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Circulating microRNAs as predictive biomarkers of myocardial infarction: Evidence from the HUNT study

Torbjørn Velle-Forbord[a,1], Maria Eidlaug[a,1], Julia Debik[a], Julie Caroline Sæther[a,b], Turid Follestad[c], Javaid Nauman[a,d], Bruna Gigante[e], Helge Røsjø[f,g], Torbjørn Omland[f,g], Mette Langaas[h], Anja Bye[a,b,*]

[a] Department of Circulation and Medical Imaging, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Norway
[b] Department of Cardiology, St. Olavs Hospital, Trondheim, Norway
[c] Department of Public Health and Nursing, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology (NTNU), Norway
[d] Institute of Public Health, College of Medicine and Health Sciences, United Arab Emirates University, Al-Ain, United Arab Emirates
[e] Unit of Cardiovascular Epidemiology, Institute for Environmental Medicine, Karolinska Institutet, Stockholm, Sweden
[f] Division of Medicine, Akershus University Hospital, Lørenskog, Norway
[g] Division of Medicine and Laboratory Sciences,The University of Oslo, Oslo, Norway
[h] Department of Mathematical Sciences, Faculty of Information Technology and Electrical Engineering, Norwegian University of Science and Technology (NTNU), Norway

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ABSTRACT

Background and aims: Several risk prediction models for coronary heart disease (CHD) are available today, however, they only explain a modest proportion of the incidence. Circulating microRNAs (miRs) have recently been associated with processes in CHD development, and may therefore represent new potential risk markers. The aim of the study was to assess the incremental value of adding circulating miRs to the Framingham Risk Score (FRS).
Methods: This is a case-control study with a 10-year observation period, with fatal and non-fatal myocardial infarction (MI) as endpoint. At baseline, ten candidate miRs were quantified by real-time polymerase chain reaction in serum samples from 195 healthy participants (60–79 years old). During the follow-up, 96 participants experienced either a fatal (n = 36) or a non-fatal MI (n = 60), whereas the controls (n = 99) remained healthy. By using best subset logistic regression, we identified the miRs that together with the FRS for hard CHD best predicted future MI. The model evaluation was performed by 10-fold cross-validation reporting area under curve (AUC) from the receiver operating characteristic curve (ROC).
Results: The best miR-based logistic regression risk-prediction model for MI consisted of a combination of miR-21-5p, miR-26a-5p, mir-29c-3p, miR-144-3p and miR-151a-5p. By adding these 5 miRs to the FRS, AUC increased from 0.66 to 0.80. In comparison, adding other important CHD risk factors (waist-hip ratio, triglycerides, glucose, creatinine) to the FRS only increased AUC from 0.66 to 0.68.
Conclusions: Circulating levels of miRs can add value on top of traditional risk markers in predicting future MI in healthy individuals.

1. Introduction

Coronary heart disease (CHD) is the most common cause of death globally. In the next decade, the number of people at risk is expected to increase, due to obesity, inactivity, diabetes and ageing [1–3]. Thus, in the years to come, it will be increasingly important to identify the individuals at risk to initiate preventive measures before a serious event occurs.

CHD is a multifactorial disease with several well-known risk factors. Numerous risk prediction models have been developed based on these risk factors to estimate individuals' 10-year risk of CHD, and especially the risk of myocardial infarction (MI) [4–10]. However, the current risk prediction models only explain a modest proportion of the incidence. It is estimated that 15–20

% of patients developing MI lack all the conventional risk factors and therefore would be classified as "low risk" by the current risk prediction models [11].

There have been several attempts during the last years to improve the risk prediction models for CHD by incorporating new biomarkers [12–23]. For instance, incorporation of C-reactive protein (CRP) and

* Corresponding author. NTNU, Department of Circulation and Medical Imaging, Postboks 8905, Medisinsk teknisk forskningssenter, 7491, Trondheim, Norway.
Email address: Anja.Bye@ntnu.no (A. Bye)
[1] These authors contributed equally to this work.

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glycated haemoglobin (HbA1c) into the Framingham risk score (FRS) was shown to improve risk prediction for both genders (the Reynolds Risk Score) [18–20]. However, despite extensive studies, there are currently no biomarkers that adequately predict the risk of developing MI. Thus, there is a clinical need for new biomarkers that could complement the assessment of traditional risk factors, to identify the individuals at risk with greater precision than today [24].

