INSTITUTE OF LIFE SCIENCES
Scuola Superiore Sant'Anna

Possono i microRNA vegetali essere nuove molecole nutraceutiche? Indizi dalla vite

Scuola Superiore Sant'Anna Pisa
Fondazione Toscana Gabriele Monasterio per la ricerca medica e di sanità pubblica
IFC CNR

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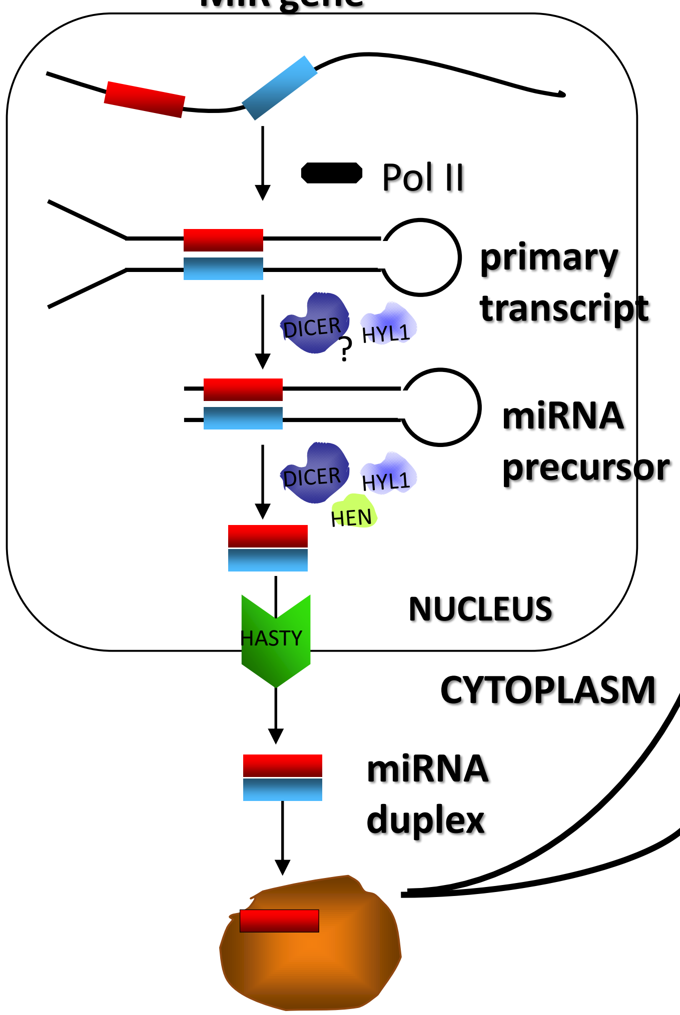
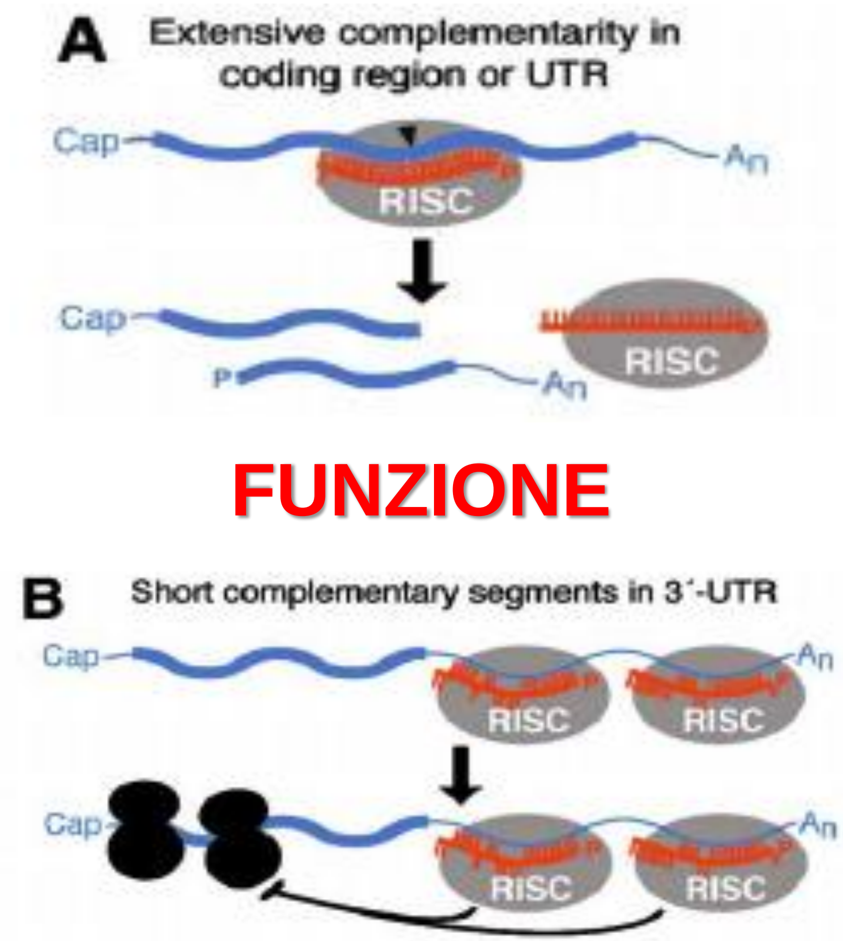
I microRNA sono una classe di molecole che regolano finemente l'espressione genica

