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1 # Circulating microRNAs as diagnostic biomarkers for cardiovascular
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Abstract

One of the major challenges in cardiovascular disease is the identification of reliable clinical biomarkers which can be measured routinely in plasma. MicroRNAs were recently discovered to circulate in the bloodstream in a remarkably stable form. Because of their stability and often tissue- and disease-specific expression, and the possibility to measure them with high sensitivity and specificity, miRNAs are emerging as new diagnostic biomarkers. In this review we will provide an overview of the potential of circulating miRNAs as biomarkers for a wide range of cardiovascular diseases such as coronary artery disease, myocardial infarction, hypertension, heart failure, viral myocarditis and type 2 diabetes mellitus. Furthermore we will discuss the challenges with regard to further validation in large patient cohorts and we will discuss how measurement of multiple miRNAs simultaneously might improve accuracy of the diagnostic test.

Key Words: Circulating miRNAs, Biomarkers, Cardiovascular disease.

Introduction

Cardiovascular diseases represent the predominant cause of human morbidity and mortality in developed countries, underscoring the need for therapeutic, diagnostic and prognostic strategies for this class of diseases.(3;22) One of the major challenges in cardiovascular disease is the identification of reliable biomarkers. MicroRNAs (miRNAs) are short, non-coding RNA sequences, that regulate gene expression at the posttranscriptional level by targeting the 3' untranslated region of mRNA sequences.(5) Gene expression studies revealed that miRNAs are differentially expressed in heart disease (46) and loss-of-function studies in mice firmly established that miRNAs control a variety of cellular processes essential to the heart.(43)

Although the presence of intact extracellular RNA in plasma was already described in 1947 (24;32), it was discovered in 2008 that miRNAs are also present in the circulation in all compartments of the blood, including plasma, platelets, erythrocytes and nucleated blood cells.(7;35) These circulating miRNAs are found to be remarkably stable in plasma even under harsh conditions as boiling, low or high pH, long-term storage at room temperature and in multiple freeze-thaw cycles.(7;35) Interestingly, circulating miRNAs are protected from endogenous RNAse activity (35) and evidence is now accumulating that this protection is achieved by packaging of plasma miRNAs into microparticles (e.g. exosomes, microvesicles or apoptotic bodies (45;57)), by binding to RNA-binding proteins (e.g. Argonaute 2 and nucleophosmin 1 (4;50)) or by linkage to high-density lipoprotein (HDL).(47) Readers interested in the cellular release mechanism of miRNAs, the nature of their stability in the

73 circulation and their possible biological role in cell-cell signaling are referred to two recent
74 reviews by Fichtlscherer et al.(17) and Creemers et al.(11)
75 Due to their stability in the circulation, miRNAs are currently explored for their potential as
76 biomarkers in a wide range of cardiovascular diseases. The ideal biomarker fulfills a number
77 of criteria: 1) accessible using non-invasive methods; 2) a high degree of sensitivity and
78 specificity to the disease; 3) allow early detection; 4) sensitivity to relevant changes in the
79 disease; 5) a long half-life within the sample; and 6) the capability of rapid and accurate
80 detection.(52) Circulating miRNAs fulfill a number of these criteria. They are stable in the
81 circulation, they are often regulated in a tissue- and pathology-specific manner, and they can
82 be detected with high sensitivity and specificity using sequence-specific amplification. These
83 qualities suggest that the discovery-validation pipeline for miRNA biomarkers will be more
84 efficient than for protein-based biomarkers, where bottlenecks at the point of specific
85 antibody generation are often encountered due to the complexity of protein composition,
86 posttranslational modifications and the low abundance of many proteins in serum and
87 plasma.(35;52)
88 In cardiovascular disease, distinctive patterns of circulating miRNAs have thus far been
89 found for coronary artery disease, myocardial infarction, hypertension, heart failure, viral
90 myocarditis and type 2 diabetes mellitus.(10;16;28;42;49;56) In this review we will
91 summarize and discuss the current knowledge regarding circulating miRNAs as putative
92 biomarkers in these specific diseases.
93

94 Coronary Artery Disease (CAD)

95 CAD is caused by atherosclerosis of the coronary arteries. Atherosclerosis is an
96 inflammatory disease of the arteries characterized by the formation of atherosclerotic
97 plaques.(30) The growth of these plaques is responsible for narrowing of the lumen of the
98 artery, whereas acute rupture of an unstable plaque can cause thrombus formation and
99 complete obstruction of the lumen.(30) All cellular components involved in plaque and
100 thrombus formation (e.g. endothelial cells, macrophages, smooth muscle cells) may
101 potentially release miRNAs in the circulation which may serve as potential biomarker for
102 atherosclerosis. Furthermore these cellular components may theoretically also take up
103 miRNAs from the circulation or simply secrete less miRNAs, which will both result in
104 decreased miRNA levels in the circulation. Loss of these miRNAs might thereby also serve
105 as potential biomarker for atherosclerosis. In the setting of coronary atherosclerosis, miRNA
106 signatures have been investigated in serum, plasma, whole blood, peripheral blood
107 mononuclear cells (PBMCs), and platelets. These studies are summarized in table 1.
108

109 *Serum / plasma miRNAs in CAD*

Fichtlscherer et al.(16) were the first to investigate miRNA signatures in plasma and serum of patients with CAD. They included patients with stable angiographically documented CAD and excluded patients with impaired ejection fraction, heart failure (HF), unstable CAD and acute myocardial infarction (MI) to reduce the influence of cell death and plaque instability on miRNA levels. Eight miRNAs selected from a microarray were determined in plasma of a cohort of 36 CAD patients and 17 controls. In this cohort the endothelial enriched miRNAs miR-126, miR-17 and miR-92a, the smooth-muscle cell enriched miR-145, and the inflammatory cell enriched miR-155 were significantly reduced, whereas the cardiomyocyte enriched miRNAs miR-133 and miR-208a were elevated in CAD patients.(16) The aberrant levels of these miRNAs was, except for miR-133 and miR-208a, confirmed in serum of a second cohort consisting of 31 CAD patients and 14 controls.

