Gene-specific DNA methylation profiles and LINE-1 hypomethylation are associated with myocardial infarction risk

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Abstract:

Background
DNA-methylation profiles are responsive to environmental stimuli and metabolic shifts. This makes DNA-methylation a potential biomarker of environmental-related and lifestyle-driven diseases of adulthood. Therefore we investigated if white blood cells' (WBCs) DNA-methylation profiles are associated with myocardial infarction (MI) occurrence.
Whole genome DNA-methylation was investigated by microarray analysis in 292 MI cases and 292 matched controls from the large prospective Italian EPIC cohort (EPICOR study). Significant signals (FDR adjusted P<0.05) were replicated by mass spectrometry in 317 MI cases and 262 controls from the Dutch EPIC cohort (EPIC-NL). LINE-1 methylation profiles were also evaluated in both groups.
Results
A differentially methylated region (DMR) within the ZBTB12 gene body and LINE-1 hypomethylation were identified in EPICOR MI cases, and replicated in the EPIC-NL sample (ZBTB12-DMR meta-analysis, effect-size±se=-0.016±0.003, 95%CI=-0.021;-0.011, P=7.54x10-10; LINE-1 methylation meta-analysis, effect-size±se=-0.161±0.040, 95%CI=-0.239;-0.082, P=6.01x10-5).
Moreover, cases with shorter time to disease had more pronounced ZBTB12-DMR hypomethylation (meta-analysis, Men: effect-size±se=-0.0059±0.0017, PTREND=5.0x10-4, Women: effect-size ±se=-0.0053±0.0019, PTREND=6.5x10-3) and LINE-1 hypomethylation (meta-analysis, Men: effect-size ±se=-0.0010±0.0003,

	PTREND=1.6x10-3, Women: effect-size ±se=-0.0008±0.0004, PTREND=0.026) than MI cases with longer time-to-disease. In the EPIC-NL replication panel, DNA-methylation profiles improved case-control discrimination and reclassification when compared with traditional MI risk factors only (Net Reclassification Improvement (95%CI) between 0.23 (0.02-0.43), P=0.034, and 0.89 (0.64-1.14), P <1x10-5). Conclusions Our data suggest that specific methylation profiles can be detected in WBCs, in a preclinical condition, several years before the occurrence of MI, providing an independent signature of cardiovascular risk. We showed that prediction accuracy can be improved when DNA-methylation is taken into account together with traditional MI risk factors, although further confirmation on a larger sample is warranted. Our findings support the potential use of DNA-methylation patterns in peripheral blood white cells as promising early biomarkers of MI.
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Response to Reviewers:	CLEP-D-15-00016R3 Reviewer #3: Indeed, also the estimation of MI risk needs to be limited to the EPIC-NL validation sample. Due to the winner's curse the large effect size in the discovery sample is not surprising and likely inflated. This is confirmed by the actual estimates as reported. Authors' Response We agree with the reviewer that MI risk estimate should not be considered for the EPICOR panel, being it the discovery panel. As such, we removed MI risk estimates from the main paper and limited MI risk estimate to the EPIC-NL replica panel. However, the analysis of the EPICOR panel provides a complementary piece of information when taken together with that of the EPIC-NL panel. In fact, even if the risk estimate should not be considered as such, this progressively modeled analysis can nevertheless provide information on the dependence/independence of DNA methylation from the TRFs. This can be seen in the analysis of both the EPIC-NL panel, and the EPICOR panel, as a complementary evidence, and it is also a confirmation of the fact that additional TRFs (other than those used in the discovery analysis on EPICOR) actually do not affect estimates also in the EPICOR discovery panel. To meet the reviewer's request, and to save the additional piece of information that is provided by the multivariate analysis on the EPICOR sample too, we modified the corresponding paragraphs in the main text and additional files. The following paragraphs have been edited: - Main Text, Results section, "DNA methylation and MI risk": page 9, line 14 - page 10, line 6 of the revised R3 paper - Main Text, Methods section, "DNA methylation and MI risk": page 19, lines 1-12 of the revised R3 paper - Additional file 2, "DNA methylation and MI risk": page 11, lines 7 – 25 of the revised R3 paper - Additional file 1: table S3A is now referred to EPIC-NL; table S3B is now referred to EPICOR.

Gene-specific DNA-methylation profiles and LINE-1 hypomethylation are associated with myocardial infarction risk

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ABSTRACT

Background

DNA-methylation profiles are responsive to environmental stimuli and metabolic shifts. This makes DNA-methylation a potential biomarker of environmental-related and lifestyle-driven diseases of adulthood. Therefore we investigated if white blood cells' (WBCs) DNA-methylation profiles are associated with myocardial infarction (MI) occurrence.

Whole genome DNA-methylation was investigated by microarray analysis in 292 MI cases and 292 matched controls from the large prospective Italian EPIC cohort (EPICOR study). Significant signals (FDR adjusted $P<0.05$) were replicated by mass spectrometry in 317 MI cases and 262 controls from the Dutch EPIC cohort (EPIC-NL). LINE-1 methylation profiles were also evaluated in both groups.

Results

A differentially methylated region (DMR) within the *ZBTB12* gene body and LINE-1 hypomethylation were identified in EPICOR MI cases, and replicated in the EPIC-NL sample (*ZBTB12*-DMR meta-analysis, effect-size±se=-0.016±0.003, 95%CI=-0.021;-0.011, $P=7.54\text{x}10^{-10}$; LINE-1 methylation meta-analysis, effect-size±se=-0.161±0.040, 95%CI=-0.239;-0.082, $P=6.01\text{x}10^{-5}$). Moreover, cases with shorter time to disease had more pronounced *ZBTB12*-DMR hypomethylation (meta-analysis, Men: effect-size±se=-0.0059±0.0017, $P_{\text{TREND}}=5.0\text{x}10^{-4}$, Women: effect-size ±se=-0.0053±0.0019, $P_{TREND}=6.5\text{x}10^{-3}$) and LINE-1 hypomethylation (meta-analysis, Men: effect-size ±se=-0.0010±0.0003, $P_{\text{TREND}}=1.6\text{x}10^{-3}$, Women: effect-size ±se=-0.0008±0.0004, $P_{TREND}=0.026$) than MI cases with longer time-to-disease.

In the EPIC-NL replication panel, DNA-methylation profiles improved case-control discrimination and reclassification when compared with traditional MI risk factors only (Net Reclassification Improvement (95%CI) between 0.23 (0.02-0.43), $P=0.034$, and 0.89 (0.64-1.14), $P<1\text{x}10^{-5}$).

Conclusions

Our data suggest that specific methylation profiles can be detected in WBCs, in a preclinical condition, several years before the occurrence of MI, providing an independent signature of cardiovascular risk. We showed that prediction accuracy can be improved when DNA-methylation is taken into account

together with traditional MI risk factors, although further confirmation on a larger sample is warranted. Our findings support the potential use of DNA-methylation patterns in peripheral blood white cells as promising early biomarkers of MI.