MicroRNAs (miRs) are small non-coding ribonucleic acid molecules that act as post-transcriptional regulators of gene expression. They have been identified as key regulators of different biological and pathological processes including ischaemic heart disease, left ventricular hypertrophy, heart failure, hypertension and arrhythmias [25]. It is now accepted that miRs are involved in almost all steps of atherogenesis and thus the development of CHD [26]. In 2008, it was discovered that stable amounts of miRs enter the circulation [27–30]. Since then, the potential of using circulating miRs as a biomarker of disease has emerged, and several studies have shown that circulating miRs have potential as biomarkers of CHD [31,32]. However, few studies have evaluated the potential of miRs in risk prediction of future cardiovascular events. Zampetaki et al. and Bye et al. previously assessed the predictive potential for CHD in a primary prevention cohort and Schulte et al. in a secondary prevention cohort [33–35].

The aim of this study was to determine the association between circulating miRs and the risk of developing MI in healthy asymptomatic individuals, and to assess the incremental value of adding circulating miRs to the FRS.

2. Material and methods

2.1. Study design and ethics

This was a case-control study with a 10-year observation period. Primary endpoints were fatal and non-fatal MI. The Nord-Trøndelag Health Study (HUNT) and Regional Ethical Committee (REK) approved the study. Patient information was stored and handled in conformity with Norwegian laws and regulations.

2.2. Subjects

The HUNT study (an acronym for the name: The Nord-Trøndelag Healthy Study) is a large population-based cohort including 125 000 Norwegian participants. So far four health surveys have been completed, HUNT1 (1984–86), HUNT2 (1995–97), HUNT3 (2006–08) and HUNT4.

(2017–19). The HUNT studies were carried out in Nord-Trøndelag county of Norway and every citizen >20 years was invited. The HUNT study includes data from surveys, interviews, clinical measurements and biological samples [36]. By using data from the *Norwegian Myocardial Infarction Registry* and the *Norwegian Cause of Death Registry,* we identified all participants in the HUNT2 cohort that suffered from fatal or non-fatal MI within the following 10 years. Among the participants reporting to be healthy at HUNT2 (baseline) aged 60–79 years, 36 participants suffered from a fatal MI and 60 participants suffered from a non-fatal MI within the next 10 years. Furthermore, we selected 99 age- and gender group-matched controls that reported to be healthy at HUNT2 (baseline) and at HUNT3 (follow-up). Participants were defined as healthy if they reported no previous or current self-reported cardiovascular disease (including stroke and angina pectoris), no previous or current use of antihypertensive drugs, any chronic kidney disease or diabetes mellitus.

2.3. Data collection

Variables needed for the calculation of FRS for hard CHD were collected from the HUNT database (age, total cholesterol, HDL cholesterol, systolic blood pressure, treatment for hypertension and smoking status, at the time of HUNT2). Information on other CHD risk factors like waist-hip ratio, serum triglycerides, glucose and creatinine was also collected. Data from the HUNT3 study was used to select controls that were defined as healthy 10 years after baseline.

2.4. Sample collection

200 μL frozen serum from each participant, collected during HUNT2 (baseline), was used for miR isolation and analysis. Standard biochemical analyses to quantify traditional risk markers were performed in non-fasting serum from fresh venous blood samples.

2.5. Blood analysis

Serum total cholesterol (mmol/L), serum HDL-cholesterol (mmol/L) and serum triglycerides (mmol/L) were measured using an enzymatic coulometric method. Serum non-fasting glucose (mmol/L) was measured by using an enzymatic hexokinase method. Serum Creatinine (μmol/L) was measured by kinetic Jaffè method with sample blank (Roche Diagnostics, Mannheim, Germany). All analyses were conducted with Hitachi 911 Autoanalyzer (Hitachi, Mito, Japan) at Levanger Hospital, Norway, using reagents from Boehringer Mannheim (Mannhein, Germany).

