Platelet mitochondrial DNA methylation predicts future cardiovascular outcome in adults with overweight and obesity

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EPIGENETICS: A PRIMER

There are many ways that epigenetic effects regulate the activation or repression of genes. Here are a few molecular tricks cells use to read off the right genetic program. **By Stefan Kubicek**

What makes the ~200 cell types in our body remember their identity? What prevents them from becoming cancer cells? Why do we inherit some traits from our father, others from our mother? How do our experiences and environment influence our thinking? Why do plants bloom in spring but not in winter? These important and quite different questions are all addressed by the field of epigenetics, which studies heritable changes in a phenotype arising in the absence of alterations in the DNA sequence. The idea of transgenerational inheritance of acquired characteristics goes back to Lamarck in the early 19th century, but still only correlative evidence exists in humans. In contrast, many cellular epigenetic phenomena are now well understood on the molecular level. In humans, they include the parent-of-origin specific expression of genes (imprinting) and the shutting-down of almost all genes on one of the two X chromosomes in females (X-chromosome inactivation).

All these epigenetic phenomena are characterized by chemical modifications to DNA itself (DNA methylation) or to histones, the proteins around which DNA is wound. These modifications change during development as stem cells give rise to liver cells and neurons, but also in response to environmental signals—in plants, for example, during the cold of winter or in humans when immune cells are activated after an infection. One of the biggest controversies in the field is whether histone modifications are inherited through cell division (called the "histone code hypothesis") or whether they only form transient indicators of transcriptional states ("signaling model").

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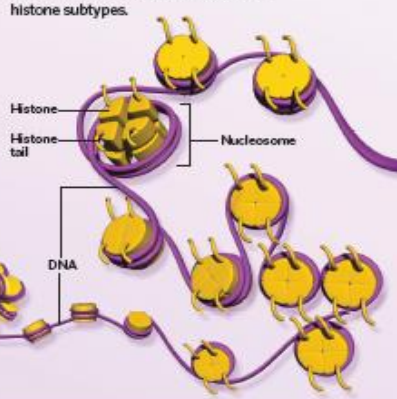
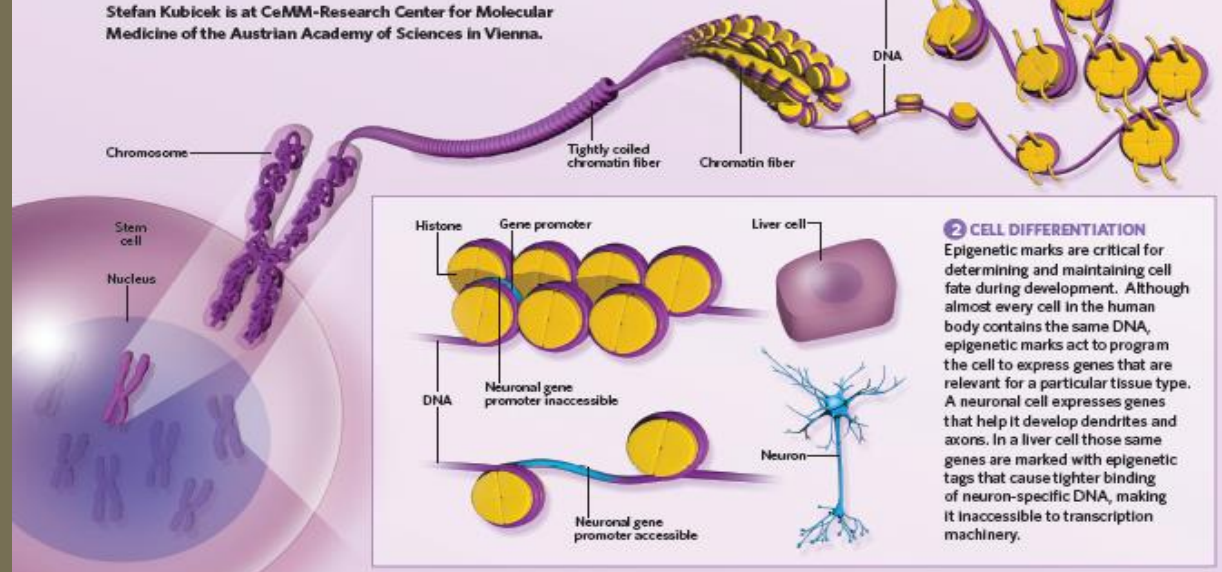
1 OVERVIEW

Epigenetic events regulate the activities of genes without changing the DNA sequence. Different genes are expressed depending on the methyl-marks attached to DNA itself and by changes in the structure and/or composition of chromatin. The main components of chromatin are histones (in bundles of eight units) around which 146 base-pairs of DNA are wound like a thread around a spool, forming a structure called the nucleosome. There are various epigenetic mechanisms that can affect the nucleosome: chemical modification (via molecular additions to histone tails or DNA), a change its positioning on DNA (via chromatin remodeling proteins), or a variation in histone subtypes.