Keywords: DNA-methylation; myocardial infarction; early biomarkers; association study; risk prediction; risk stratification

Background

Cardiovascular diseases (CVDs) are a leading cause of mortality, morbidity and hospitalization in the adult population in western countries, and a major challenge for developing countries that follow a westernized-lifestyle. Great attention has been given so far to lifestyle related CVDs risk factors, such as unhealthy diet, smoking habits, lack of physical activity, whose deleterious effects may be prevented through major lifestyle changes or medical treatments. Apart from monogenic disorders associated with cardiovascular risk (e.g., hypertrophic cardiomyopathy, familial hypercholesterolemia), there is a strong evidence that a family history of cardiovascular disease and stroke enhances individual CVD risks in relatives as compared with general population, that points out the importance of genetic factors in the etiology of CVDs.

Recent genome wide association studies (GWASs) reported several potential genetic risk factors for CVDs or intermediate disease phenotypes such as type 2 diabetes, obesity and overweight [1], hypertension [2], altered lipid profiles [3], underlying the importance of the genetic component. However, the contribution of common genetic variants to non-monogenic-CVDs is likely to act in combination with environmental factors or via epistatic (gene-gene or gene-environment) interactions. As gene-environment interactions are thought to be mediated by epigenetic modifications of the genome, epigenetic regulation can be rewarded as the boundary between the inherited genomic asset and the environment, potentially playing a major role in disease onset and severity [4]: epigenetic changes are in fact dynamic, can be modified both during the early *in utero* development stages and across lifetime by environmental factors as well as diseases, and may be reversible reflecting environmental changes [5, 6]. DNA-methylation at CpG dinucleotides is an epigenetic mechanism mainly involved in gene expression regulation. DNA-methylation patterns across the genome are not uniform: genetic regions spanning genes locations have variable DNA-methylation profiles which are linked to regulatory functions (e.g. gene promoters methylation/demethylation regulates gene expression), and structural functions in shaping local chromatin structures [7, 8]; instead, intergenic regions are usually heavily methylated, since about 45% of the mammalian genome consists of transposable and viral elements that are silenced by methylation [9]. Methylation levels of the repetitive long interspersed nuclear element-1 (LINE-1) are generally considered as a *proxy* for global

DNA methylation, as LINE-1 elements are widely distributed in the genome and usually heavily methylated in the majority of normal tissues. LINE-1 hypomethylation has previously been associated with ischemic heart disease and stroke [10], and with altered levels of LDL and HDL [11].

Altered DNA-methylation profiles have been linked to oxidative stress [12], atherosclerosis [13], ageing [14, 15] and a variety of human diseases ranging from neurological and autoimmune disorders to cancer [16-18]. In addition to individual constitutive DNA-methylation profiles, that could *per se* be associated with cardiovascular outcomes [19], subtle and progressive DNA-methylation alterations mediated by lifestyle and environmental exposures may in fact lead to dysregulation of several metabolic pathways during lifetime, and ultimately to cardiovascular damage and disease [20]. However, the few reports linking cardiovascular outcomes to DNA-methylation measured in blood cells or vascular tissue [21-23] did not provide conclusive evidences of DNA-methylation involvement in cardiovascular disease.

Apart from few reports of single CpG associations with a disease or a phenotype, it is usually the cumulative methylation profile of neighboring CpG sites to be more likely associated to a potential functional effect of the methylation status, and the search for differentially methylated regions (DMRs) able to differentiate groups of subjects with different phenotypes or outcomes of interest is a common approach. Along this line, we conducted an epigenome-wide association study (EWAS) to identify DMRs and LINE-1 methylation profiles associated to myocardial infarction (MI) risk in the cardiovascular section (EPICOR) of the Italian cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC) study, and replicated statistically significant findings in an independent case-control study nested in the Dutch EPIC cohort (EPIC-NL) with comparable biological samples and information.

Furthermore, we tested whether MI risk prediction accuracy can be improved when DNA-methylation profiles, measured at baseline in a pre-clinical condition, are taken into account together with traditional MI risk factors.

Results

Descriptive statistics of the sample are reported in Table 1. Statistically significant differences between cases and controls were found in smoking habits, body mass index (BMI) and/or waist-to-hip ratio (WHR), serum lipid profile, and blood pressure, in both the discovery (EPICOR) and the replication (EPIC-NL) studies (Table1).

After raw methylation data quality controls (QCs), and removal of cross-hybridizing and Single Nucleotide Polymorphisms-containing probes, 425,498 CpGs were included into the following analyses.

Case-control differential methylation

In the EPICOR sample, 25,376 regions with correlated methylation levels were identified with the A-clustering algorithm [24], and subsequently tested for differential methylation between cases and controls (see Methods section): the top-ranking 6 differentially methylated regions are reported in Table S1 (Additional file 1). However, only the first region reached statistical significance (FDR *Q*-value<0.05), i.e. a 15-CpGs cluster within the gene body (exon1) of the "zinc finger and BTB domain containing 12" gene (*ZBTB12*, Gene ID: 221527), that was hypomethylated in cases as compared to controls (effect-size±se=-0.019±0.004, 95%CI -0.03;-0.01, *P*-value=1.94×10^{-7}, *Q*-value=0.005). To check for sex-specific effects of the *ZBTB12*-DMR, we stratified EPICOR subjects by sex, and found the 15-CpGs cluster still significantly hypomethylated in male cases (effect-size±se=-0.023±0.005, 95%CI -0.03;-0.01, *P*-value=1.06×10^{-6}) but not in females (effect-size±se=-0.006±0.006, 95%CI -0.02;0.005, *P*-value=0.29). Details on single CpGs are reported in Table S2A (Additional file 1).

The genomic inflation factor for the overall EPICOR sample was lambda=1.023 (men, lambda=1.043; women, lambda=1.017, Q-Q plots in Supplementary Figures S1-S3).

LINE-1 differential methylation was also tested in the EPICOR overall sample by logistic regression analysis: MI cases had statistically significant LINE-1 hypomethylation as compared to controls (effect-size±se=-0.511±0.147, 95%CI -0.80;-0.22, *P*-value=5.00×10^{-4}). At a sex-stratified analysis, LINE-1 hypomethylation was still statistically significant in men (effect-size±se=-0.520±0.179,

95%CI -0.87;-0.17, *P*-value=0.004) but not in women (effect-size±se=-0.496±0.319, 95%CI -1.12;-0.13, *P*-value=0.12).

Additionally, for *ZBTB12*-DMR we found a significant sex-methylation interaction (*P*-value=0.01), whilst for LINE-1 we found no evidence of interaction.

Results were replicated on the EPIC-NL panel, where the methylation profile of the same *ZBTB12*-DMR identified in the discovery phase proved consistent with that of the EPICOR discovery sample, with a cluster of 22 contiguous CpGs significantly hypomethylated in Dutch MI cases as compared to controls (effect-size±se=-0.013±0.004, 95%CI -0.02;-0.005 *P*-value=5.82×10^{-4}). Details on *ZBTB12*-DMR single CpGs for the EPIC-NL study are reported in Table S2B (Additional file 1).

At a sex stratified analysis, *ZBTB12*-DMR was hypomethylated both in EPIC-NL men (effect-size±se=-0.014±0.007, 95%CI -0.03;-0.001, *P*-value=0.034) and women (effect-size±se=-0.012±0.004, 95%CI -0.02;-0.004, *P*-value=0.006), with effect sizes more comparable between men and women than in the EPICOR sample.

