

1 **Evaluation of the associations between circulating microRNAs and kidney**
2 **function in coronary angiography patients**

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57 **ABSTRACT**

58

59 Circulating microRNAs (miRNAs) have been linked to chronic kidney disease. Little is
60 known about the association between circulating miRNAs and kidney function in patients at
61 high cardiovascular risk. We therefore investigated the association between a ~~huge~~ panel of
62 candidate miRNAs and kidney function, based on estimated glomerular filtration rate (eGFR),
63 in two independent cohorts of patients undergoing coronary angiography. The present study
64 totally included 438 coronary angiography patients, who were divided into a discovery cohort
65 (n=120) and a validation cohort (n=318). A candidate miRNA panel comprising 50 renal
66 miRNAs were selected from the literature and expression levels of circulating miRNAs were
67 determined by real-time PCR. Out of initially tested candidate-miRNAs, 38 were sufficiently
68 detectable in plasma. Their association with kidney function was evaluated in the discovery
69 cohort. Associations of seven out of these miRNAs with eGFR were significant after multiple
70 testing correction via false discovery rate (FDR) estimation. To verify obtained results,
71 miRNAs with significant FDR were further analysed in the validation cohort. MiRNAs miR-
72 106b-5p, miR-16-5p, miR-19b-3p, miR-20a-5p, miR-25-3p, and miR-451a proved to be
73 significantly associated with eGFR also in the validation cohort (all p-values <0.001).
74 Association between identified renal miRNAs and kidney function was confirmed by
75 ANCOVA adjusting for age, gender, type 2 diabetes, hypertension, and albumin-to-creatinine
76 ratio. In conclusion, our study showed that miR-16-5p, miR-19b-3p, miR-20a-5p, miR-25-3p,
77 miR-106b-5p, and miR-451a are significantly linked to kidney function in coronary
78 angiography patients.

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80

81 INTRODUCTION

82

83 Chronic kidney disease (CKD) is increasing worldwide and is strongly linked to an elevated
84 risk of cardiovascular and all-cause mortality (31, 36). CKD is characterized by a progressive
85 loss of kidney function over months or years typically showing a long latent period when the
86 disease is clinically silent (18). Therefore, early detection of reduced kidney function is
87 essential to improve risk prediction, particularly in high-risk patients.

88

89 Recently, circulating microRNAs (miRNAs) have emerged as novel diagnostic biomarkers in
90 many diseases including kidney disease (20, 21). MiRNAs are small non-coding RNAs of
91 approximately 22 nucleotides in length that usually function as repressors of target genes by
92 either inhibiting translation or promoting degradation of mRNA (5, 30). Currently,
93 approximately 2700 mature miRNAs have been identified in humans according to database
94 miRbase, release 22.1 (15). Many of them are expressed in a tissue and/or cell-specific
95 manner playing important regulatory roles in virtually all cellular processes inclusive of
96 kidney development and function (1, 37). Notably, miRNAs can also be detected outside
97 cells, including circulating cell-free body fluids such as plasma, serum, or urine in a
98 remarkably stable form (41). It is hypothesized that miRNAs are not only passively released
99 by necrotic or injured cells but are actively secreted in membrane-bound vesicles (exosomes,
100 microvesicles) (9, 13), in apoptotic bodies (42), or in vesicle-free but protein-protected
101 protein-miRNA complexes (3). These mechanisms of miRNA packaging may protect
102 circulating miRNAs from degradation. Their highly extracellular stability together with their
103 often tissue-specific expression patterns and their feasible measurability by current techniques
104 makes circulating miRNAs highly attractive as biomarkers in biomedical research.

105

106 Several recent studies have investigated circulating miRNAs in patients with severe chronic
107 kidney disease (27, 29), end stage renal disease (10, 39), or acute kidney injury (19, 24, 38).

108 However, little is known about the association between circulating miRNAs and kidney
109 function in patients at high cardiovascular risk, such as coronary patients.

110

111 Therefore, we (i) determined the expression of 50 miRNAs, previously associated in the
112 literature with kidney function or kidney disease in a set of plasma samples obtained from
113 coronary angiography patients, (ii) identified those circulating miRNAs putatively related to
114 kidney function and (iii) validated their diagnostic value in a further independent cohort of
115 coronary angiography patients.

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119 **METHODS**

120

121 *Study subjects*

122 Patients were selected from a Caucasian patient cohort totally comprising 1048 subjects
123 referred to elective coronary angiography for the evaluation of established or suspected stable
124 coronary artery disease (CAD) at the academic teaching hospital Feldkirch.

125 The Ethics Committee of the University of Innsbruck approved the present study and written
126 informed consent was given by all participants. Detailed information on the recruitment
127 protocol and the determination of subjects characteristic has been described previously (26,
128 32). In brief, venous blood samples were collected after an overnight fast of 12 h before
129 angiography was performed. Height and weight were recorded, and body mass index (BMI)
130 was calculated as body weight (kg)/height² (m²). Hypertension was defined according to the
131 Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and