Diehl et al.(15) isolated microparticles from plasma of 5 patients with CAD and 5 patients with acute coronary syndrome and identified miR-19, miR-21, miR-146, miR-155 and miR-223 as elevated in acute coronary syndrome patients compared to the CAD patients. This suggests that the reduction in plasma miR-155 in CAD patients found by Fichtlscherer et al.(16) is due to a reduction in miR-155 in plasma microparticles.

Li et al.(29) measured serum miRNA levels in 104 patients with peripheral artery disease (arteriosclerosis obliterans) and 105 age-matched controls. They identified miR-130a, miR-21, miR-27b, and miR-210 as possible biomarkers with increased levels in peripheral artery disease compared to controls. MiR-130a and miR-27b were correlated with severity of disease indicated by Fontaine stage.(29) The lack of overlap between increased miRNAs in coronary and peripheral atherosclerosis shows the specificity of these miRNAs for the site of origin of the disease.

MiRNAs in whole blood in CAD

So far, two studies have investigated the potential of circulating miRNAs as biomarkers for CAD in *whole blood*. Taurino et al.(40) performed microarray analysis in 12 CAD patients and 12 healthy controls and found miR-140-3p and miR-182 to be enriched in CAD patients. They also performed microarrays on 10 patients before and after completion of an exercise-based rehabilitation program after surgical coronary revascularization and revealed an increase in miR-92 levels after the program.(40) This is an interesting observation regarding the fact that Fichtlscherer et al.(16) identified miR-92a to be reduced in *plasma* of CAD patients.

In the second study, Weber et al.(53) determined the levels of 16 candidate miRNAs in *whole blood* of 10 CAD patients and 15 healthy controls by qRT-PCR and found the levels of 11 miRNAs (miR-19a, miR-484, miR-155, miR-222, miR-145, miR-29a, miR-378, miR-342, miR-181d, miR-150, and miR-30e-5p) to be reduced in patients with CAD. Further analysis

147 revealed that medication with ACE inhibitors within the patient group also resulted in a
148 significant reduction of 7 miRNAs (miR-19a, miR-155, miR-145, miR-222, miR-342, miR-30e-
149 5p and miR-378).(53) Loss of miR-155 and miR-145 in whole blood of CAD patients is
150 consistent with the reduced levels found in plasma (16), but since 83% of the patients and
151 none of the controls in the study by Fichtlscherer et al.(16) was using ACE inhibitors this
152 reduction in plasma might be explained by the use of ACE inhibitors.
153
154 *MiRNA profiling in peripheral blood mononuclear cells in CAD*
155 Hoekstra et al.(21) determined the miRNA signature in peripheral blood mononuclear cells
156 (PBMCs) of CAD patients by qRT-PCR based microarrays. They included 20 healthy
157 subjects, 25 patients with stable angina pectoris (AP) and 25 patients with unstable AP and
158 revealed that miR-135 and miR-147 were downregulated both in patients with stable and
159 unstable AP. Further analysis in this same cohort revealed 3 miRNAs (miR-134, miR-370
160 and miR-198) to be significantly upregulated in the mononuclear cells of patients with
161 unstable compared to stable AP, which suggest that the expression of these miRNAs in
162 mononuclear cells might be used as biomarker to identify patients at risk for acute coronary
163 syndromes. An important limitation of this study is the pooling of RNA from 8-9 patients,
164 which results in sample sizes of 4 per group and no independent validation cohort is
165 included, implicating the need for studies with a larger patient cohort to validate these
166 findings.(21)
167 Takahashi et al.(39) measured the levels of the inflammatory-related miRNAs, miR-146a and
168 miR-146b in PBMCs of 66 stable CAD patients and 33 non-CAD controls. They found both
169 miRNAs to be significantly upregulated in CAD patients. Within 12 months of follow-up 13 of
170 the 66 CAD-patients experienced a cardiac event and miR-146a levels turned out to be an
171 independent predictor of these events.(39) This suggests that miR-146a in PBMCs might
172 serve as a prognostic biomarker to identify CAD patients at risk of cardiac events.
173 Furthermore, in circulating endothelial progenitor cells, miR-221 and miR-222 (microRNAs
174 highly expressed in endothelial cells) were found to be upregulated in 44 CAD patients
175 compared to 22 non-CAD patients.(34)
176
177 *Platelet miRNAs in CAD*
178 A miRNA signature in platelets of patients with CAD was recently investigated by
179 Sondermeijer et al.(38) Using microarrays and qRT-PCR, they found two miRNAs, miR-340*
180 and miR-624* enriched in platelets in two different cohorts consisting of 40 premature CAD
181 and 40 age-matched controls, and 27 atherosclerotic patients and 40 of their family members
182 respectively. A combination of 4 miRNAs (miR-340*, miR-624*, miR-451 and miR-454) was
183 able to distinguish the 40 premature CAD patients and 40 controls with an area under the

184 Receiver-Operating-Characteristic (ROC) curve (AUC) of 0.71. This study shows that miRNA
185 signatures in platelets differ between controls and CAD patients. Whether these miRNAs are
186 able to identify patients at risk of cardiovascular events remains to be elucidated.
187
188 In conclusion, several studies identified aberrant miRNA signatures in patients with CAD in
189 several components of the blood. The lack of overlap of identified miRNAs in the different
190 studies suggests that these aberrant miRNA signatures are specific for the investigated
191 blood components as a source of the miRNAs (table 1). Together, numerous miRNAs were
192 identified that may become helpful in diagnosis and risk stratification of patients with CAD,
193 but studies with larger patient cohorts are needed to validate the most promising miRNA or
194 combination of miRNAs and to determine the potential of these miRNAs as biomarker for
195 CAD.
196