In the EPIC-NL panel, LINE-1 mean methylation levels were lower than those of EPICOR, with an average methylation of about 0.8 in EPICOR subjects (men, mean±sd=0.844±0.007; women, mean±sd=0.843±0.007) and about 0.6 in EPIC-NL subjects (men, mean±sd=0.624±0.029; women, mean±sd=0.613±0.023). As seen in the EPICOR panel, we found LINE-1 hypomethylation also in Dutch cases as compared to controls, although with a milder effect (effect-size±se=-0.132±0.042, 95%CI -0.21;-0.05, *P*-value=0.001). The sex stratified LINE-1 analysis showed in EPIC-NL men an effect-size similar to that found in EPICOR (effect-size±se=-0.40±0.085, 95%CI -0.57;-0.23, *P*-value=2.22×10^{-6}), while in EPIC-NL women the effect was much lower and statistically non-significant (effect-size±se=-0.016±0.046, 95%CI -0.11;0.07, *P*-value=0.73).

In the Dutch panel we found no evidence of sex-methylation interaction for *ZBTB12*-DMR, while we found a statistically significant interaction for LINE-1.

The observation of sex-methylation interactions in both the discovery and replica panels, and further considerations addressed in the discussion section, suggested to consider men and women separately in all the subsequent analyses.

To achieve an overall estimate of the effects of *ZBTB12*-DMR and LINE-1 methylation across the 2 subjects panels, we performed a meta-analysis of the EPICOR and EPIC-NL studies.

The estimated *ZBTB12*-DMR effect was effect-size±se=-0.016±0.003 in the overall sample (P=7.54x10^{-10}, 95%CI=-0.021;-0.011, Cochran's Q=0.005, d.f.=1, P_{HET}=0.83), effect-size±se=-0.020±0.004 in men (P=1.82x10^{-7}, 95%CI=-0.027;-0.012, Cochran's Q=0.007, d.f.=1, P_{HET} =0.79), and effect-size±se=-0.010±0.003 in women (P=0.005, 95%CI=-0.017;-0.003, Cochran's Q=0.004, d.f.=1, P_{HET} =0.84).

The estimated LINE-1 effect was effect-size±se=-0.161±0.040 in the overall sample (P=6.01x10^{-5}, 95%CI=-0.239;-0.082, Cochran's Q=0.85, d.f.=1, P_{HET} =0.35), effect-size±se=-0.422±0.076 in men (P=3.42x10^{-8}, 95%CI=-0.572;-0.272, Cochran's Q=0.06, d.f.=1, P_{HET} =0.81), and effect-size±se=-0.025±0.046 in women (P=0.576, 95%CI=-0.115;0.064, Cochran's Q=0.70, d.f.=1, P_{HET} =0.40).

DNA-methylation and MI risk

The MI risk associated to *ZBTB12*-DMR and LINE-1 hypomethylation was estimated in the EPIC-NL replica panel: Recursively Partitioned Mixture Model (RPMM) classes and LINE-1 class (as defined in the methods section) were tested for association with MI under different models, from unadjusted to fully adjusted.

When comparing the *ZBTB12*-DMR lowest methylation class (RPMM3) with the highest methylation class (RPMM0), we found MI risk to be significantly associated with hypomethylation in the EPIC-NL women (fully adjusted, OR=2.75, 95%CI 1.39–5.45, P=0.004), whilst in EPIC-NL men the association was statistically non-significant (fully adjusted, OR=2.60, 95%CI 0.79–8.56, P=0.116), although direction and effect size were similar.

We also found a higher MI risk associated with LINE-1 lower methylation class in EPIC-NL men (fully adjusted, OR=1.95, 95%CI 1.02-3.71, P=0.043, ref. group above the median). No difference was found in EPIC-NL women (fully adjusted, OR=1.05, 95%CI 0.65–1.67, P=0.850) (Additional file 1, Table S3A).

The same analysis was performed on the EPICOR discovery sample: even though in this case the ORs cannot be considered as indicative of a true estimate of risk being EPICOR subjects the discovery

panel, the analysis was nevertheless done to assess whether the progressive inclusion in the model of additional variables, namely traditional risk factors (TRFs), could modify the estimate of risk or, on the contrary, if DNA methylation may independently contribute to MI risk. No significant evidence of inflation/deflation of the DNA-methylation-related MI risk estimate was found nor for the EPIC-NL panel nor for the EPICOR panel when progressively adding traditional risk factors (TRFs) as covariates in the model (Additional file 1: Tables S3A and B).

Discrimination, reclassification, and calibration on EPIC-NL samples

We assumed 2 models, including respectively: (1) TRFs only; (2) TRFs plus the *ZBTB12*-based RPMM classes and LINE-1 methylation class. According to the Net Reclassification Improvement (NRI) and Integrated Discrimination Improvement (IDI) indices (Table 2), a statistically significant improvement in prediction performance was achieved when adding the DNA-methylation profiles to the set of baseline predictors (i.e., TRFs), both for EPIC-NL males and females groups. Furthermore, we found an improvement in discrimination (Table 2, DeLong's test) comparing the area under Receiver Operating Curves (AUC) of the 2 models (Table 2 and Figure 1), although it was not statistically significant.

The calibration plots confirmed the goodness of fit of both the TRFs only and TRFs+Methylation models (Figure 2, Hosmer-Lemeshow test), with a better performance of the second one.

DNA-methylation and Time to Disease (TTD)

The trend test on EPICOR and EPIC-NL subjects, stratified by study and by sex, highlighted a more pronounced *ZBTB12*-DMR hypomethylation in cases with shorter time to disease (EPICOR and EPIC-NL meta-analysis, Men: P_{TREND}=0.0005, Women P_{TREND}=0.0065, Table 3). Similarly, LINE-1 was hypomethylated in cases with shorter time to disease (meta-analysis, Men: P_{TREND}=0.0016, Women P_{TREND}=0.026, Table 3).

At a *post-hoc* power analysis, our study was well powered (86% and 82% for males and females groups, respectively) to identify DMRs with effect sizes equal to half of the standard deviation, considering alpha equal to the False Discovery Rate (FDR) threshold of significance (Q=0.05).

Discussion

In this study we investigated whether WBCs DNA-methylation profiles may be associated with myocardial infarction risk. We examined clusters of adjacent CpG sites with correlated methylation levels under the assumption that they could be more reliable indicators of the underlying biological function than the single CpG methylation measurement. As we found evidences of sex-methylation interactions in both the analyzed panels, in our study the analyses were stratified by sex, in order to account for sex-related differences in DNA methylation profiles of genomic regions, of which "genomic imprinting" is a well-known example, and to account for sex specific cardiovascular risks. For coronary heart disease, sex differences in incidence, disease manifestations, and mortality are well recognized [25], and men and women seem not to share the same cardiovascular risk factors [26-29]. Moreover, patterns of sex-specific methylation have been reported in literature, and there is a general consensus on the occurrence of sex-biased autosomal DNA methylation in specific genes and regions, although with contrasting results [30-32]. Sex-associated differential DNA methylation in autosomal loci has been reported in genes associated to traits/diseases with different incidence rates according to sex [33], as well as in hormone-related genes, suggesting a differential regulation, potentially exerted via methylation [31]. Differential DNA-methylation may account for the differences in metabolic profiles of men and women, possibly leading to the different incidence, prevalence, symptoms, ages at onset and severity of cardiovascular diseases reported in literature.