2.6. Risk score calculations

FRS for hard CHD was used in this study as it corresponded best to the selected endpoints of fatal and non-fatal MI [37]. The FRS was originally developed from a white American population, and application to diverse populations has shown to overestimate the CHD risk [38]. Therefore, we performed a simple calibration after adjustment of the FRS by replacing the average Framingham underlying risk of the Cox's hazard regression model with the average event rate of the HUNT study estimated by the Kaplan-Meier estimates, and the means of the risk factors from the FRS with the means of the HUNT study [39].

2.7. MicroRNA selection

In 2016, Bye et al. published a prospective, nested case-control study with a 10-year observation period to explore the prediction potential of circulating miRs on future fatal MI in 220 apparently healthy subjects from the HUNT2 study [34]. Several circulating miRs were significantly different in blood samples from individuals who later suffered a fatal MI, compared to those who remained healthy during the follow-up. We chose a panel of ten circulating miRs based on these findings: let-7g-5p, miR-21-5p, miR-26a-5p, miR-29c-3p, miR-106a-5p, miR-144-3p, miR-151a-5p, miR-191-5p, miR-424-5p and miR-451a. miR-425-5p was included for normalization purposes.

2.8. RNA isolation from serum

Total RNA was extracted from serum using the miRCURY™ RNA isolation kit – biofluids (Exiqon, Vedbaek, Denmark). Serum samples were centrifuged at 3000 × *g* for 5 min in a 4 °C microcentrifuge. 190 μL of serum was then transferred to a new microcentrifuge tube and 60 μL of lysis solution buffer containing 1 μg carrier-RNA, and RNA spike-in

template mixture was added to the sample. The tube was vortexed and incubated for 3 min at room temperature, followed by addition of 20 μL Protein Precipitation solution buffer. The tube was vortexed, incubated for 1 min at room temperature and centrifuged at 11 000 × *g* for 3 min. The clear supernatant was transferred to a new collection tube, and 270 μL isopropanol was added. The solutions were vortexed and transferred to a binding column. The column was incubated for 2 min at room temperature, and emptied using a vacuum-manifold. 100 μL wash solution 1 buffer was added to the columns. The liquid was again removed using a vacuum-manifold, and 700 μL wash solution 2 buffer was added. The liquid was removed using a vacuum-manifold. 250 μL wash solution was added and the column was spun at 11.000 × *g* to dry the columns entirely. The dry columns were transferred to a new collection tube and 50 μL RNase free H_2O was added directly on the membrane of the spin column. The column was incubated for 1 min at room temperature prior to centrifugation at 11.000 × *g*. The RNA was stored in a −80 °C freezer. For the RNA isolation step, 3 RNA spike-ins (UniSp2, UniSp4 and UniSp5) were added as RNA isolation controls.

2.9. MicroRNA real-time quantitative PCR (qPCR)

2 μL RNA was reverse transcribed in 10 μL reactions using the miRCURY LNA™ Universal RT microRNA PCR, Polyadenylation and cDNA synthesis kit (Exiqon). cDNA was diluted 50 x and assayed in 10 μL PCR reactions according to the protocol for miRCURY LNA™ Universal RT microRNA PCR; each miR was assayed once by qPCR on the microRNA Ready-to-Use PCR, Custom Pick and Mix Panel using ExiLENT SYBR® Green master mix. Negative controls excluding template from the reverse transcription reaction was performed and profiled like the samples. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche). The amplification curves were analysed using the Roche LC software, both for determination of Cq (by the 2nd derivative method) and for melting curve analysis. For the reversed transcription step, one spike-in (UniSp6) was added to confirm that the reverse transcription and amplification occurs with equal efficiency in all samples. All assays were inspected for distinct melting curves and the melting temperature was checked to be within known specifications for the assay. Furthermore, assays had to be detected with 3 Cqs less than.

The negative control, and with Cq < 37 to be included in the data analysis. Data that did not pass these criteria were omitted from any further analysis. Cq was set at the change point for the amplification curve (2nd derivative equal zero). Using NormFinder, the best normalizer was found to be miR-425-5p, as anticipated from previous experience [34,40–42]. All data were therefore normalized to the level of miR-425–5p (normalized $\Delta Cq_{\text{candidate miR}} = Cq_{\text{miR-425-5p}} - Cq_{\text{candidate miR}}$). The difference in miR expression between cases and controls was calculated as mean ΔCq_{case} - mean $\Delta Cq_{\text{controls}} = \Delta\Delta Cq$.