132 Treatment of High Blood Pressure (34) and type 2 diabetes mellitus (T2DM) was diagnosed
133 according to American Diabetes Association (ADA) guidelines (2). Coronary angiography
134 was performed with the Judkin's technique and the severity of stenosis was assessed by visual
135 inspection by a team of two investigators, who were blinded to serologic assays as described
136 previously (8). Estimated glomerular filtration rate (eGFR) was assessed by the 'Mayo Clinic
137 Quadratic' (MQ) equation, if not otherwise noted. The MQ equation is based on sex, age, and
138 serum creatinine and has been shown to give an accurate estimate of the glomerular filtration
139 rate in patients with nearly normal renal function (33). Additionally, the 'Chronic Kidney
140 Disease Epidemiology Collaboration' (CKD-EPI) equation (17) was used to estimate
141 glomerular filtration rate. Renal function was classified as normal kidney function in subjects
142 with $eGFR \geq 90$ mL/min/1.73 m², mild impairment of kidney function in subjects with eGFR
143 60-89 mL/min/1.73 m², and chronic kidney disease in subjects with $eGFR < 60$ mL/min/1.73m²
144 (28). Urinary albumin excretion was expressed as the albumin/creatinine concentration ratio
145 (ACR) in a random fresh morning urine specimen.

146

147 *Study design*

148 In a first pre-selecting step, a candidate miRNA panel consisting of 50 miRNAs previously
149 associated in the literature with renal function and/or involved in the development and
150 progression of kidney disease (Supplemental Table S1) was analyzed in 60 plasma samples
151 obtained from patients with angiographically proven CAD to identify those miRNAs, which
152 were sufficiently detectable in plasma. MiRNAs sufficiently detectable in plasma were
153 defined as miRNAs showing raw ct-values <39 cycles in at least 70% of samples. MiRNAs
154 miR-24-3p, miR-92a-3p, and miR-222-3p were selected as endogenous reference miRNAs
155 based on previously performed experiments at our institution and proposed as normalizers in
156 other reports (22, 35, 40).

157

158 MiRNAs being detectable below selected cutoff were analyzed in 60 additional coronary
159 patients. In doing so, a final discovery set was generated totally comprising 120 patients,
160 which was used to evaluate the association between selected miRNAs and renal function.

161

162 MiRNAs providing significant association with renal function after multiple testing correction
163 in the discovery study cohort were re-tested in further 318 patients randomly selected out of
164 remaining subjects referred to coronary angiography. Finally, the association of selected
165 miRNAs with kidney function was assessed in the two combined patient cohorts totally
166 including 438 coronary angiography patients.

167

168 *Prospective study*

169 Kidney function was re-assessed based on creatinine values obtained at a follow-up visit after
170 3.6±1.2 years.

171

172 *miRNA analysis*

173 RNA was isolated from 0.2 ml plasma using the ‘miRNeasy Mini Kit’ (Qiagen, Hilden,
174 Germany) according the manufactures protocol for the purification of small RNAs from
175 plasma. Isolated miRNAs were reverse transcribed using ‘Universal cDNA Synthesis Kit’
176 (Exiqon, Vedbaeck, Denmark) according to the manufactures instructions for plasma derived
177 miRNAs. Subsequently, quantitative real-time PCR was performed using ‘Universal SYBR
178 Green master mix’ (Exiqon) and miRNA specific LNA™ PCR primer or ‘Pick-&-Mix
179 microRNA PCR Panel’ plates (Exiqon) in a 10µl volume on a LightCycler® 480 Real-Time
180 PCR System (Roche Diagnostics, Vienna, Austria). Ct values of each candidate miRNA were
181 recorded and normalized by the global mean of all miRNAs (pre-selecting study) or by the
182 mean expression of the selected reference miRNAs miR-24-3p, miR-92a-3p, and miR-222-3p
183 (discovery and validation study).

184

185 *Statistical analysis*

186 MiRNA expression levels are given as $2^{-\Delta Ct}$ values, such that increased values reflect
187 increased miRNA concentration. Normal distribution was assessed using Kolmogorov-
188 Smirnov and Shapiro-Wilk test, respectively, showing that miRNA expression levels were not
189 normally distributed. Association between miRNAs and continuous clinical/laboratory
190 parameters were explored using non-parametric Spearman's rank correlation tests. Benjamini
191 and Hochberg false discovery rate correction was used for correcting multiple testing (4). In
192 addition, analysis of covariance models (ANCOVA) were built using a general linear model
193 approach. Statistically significant differences between miRNAs and categorical variables
194 were determined by the Kruskal-Wallis test and the Mann-Whitney U test, respectively. P-
195 values <0.05 were considered significant. Statistical analyses were performed with SPSS 25.0
196 for Windows (SPSS, Inc., Chicago, IL).

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199

200 **RESULTS**

201

202 *Patients' characteristics*

203 Clinical and biochemical baseline characteristics of patients included in the discovery cohort,
204 the validation cohort as well in the combined patient cohorts are given in table 1. Study
205 cohorts showed a high proportion of patients with male sex, the metabolic syndrome, T2DM,
206 hypertension, and significant coronary artery stenoses. The discovery cohort and the
207 validation cohort showed similar eGFR values ($p=0.791$). Approximately 10% of patients
208 showed eGFR values below $60 \text{ mL/min/1.73 m}^2$. Consequently, most patients had normal
209 kidney function.