197 Myocardial Infarction (MI)

198 Several groups hypothesized that necrosis of cardiac cells after MI results in leakage of
199 miRNAs into the circulation and that miRNAs highly, and -preferably- specifically expressed
200 in the heart might be used to diagnose acute coronary events. We have summarized these
201 studies in table 2.
202
203 Several groups have investigated the time course of miRNA release after MI. For the
204 cardiomyocyte-specific miR-208, Ji et al.(23) revealed that the levels in plasma were highly
205 comparable to cardiac Troponin I (cTnI) levels in their rat model of isoproterenol-induced
206 myocardial injury. They found miR-208 undetectable at baseline, increased after 3 hrs of
207 isoproterenol treatment and significantly elevated up to 12 hours. MiR-208 was also found to
208 be rapidly induced in rodent models of MI, where it was undetectable in sham-operated
209 animals, increased at 30 minutes, peaked at 3 hours and disappeared from plasma again at
210 24 hours.(12;49) The levels of the muscle-enriched miRNAs, miR-1, miR-133a and miR-499
211 showed a rapid increase within 1 hour after MI in rats to peak between 3 to 12 hours,
212 decrease at 24 hours and return to basal levels at 3 days after MI.(9;49) Comparable time
213 courses were also found by Gidlof et al.(19) after ischemia-reperfusion injury in pigs. Also in
214 humans similar time courses of miRNA release are detected; miR-1 and miR-133a and b
215 were found to peak at 2.5 hour after the onset of symptoms in MI patients, whereas cTnI and
216 miR-499 showed slower time courses and peaked at 6 and 12 hours respectively. Elevated
217 miR-499 levels could still be detected at 48 hours after MI and after 3 days all miRNAs had
218 returned to their normal levels.(1;12)
219
220 *Diagnostic abilities of circulating miRNAs for MI*

221 The cardiac-specific miR-208 and the muscle-enriched miRNAs, miR-1, miR-133 and miR-
222 499-5p have also been investigated for their diagnostic ability in plasma of MI patients.
223 D'Alessandra et al.(12) found in a cohort of 25 MI and 17 healthy controls that miR-1, miR-
224 133a, miR-133b, and miR-499-5p were elevated in MI patients, while miR-122 and miR-375
225 were reduced. They also measured miR-208 in a subgroup of this cohort and found it only
226 detectable in 3 of the 9 examined MI patients, which may be due to the relatively late time
227 point of sample collection, which was in the whole cohort on average 9 hours after the
228 occurrence of symptoms. Wang et al.(49) confirmed that miR-208 was not detectable in
229 plasma of healthy controls or in patients with stable CAD while miR-208 could be detected in
230 91% of the MI patients. Strikingly, in a subgroup of 20 MI patients of which blood samples
231 were collected within 4 hours after the onset of symptoms, miR-208 was detected in all
232 patients, while cTnI was only detected in 85% of the patients, confirming the superior
233 sensitivity of miR-208 at early time points. In the complete cohort, miR-208 showed a
234 superior ROC curve compared to miR-1, miR-133a and miR-499 in separating 33 MI patients
235 from 33 patients with other cardiovascular diseases.(49)
236 Also miR-208b, a family member of miR-208 expressed in heart *and* skeletal muscle,
237 showed a superior performance in separating MI patients from controls with atypical chest
238 pain and no cardiac disease, when compared to miR-499, miR-1 and miR-133.(10;19)
239 Interestingly, levels of miR-499 and miR-208b could be related to disease severity, as was
240 shown by the correlation with Troponin T (TnT) and creatine phosphokinase levels.(10;19)
241 These results were further confirmed by Devaux et al.(14), who found elevated levels of miR-
242 208b and miR-499 and a correlation with TnT and creatine phosphokinase in a cohort of 510
243 MI patients and 87 healthy controls. In this cohort miR-499 showed a diagnostic accuracy
244 comparable to TnT and superior to miR-208b. Kuwabara et al.(27) were not able to detect
245 miR-208 in the majority of their 29 patients with MI, but in this study sample collection was
246 performed at a wide range of time points and it seems likely that of the 5% of patients in
247 which miR-208 was detected, blood was collected at the earliest time points.
248 The muscle enriched miRNAs, miR-1, miR-133 and miR-499 were also found to be elevated
249 in whole blood or plasma of MI patients.(2;9;51) In this regard, miR-499 was found to be
250 specifically enriched in plasma of 9 patients within 48 hr after MI, while this miRNA was
251 undetectable in the same patients at discharge from the hospital, in patients with unstable
252 AP, in patients with congestive heart failure (HF) and in subjects without cardiovascular
253 disease.(1)
254 Olivieri et al.(37) investigated plasma miRNA levels in a cohort of geriatric patients, in which
255 the diagnosis of non ST-elevation MI is challenging due to atypical symptoms and the
256 presence of a modest TnT elevation in many elderly patients, possibly due to other
257 underlying cardiac pathologies. Olivieri et al.(37) studied the miRNA levels in a cohort of 92

