Circulating microRNAs predict future fatal myocardial infarction in healthy individuals – The HUNT study

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Abstract

Coronary heart disease is the most common cause of death, and the number of individuals at risk is increasing. To better manage this pandemic, improved tool for risk prediction, including more accurate biomarkers are needed. The objective of this study was to assess the utility of circulating microRNAs (miRs) to predict future fatal acute myocardial infarction (AMI) in healthy participants.

We performed a prospective nested case-control study with 10-year observation period and fatal AMI as endpoint. In total, 179 miRs were quantified by real-time polymerase chain reaction in serum of 112 healthy participants (40-70 years) that either (1) suffered from fatal AMI within 10 years [n=56], or (2) remained healthy [n=56, risk factor-matched controls]. Candidate miRs were validated in a separate cohort of healthy individuals (n=100).

Twelve miRs were differently expressed in cases and controls in the derivation cohort (p<0.05). Among these, 10 miRs differed significantly between cases and controls in the validation cohort (p<0.05). We identified gender dimorphisms, as miR-424-5p and miR-26a-5p were associated exclusively with risk in men and women, respectively. The best model for predicting future AMI consisted of miR-106a-5p, miR-424-5p, let-7g-5p, miR-144-3p and miR-660-5p, providing 77.6% correct classification for both genders, and 74.1% and 81.8% for men and women, respectively. Adding these 5 miRs to the Framingham Risk Score, increased the AUC from 0.72 to 0.91 (p<0.001).

In conclusion, we identified several miRs associated with future AMI, revealed gender-specific associations, and proposed a panel of 5 miRs to enhance AMI risk prediction in healthy individuals.

Key words

Biomarkers, risk prediction, atherosclerosis
1. Background

Coronary heart disease (CHD) is the most common cause of death globally, with >8 million deaths (16.8%) in 2013 compared to 5.7 million deaths (12%) in 1990 [1]. Although improved treatment strategies have reduced the mortality rate in the Western world during the last decade, the number of people at risk is continuously growing [2-5]. To better manage this pandemic, improved tools for risk prediction, including more accurate biomarkers are needed.

Over the years, the knowledge of important risk factors has led to the development of several risk prediction models for determining 10-year risk of all types of cardiovascular disease (CVD) and more specifically also for acute myocardial infarction (AMI) [6-11]. However, the general use of risk prediction models has decreased in the primary care setting because current risk prediction models only explain a modest proportion of the incidence. For AMI, it is estimated that 15-20% of the patients have none of the traditional risk factors and would be classified as “low risk” by the current risk prediction scores [12]. There have been several attempts during the last years to improve the risk prediction models by incorporating new biomarkers into the currently available risk algorithms [13-18]. For instance, incorporation of C-reactive protein (CRP) and glycosylated hemoglobin (HbA1c) into the Framingham risk score were shown to improve risk prediction for both genders [19-21]. However, despite extensive studies there are currently no biomarkers that adequately predict risk of developing AMI [22]. Thus, there is a clinical need for cardiovascular biomarkers that could complement the assessment of traditional risk factors, to identify the individuals at risk with greater precision than today.

Recently, microRNAs (miRs) have emerged as potential biomarkers of disease, as small amounts of stable miRs can be detected in the circulation [23]. miRs are short, endogenous, single-stranded, non-coding RNAs that negatively regulate gene expression [24]. More than 2000 human miRs have so far been discovered, and their dysregulation have been associated with different pathologies like cancer and CVD [25-28]. miRs have large potential as diagnostic biomarkers in CVD, as studies have shown increased serum levels of miR-1 and miR-423 in patients with AMI and heart failure, respectively [29-33]. To our knowledge, only one study has previously explored the potential of circulating miRs as predictive biomarkers of AMI. In that study, which included participants with and without previous CVD (40-79 years old), incorporation of the circulating levels of miR-126, miR-197 and miR-223 into the Framingham Risk Score improved net reclassification with ~ 17% [34].

