Research article

Drug Testing and Analysis

Received: 27 June 2011 Revised: 5 August 2011 Accepted: 26 August 2011 Published online in Wiley Online Library: 24 November 2011

(wileyonlinelibrary.com) DOI 10.1002/dta.370

Circulating microRNAs as long-term biomarkers for the detection of erythropoiesis-stimulating agent abuse

Nicolas Leuenberger,[a]* Nicolas Jan,[a] Sylvain Pradervand,[b] Neil Robinson[a] and Martial Saugy[a]

MicroRNAs (miRNAs) are small, non-protein coding transcripts involved in many cellular and physiological mechanisms. Recently, a new class of miRNA called 'circulating miRNAs' was found in cell-free body fluids such as plasma and urine. Circulating miRNAs have been shown to be very stable, specific, and sensitive biomarkers.

In this paper, we investigate whether circulating miRNAs can serve as biomarkers for erythropoiesis-stimulating agent abuse. To this end, we analyzed miRNA levels in plasma by miRNA microarrays and quantitative real-time polymerase chain reaction (PCR). Plasma samples are derived from a clinical study with healthy subjects injected with erythropoiesis-stimulating agent (C.E.R.A.).

Based on microarray results, we observed a significant difference in the levels of miRNAs in plasma after C.E.R.A. injection. We demonstrated that a specific miRNA, miR-144, exhibit a high increase that lasts 27 days after C.E.R.A. stimulation. Considering the fact that miR-144 is an essential erythropoiesis agent in different organisms, these findings suggest the possibility of using miR-144 as a sensitive and informative biomarker to detect C.E.R.A. abuse. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: circulating microRNAs; doping; biomarkers; EPO

Introduction

In the last two decades, a family of short (19 to 25 nucleotides) endogenous RNAs have been identified.[1] These small, non-protein coding transcripts are called microRNAs (miRNAs). These molecules possess a single strand with a hairpin structure and are known to regulate gene expression post-transcriptionnaly.[2] Nearly a thousand miRNA have now been identified in tissues. Many studies have demonstrated that miRNAs are mainly active for down-regulating gene expression by imperfect base pairing of messenger RNAs (mRNAs) which reduces the protein output. Some studies have also showed that they are active for up-regulating gene expression by enhancing translation under specific condition.[3,4]

Since 2008, miRNAs have been found in a number of cell-free body fluids such as urine, saliva, serum, and plasma.[5] This new kind of miRNAs is called 'circulating miRNAs'. Although the physiological role played by circulating miRNAs is poorly understood, a function of hormone-like molecule is hypothesized.[6]

It has been shown that these extracellular RNAs are very stable, resistant to plasma RNases, remain stable after boiling, subject to high/low pH or after freeze-thaw cycles.[7–9]

In addition to being stable, present in lot of body fluid, and easily testable, the expression of miRNAs can be deregulated in case of cancer and organ injuries.[8,10–13] A number of publications have examined the possibility of using the circulating miRNAs as biomarkers.[14]

The fight against doping is mainly based on direct detection of a prohibited substance in an athlete's biological sample. Some methods also use indirect markers. The hematological passport is an example where blood markers are used to highlight any modification of erythropoiesis.[15] Another example is the use of methods based on proteins as an indirect marker for the detection of exogenous recombinant human growth hormone (GH) abuse (rhGH).[16]

As miRNAs can be use for medical diagnosis,[8,10,14,17–20] they may be used for the detection of doping. In comparison with protein markers, miRNAs are less complex molecules and amplification by polymerase chain reaction (PCR) can increase their detection sensitivity.

Recent research has highlighted the function of miRNAs in erythropoiesis and in hemoglobin synthesis.[21,22]

This study investigates whether circulating miRNAs can be used as biomarkers for an erythropoiesis stimulating agent (C.E.R.A.) abuse. The purpose was to highlight a difference in the expression of specific miRNAs in plasma samples after C.E.R.A. injection. In order to investigate this modification, miRNA expression profiling was performed using microarray screening. In a second step, quatitative real-time PCR (qRT-PCR) was performed on plasma samples from a clinical study conducted with healthy subjects who received a single C.E.R.A. injection.[23]