2.10. Statistical analysis

Data was analysed using the statistical packages IBM SPSS Statistics 23, Stata and R (R Core Team, 2013), with R packages foreign, bestglm (A.I. McLeod and Changjiang Xu,2017), corrplot (Taiyun Wei and Viliam Simko,2017) and pROC [43]. The Anderson-Darling test, histograms and QQ-plots were used to determine whether the data were normally distributed or not. If normally distributed, the independent sample *t*-test was used to compare baseline characteristics and miR expression between cases and controls. The Mann-Whitney *U* test was used for non-normally distributed characteristics and the Fisher's exact test for categorical variables. Correlation between traditional risk factors and miRs was tested using Pearson correlation analyses. Logistic regression was used to estimate the effect of the FRS for hard CHD and additional covariates on the 10-year risk for MI [37,44]. In the logistic regression where the miR data were included, the normalized $\Delta Cq_{\text{candidate miR}}$ for the 10 miR were used as covariates. Best subset logistic regression, using the AIC (Akaike information criterion) as model selection criterion, was applied to find the set of miRs that together with FRS for hard CHD best predicted future MI. The results are presented as estimated odds ratio with 95% Wald confidence intervals and *p* values are given for the covariates in the final model. For model evaluation, 10-fold cross-validation was used for the ROC curves and the AUC, to reflect the performance of the method on new data [45]. All parts of the model fitting, also the best subset selection, were part of the cross-validation. All AUC values were reported with 95% confidence intervals, and to compare two AUC-values a paired hypothesis test was performed in the R package pROC based on the methods of DeLong et al. [43,46].

3. Results

Table 1 shows baseline information on the 195 participants in this study. All participants were healthy at the time of blood sampling. The cases (n = 96) experienced either a fatal (n = 36) or a non-fatal (n = 60) MI during the follow-up, whereas the controls (n = 99) remained healthy. The mean time lapse between baseline (admission in cohort) and the MI in the case group was 2.6 years (range 0.25–5.25 years). Concerning traditional CHD risk factors, there were significant differences between the groups as stated in Table 1 ($p < 0.05$).

When comparing cases and controls, six out of ten miRs differed significantly between the groups (Table 2). miR-144-3p and miR-26a-5p displayed the largest differences with a ΔΔCq value of −0.64 and 0.31 ($p < 0.001$). A sub-analysis excluding the 15 smokers (all from the group of cases) provided the same significant differences in miRs between the groups.

To illustrate the independence between these miRs and the traditional risk factors for MI, a Pearson correlation analysis was conducted. No correlations were found between circulating miRs and CHD risk factors as WHR, serum glucose, triglycerides or creatinine levels (Fig. 1). However, several strong positive correlations were seen between the different circulating miRs.

The best combination of miRs to supplement the FRS in predicting 10-year risk of MI was found using best subset logistic regression with AIC as model selection criterion, testing all combinations of miRs in

Table 1
Baseline characteristics of the study participants.

	Cases (n = 96)	Controls (n = 99)	*p* value
Age (years)	66.8 ± 7.6	66.7 ± 7.1	0.974[a]
Gender (female/male)	35/61	36/63	1.000
Smokers (%)	15 (15.6)	0 (0)	<0.001
BMI (kg/m^2)	27.1 ± 3.5	25.8 ± 2.6	0.004
WHR	0.88 ± 0.07	0.86 ± 0.06	0.098[a]
Cholesterol (mmol/L)	6.9 ± 1.0	6.6 ± 1.1	0.085
HDL cholesterol (mmol/L)	1.3 ± 0.3	1.5 ± 0.4	0.0013[a]
Triglycerides (mmol/L)	2.1 ± 0.8	1.7 ± 0.8	<0.001[a]
Non-fasting glucose (mmol/L)	5.4 ± 0.9	5.4 ± 0.8	0.728[a]
Serum creatinine (mg/dL)	92.3 ± 13.9	90.4 ± 13.6	0.350
Systolic blood pressure (mmHg)	152.2 ± 20.0	142.1 ± 15.8	<0.001
Framingham Risk Score	16 ± 8	11 ± 7	0.027

Data are shown as mean ± standard deviation or n (%).
BMI: Body mass index, WHR: Waist-hip ratio, HDL: High-density lipoprotein. Framingham Risk Score: 10-year percentage risk of CHD.
[a] Non-parametric test.

