Potential chemopreventive effect of “Procont” on miRNA expression in CBA/CA mice

MÓNIKA SZIRMAI[1], KRISZTINA JUHÁSZ[1], ANDRÁS BERTHA[1], KATALIN GOMBOS[1], KATALIN GŐCZE[1], INGRID MAGDA[1], PÉTER RÉVÉSZ[2] and ISTVÁN EMBER[1]

[1]Institute of Public Health, Faculty of Medicine, University of Pécs, Pécs, Hungary;

[2] Otorhinolaryngology and Head and Neck Surgery Clinic, Clinic Center, University of Pécs, Pécs, Hungary

Correspondence to: Krisztina Juhász, Institute of Public Health, Faculty of Medicine, University of Pécs, Pécs, 12 Szigeti str., 7624 Hungary. Tel: +3672536394, e-mail: krisztina.juhasz01@gmail.com

Abstract

Background: There is emerging molecular evidence concerning the modification of miR expression pattern after the treatment with chemopreventive agents. We investigated whether a potential chemopreventive agent called "Procont" deriving from a biological system has a positive effect on the DMBA induced dysregulation of let-7 a, miR-21 and miR-146a miRNAs. *Materials and Methods:* CBA/CA $H2^k$ inbred mice were fed by Procont for 7 days before the intraperitoneal injection of DMBA. After one week of the beginning of Procont diet we analized the let7a, miR-21, miR-146a gene expression in vital organs of mice. *Results:* The Procont feeding significantly decreased all of the investigated miRNA expression compared with either the mice on a normal diet in the control group or mice were exposed to DMBA alone. *Conclusion:* The results indicate that "Procont" diet has a potential chemopreventive effect in mice exposed to DMBA.

Introduction

MicroRNAs represent a small group of small noncoding RNAs, whose mature form are 18-25 nucleotides in length (1). Over the past several years it has become clear that alterations in the expression of miRNA genes contribute to the pathogenesis of most human malignances (2).

Among many miRNA, miR-21 was found overexpressed in different type of cancers (3). Inhibiting the expression of several pro - apoptotic gene and tumor suppressor gene such as p53, PTEN, PDCD4, TPM1 miR-21 contribute to genesis and progression of many type of cancer (4). Let-7a is a member of the let-7 miRNA family and required for timing of cell fate determination. Partial upregulation of this miRNA in the stem cells is required for their terminal differentiation at the adult stage (5). Let-7a can induce apoptosis and inhibits tumorgenesis by regulating oncogens such as *RAS* and *c-myc* (6). It has been suggested that RAS and c-myc expression is negatively regulated by let-7a (7). MiR-146a is mainly involved in the activation of the NF-κB protein regulating innate and adaptive immune responses and inflammation (8). With the recognition that inflammatory conditions are often associated with carcinogenesis, it was natural to suspect that there is a link between NF-kB and carcinogenesis (9)

Recently several studies have attempted to reveal the alteration in miRNA expression profile as the early response to environmental exposures. According *Izotti et al.* the cigarette smoke induces the alterations of miRNA expression in rats after 28 days of exposure, they demonstrated considerably downregulation in the case of 24 miRNAs involving in cellular stress, cell proliferation and differentiation (10). In an another study *Progribny et al.* exposed rats to tamoxifen a potential hepatocarcinogen agent mainly showed upregulation of micro-

RNAs, functioning oncogens in hepatocellular carcinogenesis (11). *Zhang and Pan* tested the effect of hexahydro- 1, 3, 5 – trinitro – 1,3, 5 – triazine (RDX) on the micro-RNA expression in mice's liver, they found a significant upregulation of oncogenic miRNAs and a significant down-regulation of tumor-suppressing miRNAs (12).

In our previous study we investigated the effect of DMBA on the micro-RNA expression in CBA/CA $H2^k$ inbred mice after 24 hours and 1 week of the exposure (13). The expression levels of miR-21, miR-146a and let-7a were significantly higher in the vital organs of the mice after 24 hours of DMBA exposure compared to the control group. While a significant down-regulation of these miRNAs was seen 7 days after the DMBA administration. This early response to DMBA on miRNA level provide us a new in vivo model for testing chemopreventing agents and monitoring their potential chemopreventive effects via molecular epidemiological biomarkers such as onco/supressor genes and miRNAs.