* *Correspondence to: Dr Nicolas Leuenberger, Laboratoire Suisse d'Analyse du Dopage, Ch. des Croisettes 22, CH – 1066 Epalinges. Switzerland. E-mail: nicolas.leuenberger@chuv.ch*

a *Swiss Laboratory for Doping Analyses, University Center of Legal Medicine, Geneva and Lausanne, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Switzerland*

b *Genomic Technologies Facility, University of Lausanne, Switzerland*

Materials and methods

Study protocol

Details related to participants and plasma samples collection time in the study have been described elsewhere.[23] Briefly, six healthy Caucasian men having a mean age of 23.0 years (SD 2.97) and a mean body mass index (BMI) of 23.3 kg/m^2 (SD 1.48) received a single subcutaneous or intravenous injection of 200 μg C.E.R.A. (MIRCERA®, Roche Pharma AG, Reinach, Switzerland). Plasma samples were stored at −20 °C. In order to test hemolysis in plasma, hemoglobin concentration was quantified using plasma/low Hb photometer (HemoCue®, HemoCue AG, Wetzikon, Switzerland). A complete red blood cell count was performed, and the reticulocyte cell population was quantified using a Sysmex analyzer (XT-2000i analyzer, Sysmex, Norderstedt, Germany).

MicroRNA microarray

Total RNA, including miRNAs, was isolated from 200 μl plasma by using the miRNeasy kit (Qiagen) as described,[24] with minor modifications. After elution with 35 μl of Rnase-free water, eluat was concentrated to 10 μl with speedVac (UNIVAPO 150 ECH system) with a speed of 1250 rpm, a temperature of 20 °C for 15 min to increase the concentration of circulating miRNAs. In total, 100 ng RNA of each sample was labelled according to the Agilent's miRNA Microarray System protocol as described elsewhere.[25] The labelled RNAs were hybridized to Agilent human miRNA microarrays (Human miRNA Microarray Kit version 2 #G4470B, Agilent Technologies, Inc. (Santa Clara, CA, USA) according to the protocol. Signal intensities were scanned with an Agilent G2565BA fluorescent scanner and processed by Agilent Feature Extraction Software (version 10.7.1.1). Total Gene Signal from Agilent GeneView data files were imported into R (version 2.12.2, http://www.R-project.org). MicroRNAs not detected in at least one sample were discarded and data were log2 transformed after adding a small constant of 8 to avoid negative values. Then, data were normalized using an invariants-based method described previously.[26] Briefly, standard deviation (SDs) across all samples were calculated for each miRNAs and the population of miRNAs that vary the less was identified by fitting normal mixture models on the SDs, using the R package 'mclust'.[27] Here, we did not remove lowly expressed miRNAs prior to the invariants selection. Normalization coefficients were computed by robust regression using the invariants miRNAs and each array were scaled according to the regression coefficients. Differential expressed miRNAs were identified using 'limma'[28] using a linear model with the day post-injection and the subject as factors. P values were adjusted for multiple testing with the Benjamini and Hochberg method to control the false discovery rate (FDR).

MicroRNAs RT-qPCR

Extraction of miRNAs was performed as above without the concentration step. Real-time PCR for miRNAs was performed using the mercury LNA™ Universal RT microRNA PCR system (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. For qPCR, 4 μl of eluted RNA was used in a 20 μl RT reaction. The resulting cDNA was diluted 20x and 8 μl used in 20 μl PCR amplification reactions run on a Roche LightCycler 480 real-time PCR system. Non-template control was added to verify specificity of the qRT-PCR. PCR efficiency and dynamic range of quantification were similar for every tested sample. Primer set for hsa-miR-144 and hsa-miR-923 were from Exiqon library. Primers sets target the following sequence: hsa-miR-144- 5′ – uacaguauagaugauguacu – 3′ and hsa-miR-923-5′- 5′ - gucagcggaggaaaagaaacu - 3′."

Statistics

Statistical analysis was performed with 2-tailed Student's *t*-test. Quantitative data are expressed as mean ± SEM. A *P* value less than 0.05 was considered significant.

Results and discussion

The changes of plasma miRNA concentration after a single high-dose injection of erythropoiesis-stimulating agent (ESA) were investigated to study the potential of circulating miRNAs in anti-doping. The performance of this technology was evaluated using a clinical study that involved subjects receiving C.E.R.A.[23] The goal was to identify miRNAs candidates as potential long-term biomarker to detect ESA abuse. Total RNA extraction was performed with 200 μl of plasma's volunteers as depicted in Figure 1A and described in the *Material and method* section. Agilent microarrays were used to profile circulating miRNAs, 2 day and 20 days after a single injection of C.E.R.A.