210

211 *Evaluation of miRNAs levels in plasma samples*

212 A pre-selection study performed in 60 patients of the discovery study cohort showed that 12
213 miRNAs out of 50 analysed candidate miRNAs showed raw ct-values ≥ 39 cycles in at least
214 30% of samples and therefore were excluded from further investigations (Supplemental Table
215 S1). Expression data of miRNAs either normalized by the global mean or by the mean
216 expression of selected reference miRNAs miR-24-3p, miR-92a-3p, and miR-222-3p were
217 highly correlated (mean correlation coefficient = 0.950). Furthermore, selected reference
218 miRNAs were not associated with kidney function in the pre-selection set (all p-values
219 > 0.05). Therefore, miR-24-3p, miR-92a-3p, and miR-222-3p were found to be appropriate
220 endogenous reference miRNAs and were used as references in the further study. In a next
221 step, miRNAs with sufficient expression were analysed in additional 60 patients generating a
222 discovery cohort totally comprising 120 patients. Plasma levels of individual miRNAs are
223 shown in Supplemental Fig. S1. MiRNA-451a showed highest expression levels, followed by
224 miR-16-5p.

225

226 *Associations between miRNAs and eGFR in the discovery and validation cohort*

227 Associations between individual candidate miRNAs and eGFR are given in Supplemental
228 Table S2. Out of 38 miRNAs of the discovery set, 15 miRNAs were significantly associated
229 with eGFR at a nominal level of significance. Associations of seven out of these miRNAs
230 (miR-16-5p, miR-19b-3p, miR-20a-5p, miR-25-3p, miR-106b-5p, miR-320a, and miR-451a)
231 with eGFR remained significant after multiple testing correction via false discovery rate
232 (FDR) estimation. To verify obtained results, miRNAs with significant FDR and additionally
233 miR-320b showing borderline FDR significance with eGFR were further analysed in the
234 validation cohort (n=318). Table 2 shows correlation between these miRNAs and eGFR in the
235 validation study. MiRNAs miR-106b-5p, miR-16-5p, miR-19b-3p, miR-20a-5p, miR-25-3p,

10

236 and miR-451a, but not miR-320a and miR-320b, proved to be significantly associated with
237 eGFR also in the validation study cohort.

238

239 *Associations between identified renal miRNAs and kidney function in the total study cohort*

240 The association of identified renal miRNAs with traits of kidney function was further
241 investigated in the combined study cohorts totally comprising 438 subjects. As to be expected,
242 the associations between identified renal miRNAs and eGFR were significant also in the total
243 study cohort. MiRNAs also remained significantly associated with kidney function
244 alternatively using the CKD-EPI equation (17) instead of the Mayo Clinic Quadratic
245 equation to estimate glomerular filtration rate (Supplemental Table S3). Identified renal
246 miRNAs were able to significantly discriminate between normal and mildly impaired
247 glomerular filtration rate (figure 1). Furthermore, a significant association between identified
248 renal miRNAs and eGFR was proved by ANCOVA adjusting for age, gender, T2DM,
249 hypertension, and ACR (Supplemental Table S4).

250

251 Spearman correlation analysis revealed a high correlation of identified renal miRNAs with
252 each other (Supplemental Table S5). In ANCOVA adjusting for each identified renal miRNA,
253 only associations between the miR-19b-3p, miR-106b-5p and miR-451a with eGFR remained
254 significant (Supplemental Table S6).

255

256 To further elucidate the impact of the identified renal miRNAs on kidney function, the
257 association between identified renal miRNAs and parameters, which were associated with
258 kidney function, was assessed. Results of correlation analysis are given in table 3. The
259 identified renal miRNAs were significantly associated with age and established renal markers
260 including ACR, urea, and FGF23 serum levels. However, after adjustment for eGFR in

261 ANCOVA, the associations between miRNAs and these variables did not remain significant
262 (all p-values >0.05).

263

264 *Associations between identified renal miRNAs and future kidney function*

265 Creatinine serum concentrations and eGFR assessments were available from 271 subjects out
266 of the 438 initially included patients from a follow-up visit after 3.6 ± 1.2 years. All baseline
267 miRNAs were significantly associated with eGFR also at follow up (Supplemental Table S7).

268

269

270 **DISCUSSION**

271

272 In the present work we report a strong correlation of the six plasma-derived miRNAs miR-16-
273 5p, miR-19b-3p, miR-20a-5p, miR-25-3p, miR-106b-5p, and miR-451a with kidney function
274 in coronary patients. Our findings are based on a multi-step strategy, including a discovery
275 study to identify circulating miRNAs significantly associated with kidney function as well as
276 an independent validation study to verify obtained results. Identified renal miRNAs were even
277 able to significantly discriminate between normal and mildly impaired eGFR.

278

279 Our observations are in line with previous studies also demonstrating a significant reduction
280 of circulating miRNAs in patients with CKD. In this regard, Neal et al. (29) showed that in
281 patients with severe chronic kidney disease, circulating levels of total and specific miRNAs,
282 including miR-16-5p, are reduced in comparison to patients with mild renal impairment or
283 normal renal function. Furthermore, Lee et al. (16) reported that circulating miR-20a-5p and
284 miR-106b-5p were significantly lower in CKD patients than in healthy subjects. Notably, the
285 association between plasma-derived miR-19b-3p, miR-25-3p, and miR-451a and kidney
286 function in humans, as described in our study, is new.

