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Cardiospecific miRNA plasma levels correlate with troponin and cardiac function in patients with ST-elevation myocardial infarction, are selectively dependent on renal elimination and can be detected in urine samples

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Abstract

Objectives: Circulating miRNAs are promising as biomarkers for various diseases. We examined release patterns of cardiospecific miRNAs in a closed chest, large animal ischemia-reperfusion model and in patients with ST-elevation myocardial infarction (STEMI).

Methods: 6 anesthetized pigs were subjected to coronary occlusion/reperfusion. Plasma, urine and clinical parameters were collected from twenty-five STEMI patients undergoing primary PCI. miRNA was extracted and measured with qPCR.

Results: In the pig reperfusion model miR-1, -133a and -208b increased rapidly in plasma with a peak at 120 min, while miR-499-5p remained elevated longer. In patients with STEMI all four miRNA increased abruptly 70-3000-fold in plasma with a peak within 12 hours ($p<0.01$). miR-1 and miR-133a both correlated strongly with GFR, indicating renal elimination. This was confirmed by detection of miR-1 and miR-133a, but not miR-208b or miR-499-5p in urine. Peak values of miR-208b correlated with peak troponin and ejection fraction.

Conclusion: We demonstrate a distinct and rapid increase in levels of cardiac-specific miRNA in the circulation after myocardial infarction. Release of miRNAs correlated with cardiomyocyte necrosis markers, ejection fraction and glomerular filtration rate, indicating a possible role for these molecules as biomarkers for diagnosis of STEMI as well as prediction of long-term complications.

Introduction

Ischemic heart disease is the leading cause of death in developed countries and each year more than 3 million people are estimated to have an acute ST-elevation myocardial infarction (STEMI).[1] An early diagnosis and swift reperfusion of the occluded coronary vessel is essential for limiting myonecrosis and reducing morbidity and mortality. Cardiac biomarkers, i.e. proteins that leak out into the blood stream from necrotic cardiomyocytes, are of great clinical use for establishing a timely and correct diagnosis. Cardiac troponin T and I are detectable in the blood within 3-6 hours of myocardial damage and are widely accepted as the most reliable biomarkers for diagnosis of myocardial infarction today.[2-4]

MicroRNAs (miRNAs) are short (~22 nucleotide) endogenous RNAs that are essential modulators of gene expression. These non-coding RNA molecules act by pairing with complimentary regions in the 3' untranslated region of target mRNA, thereby suppressing gene expression.[5] At present more than 900 human miRNA species have been reported in miRBase[6] release 15 (http://mirbase.org) and many of them appear to be expressed in a tissue-specific manner.[7] Differential expression of certain miRNA species have been linked with pathological processes such as cancer[8], inflammation[9] and cardiovascular disease.[10-12] MiRNA has been shown to be important for cardiac development and knockout of miR-1 results in cardiomyopathy.[13] The remarkable stability of miRNAs in blood[14] and urine[15] have also made them interesting candidates as biomarkers for various pathological conditions. Certain miRNA species that have been shown to be highly enriched either in cardiac and skeletal muscle in general (miR-1, -133a and -499-5p) or specifically in cardiomyocytes (miR-208a and -208b) are released into the circulation following myocardial infarction. Several recent studies have aimed at demonstrating the usefulness of some of these miRNAs as cardiac biomarkers with promising results.[16-20] The aim of our study was to investigate the levels of cardiospecific miRNAs in plasma

following a myocardial infarction and to evaluate their usefulness as markers of cardiac cell death and cardiac function.

Materials and Methods

Porcine model of myocardial infarction

The procedure has been described in detail previously.[21] Briefly, six healthy 40-50 kg domestic pigs were anesthesized and a 3.0-3.5 mm x 20 mm Maverick Monorail angioplasty balloon (Boston Scientific Scimed, Maple Grove, MN) was positioned in the proximal LAD. Ischemia was induced by inflation of the angioplasty balloon for 40 minutes. Total occlusion of the LAD during inflation of the balloon and restoration of blood flow after deflation was verified with angiograms. Blood samples were drawn before occlusion and every 30 minutes for a total of 150 minutes after inflation of the balloon. All pigs were sacrificed after 4 hours.

Patients

Patients eligible for inclusion were those undergoing primary-PCI at Skåne University Hospital Lund due to a ST-elevation myocardial infarction. All patients gave their written approval of participating in the study. The study group included 25 patients of whom 20 (80%) were men. The mean age of patients were 64,56 years (SEM=2,70). Patient characteristics are summarized in table 1. Healthy volunteers recruited at the Biomedical Centre in Lund, Sweden were used as controls. The control group included 7 men (64%) and 4 women (36%) with a mean age of 65,09 (SEM=3,51).

This study was carried out according to the principles of the Declaration of Helsinki and approved by the local ethics committee at Skane University Hospital.

Sample collection and handling

The first blood sample was obtained by venipuncture within 24 hours after the onset of symptoms, the second within 48 hours and the third within 72 hours. The elevation of cardiac-specific microRNAs in the setting of a myocardial infarction has previously been shown to rise rapidly, peaking within hours of the presentation of symptoms.[16] The initial samples were therefore divided into two groups depending on whether they were collected before or after a cut-off value of 12 hours (n=9 <12 hours and n=16 >12 hours). After discharge, additional blood samples were collected after 3-6 months. The samples were centrifuged at 1600 g for 15 minutes, within two hours of collection, followed by aspiration of the plasma, which was stored at -80 °C. A urine sample was collected in a subset of STEMI patients (n=8) within 24 hours of onset of symptoms and stored at -80 °C. To test the stability of miRNA in urine during freezing/thawing, urine samples from three patients were subjected to four cycles of freezing/thawing and aliquots were taken in each cycle for RNA preparation.

