D'Alessandra *et al*. Circulating microRNAs are new and sensitive biomarkers of myocardial infarction

Supplementary Data Online

Supplementary Materials and Methods

Patients characteristics and blood samples collection

Blood samples were obtained from 17 healthy donors and 2 different groups of patients. STEMI patients in group 1 (n=25; Suppl. Table S1) were enrolled according to specific inclusion criteria (Suppl. Table S2); blood samples were obtained 517 ± 309 min after the onset of symptoms and, again, at day 5 and 30 after MI. All patients underwent PCI ~ 30 min prior to the first blood sampling. STEMI patients in group 2 (n=8; Suppl. Table S3) were enrolled and blood samples for simultaneous miRNA and TnI determination were obtained 156 ± 72 min after the onset of symptoms and, again, 3, 9, 15, 21, 33, 45 and 69 hr therafter: four of these patients were treated with thrombolysis and four with PCI ~ 30 min prior to the first blood collection. K2-EDTA- and thrombin-coated tubes (Beckton Dickinson, BD) were used for plasma and serum preparation, respectively. Whole blood was allowed to clot for 10 minutes at 21° C in order to obtain the serum sample. Cell- and platelet-free plasma and serum were prepared following a 2 step centrifugation protocol: samples were initially centrifuged at 1.500g for 15' at 4°C. The supernatant was collected and centrifuged again at 14.000g for 15' at 4°C; thereafter it was aliquoted and stored at -80°C.

Mouse models of MI and hindlimb ischemia

C57BL/6 female mice, 2-3 months of age, were used for all experiments.
MI was induced as previously described (20). Briefly, mice kept under anesthesia were mechanically ventilated; thoracotomy via the third left intercostal space was performed, the heart was visualized and the left coronary artery was ligated. The chest was closed and the mice were allowed to recover. Sham operated mice were treated similarly without coronary artery ligation. Blood was collected just prior to euthanasia, 15, 30, 60, 90 120, 150 min, 3, 6, 18, 24 hr, 2, 3 and 5 days after surgery. Both the border and infarcted regions of mice with MI and the left ventricle of sham operated mice were collected 3 and 6 hr after coronary artery ligation and stored at –80°C to be processed for RNA isolation.

Hindlimb ischemia was induced as previously described (21). Briefly, mice were kept under anesthesia, the left femoral artery was exposed, dissected free, and excised. Sham operated mice were treated similarly, without femoral artery excision. Blood was collected just prior to euthanasia, 6 and 24h after femoral artery dissection. The adductor and gastrocnemius skeletal muscles were obtained both from the ischemic and the contralateral, normoperfused, limb, 6 and 24 hr after acute ischemia and stored at –80°C to be processed for RNA isolation.
All blood samples were collected in K2-EDTA tubes and processed as described above to obtain cell- and platelet-free plasma.
All procedures complied with the Guidelines of the Italian National Institutes of Health, with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee at IDI-IRCCS.

RNA isolation
Total RNA was isolated from donor plasma using mirVana PARIS isolation kit (Ambion, Austin, Texas) according to manufacturer instructions for liquid samples. In 6 control subjects both plasma and serum were used to establish whether any difference in miRNAs levels existed between the two samples. Briefly, 4 aliquots, each of 0.6 ml for a total of 2.4 ml, of human plasma or serum were used to extract total RNA. Each sample was eluted in 100 µl of RNAse-free water. Then RNAs derived from the same plasma/serum sample were pooled and concentrated to a volume of 50 µl using a SC110 SpeedVac Plus (Thermo Scientific, Waltham, MA). In mice, total RNA was obtained using TRIZOL (Invitrogen, Carlsbad, CA) extraction procedure. Briefly, 1ml of TRIZOL was used for 50 - 400µl of plasma followed by phase separation. RNA was precipitated by adding 30µg of glycogen (Ambion) and 1 ml of isopropanol to the collected aqueous phase. Pellets were resuspended in 100 µl of RNAse-free water.
Total RNA was extracted from human LV and mouse cardiac and skeletal muscle samples using TRIZOL according to manufacturer instructions.
Spectrophotometric RNA quantification was not reliable in samples obtained from plasma and serum because of undetermined contaminants with an absorbance peak at 270 nm. However, we found that qPCR was not affected. Thus, with the exception of the samples used for profiling experiments, all RNA samples were analyzed for miR-17-5p expression to assess an approximate yield of RNA extraction and to ensure that comparable amounts of starting material were used in each RT reaction.