Table 2
Serum levels of microRNAs (quantified as ΔCq) and difference in cases versus controls (quantified as ΔΔCq).

MicroRNA	Cases ΔCq ± SD	Controls ΔCq ± SD	ΔΔCq (95% CI)	*p* value
n	96	99		
let-7g-5p	2.41 ± 0.59	2.20 ± 0.41	0.21 (0.07, 0.36)	0.004
miR-21-5p	5.22 ± 0.50	5.13 ± 0.40	0.09 (−0.04, 0.22)	0.162
miR-26a-5p	1.45 ± 0.54	1.15 ± 0.54	0.31 (0.15, 0.46)	<0.001
miR-29c-3p	0.74 ± 0.57	0.97 ± 0.52	−0.22 (−0.38, −0.07)	0.005
miR-106a-5p	2.86 ± 0.52	2.78 ± 0.44	0.08 (−0.05, 0.22)	0.232
miR-144-3p	2.57 ± 0.92	3.21 ± 0.66	−0.64 (−0.86, −0.41)	<0.001
miR-151a-5p	1.43 ± 0.59	1.33 ± 0.52	0.11 (−0.05, 0.26)	0.187
miR-191-5p	1.55 ± 0.54	1.41 ± 0.41	0.15 (0.01, 0.28)	0.035
miR-424-5p	0.63 ± 0.82	0.55 ± 0.92	0.08 (−0.17, 0.33)	0.519
miR-451a	7.80 ± 0.61	8.07 ± 0.54	−0.26 (−0.43, −0.10)	0.002

ΔCq values are shown as mean ± standard deviation, and ΔΔCq values are shown with 95% confidence intervals. A positive ΔΔCq value indicates increased miR expression in the cases and a negative ΔΔCq value indicates decreased miR expression in the cases.
SD: standard deviation, CI: confidence interval.

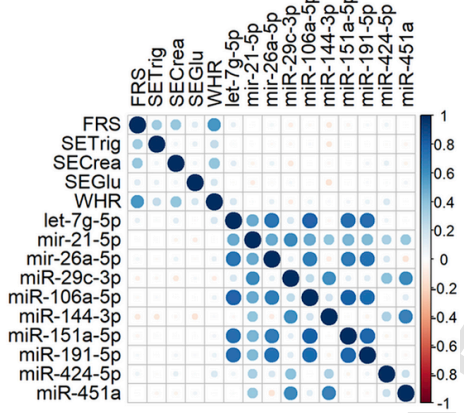

Fig. 1. Correlation plot (Pearson correlation) for all the quantified miRs, FRS and CHD-risk factors. Positive correlations are displayed in blue and negative correlations are displayed in red. The circle sizes and colour intensities are proportional to the absolute value of the corresponding correlation coefficients. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2. Adding a combination of miR-21-5p, miR-26a-5p, miR-29c-3p, miR-144-3p and miR-151a-5p to the FRS represented the best model for predicting 10-year risk of MI (results are presented as estimated odds ratio with 95% Wald confidence intervals and *p* values in the final model (Table 3)). By adding these 5 miRs to the FRS, the AUC increased from 0.66 [95% CI 0.59–0.74] to 0.80 [95% CI 0.73–0.86] (evaluated by 10-fold cross-validation). This 0.14 unit increase in AUC was significant ($p < 0.001$) and represented a substantial improvement in model performance. The performance of the FRS for hard CHD (**Model 1**) in comparison with the miR-FRS model (**Model 2**) is shown in Fig. 2 as ROC-curves (10-fold cross-validation). Furthermore, to test whether adding other well-known risk factors to the FRS could improve the prediction model to the same extent as by adding miRs, we made a new model incorporating information on waist-hip ratio, triglycerides, glucose, and creatinine to the FRS (**Model 4**). As seen from Fig. 2, this

Table 3
Results of the best subset logistic regression analysis with AIC as model selection criterion, using case/control as the response value.