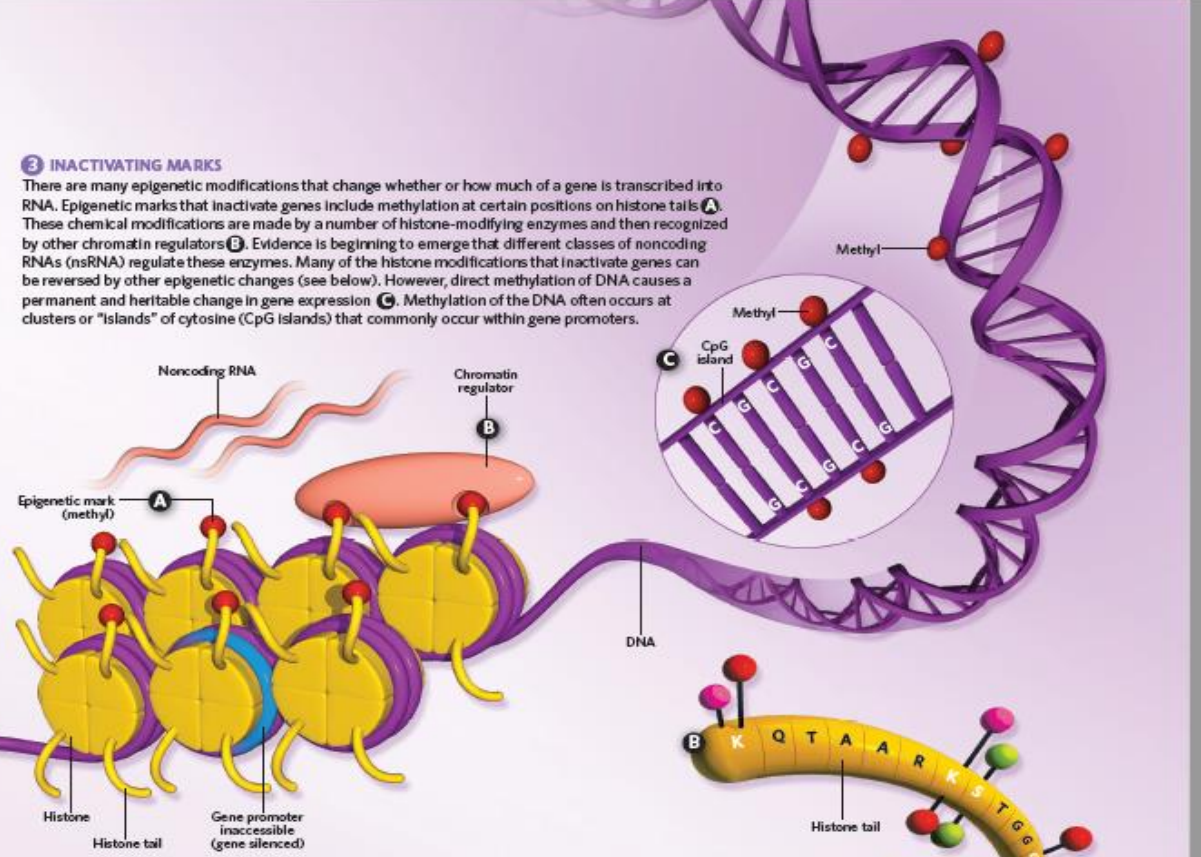


2 CELL DIFFERENTIATION

Epigenetic marks are critical for determining and maintaining cell fate during development. Although almost every cell in the human body contains the same DNA, epigenetic marks act to program the cell to express genes that are relevant for a particular tissue type. A neuronal cell expresses genes that help it develop dendrites and axons. In a liver cell those same genes are marked with epigenetic tags that cause tighter binding of neuron-specific DNA, making it inaccessible to transcription machinery.

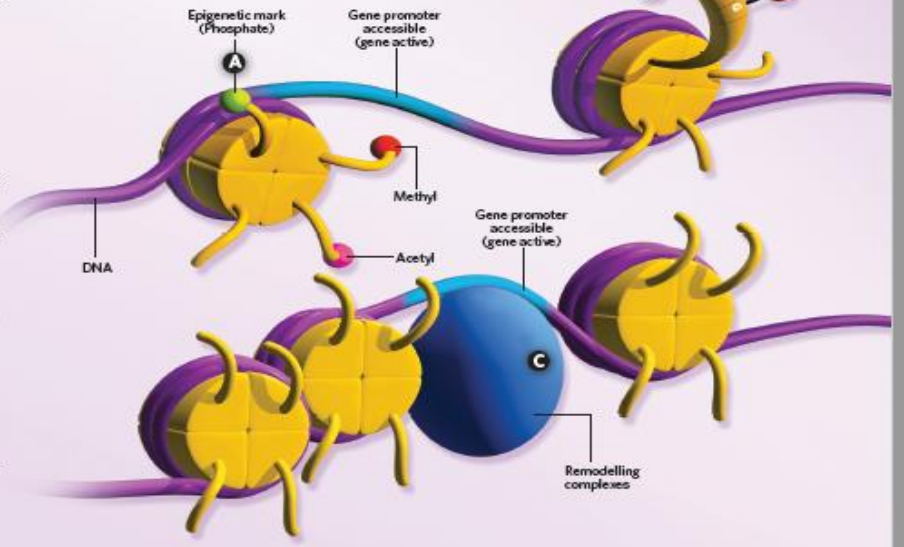
3 INACTIVATING MARKS

There are many epigenetic modifications that change whether or how much of a gene is transcribed into RNA. Epigenetic marks that inactivate genes include methylation at certain positions on histone tails (A). These chemical modifications are made by a number of histone-modifying enzymes and then recognized by other chromatin regulators (B). Evidence is beginning to emerge that different classes of noncoding RNAs (nsRNA) regulate these enzymes. Many of the histone modifications that inactivate genes can be reversed by other epigenetic changes (see below). However, direct methylation of DNA causes a permanent and heritable change in gene expression (C). Methylation of the DNA often occurs at clusters or "islands" of cytosine (CpG islands) that commonly occur within gene promoters.