miRNAs profiling and validation
TaqMan Human MicroRNA A and B Arrays version 2.0 (Applied Biosystems, Foster City, CA) were used for miRNA expression screening of 667 miRNAs. Reverse transcription (RT) and pre-amplification steps were performed using the same volume of total RNA from Group 1 patients (n=6 card A, n=4 card B) and controls (n=6 card A, n=4 card B), according to the manufacturer protocol. All steps were performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems); results were expressed as Cts and normalized on the calculated median Ct of each sample (ΔCt). Relative expression was calculated using the comparative Ct method ($2^{-[\Delta][\Delta]Ct}$). All miRNAs present in at least 5 out of 6 samples for card A and in all 4 samples for card B and that showed a Ct ≤ 39 were considered as expressed.
To minimize the number of false positives, only miRNAs that significantly differed from control more than 3 ΔΔCt, i.e. 8-fold, were considered for the subsequent validation step by real time RT-

PCR (qRT-PCR). In single TaqMan microRNA assays miR-17-5p was used as internal reference. Single miRNAs expression was determined using TaqMan microRNA assays (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. The analysis of mice plasma and tissue samples was limited to those miRNAs which were significantly modulated in human plasma.

Troponin I determination

Plasma Troponin I (TnI) level was determined with commercially available ELISA assays for human (RF421C, Siemens Healthcare) and mouse (HK5310-CTNI, Heska Corporation) using a DimensionRXLMax system (Siemens Healthcare) and an i-STAT 1 analyzer (Heska Corporation) for human and mouse samples, respectively.

The upper limit for the normal reference range was 0.06 ng/ml.

Supplementary Results

Validation of miR-17-5p as internal reference for qRT-PCR normalization.

The data from the screening analysis were normalized on the median Ct for each sample so that all subsequent calculations were independent from RNA amount of the samples. Profiling data analysis showed that miR-17-5p was readily detectable, displayed minimal variability and non-significant differences between STEMI patients and controls (Supplemental Table 5). Further, no other study on circulating miRNAs to-date, regardless of the disease under evaluation, has shown an increase in circulating miR-17-5p. Thus, this miRNA was selected for normalization purposes in qRT-PCR single assay experiments. In order to validate miR-17-5p as internal reference, its stability during extraction was compared to that of synthetic miR-39, a miRNA of *C. elegans* that is not present in humans. As previously described by Mitchell *et al.* (14), 25 fmol of synthetic cel-miR-39 were spiked after addition of TRIZOL to the plasma samples to avoid degradation by endogenous RNases and RNA was extracted. For each sample we measured the expression of cel-miR-39, miR-17-5p and two miRNAs among those that in the screening procedure were found to be expressed and modulated by STEMI, i.e. miR-133a and miR-499-5p. We compared the expression of these miRNAs in a group of 5 control vs 7 patients and obtained the same differences in miR-133a and 499-5p expression regardless of whether miR-17-5p or cel-miR-39 were used as normalizer (not shown). In addition, we analyzed the expression of 3 additional miRNAs that, in the screening analysis, exhibited good stability between patients and controls; miR-16, which has been used as a normalizer in previous studies (39), miR-106a and miR-30c which is highly expressed in human heart. Therefore, miR-16, -106a, -30c as well as miR-17-5p were all used to normalize the signals of miR-1. -133a, -133b, -599-5p, -122, -375 on samples from 8 healthy donors and 8 patients at day 0, shortly after the onset of acute STEMI. Interestingly, the results obtained after normalization with miR-17-5p, -16, -106a and -30c were almost identical for each of the 6 miRNAs analyzed (not shown).

Plasma miRNA stability.