287

288 That said, it remains unclear whether the identified circulating renal miRNAs are directly
289 involved in kidney function or are a result of impaired kidney function. MiR-20a-5p together
290 with miR-19b-3p and several other miRNAs belongs to the miR-17~92 cluster, which is
291 highly conserved in vertebrates. MiR-106b-5p and miR-25-3p are members of the
292 miR106b~25 cluster, a paralog of the miR-17~92 cluster originated by gene duplication and
293 deletion events during vertebrate evolution (7). Both clusters are essential for development
294 and homeostasis promoting cell division and resistance to apoptosis (7). Apoptosis promotes
295 loss of renal epithelial cells that characterizes acute and chronic kidney disease. Therefore, it
296 may be hypothesized that the observed reduced circulating levels of miRNAs miR-19b-3p,
297 miR-20a-5p, miR-25-3p, and miR-106b-5p as members of the miR-17~92 cluster family
298 reflect an increase in apoptotic processes in the kidneys accelerating renal dysfunction. The
299 supposed impact of the 17~92 cluster family on renal function is supported by animal studies.
300 In this regard, Marrone et al. showed that the miR-17~92 cluster is essential in renal
301 development and that its loss leads to the development of renal disease in mice (23).
302 However, the individual mechanisms by which low plasma levels of members of the miR-
303 17~92 cluster family contribute to renal function remain to be elucidated.

304

305 Interestingly, renal miRNAs of the miR-17~92 cluster family were not only highly correlated
306 to each other but also to miR-16-5p and miR-451a, which belong to the miR-15~16 cluster
307 and the miR-144~451 cluster, respectively. The high correlation between circulating miR-16-
308 5p and miR-19b-3p has also been observed by Zhang et al., linking low levels of plasma-
309 derived miR-16-5p and miR-19b-3p to gastric cancer (43). In this regard, the association
310 between miR-16-5p and renal function was no longer significant after adjusting for other
311 identified renal miRNA. Therefore, the significant association between circulating miR-16-5p

312 and eGFR observed in our study and by others (29) appears to be mediated by other miRNAs,
313 closely correlated to miR-16-5p, such as miR-19b-3p.

314

315 However, a direct impact of circulating miR-451-5p on kidney function cannot be excluded.
316 Animal studies showed that miR-451-5p is downregulated in diabetic kidney disease
317 suggesting a protective role of miR-451-5p in kidney tissue (25, 44). It has been shown that
318 overexpression of miR-451-5p inhibits glomerular mesangial cell hypertrophy (44), a key
319 event occurring at a very early stage of diabetic nephropathy. That said, in the present study
320 the association between circulating miR-451-5p and kidney function was independent from
321 the presence of T2DM.

322

323 Notably, miR-16-5p together with miR-451-5p showed highest expression levels among the
324 investigated candidate miRNA panel (Supplemental Fig. S1), indicating that miR-16-5p and
325 miR-451-5p account for a significant proportion of total circulating miRNA. Low levels of
326 miR-16-5p or miR-451-5p may therefore reflect reduced levels of total circulating miRNA,
327 which by itself has been associated with reduced kidney function (29). However, the
328 biological background behind the association between reduced miRNA levels and reduced
329 kidney function is still unclear. In this context, it has been shown that subjects with renal
330 dysfunction show enhanced levels of RNases (12, 14) probably leading to increased
331 degradation and, consequently, reduced levels of circulating miRNAs. That said, this
332 hypothesis has been rejected by several authors due to the given protection of circulating
333 miRNAs from degradation by different mechanisms of miRNA packaging such as the
334 incorporation of miRNAs into vesicles or the formation of protein-miRNA complexes (11,
335 29). Also, the question remains, why some plasma miRNAs are associated with eGFR while
336 others are not.

337

338 Evidence suggests that different miRNA transport forms are associated with distinct miRNA
339 signatures (6). Certain miRNAs were mainly detected in microvesicles, whereas others were
340 associated with the RNA binding protein Argonaute 2 (3), which is part of the RNA-induced
341 silencing complex. It may be hypothesized that the kind of extracellular miRNA stabilization
342 contributes to our observation that a specific signature of abundant plasma miRNAs is
343 associated with eGFR, while other common miRNAs (such as miR-223-3p or miR 486-5p)
344 are not. However, sub-classes of miRNA carriers were not determined in our study and,
345 therefore, any conclusions in that regard remain speculative.

346

347 Our study has strengths and limitations. One strength of our study is the two-step strategy to
348 identify circulating miRNAs associated with renal function. Significant associations between
349 six miRNAs and eGFR found in a discovery study could be confirmed in a further
350 independent study cohort. However, limited sample size of the discovery study might have
351 reduced the chance of detecting true associations between other candidate miRNAs and
352 kidney function. Another limitation is that GFR was not measured directly, but was estimated
353 based on serum creatinine levels by the Mayo Clinic Quadratic equation. Notably, the Mayo
354 Clinic Quadratic equation has been shown to give an accurate estimate of GFR in patients
355 with nearly normal renal function (33), which was present in the majority our patients. Results
356 could also be reproduced employing the more frequently used CKD-EPI equation. Creatinine
357 values used to estimate GFR were based on a single measurement. Consequently, a non-
358 steady state of kidney function indicated by varying creatinine levels over time cannot be
359 excluded for all of our patients. However, per study design patients included in our study were
360 not acutely ill or hospitalized. Therefore, a steady state of kidney function making eGFR
361 interpretable appears likely at least in most of our patients. Moreover, identified renal
362 miRNAs were significantly linked with eGFR assessments based on creatinine values
363 determined nearly four years after baseline examination confirming their association with

364 kidney function. Our study participants were a selected group as all of them were referred to
365 coronary angiography for the evaluation of CAD. That said, due to the close correlation
366 between even mild-to-moderate deterioration of kidney function and morbidity or mortality in
367 cardiovascular risk patients, the coronary angiography patients we chose to investigate are of
368 particular clinical interest. The impact of identified renal miRNAs on the incidence of future
369 events has to be investigated in prospective studies.