Covariate	OR	95% CI	*p*-value
Framingham Risk Score (FRS)	1.10	1.05, 1.15	<0.001
miR-21–5p	11.3	2.82, 50.8	<0.001
miR-26a-5p	3.18	1.24, 8.56	0.018
miR-29c-3p	0.36	0.13, 0.94	0.039
miR-144-3p	0.29	0.15, 0.51	<0.001
miR-151a-5p	0.37	0.14, 0.92	0.038

OR: Odds ratio, where OR > 1, OR = 1 and OR < 1 indicate positive, no or inverse association between the covariate and MI, respectively, CI: confidence interval for the odds ratio.

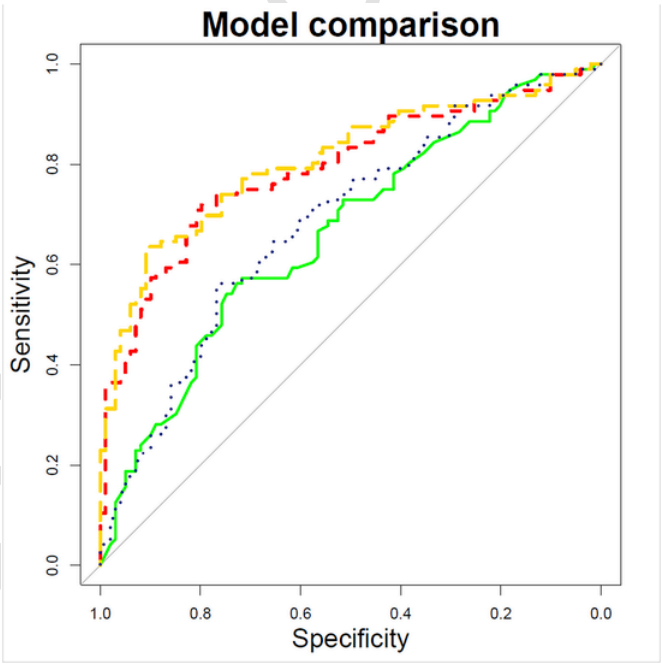

Fig. 2. Model performance illustrated with ROC-curves. The performance of the FRS (Model 1, green, solid), FRS + miRs (Model 2, red, short dash), FRS + miRs + other risk factors (Model 3, yellow, long dash) and FRS + other risk factors (Model 4, blue, dotted). miR: microRNA, FRS: Framingham risk score. The ROC-curves are based on 10-fold cross-validation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

only provided a minor improvement in performance, as AUC increased from 0.66 to 0.68 [95% CI 0.61–0.76, $p = 0.2733$], i.e. 0.02 units. As no strong correlations were found between miRs and waist-hip ratio, triglycerides, glucose, creatinine (Fig. 1), we tested whether adding information on the same risk factors to the miR-FRS model could improve performance additionally (**Model 3**). However, this model only increased AUC by 0.01 (from 0.80 to 0.81, $p = 0.1719$).

Due to the strong correlation between several of the miRs quantified in this study, several combinations of miRs will improve the precision of FRS in almost a similar manner. For instance, adding a combination of miR-106a-5p, miR-144-3p and miR-424-5p resulted in an AUC of 0.79 [95% CI 0.72–0.86].

4. Discussion

In this present study, we confirm and extend prior observations concerning the association between circulating levels of microRNAs (miRs) and the risk of developing myocardial infarction (MI) in healthy, asymptomatic individuals. By exploring ten miRs previously associated

with MI-risk, six miRs were found significantly related to 10-year risk of MI in this present cohort. The main intention of this study was to test whether incorporation of a miR-signature into the Framingham Risk Score (FRS), could improve prediction of MI in a clinically relevant manner. In this study, the best combination of miRs to supplement the FRS in predicting 10-year risk of MI included miR-21-5p, miR-26a-5p, miR-29c-3p, miR-144-3p and miR-151a-5p. The new model improved the accuracy of risk prediction, with an increase from

0.66 to 0.80 in the AUC, which is highly relevant in a clinical setting. In comparison, adding other well-known risk factors to the FRS only provided a minor improvement in AUC.