Based on data, confirmed the early modification of miR expression after carcinogenic exposure, our research group elaborated an animal model in order to reveal the potential chemopreventive effect of „Procont" consumption on miRNA expression in mice exposed to DMBA. „Procont" is a protein complex isolated from colostrum of cows with leucosis. The Hungarian patent of Procont (P0200172) was registered under international patent number: PCT/HU0300004 in 2003. Bioactive compounds of Procont include 40 and 60 kDA proteins. These proteins is degraded enzymatically in stomach and is accompanied by the release of 9-12 kDa biologicaly active fragments.

Materials and methods

6-week-old CBA/CA $H2^k$ haplotype mice of both sex were used in this experiment. The average animal weight were 20 g. Mice were divided into three groups (6-6 in each group, male and female). The preparation of groups is shown in *Table 1*.

The first group was the control group where animals consumed the standard laboratory chew pellet and tap water ad libitum. The second group received intraperitoneal injection of 7, 12 – dimethylbenz(α)anthracene in a single 20 mg/kg animal weight dose (0,4 mg DMBA dissolved in 0,1 ml corn oil) at the start of the examination. Intraperitoneal gavage decreased the first pass elimination of DMBA in the liver. The animals belonging to the third group consumed „Procont" for 7 day before the exposure of DMBA. The feed were provided ad libitum and prepared according to the instructions of the manufacturers (30 g of the agent was added to 90 g vehicle and 1500 g powder feed, thoroughly mixed, reshaped into food pellets). At the seventh day of the experiment the mice from the third group received intraperitoneal injection of DMBA in a single 20 mg/kg animal weight dose (0,4 mg DMBA dissolved in 0,1 ml corn oil).

7 days after the onset of the examination the mice of the first and the third groups were autopsied. 24 hours after DMBA administration mice in the second group were autopsied. Liver, spleen, lungs and kidneys of the animals were removed during autopsies.

Tissue samples from the dissected organs were homogenized. MiRNA was isolated with RNAzol solution (Molecular Research Center Inc., Cincinnati, OH, USA) according the manufacturer's instruction. Quality of the isolated RNA was checked by absorption photometry at 260/280nm. Optical density of the RNA was between 1.9 and 2.1.

High purity miRNA was used in reverse transcription followed by nucleic acid amplification with a one-step RNA amplification kit: Light Cyler RNA Master SYBR Green I kit (Roche, Berlin, Germany) containing SYBR green fluorescent labelling. The PCR reaction mix included: 8,2 µl H_2O, 1,3 µl Mn(OAc)2 stock solution, 7,2 µl LightCycler RNA master

SYBR Green I fluorescent labeled dye, 2µl specific primer at 0.5 µM final concentration and 1µl template miRNA in 20µl final volume. PCR amplifications were carried out in LightCycler 2.0 carousel based PCR system (Roche).

PCR settings were the followings: Reverse transcription at 61C° for 20 minutes, pre-incubation (1 cycle) 30 s at 95 C°, amplification (45 cycles) : denaturation 95C° 5s, annealing 50C° 15 s, extension 72C° 5s, melting curves (1 cycle) denaturation at 95C° for 0.1s, annealing at 65C° for 5s melting curve detection at 95C° at 0.1 ramp rate for 8s.

Sequence specific primers for let-7a, miR-21 and miR-146a were synthesized by TIB Molbiol, ADR Logistics, (Roche Warehouse, Budapest, Hungary): let-7a forward: 5'-GCCGCTGAGGTAGTAGGTTGTA-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; miR-21 forward: 5'-GCCCGCTAGCTTATCAGACTGATG-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; miR-146a forward: 5'-GCCGCCCTGTGAAATTCAGTT-3', reverse: 5'-GTGCAGGGTCCGAGG -3'.The gene expression was determined by absolute nucleic acid quantification method in the case of miRNAs, with 4.0 Light Cycler software.

Student's t test was performed between the groups and 95 % confidence interval of the differendce was calculated in the case of each organ. Values were expressed as the mean ± 2 SD. The calculation was performed using Statistical Program for Social Science 19.0 (SPSS) software.

Results

In this study we compared the effect of Procont consumption on mice exposed to DMBA with the mice were treated with DMBA alone as shown *Figure 1-3.* The results of the statistical analysis are shown in *Table 2*.