RNA isolation and real-time quantitative RT-PCR (qRT-PCR)

Plasma or urine was mixed with TRIzol LS (Invitrogen, Carlsbad, CA) in a 1:3 ratio and the samples were homogenized by vortexing >30 s. RNA was then isolated with the miRNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. RNA quantity and quality were assessed by NanoDrop (Nanodrop Products, Wilmington, DE) and Bioanalyzer (Agilent, Santa Clara, CA) using the 2100 Small RNA assay. cDNA was synthesized from 20 ng of total RNA using the miRCURY LNA Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark) according to the manufacturers instructions. qRT-PCR was carried out in 20 μl triplicate reactions with Fast SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and LNA primer sets (Exiqon) specific for miR-1, -133a, -208a, -

208b, -499-5p and -16 according to the manufacturers protocols on a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA).

Analysis of qRT-PCR data

The threshold cycle (Ct) is defined as the fractional PCR cycle number where the fluorescence reaches a given threshold. For the plasma samples, Ct-values for the miRNAs of interest were normalized against the arbitrary value 40 and expressed relative to the mean of the control samples according to the $2^{-\Delta\Delta Ct}$-method. The levels of miRNA in urine samples were normalized to an exogenous miRNA spike-in (Exiqon) and expressed as relative quantities, $2^{-\Delta Ct}$. The porcine miRNA data was normalized against baseline values and the maximum value in each pig during the time course. Samples where no amplification could be detected were defined as having a Ct-value of 40. An inter-plate calibrator sample was used to adjust for run-to-run variation.

Statistical analysis

All statistical analysis was carried out using the GraphPad Prism 4.0 software (GraphPad Software Inc. La Jolla, CA). Differences in miRNA levels were analyzed using one-way ANOVA with Bonferroni's multiple comparison test *post hoc*. Normality tests of all parameters were performed using the D'Agostino-Pearson omnibus method. Correlations between circulating miRNA levels and clinical parameters were performed using Spearman's rank correlation coefficient. All p-values <0,05 were considered statistically significant. All error bars represent the standard error of the mean (SEM).

Results

Levels of miRNA in a porcine myocardial infarction model

We wanted to study the levels of cardiospecific miRNAs following induction of cardiac ischemia in a large animal model and evaluate how rapidly they are released and eliminated. In order to accurately reflect the therapy given to patients in the clinic, we used an occlusion-reperfusion protocol in a closed chest pig model. We observed a significant increase in miR-1, -133a, -208b and -499-5p after cardiac ischemia (figure 1). None of the miRNAs could be detected during the 40 min occlusion period, but all started to increase 20 min after reperfusion (the 60 min time point in fig 1). The dynamics of miR-1, -133 and -208b were especially rapid, peaking after 120 minutes, followed by a fast 25-50 percent reduction already 30 min later. The level of miR-499-5p however remained high even at 150 minutes, indicating slower elimination rates. miR-208a could not be detected.

Levels of circulating miR-1, miR-133a, -208b and miR-499-5p are elevated in patients with STEMI

The levels of circulating miR-1, -133a, -208b and -499-5p were elevated within 12 hours of onset of symptoms in STEMI-patients (figure 2). miR-1 was increased 300-fold ($p<0.01$), miR-133a 70-fold ($p<0.01$), miR-208b 3000-fold ($p<0.001$) and miR-499-5p 250-fold ($p<0.01$) as compared to healthy controls. The levels of miR-208b and -499-5p remained 320- and 50-fold elevated after two days ($p=0.10$ and $p=0.32$, respectively) and miR-208b was still 70-fold higher than baseline after three days ($p=0.20$). The level of miR-16, which is not tissue-specific, did not change significantly during the time course (data not shown). miR-208a could not be detected. Peak levels for all pairs of miRNAs except miR-1 versus miR-208b or 499-5p correlated at day 1. At day 3, miR-1 and miR-133a correlated very strongly with each other ($p<0.0001$), as did miR-208b with miR-499-5p ($p=0.0033$). All correlation data are summarized in Table 2.

miR-1 and -133 are detectable in urine after a myocardial infarction

The rapid clearance of circulating miRNA after 12 hours following a myocardial infarction led us to speculate whether these miRNAs are excreted in the urine. We were able to detect miR-1 and miR-133a, but not miR-499-5p or miR-208b in urine of STEMI-patients (n=8) within 24 hours of onset of symptoms (Figure 3). These results suggest that the route of degradation and/or excretion of miR-499-5p and miR-208b differ from that of miR-1 and miR-133a.

To ensure that miRNA stability in urine was not affected negatively by freezing and thawing we extracted miRNA from urine samples subjected to four consecutive freeze-thaw cycles and analyzed the abundance of miR-1 and miR-16 by qRT-PCR. The levels of these miRNA species were not affected significantly by the treatment. Mean Ct for miR-1 changed from 34.42 (SEM=0.90) at cycle 1 to 34.79 (SEM=0.89) at cycle 4 and from 25.72 (SEM=0.16) to 25.93 (SEM=0.34) for miR-16, n=3.

Clinical correlations

To evaluate the significance of increased levels of circulating cardio-miRs following a myocardial infarction we analysed whether the levels of these miRNAs correlated with a set of clinical parameters. We found that the peak level of circulating Troponin T correlated significantly with the peak values of miR-208b ($p=0.01$, $r^2=0.25$) at day 1 (figure 4a). Furthermore, we found negative correlations between ejection fraction and peak miR-208b ($p=0.01$, $r^2=0.32$) at day 1 (Figure 4b). However, neither of the other miRNAs correlated with troponin or ejection fraction. Peak Troponin T correlated significantly with ejection fraction ($p=0.01$, $r^2=0.33$).