370

371 In conclusion, our study showed that decreased circulating levels of miR-16-5p, miR-19b-3p,
372 miR-20a-5p, miR-25-3p, miR-106b-5p, and miR-451a are significantly linked to reduced
373 kidney function in coronary angiography patients. Their close correlation to each other as well
374 as to kidney function should be considered in future studies. Further studies are needed to
375 clarify the pathophysiological background behind the observed association between reduced
376 levels of identified circulating renal miRNAs and kidney dysfunction.

377

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386 **References**

387

- 388 1. **Aguado-Fraile E, Ramos E, Conde E, Rodríguez M, Liaño F, García-Bermejo**
389 **ML.** MicroRNAs in the kidney: novel biomarkers of acute kidney injury. *Nefrologia*
390 33: 826–34, 2013.
- 391 2. **American Diabetes Association.** Standards of medical care in diabetes-2015. *Diabetes*
392 *Care* 38: S1–S93, 2015.
- 393 3. **Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, Mitchell**
394 **PS, Bennett CF, Pogosova-Agadjanyan EL, Stirewalt DL, Tait JF, Tewari M.**
395 Argonaute2 complexes carry a population of circulating microRNAs independent of
396 vesicles in human plasma. *Proc Natl Acad Sci U S A* 108: 5003–8, 2011.
- 397 4. **Benjamini Y, Hochberg Y.** Controlling the False Discovery Rate: A Practical and
398 Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B* 57 WileyRoyal
399 Statistical Society: 289–300, 1995.
- 400 5. **Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W.** Relief of
401 microRNA-mediated translational repression in human cells subjected to stress. *Cell*
402 125: 1111–24, 2006.
- 403 6. **Boon RA, Vickers KC.** Intercellular Transport of MicroRNAs. *Arterioscler Thromb*
404 *Vasc Biol* 33: 186–192, 2013.
- 405 7. **Concepcion CP, Bonetti C, Ventura A.** The MicroRNA-17-92 Family of MicroRNA
406 Clusters in Development and Disease. *Cancer J* 18: 262–267, 2012.
- 407 8. **Drexel H, Amann FW, Beran J, Rentsch K, Candinas R, Muntwyler J, Luethy A,**
408 **Gasser T, Follath F.** Plasma triglycerides and three lipoprotein cholesterol fractions
409 are independent predictors of the extent of coronary atherosclerosis. [Online].
410 *Circulation* 90: 2230–5, 1994. <http://www.ncbi.nlm.nih.gov/pubmed/7955178> [28 Jun.

- 411 2018].
- 412 9. **Eldh M, Ekström K, Valadi H, Sjöstrand M, Olsson B, Jernås M, Lötvall J.**
- 413 Exosomes communicate protective messages during oxidative stress; possible role of
- 414 exosomal shuttle RNA. *PLoS One* 5: e15353, 2010.
- 415 10. **Emilian C, Goretti E, Prospert F, Pouthier D, Duhoux P, Gilson G, Devaux Y,**
- 416 **Wagner DR.** MicroRNAs in patients on chronic hemodialysis (MINOS study). *Clin J*
- 417 *Am Soc Nephrol* 7: 619–623, 2012.
- 418 11. **Fourdinier O, Schepers E, Metzinger-Le Meuth V, Glorieux G, Liabeuf S,**
- 419 **Verbeke F, Vanholder R, Brigant B, Pletinck A, Diouf M, Burtsey S, Choukroun**
- 420 **G, Massy ZA, Metzinger L, European Uremic Toxin Work Group-EUTox.** Serum
- 421 levels of miR-126 and miR-223 and outcomes in chronic kidney disease patients. *Sci*
- 422 *Rep* 9: 4477, 2019.
- 423 12. **Humphrey RL, Karpetsky TP, Neuwelt EA, Levy CC.** Levels of serum ribonuclease
- 424 as an indicator of renal insufficiency in patients with leukemia. [Online]. *Cancer Res*
- 425 37: 2015–22, 1977. <http://www.ncbi.nlm.nih.gov/pubmed/266415> [22 Jul. 2019].
- 426 13. **Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee**
- 427 **M-LT, Schmittgen TD, Nana-Sinkam SP, Jarjoura D, Marsh CB.** Detection of
- 428 microRNA Expression in Human Peripheral Blood Microvesicles. *PLoS One* 3: e3694,
- 429 2008.
- 430 14. **Karpetsky TP, Humphrey RL, Levy CC.** Influence of Renal Insufficiency on Levels
- 431 of Serum Ribonuclease in Patients With Multiple Myeloma 2. *JNCI J Natl Cancer Inst*
- 432 58: 875–880, 1977.
- 433 15. **Kozomara A, Griffiths-Jones S.** miRBase: annotating high confidence microRNAs
- 434 using deep sequencing data. *Nucleic Acids Res* 42: D68-73, 2014.
- 435 16. **Lee MS, Lee F-Y, Chen Y-L, Sung P-H, Chiang H-J, Chen K-H, Huang T-H,**
- 436 **Chen Y-L, Chiang JY, Yin T-C, Chang H-W, Yip H-K.** Investigated the safety of