The DMBA exposition caused increased expression of the let-7a and miR-21 genes by 1,1 – 3,02 fold change in all examined organs compared to the controls *(Figure 1, 2).* On the other hand, there was no significant effect of DMBA on the expression of miR-146 a gene except for lungs *(Figure 3*).

Dietary administration of Procont before DMBA treatment caused a strongly decrease in the level of miRNAs compared to the animals were treated with the carcinogen agent alone *(Figure 1-3).* The miR-146a level in kidneys was detected to be more than six times higher in DMBA treated group than in mice consumed Procont before DMBA gavage. We observed the lowest expression of let-7a in spleen and lung tissues at the DMBA exposed mice treated with Procont. The mir-21 and miR-146a gene expression also showed markedly reduction in mice lung relative to the other organ *(Figure 2, 3).* Especially the expression of miR-21, where we found nine times lower expression (*Figure 2*).

Discussion

It has been demonstrated that administration of chemopreventive agents can target multiple cellular signaling patways. Several animal model was developed by our research group to evaluate the potential chemopreventive effect of different compounds by analysing the changes in oncogene and tumor supressor gene expression. The results of one of these studies supported, that the coadministration of the chemopreventive agent „Afobazole" and carcinogenic agents DMBA resulted in decrease of DMBA induced overexpression of *Ha-ras* and *p53* gene (14). In an other study we examined the effect of „CoD ^{TM} tea" on the expression pattern of *c-myc, Ha-ras, Bcl-2* and *K-ras* proto-oncogene and p53 tumor supressor gene. According to the results the experimental agent was able to decrease the DMBA-induced overexpression of onco- and tumor supressor genes (15).

Firstly *Izotti et al.* was concerned with the investigation of the effectiveness of different chemopreventive agents by molecular biological methods based on miRNAs (16). They registered the effect of chemopreventive agents such as phenethyl isothiocyanate (PEITC), indole-3-carbinol (I3C), synthetic flavone 5, 6 benzoflavone (BF), N-acetyl-L-cysteine (NAC) and oltipraz (OPZ) and also invetigated their combination: PEITC and I3C, OPZ and NAC in vivo. The rats were administered orally the chemopreventive agents before they were exposed to enviromental cigarette smoke. After the analisis of the expression of 484 miRNA genes they found, that compared to the ECS induced downegulation, in the group of rats exposed to ECS and received chemopreventive treatment all agents tended to upregulate the investigated miRNAs. The most strikingly upregulated miRNAs in this group included let-7a, miR-26, miR-34c, miR-123-prec, miR-146, miR-294. In comparison to this study, the expression of let7-a and miR-146a were found to be opposite in our study.

When Melkau et al. investigated the alteration of microRNA induced by indole-3-carbinol (I3C) in vinyl carbamate-induced murine lung tumors. The analysis of miRNAs by microarray method revealed a significant upregulation of miR-21, miR-31, miR-130a and miR-146b in lung tumors of mice. While in mice treated with vinyl carbamate and given I3C diet the expression level of these miRNAs were significantly reduced relative to the expression level of mice were treated with vinyl carbamate alone (17). Our study showed parallel results regarding the expression tendency of miR-21and miR-146a. The similarity concerning the onco-miR-21expression also corresponds with the fact that there is a link between the overexpression of miR-21 and pathogenesis of many type of cancers (18).

Conclusion

In summary, the analysis of miRNA expression can be considered as promising tool for recognition of early stage of chemically induced carcinogenesis as well as useful method

for assessing the capability of a chemopreventive agent to modulate the altered miRNA expression profile induced by environmental carcinogens.

Based on our data, Procont seems to induce a very characteristic decrease in the expression level of let-7a, miR-146a and onco- miR-21 after just 7 days consumption in mice were exposure to a potential carcinogenic agent. According the results of the present study the potential chemopreventive effect of „Procont" could be confirmed. We have several undergoing experiments that clarifying the chemoprentive effects of „Procont" and could give deeper insight into the biochemical action of Procont.

Acknowledgements

This work was supported by NKTH, TECH -09-A4-2009-0133, BDREVAM2, Hungary.

Conflicts of interest

No conflicts of interest were declared in relation to this paper.

References

1 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116(2):281-97.

