Circulating MicroRNA-210 Concentrations in Patients with Acute Heart Failure: Data from the Akershus Cardiac Examination 2 Study

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BACKGROUND: MicroRNA (miR)-210 expression is induced by acute and chronic hypoxia and provides prognostic information in patients with aortic stenosis and acute coronary syndrome. We hypothesized that circulating miR-210 concentrations could provide diagnostic and prognostic information in patients with acute heart failure (HF).

METHODS: We measured miR-210 concentrations in serum samples on admission from 314 patients hospitalized for acute dyspnea and 9 healthy control subjects. The diagnostic and prognostic properties of miR-210 were tested in patients after adjudication of all diagnoses and with median follow-up of 464 days.

RESULTS: All patients and control subjects had miR-210 concentrations within the range of detection, and the analytical variation was low as the coefficient of variation of synthetic spike-in RNA was 4%. Circulating miR-210 concentrations were increased in patients with HF compared to healthy control subjects, but miR-210 concentrations did not separate patients with acute HF (n = 143) from patients with non-HF-related dyspnea (n = 171): the area under the curve was 0.50 (95% CI 0.43–0.57). Circulating miR-210 concentrations were associated with mortality (n = 114) after adjustment for clinical risk factors (hazard ratio 1.65 [95% CI 1.03–2.62] per unit miR-210 increase), but this association was attenuated and not significant after adjustment for established cardiac protein biomarkers.

CONCLUSIONS: Circulating miR-210 concentrations are associated with mortality, but do not add to established protein biomarkers for diagnosis or prognosis in patients with acute dyspnea.

Introduction

Dyspnea is a common symptom in patients admitted to hospital emergency departments and is associated with high probability of serious illness (1). Acute heart failure (HF) is a prevalent condition in patients with dyspnea, and measurement of cardiac protein biomarkers has been found to improve diagnostic accuracy for acute HF and to risk stratify patients with dyspnea (2).

MicroRNAs (miRs) are small noncoding double-stranded RNA molecules that serve as negative sequence-specific regulators of transcription. One gene may be regulated by several miRs, and most miRs have multiple targets (3). miRs may either repress transcription or promote degradation of mRNA template. However, as miRs normally only suppress and do not completely block gene expression, the final result is often a percent reduction of yield (4). Many miRs are found in peripheral blood samples, and a number of specific miRs have been shown to have potential as biomarkers for cardiovascular disease. A particular subset of miRNAs, the so-called hypoxamirs, are associated with hypoxic conditions (5). miR-210 is a principal hypoxamir (6) and circulating miR-210 concentrations provide prognostic information in patients with coronary artery disease and aortic stenosis (7, 8). Hence, miR-210 appears to be a primary candidate as a novel cardiovascular biomarker among all circulating miRs. Still,

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whether circulating miR-210 concentrations improve diagnosis or risk stratification in patients with acute dyspnea is unknown. Since miR-10 previously has been linked to cardiovascular disease, we hypothesized that miR-210 concentrations could provide additional diagnostic and prognostic information in unselected patients hospitalized with acute dyspnea, and especially in acute HF. We also included information about miR-210 in patients with acute exacerbation of chronic obstructive pulmonary disease (AECOPD), because a large group of patients with dyspnea are diagnosed with AECOPD and no biomarker is currently in clinical use to diagnose or risk stratify these patients (9).

Methods

PATIENT MATERIAL: AKERSHUS CARDIAC EXAMINATION (ACE) 2 STUDY

The Akershus Cardiac Examination (ACE) 2 Study was performed at Akershus University Hospital, a Norwegian teaching hospital, from June 2009 to November 2010. The study complied with the Declaration of Helsinki and the protocol was approved by the Regional Ethics Committee. All study participants provided written informed consent before study commencement. The study included 314 patients admitted through the Emergency Department with shortness of breath as the chief complaint. Study inclusion criteria were acute dyspnea as the cause of index hospitalization, age at least 18 years, ability to provide informed consent, and the ability to obtain patient approval and draw blood samples of sufficient size and quality within 24 h of admission. Study exclusion criteria were dementia or other diseases that made informed consent impossible, coronary intervention or major surgery during the last 2 weeks, and disseminated cancer or other pre-terminal or terminal somatic disease.

Following written informed consent, we also included 9 healthy volunteers for sampling of serum used to determine control miR-210 concentrations during the same study period. The healthy volunteers had a median age of 30 years (IQR 6), 4 were males and 5 were females. Nine completely healthy controls were only included to provide a background reference population to normalize miRNA concentrations.

DATA COLLECTION AND ADJUDICATION OF DIAGNOSIS AND FOLLOW-UP IN THE ACE 2 STUDY

Patients were included Monday to Thursday from 08:00–14:00 due to study logistics. All blood samples were obtained within 24 h of admission. The patient records were used to collect information on clinical variables, including admission blood pressure, heart rate, body temperature, electrocardiogram, and previous medical history. Body mass index was calculated by body weight/(height × height) (kg/m^2). Coronary artery disease was defined as history of myocardial infarction or coronary intervention. Paroxysmal, persistent, or chronic atrial fibrillation (AF) were classified together as AF. The index diagnosis for the patients was determined by 2 senior physicians, who independently went through the patients' medical records, including results of supplementary examinations and follow-up data with median follow-up of 464 days [interquartile (IQR) 401] from index hospitalization to adjudication. Survival data were obtained until November 1, 2012, from electronic hospital records, synchronized with Statistics Norway. The patients were first categorized as suffering from HF or non-HF related dyspnea, and then assessed whether non-HF related dyspnea was caused by AECOPD. The diagnosis of HF was established according to the criteria outlined by the European Society of Cardiology (2). The diagnosis of AECOPD was based on criteria defined by the Global Initiative for Chronic Obstructive Lung Disease (10). Discordant diagnoses were resolved by consensus. Study-specific biomarker measurements were not available to the adjudication committee when they classified patients as HF or non-HF-related. Still, prior N-terminal pro-B-type natriuretic peptide (NT-proBNP) measurements and other biomarker measurements ordered as part of clinical routine during hospitalization, were made available to the adjudication committee.