- 437 intra-renal arterial transfusion of autologous CD34+ cells and time courses of
438 creatinine levels, endothelial dysfunction biomarkers and micro-RNAs in chronic
439 kidney disease patients-phase I clinical trial. *Oncotarget* 8: 17750–17762, 2017.
- 440 17. **Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, Feldman HI, Kusek**
441 **JW, Eggers P, Van Lente F, Greene T, Coresh J, CKD-EPI (Chronic Kidney**
442 **Disease Epidemiology Collaboration).** A new equation to estimate glomerular
443 filtration rate. *Ann Intern Med* 150: 604–12, 2009.
- 444 18. **Lopez-Giacoman S, Madero M.** Biomarkers in chronic kidney disease, from kidney
445 function to kidney damage. *World J Nephrol* 4: 57, 2015.
- 446 19. **Lorenzen JM, Kielstein JT, Hafer C, Gupta SK, Kumpers P, Faulhaber-Walter R,**
447 **Haller H, Fliser D, Thum T.** Circulating miR-210 Predicts Survival in Critically Ill
448 Patients with Acute Kidney Injury. *Clin J Am Soc Nephrol* 6: 1540–1546, 2011.
- 449 20. **Lorenzen JM, Martino F, Thum T.** Detection and transport mechanisms of
450 circulating microRNAs in neurological, cardiac and kidney diseases. [Online]. *Curr*
451 *Med Chem* 20: 3623–8, 2013. <http://www.ncbi.nlm.nih.gov/pubmed/23834179> [18 Jan.
452 2018].
- 453 21. **Lorenzen JM, Thum T.** Circulating and urinary microRNAs in kidney disease. *Clin J*
454 *Am Soc Nephrol* 7: 1528–33, 2012.
- 455 22. **Marabita F, de Candia P, Torri A, Tegnér J, Abrignani S, Rossi RL.**
456 Normalization of circulating microRNA expression data obtained by quantitative real-
457 time RT-PCR. *Brief Bioinform* 17: 204–12, 2016.
- 458 23. **Marrone AK, Stolz DB, Bastacky SI, Kostka D, Bodnar AJ, Ho J.** MicroRNA-
459 17 92 Is Required for Nephrogenesis and Renal Function. *J Am Soc Nephrol* 25: 1440–
460 1452, 2014.
- 461 24. **Martino F, Lorenzen J, Schmidt J, Schmidt M, Broll M, Görzig Y, Kielstein JT,**
462 **Thum T.** Circulating MicroRNAs Are Not Eliminated by Hemodialysis. *PLoS One* 7:

- 463 e38269, 2012.
- 464 25. **Mohan A, Singh RS, Kumari M, Garg D, Upadhyay A, Ecelbarger CM, Tripathy**
465 **S, Tiwari S.** Urinary Exosomal microRNA-451-5p Is a Potential Early Biomarker of
466 Diabetic Nephropathy in Rats. *PLoS One* 11: e0154055, 2016.
- 467 26. **Muendlein A, Leiherer A, Saely CH, Rein P, Zanolin D, Kinz E, Brandtner E-M,**
468 **Fraunberger P, Drexel H.** Common single nucleotide polymorphisms at the NPC1L1
469 gene locus significantly predict cardiovascular risk in coronary patients.
470 *Atherosclerosis* 242: 340–345, 2015.
- 471 27. **Muralidharan J, Ramezani A, Hubal MJ, Knoblach S, Shrivastav S, Karandish S,**
472 **Scott R, Maxwell N, Ozturk S, Beddhu S, Kopp JB, Raj DS.** Extracellular
473 microRNA signature in chronic kidney disease. *Am. J. Physiol. Renal Physiol.* (
474 January 2017). doi: 10.1152/ajprenal.00569.2016.
- 475 28. **National Kidney Foundation.** K/DOQI clinical practice guidelines for chronic kidney
476 disease: evaluation, classification, and stratification. [Online]. *Am J Kidney Dis* 39: S1-
477 266, 2002. <http://www.ncbi.nlm.nih.gov/pubmed/11904577> [24 Jan. 2018].
- 478 29. **Neal CS, Michael MZ, Pimlott LK, Yong TY, Li JYZ, Gleadle JM.** Circulating
479 microRNA expression is reduced in chronic kidney disease. *Nephrol Dial Transplant*
480 26: 3794–3802, 2011.
- 481 30. **Pillai RS, Bhattacharyya SN, Filipowicz W.** Repression of protein synthesis by
482 miRNAs: how many mechanisms? *Trends Cell Biol* 17: 118–126, 2007.
- 483 31. **Rein P, Saely CH, Vonbank A, Boehnel C, Drexel H.** Usefulness of Serial Decline of
484 Kidney Function to Predict Mortality and Cardiovascular Events in Patients
485 Undergoing Coronary Angiography. *Am J Cardiol* 113: 215–221, 2014.
- 486 32. **Rein P, Saely CH, Vonbank A, Fraunberger P, Drexel H.** Is albuminuria a
487 myocardial infarction risk equivalent for atherothrombotic events? *Atherosclerosis* 240:
488 21–25, 2015.