In the EPICOR discovery panel, we identified a 15-CpGs cluster within the *ZBTB12* gene that was significantly differentially methylated in Italian MI cases and controls, and that was also significantly hypomethylated in MI cases in the independent Dutch panel. Moreover, *ZBTB12*-DMR showed a trend towards more pronounced hypomethylation in subjects with a short TTD both in the Italian and in the Dutch sample.

ZBTB12-DMR spans a ~250bp region in *ZBTB12* exon 1: although the role of gene-body methylation in transcriptional regulation is not fully understood, yet there are evidences of a role of the first exon's DNA-methylation in transcriptional silencing and, putatively, in alternative splicing [34]. All of our samples belong to the EPIC cohort, for which no biospecimen suitable for transcriptome analyses is available to address the relationship between *ZBTB12* methylation and gene expression levels. To cope with this issue, we explored *ZBTB12* DNA-methylation/gene-expression relationship in cryopreserved peripheral blood mononuclear cells from ~80 healthy young subjects belonging to another ongoing study, for which we already measured methylation and gene-expression levels: in our data, *ZBTB12* mRNA abundance was below the background level (as assessed by Illumina HumanHT12 gene-expression BeadChip), while *ZBTB12* methylation levels were comparable to that of EPICOR and EPIC-NL controls (data not shown). No relationship was found also with the gene-expression levels of the nearby genes (data not shown). Data mining in freely available resources (e.g. BioGPS, AceView, ProteinAtlas, Genome Atlas) confirmed the generalized low *ZBTB12* mRNA level in tissues and cell types, although ZBTB12 protein is detectable in many tissues, including cardiovascular tissues. Although no clear function is described for ZBTB12, this protein is probably involved in transcriptional regulation, like other members of the ZBTB family of methyl-CpG binding proteins (MBPs). This is also supported by its mainly nuclear localization. MBPs bind to methylated DNA and recruit chromatin remodeling co-repressor complexes, resulting in compaction of chromatin into its transcriptionally inactive state [35]. Specifically, members of the ZBTB family function as mediators of epigenetically controlled gene silencing by recognizing symmetrically methylated CpG sites and sequence specific non methylated sites [8, 35].

According to the Human Protein Reference Database [36], ZBTB12 (HPRD ID: 15691) directly interacts with Harvey Rat Sarcoma Viral Oncogene Homolog (HRAS) and RAP1 GTPase Activating Protein1 (RAP1GAP). RAP1GAP down-regulates the activity of Ras -associated protein 1 (RAP1), a small GTPase involved in several aspects of cell adhesion, including angiogenesis [37]. HRAS, a member of the RAS oncogene family, is a key transducer in several growth-signaling events that may trigger cardiovascular complications such as angiogenesis and vascular permeability [38], and may be involved in inflammatory proliferative arterial diseases, including atherosclerosis and restenosis after

angioplasty [39]. The RAS-MEK-ERK cascade has been described as implicated in cardiac hypertrophy and heart failure, and ERK signal transduction pathways were associated with cardiac hypertrophy [40].

In addition to gene/region specific DNA-methylation, we investigated the cumulative DNA-methylation profile of LINE-1 repetitive sequences and found LINE-1 hypomethylation in MI cases, statistically significant in men in both panels, but not in women. LINE-1 hypomethylation was associated to cardiovascular-related traits in previous studies [10, 11], and it is associated to MI and shorter TTD in the present study. DNA hypomethylation is regarded as a cause of genomic instability, and as a matter of fact LINE-1 hypomethylation was found in several conditions, including cancer [41], autoimmune diseases [42] and cardiovascular diseases [10]. Specifically, global hypomethylation of genomic DNA and gene-specific methylation profiles have been associated to conditions already known to predispose to cardiovascular diseases, such as cellular ageing [43], atherosclerotic plaques [44], menopausal state and osteoporosis [45]. On the other hand, LINE-1 hypomethylation could simply be a marker of increased white blood cells proliferation due to inflammatory or immunological responses which are known to be active during cardiovascular pathogenic processes [10]. *In vitro* experiments on mouse embryonic stem cells showed that folate deficiency affected the homeostasis of folate-mediated one-carbon metabolism, leading to reduced LINE-1 methylation [46]. In a targeted analysis, we recently demonstrated on a subset of the EPICOR cohort (206 MI cases and 206 matched controls), an inverse relationship between B-vitamins intake and DNA-methylation of genes belonging to One Carbon Metabolism and Homocysteine pathways [20]. These previous observations, together with our current finding of LINE-1 hypomethylation in cases compared to healthy controls, suggest a link between DNA-methylation patterns and CVD risk conferred by low folate and B-vitamins intake, that is worthy of further investigation.

Overall, this study analyzed 609 cases and 554 controls, and was sufficiently powered to detect effects of the magnitude we found. The discovery and the replica panels share homogeneous features: both belong to the European EPIC cohort, subjects were all enrolled in the nineties, and biosamples were collected and stored at enrollment according to shared standard protocols [47]. Nevertheless, a

limitation of the study is that while EPICOR cases and controls were matched by age, sex, center and season of recruitment, this could not be achieved for the EPIC-NL sample, since a DNA sample suitable for methylation analysis was not available for all the subjects enrolled in the Dutch EPIC cohort.

Another limitation is that the assessment of the methylation levels was done with different methods for the two panels. However, our approach that considered the regional methylation profile as a whole instead of single CpGs may contribute to overcome the bias due to measure errors at single CpGs level, as highlighted by the correlation between the methylation measures of 16 control samples assayed with both BeadChip and MassArray Assay (Additional file 2: Supplementary Methods). Moreover, although the CpGs positions assayed with the 2 methods are not exactly the same due to technical constrains (Additional file 2: Supplementary Methods, and Figure S4), still the analysis of methylation data collected with each one of the two different techniques highlighted a cluster of CpGs with correlated methylation levels within exon1 of *ZBTB12*, hypomethylated in MI cases *vs* controls. This complies with our study design assumption that the methylation status of multiple CpGs with correlated methylation could better describe the cumulative methylation status of the underlying region, and that this could be potentially related to the underlying biological function, if any. The same goes for LINE-1 methylation, which is defined as the cumulative DNA-methylation status of the several CpGs located in LINE-1 sequences across the genome. Also in this case, different portions of LINE-1 sequence were investigated with the 2 techniques, i.e. CpGs scattered across the whole LINE-1 sequence were analyzed on the BeadChip, whereas CpGs within base pairs 335–767 of the LINE-1 promoter (Gen-Bank accession number X58075.1) were analyzed by MassArray according to Wang et al. [48] (Figure S4).

Despit**e** slight differences between EPICOR and EPIC-NL panels in LINE-1 average methylation levels, arguably due to the use of different methods and different assayed CpGs, our results highlighted the same effect trend in both the EPIC sub-cohorts.

When included in the same multivariate models, the estimated risks associated to *ZBTB12*-DMR and LINE-1 methylation profiles were not attenuated by the adjustment for known risk factors (Additional file 1: Table S3A and B), suggesting that they independently contribute to MI risk estimate.

Moreover, we observed that discrimination between MI cases and controls and prediction accuracy both improved when DNA-methylation was taken into account together with traditional risk factors, suggesting the DNA-methylation could be an independent predictor of MI risk, although further confirmation on a larger sample is warranted.