The large biobank established during the Nord-Trøndelag Health Study (HUNT), provides a unique opportunity to identify new biomarkers that can predict AMI, and to develop more accurate, gender-specific risk prediction models. Accordingly, the aim of this study was to assess the utility of circulating miRs to predict future fatal AMI in participants that were apparently healthy at the time of blood sampling.
2. Methods

2.1 Design
This study has a prospective nested case-control design with fatal AMI as endpoint (I21, ICD-10) and with 10-year follow-up. To increase the chance of finding new and independent biomarkers, the cases and controls were directly matched for traditional risk factors such as BMI, total cholesterol and HDL-C. The study was in conformity with Norwegian laws, the Declaration of Helsinki, and was approved by the Regional Ethical Committee (REK, 2011/136-3) and HUNT (2011/5014/MCS).

2.2 Subjects
Data collection in the HUNT studies were carried out in the Nord-Trøndelag County in Norway in three waves, the first in 1984–1986 (HUNT1), the second in 1995–1997 (HUNT2) and the third in 2006–2008 (HUNT3). All inhabitants, ≥20 years in the county were invited to participate, with approximate overall participant rates in HUNT1, HUNT2 and HUNT3 of 88%, 70% and 56%, respectively. Information on anthropometry, blood pressure, blood lipids and glucose were measured. Participants also completed questionnaires about health status, lifestyle variables (e.g. physical activity, smoking, alcohol consumption) and background variables (e.g. education, occupation, marital status) [35].

In the derivation cohort, we included 112 apparently healthy men and women (40-70 years) attending the HUNT2 study, that either (1) suffered from fatal AMI within the 10 year follow-up (n=56), or (2) reported no cardiovascular events during the 10 year follow-up (n=56, risk factor-matched controls). In a separate validation cohort, we included 100 apparently healthy men (n=56) and women (n=44) (40-70 years) from the HUNT2 study that either (1) suffered from fatal AMI within 10 years [n=50], or (2) reported no cardiovascular events during the 10 year follow-up [n=50, risk factor-matched controls]. Exclusion criteria were previous diagnosis of AMI, stroke, angina pectoris, diabetes, hypertension, severe obesity (BMI>35), cancer, rheumatic disease, hypo/hyperthyroidism, medication for these conditions, or other self-reported chronic medical conditions. A link was made between the HUNT2 Registry and the Cause of Death Registry at Statistics Norway to select the cases. Self-reported information of current or previous diseases reported in the HUNT3 study (collected 10 years after the HUNT2 study) were used to select controls that have remained healthy during the 10 years after HUNT2.

2.3 Blood analysis
Standard biochemical analyses were performed on fresh venous non-fasting blood samples at Levanger Hospital, Norway. Non-fasting glucose was analyzed by Hexokinase/G-G-PDH methodology reagent kit 3L82-20/3L82-40 Glucose (Abbott Diagnostics, Abbott Park, IL, USA), high-density lipoprotein (HDL) cholesterol by the Accelerator selective detergent methodology reagent kit 3K33-20 Ultra HDL (Abbott Diagnostics, Abbott Park, IL, USA), and triglycerides by
Glycerol Phosphate Oxidase methodology reagent kit 7D74 Triglyceride (Abbott Diagnostics, Abbott Park, IL, USA). Serum creatinine was analyzed by Alkaline Phosphate methodology kit 7D65-20 Creatinine (Abbott Diagnostics, Abbott Park, IL, USA).

2.4 RNA isolation from serum
Total RNA was extracted from serum using the miRCURY™ RNA isolation kit for biofluids (Exiqon, Vedbaek, Denmark). 200 µl of serum was mixed with 60 µl of lysis solution containing 1 µg carrier-RNA. An RNA spike-in kit was used for quality control of the RNA isolation and cDNA synthesis (Exiqon, Vedbaek, Denmark). The kit included the following synthetic RNA spike-ins: UniSp2, UniSp4, UniSp5, UniSp6 and cel-miR-39-3p, which allows the users to apply only the spike-ins needed for the particular study. 1 µL synthetic UniSp2 (2 fmole/µl), UniSp4 (0.02 fmole/µl), UniSp5 (0.0002 fmole/µl) RNA spike-in mix was introduced per RNA sample. Briefly, the samples were vortexed and incubated for 3 min at room temperature, followed by the addition of 20 µL protein precipitation solution. The samples were vortexed, incubated for 1 min at room temperature and centrifuged at 11,000 rcf for 3 min. The clear supernatants were transferred to a new collection tubes with 270 µl isopropanol and the solutions were vortexed and loaded on binding columns. The columns were incubated for 2 min at room temperature and emptied using vacuum. In a stepwise procedure the columns were washed 3 times with wash solution, before the columns were spun at 11,000 rcf to dry completely. The dry columns were transferred to new collection tubes and 50 µl RNase free H₂O was added directly on the membrane of the spin columns. The columns were incubated for 1 min at room temperature prior to centrifugation at 11,000 rcf. The RNA was stored at -80°C.

