard3 MarkDuplicates tool, producing an alignment file with all potential duplicate reads marked for exclusion in downstream analyses. Upon completion of variant calling, individual sample BAM files were converted to fully lossless CRAM files using samtools.

Following completion of sample sequencing, samples showing disagreement between genetically determined and reported sex, high rates of heterozygosity/contamination (D-stat > 0.4), low sequence coverage (less than 85% of targeted bases achieving 20X coverage) or genetically identified sample duplicates, and WES variants discordant with genotyping chip were excluded. The CRAM files were then submitted by the Regeneron Genetics Center to the UK Biobank data repository for distribution.

PROMIS

Exome sequencing was performed at the Broad Institute. Briefly, samples were shipped to the Biological Samples Platform laboratory at the Broad Institute of MIT and Harvard (Cambridge, MA, USA). DNA concentration was determined by PicoGreen (Invitrogen; Carlsbad, CA, USA) prior to storage in 2D-barcoded 0.75 ml Matrix tubes at −20 °C in the SmaRTStore (RTS, Manchester, UK) automated sample handling system. Initial quality control (QC) on all samples involving sample quantification (PicoGreen), confirmation of high-molecular weight DNA and fingerprint genotyping and gender determination (Illumina iSelect; Illumina; San Diego, CA, USA). Samples were excluded if the total mass, concentration, integrity of DNA or quality of preliminary genotyping data was too low. Multiplexed DNA libraries were created with in-solution hybrid selection using capture using either Affymetrix reagents or Illumina's Rapid Capture Exome Kit or Illumina's Nextera Exome kit. For adapter ligation, Illumina paired end adapters were replaced with palindromic forked adapters, purchased from Integrated DNA Technologies, with unique 8 base molecular barcode sequences included in the adapter sequence to facilitate downstream pooling. With the exception of the palindromic forked adapters, the reagents used for end repair, A-base addition, adapter ligation, and library enrichment PCR were purchased from KAPA Biosciences (Wilmington, MA, USA) in 96-reaction kits. All hybridization and capture steps were automated on the Agilent Bravo liquid handling system. Following post-capture enrichment, libraries were quantified using quantitative PCR (KAPA Biosystems) with probes specific to the ends of the adapters. This assay was automated

using Agilent's Bravo liquid handling platform. Based on qPCR quantification, libraries were normalized to 2nM and pooled by equal volume using the Hamilton Starlet. Pools were then denatured using 0.1 N NaOH. Finally, denatured samples were diluted into strip tubes using the Hamilton Starlet. Cluster amplification of denatured templates was performed according to the manufacturer's protocol (Illumina) using HiSeq v3 cluster chemistry and HiSeq 2000 or 2500 flowcells. Flowcells were sequenced on HiSeq 2000 or 2500 using v3 Sequencing-by-Synthesis chemistry, then analyzed using RTA v.1.12.4.2. Each pool of whole exome libraries was run on paired 76bp runs, with and 8 base index sequencing read was performed to read molecular indices, across the number of lanes needed to meet coverage for all libraries in the pool.

Fingerprint concordance between sequence data and fingerprint genotypes was evaluated. Variant calls were evaluated on both bulk and per- sample properties: novel and known variant counts, transition–transversion (TS–TV) ratio, heterozygous–homozygous non-reference ratio, and deletion/insertion ratio. Both bulk and sample metrics were compared to historical values for exome sequencing projects at the Broad Institute. No significant deviation of from historical values was noted.

Variant Identification

Putative somatic SNPs and short indels were called from the CRAM files with GATK (version 4.1.1.0) Mutect2 (https://software.broadinstitute.org/gatk). Briefly, Mutect2 searches for sites where there is evidence for variation, and then performs local reassembly. It uses an external reference of recurrent sequencing artifacts termed a "panel of normal" derived from 100 sequenced samples to filter out these sites, and calls variants at sites where there is evidence for somatic variation. An external reference of germline variants from the gnomad resource (https://gnomad.broadinstitute.org/) was provided to filter out likely germline calls. We deployed this variant calling process on Google Cloud using the Terra platform (www.terra.bio). The cloud workflow scripts are available from download from the github repository (https://github.com/gatk-workflows/gatk4-somatic-snvs-indels) and docker images with configured cloud software are available at the dockstore repository (https://dockstore.org/workflows/github.com/gatk-workflows/gatk4-somatic-snvs-indels/mutect2_nio:2.5.0?tab=info). The caller was run individually for each sample with the same settings.

To identify samples with CHIP, we utilized a previously published pre-specified list of variants in *DNMT3A* and *TET2* known to be recurrent drivers of myeloid malignancies. The variants were selected on the basis of being reported in the literature and/or the Catalog of Somatic Mutations in Cancer (COSMIC, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). We used minimum variant read counts of 3 reads and required evidence of a variant on both forward and reverse reads. Frameshift, nonsense, and splice-site mutations were further excluded if they occurred in the first or last 10% of the gene open reading frame, unless mutations in those regions had been previously reported, (e.g. *DNMT3A*). Frameshift mutations were also excluded if the insertions/deletions occurred in homo-polymer repeats (5 consecutive reads of the same nucleotide). For *TET2*, all missense variants in particular regions were considered somatic if the variant allele fraction (VAF) significantly deviated from the expected distribution for a germline allele (defined as a p-value from a binomial test of less than 0.001 assuming a probability of success in a single Bernoulli experiment of 0.5 and using the alternate allele read count as the

number of successes and the alternate allele read count + reference allele read count as the number of trials).