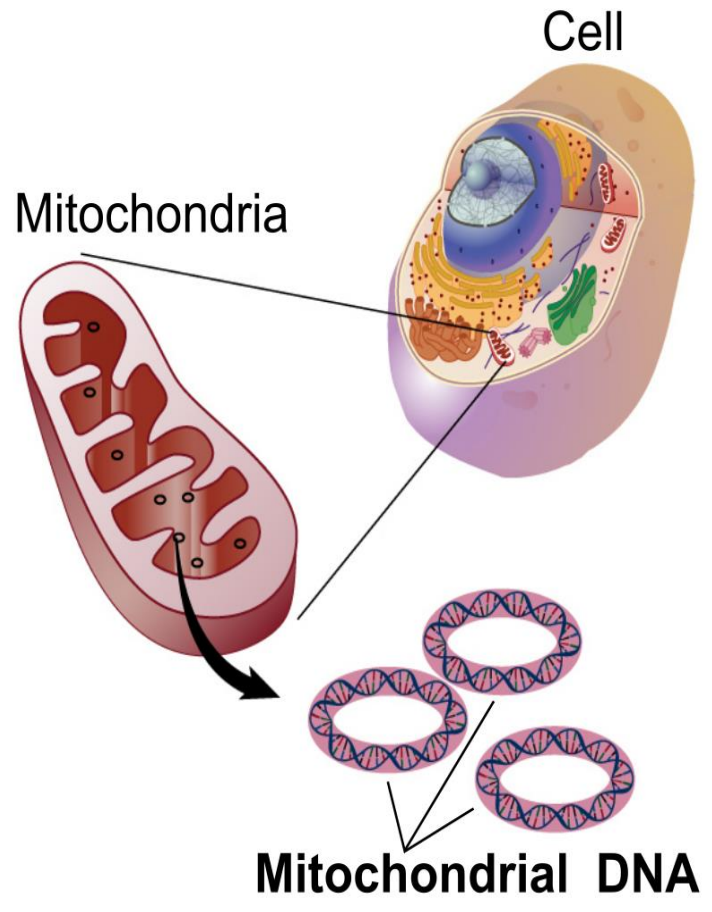


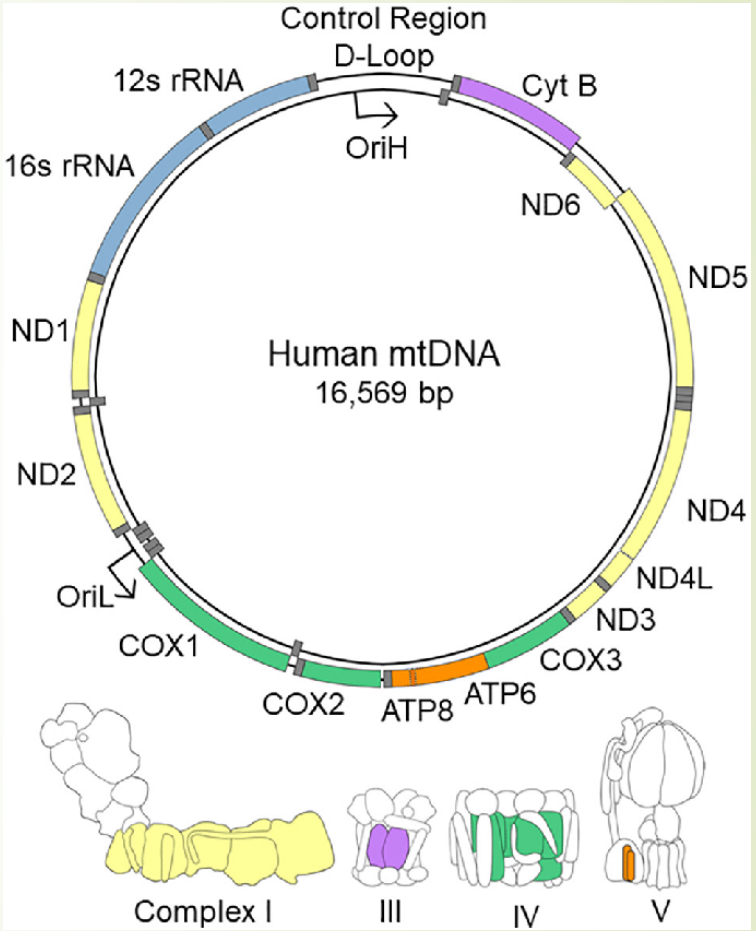
4 ACTIVATING MARKS

The heritability of DNA methylation, which often occurs in the early stages of development, allows cells to keep irrelevant genes silenced in successive generations of liver or skin cells. However some genes—such as the plant genes that govern winter dormancy and springtime flowering—require silenced genes to be reactivated. Several modifications, including the acetylation, phosphorylation, as well as methylation of certain positions on a histone tail (A), can cause DNA to unwind, releasing the genes that are otherwise inaccessible. These modifications occur mostly at specific positions on the accessible tails of the histones, and subsequently recruit additional activating proteins (B). Histone-remodeling complexes, which slide histones in one direction or another, can also make genes accessible to transcription (C).



Mitochondria





generate most of the chemical energy needed to power the cell's biochemical reactions

Mitochondrial DNA methylation

- DNA methylation can happen not only in nuclear DNA but also in mitochondrial DNA that plays an important role in:
- mitochondrial genome stability
- mitochondrial gene expression
- mitochondrial function
- the pathophysiological processes of many diseases

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Clinical Epigenetics

RESEARCH Open Access

Platelet mitochondrial DNA methylation predicts future cardiovascular outcome in adults with overweight and obesity

Sarah Corsi[1], Simona Iodice[2], Luisella Vigna[3], Akin Cayir[4], John C. Mathers[1], Valentina Bollati[2] and Hyang-Min Byun[1*]

Back Ground

- Mitochondrial dysfunction and damage have been implicated in obesity
- platelet mitochondria are important in maintaining thrombosis and hemostasis
- mitochondrial DNA (mtDNA) in platelets is aberrantly methylated in CVD patients

Back Ground

- The association between obesity and cardiovascular disease (CVD) is proven
- why some adults with obesity develop CVD

Hypotheses and objectives

- Epigenetic changes in the mitochondrial epigenome may be early events related to CVD development
- Aberrant platelet mtDNA methylation occurs in at-risk individuals, such as adults with obesity, prior to developing CVD and may therefore serve as a biomarker of CVD risk.
- Determining the utility of platelet mtDNA methylation to predict future CVD events in adults with overweight or obesity