The hybridization intensity of circulating miRNAs was calculated and most miRNAs were either not expressed or expressed at very low levels. Indeed, out of 961 miRNAs measured, only 167 non-control miRNAs were called 'present' in at least one sample and 63 miRNAs were commonly detected in all samples. This number of detectable circulating miRNAs is consistent with a previous study.[29] Subjects A and B were injected intravenously or subcutaneously, respectively. The number of present miRNAs is higher for the late time points, suggesting that the number of different circulating miRNAs increases after stimulation (Figure 1B). No significant change was observed between intravenous and subcutaneous administration routes. Among the miRNAs showing at least 2-fold change between late and early time points and a false discovery rate (FDR) $< 5\%$, miR-144 showed the largest and most significant fold change (9.4-fold; $P < 0.0005$) (Figure 1C, Table 1 and supplementary Table 1). MiR-144 is an interesting candidate, because it has been shown to be essential in erythropoiesis in different organisms such as zebrafish, mice, and humans.[22,30,31] MiR-451 has also been shown to respond to erythropoiesis stimulation and is another potential biomarker with a 2-fold change increase at day 20 after C.E.R.A. injection.[32–33] However, miR-451 was not significant (FDR = 0.30). Human miR-923 was the most expressed circulating miRNA and it presented only a moderate non-significant change (Figure 1C). The high plasmatic expression of miR-923 was reported previously.[5] Since miR-923 had also a low coefficient of variation across all samples (0.031), it was considered as invariant and used as an internal control for RT-qPCR validation of microarray results.

Plasma hemoglobin was measured in the samples to ensure that hemolysis did not influence miR-144 concentration in plasma. We observed no difference in hemolysis between the samples (data not shown). Different time points after C.E.R.A. injection were analyzed by RT-qPCR to validate microarray results for miR-144. An increase of the miR-144/miR-923 ratio was observed up to 27 days after C.E.R.A. single injection (Figure 2A). At Day 27, the miR-144/miR-923 ratio showed an average increase of 5.13-fold ($P \leq 0.05$) compared to Day 0 (day prior to injection). The variation of miR-144 measurement was quite large, which was caused by different degrees of response to C.E.R.A.