PROMIS replication analysis

Percent missing was computed for each covariate and missing data patterns were examined using cluster analysis of variables usually missing together. Observed patterns were suggestive of data being missing at random.

Multiple imputation for the missing values was performed using the aregImpute algorithm in the Hmisc package in R (version 3.2). The algorithm accounts for all aspects of uncertainty in the imputations by boot-strapping to approximate the process of drawing predicted values from a full Bayesian predictive distribution. Different bootstrap resamples are used for each of the multiple imputations. A flexible additive model is fit on a sample with replacement from the original data and this model is used to predict all of the original missing and non-missing values for the target variable, then the imputation models are run. In the imputation model, linearity is assumed for target variables (variables being imputed) while continuous predictors on the right-hand side of the model are transformed using restricted cubic splines with 5 knots. The algorithm uses predictive mean matching with weighted probability sampling of donors to fill-in the missing data.

Five imputations were performed, creating 5 complete datasets. The regression model (comprising all covariates included in the imputation model) was fit on each complete dataset and the regression coefficients were averaged over the multiple imputations. The distributions of measured and imputed values were highly comparable and the variance inflation due to the missing variables was modest.

Supplemental Tables

Supplemental Table 1. CHIP Variants queried in DNMT3A and TET2

Gene name/ Accession	Reported mutations used for variant calling
DNMT3A **NM_022552**	Frameshift/nonsense/splice-site, F290I, F290C, V296M, P307S, P307R, R326H, R326L, R326C, R326S, G332R, G332E, V339A, V339M, V339G, L344Q, L344P, R366P, R366H, R366G, A368T, A368V, R379H, R379C, I407T, I407N, I407S, F414L, F414S, F414C, A462V, K468R, C497G, C497Y, Q527H, Q527P, Y533C, S535F, C537G, C537R, G543A, G543S, G543C, L547H, L547P, L547F, M548I, M548K, G550R, W581R, W581G, W581C, R604Q, R604W, R635W, R635Q, S638F, G646V, G646E, L653W, L653F, I655N, V657A, V657M, R659H, Y660C, V665G, V665L, M674V, R676W, R676Q, G685R, G685E, G685A, D686Y, D686G, R688H, G699R, G699S, G699D, P700L, P700S, P700R, P700Q, P700T, P700A, D702N, D702Y, V704M, V704G, I705F, I705T, I705S, I705N, G707D, G707V, C710S, C710Y, S714C, V716D, V716F, V716I, N717S, N717I, P718L, R720H, R720G, K721R, K721T, Y724C, R729Q, R729W, R729G, F731C, F731L, F731Y, F731I, F732del, F732C, F732S, F732L, E733G, E733A, F734L, F734C, Y735C, Y735N, Y735S, R736H, R736C, R736P, L737H, L737V, L737F, L737R, A741V, P742P, P743R, P743L, R749C, R749L, R749H, R749G, F751L, F751C, F752del, F752C, F752L, F752I, F752V, W753G, W753C, W753R, L754P, L754R, L754H, F755S, F755I, F755L, M761I, M761V, G762C, V763I, S770L, S770W, S770P, R771Q, F772I, F772V, L773R, L773V, E774K, E774D, E774G, I780T, D781G, R792H, W795C, W795L, G796D, G796V, N797Y, N797H, N797S, P799S, P799R, P799H, R803S, R803W, P804L, P804S, K826R, S828N, K829R, T835M, N838D, K841Q, Q842E, P849L, D857N, W860R, E863D, F868S, G869S, G869V, M880V, S881R, S881I, R882H, R882P, R882C, R882G, A884P, A884V, Q886R, L889P, L889R, G890D, G890R, G890S, V895M, P896L, V897G, V897D, R899L, R899H, R899C, L901R, L901H, P904L, F909C, P904Q, A910P, C911R, C911Y
TET2 **NM_001127208**	Frameshift/nonsense/splice-site, missense mutations in catalytic domains (p.1104-1481 and 1843-2002)

Supplemental Table 2. Billing codes used to define outcomes

Outcome	ICD Codes	UK Biobank data-fields
Primary outcome (any CVD event)	Any from “MI”, “CAD”, “Stroke”, or “Death” below	Any from “MI”, “CAD”, “Stroke”, or “Death” below
MI		42001 (values: 1,2)
CAD (Revascularization)	K40.1, K40.2, K40.3, K40.4, K41.1, K41.2, K41.3, K41.4, K45.1, K45.2, K45.3, K45.4, K45.5, K49.1, K49.2, K49.8, K49.9, K50.2, K75.1, K75.2, K75.3, K75.4, K75.8, K75.9	42001 (values: 1,2)
Stroke		42007 (values: 1,2)
Death		40020 (any value)
Type 2 Diabetes	E11, E11.0, E11.1, E11.2, E11.3, E11.4, E11.5, E11.6, E11.7, E11.8, E11.9	20002 (values: 1223)
Hypertension	ICD9: 401, 4010, 4011, 4019, 402, 4020, 4021, 4029, 403, 4030, 4031, 4039, 404, 4040, 4041, 4049, 405, 4050, 4051, 4059 ICD10: I10, I11, I11.0, I11.9, I12, I12.0, I12.9, I13, I13.0, I13.1, I13.2, I13.9, I15, I15.0, I15.1, I15.2, I15.8, I15.9	20002 (values: 1065, 1072) 6150 (values: 4)
Myeloproliferative neoplasm	D45, D46.9, D471.1, D47.3	
Myeloid Leukemia	C92, C92.0, C92.1, C92.3, C92.4, C92.5, C92.7, C92.9	
Sepsis	A40.0, A40.1, A40.2, A40.3, A40.8, A40.9, A41.0, A41.1, A41.2, A41.3, A41.4, A41.5, A41.8, A41.9	