Finally, we found significant negative correlations between glomerular filtration rate (GFR) and the day 3 levels of miR-1 ($p=0.01$, $r^2= 0.27$) and miR-133a ($p=0.04$, $r^2=0.17$,

figure 5), but no correlation with miR-208b (p=0.09, r^2=0.12) or miR-499-5p (p=0.14, r^2=0.097). Neither peak TnT nor EF correlated significantly with GFR.

Receiver operating characteristic (ROC) analysis

To evaluate the usefulness of circulating miRNA as biomarkers for STEMI, we performed ROC analysis (Figure 6). Data from STEMI patients where samples were taken within 12 hours (n=9) and healthy controls (n=11) was included in the analysis. The ROC curves strongly distinguished between the two groups. miR-208b stood out as the best candidate with 100% specificity and sensitivity. The other miRNAs had an area under the curve (AUC) of 0.98 (95% CI 0.93-1.03) for miR-1, 0.86 (95% CI 0.69-1,02) for miR-133a and 0.98 (95% CI 0.93-1.03)

Discussion

The observation that miRNAs are stable and present in the circulation has led to a rapidly growing number of reports on the use of these molecules as biomarkers for various diseases. In the case of coronary artery disease miR-1, -133a, -208a, -208b and -499-5p are considered cardio- or skeletal muscle specific and are all candidates as biomarkers for MI. This led us to investigate the dynamics of these miRNAs in the immediate response to myocardial infarction in a closed chest ischemia-reperfusion pig model as well as patients with STEMI. To our knowledge, no previous studies have been conducted on circulating miRNA following myocardial infarction in a large animal model. The studies performed so far on circulating miRNA following cardiac ischemia in animal models[16,18] induce myocardial infarction by permanent ligation of a coronary artery. In contrast, we chose to induce myocardial infarction in an occlusion-reperfusion manner that we believe better reflect the treatment given to patients in the clinic. In addition, we observed faster kinetics with

regard to miR-1 and -133a than previously reported, probably secondary to reperfusion. The results showed that miR-1, -133a and -208a increased rapidly and peaked within 120 minutes whereas miR-499-5p continued to increase even after 150 minutes following onset of cardiac ischemia. In patients with STEMI there was a massive increase in the levels of the cardiospecific miRNAs in the circulation compared to healthy controls, followed by a return to near baseline of miR-1 and -133a after 12 hours of onset of symptoms. The levels of miR-208b and miR-499-5p decreased more slowly and was still elevated 300 and 50-fold respectively, two days after the STEMI. These findings are concurrent with the work of D'Alessandra et al (2010), which suggest that miR-499-5p has a slower dynamic than miR-1 and miR-133a.

In a very recent study, miR-208b was shown to be elevated in plasma following myocardial infarction [22]. Of the miRNAs included in this study, miR-208b showed the most marked increase (3000-fold) and was still elevated 70-fold after three days.

miR-208a has recently been proposed as an ideal biomarker for diagnosis of myocardial infarction.[16,17] The expression of miR-208a is highly restricted to cardiomyocytes[16] and it has been shown to be present in the circulation of patients with AMI. However, in concordance with a recent report[18] we could not readily detect miR-208a neither in the blood of these patients nor in the porcine ischemia-reperfusion model. This might be explained by an extremely rapid half-life of circulating miR-208a. The earliest patient samples in this study were taken within 12 hours of the onset of symptoms, in contrast to the work of Wang et al (2010) where the first sample was taken within 4 hours. Thus, it is feasible that the levels of circulating miR-208a might have sunken below the detection limit of our assay even in the earliest samples. Nevertheless, a molecule with such a short half-life might not be optimally suited as a biomarker.

Cardiac troponins are considered the gold standard as biomarkers for diagnosis of MI at the time being. We found that peak levels of miR-208b correlated with peak levels of Troponin T, suggesting that it can be used as markers of acute cardiomyocyte cell death. ROC analysis showed that miR-208b had 100% sensitivity and specificity in distinguishing between STEMI patients where samples were taken within 12 hours and healthy controls, albeit in a rather limited study material. This suggests that miR-208b is the most reliable biomarker for STEMI of the miRNAs included in this study.

We show for the first time a correlation between circulating miRNA levels and ejection fraction of patients after myocardial infarction. The peak amount of miR-208b in the blood correlated negatively with ejection fraction. Thus, it is possible that quantifying cardio-specific miRs in the circulation might not only be of use in diagnosing cardiac ischemia but could potentially also be helpful in prognosticating long term cardiac function and the risk of developing heart failure. However, the significance of this finding needs to be confirmed in a larger patient material and the possible biological link between miRNA and ejection fraction remains to be elucidated. Troponin T did also correlate significantly with ejection fraction, as previously described [23]. miR-1, -133a and -499-5p did not correlate with either TnT or EF, indicating that they do not reflect cardiac damage as good as miR-208b.

miR-208b and -499-5p remained markedly elevated in patients even two or three days after STEMI. The time profile indicates a reduced elimination rate compared to miR-1 and miR-133a. Interestingly, we found negative correlations between GFR and the amount of miR-1 and miR-133a, but not miR-208b or miR-499-5p, in the blood three days after STEMI, indicating possible renal elimination of the former two. This was confirmed by detecting miR-1 and miR-133a (but not miR-208a or miR-499-5p) in urine from patients within one day after their STEMI. Intrarenal expression of miR-200a and miR-205 correlate with GFR in patients with hypertensive nephrosclerosis[24] but no association have

previously been made between circulating miRNA and kidney function. Renal failure increases mortality substantially after a myocardial infarction[25] and the link between kidney function and circulating miRNAs merits more extensive study.