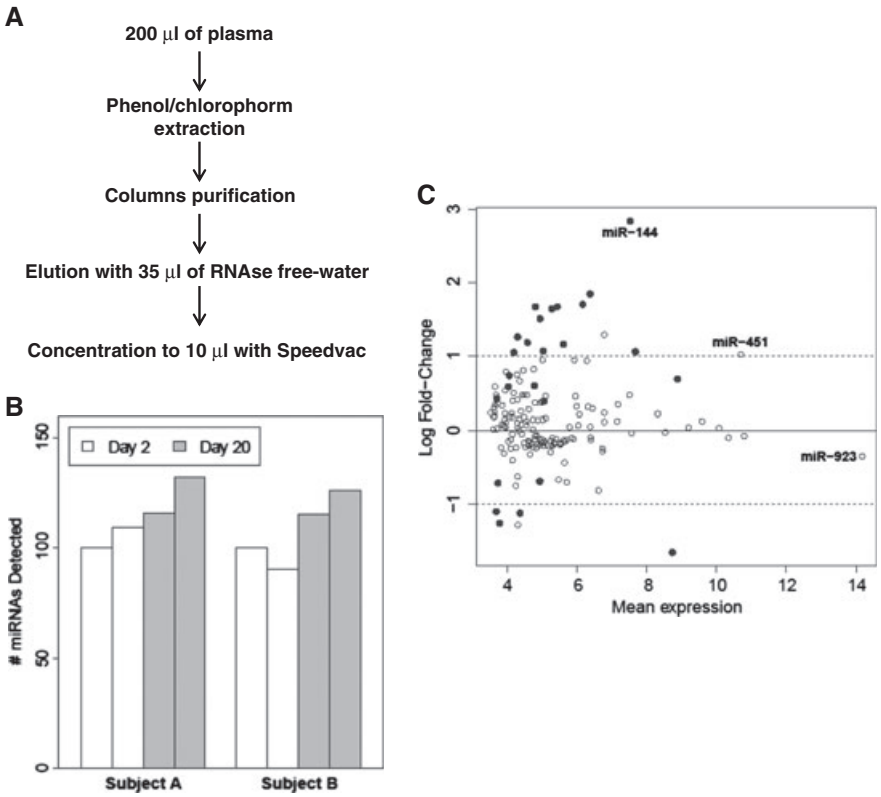

Figure 1. (A) Strategy for extracting circulating miRNAs from plasma. (B) Number of miRNAs detected by the Agilent microarray platform from plasma of the two subjects tested. Values are shown in duplicate. (C) Log-fold change versus mean expression for the 167 non-control miRNAs called 'present' in at least one sample. Fold changes are log2 differences between expression at Day 20 and Day 2 after ESA stimulation. Normalized expression of the 8 microarray samples is averaged to calculate the mean. Significant miRNAs are indicated with the closed circles. Fold change and mean of expression are based on microarray hybridization intensity.

administration among different individuals as observed in other studies.[18,20] In comparison, hematological biomarkers such as the percentage of reticulocytes showed a pick at 6–8 days and a return to the baseline 16 days after C.E.R.A. injection (Figure 2B).

Table 1. List of most affected miRNAs by C.E.R.A. stimulation. FC = Fold change (log2) injected/control; Mean Exp. = mean expression. Mean of expression are based on microarray hybridization intensity. FDR = false discovery rate. Selected MicroRNAs were detectable in every sample

miRNA name	FC	Mean Exp.	P Value	FDR
hsa-miR-144	2.84	7.52	0.0023	0.0218
hsa-miR-19b	1.85	6.38	0.0004	0.0065
hsa-miR-106b	1.71	6.16	0.0001	0.0049
hsa-miR-93	1.68	4.79	0.0002	0.0064
hsa-miR-101	1.68	5.43	0.0005	0.0068
hsa-miR-185	1.64	5.27	3.22E-05	0.0026
hsa-miR-19a	1.51	4.93	0.0004	0.0067
hsa-miR-1246	1.29	6.77	0.0693	0.1645
hsa-miR-140-3p	1.26	4.29	0.0003	0.0064
hsa-miR-142-5p	1.19	4.58	0.0008	0.009
hsa-miR-25	1.16	5.61	0.0004	0.0065
hsa-miR-29c	1.07	5.03	0.0006	0.0073
hsa-miR-92a	1.06	7.67	2.93E-05	0.0026
hsa-miR-17	1.05	4.18	0.0041	0.0333
hsa-miR-451	1.03	10.7	0.1937	0.3028
hsa-miR-874	−1.66	8.73	0.004	0.0333

No significant hemoglobin concentration change was observed throughout this study (data not shown). These data further suggested that specific circulating miRNAs, such as miR-144, might be more sensitive indirect biomarkers than the current hematological parameters such as the reticulocyte count.

To investigate the utilization of circulating miR-144 as a long-term biomarker to detect erythropoiesis-stimulating agents (ESA) abuse with more statistical significance, we assessed miR-144 expression in 18 individuals in early and late time points after C.E.R.A. injection (Figure 3A). MiR-144 showed an average of 3.5-fold change compared to control days ($P < 0.01$). In contrast, no significant difference of quantitative cycle (Cq) was observed for miR-923 that demonstrate that miR-923 is an ideal endogenous control (Figure 3B). Taken together, miR-144 represents a potential long-term biomarker to detect C.E.R.A. abuse from plasma samples.

Besides being recognized as key molecules in intracellular regulatory networks for gene expression, the spectra and levels of some miRNAs are emerging as biomarkers for various pathological conditions.[34] In recent years, some findings suggest that circulating miRNAs may be plasma biomarkers for cancer diagnosis and organ injuries.[8,10,14,17–20] In this study, we hypothesized that circulating miRNAs in plasma could be very specific and sensitive for the detection of ESA agent abuse in the anti-doping field. This report demonstrates circulating miRNAs changes of in plasma resulting from ESA injection. We used plasma from healthy volunteers as a model system and showed changes in spectra and levels of circulating miRNA as a result of a single injection of C.E.R.A. As the same samples were used in other study