2.5 Circulating miR exploration
2 µl RNA was reverse transcribed in 10 µl reactions using the miRCURY LNA™ Universal RT miR PCR, Polyadenylation and cDNA synthesis kit (Exiqon, Vedbaek, Denmark). 0.5 µl synthetic spike-in mix including UniSp6 (0.002 fmole/ µL) and cel-miR-39-3p (0.625 ng/ µl) was added to each RT reaction. cDNA was diluted 50 x and assayed in 10 µl PCR reactions according to the protocol for miRCURY LNA™ Universal RT miR PCR. Each miR was assayed once by qPCR on the miR Ready-to-Use PCR, using ExiLENT SYBR®Green master mix. We included negative controls that excluded the template from the reverse transcription reaction in the analyses. The amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) in 384 well plates. The amplification curves were analyzed using the Roche LC software, both for determination of Cq (by the 2nd derivative method) and for melting curve analysis. For exploration, 179 miRs were analyzed using the Serum Focus miR PCR Panels (Exiqon, Vedbaek, Denmark). The panels contain 7 reference genes for normalization and quality control, and assays for 5 synthetic RNAs in the RNA Spike-in kit (cel-miR-39-3p, UniSp2, UniSp4, UniSp5 and UniSp6) to monitor RNA purification and cDNA synthesis. Each panel also contains an inter-plate calibrator in triplicate and an empty negative control. For the validation cohort, 16 miRs were analyzed using the miR Pic-and-mix System (Exiqon, Vedbaek, Denmark): 12 candidate miRs
(let-7d-5p, let-7g-5p, miR-26a-5p, miR-29c-3p, miR-101-3p, miR-103a-3p, miR-106a-5p, miR-144-3p, miR-148b-3p, miR-151a-5p, miR-424-5p, miR-660-5p), 2 potential endogenous reference miRs based on the previous derivation cohort (miR-191-5p, miR-425-5p), and 2 hemolysis miRs (miR-23a-3p, miR-451) [36].

2.6 Data analysis
The amplification efficiency was calculated using algorithms similar to the LinReg software. All assays were inspected for distinct melting curves and the Tm was checked to be within known specifications for the assay. Furthermore, we only considered miRs that had at least 3 Cq’s lower than the negative control and also Cq<37. Raw Cq values for the miRs selected for the validation cohort are shown in supplementary Table I. Data that did not pass these criteria were omitted from any further analysis. Cq was calculated as the 2nd derivative. NormFinder determined that the best strategy for normalization were the global mean. Hence, all data was normalized to the average of assays detected in all samples (average – assay Cq). In the validation cohort, we tested normalization to both the global mean, and to the levels of miR-425-5p, as the expression levels of the global mean and miR-425-5p were almost similar (Supplemental figure I). miR-425-5p has previously been found to be a stable expressed miR in other cohorts [36, 37]. After normalization of data to miR-425-5p or global mean, a ΔCq value is obtained (ΔCq). Differences in expression levels were calculated as ΔCq (cases) - ΔCq (controls) = ΔΔCq. To convert this to fold change, the formula $2^{\Delta\Delta C_q}$ was used.