Our results highlight the possibility to identify MI-related methylation marks on DNA from blood samples drawn in a preclinical condition, for some subjects many years before the MI. Unfortunately, due to the initial EPIC study design that envisaged only one blood sampling at enrollment time, it was not possible to monitor individual DNA-methylation level changes at different time points. Further replication in additional cohorts with prospective design and biospecimens sampled at multiple points along time is warranted to elucidate DNA-methylation changes across time, from 'healthy' status to MI. This will allow a better estimation of the *ZBTB12*-DMR and LINE1 de-methylation rates associated with increased MI risk, in the view of a personalized risk assessment that will take into account TRFs and MI risk biomarkers, such as DNA-methylation profiles.

Conclusions

To the best of our knowledge, this is the first paper reporting an association between MI risk and DNA-methylation profiles identified from epigenome-wide data in prospectively collected subjects with well-recorded clinical endpoints, and replicated in an independent sample form the same large European prospective cohort.

Taken together, the reported results suggest the possible role of DNA-methylation patterns in peripheral blood white cells as promising early MI biomarkers to be potentially used, together with TRFs, for individual MI risk assessment.

Methods

Study population

For the discovery phase, 292 MI cases and 292 matched healthy controls were recruited among those enrolled in the EPICOR study [49], a case-cohort study nested within the EPIC-Italy prospective cohort (~50.000 participants) [50]. All EPICOR cases developed MI after recruitment (average time

to diagnosis 6.90 years). Cases were identified at cohort follow-up from hospital discharge databases, and were then matched with healthy controls from the same cohort without evidence of MI at follow-up. Matching parameters were age at recruitment (±1.5 years), sex, center and season of recruitment. Results from the discovery phase were replicated in an independent sample of 317 Dutch subjects from the prospective EPIC-NL cohort [51] who developed MI during follow-up (average time to diagnosis 5.64 years) and 262 unmatched healthy controls from the same cohort. Details on anthropometrics, lifestyle, biochemical measurements, and MI definition are provided in Additional file 2: Supplementary Methods.

Ethical considerations

Our study complies with the Declaration of Helsinki principles, and conforms to ethical requirements. All volunteers signed an informed consent form at enrollment in the respective studies. The EPIC study protocol was approved by Ethics Committees of the International Agency for Research on Cancer (Lyon, France), as well as by local Ethical Committees of the participant centers. The EPICOR study was approved by the Ethical Committee of the Human Genetics Foundation (Turin, Italy). For the Dutch EPIC samples, approval was obtained by the Institutional Review Board of the University Medical Center Utrecht (Utrecht, the Netherlands) and the Medical Ethical Committee of TNO Nutrition and Food Research (Zeist, the Netherlands).

DNA-methylation measurement

DNA-methylation was measured in DNA from WBCs collected at subject enrollment into EPIC and stored in liquid nitrogen [47]. The Infinium HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA) and the MALDI-TOF mass spectrometry methylation assay (Sequenom Inc., S. Diego, CA, USA) were used for the discovery phase and the replication phase, respectively. Laboratory methods for DNA-methylation levels measurement, are detailed in Additional file 2: Supplementary Methods.

Whole-genome methylation data quality control (QC) and normalization procedures

DNA-methylation levels were measured as Beta-values, ranging from 0 to 1. We excluded from the analyses: i) single Beta-values with detection p-value≥0.01; ii) CpG loci with detection p-value≥0.01 in more than 20% of the assayed samples; iii) probes containing SNPs with MAF≥0.05 in the CEPH (Utah residents with ancestry from northern and western Europe, CEU) population; iv) samples with a global call rate ≤95%.

From the 435,457 CpGs that passed QCs (~95% of BeadChip content), we further removed 9,959 CpGs whose methylation signal was detected by cross-hybridizing and SNPs-containing probes [52]. A total of 292 matched case-control pairs and 425,498 CpG sites were used in the following analyses. Background normalization was performed on raw methylation data according to Marabita et al. [53].

Statistical analyses

Statistical analyses were conducted using the open source R v3.0.1 package [54].

Analyses were performed stratifying by sex, in order to account for the occurrence of sex-specific DNA-methylation, and for the different cardiovascular risk profiles between men and women (see Discussion). Descriptive statistics of sample characteristics, anthropometrics, lipid profiles, hypertension, and lifestyle habits (smoke, alcohol consumption) was performed.

Case-control DMRs analysis

We analyzed the EPICOR methylation data (discovery phase, 425,498 CpGs) with the A-clustering algorithm [24] to identify clusters of 2 or more neighboring CpGs with correlated methylation levels. The association between each one of the identified methylation clusters and case-control status was tested by Generalized Estimating Equations (GEE) [55] to identify DMRs between MI cases and controls. We adjusted the analyses for matching variables (age at recruitment, center, season of recruitment, sex in the overall analyses), estimated WBC composition (for the EPICOR panel only), and for the major cardiovascular risk factors [56] i.e. smoking status, BMI, blood pressure, physical activity (for the EPICOR panel only). EPICOR sample analyses were additionally adjusted for 'control probes' Principal Components, while EPIC-NL analyses did not require batch correction (see Additional file 2, Removal of technical biases).

As fasting glucose measurement was missing for >20% of the EPICOR and EPIC-NL samples, glucose level was excluded from the adjustment covariates. Lipid levels were missing for 48 EPICOR subjects: lipid levels were omitted as covariates too, after verification that inclusion or exclusion of this parameter did not substantially affected the results (Additional File 2: Supplementary Methods). Due to the small number of subjects with incident diabetes identified at follow-up (n=9), diabetes was not included in the covariate list.

DMRs with FDR *Q*-value<0.05 were considered statistically significant and investigated in the EPIC-NL sample with the same statistical approach. The Q statistic [57] was used to assess heterogeneity between the two sample panels: provided no heterogeneity was found, an inverse variance weighted fixed effect meta-analysis, was additionally carried out to achieve an overall estimate of the two studies.

Case-control LINE-1 methylation analyses

To analyze LINE-1 methylation levels from BeadChip data we first identified all the BeadChip's CpGs lying in LINE-1 sequences according to the UCSC Genome Browser database. The cumulative DNA-methylation level of LINE-1 sequences was computed, for each subject, as the average methylation level across the 12,762 CpGs, out of the >450K assayed on the BeadChip, that were annotated in LINE-1 sequences. Case-control differences were assayed by logistic regression, with methylation levels as a continuous variable, and the same adjustment used for the case-control DMRs discovery and replication analyses. For replication purposes, the same analysis was performed on the EPIC-NL samples using LINE-1 methylation data from MassARRAY analysis (Additional file 2: Supplementary Methods). A LINE-1 methylation meta-analysis of the two studies was also done as described above.