CVD = cardiovascular disease, MI = myocardial infarction

Supplemental Table 3. CHIP carrier status and covariates, by VAF group

Trait	CHIP noncarriers	CHIP carriers, VAF <= 0.1	CHIP carriers, VAF > 0.1	P value
N	34337	647	432	
Women	18823 (54.8)	361 (55.8)	238 (55.1)	8.80E-01
Age at exome sequencing	57.1±7.87	60.1±6.88	61.5±6.45	6.44E-49
Age at the start of follow-up	58.8±8.08	61.6±7.01	62.8±6.7	4.74E-39
BMI (kg/m2)	27.3±4.75	27.2±4.62	27.8±4.73	1.22E-01
Height (cm)	169±9.21	169±9.08	169±8.93	4.63E-01
Weight (kg)	78.3±15.9	77.6±15.2	79.2±15.8	2.80E-01
Systolic blood pressure (mmHg)	137±18.2	140±18.5	141±18.6	6.75E-09
Diastolic blood pressure (mmHg)	81.9±9.95	82.6±9.79	82.6±8.99	6.02E-02
Type 2 diabetes mellitus	727 (2.12)	28 (4.33)	7 (1.62)	4.70E-04
Hypertension	9743 (28.4)	207 (32)	147 (34)	4.95E-03
Hypercholesterolemia	5059 (14.7)	105 (16.2)	78 (18.1)	9.08E-02
Standard drinks/week	12.1±10.5	12.2±9.58	12.5±11.8	8.09E-01
Pack years of tobacco	6.07±13.1	7.05±13.6	7.52±14.2	1.35E-02
Currently using tobacco at enrollment	2976 (8.67)	60 (9.27)	53 (12.3)	2.73E-02
Total cholesterol	5.75±1.11	5.78±1.1	5.83±1.21	2.88E-01
LDL cholesterol, direct	3.58±0.82	3.6±0.83	3.64±0.91	3.09E-01
HDL cholesterol	1.49±0.36	1.51±0.35	1.48±0.35	3.08E-01
Triglycerides	1.7±0.95	1.68±0.9	1.74±0.89	6.08E-01
Apolipoprotein A1	1.56±0.27	1.58±0.26	1.57±0.27	3.22E-01
Apolipoprotein B	1.04±0.23	1.04±0.23	1.06±0.26	2.33E-01
Lipoprotein (a)	43.6±49.1	48.5±51.8	40.6±47.7	5.22E-02
Statin-adjusted LDL cholesterol	3.77±0.83	3.83±0.82	3.89±0.88	3.78E-03
High-sensitivity C-reactive protein	2.49±4.26	2.91±5.05	2.75±5.37	2.93E-02
Prescribed a statin at enrollment	4846 (14.1)	112 (17.3)	81 (18.8)	1.79E-03
Prescribed a blood-pressure lowering medication at enrollment	6184 (18)	138 (21.3)	98 (22.7)	4.43E-03
LDL cholesterol polygenic score	-0.01±1	0.04±1.01	0±1.05	3.83E-01

LDL cholesterol = low-density lipoprotein cholesterol. CHIP carriers are divided into variant allele fraction (VAF) subgroups: those with VAF less than or equal to 10%, versus those with VAF greater than 10%. P-values calculated using the F-test from an ANOVA model.

Supplemental Table 4. Complete blood cell and differential indices in UK Biobank by CHIP carrier status

Trait	No CHIP	CHIP	P value
White blood cell count (x10_3 cells/mm_3)	6.97±2.1	7.05±1.87	0.395
Hemoglobin concentration (g/dL)	14.3±1.23	14.4±1.15	0.010
Mean corpuscular volume (µm_3)	92±4.23	91.9±4.44	0.620
Red blood cell distribution width (%)	13.5±0.94	13.6±0.91	0.021
Platelet count (x10_3 cells/mm_3)	244±57.1	241±53.2	0.290
Lymphocyte count (x10_3 cells/mm_3)	2±1.25	2±0.66	0.855
Monocyte count (x10_3 cells/mm_3)	0.48±0.23	0.5±0.23	0.204
Neutrophill count (x10_3 cells/mm_3)	4.28±1.39	4.35±1.48	0.324
Eosinophill count (x10_3 cells/mm_3)	0.17±0.13	0.16±0.11	0.004
Basophill count (x10_3 cells/mm_3)	0.04±0.05	0.04±0.06	0.238
Reticulocyte count (%)	0.06±0.03	0.06±0.03	0.156

P-value calculated using a two-sample t-test. To account for multiple-hypothesis testing, statistical significance was assigned at alpha = 0.05 / 11 = 0.0045.