2.7 Statistical analysis
Data were analyzed using the statistical packages IBM SPSS Statistics 22 (SPSS Inc., Chicago, USA), STATA version 12IC (StataCorp, Texas, USA) and R (R Development Core Team, USA). Paired sample t-tests were used to analyze differences in participant characteristics between those who developed MI during follow-up and those who remained healthy. Correlations between miRs were assessed using Pearson correlation coefficients, and differently expressed miRs between cases and controls were identified using paired sample t-tests. Odds ratios were calculated using conditional logistic regression, and precision of estimates were reported as 95% confidence interval. A significance level of 0.05 was used if not otherwise stated. The set of miRs with the highest predictive ability for future MI were identified using best subset regression. Conditional logistic regression models were fitted for all possible subsets of the set of miRs found to be differentially expressed between cases and controls. The models were compared according to their values of the Akaike information criterion (AIC), and the model with the lowest AIC was selected. Classification into cases and controls based on the selected model was performed using a cut-off of 0.5 for the predicted probabilities. Predictive accuracy for miR-based models and addition of the miR-based prediction model to the Framingham Risk Score (FRS) were assessed by calculating the area under the receiver-operating characteristic curve (AUC) [38]. p values <0.05 were considered significant.
3. Results

Baseline characteristics of the 212 participants are shown in Table 1. All participants were healthy at the time of blood sampling, but the cases suffered from fatal AMI within 1-10 years, whereas the matched controls remained healthy. No significant group differences in CVD risk factors were detected between the cases and matched controls (Table 1).

Table 1: Characteristics of the study participants.

<table>
<thead>
<tr>
<th></th>
<th>Derivation cohort</th>
<th>Validation cohort</th>
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<tbody>
<tr>
<td></td>
<td>Cases (n=56)</td>
<td>Controls (n=56)</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>60.2 ± 8.4</td>
<td>60.0 ± 8.3</td>
</tr>
<tr>
<td>Gender (female/male)</td>
<td>10/46</td>
<td>10/46</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>55</td>
<td>52</td>
</tr>
<tr>
<td>Years from baseline to AMI</td>
<td>2.8 ± 1.6</td>
<td>-</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.9 ± 3.5</td>
<td>26.4 ± 3.0</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>6.6 ± 1.0</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.9 ± 1.0</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.5 ± 0.9</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.3 ± 0.4</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>87.7 ± 13.7</td>
<td>86.3 ± 10.6</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>146 ± 20</td>
<td>142 ± 19</td>
</tr>
</tbody>
</table>

Data are shown as mean ± standard deviation, MI: myocardial infarction, BMI: body mass index, HDL: high-density lipoprotein

The miR isolation and quantification of the samples from the derivation and validation cohort were successfully accomplished, as controls and RNA spike-ins (UniSp2 and UniSp6) indicated good technical performance. None of the samples showed signs of being affected by hemolysis as measured by the miR-23a-3p/miR-451 ratio. Out of the 179 miRs analyzed in the derivation cohorts, 123 miRs were detectable in more than 90% of the samples, and 76 miRs were detected in all samples. We selected 12 miRs that were expressed in all samples and differed significantly between cases and controls (p<0.05) for validation in a separate cohort (Table 2). In the validation cohort, we tested two different approaches for normalization of miR levels; global mean normalization and normalization to the proposed endogenous reference, miR-425-5p. Both normalization strategies provided almost similar results, as the expression levels are highly correlated (Supplementary Figure I). In the following section, we chose to present the data normalized to the global mean.

Ten of the 12 candidate miRs from the derivation cohort were found to differ significantly between cases and controls in the validation cohort (p<0.05). In addition, miR-144-3p was significantly different between women cases and controls. The fold-change difference in miR-level between cases and controls are shown in Figure 1. Sub-groups of those suffering from early (AMI within 0-5 years) or late events (AMI within 6-10 years) are shown in Table 2. For the most significant miRs, the odds ratios per standard deviation higher miR level are shown in Figure 2.
Table 2: Difference in microRNA expression in cases versus controls in the derivation and validation cohorts as well as sub-groups of the validation cohort, shown as ΔΔCq-values normalized to the global mean.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Derivation cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>N</td>
<td>112</td>
<td>100</td>
</tr>
<tr>
<td>let-7d-5p</td>
<td>-0.4*</td>
<td>-0.2*</td>
</tr>
<tr>
<td>let-7g-5p</td>
<td>-0.3**</td>
<td>-0.2**</td>
</tr>
<tr>
<td>miR-26a-5p</td>
<td>-0.3**</td>
<td>-0.3**</td>
</tr>
<tr>
<td>miR-29c-3p</td>
<td>0.4**</td>
<td>0.1*</td>
</tr>
<tr>
<td>miR-101-3p</td>
<td>0.4**</td>
<td>0.1</td>
</tr>
<tr>
<td>miR-103a-3p</td>
<td>-0.3**</td>
<td>-0.2*</td>
</tr>
<tr>
<td>miR-106a-5p</td>
<td>-0.2**</td>
<td>-0.2**</td>
</tr>
<tr>
<td>miR-144-3p</td>
<td>0.6*</td>
<td>0.2</td>
</tr>
<tr>
<td>miR-148b-3p</td>
<td>-0.4**</td>
<td>-0.2*</td>
</tr>
<tr>
<td>miR-151a-5p</td>
<td>-0.3**</td>
<td>-0.2**</td>
</tr>
<tr>
<td>miR-424-5p</td>
<td>0.6**</td>
<td>0.3*</td>
</tr>
<tr>
<td>miR-660-5p</td>
<td>0.5**</td>
<td>0.2*</td>
</tr>
</tbody>
</table>