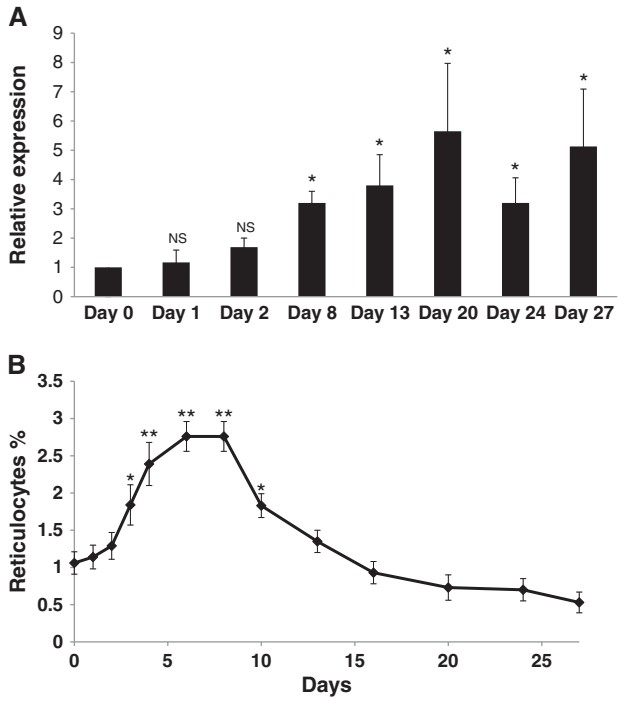

Figure 2. (A) Levels of miR-144 in plasma samples collected from healthy volunteers after injection of C.E.R.A. The collection days are indicated on x-axis. The relative change of miR-144 level is compared with day 0. Data were normalized to miR-923, which remained unchanged after C.E.R.A. injection. The values of miRNA fold change are the average of 6 independent samples from each time point, values are presented as mean ± SEM. *$P \leq 0.05$ versus day 0. (B) Percentage of reticulocytes was determined after CERA injection. The collection days are indicated on x-axis. Blood samples are the same than collected plasma in (A). Values are presented as mean ± SEM. *$P \leq 0.05$ and **$P \leq 0.01$ versus day 0.

that described a direct assay to detect C.E.R.A. in serum,[23] the detection window can now easily be compared with this report. In that study, the pick of C.E.R.A. concentration in serum in subjects was between 3 to 5 days after injection and the detection window around 15 days for the most of volunteers. In addition, the highest level of reticulocytes was observed 8 days after C.E.R.A. injection (Figure 2B). In our study, we were able to detect a change of a specific miRNA 27 days after C.E.R.A. injection. Due to the high level of specific of miRNA at this time point, it is likely that the detection window could be longer. Thus, the level of a specific plasma miRNA is more sensitive than the current markers for detecting C.E.R.A. abuse in athletes.

Previous studies reported a general increase of detectable miRNA after drug injection or in leukemic patient compared to control.[19,20] We observed the same trend in our study where the number of miRNAs called present is higher in all the late time point samples compared to early time points.

The highest observed increase microRNA following C.E.R.A. injection was the circulating miR-144. Interestingly, miR-144 has been shown to be essential in erythropoiesis in different organisms such as zebrafish, mice, and humans.[22,30,31] In addition, miR-451 that increases 2-fold in our study was demonstrated to be a key molecule in normal human erythroid differentiation.[32,33] Indeed, the results of our *in vivo* study could enhance the understanding in human hematopoiesis field. C.E.R.A. injection induces a significant change in the expression levels of a number of miRNAs in plasma. For this study, we focused on miR-144 due to its highest change. However, characterization of other C.E.R.A. stimulated-circulating miRNAs is under investigation in our laboratory.

The lack of a standard house-keeping miRNA for normalization is a major technique issue in the quantification of circulating miRNAs. A common strategy is to use spiked exogenous control from *C.elegans* origin.[8] Previously, a study showed that normalizing results to those of spiked exogenous control miRNAs was not optimal.[35] Reproducibility studies emphasized the importance of choosing an endogenous control to counteract the physiological variance between individuals. Circulating miR-16 has been used to normalize the results of miRNAs of interest in many studies.[14] However, this miRNA was shown to be particularly susceptible to hemolysis.[35] In our study, miR-923 was chosen as endogenous control to normalize data, due to its highest plasmatic quantity[5] and its low coefficient of variation across all samples. This low variation was confirmed with RT-qPCR (Figure 3B). Thus, miR-923 could be considered as potential internal control in the future.