258 patients with non ST-elevation MI, 81 patients with acute HF, and 99 healthy controls and
259 found miR-133a, miR-1, miR-499-5p, miR-21 and miR-423-5p elevated in the MI patients
260 compared to the controls and, except miR-1, in HF patients compared to controls. MiR-21
261 and miR-499-5p were elevated in MI compared to HF patients and miR-423-5p was higher
262 elevated in HF patients than in MI patients. As miR-423-5p has been proposed as a
263 biomarker for HF and 74% of MI patients in this population experience HF, we suggest that
264 the elevation of miR-423-5p in the MI patients is due to the presence of HF in this population.
265 Meder et al.(33) performed a microarray on whole blood of 20 MI patients compared to 20
266 control patients. Strikingly, miR-208a/b, miR-133a/b, miR-1 and miR-499 were not among the
267 20 most highly enriched miRNAs detected in this study. This can not be explained by the
268 time of sample collection as blood was drawn within 3 hours after onset of symptoms.(33)
269 This indicates that miRNAs in plasma are only a small fraction of the miRNAs in whole blood,
270 which may lead to biomarkers specific for the different components of blood. However Meder
271 et al.(33) did find 121 miRNAs significantly changed, of which miR-1291 and miR-663b
272 showed the highest AUC of 0.91 and 0.94 respectively, and miR-145 and miR-30c showed
273 the highest correlation (positive) with TnT as a measure of infarct size. A combination of 20
274 miRNAs even showed an AUC of 0.99, indicating that a miRNA signature might be superior
275 as a diagnostic biomarker for MI.
276
277 The different groups investigating circulating miRNAs after MI do not all agree on the
278 suitability of miR-208a as diagnostic biomarker after MI. While its cardiac-specific expression
279 results in high specificity, it might also result in relatively low levels of this miRNA in the blood
280 compared to miRNAs released by other sources and therefore it might be that miR-208a was
281 below the detection limit in some studies resulting in a low sensitivity. Another explanation
282 might be that blood collection in some studies was too long after onset of symptoms, as miR-
283 208a is shown to have an early peak and fast reduction to normal levels in animal models of
284 MI.(12;49)
285
286 *Source of released miRNAs*
287 The first indirect indication that the elevated plasma levels of miR-1, miR-133a, miR-208 and
288 miR-499 in MI patients are released by the injured myocardium is that the levels of these
289 miRNAs are reduced within the infarcted myocardium of mice compared to myocardium from
290 sham operated control mice.(27) More direct evidence was obtained by De Rosa et al.(13),
291 who measured miRNA levels in plasma simultaneously obtained from the aorta and coronary
292 venous sinus of patients with acute coronary syndromes. MiR-133a and miR-499 showed an
293 increased level in the coronary venous sinus compared to the aorta samples in 19 patients
294 with acute coronary syndromes, which correlated with the increased levels of TnT and

295 suggests their release to reflect the extent of myocardial injury. No differences in coronary
296 venous sinus and aorta plasma levels were demonstrated for miR-208, which was most likely
297 due to the low concentrations in plasma limiting precise quantitative assessment.
298 Interestingly, not all myocardial miRNAs leak into the circulation with the same kinetics. The
299 slower release of miR-499 into the bloodstream compared to several other myocardial
300 miRNAs may suggest that the different miRNAs are bound to different proteins within the
301 cell. It is also striking that two other highly expressed cardiac miRNAs, miR-30c and miR-24,
302 which are expressed at even higher levels than miR-208 in cardiomyocytes, failed to
303 increase in plasma after MI.(12) The observation that miR-30c was increased in whole blood
304 after MI and correlated with TnT levels(33), suggests that this elevation is due to
305 upregulation in blood cells instead of leakage by cardiomyocytes.
306
307 *Prognostic abilities of circulating miRNAs after MI*
308 The studies described above are based on low patient numbers and therefore not able to
309 assess the relation of miRNAs to clinical characteristics and their potential prognostic value.
310 Widera et al.(54) determined levels of 6 candidate miRNAs in plasma of 444 patients with
311 acute coronary syndromes and found increased levels of miR-1, miR-133a, and miR-208b in
312 plasma of patients with MI (n=327) compared to unstable AP (n=117) and no differences in
313 miR-133b, miR-208a and miR-499. During 6 months of follow-up, 34 patients died and miR-
314 133a and miR-208b were significantly related to all-cause mortality. Both miRNAs were not
315 able to enhance the discriminatory ability of high sensitive (hs)TnT between survivors and
316 non-survivors.(54)
317
318 In conclusion, several studies fuel the notion that circulating miRNAs might be useful as
319 diagnostic and prognostic biomarkers for MI. In a clinical setting the differences in time
320 courses of release between specific miRNAs and cTnI might be valuable. Especially
321 considering the fact that the cTnI levels begin to rise only 4 to 8 hours after MI (49),
322 diagnosis via biomarkers with a faster cardiac release, such as miR-208, miR-1 and miR-133
323 might be beneficial. The slow time-course of miR-499 might lead to increased diagnostic
324 performance at late time-points after MI when cTnI has already returned back to normal
325 levels. Therefore, it may be expected that in the future, a panel of miRNAs, probably in
326 combination with cTnI, has a better potential to offer sensitive and specific diagnostic tests
327 for acute coronary syndromes.
328

329 **Essential Hypertension**

330 Essential hypertension is a predisposing risk factor for stroke, MI, HF, arterial aneurysm and
331 chronic renal failure. Li et al.(28) have investigated whether a specific miRNA signature could

be identified in patients with essential hypertension (detailed methods are included in table 4). After initial miRNA array analysis in plasma of hypertensive patients and controls, they were able to confirm the different levels of three miRNAs (hcmv-miR-UL112, let-7e, miR-296-5p) in a cohort of 127 hypertensive patients and 67 control subjects. Interestingly, one of the successfully validated miRNAs appeared to be a human cytomegalovirus (HCMV)-encoded miRNA, which suggests a novel link between HCMV infection and essential hypertension. Li et al.(28) subsequently measured HCMV titers in their hypertensive patients and controls and showed elevation of HCMV titers in the hypertensive patients and a correlation between the HCMV titers and the levels of hcmv-miR-UL112 in plasma.

In conclusion, miRNA profiling in plasma of hypertensive patients reveals a possible involvement of HCMV in the pathogenesis of essential hypertension. A possible causal link between HCMV and blood pressure is recently found in mice, where infection with mouse HCMV resulted in higher blood pressure.(8) However, as indicated by Li et al.(28) a high degree of interpatient variation was detected in miRNA levels in plasma, which will make it difficult to use these miRNAs as biomarkers for hypertension.

Heart Failure (HF)

HF is defined as a complex clinical syndrome that can result from any structural or functional disorder that impairs the ability of the ventricles to fill with or eject blood. Table 3 contains an overview of the published studies that report on circulating miRNAs in HF.