Positive ΔΔCq values indicates that the microRNA is increased in cases, and negative ΔΔCq values indicates that the microRNA is decreased in cases. *p<0.05, **p<0.01 between cases and controls.

Figure 1: Fold-change differences in microRNA-levels between cases and controls in the validation cohort. n=100, *p<0.05, **p<0.01
microRNAs predicting MI

Figure 2: Risk estimates for acute myocardial infarction based on single microRNA-level. Odds ratio for fatal acute myocardial infarction within 10 years determined per standard deviation higher microRNA concentration with 95% confidence interval in all participants and men and women separately. Only the most significantly differentially expressed microRNAs when comparing all participant or men and women separately are shown in this figure.

There were significant correlations between the circulating levels of several miRs. Moderate correlations were observed between miR-106a-5p, miR-151a-5p, let-7g-5p and miR-26a-5p (correlation coefficients in the range of 0.42-0.70, p<0.0005 for all, Supplementary Table II). We also found significant negative correlations between miR-106a-5p, let-7g-5p, miR-26a-5p and age (correlation coefficients -0.22, -0.22, -0.26, respectively, p<0.01). Significant negative correlations were also seen between miR-151a-5p, miR-26a-5p and systolic BP (correlation coefficients -0.27, -0.26, respectively, p<0.01). For the cases, we found correlation between miR-26a and years from blood sampling to AMI (0.34, p<0.05). We found no correlations between any miRs and indices of renal function (serum creatinine). The only miR that were differently expressed between genders were miR-660-5p, showing higher expression in men (p<0.05).

To find the best combination of miRs for predicting future AMI, we used conditional logistic regression, testing all combinations of miRs in Table 2, except for miR-101-3p, that were found unrelated to future AMI in all participants, and men and women separately in the validation cohort. This resulted in 4095 possible combinations, and the model with the lowest AIC (Akaike Information Criterion) was miR-106a-5p, miR-424-5p, let-7g-5p, miR-144-3p and miR-660-5p.
microRNAs predicting MI

(AIC=47.1), providing 78% overall correct classification. The model was better for predicting AMI in women, providing 82% overall correct classification. For men, the model provided 74% overall correct classification. Addition of the 5 miRs identified by the best subset regression approach to a conditional logistic regression model that includes the Framingham Risk Score (FRS) for hard CHD as an endpoint, increased the AUC from 0.72 (95% CI: [0.61, 0.82]) to 0.91 (95% CI: [0.85, 0.96]) (Δ0.19, p<0.001). The ROC-curves in Figure 3 illustrates the performance of the FRS alone and for a model including the 5 miRs identified in addition to the FRS for all participants, and men and women separately.

Figure 3: ROC-curves for illustration of model performance. Comparing the performance of the Framingham Risk Score and the microRNA model added to the Framingham Risk Score in A) all participants from the validation cohort (n=100), B) women from the validation cohort (n=44) and C) men from the validation cohort (n=56). FRS: Framingham Risk Score
4. Discussion
During the last years, several new biomarkers have been suggested as risk indicators of CVD, including CRP, natriuretic peptides, homocysteine, interleukin 6, and cardiac-specific troponins. However, their incremental predictive value beyond classic risk factors has been minor [15, 39, 40]. To increase the chance of identifying new biomarkers that are independent of and may add prognostic information to traditional risk factors, we designed this nested case-control study where cases and controls were directly matched on traditional risk factors. The nested case-control design is generally more efficient than a case-cohort design with the same number of selected controls [41]. Interestingly, several circulating miR differed significantly between individuals that subsequently suffered a fatal AMI and those that remained healthy during the 10-year follow-up period. Common features for several of these miRs are their relevance for the different steps of the atherosclerotic process [42-48].