MEASUREMENT OF CARDIAC TROPONIN T, BNP, AND NT-PROBNP

For cardiac troponin T (cTnT) concentrations, we employed a high sensitivity assay (Elecsys TnT hs stat, Roche Diagnostics, Penzberg, Germany) on a Cobas 8000 Platform (Roche Diagnostics) at Akershus University Hospital, Lørenskog, Norway. This assay has a range of detection from 3 to 10000 ng/L. hs-cTnT values that fell below the limit of detection were assigned the value of 3 ng/L. NT-proBNP was measured with the proBNP II assay (Roche Diagnostics) on the Cobas 8000 Platform at Akershus University Hospital. The assay has a range of detection from 5 to 35000 ng/L.

MIRNA EXTRACTION

The investigator performing molecular analyses was blinded to the clinical characteristics of the patients. In total, RNA from 323 serum samples (including 9 controls, 200 μL per sample) were extracted. A slightly optimized Qiazol-based protocol for the miRNeasy Serum/Plasma kit (#217184, Qiagen, Netherlands) was used, with the following modifications. One microgram MS2 RNA (#10165948001, Roche Diagnostics, Switzerland)

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was added as a carrier RNA to Qiazol mastermix. Following 5 min incubation at room temperature, all samples were spiked with 5 μL of 5 nM synthetic *Caenorhabditis elegans* (cel)-miR-39. These stages were followed by chloroform addition and phase-separation stage as per the Qiazol protocol. Randomly selected samples were chosen for quality control assessment by the use of the Bioanalyzer 2100 platform and the small RNA kit (#5067-1548, Agilent Technologies, USA). All of the tested samples were found to have good quality with the median miRNA yield 38% (IQR 3%).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-QPCR)

For each sample, cDNA synthesis was performed in duplicates according to the protocol for the Universal cDNA synthesis kit (#203300, Exiqon) at Akershus University Hospital, Lørenskog, Norway. RT-qPCR analysis was performed using the HT 7900 Fast Real-Time PCR System (Applied Biosystems, Thermo Fischer, USA). In short, the 10x diluted cDNA samples were mixed with SYBR green master mix, Universal RT from Exiqon (#203450) prior to loading onto 96-well plates in duplicates. Negative controls, where the synthesis mix did not contain reverse transcriptase enzyme (RT), were included in all plates. The assays for miR-210 and miR-425 were purchased as pre-made assays (cat#204333 and cat#204337, Exiqon, Vedbaek, Denmark), while the cel-miR-39 assay was ordered custom-made from Exiqon. To enable interplate calibration, one sample was chosen and added onto all the plates that were analyzed with the same assay. The expression levels of miR-210, miR-425, and cel-miR-39 were measured in all samples. The expression values for all 3 assays were interplate calibrated at the quantification cycle (Cq) level. Relative quantification calculations were done using the SDS 2.4 software (Applied Biosystems), and according to the 2–δδCt method (11). All RT-qPCR runs for the same assay were analyzed with the same threshold and background setting values. miR-210 expression levels were normalized against the miR-425 expression levels as previously described (7, 12–14). For all 3 assays, the sample with the highest Cq value was chosen as the internal calibrator sample for the relative quantification.

STATISTICAL ANALYSIS

The data are presented as medians with interquartile range (IQR) and absolute numbers with proportions unless stated otherwise. Comparison between groups of patients with dyspnea and healthy subjects with regard to miR-210 expression levels was carried out using one-way ANOVA with Dunn's post-test. Descriptive statistics were performed using Mann-Whitney U test for continuous variables and Fisher exact test for categorical variables. Correlation and association of miR-210 concentrations with clinical indices was analyzed by Spearman rank correlation and linear regression, respectively. Variables significantly associated with miR-210 concentrations in the univariable linear regression analyses were selected for multivariable analysis using forward selection. Survival in groups by quartiles of miR-210 concentrations were compared by Mantel-Cox log-rank test and visualized by Kaplan-Meier plots. Diagnostic and prognostic accuracy of miR-210 and NT-proBNP were assessed by receiver operating characteristics analysis with area under the curve (ROC-AUC). Biomarker concentrations (miR-210, NT-proBNP, and cTnT) were transformed using the natural logarithm prior to regression analyses due to right-skewed distributions. Predictors of mortality were evaluated by Cox proportional hazards regression models. Variables significantly associated with mortality in the univariate analyses were selected for multivariable analysis, utilizing forward selection. Statistical significance was assumed at a 2-side P-value <0.05. All data were analyzed using SPSS software for Mac (IBM).

Results

BASELINE CHARACTERISTICS

We assessed 468 patients presenting with acute dyspnea at the Emergency Department of Akershus University Hospital and included 314 patients into the study (Fig. 1). Among the patients included, 143 (46% of total) had HF as the adjudicated cause of admission, while 84 (27% of total) within the remaining 171 patients were adjudicated as AECOPD. The endpoint committee agreed on the diagnosis in 95% of the cases.