The development of minimally invasive test for the detection of forbidden substance in blood could greatly help the fight against doping in general.

In this paper, our results lay the foundation for the development of microRNAs as a novel class of blood-based doping biomarkers. Experience from Athlete's hematological passport shows the necessity to use a combination of different biomarkers to detect doping in sport. In this line, circulating miRNAs could serve as additional

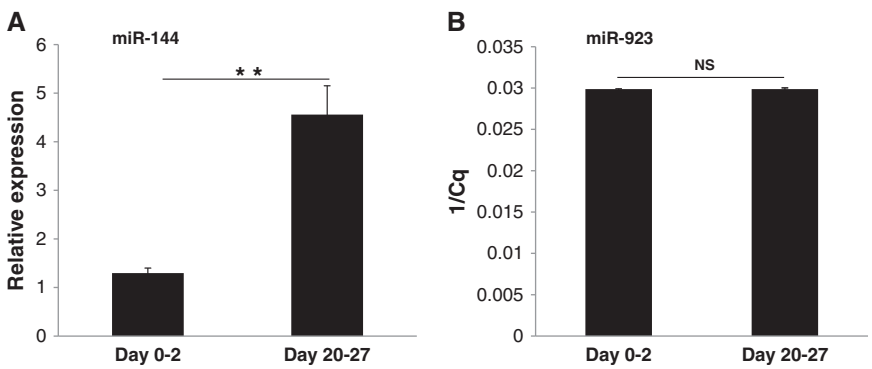

Figure 3. (A) Level of miR-144 between early time points (day 0–2) after C.E.R.A. injection compared to late time points (day 20–27). Circulating miR-923 is used as internal control. The values of miRNA fold change are the average of 15 independent samples, value are presented as mean ± SEM. **$P \leq 0.01$ versus early times points. B. Level of miR-923 between early time points (day 0–2) after CERA injection compared to late time points (day 20–27). Average Cq (quantification cycle) values is used for miR-923 comparison. NS = non-significant change.

genomic biomarkers to optimize the fight against doping. However, influence of different parameters such as high altitude training, effort and robustness on miR-144 have to be tested.

Circulating miRNAs may be excellent biomarkers in anti-doping compared to proteins. MiRNAs are not known to undergo any post-processing modifications and due to their size, their chemical composition is much less complex than most other biological molecules. Detecting specific miRNA species, although somewhat challenging, is inherently a much easier task than detecting proteins.[36] A synthetic complementary oligonucleotide should deliver sufficient specificity, and a standard qRT-PCR assay can be used for amplification to increase the detection sensitivity.

Circulating miRNAs can be directly detected in less than 1 µl of serum by RT-qPCR.[17] Thus, smaller volumes of plasma sample need to be collected during a doping control. In addition, it has been demonstrated that miRNAs could be detected in urine.[5,37,38] The use of urinary miRNAs to detect ESA abuse is under investigation in our laboratory.

Our results, although preliminary, strongly suggest that circulating miRNAs could be applicable in many doping trends such as autologous blood transfusion or growth hormone abuse.

Conclusion

In summary, this study showed an increase of a specific circulating miRNA, miR-144, in plasma samples after a single C.E.R.A. injection. Moreover, this increase indicated that the miRNA method might be use for long-term detection.

Acknowledgements

We acknowledge Annie Ferrari, Mélanie Johner, Rita Saugy for their excellent technical assistance. The Genomic Technology facility from Lausanne University is acknowledged for the technical support in microarray technology. We are grateful to the World Anti-Doping Agency (WADA) for providing funds (10C16NL grant).