Our laboratory investigated whether circulating miRNAs show aberrant profiles in HF and can be used as a biomarker for this disease.(42) In a cohort of 39 healthy controls and 50 dyspnea patients, 30 of whom were diagnosed to have dyspnea due to HF and 20 due to other causes, we determined the levels of 16 miRNAs selected from a microarray. Seven miRNAs were validated to be enriched in plasma of HF patients in this cohort (miR-423-5p, miR-18b*, miR-129-5p, HS_202.1, miR-622, miR-654-3p, and miR-1254), among which miR-423-5p was most strongly related to the clinical diagnosis of HF. MiR-423-5p distinguished HF patients from healthy controls with an AUC of 0.91 and from dyspnea patients without HF with an AUC of 0.83.(42) The circulating levels of miR-423-5p were related to disease severity as shown by an inverse correlation with ejection fraction and higher levels of miR-423-5p in patients with a higher New York Heart Association (NYHA) classification. MiR-423-5p was also correlated to the levels of the current clinically used biomarker N-terminal pro brain natriuretic peptide (NT-proBNP). The elevation of circulating miR-423-5p levels in HF was confirmed by Goren et al.(20), who determined the levels of 186 miRNAs in serum of 30 chronic HF patients compared to 30 age-, gender- and ethnically-matched healthy controls. They found 26 miRNAs to show significantly different levels in HF patients, of which miR-423-5p showed the strongest increase. In this study miR-423-5p was able to distinguish HF-

369 patients from healthy controls with an AUC of 0.88. A direct correlation between circulating
370 miR-423-5p levels and BNP was also detected in this population. However, no relation to
371 disease severity was found, shown by a lack of correlation between miR-423-5p and ejection
372 fraction or NYHA class.(20)
373 In contrast to these findings in *left* ventricular HF, Tutarel et al.(44) do not find elevations of
374 miR-423-5p in *right* ventricular HF. They studied 41 patients with congenital transposition of
375 the aorta and pulmonary artery where the systemic circulation is supported by the right
376 ventricle. Compared to 10 age- and sex-matched controls they did not find any differences in
377 circulating miR-423-5p between patients and controls. Two possible explanations for these
378 differences could be postulated: 1) the difference in pathophysiology of systemic right
379 ventricular HF compared to left ventricular HF(44) and 2) the difference in severity of affected
380 patients, as the patients in the studies of Tijsen et al.(42) and Goren et al.(20) were more
381 severely affected compared to the study by Tutarel et al. These findings indicate the
382 specificity of miR-423-5p elevation for left ventricular HF. Unfortunately no other miRNAs
383 were measured in patients with right ventricular HF, as this could have resulted in promising
384 biomarkers for this specific disease.
385 Several important questions about miR-423-5p remain, as it is unanswered how this miRNA
386 is released into the circulation and by which cell type. It has been shown that in the
387 circulation of healthy subjects, miR-423-5p is specifically bound to Argonaute2 complexes
388 and not associated with microvesicles.(4) On the other hand, Goren et al.(20) did find
389 enrichment of miR-423-5p in the exosomal fraction of HF patients, indicating that the
390 mechanism of miR-423-5p release may be different in HF patients compared to healthy
391 controls. In the pig, miR-423-5p was shown to be ubiquitously expressed, with high levels in
392 heart, liver and brain.(55) Together with the detected upregulation of miR-423-5p in human
393 failing myocardium(41;42), this suggests that circulating miR-423-5p in HF is derived from
394 the myocardium. Interestingly, miR-423-5p levels were also found to be increased in serum
395 of patients with specific forms of cancer such as non-small cell lung cancer (7), and gastric
396 cancer.(31) The fact that levels of miR-423-5p were not elevated in dyspnea patients without
397 HF argues against the possibility that (damage to) the lung is a source of miR-423-5p release
398 during HF.(42)
399 Three other circulating miRNAs are linked to the diagnosis of HF. The endothelium-specific
400 miR-126 was found to be negatively correlated with age, BNP and NYHA class in 10 HF
401 patients and 17 asymptomatic controls.(18) Corsten et al.(10) found both miR-499 and miR-
402 122 to be enriched in the plasma of 33 acute HF patients compared to 34 healthy controls, of
403 which miR-499 is probably myocardium-derived and miR-122 might possibly reflect hepatic
404 venous congestion, as miR-122 is enriched in the liver.
405

406 Voellenkle et al.(48) studied the miRNA signature of HF patients in peripheral blood
407 mononuclear cells. In a cohort of 19 control subjects, 19 subjects with non-ischemic dilated
408 cardiomyopathy (NIDCM), and 15 subjects with ischemic cardiomyopathy (ICM) miR-107,
409 miR-142-5p, and miR-139 were downregulated in both classes of HF, miR-125b and miR-
410 497 in ICM only and miR-142 and miR-29b upregulated in NIDCM only.(48) That none of the
411 plasma/serum miRNAs were significantly different within these mononuclear cells suggests
412 that these miRNAs are not released by these cells and that the results of the other studies
413 are not influenced by lysis of these cells during sample preparation.
414
415 One of the characteristics of an ideal biomarker is changing levels as disease severity
416 changes in response to therapy. For two of the identified circulating miRNAs in HF, miR-499-
417 5p and miR-423-5p, this response to therapy was shown by Montgomery et al.(36) in a rat
418 model of HF. In Dahl salt-sensitive rats HF was hypertension-induced by a high salt diet and
419 treatment with antimiR-208a resulted in improved cardiac function and survival. This antimiR
420 treatment also blunted the increase of circulating miR-499-5p and miR-423-5p levels.(36)
421 Although this response to therapy makes miR-499-5p and miR-423-5p promising biomarkers
422 for HF, the diagnostic performance of these miRNAs is only tested in relatively small patient
423 cohorts, therefore larger studies are needed to confirm their diagnostic capability.
424

425 **Viral Myocarditis (VM)**

426 Myocarditis is an acute or chronic inflammatory disease of the myocardium, which can be
427 caused by viral infections, postinfectious immune reactions or organ-specific autoimmune
428 reactions. Corsten et al.(10) determined circulating miRNA levels in plasma of 14 patients
429 with acute VM, 20 patients in the post-VM phase and 20 healthy control subjects. They
430 identified two cardiac-enriched microRNAs, miR-208b and miR-499-5p, specifically elevated
431 in patients with acute VM compared to both control groups. Subdivision of patients in the
432 acute VM group into mild, moderate and severe VM based on TnT levels and ejection
433 fraction revealed that the levels of miR-208b and miR-499-5p are associated with disease
434 severity.(10) Detailed methods of this study can be found in table 4. Both miRNAs, miR-208b
435 and miR-499-5p are found to be elevated in other cardiovascular diseases as well; miR-208b
436 in MI and miR-499 in MI and HF.(10;49) This may suggest that the release of these miRNAs
437 into the circulation is an indication of cardiac damage and they are therefore not very specific
438 as a biomarker for the diagnosis of VM.
439