The baseline characteristics of patients are shown in Table 1. Patients with adjudicated admission diagnosis of HF ($n = 143$) were older, had higher body mass index, lower creatinine clearance, and were more frequently male with an abnormal electrocardiogram than patients with non-HF-related dyspnea. More patients with HF had history of coronary artery disease, hypertension, HF, diabetes mellitus, and atrial fibrillation. Current smoking was more common in the non-HF group, as well as a history of COPD.

EXPRESSION OF MIR-210 IN PATIENTS WITH ACUTE DYSPNEA AND IN HEALTHY SUBJECTS

All patients and control subjects had circulating miR-210 concentrations within the range of detection ($Cq < 35$; range Cq 26–32) for the RT-qPCR analysis. The coefficient of variation for the spiked-in control synthetic cel-miR-39 was 3.7% in the serum from patients with HF and 4.2% in patients without HF

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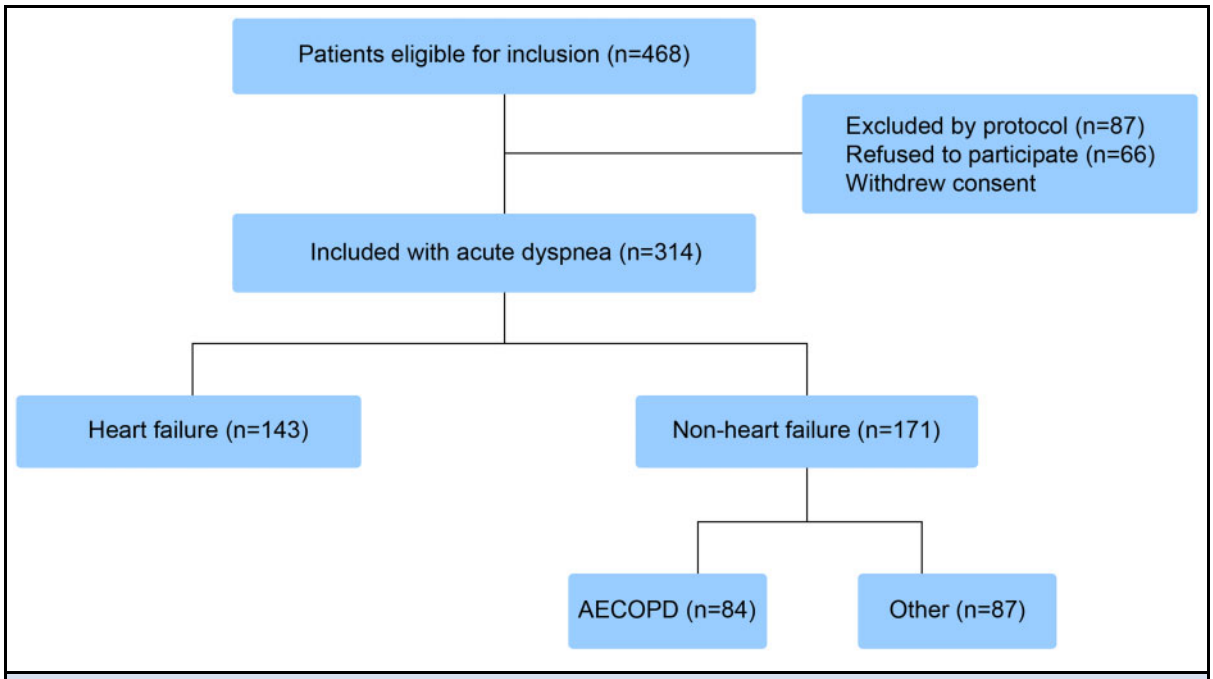

Fig. 1. Flow chart of study inclusion.

(Supplemental Fig. 1), which supported stable and uniform quality of miR isolation and preparation.miR-210 concentrations were increased in serum of patients with acute dyspnea compared to healthy subjects (relative quantity 3.99 [IQR 2.21] vs 1.53 [IQR 0.48], $P < 0.001$), as well is in patients with both acute HF (3.98 [IQR 2.5] vs 1.53 [IQR 0.48], $P < 0.001$) and AECOPD (4.0 [IQR 1.74] vs 1.53 [IQR 0.48], $P < 0.001$) (Fig. 2). In contrast, there was no significant difference in circulating miR-210 concentrations between patients with acute HF and AECOPD, $P = 0.43$. ROC-AUC of miR-210 to separate patients with HF from patients with non-HF-related dyspnea was 0.50 (95% CI 0.43–0.57). In contrast, as previously also reported (15), the AUC of NT-proBNP to diagnose acute HF in this cohort was 0.85 (0.81–0.89).miR-210 concentrations in the total cohort correlated with age, heart rate on admission, hemoglobin concentrations, and with concentrations of NT-proBNP and cTnT. In contrast, no correlation was found with severity of dyspnea as measured by New York Heart Association functional class IV (Supplemental Table 1).

In the total cohort of patients with acute dyspnea, lower heart rate (unstandardized coefficient [B] -0.004, SE 0.001, $P = 0.003$) and higher NT-proBNP concentrations (B 0.006, SE 0.014, $P < 0.001$) were associated with miR-210 concentrations in multivariable analyses (Supplemental Table 2). In patients with adjudicated diagnosis of HF, independent determinants of increasing miR-210 concentrations were age, heart rate, and increasing NT-proBNP concentrations (Table 2).