Elucidating the possible biological role of cardiac-specific miRNAs in the circulation after STEMI is beyond the scope of this paper. However, considering the recent reports describing cell-to-cell transport of miRNAs[26] and of miRNAs as paracrine signalling molecules [27] one might speculate that the presence of miRNAs in circulation is not merely a by-product of myocardial necrosis but also implies a functional role for these molecules. miR-1 and miR-133a have been shown to regulate cardiomyocyte apoptosis through different mechanisms [28,29] whereas miR-208b and miR-499 have been shown to affect muscle function and performance by regulating myosin gene expression [30]. This might point towards a cardioprotective role for these miRNAs in the setting of myocardial infarction. These questions need to be addressed thoroughly in future studies.

Our results demonstrate that there are clear differences for the properties of cardiac-specific miRNA. The release pattern and correlation with cardiomyocyte necrosis markers and ejection factor was restricted to miR-208b. miR-208b also exhibited by far the highest raise in man, increasing more than 3000-fold and correlated with troponin. It may be the most sensitive miR for detection of cardiac ischemia, as suggested by the results of the ROC analysis. Furthermore, elimination pattern differed in that miR-1 and miR-133a seem to be dependent on renal elimination and are detectable in urine, while miR-208b and -499-5p are not. This could be important for understanding miRNAs and their roles in cardiovascular disease in the future.

We could detect cardio-specific miRNAs in the circulation as early as 90 minutes after occlusion of the LAD in the pig model of MI. Assuming that the time frame is comparable in humans this could mean a reduction in the time to determine diagnosis and

could possibly reduce morbidity and mortality of MI. Moreover, the finding that circulating cardio-specific miRNAs correlate with EF and GFR might possibly imply a broader role for these molecules as biomarkers for MI, not only as tools for diagnosis but also for estimating risk of long-term complications. Nevertheless, the data presented here is based on a rather limited study population and the results needs to be confirmed in a larger material.

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Tables

Table 1. Patient Characteristics

Number of patients	25
Women no (%)	5 (20)
Hypertension no (%)	9 (36)
Diabetes Mellitus no (%)	3 (12)
Hyperlipidemia at admission no (%)	0 (0)
Current smoker no (%)	10 (40)
Infarct related artery	
LAD no (%)	14 (56)
Cx no (%)	5 (20)
RCA no (%)	6 (24)
Symptoms-to-reperfusion time (min) no (%)	
<100	4 (16)
100-180	10 (40)
181-360	5 (20)
361-600	2 (8)
>600	1 (4)
Unknown	3 (12)
TIMI 3 flow post PCI no (%)	24 (96)
Ejection fraction	
> 55%	11 (44)
45-55%	2 (8)
35-45	10 (40)
25-35%	2 (8)
<25%	0
Cardiovascular death within 3 month after AMI no (%)	1 (4)
Rehospitalistion for ACS within 3 month after AMI no (%)	0 (0)
Glomerular filtration rate (GFR) ml/min	
>90 no (%)	10 (40)
60-90 no (%)	11 (44)
GFR 30-59 no (%)	3 (12)
< 30 no (%)	1 (4)

LAD, left anterior descending; Cx, circumflex; RCA, right coronary artery; TIMI, thrombolysis in myocardial infarction; PCI, percutaneous coronary intervention; AMI, acute myocardial infarction; ACS, acute coronary syndome. All patients received hyperlipidemia treatment at admission.

Table 2. Intercorrelations of circulating miRNAs at day 1 and 3 following STEMI.

	Day 1		**Day 3**	
	p	*r*	*p*	*r2*
miR-1 vs miR-133a	**0,0008**	0,6358	**<0,0001**	0,7738
miR-1 vs miR-208b	0,0732	0,3723	0,1206	0,3186
miR-1 vs miR-499-5p	0,1569	0,2983	0,9774	-0,005965
miR-133a vs miR-208b	**0,0001**	0,6945	**0,0101**	0,5046
miR-133a vs miR-499-5p	**0,002**	0,5878	0,1533	0,2943
miR-208b vs miR-499-5p	**0,0084**	0,5155	**<0.0033**	0,5643

Statistically significant correlations are depicted in bold text.

Figure legends

Figure 1. miRNA release in a closed chest pig myocardial infarction model (n=6). All data are normalized to baseline values and to the maximum value that each miRNA reached in each pig. *p<0.05, **p<0.01, ***p<0.001. Number of non-detected samples: miR-1, 1 at 0 minutes and 3 at 30 minutes; miR-133a, 1 at 0 minutes and 1 at 30 minutes. miR-208b, 5 at 0 minutes, 5 at 30 minutes, 1 at 90 minutes and 1 at 150 minutes; miR-499-5p, 1 at 0 minutes and 3 at 30 minutes.

Figure 2. Dynamic changes of circulating miRNA from <12 h to >1 month in patients with STEMI (n=25). All data are normalized against the mean of the control samples (n=11). **p<0.01, **p<0.001. Number of non-detected samples: miR-499-5p, 5 in the control group, 2 at day 2, 1 at day 3, 7 at >1 month; miR-208b, 3 in the control group, 1 at day 3, 8 at >1 month.