Today, there is little consensus concerning the specific miRs reported to improve risk prediction in CHD. For instance, Zampetaki et al. reported that adding miR-126, miR-197 and miR-223 to the FRS improved risk prediction, whereas a previous study from our research group suggested using a combination of miR-106a-5p, miR-424-5p, let-7g-5p, miR-144-3p and miR-660-5p to improve the precision of the FRS [33,34]. One possible reason for the discrepancy between studies is the strong correlation that exists between the levels of circulating miRs (Fig. 1). Thus, there is a chance that a miR reported in one study is strongly correlated with a miR reported in another study, thus, the results could be approximately the same independent of which of the miRs that are chosen to be included in the model. Furthermore, Schulte et al. were able to identify miR-197 and miR-223 as predictors for cardiovascular death in a cohort of documented CHD patients, and thus extended the applicability of circulating miRs towards secondary prevention [35]. An issue with the latter study was the low number of events (2.1%), which calls for further evaluation.

Obesity is a well-known risk factor for CHD [47]. As the FRS does not include waist-hip-ratio (WHR) or other obesity-related parameters (e.g. waist, BMI), we assumed that some of the miRs predicting MI were reflecting obesity status [48]. We therefore tested whether adding other well-known CHD risk factors to the FRS could obtain similar improvements in risk prediction, as could be obtained by adding miRs. Surprisingly, adding information on serum triglycerides, serum glucose, serum creatinine, and WHR barely improved risk prediction.

The discovery of new biomarkers able to improve risk prediction has several relevant clinical applications such as early identification of individuals eligible for primary prevention and better risk stratification of individuals at intermediate risk. Identification of new biomarkers can also contribute to new knowledge on signalling pathways that may represent new therapeutic targets. Two of the miRs associated with future MI in this present study, miR-144-3p and miR-451a, are transcribed from the same precursor RNA, and make up the miR-144/451-cluster. The miR-144/451-cluster is controlled by the important transcription factor GATA-4 in cardiomyocytes [49]. A previous study have reported that the miR-144/451-cluster is dysregulated in ischemic hearts, and that high levels of these miRs protect the heart from ischemic injury [49]. Our results supports this, as we found significantly lower levels of circulating miR-144-3p and miR-451a in the group of participants developing MI during the 10-year follow-up period.

One additional miR was down-regulated in the group of participants who later developed MI; miR-29c-3p. This miR showed a moderate positive correlation with miR-144. Previous studies in cultured endothelial cells indicated that miR-29a-3p is required for normal endothelial.

Function and that delivery of miR-29 have been shown to restore endothelium-dependent vasodilation [50]. The same study showed that miR-29 promotes endothelium-dependent vasodilation in both human and rat arterioles. In contrast, a previous study from our research group involving younger and healthier participants, reported that high baseline levels miR-29a-3p were associated with increased risk for fatal MI [34]. The reason for this discrepancy is uncertain, however, the differences in age and the presence of other CHD risk factors might influence the result.

Another miR that was differently expressed between the cases and controls and were included in the prediction model was miR-26a-5p. High levels of circulating miR-26a-5p was associated with increased risk for MI. Decreasing systemic levels of miR-26a in mice has been shown to protect the heart form ischemic injury [51]. The same study also revealed that miR-26a-5p levels are increased patients with stenotic lesions >70% compared to patients with <20% lesions. Another study reported that miR-26a levels are increased in endothelial progenitor cells from patients with atherosclerosis compared to healthy controls, and that the overexpression is associated with dysfunction of these cells [52]. Surprisingly, in a previous study from our research group involving younger and healthier participants, we found that low baseline levels of miR-26a were associated with increased risk for MI [34]. There may be consensus that miR-26a-5p plays a major role in the preservation of cardiovascular health, but the knowledge of its role as a biomarker of CHD is still sparse [53].

One of the miRs that were selected for the prediction model, despite being significantly differently expressed in the cases and controls were miR-21-5p. This miR has previously been associated with atherosclerosis, endothelial damage and dysfunction [26]. High circulating levels of miR-21-5p have been found in patients with atherosclerosis obliterans, cerebrovascular disease and chronic CHD [54–56]. In our study, participants developing MI during the follow-up had a tendency towards higher levels of circulating miR-21-5p compared to the controls, although not significant ($p = 0.16$). This could reflect asymptomatic atherosclerosis at the time of inclusion, and thereby increased risk of MI during follow-up.