MIR-210 CONCENTRATIONS AND PROGNOSIS

During median 464 days of follow-up, a total of 114 patients (36%) died. Of these, 66 patients (59%) were diagnosed with acute HF at study inclusion and 35 patients (31%) were diagnosed with AECOPD as the cause for hospitalization. Prognosis in patients with HF was progressively worse according to the quartiles of miR-210 concentrations, (P by log-rank test = 0.017; Fig. 3, A), while mortality rates did not vary according to the concentrations of miR-210 in patients with AECOPD ($P = 0.15$ by log-rank test; Supplemental Fig. 2).

In the total cohort, miR-210 concentrations predicted mortality with unadjusted hazard ratio (HR) 2.25 (95% CI 1.52–3.31). In patients with HF, miR-210 concentrations predicted mortality with unadjusted HR 2.32 (95% CI 1.48–3.65). Adjusted for clinical variables, miR-210 concentrations predicted mortality with HR 1.65 (95% CI 1.03–2.62; Table 3). However, adjusting also for cTnT and NT-proBNP concentrations attenuated the association between miR-210 and mortality, which was no longer significant. miR-210 concentrations did not predict mortality in patients without HF (unadjusted HR 1.79, 95% CI 0.90–3.54;

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Table 1. Baseline characteristics of patients included in the study.

Continuous	HF (n = 143)	Non-HF (n = 171)	*P*-value
Age, years	78 (68–83)	67 (61–77)	<0.001
Weight, kg	80 (64–90)	71 (58–90)	0.026
BMI, kg/m^2	26.5 (22.2–29.4)	24.8 (21.1–30.3)	0.29
Systolic BP, mmHg	144 (123–166)	140 (129–157)	0.67
Diastolic BP, mmHg	80 (69–92)	77 (67–88)	0.12
Heart rate, beats/min	88 (74–109)	93 (79–107)	0.19
LVEF, %	40 (30–55)	...	...
Hb, g/dL	13.3 (12.1–14.5)	13.6 (12.6–14.7)	0.13
Leukocytes, *10^9/L	8.1 (6.7–11)	10.4 (8.1–13.1)	<0.001
CRP, mg/L	13 (5.0–32.0)	22 (5–80)	0.026
Creatinine clearance, mL/min	59 (41–82)	79 (62–103)	<0.001
NT-proBNP, ng/L	3600 (1601–8396)	348 (119–1139)	<0.001
cTnT, ng/L	37.9 (21.8–75.3)	13.4 (4.2–25.5)	<0.001
miR-210 (RQ)	3.98 (2.86–5.45)	4.01 (3.08–4.91)	0.68
Categorical	%	%	***P*-value**
Male sex	90 (63%)	74 (43%)	0.001
Current smoking	30 (21%)	55 (32%)	0.026
History of:			
heart failure	87 (61%)	14 (8%)	<0.001
coronary disease	77 (54%)	33 (19%)	<0.001
hypertension	69 (48%)	51 (30%)	0.001
diabetes	43 (30%)	25 (15%)	0.001
COPD	61 (43%)	94 (55%)	0.030
− atrial fibrillation	68 (48%)	27 (16%)	<0.001
Fever (>38°C)	9 (6%)	28 (16%)	0.006
NYHA class IV	65 (45%)	71 (42%)	0.48
Abnormal ECG	118 (82%)	105 (61%)	<0.001

Abbreviations: BMI, body mass index; BP, blood pressure; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; cTnT, high sensitivity cardiac troponin T; ECG, electrocardiography; Hb, hemoglobin; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal B-type natriuretic peptide; NYHA, New York Heart Association dyspnea scale (I-IV); RQ, relative quantity.

P for interaction = 0.53) or patients with AECOPD (unadjusted HR 1.38, 95% CI 0.65–2.93; *P* for interaction = 0.12).

The ROC-AUC for miR-210 to predict mortality in the total cohort was 0.61 (95% CI 0.54–0.67) and 0.63 (95% CI 0.54–0.72) in patients with acute HF (Fig. 3, B, C). The ROC-AUC of miR-210 to predict mortality in AECOPD was 0.50 (0.42–0.58).

Discussion

The main findings of the current study are that although analytical variation was low, which was reflected by low coefficient of variation of spike-in RNA and consistent RNA yield, and patients had miR-210 concentrations within the range of detection, circulating miR-210 concentrations did not improve diagnostic accuracy or risk stratification over the established cardiac protein biomarker NT-proBNP in patients with acute dyspnea.

There is an on-going process to identify novel biomarkers in patients with acute HF and AECOPD. Multiple biomarkers have been proposed for HF, including miRs that are small noncoding RNAs involved in translational suppression and mRNA degradation (3). Some miRs make their way into the circulation, where they can be accurately measured, thanks to their stability

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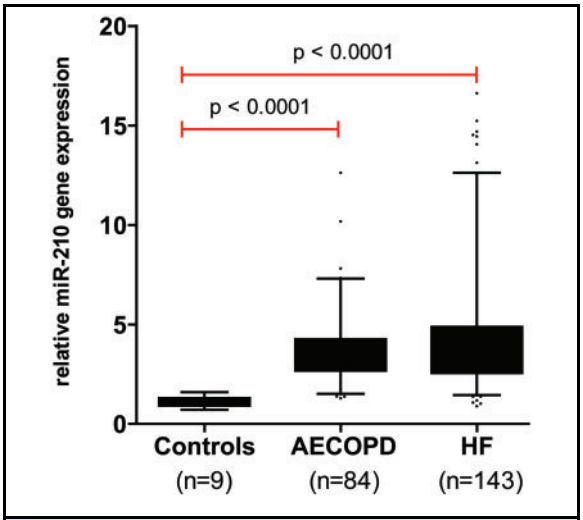