References

[1] R. C. Lee, R. L. Feinbaum, V. Ambros. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **1993**, *75*, 843.
[2] A. Esquela-Kerscher, F. J. Slack. Oncomirs – microRNAs with a role in cancer. *Nat. Rev. Cancer* **2006**, *6*, 259.
[3] C. L. Holley, V. K. Topkara. An introduction to small non-coding RNAs: miRNA and snoRNA. *Cardiovasc. Drug Ther.* **2011**, *25*, 151.
[4] S. Vasudevan, Y. Tong, J. A. Steitz. Switching from repression to activation: microRNAs can up-regulate translation. *Science* **2007**, *318*, 1931.
[5] J. A. Weber, D. H. Baxter, S. Zhang, D. Y. Huang, K. H. Huang, M. J. Lee, D. J. Galas, K. Wang. The microRNA spectrum in 12 body fluids. *Clin. Chem.* **2010**, *56*, 1733.
[6] M. A. Cortez, C. Bueso-Ramos, J. Ferdin, G. Lopez-Berestein, A. K. Sood, G. A. Calin. MicroRNAs in body fluids-the mix of hormones and biomarkers. *Nat. Rev. Clin. Oncol.* **2011**, *8*, 467. doi: 10.1038/nrclinonc.2011.76
[7] X. Chen, Y. Ba, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, Y. Zhang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, J. Wang, K. Zen, J. Zhang, C. Y. Zhang. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and diseases. *Cell Res.* **2008**, *18*, 997.
[8] P. S. Mitchell, R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. O'Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher, B.S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin, M. Tewari. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 10513.
[9] D. C. Yu, Q. G. Li, X. W. Ding, Y. 1T. Ding. Circulating MicroRNAs: Potential Biomarkers for Cancer. *Int. J. Mol. Sci.* **2011**, *12*, 2055.
[10] N. Kosaka, H. Iguchi, T. Ochiya. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. *Cancer Sci.* **2010**, *101*, 2087.
[11] N. Scholer, C. Langer, H. Dohner, C. Buske, F. Kuchenbauer. Serum microRNAs as a novel class of biomarkers: a comprehensive review of the literature. *Exp. Hematol.* **2010**, *38*, 1126.
[12] H. Weng, C. Shen, G. Hirokawa, X. Ji, R. Takahashi, K. Shimada, C. Kishimoto, N. Iwai. Plasma miR-124 as a biomarker for cerebral infarction. *Biomed. Res.* **2011**, *32*, 135.
[13] Y. Zhang, Y. Jia, R. Zheng, Y. Guo, Y. Wang, H. Guo, M. Fei, S. Sun. Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. *Clin. Chem.* **2010**, *56*, 1830.
[14] G. Reid, M. B. Kirschner, N. van Zandwijk, Circulating microRNAs: Association with disease and potential use as biomarkers. *Crit. Rev. Oncol. Hematol.* **2010**. In press.
[15] N. Robinson, M. Saugy, A. Vernec, P.-E. Sottas. The athlete biological passport: An effective tool in the fight against doping. *Clin. Chem.* **2011**, *57*, 830.
[16] R. I. Holt, I. Erotokritou-Mulligan, C. McHugh, E. E. Bassett, C. Bartlett, A. Fityan, J. L. Bacon, D. A. Cowan, P. H. Sonksen. The GH-2004 project: the response of IGF1 and type III pro-collagen to the administration of exogenous GH in non-Caucasian amateur athletes. *Eur. J. Endocrinol.* **2010**, *163*, 45.
[17] S. Asaga, C. Kuo, T. Nguyen, M. Terpenning, A. E. Giuliano, D. S. B. Hoon. Direct serum assay for microRNA-21 concentrations in early and advanced breast cancer. *Clin. Chem.* **2011**, *57*, 84.
[18] O. F. Laterza, L. Lim, P. W. Garrett-Engele, K. Vlasakova, N. Muniappa, W. K. Tanaka, J. M. Johnson, J. F. Sina, T. L. Fare, F. D. Sistare, W. E. Glaab. Plasma MicroRNAs as sensitive and specific biomarkers of tissue injury. *Clin. Chem.* **2009**, *55*, 1977.
[19] E. Moussay, K. Wang, J.-H. Cho, K. van Moer, S. Pierson, J. Paggetti, P. V. Nazarov, V. Palissot, L. E. Hood, G. Berchem, D. J. Galas. MicroRNA as biomarkers and regulators in B-cell chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 6573.
[20] K. Wang, S. Zhang, B. Marzolf, P. Troisch, A. Brightman, Z. Hu, L. E. Hood, D. J. Galas. Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 4402.
[21] J. C. Byon, T. Papayannopoulou. MicroRNAs: Allies or foes in erythropoiesis? *J. Cell. Physiol.* **2011**. doi: 10.1002/jcp.22729
[22] Y. F. Fu, T. T. Du, M. Dong, K. Y. Zhu, C. B. Jing, Y. Zhang, L. Wang, H. B. Fan, Y. Chen, Y. Jin, G. P. Yue, S. J. Chen, Q. H. Huang, Q. Jing, M. Deng, T. X. Liu. Mir-144 selectively regulates embryonic alpha-hemoglobin synthesis during primitive erythropoiesis. *Blood* **2009**, *113*, 1340.
[23] S. Lamon, S. Giraud, L. Egli, J. Smolander, M. Jarsch, K.-G. Stubenrauch, A. Hellwig, M. Saugy, N. Robinson. A high-throughput test to detect C.E.R.A. doping in blood. *J. Pharm. Biomed. Anal.* **2009**, *50*, 954.
[24] D. Andreasen. Improved microRNA quantification in total RNA from clinical samples. *Methods* **2010**. PMID : 20215018.
[25] H. Wang, R. A. Ach, B. Curry. Direct and sensitive miRNA profiling from low-input total RNA. *RNA* **2007**, *13*, 151.
[26] S. Pradervand, J. Weber, J. Thomas, M. Bueno, P. Wirapati, K. Lefort, G. P. Dotto, K. Harshman. Impact of normalization on miRNA microarray expression profiling. *RNA* **2009**, *15*, 493.
[27] K. Y. Yeung, C. Fraley, A. Murua, A. E. Raftery, W. L. Ruzzo. Model-based clustering and data transformations for gene expression data. *Bioinformatics* **2001**, *17*, 977.
[28] G. K. Smyth. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* **2004**, *3*, 3.
[29] M. Tanaka, K. Oikawa, M. Takanashi, M. Kudo, J. Ohyashiki, K. Ohyashiki, M. Kuroda. Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients. *PLoS One* **2009**, *4*, e5532.
[30] K. D. Rasmussen, S. Simmini, C. Abreu-Goodger, N. Bartonicek, M. Di Giacomo, D. Bilbao-Cortes, R. Horos, M. Von Lindern, A.J. Enright, D. O'Carroll. The miR-144/451 locus is required for erythroid homeostasis. *J. Exp. Med.* **2010**, *207*, 1351.
[31] C. Sangokoya, M. J. Telen, J. T. Chi. microRNA miR-144 modulates oxidative stress tolerance and associates with anemia severity in sickle cell disease. *Blood* **2010**, *116*, 4338.
[32] S. Masaki, R. Ohtsuka, Y. Abe, K. Muta, T. Umemura. Expression patterns of microRNAs 155 and 451 during normal human erythropoiesis. *Biochem. Biophys. Res. Commun.* **2007**, *364*, 509.