miR-106a-5p was found to be a highly significant marker in both genders (p<0.01), and low levels were associated with increased risk for AMI. Low serum levels of miR-106a-5p have previously been reported in patients with acute heart failure [49]. Furthermore, the level of miR-106a-5p has previously been shown to decrease with age, which was also confirmed by our study [50]. A previous study has also linked this miR to the atherosclerotic process, as it was shown to be an important regulator of the macrophage inflammatory response [45].

Another miR that was highly associated with risk for AMI in men was miR-424-5p. Interestingly, this miR is directly involved in the atherosclerotic development by driving the monocyte-to-macrophage differentiation process [43]. The cholesterol-lowering drug ezetimibe has actually been shown to inhibit monocyte-to-macrophage differentiation through down-regulation of miR-424 and other miRs in the monocytes [43]. Several studies in experimental models show that miR-424-5p also is up-regulated in endothelial cells by vascular injury and by hypoxic conditions, as well as in the in the ischemic heart [51, 52]. Upregulation of miR-424-5p promotes downstream processes associated with hypoxia, like angiogenesis and erythropoiesis to overcome perfusion deficits [52]. Circulating miR-424-5p has previously also been considered as a marker for inflammation, and high levels have been detected after a marathon race [53]. Despite small sub-groups, miR-424-5p seems to be more closely associated with early events (AMI that occurs 0-5 years from blood sampling) compared to later events (MI that occurs 6-10 years from blood sampling), but this has to be further investigated in larger populations.

Interestingly, gender-specific association were observed between AMI-risk and miR-424-5p and miR-26a-5p. Gender differences in miR expression can often be explained by hormonal and genetic differences, as hormones and X-linked genes can influence the regulation of miRs [54]. However, there are just a few studies highlighting the role of miRs in sexually dimorphic disease [55, 56]. miR-424-5p is located at the X chromosome, and may potentially be among the 15% of X-linked genes that escape female X chromosome inactivation, causing higher expression in women [57]. However, no differences were found in miR-424-5p when comparing serum from men and women at baseline, independent of outcome.
Two members of the well-studied let-7 family, let-7d-5p and let-7g-5p, differed significantly between cases and controls in the validation cohort. Several studies have shown that let-7 family members have athero-protective effects. For instance, circulating levels of let-7i and let-7g-5p are decreased in patients with CHD and hypercholesterolemia, respectively [58, 59]. Furthermore, decreased let-7g-5p levels have been shown to impair endothelial function and to increase the risks of CVD through targeting TGF-β signaling [60].

A common feature for several of the differentially expressed miRs (miR-26a-5p, miR-106a-5p, miR-144-3p and miR-148b-3p) are their mutual target, the ATP-binding cassette, sub-family A, member 1 (ABCA1) [46, 61-64]. ABCA1 is the major regulator of cellular cholesterol and fundamental to the initiation and progression of atherosclerosis. In our study, we found increased circulating levels of miR-144-3p in the participants that later suffered from fatal AMI. In apoE−/− mice increased levels of miR-144-3p in macrophage-derived foam cells inhibited cholesterol efflux, decreased circulating levels of HDL and impaired reverse cholesterol transport in vivo, thereby accelerating atherosclerosis [46, 48]. Increasing the levels of miR-144-3p, by mimics and agomirs, has been shown to enhance the expression of inflammatory factors, including IL-1β, IL-6 and TNF-α, in vivo and in vitro. These findings indicate that miR-144-3p may be a potential therapeutic target of atherosclerosis. Furthermore, clinical studies have shown a positive correlation between circulating miR-144-3p and serum creatine kinase, lactate dehydrogenase and aspartate aminotransferase in subjects with AMI [46].