- 489 33. **Rule AD, Larson TS, Bergstralh EJ, Slezak JM, Jacobsen SJ, Cosio FG.** Using
490 serum creatinine to estimate glomerular filtration rate: accuracy in good health and in
491 chronic kidney disease. [Online]. *Ann Intern Med* 141: 929–37, 2004.
492 <http://www.ncbi.nlm.nih.gov/pubmed/15611490> [15 Dec. 2017].
- 493 34. **Scanlon PJ, Faxon DP, Audet AM, Carabello B, Dehmer GJ, Eagle KA, Legako**
494 **RD, Leon DF, Murray JA, Nissen SE, Pepine CJ, Watson RM, Ritchie JL,**
495 **Gibbons RJ, Cheitlin MD, Gardner TJ, Garson A, Russell RO, Ryan TJ, Smith**
496 **SC.** ACC/AHA guidelines for coronary angiography. A report of the American College
497 of Cardiology/American Heart Association Task Force on practice guidelines
498 (Committee on Coronary Angiography). Developed in collaboration with the Society
499 for Cardiac Angiography and Interventions. [Online]. *J Am Coll Cardiol* 33: 1756–824,
500 1999. <http://www.ncbi.nlm.nih.gov/pubmed/10334456> [5 May 2017].
- 501 35. **Tay JW, James I, Hughes QW, Tiao JY, Baker RI.** Identification of reference
502 miRNAs in plasma useful for the study of oestrogen-responsive miRNAs associated
503 with acquired Protein S deficiency in pregnancy. *BMC Res Notes* 10: 312, 2017.
- 504 36. **Tonelli M, Wiebe N, Culleton B, House A, Rabbat C, Fok M, McAlister F, Garg**
505 **AX.** Chronic Kidney Disease and Mortality Risk: A Systematic Review. *J Am Soc*
506 *Nephrol* 17: 2034–2047, 2006.
- 507 37. **Trionfini P, Benigni A, Remuzzi G.** MicroRNAs in kidney physiology and disease.
508 *Nat Rev Nephrol* 11: 23–33, 2015.
- 509 38. **Vliegenthart ADB, Shaffer JM, Clarke JI, Peeters LEJ, Caporali A, Bateman DN,**
510 **Wood DM, Dargan PI, Craig DG, Moore JK, Thompson AI, Henderson NC,**
511 **Webb DJ, Sharkey J, Antoine DJ, Park BK, Bailey MA, Lader E, Simpson KJ,**
512 **Dear JW.** Comprehensive microRNA profiling in acetaminophen toxicity identifies
513 novel circulating biomarkers for human liver and kidney injury. *Sci Rep* 5: 15501,
514 2015.

- 515 39. **Wang H, Peng W, Ouyang X, Dai Y.** Reduced Circulating miR-15b Is Correlated
516 with Phosphate Metabolism in Patients with End-Stage Renal Disease on Maintenance
517 Hemodialysis. *Ren Fail* 34: 685–690, 2012.
- 518 40. **Wang Z, Lu Y, Zhang X, Ren X, Wang Y, Li Z, Xu C, Han J.** Serum microRNA is
519 a promising biomarker for osteogenesis imperfecta. *Intractable rare Dis Res* 1: 81–5,
520 2012.
- 521 41. **Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, Galas DJ, Wang**
522 **K.** The microRNA spectrum in 12 body fluids. *Clin Chem* 56: 1733–41, 2010.
- 523 42. **Zernecke A, Bidzhekov K, Noels H, Shagdarsuren E, Gan L, Denecke B, Hristov**
524 **M, Köppel T, Jahantigh MN, Lutgens E, Wang S, Olson EN, Schober A, Weber C.**
525 Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular
526 protection. *Sci Signal* 2: ra81, 2009.
- 527 43. **Zhang J, Song Y, Zhang C, Zhi X, Fu H, Ma Y, Chen Y, Pan F, Wang K, Ni J, Jin**
528 **W, He X, Su H, Cui D.** Circulating MiR-16-5p and MiR-19b-3p as Two Novel
529 Potential Biomarkers to Indicate Progression of Gastric Cancer. *Theranostics* 5: 733–
530 745, 2015.
- 531 44. **Zhang Z, Luo X, Ding S, Chen J, Chen T, Chen X, Zha H, Yao L, He X, Peng H.**
532 MicroRNA-451 regulates p38 MAPK signaling by targeting of Ywhaz and suppresses
533 the mesangial hypertrophy in early diabetic nephropathy. *FEBS Lett* 586: 20–26, 2012.
534

535 **FIGURE LEGENDS**

536

537 **Figure 1: Associations between identified renal miRNAs and kidney function evaluated**
538 **in the total study cohort (n=438).** Figure 1 shows relative plasma expression levels of miR-
539 16-5p (A), miR-19b-3p (B), miR-20a-5p (C), miR-25-3p (D), miR-106b-5p (E), and miR-
540 451a (F) in patients with normal kidney function (eGFR ≥ 90 mL/min/1.73 m²), mild
541 impairment of kidney function (eGFR $< 90-60$ mL/min/1.73 m²), and kidney disease (eGFR
542 < 60 mL/min/1.73 m²). Expression levels are given as $2^{-\Delta C_t}$ values (median and interquartile
543 range), such that increased y-axis values reflect increased miRNA concentration; Ct values
544 were normalized by the mean expression of miR-24-3p, miR-92a-3p, and miR-222-3p.
545 Statistically significant differences were determined by the Kruskal-Wallis test and the Mann-
546 Whitney U test, respectively. P-values between stages were given either as n.s. (non
547 significant): $P \geq 0.05$, *: $P < 0.05$, **: $P < 0.01$, or ***: $P < 0.001$.