Research design

prospective nested case-control study

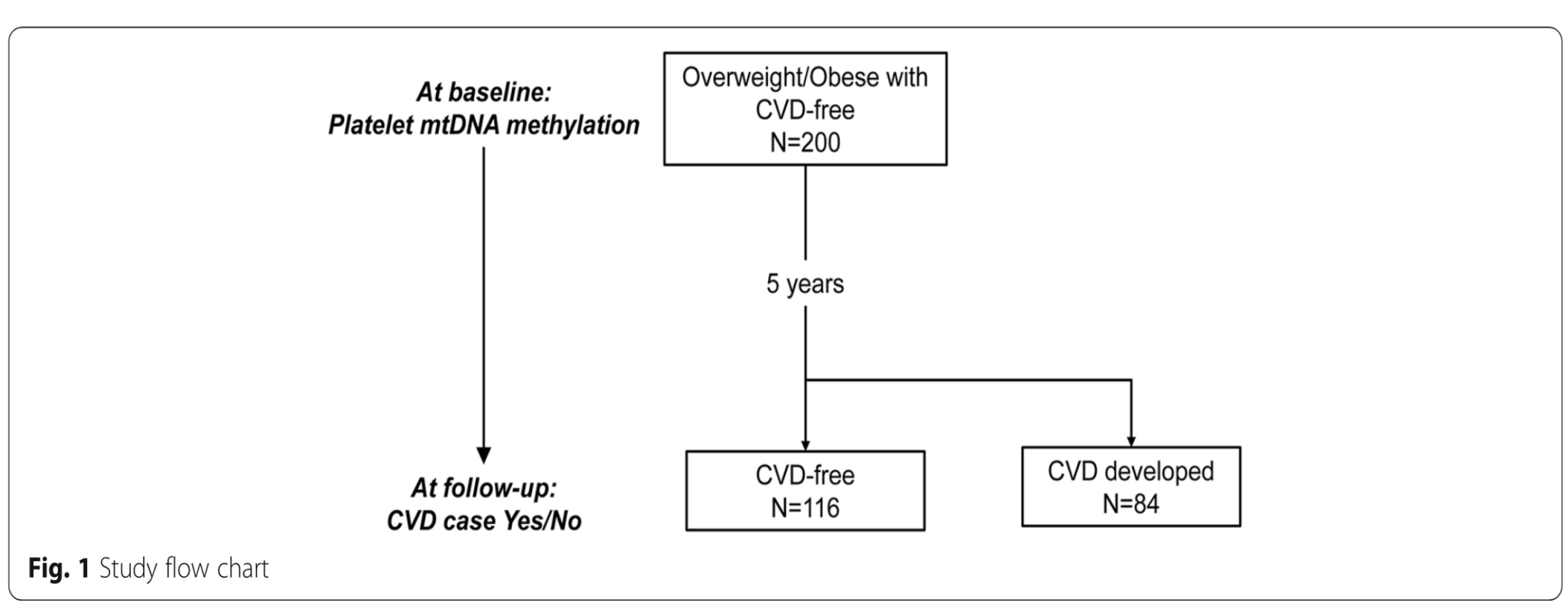


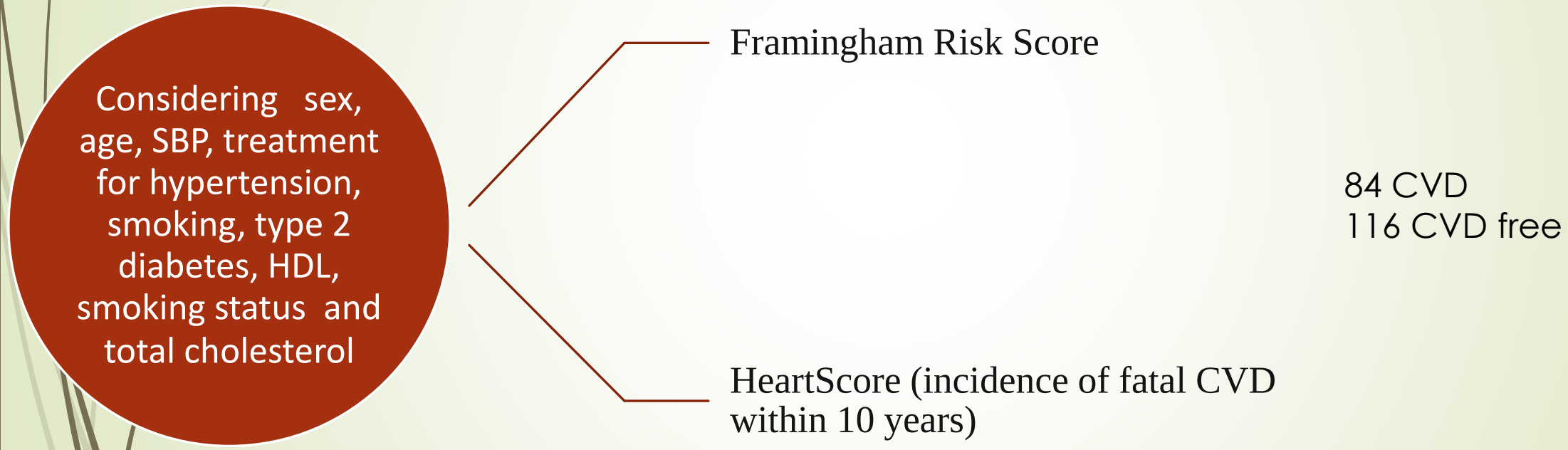
Fig. 1 Study flow chart

The demographic and clinical characteristics of these participants

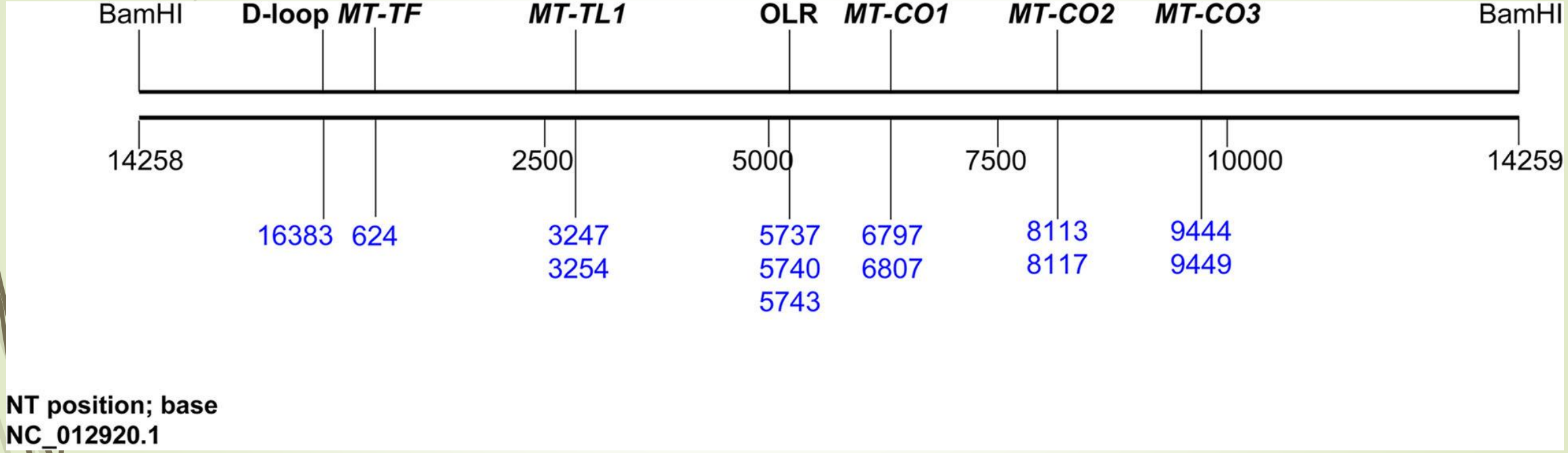
Table 1 Participant characteristics at baseline

Variable	All participants (n = 200)	CVD-free at the follow-up (n = 116)	CVD-developed at the follow-up (n = 84)	P value
Sex (n, %)				
Male	78 (39%)	44 (38%)	34 (40%)	0.716
Female	122 (61%)	72 (62%)	50 (60%)	
Age (mean, SD)	62.5, ± 10	61.7, ± 9.5	63.5, ± 10.6	0.210
BMI (mean, SD)	35.5, ± 5.1	35.4, ± 4.9	35.5, ± 5.4	0.936
BMI categorical (n, %)				
25.1–30.0 (overweight)	34 (17%)	22 (19%)	12 (14%)	0.762
30.1–34.9 (obesity I)	62 (31%)	33 (28%)	29 (35%)	
> 35.1 (obesity II and III)	104 (52%)	61 (53%)	43 (51%)	
Smoking status (n, %)				
Never	89 (45%)	53 (46%)	36 (43%)	0.859
Former	91 (46%)	50 (43%)	41 (49%)	
Current	19 (10%)	13 (11%)	6 (7%)	
Education, years of education (n, %)				
Primary school and other (< 5 years)	34 (17%)	18 (16%)	16 (19%)	0.297
Secondary school and high school (< 13 years)	129 (65%)	76 (66%)	53 (63%)	
University degree (> 14 years)	32 (16%)	21 (18%)	11 (13%)	
SBP, mmHg (mean, SD)	128.2, ± 13.7	129.1, ± 13.3	127, ± 14.1	0.268
DBP, mmHg (mean, SD)	78.9, ± 8.5	79.2, ± 8.5	78.4, ± 8.5	0.517
Fasting blood glucose, mmol/L (mean, SD)	5.9, ± 1.4	5.8, ± 1.4	6.0, ± 1.4	0.384
Total cholesterol, mg/dL (mean, SD)	206.6, ± 42.9	204.5, ± 42.4	209.5, ± 43.8	0.421
HDL cholesterol, mg/dL (mean, SD)	58.6, ± 15.0	60.0, ± 15.5	56.8, ± 14.3	0.141
LDL cholesterol, mg/dL (mean, SD)	128.3, ± 37.1	127.6, ± 36.0	129.1, ± 38.8	0.777
Triglyceride (TC), mg/dL (mean, SD)	126.4, ± 61.6	121.0, ± 57.6	133.7, ± 66.2	0.153
TC/HDL ratio (mean, SD)	3.7, ± 1.1	3.6, ± 0.9	3.9, ± 1.2	0.039
Framingham Risk Score, median (Q1, Q3)	18.2 (9.3, 28.9)	17.9 (9.6, 26.2)	18.3 (8.8, 30.5)	0.636
HeartScore, median (Q1, Q3)	2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	2.0 (1.0, 4.0)	0.232
Medication usage (n, %)				
Not available	19 (9%)	6 (5%)	13 (16%)	0.039
Yes	46 (23%)	30 (26%)	16 (19%)	
No	135 (68%)	80 (69%)	55 (65%)	

Assessment of CVD risk at baseline and CVD events at follow-up



Platelet mtDNA preparation and DNA methylation measurement



Statistical analysis

- Data for CVD-free and CVD-developed participants at follow-up were compared

using the $\chi 2$ test for categorical data and Student's t test for continuous variables

- Multivariate logistic regression, adjusted for age, BMI, fasting blood glucose, cholesterol

ratio (TC/HDL), SBP, and DBP

- ROC curves were generated to evaluate the diagnostic ability of the cholesterol ratio and mtDNA loci to distinguish between participants who were CVD-free and those in whom CVD-developed at follow-up