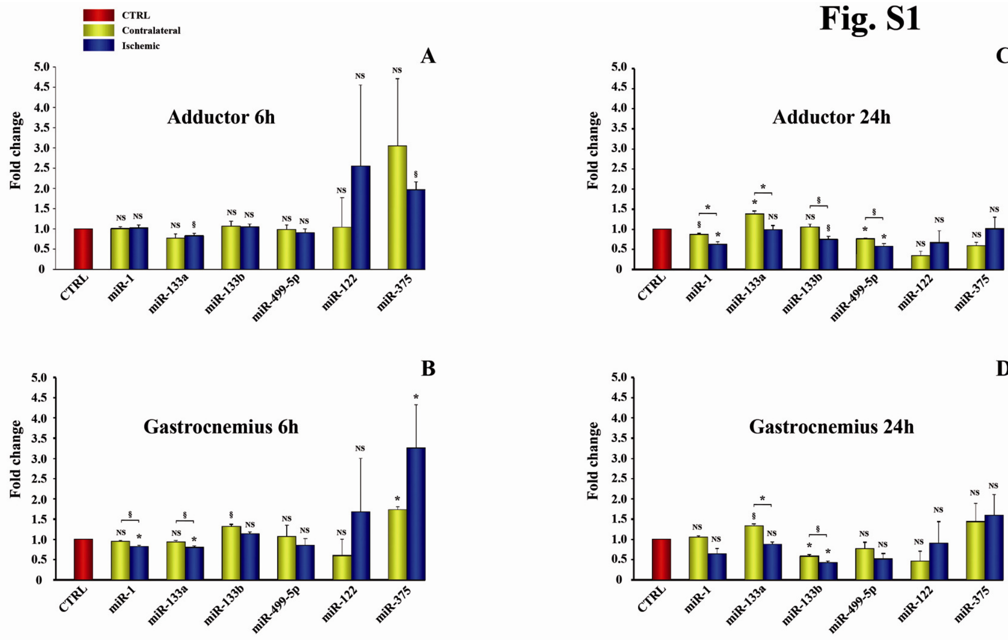
Human plasma from 4 control individuals was isolated and 400 µl aliquots were maintained at room temperature for 2h, 6h and 24h. Immediately after incubation samples were processed for RNA isolation. In keeping with previous observations (14), it was found that miR-17-5p, -1 and -133b displayed similar Ct values at all timepoints (Suppl. Table S6). Thus, plasma miRNAs are stable in the observed time periods.

To asses the stability of miRNAs in plasma after multiple freeze/ thaw cycles, we proceeded as described by Mitchell *et al.*(14). RNA was extracted from 400 µl plasma aliquots obtained from the same 4 individuals used for room temperature incubation experiments described above. The samples underwent 0, 4 and 8 freeze/ thaw cycles. As previously observed, (14), it was found that miR-17-5p, -1and -133b displayed similar Ct values regardless of the number of freeze/thaw cycles (Suppl. Table S6).

Supplementary Figure Legends

Supplementary Figure S1: miRNAs skeletal muscle levels in mice with acute hindlimb ischemia

miR-1, -133a, -133b, -499-5p, -122 and -375 skeletal muscle levels in mice with acute hindlimb ischemia. Measurements were made 6 and 24 hr after surgery in the adductor (Panels A and C) and gastrocnemius (Panels B and D) skeletal muscles of both the ischemic and of the contralateral, non-ischemic limb, as well as in sham operated mice (CTRL, arbitrarily set at 1). Differences were considered of interest when they occurred both between the ischemic muscle and the contralateral normoperfused muscle as well as between the ischemic muscle and the normoperfused muscle in sham operated mice. miR-1 was lower than both controls in the gastrocnemius muscle at 6 hr, and in the adductor muscle at 24hr. miR-133a was lower than both controls only in the gastrocnemius muscle at 6 hr. miR-133b was lower than both controls in the adductor and gastrocnemius muscles only at 24 hr. mir-499-5p was lower than both controls in the adductor muscle at 24 hr. (n = 5 for hindlimb ischemia in each group and n = 5 for sham operated control mice; values indicate fold changes of each miRNA vs its level in sham operated mice, arbitrarily set at 1 as indicated by the red bar, at the same time point; results for each miRNA are reported as mean ± SEM; not significant = NS; * indicates $p \leq 0.01$; § indicates $p \leq 0.05$ vs sham operated and, when reported above the horizontal bar, vs the contralateral limb).


CTRL
Contralateral
Ischemic
Fig. S1
A
Adductor 6h
B
Gastrocnemius 6h
C
Adductor 24h
D
Gastrocnemius 24h
Fold change
CTRL
miR-1
miR-133a
miR-133b
miR-499-5p
miR-122
miR-375