440 **Diabetes Mellitus type 2 (DM)**

441 Type 2 DM is a disease characterized by chronic elevation of blood glucose levels and is one
442 of the major risk factors for cardiovascular disease.(56) We have summarized the studies
443 investigating circulating miRNAs in DM in table 5.
444 Based on network analysis of microarray results Zampetaki et al.(56) selected 13 miRNAs for
445 validation in a cohort of 80 DM patients of the Bruneck study and 80 age- and sex-matched
446 controls. In this cohort 12 miRNAs were significantly associated with DM in a multivariate
447 analysis (miR-24, miR-21, miR-20b, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-
448 320, miR-486, miR-150 and miR-28-3p). In a second validation step miR-126 emerged as a
449 significant predictor of DM in the entire Bruneck cohort (n=822) and showed a gradual
450 decrease from normal glucose tolerance via impaired glucose tolerance to DM. Using the
451 expression profiles of the 5 most significantly changed miRNAs (miR-15a, miR-126, miR-
452 320, miR-223, miR-28-3p) they were able to distinguish DM patients from healthy controls
453 with a sensitivity of 70% and a specificity of 92%.(56) Interestingly, levels of miR-126, miR-
454 15a, miR-29b, miR-223 and miR-28-3p were already altered before manifestation of the
455 disease.
456 In a study population of 19 patients susceptible for DM (high BMI and/or family history of
457 DM), 19 pre-diabetic patients (impaired glucose tolerance and/or impaired fasting glucose),
458 and 18 patients with type 2 DM, Kong et al.(26) measured the serum levels of seven miRNAs
459 previously reported to be involved in insulin regulation (miR-9, miR-29a, miR-30d, miR-34,
460 miR-124, miR-146a, and miR-375, but unfortunately not miR-126). Strikingly, all seven
461 miRNAs were found to be elevated in type 2 DM patients and not in pre-diabetic patients
462 compared to the susceptible controls. Karolina et al.(25) used microarrays to investigate the
463 miRNA signature in whole blood in a cohort of 14 patients with impaired fasting glucose, 21
464 patients with DM type 2 and 15 healthy subjects. They identified circulating miR-144, miR-
465 192 and miR-29a as increased in impaired fasting glucose patients and further increased in
466 type 2 DM patients, miR-146 as decreased in both disease states with higher fold change in
467 type 2 DM, miR-150 and miR-320 were decreased in patients with impaired fasting glucose
468 and increased in type 2 DM, and this was the opposite for miR-182 and miR-30d.(25) MiR-
469 126, the miRNA that was most strongly related to DM type 2 in the Bruneck population (56)
470 was not changed, which might be explained by the different sample characteristics (plasma
471 versus whole blood) between the two studies.
472 Caporali et al.(6) identified miR-503, a miRNA involved in diabetic endothelial dysfunction, to
473 be enriched in plasma of 11 DM patients with critical ischemia compared to 11 control
474 subjects. That this miRNA is not detected in the other studies(25;26;56) on circulating
475 miRNAs in DM suggests that miR-503 might be a biomarker for ongoing ischemia in DM.
476

477 In conclusion, several circulating miRNAs are reported to show aberrant levels in type 2 DM
478 patients, of which 3 miRNAs (miR-29a, miR-30d, miR-146a) were shared between the
479 studies by Kong et al.(26) and Karolina et al.(25) The diagnostic ability of miR-126 was
480 successfully validated in a large prospective cohort of 822 individuals. Furthermore this
481 miRNA was already regulated years before manifestation of the disease and therefore
482 possibly useful for risk prediction.
483

484 Future Perspective

485 *Challenges for circulating miRNAs as biomarker*

486 Circulating miRNAs are emerging as blood-based biomarkers for cardiovascular diseases,
487 since they offer many attractive features of biomarkers. They are stable in the circulation,
488 their sequences are evolutionarily conserved, their expression is often tissue- or pathology-
489 specific, and their detection is based on sequence-specific amplification, features that are
490 helpful in the development of sensitive and specific assays. As discussed in this review,
491 initial candidate miRNAs have been proposed as biomarker for CAD, MI, hypertension, HF,
492 VM and type 2 DM. However, there are also challenges associated with the discovery-
493 validation pipeline for circulating miRNAs as biomarkers for disease. First, most studies
494 performed to date are evaluated in populations with less than 100 subjects. Although several
495 candidate miRNAs are confirmed in more than one study, further validation in larger patient
496 cohorts is needed. In these larger studies one should not only focus on the diagnostic
497 performance of these miRNAs, but also investigate the usefulness of the miRNAs in
498 predicting the prognosis of patients. For instance, it will be interesting to identify a set of
499 miRNAs that may be able to predict which CAD patients are at risk for developing MI.
500 Furthermore, future studies should also clarify whether miRNAs are useful to monitor the
501 response to therapy, as suggested for miR-499-5p and miR-423-5p, which are reduced in
502 rats with HF upon treatment with antimiR-208.(36)
503 Validation of candidate miRNAs in larger patient cohorts is not the only challenge for
504 circulating miRNAs to become clinically useful as biomarker. The other challenges mainly
505 relate to the low amount of total RNA in plasma or serum, which makes miRNA amplification
506 often necessary to measure circulating miRNAs. The low amount of RNA in plasma and
507 serum makes it virtually impossible to measure concentration and quality of the isolated
508 RNA, and as a consequence, variances based on the amount of starting material and miRNA
509 extraction might occur. Therefore normalization is an important aspect in the measurement of
510 circulating miRNAs, but at the moment no satisfying ‘housekeeping’ circulating miRNA has
511 been identified. Several reports use the small nucleolar RNA U6 or other miRNAs as an
512 internal control and although these miRNAs may be stable in some studies, they may change
513 in other pathological conditions and are therefore not suitable as internal control in all

studies. Another widely used method for normalization is the addition of synthetic spike-in miRNAs, mainly *Caenorhabditis elegans* miRNAs without homology to human miRNAs, during the purification process. This method worked well for Mitchell et al.(35), who reported it first, but they also found these synthetic miRNAs to be unstable in crude plasma. Therefore, the moment of adding the spike-in miRNAs to the plasma is of crucial importance since plasma RNAse activity should be fully inactivated before the synthetic spike-in RNAs are added to the sample. Cheng et al.(9) reported that correcting for plasma volume is the best method of normalization, as volume of plasma is clinically standard for other biomarkers. Future studies are needed to compare these different methods and identify the most reliable method of normalization, which might be specific for the release route of the miRNAs (microparticles or protein-bound).