Figure 3. Detection of cardiospecific miRNAs in urine after STEMI. Data are expressed relative to the amount of exogenous miRNA spike-in. n=8. Number of non-detected samples: 1 for miR-1, 3 for miR-133a. N.D., not detectable.

Figure 4. Correlation of levels of miR-208b at day 1 with a) Troponin T (p=0.01, r^2=0.25) and b) ejection fraction (p=0.01, r^2=0.32) in STEMI patients (n=25).

Figure 5. Correlation of GFR with a) miR-1 (p=0.01, r^2= 0.27) and b) miR-133 (p=0.04, r^2=0.17), at day 3 in STEMI patients (n=25).

Figure 6. ROC curves for circulating miRNA within 12 hours of STEMI. Patients: n=9, controls; n=11.

Figure 1.

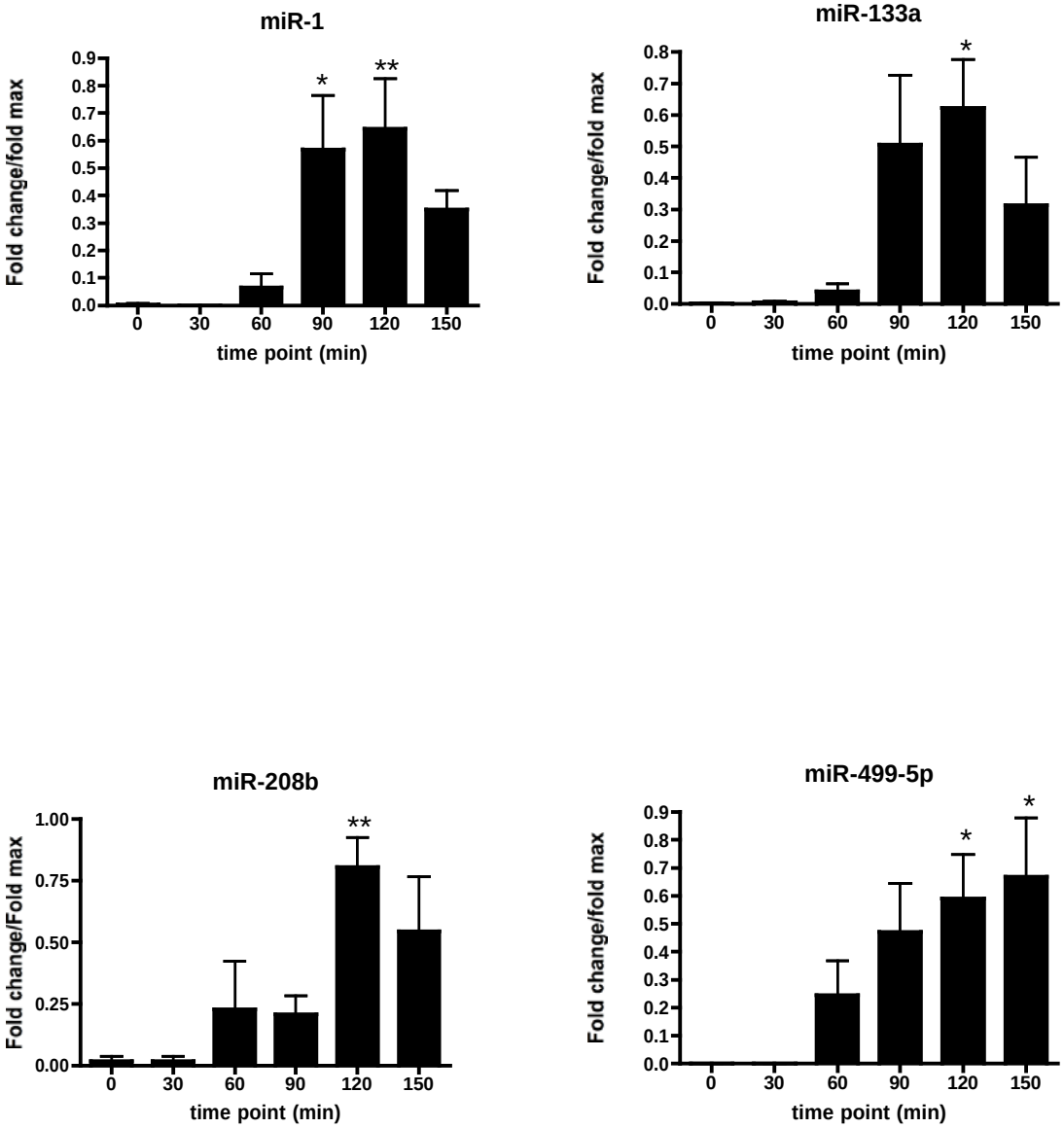

miR-1
Fold change/fold max
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
*
**
0
30
60
90
120
150
time point (min)
miR-133a
Fold change/fold max
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
*
0
30
60
90
120
150
time point (min)
miR-208b
Fold change/Fold max
0.00
0.25
0.50
0.75
1.00
**
0
30
60
90
120
150
time point (min)
miR-499-5p
Fold change/fold max
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
*
*
0
30
60
90
120
150
time point (min)

Figure 2.