Methylation at baseline was in those participants who remained CVD-free compared with those who developed CVD during follow-up

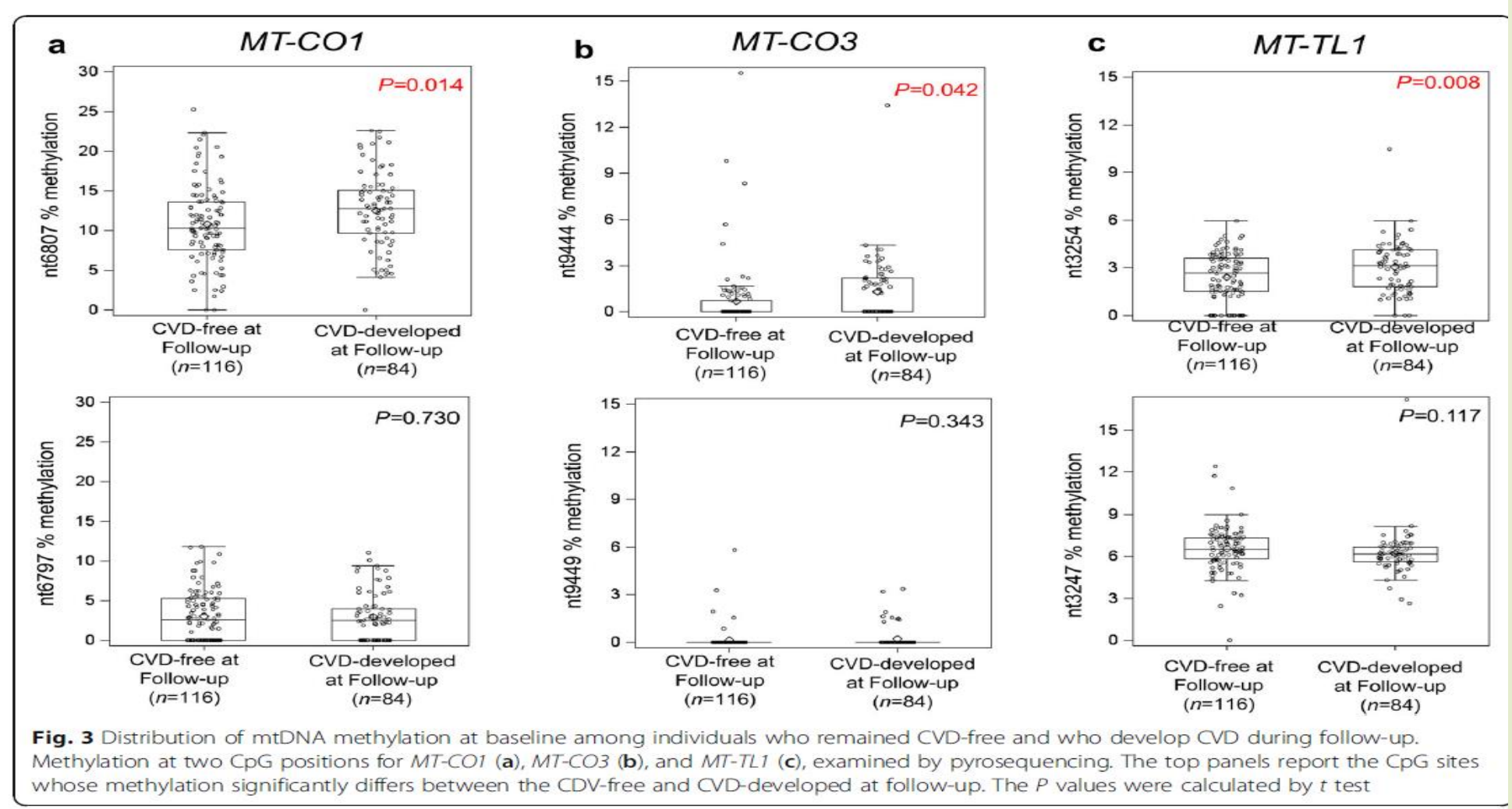


Fig. 3 Distribution of mtDNA methylation at baseline among individuals who remained CVD-free and who develop CVD during follow-up. Methylation at two CpG positions for *MT-CO1* (**a**), *MT-CO3* (**b**), and *MT-TL1* (**c**), examined by pyrosequencing. The top panels report the CpG sites whose methylation significantly differs between the CDV-free and CVD-developed at follow-up. The *P* values were calculated by *t* test

methylation at the three loci in relation to the development of CVD during follow-up