Clinical application

At the moment most studies are investigating the usefulness of individual miRNAs as biomarker for disease, but it is expected that a combination of multiple miRNAs may provide greater accuracy. For example, a combination of miR-208a, miR-133, miR-1 and miR-499-5p in one test will result in a test able to identify MI patients in a broader time range after onset of complaints, as the first three miRNAs peak at 3 hours after MI and miR-499 at 12 hours.(12;49) Furthermore, a combination of miRNAs in a diagnostic test may provide better diagnostic accuracy since different causes for the disease might result in different levels of plasma miRNAs. This might be the case in atherosclerotic and non-atherosclerotic forms of HF and in right and left ventricular HF. An indication that a panel of miRNAs provides better diagnostic accuracy is described in type 2 DM by Zampetaki et al.(56), where a combination of 5 miRNAs resulted in a diagnostic test with high sensitivity and specificity.

In conclusion, the identification of stable circulating miRNAs launches a new generation of potential biomarkers, for which assays can be developed with relative ease, at a relatively low expense, but with potentially unrivaled specificity and sensitivity. These assays could easily be designed to combine a large number of circulating miRNAs, which could drastically change the use and interpretation of circulating biomarkers as we know them.

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Dr. Pinto is a cofounder of and holds less than 5% equity in ACS Biomarker BV, a company that commercializes cardiovascular biomarkers.

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760 **Table 1: Circulating miRNAs as biomarkers for coronary artery disease.**

Groups	miRNAs	Source	RNA isolation	miRNA detection	Normali-zation	Age/sex differences	Multivariate analysis	ref
31 controls; 67 CAD	miR-126, miR-17, miR-92a, miR-145, miR-155	EDTA plasma	Trizol and miRNeasy kit	Taqman probes	cel-miR-39 spike in	Controls >30 years younger, more females	no	(16)
12 controls; 12 CAD	miR-140-3p, miR182	Whole blood	miRNeasy kit	Microfluidic array LC sciences	No	No	No	(40)
15 healthy control; 10 CAD	miR-19a, miR-584, miR-155, miR-222, miR-145, miR-29a, miR-378, miR-342, miR-181d, miR-150, miR-30e-5p	Whole blood	PAXgene miRNA kit	Taqman probes	RNU44	Age and gender matched	No	(53)
40 premature CAD; 40 healthy controls and 27 atherosclerosis; 40 family members	miR-340*, miR-624*	platelets	mirVana PARIS	Sybr-green based, taqman probes	Platelet count, miR-223	Age matched and males only; Controls >20 years younger, sex unknown	No	(38)
20 healthy controls; 25 unstable AP; 25 stable AP;	miR-135, miR-147	PBMC	Guanidium-thiocyanate	Taqman array	let-7a, miR-16	Age, sex, ethnically, smoking matched	No	(21)

33 non-CAD; 66 stable CAD	miR146a/b	PBMC	mirVana PARIS	Taqman probes	RNU6	No	Yes*	(39)
22 non-CAD; 44 stable CAD	miR-221, miR-222	Endothelial progenitors	mirVana PARIS	Taqman probes	RNU6	No	No	(34)
5 CAD; 5 acute coronary syndrome	miR-19, miR-21, miR-146, miR-155, miR-223	Microparticles from plasma	miRNeasy kit	Sybr-green based	No	No	No	(15)
104 arteriosclerosis obliterans; 105 controls	miR-130a, miR-27b, miR-210, miR-21	Serum	QIAamp circulating nucleic acid kit	Sybr-green based	No	Age-matched	No	(29)

761 CAD, coronary artery disease; AP, angina pectoris; PBMC, peripheral blood mononuclear cells.

762 * Age, gender, culprit lesion, smoking, blood pressure, fasting glucose, HbA_{1c}, LDL-cholesterol, high sensitive C-reactive protein and history of

763 hypertension, DM and CAD corrected.

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766 **Table 2: Circulating miRNAs as biomarkers for myocardial infarction.**

Groups	miRNAs	Source	RNA isolation	miRNA detection	Normali-zation	Age/sex differences	Multivariate analysis	ref
7 no CAD; 31 stable CAD; 19 ACS	miR-499, miR-133, miR-208a, miR-126	EDTA plasma	Tri reagent	Taqman probes	cel-miR-39 spike-in	No	No	(13)
36 atypical chest pain; 32 MI	miR-1, miR-133, miR-208b, miR-499	Citrate plasma	mirVana PARIS kit	Sybr-green based	3 *c.elegans* spike-ins	No	No	(10)
117 unstable AP; 327 MI	miR-1, miR-133, miR-208b	plasma	MasterPure kit	Taqman probes	cel-miR-54 spike-in	No	Yes*	(54)