Fig. 2. Serum concentrations of miR-210 in patients with acute dyspnea and in healthy subjects. COPD (chronic obstructive lung disease), serum from patients presenting with acute dyspnea where COPD was the adjudicated index diagnosis. HF, serum from dyspnea patients with adjudicated index diagnosis of heart failure, presented as 75% percentile box plots with outliers, analyzed with one-way ANOVA with Dunn's post-test.

in fractionated samples (16). This makes them attractive as potential biomarkers for human disease. A recent large cohort study demonstrated that a panel of 8 miRs combined with NT-proBNP provided better clinical sensitivity and specificity in diagnosing nonacute heart failure than NT-proBNP alone (17), which reflects the potential of circulating miRs also in patients with HF.

In the current study we aimed to test the prognostic and diagnostic properties of a single miR in patients with acute dyspnea. We selected miR-210 as a prominent "hypoxamir"—a miR that is transcriptionally activated in hypoxic conditions (6). We hypothesized that it may be relevant to monitor in patients with acute dyspnea, especially since we and other groups previously have found miR-210 to provide prognostic information in patients with cardiovascular disease. For example, an earlier study showed that circulating miR-210 concentrations provided strong and additional prognostic information to established risk indices in patients with moderate to severe aortic stenosis (7). It has also been reported that miR-210 concentration provides prognostic information in patients with acute coronary syndrome (8).

Prior studies of miR-210 in heart failure seem to have been performed only in small cohorts and have other additional limitations. In a sample of 13 patients with HF, miR-210 concentrations in mononuclear cells separated patients with advanced heart failure and healthy controls from patients in NYHA class II (18). In another cohort (n = 39) of outpatient NYHA II patients in the same study, lower concentrations of miR-210 were associated with clinical improvement over 3-months follow-up (18). A 2013 study compared miR-210 concentrations in patients with HF presenting with NYHA class III and IV with patients with paroxysmal supraventricular tachycardia but normal heart structure and function (19). This study found increased concentrations of miR-210 in patients with HF, as well as a bell-shaped correlation of miR-210 with NT-proBNP. In patients with acute coronary syndrome, miR-210 increase emerged as a strong predictor of mortality after adjustment for age, sex, and one of the following—cTnT, NT-proBNP, left ventricular ejection fraction, or the number of affected coronary vessels (8). We also adjusted for NT-proBNP and other risk indices in our prior study of patients with aortic stenosis (7), but both this study and the current investigation are limited by a moderate sample size. Clearly, there is a need for studies in other and larger cardiovascular cohorts, including patients with HF.

Our study systematically addresses the strengths and limitations of miR-based biomarker strategy in patients with acute dyspnea, including a large proportion of patients with acute HF. Having access to a number of accurately measured clinical characteristics in our patient cohort, we have been able to subject the miR-210 expression level to a stringent and systematic validation (20). Our spike-in control miR-39 from *C. elegans* showed very little expression variation between samples, indicating excellent preanalytical and analytical handling of the samples. Further, the Cq values of both miR-210 and the housekeeping gene (miR-425) were well within the detection concentrations by RT-q-PCR, with mean Cq of 29.0 and 27.2, respectively. As in our previous studies, we employed both a synthetic spike-in miR and the housekeeping gene (miR-425) for quality control and normalization, respectively (7). A recent publication also verified miR-425 as the best candidate miR for normalization after a screening among 179 circulating miRs in a cohort of plasma samples from patients with acute myocardial infarction (21). In addition, miR-425 has been reported as the most robust miR for normalization in breast cancer patient material (13) and vulvar carcinomas (14). Finally, miR-425 was among the top 6 most stably expressed miRs in a colorectal cancer study when comparing tumor versus normal tissue samples (22).

We found that miR-210 concentrations were increased in patients with dyspnea compared to healthy control subjects, but that miR-210 concentrations did not separate between patients with acute HF and non-HF-related dyspnea. In contrast, NT-proBNP provided diagnostic information among patients hospitalized with

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Table 2. Linear determinants of increased concentration of miR-210 in patients with HF (n = 143).*

Univariate analysis	B	Standard error	*P*-value	t
Age	0.014	0.004	<0.001	3.57
Male sex	−0.162	0.097	0.10	−1.68
BMI	−0.016	0.008	0.040	−2.07
Current smoking	0.016	0.116	0.89	0.14
Systolic BP	−0.002	0.001	0.13	−1.53
Diastolic BP	−0.004	0.003	0.10	−1.64
Heart rate	−0.004	0.002	0.024	−2.29
Creatinine clearance	−0.005	0.001	<0.001	−4.10
NYHA class IV	0.249	0.092	0.008	2.69
History of:				
heart failure	0.207	0.095	0.031	2.18
coronary disease	0.164	0.093	0.08	1.75
hypertension	−0.006	0.094	0.95	−0.06
diabetes mellitus	0.034	0.103	0.74	0.33
COPD	0.041	0.095	0.66	0.44
atrial fibrillation	0.088	0.094	0.35	0.94
$_{ln}$cTnT	0.101	0.047	0.032	2.17
$_{ln}$NT-proBNP	0.151	0.033	<0.001	4.58
Multivariate analysis	**B**	**Standard error**	***P*-value**	**T**
$_{ln}$NT-proBNP	0.135	0.033	<0.001	4.09
Heart rate	−0.004	0.002	0.022	−2.32
Age	0.009	0.004	0.027	2.24