Supplemental Table 5. Association of interaction term (CHIP**IL6R* **p.Asp358Ala) across all of the sub-components of the primary outcome**

	Hazard Ratio	95% CI	P value
MI	0.267	(0.089, 0.804)	0.019
Stroke	1.093	(0.726, 1.657)	0.858
CAD	0.346	(0.145, 0.823)	0.016
Death	0.395	(0.206, 0.758)	0.005

MI, myocardial infarction. CAD, coronary artery disease.

Supplemental Table 6. Association of interaction term for CVD outcomes, stratified by *DNMT3A* vs *TET2*

Outcome	Gene	Hazard Ratio	95% CI	P value
Composite	*DNMT3A*	0.481	(0.275, 0.842)	0.01
Composite	*TET2*	0.498	(0.240, 1.033)	0.06
CAD	*DNMT3A*	0.619	(0.222, 1.727)	0.36
CAD	*TET2*	0.084	(0.011, 0.650)	0.02
Death	*DNMT3A*	0.362	(0.159, 0.821)	0.02
Death	*TET2*	0.451	(0.159, 1.279)	0.13
MI	*DNMT3A*	0.314	(0.086, 1.145)	0.08
MI	*TET2*	0.155	(0.019, 1.298)	0.09
Stroke	*DNMT3A*	1.022	(0.330, 3.160)	0.97
Stroke	*TET2*	1.260	(0.197, 8.071)	0.81

MI, myocardial infarction. CAD, coronary artery disease.

Supplemental Table 7. Consistent incident effects observed when including carriers of *JAK2* or *ASXL1* CHIP.

Exposures	Association with incident CVD events		
	HR	95% CI	P
DNMT3A/*TET2*/*JAK2*/*ASXL1* CHIP	1.26	1.03-1.54	0.023
DNMT3A/*TET2*/*JAK2*/*ASXL1* Large (VAF>10%) CHIP	1.55	1.18-2.03	0.0015
DNMT3A/*TET2*/*JAK2*/*ASXL1* Large (VAF>10%) CHIP x *IL6R* p.Asp358Ala Interaction†	0.47	0.30-0.73	7.9×10^{-4}

†Hazard ratio and p value for the reflect the interaction term. Here, this represents the risk difference conferred by IL6R p.Asp358Ala between *DNMT3A*/*TET2*/*JAK2*/*ASXL1* large CHIP versus all others. CHIP, clonal hematopoiesis of indeterminate potential. CI, confidence interval. CVD, cardiovascular disease. HR, hazard ratio. VAF, variant allele fraction.

Supplemental Table 8. Evaluation of CRP-associated variants near IL-6 signaling pathway genes for CVD risk difference between large CHIP carriers versus all others.

		Association of genetic instrument with CVD event among all individuals			Association of genetic instrument with CVD event for the interaction of large (VAF >10%) CHIP carriers versus all others		
Genetic instrument	**EAF**	**HR**	**95% CI**	**P**	**$HR_{interaction}$**	**$95\%CI_{interaction}$**	**$P_{interaction}$**
IL6 (rs1880241)	0.495	0.98	0.93-1.05	0.69	0.64	0.44-0.95	0.025
IL1RN-IL1F10 (rs6734238)	0.398	1.03	0.97-1.10	0.32	1.14	0.76-1.71	0.51
IL1R1 (rs9284725)	0.763	1.05	0.98-1.14	0.13	0.74	0.47-1.18	0.21

Variants near IL-6 signaling pathway genes previously significantly associated with CRP were identified. The top associated variant is the *IL6R* p.Asp358Ala variant used as the primary genetic proxy; the other variants are listed here. First, incident CVD effects were estimated for each variant across all individuals.

We then evaluated whether incident CVD risk was different for large CHIP carriers compared to all others. Hazard ratios and p values represent the relative effect difference between large CHIP carriers versus all others.

CHIP, clonal hematopoiesis of indeterminate potential. CVD, cardiovascular disease. EAF, effect allele frequency. HR, hazard ratio. VAF, variant allele fraction.