Interestingly, we found none of the typically reported atherosclerosis-related miRs, as miR-126, miR-21, miR-92a, miR-19a, miR-221 and miR-222 (reviewed in [65]), to be associated with future AMI in our study. We believe the reason might, in part, be explained by the platform used for the miR analysis and also the choice of normalization method. To our knowledge, there is only one other study that previously has explored the value of circulating miRs for the prediction of future AMIs and this study showed an association between miR-126, miR-223, miR-197 and disease risk [34]. Of note, this study used another platform for miR analysis (Taqman qPCR assay) and normalized against U6 [34]. Several studies has in the last years concluded that U6 is not a suitable endogenous control for the quantification of circulating miRs [66, 67].

Based on the data in this study, we suggest using a combination of miR-106a-5p, miR-424-5p, let-7g-5p, miR-144-3p and miR-660-5p to predict the risk for AMI. This model provided 77.6% correct classification for both genders, and 74.1% and 81.8% for men and women, respectively. When adding this miR-based model to the FRS for hard CHD as endpoint, we were able to significantly improve the model performance increasing the AUC from 0.72 to 0.91. This indicates that the miR-model is an independent predictor of future AMI that may add predictive information to the traditional risk factors included in the FRS. However, caution should be made when interpreting these results due to the small and selected sample size. Further studies in larger populations are needed to determine the clinical potential of measure these miR in addition to the classical CVD risk factors.
Indeed, the stability of circulating miRs, their non-invasive sampling techniques and their proven dysregulation in CVD make them promising candidate biomarkers. An ideal predictive biomarker should allow for early identification of individuals at risk for a given outcome, and the biomarker should also be relatively easy to measure with acceptable costs. To be able to use miRs as biomarkers in the clinical setting, reliable measurement is essential. Since RT-qPCR is highly sensitive and specific and has a high reproducibility, this is the gold-standard method to quantify circulating miRs. To achieve reliable quantification of circulating miRs, possible processing variations between samples have to be corrected by normalization [68]. Many different methods for normalization of miR data have been suggested, but currently there is no consensus on the most appropriate method [69-71]. The 3 most commonly used normalization strategies include normalization to the global mean of all detected miRs, normalization to one or more stably-expressed endogenous miRs, and the use of a synthetic or biologic spike-in [72, 73]. In this study, we tested both normalization using global mean and the endogenous miR-425-5p, since the latter has shown stable expression in previous studies [36, 37, 74]. The potential use of miR-425-5p as an endogenous miR was replicated in this study, as miR-425-5p was expressed close to the global mean in both the derivation and validation cohort. To our knowledge, no study has yet to report that circulating miR-425-5p levels are influenced by CVD, thus, miR-425 may represent a potential strategy for normalization of circulating miR levels. However, this needs to be further investigated in additional studies. Moreover, our results were obtained by the use of LNA-enhanced oligos and the platform by Exiqon, and should also be validated in other systems and platforms.

A potential limitation of the study is that mean time to event were shorter in the derivation cohort compared to the validation cohort. However, shorter time to events in the validation cohort should mainly make replication more difficult in the validation cohort than lead to false positive associations between individual miRNAs and incident AMI in the second cohort. Accordingly, as we were able to validate most of the results from the derivation cohort in the validation cohort, we do not see that the differences in times to event between the two cohorts are responsible for our results. Furthermore, the lack of consensus on the most appropriate method for normalization represents a major limitation that may explain some of the confliction data reported in circulating miR studies.

5. Conclusions
Based on blood samples from 212 well-characterized healthy subjects, we identified and verified significant associations between the level of 10 different circulating miRs and subsequent fatal AMI. These miRs may represent independent new risk markers of future AMI, based on the risk factor-matched case-control design. The best prediction of AMI-risk was achieved using a combination of miR-106a-5p, miR-424-5p, let-7g-5p, miR-144-3p and miR-660-5p, providing 77.6% correct classification for both genders, and 74.1% and 81.8% for men and women, respectively. When adding this miR-based model to the FRS, the model performance significantly improved. However, larger studies are needed to determine if these miRs can provide additional
predictive value on top of traditional risk factors in the currently used algorithms for assessing CVD risk.

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7. References
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microRNAs predicting MI


Highlights:

- Circulating microRNAs can predict future myocardial infarction in healthy subjects
- A combination of 5 microRNAs provided the best prediction model
- The microRNA risk prediction model provided 77.6 % correct classification