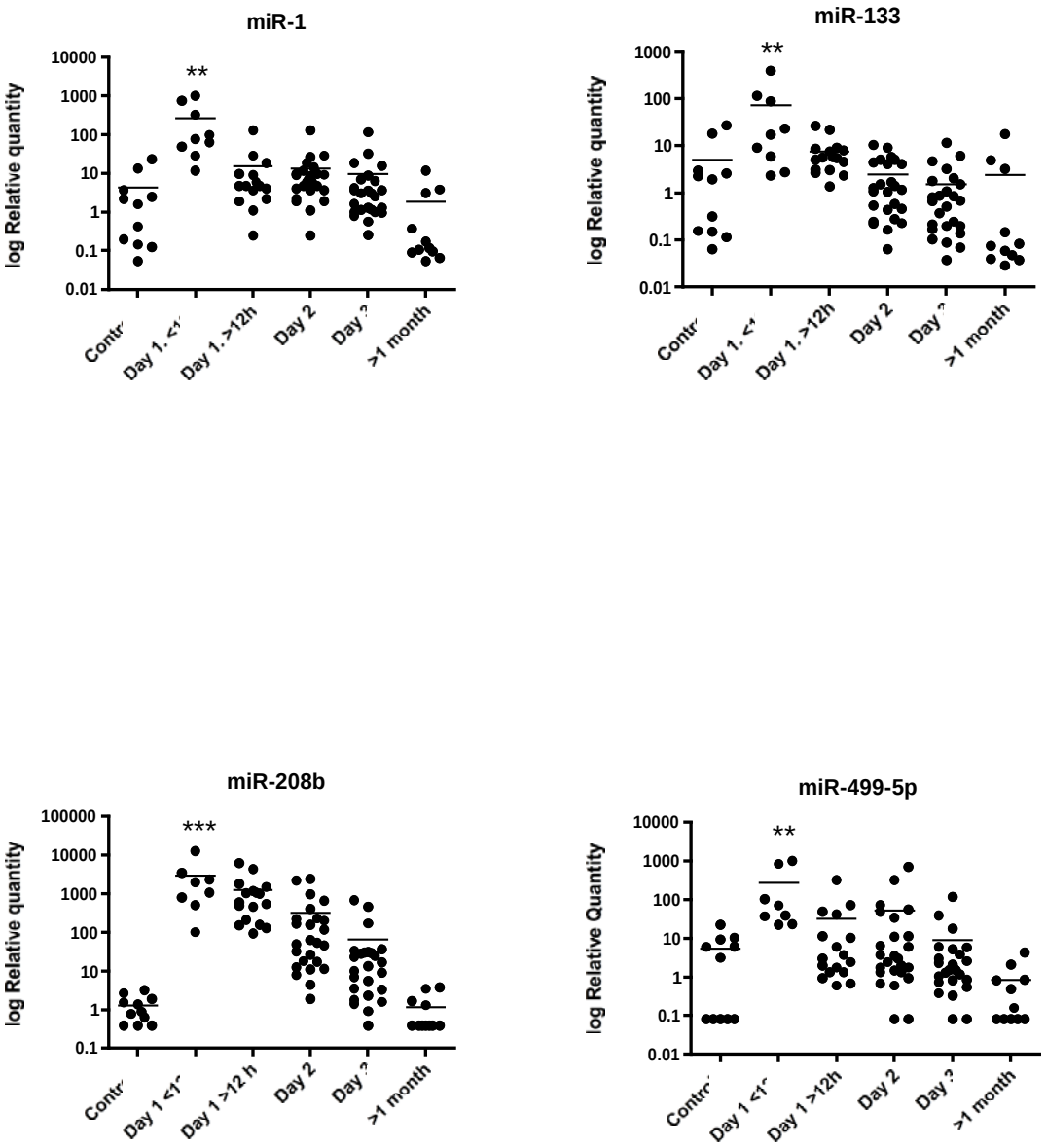

miR-1
log Relative quantity
10000
1000
100
10
1
0.1
0.01
**
Day 1, >12h
Day 2
>1 month
miR-133
log Relative quantity
1000
100
10
1
0.1
0.01
**
Day 1, >12h
Day 2
>1 month
miR-208b
log Relative quantity
100000
10000
1000
100
10
1
0.1
***
Day 1 >12 h
Day 2
>1 month
miR-499-5p
log Relative Quantity
10000
1000
100
10
1
0.1
0.01
**
Day 1 >12h
Day 2
>1 month

Figure 3.