Table 1: Baseline patients' characteristics

Baseline characteristics	Discovery cohort n=120	Validation cohort n=318	Total cohort n=438
Age (years)	67.5 ± 9.8	67.1 ± 9.8	67.2 ± 9.6
Male gender, n (%)	67 (55.8)	173 (54.4)	240 (54.8)
Body mass index (kg/m ²)	28.7 ± 5.0	28.0 ± 4.6	28.2 ± 4.7
Metabolic syndrome, n (%)	65 (54.2)	135 (42.5)	200 (45.7)
Type 2 diabetes, n (%)	64 (55.3)	131 (42.2)	195 (44.5)
Hypertension, n (%)	92 (67.7)	247 (77.7)	339 (77.4)
History of smoking, n (%)	73 (60.8)	173 (54.4)	246 (56.2)
Significant stenoses, n (%)	81 (67.5)	164 (51.6)	245 (55.9)
Total cholesterol (mg/dl)	196.1 ± 41.5	196.0 ± 47.6	196.1 ± 46.0
HDL-cholesterol (mg/dl)	56.0 ± 15.3	58.6 ± 17.4	57.9 ± 16.9
Triglycerides (mg/dl)	143.5 ± 105.6	135.4 ± 78.7	137.6 ± 86.9
Statin use, n (%)	60 (50.0)	161 (50.6)	221 (50.5)
eGFR (ml/min/1.73m ²)	91.8 ± 22.2	91.1 ± 19.8	91.3 ± 20.5
eGFR 89 – 60 ml/min/1.73m ² , n (%)	42 (35.0)	124 (38.8)	165 (37.8)
eGFR < 60 ml/min/1.73m ² , n (%)	12 (10.0)	28 (8.8)	40 (9.2)
ACR (mg/g)	181.7 ± 429.7	71.9 ± 205.8	100.8 ± 285.8

eGFR, estimated glomerular filtration rate; ACR albumin to creatinine concentration ratio.

Table 2: Associations of renal miRNAs identified in the discovery study with eGFR in the validation study

	rho	p-value
miR-16-5p	0.239	<0.001
miR-19b-3p	0.301	<0.001
miR-20a-5p	0.292	<0.001
miR-25-3p	0.196	<0.001
miR-106b-5p	0.343	<0.001
miR-320a	-0.054	0.338
miR-320b	-0.015	0.788
miR-451a	0.361	<0.001

Statistically significant differences were determined by the Spearman's rho correlation test.

Table 3: Associations between identified renal miRNAs and anthropometrics and laboratory parameters

microRNA	miR-16-5p		miR-19b-3p		miR-20a-5p		miR-25-3p		miR-106b-5p		miR-451a	
	rho	p-value	rho	p-value	rho	p-value	rho	p-value	rho	p-value	rho	p-value
Age	-0.115	0.017	-0.115	0.017	-0.155	0.001	-0.130	0.006	-0.175	<0.001	-0.202	<0.001
BMI	-0.018	0.701	-0.046	0.341	-0.040	0.408	0.050	0.299	-0.051	0.290	0.012	0.797
Glucose	0.069	0.148	0.032	0.507	0.031	0.513	0.116	0.015	-0.031	0.513	0.122	0.011
HbA1c	0.044	0.361	-0.016	0.747	0.027	0.575	0.099	0.038	-0.019	0.691	0.086	0.071
SBP (mm Hg)	-0.036	0.452	-0.088	0.069	-0.069	0.151	-0.013	0.787	-0.056	0.244	-0.042	0.379
DBP (mm Hg)	-0.038	0.428	-0.102	0.034	-0.028	0.561	-0.005	0.918	-0.029	0.551	-0.007	0.881
ACR	-0.215	<0.001	-0.153	0.008	-0.172	0.003	-0.137	0.017	-0.195	0.001	-0.129	0.024
Urinary albumin	-0.071	0.149	-0.061	0.213	-0.102	0.039	-0.016	0.744	-0.116	0.018	-0.011	0.826
Serum urea	-0.081	0.089	-0.147	0.002	-0.161	0.001	-0.070	0.144	-0.214	<0.001	-0.154	0.001
Serum uromodulin	0.009	0.879	-0.012	0.838	0.091	0.114	-0.028	0.627	0.114	0.048	0.037	0.521
PTH	-0.091	0.067	-0.096	0.055	-0.130	0.009	-0.076	0.126	-0.096	0.053	-0.092	0.064
FGF23	-0.096	0.071	-0.180	0.001	-0.137	0.010	0.008	0.886	-0.158	0.003	-0.139	0.009
Klotho	0.014	0.780	0.078	0.125	0.014	0.786	-0.066	0.196	0.041	0.424	0.043	0.395

Associations of identified renal miRNAs with anthropometrics and laboratory parameters were evaluated in the total study cohort (n=438).

Statistically significant differences were determined by the Spearman's rho correlation test. Significant associations are indicated in bold. BMI, body

mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACR, albumin-to-creatinine ratio; PTH, Parathyroid hormone; FGF23, Fibroblast growth factor 23.