Abbreviations: BMI, body mass index; BP, blood pressure; COPD, chronic obstructive pulmonary disease; cTnT, high sensitivity troponin T; NT-proBNP: N-terminal B-type natriuretic peptide; NYHA, New York Heart Association dyspnea grading scale (I-IV); RQ, relative quantity.
*B, unstandardized coefficient; t,coefficient divided by standard error.

dyspnea. Furthermore, although circulating miR-210 concentrations correlated with prognosis in the patients hospitalized with acute dyspnea, miR-210 concentrations were not associated with mortality in analysis that adjusted for NT-proBNP and cTnT. Hence, we find the prognostic merit of miR-210 to be inferior to established cardiac protein biomarkers. Whether miR-210 still could have a role in a multimarker panel among patients with dyspnea will need to be examined in additional studies.

Our study has some strengths and limitations. One limitation is the measurement of only miR-210; this was a result of our interest in miR-210 based on previous reports (14, 23). The number of patients included in the studies is also modest, but comparable to the number of patients included in similar miR biomarker studies. This sample size was determined by the massive work required to obtain miRs from serum samples and to manually set up all the RT-qPCRs. An important variable not assessed in the study is the timing of miR-210 measurements in the circulation in relation to the onset of the supposed hypoxic trigger manifested by dyspnea. To date, no studies have addressed this question in patients. Experiments with mice placed in hypoxic conditions demonstrated an increase of miR-210 concentrations in plasma over the first 2 weeks of exposure with a subsequent decrease after 2 weeks (24). Hence, miR-210 seems upregulated in both acute and chronic hypoxia, and timing of blood sampling could influence the miR concentration. Finally, echocardiography was not universally performed in all patients included in our study, limited to patients with a clinical suspicion of HF. Hence, all patients with adjudicated HF diagnosis were subjected to echocardiography. Still, we cannot exclude that some patients with HF may have been missed. Of note, the lack of echocardiography in some individuals with non-HF-related dyspnea is similar to the situation in the Breathing Not Properly Study (25)

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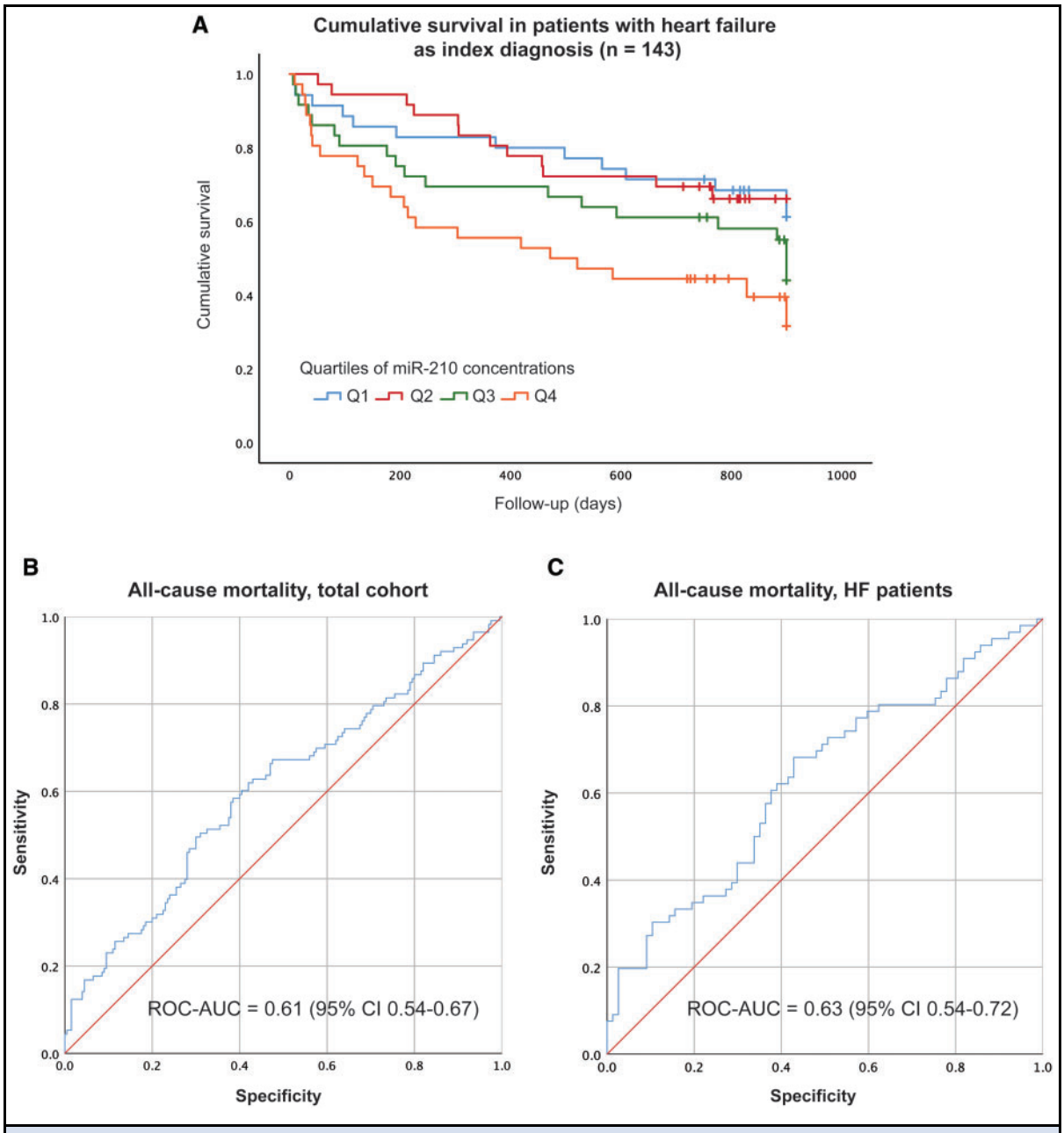