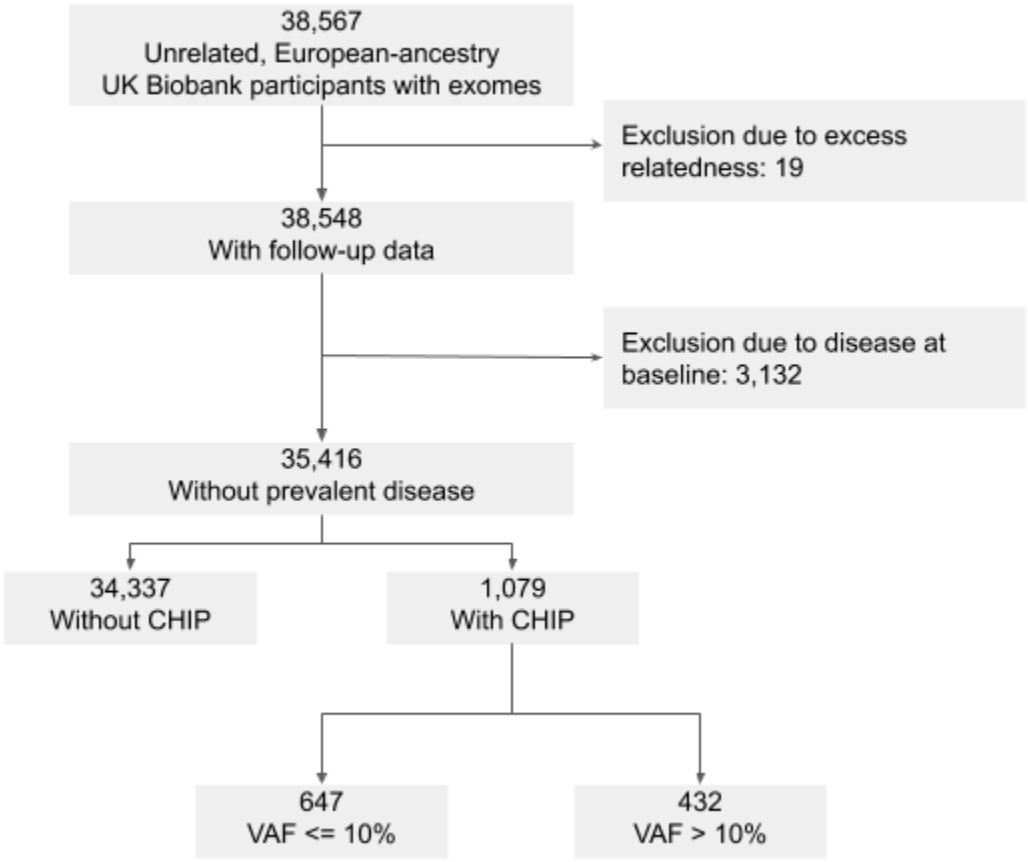
Supplemental Table 9. Baseline characteristics of PROMIS participants.

Trait	No CHIP	Large CHIP	P value
N	16,234	99	
Female	3,139 (19.3)	18 (18.2)	0.871
Myocardial infarction	7,981 (49.2)	63 (63.6)	0.006
Age (years)	60.00 (6.57)	62.37 (6.38)	<0.001
LDL cholesterol (mg/dl)	120.70 (48.45)	119.91 (43.57)	0.881
Triglycerides (mg/dl)	190.57 (114.53)	167.85 (97.90)	0.066
Diabetes mellitus, type 2	5,094 (32.5)	32 (33.3)	0.95
Body-mass index (kg/m$_2$)	25.80 (4.36)	25.87 (4.59)	0.905
SBP (mmHg)	127.74 (18.23)	125.71 (18.38)	0.285
Current smoker	5,622 (36.1)	34 (35.8)	1
Former smoker	1,313 (8.4)	10 (10.5)	0.581
Hypertension	5,506 (35.5)	32 (33.7)	0.795
***IL6R* p.Asp358Ala genotype**			0.646
Asp/Asp	7,766 (47.9)	49 (50.0)	
Asp/Ala	6,827 (42.1)	42 (42.9)	
Ala/Ala	1,613 (10.0)	7 (7.1)	

Categorical data are expressed as N (%). Continuous data are expressed as mean (standard deviation). CHIP = clonal hematopoiesis of indeterminate potential; LDL = low-density lipoprotein; SBP = systolic blood pressure. P-values calculated with two-sample t-test for continuous traits or chi-square test for categorical values.