2 Zhang B, Pan X, Cobb GP, Anderson TA: microRNAs as oncogenes and tumor suppressors. Developmental Biology. 2007; 302(1):1-12.

3 Croce CM: Causes and consequences of microRNA dysregulation in cancer. Nat Reviews Genetics. 2009; 10(10):704-14.

4 17 Zhu S, Wu F, Nie D, Sheng S, Mo YY: Micro-RNA-21 targets tumor suppressor genes in invasion and metastases. Cell Research. 2008; 18: 350-9.

5 Pasquinelli, A., Reinhart, B., Slack, F., Maller, B., and Ruvkun, G. (2000): Conservation across animal phylogeny of the sequence and accession numbers AY941100 and AY941101 temporal regulation of the 21 nucleotide C. elegans *let-7* heterochronic regulatory RNA. Nature. 2000; 408: 86–89.

6 Akao Y, Nakagawa Y, Naoe T: let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. Biol Pharm Bull. 2006; 29(5):903-6.

7 Steven M. Johnson, Helge Grosshans, Jaclyn Shingara, Mike Byrom, Rich Jarvis, Angie Chang, Emmanuel Labourier, Kristy L. Reinert, David Brown, Frank J. Slack: RAS is regulated by the let-7 micrRNA family. Cell. 2005; 120:635-647.

8 Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM, Baltimore D: NF-{kappa}B dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. Proc Natl Acad Sci U S A. 2011; 108(22):9184-9.

9 Naugler WE, Karin M: NF-kappaB and cancer-identifying targets and mechanisms. Curr Opin Genet Dev. 2008; 18(1):19-26.

10 Alberto Izzotti, George A. Calin,Patrizio Arrigo,Vernon E. Steele, Carlo M. Croce, Silvio De Flora: Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. FASEB J. 2009; 23(3): 806–812.

11 Pogribny IP, Tryndyak VP, Boyko A, Rodriguez-Juarez R, Beland FA, Kovalchuk O: Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure. Mutation Research. 2007; 619(1-2):30-7.

12 Zhang B, Pan X: RDX induces aberrant expression of microRNAs in mouse brain and liver. Environ Health Perspect. 2009; 117(2):231-40.

13 Krisztina Juhász, Katalin Gombos, Mónika Szirmai, Péter Révész, Ingrid Magda, Katalin Gőcze, István Ember: DMBA Induces Deregulation of miRNA Expression of *let-7*, *miR-21* and *miR-146a* in CBA/CA Mice. In vivo. 2012; 26(1):113-7.

14 István Szanyi, László Lujber, Imre Gerlinger, József Pytel, Miklós Bauer, András Csejtey, Eszter Szele, Katalin Gombos, István Kiss, Sergey Seredenin, Milada Yarkova, István Ember: In vivo effects of Afobazole (2-Mercaptobenzimidazole Derivative) on the 7,12-Dimethylbenz(a)anthracene-induced oncogene and suppressor gene expression. In vivo. 2007; 21:1059-1064.

15 Ferenc Budán, István Szabó, Tímea Varjas, Ghodratollah Nowrasteh, Tamás Dávid, Péter Gergely, Zsuzsa Varga, Kornélia Molnár, Balázs Kádár, Zsuzsa Orsós, István Kiss, István Ember: Mixtures of Uncaria and Tabebuia extracts are potentially chemopreventive in CBA/CA mice: a long term experiment. Phytotherapy Research. 2011; 25:493-500.

16 Izzotti A, Calin GA, Steele VE, Cartiglia C, Longobardi M, Croce CM, De Flora S: Chemoprevention of cigarette smoke-induced alterations of MicroRNA expression in rat lungs. Cancer Prev Res. 2010; 3(1):62-72.

17 Tamene Melkamu, Xiaoxiaou Zhang, Jiankang Tan, Yan Zeng, Fekadu Kassie: Alteration of miRNA expression in vinyl carbamate-induced mouse lung tumors and modulationby chemopreventive agent indole-3-carbinol. Carcinogenesis. 2010; 31(2):252-258.

18 Zhu S, Wu F, Nie D, Sheng S, Mo YY: Micro-RNA-21 targets tumor suppressor genes in invasion and metastases. Cell Research. 2008; 18: 350-9.