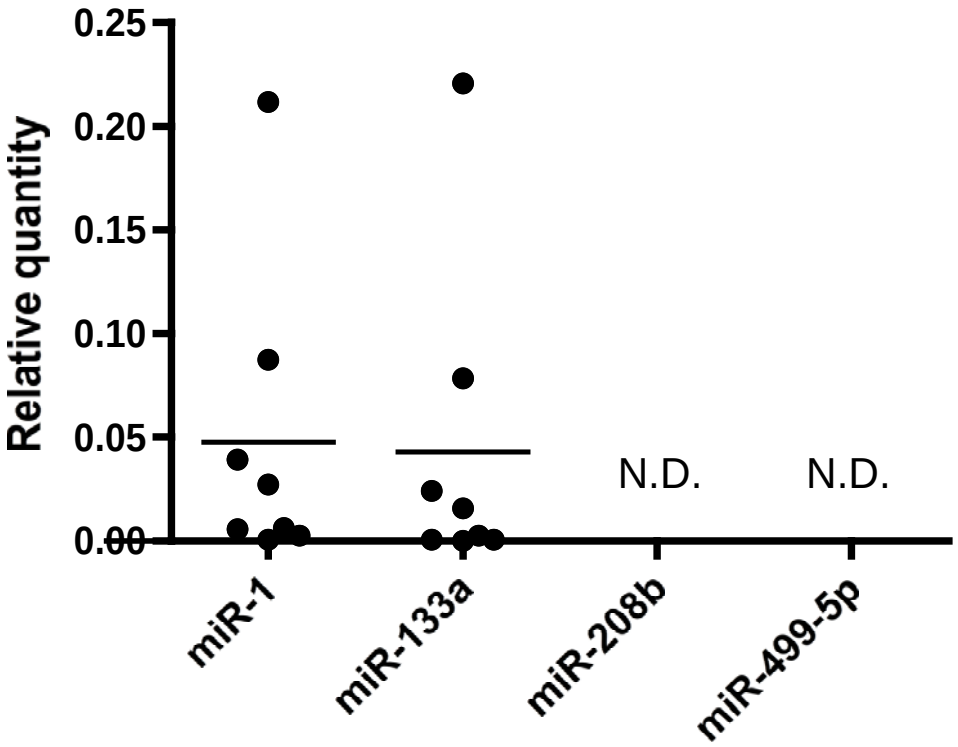

0.25
0.20
0.15
0.10
0.05
0.00
Relative quantity
N.D.
N.D.
miR-1
miR-133a
miR-208b
miR-499-5p

Figure 4.

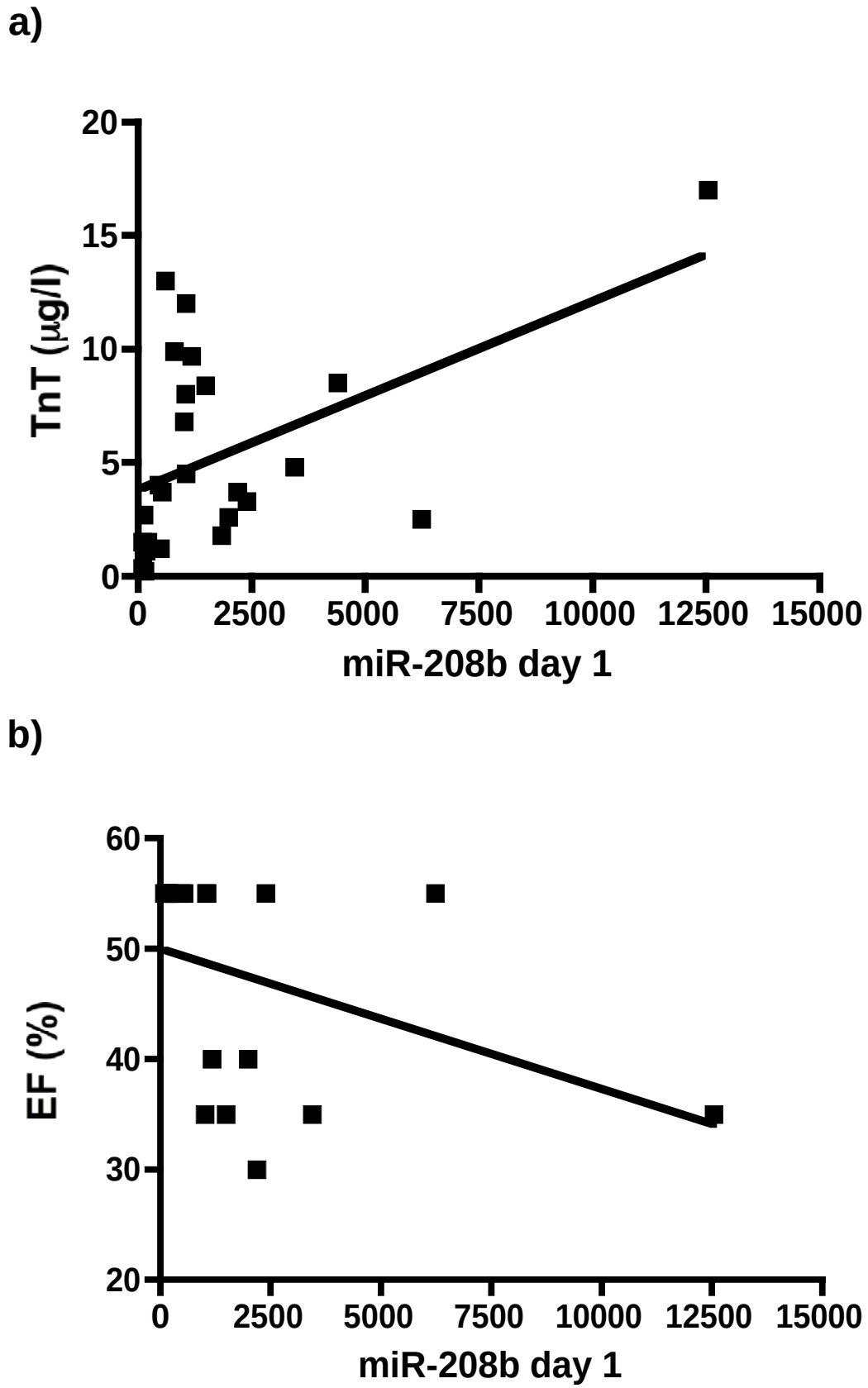

a)
20
15
10
5
0
TnT (µg/l)
0
2500
5000
7500
10000
12500
15000
miR-208b day 1
b)
60
50
40
30
20
EF (%)
0
2500
5000
7500
10000
12500
15000
miR-208b day 1

Figure 5.