DNA methylation and MI risk

EPICOR and EPIC-NL subjects, stratified by sex and by study, were clustered with a RPMM algorithm [58] into 4 classes according to their *ZBTB12*-DMR methylation profile, irrespective of case-control status. Each subject was also allocated to a LINE-1 methylation class (above/below the

median). The association between MI and DNA-methylation (as RPMM class, or LINE-1 methylation profile) was evaluated on the EPIC-NL panel by logistic regression analysis, stratifying by sex. Moreover, to test the dependence/independence of the DNA-methylation effects from the traditional risk factors, we compared the ORs associated to each RPMM class and to LINE-1 methylation status under three logistic regression models, progressively including additional covariates at each step. To this purpose, the same analysis was done on the EPICOR discovery panel as well, under the caveat that the estimated ORs in this case should not be considered as a risk estimate, being assessed in the discovery panel and, as such, putatively inflated. Briefly, Model 1 included the matching variables only, Model 2 included the whole set of covariates used for the case-control DMRs discovery and replication analyses, and Model 3 was fully adjusted with the comprehensive set of variables as available in the 2 studies. Further methodological details are provided in Additional file 2: Supplementary Methods.

Discrimination, reclassification, and calibration

We tested for the improvement in the performance of MI risk prediction when including DMRs and LINE-1 profiles identified in the EPICOR dataset (discovery phase) by running discrimination and reclassification analyses on the independent EPIC-NL dataset. Two models were compared: the first one included only TRFs that were significantly associated to MI in our study or reported in the literature to be associated to MI (Figure 1, legend); the second one comprised TRFs as model 1 plus *ZBTB12*-RPMM classes and LINE-1 methylation class.

For discrimination, we compared the AUC of the two models by the DeLong test [59]. For reclassification, we computed the NRI and IDI indices [60]. The goodness-of-fit was evaluated by the Hosmer-Lemeshow (HL) test [61] in order to assess the proper calibration of the model.

DNA-methylation and TTD

Being EPICOR and EPIC-NL prospective cohorts with incident MI cases identified during cohort follow-up, we investigated the relationship between methylation and TTD, i.e. the time lapse between blood collection and the MI event. EPICOR and EPIC-NL cases, stratified by study and by sex, were

divided in tertiles according to TTD. Control groups were used as reference. The occurrence of a linear trend between DNA-methylation levels and TTD, as ordinal categorical variable, was tested by GEE (details in Additional file 2).

Abbreviations

WBCs: white blood cells

MI: myocardial infarction

DMR: differentially methylated region

CVD: cardiovascular disease

LINE-1: long interspersed nuclear element-1

EWAS: epigenome-wide association study

EPIC: European Prospective Investigation into Cancer and Nutrition

BMI: body mass index

WHR: waist-to-hip ratio

QC: quality control

FDR: false discovery rate

TRFs: traditional risk factors

NRI index: net reclassification improvement index

IDI index: integrated discrimination improvement index

AUC: area under receiver operating curve

RPMM: recursively partitioned mixture model

TTD: time to disease

MBPs: methyl-CpG binding proteins

GEE: generalized estimating equations

Beta-value: estimate of methylation level at each CpG

Competing interests

The author(s) declare that they have no competing interests

Authors' contribution

GM, SPa, VK, CS, SG and GFi conceived the study.

SPa, AM, PC, VK, CA, RT, GFr, PV, FRi, CS and GM for EPIC-Italy (EPICOR), and MWMV, JMAB, NCOM and YTvdS for EPIC-NL enrolled the subjects, managed personal information databases (data from questionnaires, clinical data, cohort follow up data) as responsible of the respective cohorts, coordinated biospecimens storage, retrieval, and shipment to the analytical laboratories at HuGeF;

GFi, PC, and FRi contributed to sample selection and EPIC-Italy (EPICOR) database management;

SG, AA, AR and SPo, carried out all the laboratory analyses from DNA extraction (EPICOR) to DNA-methylation analyses (EPICOR and EPIC-NL);

LI carried out all the biochemical measurements on the EPICOR samples;

GFi carried out all the statistical analyses;

SG, AA, AR, CDG, GM substantially contributed to the interpretation of results;

SG wrote the manuscript;

GM, GFi, CDG, MWMV, JMAB, NCOM, YTvdS, PV, CS, LI, VK and SPa critically revised the manuscript content and provided important intellectual content;

All authors read and approved the final manuscript.

Additional files

Additional file 1: Supplemental Tables S1, S2, S3, S4

A document with all supplementary Tables (S1 to S4). **Table S1:** Top 6 genic DMRs in EPICOR MI overall cases *vs* controls. **Table S2A:** Details of the ZBTB12-DMR CpGs in EPICOR subjects. **Table S2B:** Details of the *ZBTB12*-DMR CpGs in EPIC-NL subjects. **Table S3A:** EPIC-NL MI risk, Adjusted Models. **Table S3B:** EPICOR, Adjusted Models. **Table S4A:** EPICOR case-control differential methylation analysis: comparison of models with and without lipids adjustment. **Table S4B:** EPIC-NL case-control differential methylation analysis: comparison of models with and without lipids, batch, and WBCs adjustments.

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Additional file 2: Supplementary Methods

A document with supplementary materials, including: (1) **Subjects:** Cohort details; Lifestyle, anthropometrics, and biochemical measurements; Outcome definition; (2) **Laboratory methods:** EPICOR Sample preparation; Discovery phase: Illumina Human450K Methylation Assay; Replication phase on EPIC-NL sample: Sequenom MassArray; (3) **Supplementary statistical methods:** Case-control differential methylation; Removal of technical biases; DNA methylation and MI risk; DNA-methylation and time to disease (TTD); Supplementary References.

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Figure Legends

Figure 1. Receiver Operating Curves (ROC), EPIC-NL validation sample

Model1 (TRFs, dotted line) includes age, sex, center of recruitment, smoking habits, BMI, WHR, lipid levels, blood pressure, menopausal status in women.

Model2 (TRFs+Meth., solid line), as Model1 plus *ZBTB12*-RPMM classes, LINE-1 methylation profile.

Panel A: EPIC-NL Men; B: EPIC-NL Women. Statistics in Table 2.

Figure 2. Calibration Plots, EPIC-NL validation sample

Goodness of Fit, Model1 (TRFs, triangles) *vs* Model2 (TRFs+Meth., dots).

Hosmer-Lemeshow test: Men: P_{TRF}=0.118, P_{TRF+M}=0.414; Women: P_{TRF}=0.636, P_{TRF+M}=0.724

Panel A: EPIC-NL Men; B: EPIC-NL Women. Statistics in Table 2.

Supplementary Figures Legends

Figure S1: Quantile-Quantile plot, EPICOR overall subjects

Figure S2: Quantile-Quantile plot, EPICOR men

Figure S3: Quantile-Quantile plot, EPICOR women

Figure S4: Locations of *ZBTB12* and LINE-1 CpG sites investigated by Sequenom MassARRAY

CpGs (in red) investigated within ZBTB12-DMR, LINE-1, and flanking primers (upper case: complementary to DNA; lower case: T7-promoter sequence and 10mer tag).

CpG sites that could not be tested individually due to MassArray technology constrains, but had to be tested jointly with neighboring CpGs as a single unit, are underlined: the methylation level is the cumulative value of all the sites within the CpG unit.