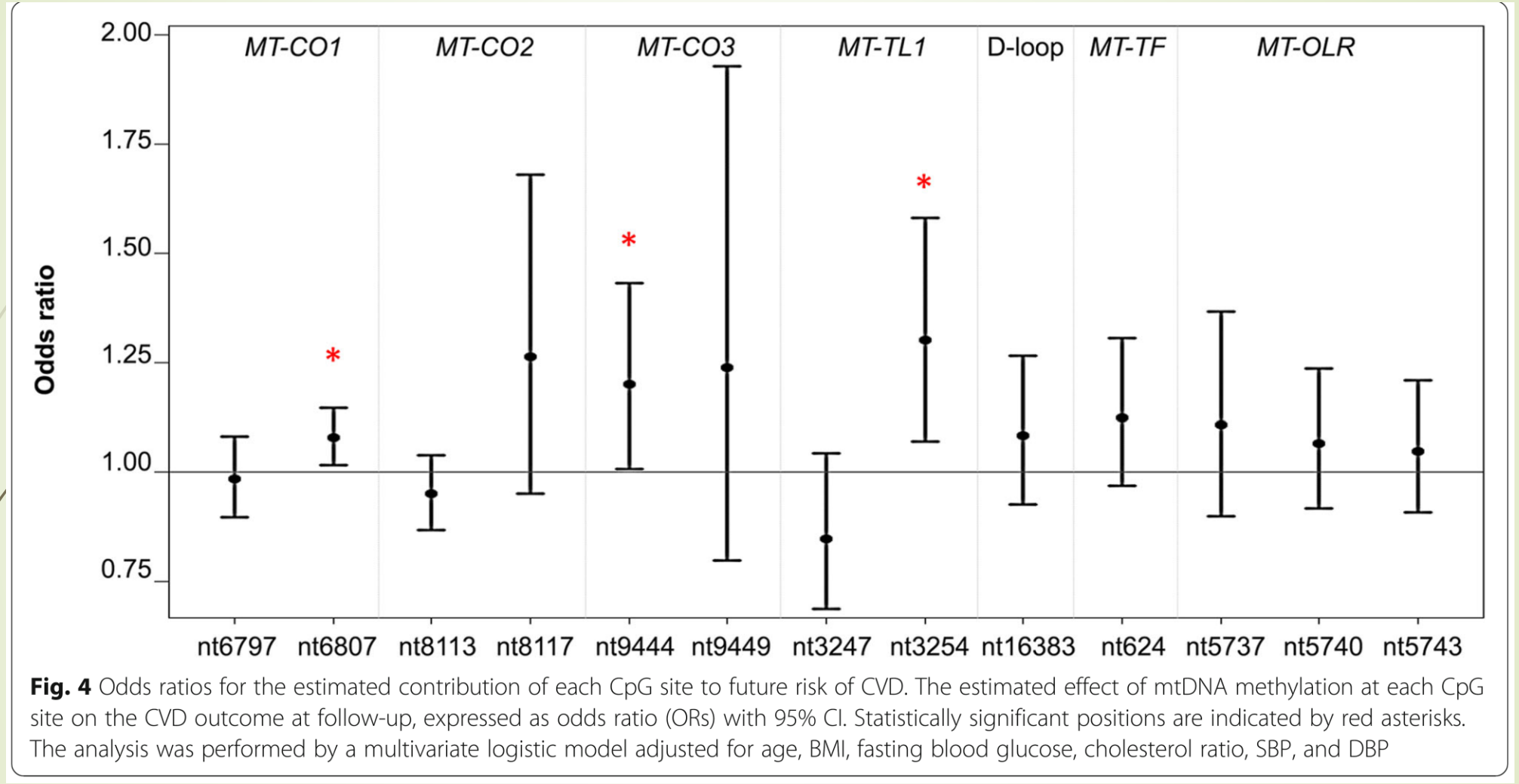


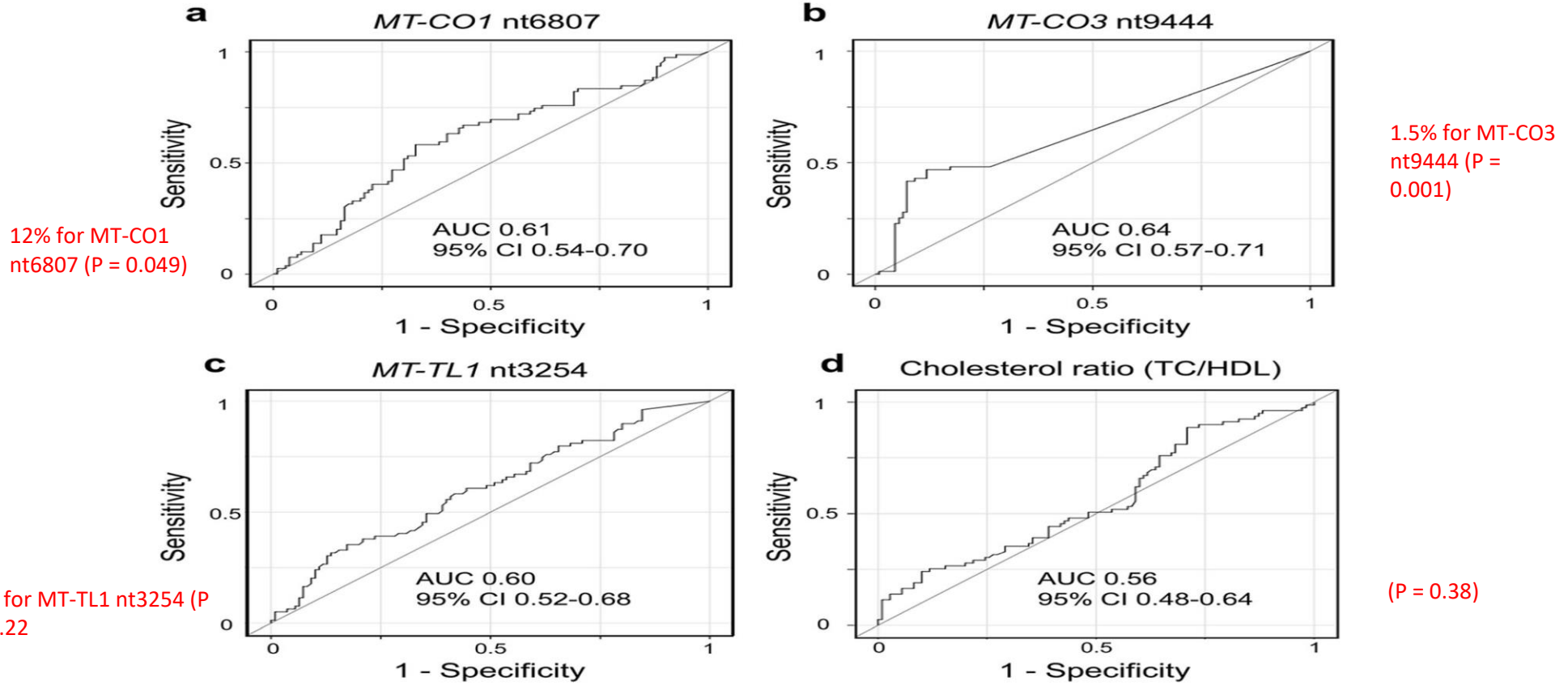
Fig. 4 Odds ratios for the estimated contribution of each CpG site to future risk of CVD. The estimated effect of mtDNA methylation at each CpG site on the CVD outcome at follow-up, expressed as odds ratio (ORs) with 95% CI. Statistically significant positions are indicated by red asterisks. The analysis was performed by a multivariate logistic model adjusted for age, BMI, fasting blood glucose, cholesterol ratio, SBP, and DBP

Not find any association between mtDNA methylation level and the most common CVD risk factors including age, BMI, blood pressure, blood glucose concentration, cholesterol, and uric acid in individuals with overweight and obesity

Table S1. Multivariate analysis for the association between potential risk factors and mitochondrial DNA methylation at baseline.

Variable	*MT-CO1* nt6807				*MT-CO3* nt9444				*MT-TL1* nt3254			
	Estimate	CI	SE	P value	Estimate	CI	SE	P value	Estimate	CI	SE	P value
Age	-0.001	-0.006, 0.003	0.002	0.524	-0.003	-0.013,0.007	0.005	0.582	-0.003	-0.006, 0.000	0.002	0.083
BMI	0.001	-0.007, 0.009	0.004	0.783	-0.007	-0.028, 0.013	0.010	0.492	0.005	-0.002, 0.011	0.003	0.148
Serum Uric Acid	0.010	-0.023, 0.042	0.016	0.554	-0.024	-0.107, 0.059	0.042	0.564	-0.012	-0.036, 0.012	0.012	0.330
ALT	0.000	-0.002, 0.003	0.001	0.760	-0.002	-0.007, 0.002	0.002	0.334	-0.001	-0.002, 0.001	0.001	0.539
AST	0.001	-0.003, 0.005	0.002	0.478	-0.005	-0.012, 0.002	0.003	0.133	-0.001	-0.004, 0.002	0.002	0.385
Basophil	-1.406	-3.819, 1.007	1.222	0.252	-5.863	-11.877, 0.151	3.005	0.056	1.238	-0.574, 3.050	0.917	0.179
Basophil percentage	-0.113	-0.283, 0.058	0.086	0.194	-0.475	-0.913, -0.036	0.219	**0.034**	0.052	-0.075, 0.179	0.064	0.419
Waist circumference	0.001	-0.002, 0.005	0.002	0.520	-0.005	-0.016, 0.005	0.005	0.287	0.000	-0.003, 0.003	0.001	0.941
HDL cholesterol	-0.002	-0.006, 0.001	0.002	0.170	0.007	-0.003, 0.018	0.005	0.181	0.000	-0.003, 0.003	0.001	0.995
LDL cholesterol	-0.001	-0.002, 0.001	0.001	0.250	0.000	-0.004, 0.004	0.002	0.982	-0.001	-0.002, 0.001	0.001	0.326
Total cholesterol	-0.001	-0.002, 0.000	0.001	0.192	0.001	-0.002, 0.004	0.002	0.628	0.000	-0.001, 0.001	0.000	0.790