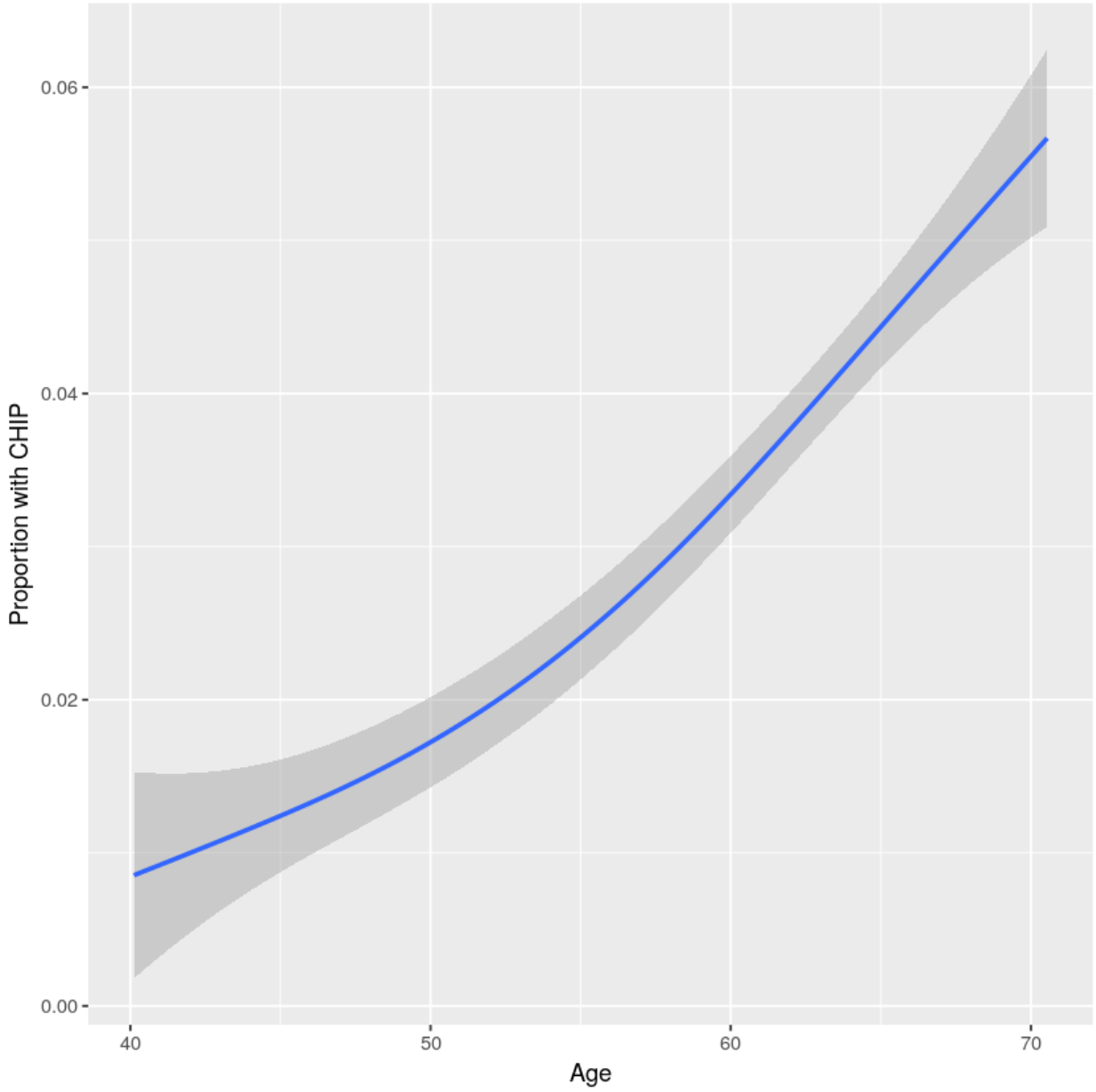
Supplemental Figures

Supplemental Figure 1. Cohort selection flow diagram



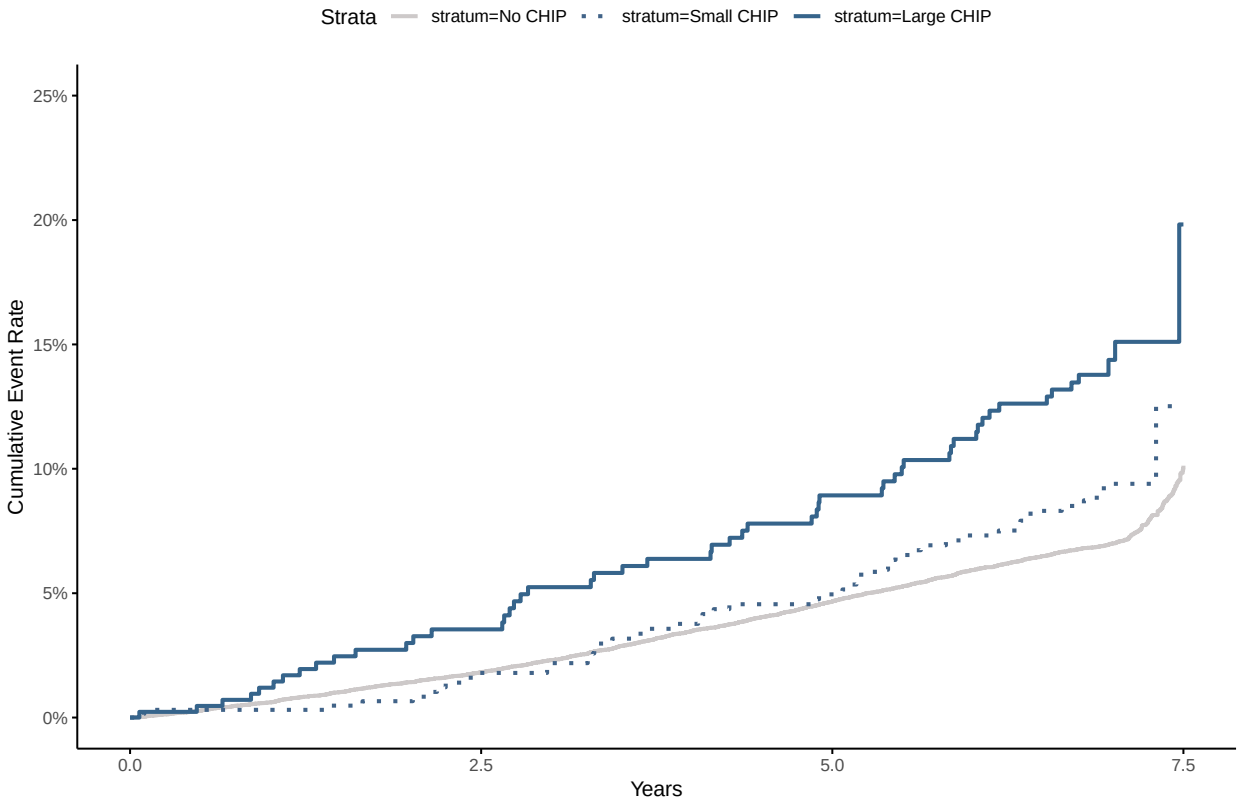
CHIP = clonal hematopoiesis of indeterminate potential. VAF = variant allele fraction.