Another miR that was enriched in the group of participants that developed MI during follow up was miR-191-5p. This miR has previously been reported to be abnormally expressed in several cancers, as well as in diabetes type 2 and pulmonary hypertension [57]. A study of diabetic patients showed that circulating levels of miR-191-5p correlate positively with C-reactive protein, and thus make a possible link to the atherosclerotic process [58]. Exposure to smoking and anti-platelet therapy have been shown to influence the circulating levels of miR-191-5p [59,60].

As previously mentioned, there is a lack of consensus regarding the specific miRs reported to improve risk prediction in CHD. In our previous study exploring miRs predicting MI, we found other miRs capable of improving risk prediction [61]. However, due to the strong correlation between several of the miRs quantified, several combinations of miRs will improve the precision of FRS in almost a similar manner. To investigate this we fitted our logistic regression to the FRS and miR-106a-5p, miR-424-5p and miR-144-3p. These miRs were chosen since they are highly correlated with the five miRs presented in Table 3 (see Fig. 1 for the correlation plot). The 10-fold CV AUC for this model was 0.79, indicating that these alternative miR had the capability of improving risk prediction to almost a similar extent.

One of the major limitations in the field of circulating miR is the lack of a common strategy for normalization. This represents a substantial disadvantage when comparing expression levels between different studies [62]. Furthermore, antiplatelet therapy, heparin, smoking and a plethora

of diseases have been shown to alter the miR levels in blood [59,60,63]. Caution must therefore be made when interpreting existing studies, and especially when designing new ones. Unfortunately, detailed information on pharmacological therapy like the use of antiplatelet drugs was not available in the HUNT-cohort. Hence, we cannot rule out the possibility of medication-induced alterations of miR levels in individual patients that are not associated with the development of the disease itself. However, as blood samples were collected in relatively healthy participants with no previous heart disease, stroke or angina pectoris, we anticipate that this would not have dramatic effects on the results.

Another limitation of this study was the use on non-fasting blood samples in analysis of lipids and glucose. However, non-fasting blood samples was considered to be the best approach for the HUNT-studies to ensure high participation rate and avoid potential fasting-evoked hypoglycemic events in diabetic patients. Moreover, a recent study has shown that most lipid levels differ minimally after a meal compared with fasting [64]. In addition, numerous large prospective studies have shown that non-fasting lipids are sufficient for general screening of CHD risk [65–67]. A meta-analysis of 68 prospective studies, 20 of which used non-fasting blood samples, found no attenuation of lipid relationships with predicting incident events for non-fasting lipids [67]. Based on this, we believe that our results would not be significantly interfered by the fasting-status of the participants.

One more limitation of this study is the lack of a mechanistic pathway insight. Such information could provide knowledge on signalling pathways that may represent new therapeutic targets of CVD. However, as the circulating miRs may originate from different tissues/organs, pathway analyses could be difficult to interpret.

Finally, due to the lack of data on some of the standardized, already clinically introduced risk factors (e.g. hsCRP), we cannot rule out the possibility that adding information on other competing risk factors potentially could improve the risk prediction model to the same extent as by adding miRs. This needs to be addressed in future studies.

In conclusion, our findings support previous studies showing that circulating miR can add value on top of traditional risk factors in risk prediction algorithms for CHD. However, discrepancies with previous studies indicates that there are still methodological issues to solve before circulating miRs can be considered as reliable biomarkers in disease.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contribution

Study concept and design: TV, ME, TF, BG, HR, TO, AB. Data acquisition: TV, ME, AB.

Data interpretation: TV, ME, JCS, BG; HR, TO, AB. Statistical analyses: TV, ME, JD, TF, JN, ML, AB.

Critical review of the manuscript throughout the editorial process: TV, ME, JD, JCS, TF, JN, BG, HR, TO, ML, AB.

Approval of the final manuscript draft submitted for publication: TV, ME, JD, JCS, TF, JN, BG, HR, TO, ML, AB.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.07.024.

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