Evaluate the diagnostic ability of the cholesterol ratio and mtDNA loci to distinguish between participants who were CVD-free and those in whom CVD-developed at follow-up



12% for MT-CO1 nt6807 (P = 0.049)

1.5% for MT-CO3 nt9444 (P = 0.001)

3% for MT-TL1 nt3254 (P = 0.22

(P = 0.38)

Fig. 5 ROC curves for methylation at three loci and cholesterol ratio for prediction of CVD outcome. Discrimination ability of methylation at three CpG sites (*MT-CO1* nt6807, *MT-CO3* nt9444, and *MT-TL1* nt3254) (**a**–**c**) and the cholesterol ratio (TC/HDL) (**d**) to predict CVD incidence within 5 years of baseline. Area under the ROC curve (AUC) and 95% CI values are annotated

- Methylation not above the thresholds at any of the three loci (score 0)
- methylation above the threshold at any one locus(score 1)
- methylation above the threshold at any two or all three loci (score 2)

Table 2 MtDNA methylation thresholds for each CpG site and score for predicting CVD outcome

a. Threshold for each CpG site

	Methylation	Median survival time (months)*	At risk	CVD during follow-up	CVD free	Log-rank *P* value
All patients		43.8	200	84	116	
MT-CO1 nt6809 (% methylation)	< 12.0	47.5	114	35	79	**0.049**
	≥ 12.0	38.3	83	47	36	
MT-CO3 nt9444 (% methylation)	< 1.5	47.0	146	44	102	**0.001**
	≥ 1.5	33.0	51	38	13	
MT-TL1 nt3254 (% methylation)	< 3.0	45.7	105	37	68	0.22
	≥ 3.0	42.1	94	46	48	
Cholesterol ratio	< 3.5	42.1	102	43	59	0.38
	≥ 3.5	45.3	94	41	53	

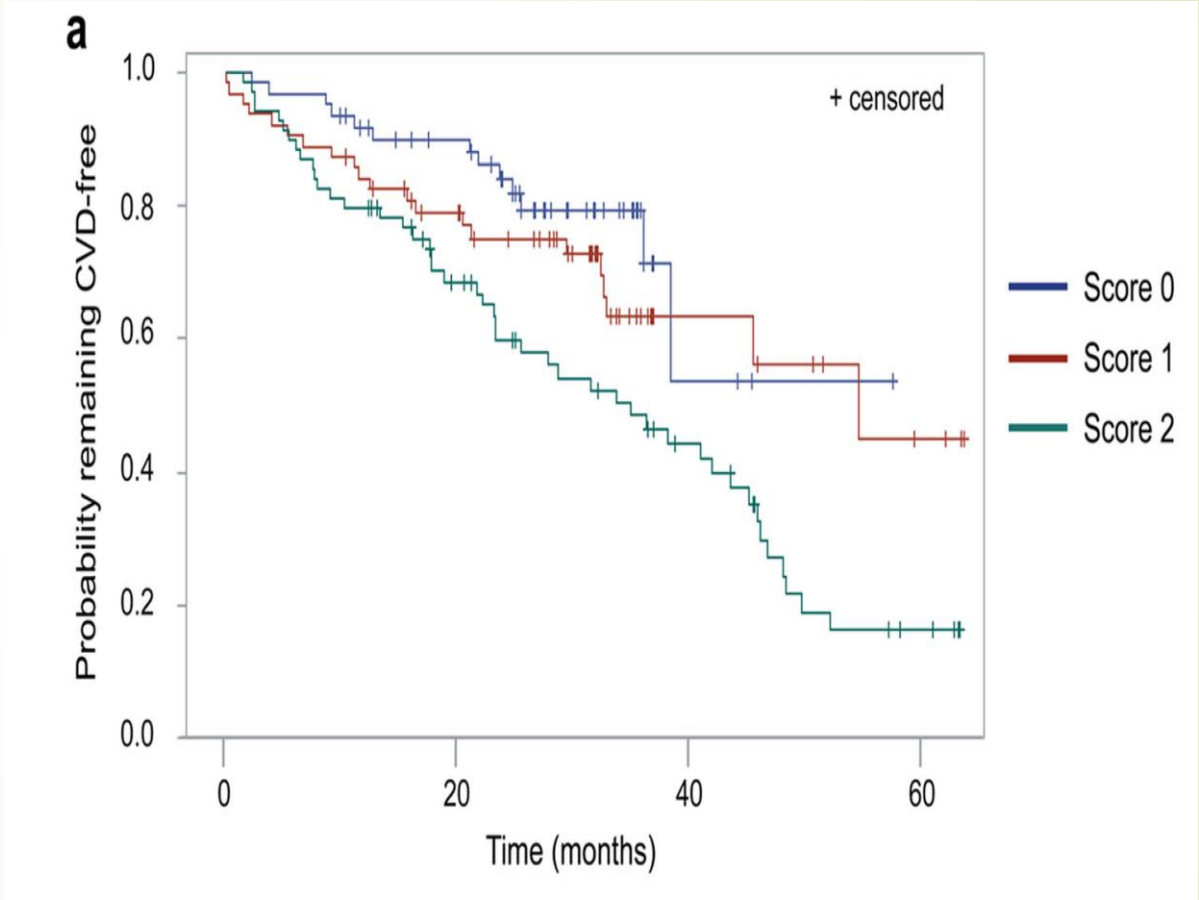
b. Score for predicting the CVD outcome

Score**	Median survival time (months)	At risk	CVD during follow-up	CVD-free	% CVD-developed at follow-up	Log-rank *P* value
0	~ 60	61	13	48	21%	**0.003**
1	54.8	63	21	42	33%	
2	35.1	69	45	24	65%	

Evaluate the independent prognostic value of each single locus and of their combination on future CVD cases