Supplemental Figure 2. CHIP prevalence vs Age in UK Biobank



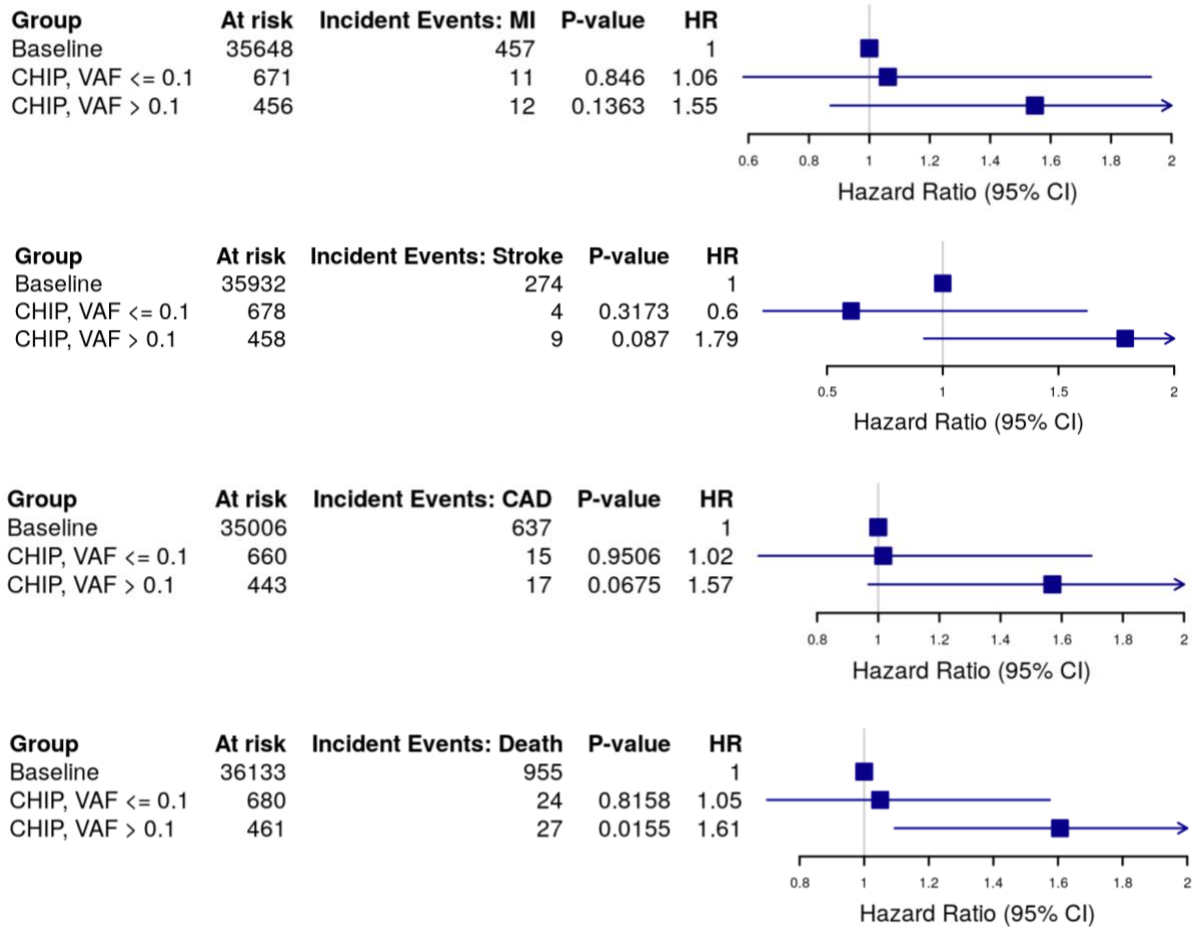
The proportion of the samples in the UK Biobank having *DNMT3A* or *TET2* CHIP increases with age at the time of exome sequencing. Data is fit with the general additive model using cubic regression splines from the mgcv package and plotted with the ggplot2 package in R. Shaded band displays the modeled standard deviation. CHIP, clonal hematopoiesis of indeterminate potential.

Supplemental Figure 3. Cumulative CVD event rate binned by no CHIP, small CHIP, and large CHIP



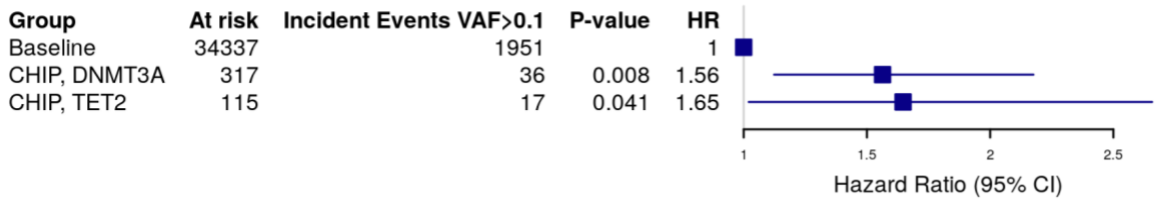
Small CHIP refers to CHIP with VAF ≤ 10%. Large CHIP refers to CHIP with VAF > 10%.