TABLE 1. EPICOR and EPIC-NL sample descriptive

	EPICOR MEN		EPICOR WOMEN		EPIC-NL MEN		EPIC-NL WOMEN	
	CASES (N=188)	**CONTROLS (N=188)**	**CASES (N=104)**	**CONTROLS (N=104)**	**CASES (N=116)**	**CONTROLS (N=83)**	**CASES (N=201)**	**CONTROLS (N=179)**
	N (%)	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
Centre								
Varese	42 (22.34)	42 (22.34)	67 (65.69)	67 (65.69)				
Ragusa	19 (10.11)	19 (10.11)	3 (2.94)	3 (2.94)				
Turin	127 (67.55)	127 (67.55)	23 (22.55)	23 (22.55)				
Naples	--	--	11 (10.78)	11 (10.78)				
Utrecht					--	--	149 (74.13)	140 (78.21)
Bilthoven					116 (100)	83 (100)	52 (25.87)	39 (21.79)

Smoking status								
Never	33 (17.55)	50 (26.60)	53 (50.96)	73 (70.19)	19 (16.38)	18 (21.69)	60 (29.85)	85 (47.49)
Former	70 (37.23)	87 (46.28)	11 (10.58)	13 (12.50)	34 (29.31)	32 (38.55)	48 (23.88)	48 (26.81)
Current	85 (45.21)	51 (27.13)	40 (38.46)	18 (17.31)	62 (53.45)	33 (39.76)	89 (44.28)	45 (25.14)
NA					1 (0.86)		4 (1.99)	1 (0.56)
Menopausal Status								
Pre-menopause			27 (25.96)	26 (25.00)			43 (21.39)	42 (23.46)
Post-menopause			77 (74.04)	78 (75.00)			158 (78.61)	137 (76.54)
	Mean±SD	**Mean±SD**	**Mean±SD**	**Mean±SD**	**Mean±SD**	**Mean±SD**	**Mean±SD**	**Mean±SD**
Age at recruitment (years)	50.98±6.93	50.92±7.01	55.01±7.40	55.01±7.51	51.51±7.68	51.11±8.30	58.56±8.74	59.30±8.12
Avg. Follow -up (years)	12.98±2.29	13.26±2.06	12.24±1.97	12.66±1.16	13.07±5.21	15.25±2.31†	11.61±4.92	14.27±2.62†
Avg. TTD (years)	7.14±3.88	--	6.54±3.57	--	5.44±3.22	--	5.76±3.06	--

BMI (kg/m2)	27.05±2.96	26.35±3.12†	26.95±4.69	25.93±5.11	27.12±3.30	27.08±3.15	26.47±4.24	26.05±4.17
WHR	0.94±0.06	0.93±0.06†	0.83±0.06	0.79±0.06†	0.95±0.08	0.94±0.08	0.82±0.07	0.80±0.07†
Total Cholesterol (mmol/L)	6.10±1.12	5.86±1.22	6.42±1.23	6.36±1.16	6.24±0.95	5.79±0.97†	5.51±0.98	5.27±0.98†
LDL Cholesterol (mmol/L)	3.94±1.00	3.60±1.01†	4.07±1.15	3.97±1.01	3.60±0.94	3.34±0.92	3.44±0.82	3.19±0.77†
HDL Cholesterol (mmol/L)	1.30±0.29	1.48±0.37†	1.55±0.39	1.76±0.41†	1.11±0.28	1.12±0.28	1.16±0.33	1.27±0.36†
Triglycerides (mmol/L)	1.89±0.99	1.71±1.04	1.74±1.43	1.38±0.57†	2.28±1.27	2.28±1.46	1.77±0.98	1.54±0.91†
SBP (mmHg)	137.47±16.56	135.04±19.03	144.36±9.77	136.76±10.08†	134.43±17.61	128.51±14.93†	138.50±22.23	133.59±20.76†
DBP (mmHg)	85.38±8.96	84.97±10.80	86.08±22.13	84.97±19.02	84.96±10.71	80.60±10.19†	81.99±11.20	79.22±11.18†
Alcohol (gr/day)	23.46±20.26	24.98±20.90	6.29±10.78	8.76±15.43	19.42±21.89	18.41±22.27	7.55±11.24	8.44±12.21

*Chi-Squared test P<0.05

†T-Test P<0.05

LDL = Low Density Lipoprotein; HDL = High Density Lipoprotein; SBP = Systolic Blood Pressure; DBP = Diastolic Blood Pressure; TTD: time to disease.

TABLE 2. Discrimination and reclassification indices, EPIC-NL validation sample

	AUC_{TRF} (95%CI)	**AUC_{TRF+M} (95%CI)**	**DeLong's test *P***	**NRI (95%CI)**	**P_{NRI}**	**IDI (95%CI)**	**P_{IDI}**
EPIC-NL MEN	0.66 (0.58-0.74)	0.70 (0.63-0.78)	0.147	0.47 (0.19-0.76)	0.001	0.04 (0.01-0.08)	0.004
EPIC-NL WOMEN	0.66 (0.61-0.72)	0.69 (0.63-0.74)	0.095	0.23 (0.02-0.43)	0.034	0.03 (0.01-0.05)	0.001

Table 3. DNA-methylation and time-to-disease (TTD)

		ZBTB12					LINE-1				
TTD Class*	**Range‡**	**Effect-size**	**95%CI**	**se**	**P_{TREND}**	**Cochran's Q**	**Effect-size**	**95%CI**	**se**	**P_{TREND}**	**Cochran's Q**
EPICOR MEN											
TTD class1	8.89-14.66										
TTD class2	5.23-8.88	-0.0054	-0.0090;-0.0018	0.0018	0.0036		-0.0009	-0.0016;-0.0003	0.0003	0.0044	
TTD class3	0.26-5.20										
EPIC-NL MEN											
TTD class1	6.97-12.31										
TTD class2	3.53-6.86	-0.0093	-0.0182;-0.0005	0.0045	0.0389		-0.0035	-0.0070;0.00003	0.0018	0.0537	
TTD class3	0.23-3.52										
Meta-analysis		-0.0059	-0.0093;-0.0027	0.0017	0.0005	0.65†	-0.0010	-0.0017;-0.0004	0.0003	0.0016	2.03†
EPICOR WOMEN											
TTD class1	8.16-14.02										
TTD class2	4.40-8.06	-0.0056	-0.0106;-0.0007	0.0025	0.0250		-0.0008	-0.0017;0.00004	0.0004	0.0636	
TTD class3	0.33-4.30										
EPIC-NL WOMEN											

TTD class1	7.35-12.30										
TTD class2	4.40-7.21	-0.0047	-0.0106;0.0011	0.0030	0.1147		-0.0011	-0.0032;0.0010	0.0011	0.2970	
TTD class3	0.04-4.38										
Meta-analysis		-0.0053	-0.0091;-0.0015	0.0019	0.0065	0.05†	-0.0008	-0.0016;-0.0001	0.0004	0.0263	0.07†

*Healthy controls (TTD class 0) were used as reference group. Cases were divided in tertiles (TTD classes 1 to 3)

‡Minimum and maximum TTD (i.e. time-lapse in years from enrollment to occurrence of MI) for each class