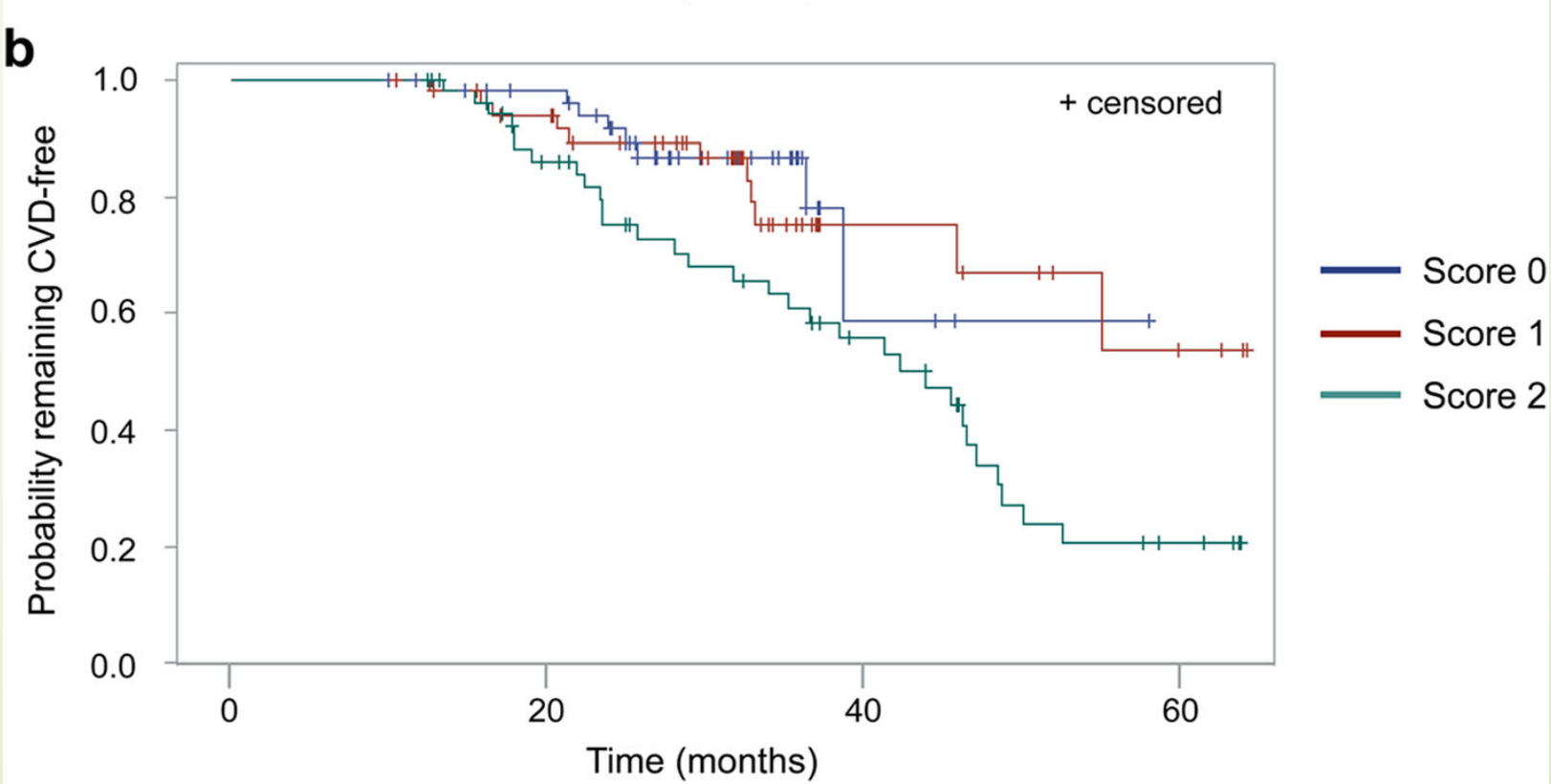
- The hazard ratio (HR) for developing CVD for score 1 was 1.38 to score 0 (95% CI, 0.68–2.78)
- score 2 was 2.68 (95% CI, 1.41–5.08)
- 65% of the individuals with score 2 developed CVD
- 21% of individuals with score 0 developed CVD

- Participants with score 2 had a lower median time without-CVD than participants with score 1
- More than half of the participants with score
- 0 were CVD-free at the end of the follow-up period.



sensitivity analysis

- Excluding participants who developed CVD within a year from baseline
  - The HR for those who scored 2 remained significantly higher than those who scored 1 (HR = 2.17, 95% CI 1.06–4.47)
  - The HR for those who scored 2 remained in comparison with those who scored 0 (HR = 2.53, 95% CI1.12–5.72)



sensitivity analysis

- Stratifying the CVD cases into “Mild,” such as hypertension (n = 51), and “Severe” events, such as ischemic heart diseases (n = 33)
  - mtDNA methylation score was a significant (P <0.001) predictor of future risk of developing CVD in mild group
  - The HR for those who scored 2 was significantly higher than for those scored 1 (HR = 2.27, 95% CI 1.13–4.44, P =0.021) and those who scored 0 (HR = 4.34, 95% CI 1.76–10.73, P < 0.002)

Discussion & conclusion

- In this nested case-control study of 200 adults with overweight and obesity,

higher mtDNA methylation at three loci (MT-CO1 nt6807,MT-CO3 nt9444, and MT-TL1 nt3254) in platelets was associated with higher risk of developing CVD within 5 years

- participants with score 2 (high methylation at two or three loci) developed CVD significantly sooner than the participants with score 1 and score 0

- mtDNA methylation at the three loci may be a novel predictive biomarker for the future risk of developing CVD

Discussion & conclusion

- No association between mtDNA methylation level and CVD risk factors in individuals with overweight and obesity.

- Supports the idea that altered mtDNA methylation in platelets precedes the development of CVD, and may serve as a non-invasive BIOMARKERS to distinguish individuals with higher CVD risk.

limitation

- The outcome in this study was diagnosis of any of a heterogeneous group of CVDs that ranged from mild to more severe events
- Their model remained strong in predicting the “mild” CVD events, but the lack of

  statistical power prevented examination of its ability to predict more “severe” cases
- To replication the finding, access to data and samples from a cohort that had collected plasma or platelets and had follow-up data on CVD incidence as part of a prospective study of individuals with overweight and obesity is required
- most of the participants were Caucasian, additional studies are needed to validate these findings in individuals with different ethnicities
- hospital discharge records were utilized, the use of thoroughly validated administrative
- databases may strengthen future studies

The use of thoroughly validated administrative databases may strengthen future studies

- Multivariate logistic regression,
- adjusted for age, BMI, fasting blood glucose, cholesterol
- ratio (TC/HDL), SBP, and DBP, was performed to investigate
- the association between DNA methylation at each
- locus (CpG site) and the risk of CVD during follow-up.

- 2000 participants with overweight (25 < BMI < 30 kg/m2) and obesity (BMI ≥ 30 kg/m2) in Milan, Italy
- For those who developed CVD, the
- follow-up stopped after the first CVD diagnosis

- for
- those who remained CVD-free, the follow-up lasted until
- the last update from the Italian National Health Service.
- We selected 84 individuals who developed CVD in the
- follow-up period

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- follow-up stopped after the first CVD diagnosis; for
- those who remained CVD-free, the follow-up lasted until
- the last update from the Italian National Health Service
- 84 individuals who developed CVD in the
- follow-up period, and these were sex- and BMI-matched
- with 116 individuals who remained CVD-free.

- Assessment of CVD risk at baseline and CVD events at
- follow-up
- To estimate individual CVD risk at baseline, we calculated
- the Framingham Risk Score which uses information
- on sex, age, SBP, treatment for hypertension,
- smoking, type 2 diabetes, HDL, and total cholesterol
- HeartScore to predict the
- incidence of fatal CVD within 10 years [36, 47] using
- age, sex, SBP, cholesterol, HDL cholesterol, BMI, and
- smoking status.

Table 1 Participant characteristics at baseline

Variable	All participants (n = 200)	CVD-free at the follow-up (n = 116)	CVD-developed at the follow-up (n = 84)	P value
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Framingham Risk Score, median (Q1, Q3)	18.2 (9.3, 28.9)	17.9 (9.6, 26.2)	18.3 (8.8, 30.5)	0.636
HeartScore, median (Q1, Q3)	2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	2.0 (1.0, 4.0)	0.232
Medication usage (n, %)				
Not available	19 (9%)	6 (5%)	13 (16%)	0.039
Yes	46 (23%)	30 (26%)	16 (19%)	
No	135 (68%)	80 (69%)	55 (65%)	