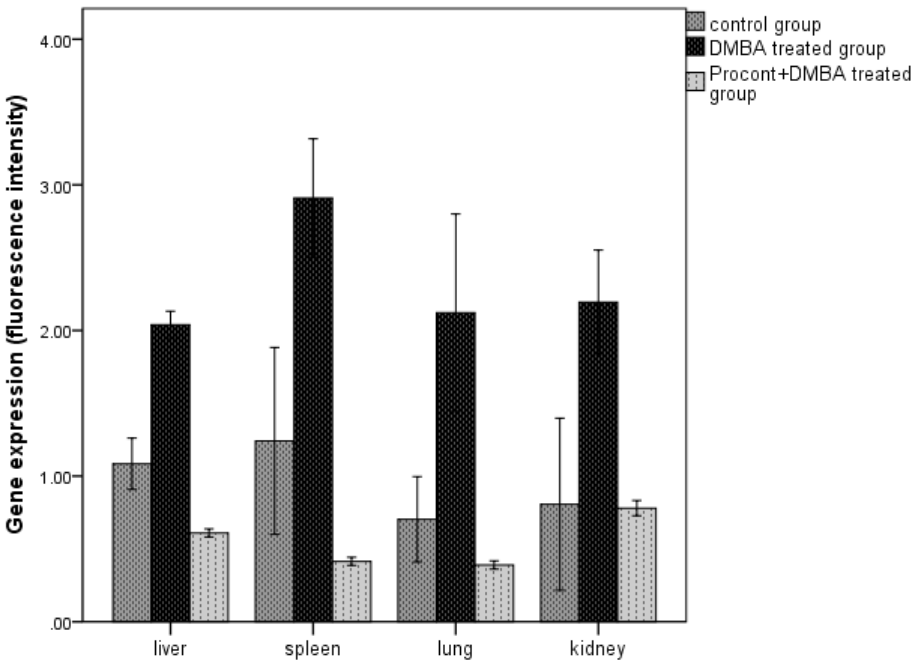

Figure 1. *Let-7a* gene expression pattern in liver, spleen, lung and kidney of mice.

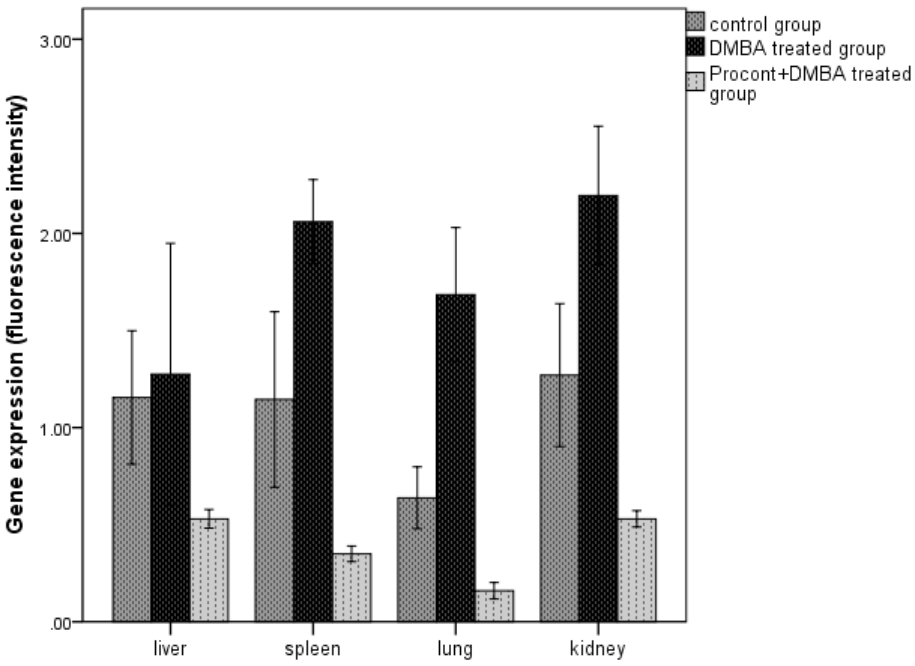

Figure 2. *miR-21* gene expression pattern in liver, spleen, lung and kidney of mice.