Fig. 3. miR-210 and mortality in cohort 2. (A) Association between miR-210 concentrations and mortality during follow-up in patients with acute heart failure. miR-210 concentrations were stratified by quartiles. The data are shown as Kaplan-Meier survival plot, analyzed by Mantel-Cox log-rank test ($P = 0.017$). (B) The ROC-AUC of miR-210 to predict mortality in total cohort of patients with acute dyspnea. (C) The ROC-AUC of miR-210 to predict mortality in total cohort of patients with acute HF. ROC-AUC, receiver operating characteristics with area under the curve.

and the PRIDE Study (26). This has also previously been discussed in other publications from the ACE 2 Study (27).

Strengths of the study are the low variation between the duplicates and between the expression level of spiked-in cel-miR-39, indicating that we have implemented reliable routines for RNA isolation, reverse transcription, and RT-qPCR analysis. Other strengths of the study are adjudication of all diagnoses among patients with acute dyspnea, complete data coverage on

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Table 3. Predictors of mortality in HF only patients.

Univariate analysis	Hazard ratio	95% CI	*P*-value	Wald
Age	1.04	1.01–1.07	0.002	9.446
Male sex	0.53	0.32–0.86	0.010	6.621
Systolic BP	0.99	0.98–0.996	0.004	8.341
Diastolic BP	0.97	0.96–0.99	0.001	11.279
BMI	0.94	0.89–0.99	0.012	6.349
Creatinine clearance	0.98	0.97–0.99	<0.001	8.01
NYHA class IV	2.01	1.23–3.29	0.006	7.83
History of DM	1.78	1.08–2.95	0.024	5.107
History of COPD	1.85	1.14–3.00	0.013	6.105
$_{ln}$NT-proBNP	1.53	1.24–1.89	<0.001	15.989
$_{ln}$cTnT	1.37	1.10–1.71	0.005	7.992
$_{ln}$miR-210 (RQ)	2.32	1.48–3.65	<0.001	12.91
Multivariate analysis	**Hazard ratio**	**95% CI**	***P*-value**	**Wald**
$_{ln}$NT-proBNP	1.75	1.38–2.22	<0.001	21.631
Systolic BP	0.98	0.98–0.99	<0.001	12.686
History of DM	2.62	1.51–4.52	0.001	11.851
History of COPD	2.49	1.45–4.28	0.001	10.952
Age	1.05	1.02–1.08	0.002	9.293
Male sex	0.59	0.35–0.97	0.039	4.256
Multivariate analysis, proBNP and cTnT excluded:	**Hazard ratio**	**95% CI**	***P*-value**	**Wald**
Male sex	0.58	0.35–0.96	0.036	4.415
Diastolic BP	0.98	0.97–1.00	0.067	3.356
Creatinine clearance	0.98	0.97–0.995	0.005	7.805
History of diabetes	2.21	1.28–3.81	0.005	8.067
History of COPD	1.90	1.13–3.19	0.015	5.875
$_{ln}$miR-210 (RQ)	1.65	1.03–2.62	0.036	4.399

Abbreviations: BMI: body mass index; BP, blood pressure; COPD, chronic obstructive pulmonary disease; cTnT, high sensitivity troponin T; DM, diabetes mellitus; NT-proBNP, N-terminal B-type natriuretic peptide; NYHA, New York Heart Association dyspnea grading scale (1–4); RQ, relative quantity.

all patients, and the stringent assessment of circulating miR-210 as a biomarker, including head-to-head comparison with the established cardiac protein biomarker NT-proBNP.

In conclusion, we demonstrate that miR-210 concentration is increased in patients with acute dyspnea and associated with inferior prognosis in patients with acute HF. However, this association was attenuated and no longer significant after adjustment for established cardiac protein biomarkers. This largely rules out miR-210 alone as a diagnostic or prognostic biomarker in unselected patients with acute dyspnea.

Nonstandard Abbreviations ACE, Akershus Cardiac Examination; AECOPD, acute exacerbation of chronic obstructive pulmonary disease; AF, atrial fibrillation; AUC, area under curve; BMI, , body mass index; BNP, B-type natriuretic peptide; BP, blood pressure; cDNA, complementary DNA; CI, confidence interval; Cq, quantification cycle; cTnT, cardiac troponin T ECG, electrocardiogram; Hb,

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hemoglobin (concentrations in blood); HF, heart failure; HR, hazard ratio; IQR, interquartile; LVEF, left ventricular ejection fraction (by echocardiography); miR, ,microRNA; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NTHA, New York Heart Association (dyspnea grading scale); PCR, polymerase chain reaction; ROC-AUC, receiver operating characteristics with area under curve; RQ, relative quantity (measured by RT-qPCR); RT, reverse transcription; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SE, standard error.