Supplemental Figure 4. Risk of sub-components of the primary outcome stratified by CHIP clone size



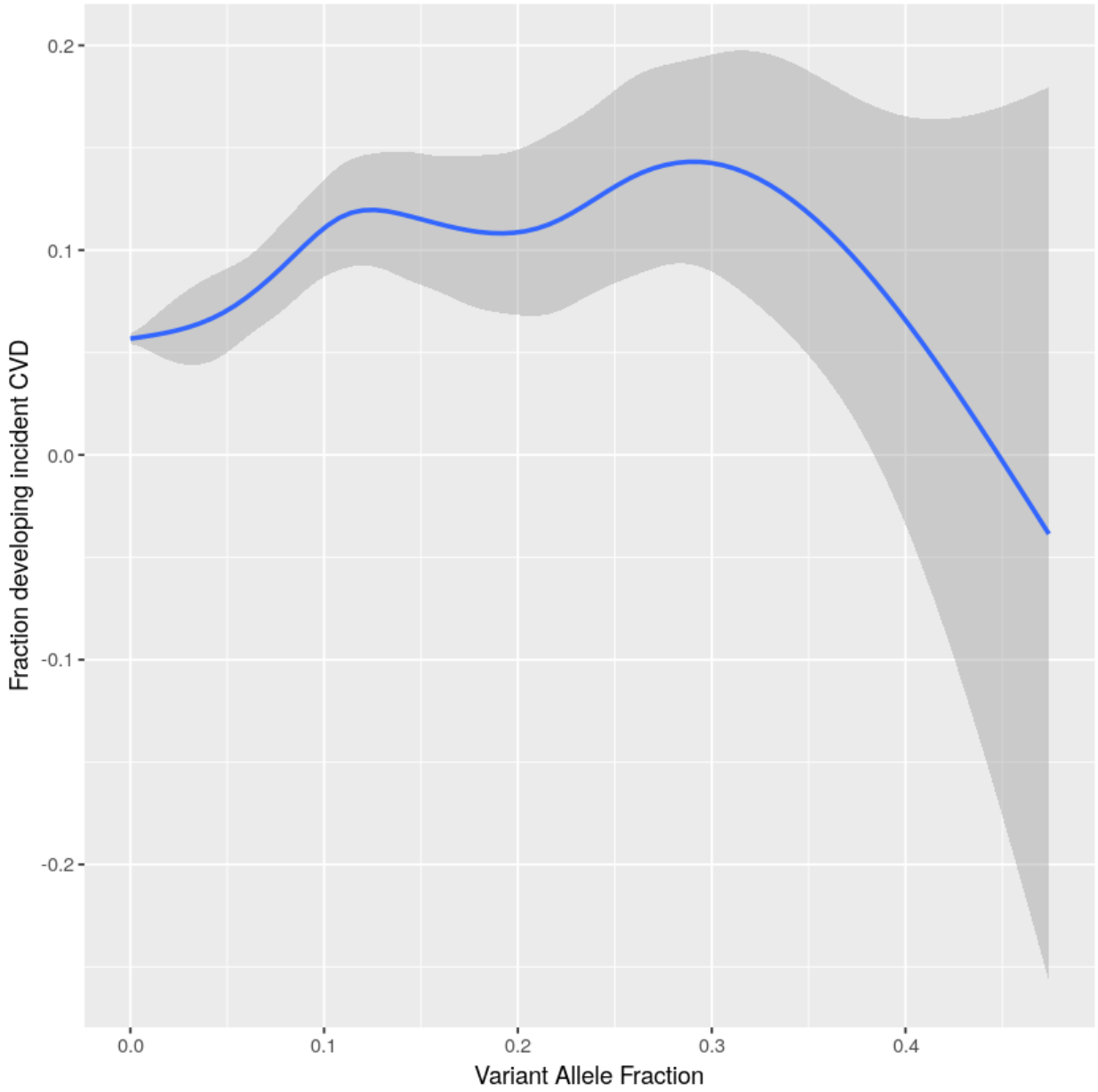
Large CHIP clones lead to similar risk estimates across the sub-components of the primary outcome.

Supplemental Figure 5. Primary CVD outcome stratified by CHIP gene, VAF >10%



Large CHIP clones lead to similar risk estimates of the primary outcome, whether identified in *DNMT3A* or *TET2*. CHIP = clonal hematopoiesis of indeterminate potential. VAF = variant allele fraction.

Supplemental Figure 6. Disease risk conferred by CHIP is not linear with the variant allele fraction



CHIP clones smaller than a VAF of approximately 10% show little association with incident CVD, while CHIP clones larger than 10% appear to confer a similar risk to one another. Few participants have CHIP clones larger than 35%, as revealed by the increasingly wide shaded bands at the tail. Data is fit with the general additive model using cubic regression splines from the mgcv package and plotted with the ggplot2 package in R. Shaded band displays the modeled standard deviation. CHIP = clonal hematopoiesis of indeterminate potential. VAF = variant allele fraction.