†d.f.=1, *P*=ns

Figure 1 - Receiver Operating Curves

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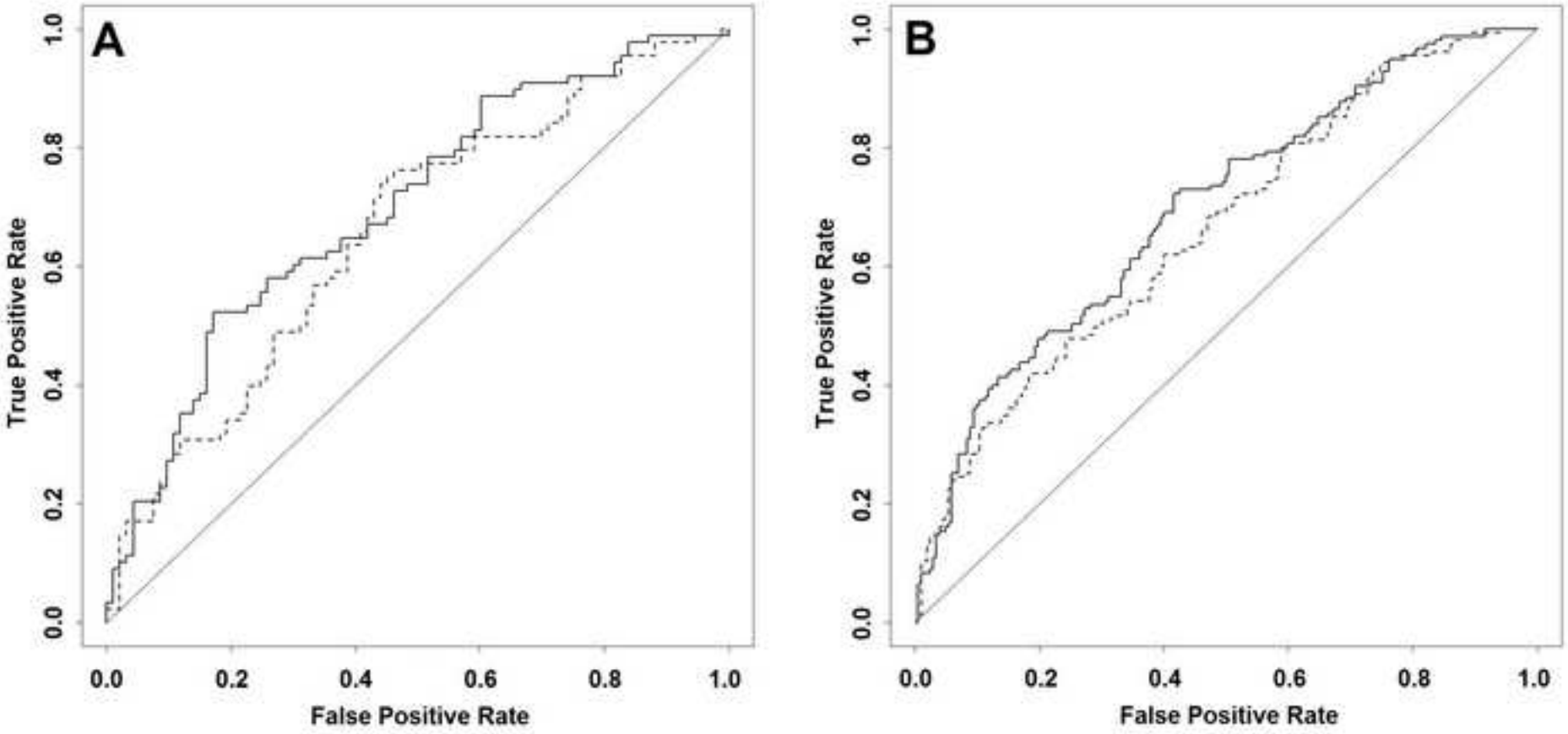

Figure 2 - Calibration Plots

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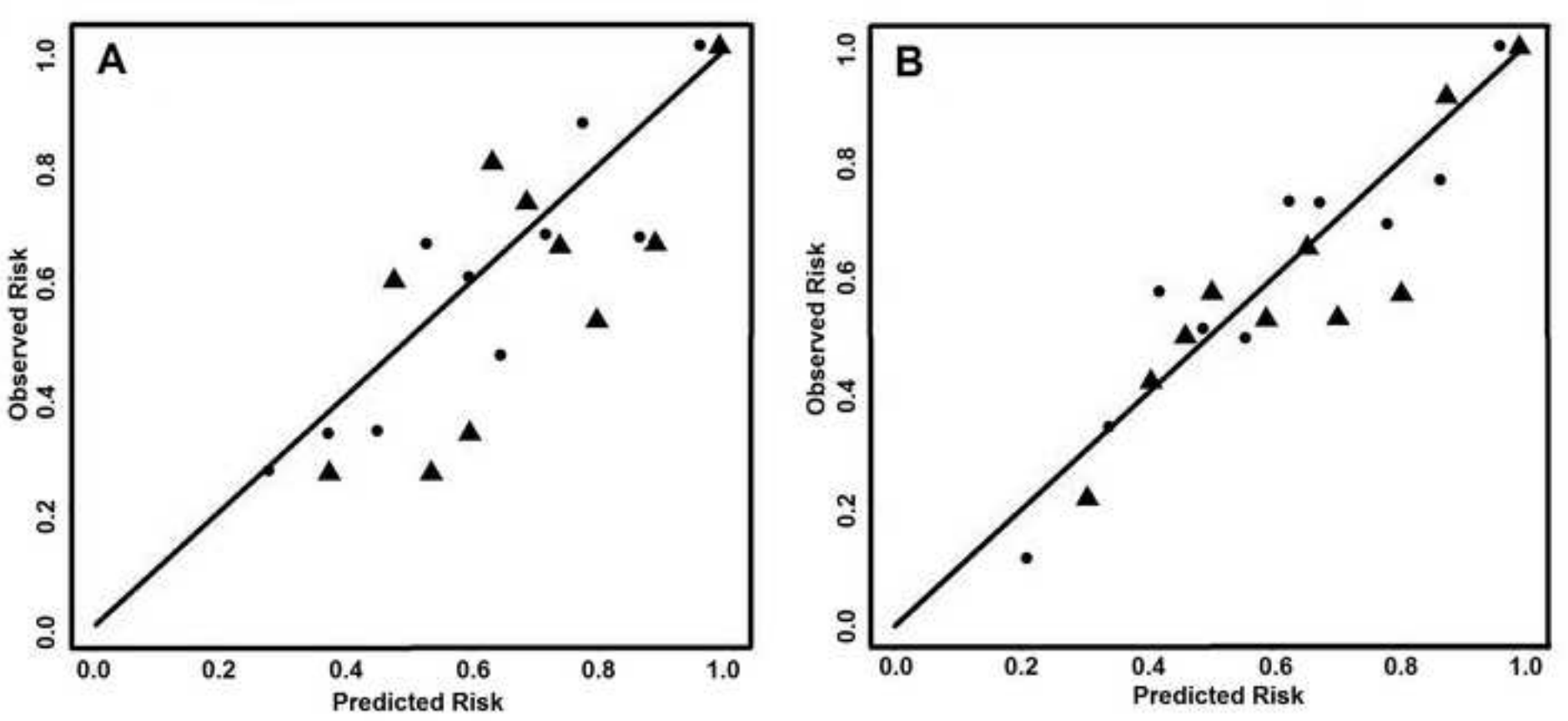

Suppl Figure S1 - QQplot_EPICOR overall

Suppl Figure S2 - QQplot_EPICOR men

Suppl Figure S3 - QQplot_EPICOR women