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References

1. Johnson MJ, Hutchinson A. Breathlessness in the emergency care setting. Curr Opin Support Palliat Care 2018; 3:232–6.
2. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JGF, Coats AJS, et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Eur Heart J 2016;37: 2129–200.
3. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
4. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature 2008;455:64–71.
5. Chan SY, Loscalzo J. MicroRNA-210: A unique and pleiotropic hypoxamir. Cell Cycle 2010;9:1072–83.
6. Chan YC, Banerjee J, Choi SY, Sen CK. miR-210: the master hypoxamir. Microcirculation 2012;19:215–23.
7. Rosjo H, Dahl MB, Bye A, Andreassen J, Jorgensen M, Wisloff U, et al. Prognostic value of circulating microRNA-210 levels in patients with moderate to severe aortic stenosis. PLoS One 2014;9:e91812.
8. Karakas M, Schulte C, Appelbaum S, Ojeda F, Lackner KJ, Munzel T, et al. Circulating microRNAs strongly predict cardiovascular death in patients with coronary artery disease-results from the large AtheroGene study. Eur Heart J 2017;7:516–23.
9. Mannino DM. Biomarkers for chronic obstructive pulmonary disease diagnosis and progression: insights, disappointments and promise. Curr Opin Pulm Med 2019; 25:144–9.
10. Vestbo J, Hurd SS, Agusti AG, Jones PW, Vogelmeier C, Anzueto A, et al. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 2013;187:347–65.
11. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;4: 402–8.
12. Velle-Forbord T, Eidlaug M, Debik J, Sæther JC, Follestad T, Nauman J, et al. Circulating microRNAs as predictive biomarkers of myocardial infarction: Evidence from the HUNT study. Atherosclerosis 2019;289:1–7.
13. McDermott AM, Kerin MJ, Miller N. Identification and validation of miRNAs as endogenous controls for RQ-PCR in blood specimens for breast cancer studies. PLoS One 2013;8:e83718.
14. Zalewski K, Misiek M, Kowalik A, Bakuła-Zalewska E, Kopczyński J, Zielińska A, et al. Normalizers for microRNA quantification in plasma of patients with vulvar intraepithelial neoplasia lesions and vulvar carcinoma. Tumour Biol 2017;39:101042831771714.
15. Rosjo H, Dahl MB, Jorgensen M, Roysland R, Brynildsen J, Cataliotti A, et al. Influence of glycosylation on diagnostic and prognostic accuracy of N-terminal pro-B-type natriuretic peptide in acute dyspnea: data from the Akershus Cardiac Examination 2 Study. Clin Chem 2015;61:1087–97.
16. Glinge C, Clauss S, Boddum K, Jabbari R, Jabbari J, Risgaard B, et al. Stability of circulating blood-based microRNAs–pre-analytic methodological considerations. PLoS One 2017;12:e0167969.
17. Wong LL, Zou R, Zhou L, Lim JY, Phua DCY, Liu C, Chong JPC, et al. Combining circulating microRNA and NT-proBNP to detect and categorize heart failure subtypes. J Am Coll Cardiol 2019;73:1300–13.
18. Endo K, Naito Y, J I. X, Nakanishi M, Noguchi T, Goto Y, et al. MicroRNA 210 as a biomarker for congestive heart failure. Biol Pharm Bull 2013;1:48–54.
19. Zhao DS, Chen Y, Jiang H, Lu JP, Zhang G, Geng J, et al. Serum miR-210 and miR-30a expressions tend to revert to fetal levels in Chinese adult patients with chronic heart failure. Cardiovasc Pathol 2013;22:444–50.
20. Morrow DA, de Lemos JA. Benchmarks for the assessment of novel cardiovascular biomarkers. Circulation 2007;8:949–52.
21. Coelho-Lima J, Mohammed A, Cormack S, Jones S, Das R, Egred M, et al. Overcoming heparin-associated RT-qPCR inhibition and normalization issues for microRNA quantification in patients with acute myocardial infarction. Thromb Haemost 2018;118:1257–69.
22. Chang KH, Mestdagh P, Vandesompele J, Kerin MJ, Miller N. MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. BMC Cancer 2010;10:173.
23. Bye A, Rosjo H, Aspenes ST, Condorelli G, Omland T, Wisloff U. Circulating microRNAs and aerobic fitness–the HUNT-Study. PLoS One 2013;8:e57496.
24. Gou D, Ramchandran R, Peng X, Yao L, Kang K, Sarkar J, et al. miR-210 has an antiapoptotic effect in pulmonary artery smooth muscle cells during hypoxia. Am J Physiol Lung Cell Mol Physiol 2012;303:L682–91.
25. Maisel AS, Krishnaswamy P, Nowak RM, McCord J, Hollander JE, Duc P, et al. Rapid measurement of B-type natriuretic peptide in the emergency diagnosis of heart failure. N Engl J Med 2002;347:161–7.
26. Januzzi JL, Jr., Camargo CA, Anwaruddin S, Baggish AL, Chen AA, Krauser DG, Tung R, et al. The N-terminal Pro-BNP investigation of dyspnea in the emergency department (PRIDE) study. Am J Cardiol 2005; 95:948–54.
27. Lyngbakken MN, Pervez MO, Brynildsen J, Pedersen MH, Solvernes J, Christensen G, et al. Fibroblast growth factor 23 in patients with acute dyspnea: Data from the Akershus Cardiac Examination (ACE) 2 Study. Clin Biochem 2018;52:41–7.

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