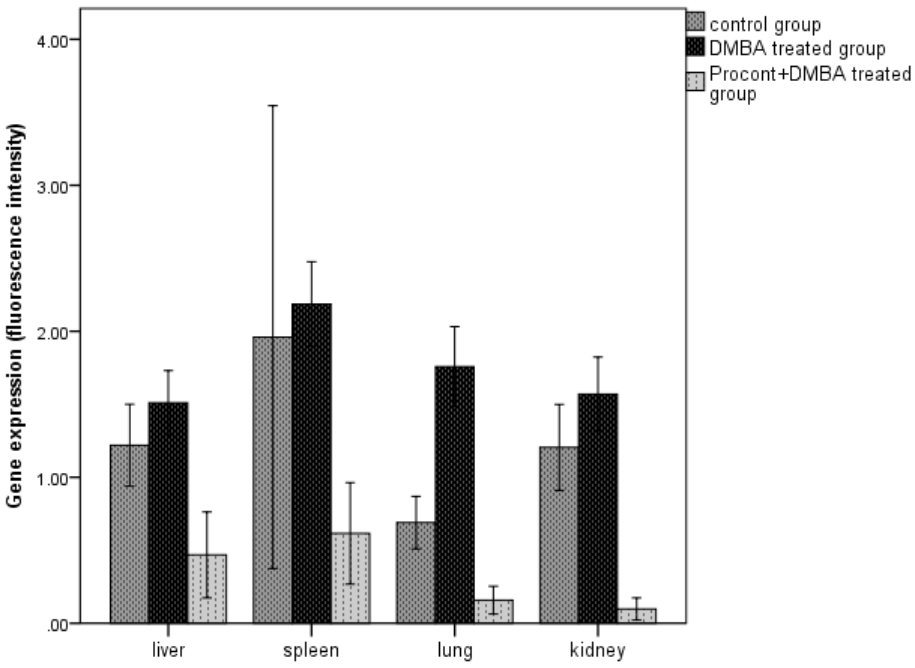

Figure 3. *miR-146* gene expression patten in liver, spleen, lung and kidney of mice.

Table 1. Preparation of groups

Groups	**Treatment**	**Autopsy**
Control group	Consumption of laboratory chew pellet and tap water ad libitum	7th days after the onset of the examination
DMBA treated group	Intraperitoneal injection of DMBA in a single 20 mg/kg animal weight dose (0,4 mg DMBA dissolved in 0,1 ml corn oil)	24 hours after the DMBA administration
Procont + DMBA treated group	Consumption of „Procont" for seven days (30 g of the experimental agent was added to 90 g vehicle and 1500 g powder feed) At the 7th day mice received a single injection of DMBA (0,4 mg DMBA dissolved in 0,1 ml corn oil)	7th days after the onset of the examination

Table 2. Results of statistical analysis.
* 95 % confidence interval of the difference

	miRNA		***DMBA treatment/control***	***Procont+DMBA treatment/DMBA treatment***
Liver	*let-7a*	*fold change 95% CI **	1,89 [0,774 - 1,135]	0,30 [1,342 - 1,517]
	miR-21	*fold change 95% CI**	1,10 [-0,568 - 0,808]	0,42 [0,13 - 0,136]
	miR146a	*fold change 95% CI**	1,24 [-0,034 – 0,614]	0,31 [0,707 – 1,376]
Spleen	*let-7a*	*fold change 95% CI**	2,34 [0,977 – 2,358]	0,14 [2,124 – 2,865]
	miR-21	*fold change 95% CI**	1,8 [0,46-1,37]	0,86 [1,509 – 1,91]
	miR-146a	*fold change 95% CI**	1,12 [-1,238 – 1,692]	0,28 [1,161 – 1,982]
Lung	*let-7a*	*fold change 95% CI**	3,02 [0,746 – 2,09]	0,18 [1,114 – 2,348]
	miR-21	*fold change 95% CI**	2,64 [0,71 – 1,392]	0.09 [1,208 – 1,841]
	miR-146a	*fold change 95% CI**	2,31 [0,502 – 1,302]	0,10 [1,058 – 1,808]
Kidney	*let-7a*	*fold change 95% CI**	2,72 [0,76-2,016]	0,36 [1,087 – 1,742]
	miR-21	*fold change 95% CI**	1,72 [0,459 – 1,39]	0,24 [1,338 – 1,991]
	miR-146a	*fold change 95% CI**	1,30 [0,011 – 0,718]	0,06 [1,231 – 1,712]