[33] M. Zhan, C. P. Miller, T. Papayannopoulou, G. Stamatoyannopoulos, C.-Z. Song. MicroRNA expression dynamics during murine and human erythroid differentiation. *Exp. Hematol.* **2007**, *35*, 1015.
[34] S. A. Waldman, A. Terzic. MicroRNA signatures as diagnostic and therapeutic targets. *Clin. Chem.* **2008**, *54*, 943.
[35] J. S. McDonald, D. Milosevic, H. V. Reddi, S. K. Grebe, A. Algeciras-Schimnich. Analysis of Circulating MicroRNA: Preanalytical and Analytical Challenges. *Clin. Chem.* **2011**.
[36] M. L. Cowan, J. Vera. Proteomics: advances in biomarker discovery. *Expert Rev. Proteomics* **2008**, *5*, 21.
[37] S. Gilad, E. Meiri, Y. Yogev, S. Benjamin, D. Lebanony, N. Yerushalmi, H. Benjamin, M. Kushnir, H. Cholakh, N. Malamed, Z. Bentwich, M. Hod, Y. Goren, A. Chajut. Serum microRNAs are promising novel biomarkers. *PLoS One* **2008**, *3*, e3148.
[38] G. Wang, L. S. Tam, E. K. Li, B. C. Kwan, K. M. Chow, C. C. Luk, P. K . Li, C. C. Szeto. Serum and urinary cell-free MiR-146a and MiR-155 in patients with systemic lupus erythematosus. *J. Rheumatol.* **2010**, *37*, 2516.