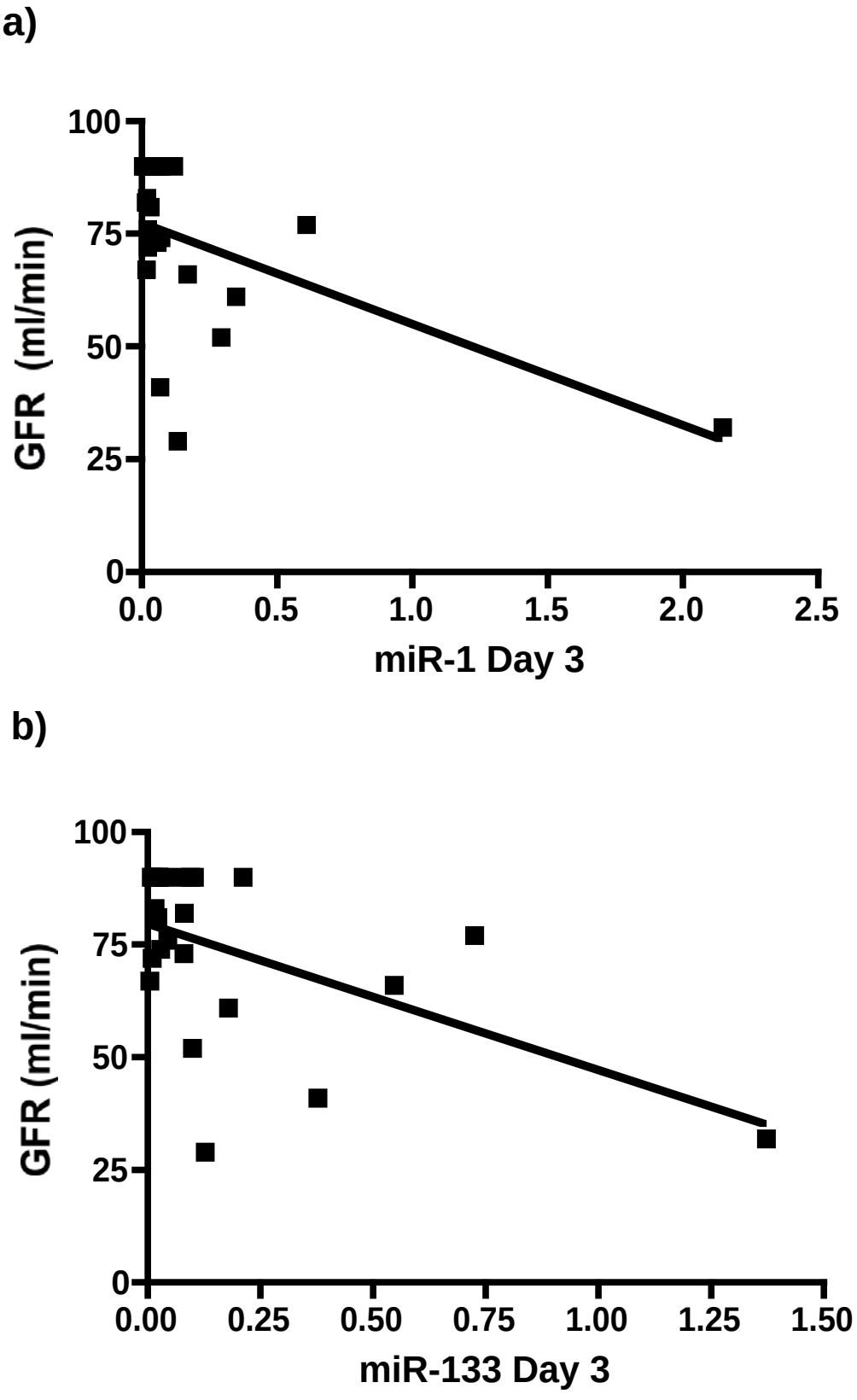
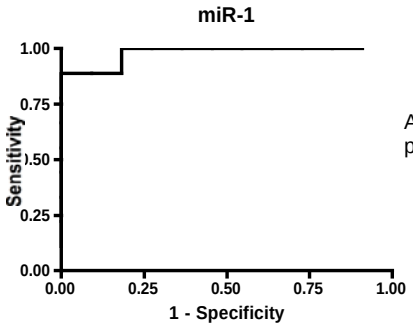
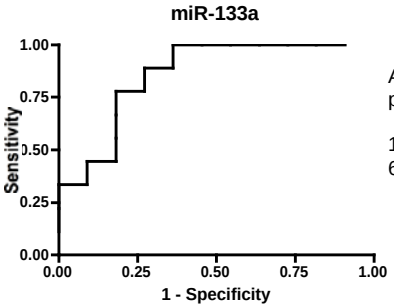

a)
100
75
50
25
0
GFR (ml/min)
0.0
0.5
1.0
1.5
2.0
2.5
miR-1 Day 3
b)
100
75
50
25
0
GFR (ml/min)
0.00
0.25
0.50
0.75
1.00
1.25
1.50
miR-133 Day 3

Figure 6.

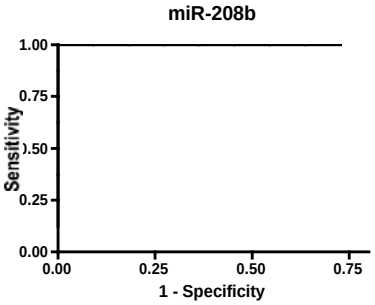


AUC=0.9798
p=0.0003



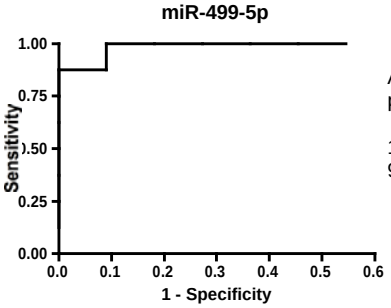
AUC=0.8586
p=0.007

100% Sensitivity
63% Specificity



AUC=1.0
p=0.0003

100% Sensitivity
100% Specificity



AUC=0.9886
p=0.0004

100% Sensitivity
91% Specificity