10 controls; 9 MI; 5 unstable AP; 15 HF	miR-499	EDTA plasma	mirVana PARIS	Taqman probes	synthetic miRNA	Controls >30 years younger	No	(1)
66 healthy control; 93 MI	miR-1	Citrate plasma	mirVana PARIS	Sybr-green based	RNU6	No	No	(2)
20 controls; 31 MI	miR-1	serum	miRNA isolation kit	Sybr-green based	No	Age, sex matched	No	(9)
17 healthy control; 33 MI	miR-1, miR-133a, miR-133b, miR-499-5p, miR-208, miR-122, miR-375	EDTA plasma	mirVana PARIS	Taqman probes	miR-17-5p	Controls >10 years younger	Age	(12)
30 healthy control; 33 MI, 16 CAD, 17 other CVD	miR-1, miR-133a, miR-499-5p, miR-208	plasma	Tri reagent	Taqman probes	cel-miR-39	No	No	(49)
29 MI; 42 non-MI	miR-1, miR-133a	Serum	Trizol LS	Taqman probes	No	No	No	(27)
11 healthy control; 9 MI	miR-208b, miR-1, miR-133, miR-499-5p	plasma	Trizol LS, miRNeasy kit	Sybr-green based	No	No	No	(19)
28 control; 51 MI	miR-133, miR-328	EDTA plasma, whole blood	Trizol LS	Sybr-green based	RNU6	No	No	(51)
20 control patients; 20 MI	miR-1291, miR-663b, miR-145, miR-30c	Whole blood	miRNeasy kit	Microarray	No	No	No	(33)
92 elderly MI; 81 HF; 99 healthy control	miR-1, miR-133a, miR-423-5p, miR-21, miR-499-5p	EDTA plasma	mirVana PARIS	Taqman probes	miR-17, cel-miR-39	Age-matched	Yes†	(37)
510 MI; 87 control	Mir-208b, miR-499	Citrate	mirVana	Sybr-green	3 *c. elegans*	No	Yes‡	(14)

		plasma	PARIS	based	spike-ins			

CAD, coronary artery disease; ACS, acute coronary syndrome; MI, myocardial infarction; AP, angina pectoris; HF, heart failure; CVD, cardiovascular disease.

* Age, sex, and hsTnT corrected

† Age and sex corrected

‡ Age, sex, hypertension, hypercholesterolemia, smoking and hs-TnT corrected

Table 3: Circulating miRNAs as biomarker for heart failure.

Groups	miRNAs	Source	RNA isolation	miRNA detection	Normali-zation	Age/sex differences	Multivariate analysis	ref
39 healthy controls; 20 HF cases; 20 non-HF cases with dyspnea	miR-423-5p, miR-18b*, miR-129-5p, HS_202.1, miR-622, miR-654-3p, miR-1254	Citrate plasma	mirVana PARIS kit	Sybr-green based	miR-1249	Controls >10 years younger	Yes*	(42)
30 chronic HF; 30 healthy controls	miR-423-5p, miR-320a, miR-22, miR-92b	Serum	phenol: chloroform purification	Taqman probes	Based on mean Ct of all miRNAs	Age, sex, ethnically matched	No	(20)
41 right ventricular HF; 10 healthy controls	miR-423-5p	plasma	MasterPure kit	Taqman probes	cel-miR-54 spike-in	Age and sex matched	No	(44)
10 HF; 17 asymptomatic control	miR-126	EDTA plasma	mirVana PARIS	Taqman probes	Synthetic spike-in	Controls >25 years younger	No	(18)
33 acute HF; 34 healthy controls	miR-499, miR-122	EDTA plasma	mirVana PARIS	Sybr-green based	3 *c. elegans* spike ins	Controls >40 years	No	(10)

						younger		
19 healthy controls; 19 NIDCM; 15 ICM	miR-107, miR-139, miR-142-5p, miR-142-3p, miR-19b, miR-125b, miR-497	PBMC	Trizol	Taqman probes	miR-16	Unknown	No	(48)

775 HF, heart failure; NIDCM, non-ischemic dilated cardiomyopathy; ICM, ischemic cardiomyopathy; PBMC, peripheral blood mononuclear cells.

776 * age and sex corrected

777

778 **Table 4: Circulating miRNAs as biomarker for hypertension and viral myocarditis**

Disease	Groups	miRNAs	Source	RNA isolation	miRNA detection	Normali-zation	Age/sex differences	Multivariate analysis	ref
Essential Hypertension	89 controls; 151 hypertension	hcmv-miR-UL112, let-7e, miR-296-5p	EDTA plasma	Tri reagent and RNeasy mini kit	Taqman probes	No	No	Yes*	(28)
Viral Myocarditis	14 acute VM; 20 post-VM; 20 healthy controls	miR-208b, miR-499	EDTA plasma	mirVana PARIS kit	Sybr-green based	3 *c. elegans* spike-ins	Age matched	No	(10)

779 VM, viral myocarditis

780 * age, sex, BMI, diabetes mellitus, hyperlipidemia, and history of CAD corrected

781

782 **Table 5: Circulating miRNAs as biomarker for diabetes mellitus.**

Groups	miRNAs	Source	RNA isolation	miRNA detection	Normali-zation	Age/sex differences	Multivariate analysis	ref
80 DM; 80 controls	miR-15a, miR-126, miR-29b, miR-223, miR-28-3p	plasma	miRNeasy kit	Taqman probes	Unadjusted, miR-454,	Age and sex	Yes*	(56)

					RNU6b	matched		
580 controls; 162 impaired glucose; 80 DM	miR-126	plasma	miRNeasy kit	Taqman probes	Unadjusted, miR-454, RNU6b	Unknown	Yes*	(56)
19 susceptible; 19 pre-diabetic; 18 DM	miR-29, miR-34a, miR-146a, miR-375, miR-9, miR-30d, miR-124	serum	mirVana miRNA isolation kit	Taqman probes	RNU6b	No	No	(26)
15 healthy controls; 14 impaired glucose tolerance; 21 DM	miR-146a, miR-182, miR-30d, miR-144, miR-150, miR-192, miR-29a, miR-320	Whole blood	RiboPure Blood kit	Taqman probes	RNU6	Age matched; only males	No	(25)
11 controls; 11 DM with critical ischemia	miR-503	Plasma	Trizol	Taqman probes	RNU6	No	No	(6)

DM, diabetes mellitus

* Social status, DM family history, BMI, waist to hip ratio, smoking, alcohol, C-reactive protein, physical activity, age, and sex corrected