BIOGENESI





FUNZIONE

- Geni specifici
- Molecola matura (20-24 nucleotidi)

- Complesso RNA – proteine interagisce con i bersagli (mRNA)

- Molecole di regolazione genica

PRESENTI IN ANIMALI e PIANTE con CARATTERISTICHE DIFFERENTI

Perché Interessarsi dei miRNA in Prospettiva Nutraceutica?

Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA

Cell Research (2012) 22:107-126.

Lin Zhang[1,*], Dongxia Hou[1,*], Xi Chen[1,*], Donghai Li[1,*], Lingyun Zhu[1,2], Yujing Zhang[1], Jing Li[1], Zhen Bian[1], Xiangying Liang[1], Xing Cai[1], Yuan Yin[1], Cheng Wang[1], Tianfu Zhang[1], Dihan Zhu[1], Dianmu Zhang[1], Jie Xu[1], Qun Chen[1], Yi Ba[3], Jing Liu[1], Qiang Wang[1], Jianqun Chen[1], Jin Wang[1], Meng Wang[1], Qipeng Zhang[1], Junfeng Zhang[1], Ke Zen[1], Chen-Yu Zhang[1]

Our previous studies have demonstrated that stable microRNAs (miRNAs) in mammalian serum and plasma are actively secreted from tissues and cells and can serve as a novel class of biomarkers for diseases, and act as signaling molecules in intercellular communication. Here, we report the surprising finding that exogenous plant miRNAs are present in the sera and tissues of various animals and that these exogenous plant miRNAs are primarily acquired orally, through food intake. MIR168a is abundant in rice and is one of the most highly enriched exogenous plant miRNAs in the sera of Chinese subjects. Functional studies *in vitro* and *in vivo* demonstrated that MIR168a could bind to the human/mouse low-density lipoprotein receptor adapter protein 1 (LDLRAP1) mRNA, inhibit LDLRAP1 expression in liver, and consequently decrease LDL removal from mouse plasma. These findings demonstrate that exogenous plant miRNAs in food can regulate the expression of target genes in mammals.

***Keywords*:** microRNA; MIR168a; LDLRAP1; low-density lipoprotein; microvesicle; cross-kingdom

Cell Research (2012) **22**:107-126. doi:10.1038/cr.2011.158; published online 20 September 2011

Multidisciplinary Approches

BIOINFORMATICS to identify putative grapevine miRNA targets in mouse and humans

MOUSE CELLULAR SYSTEM to evaluate cellular response in physiological and oxidative condition after exposition of grapevine small RNAs

MOUSE MODEL of myocardial infarction to evaluate the effect of Sangiovese grape juice on infarcted heart

Bioinformatics: Results

BIOINFORMATICS to identify putative grapevine miRNA targets in mouse and humans

- **36 miRNAs highly expressed in mature berry**
- **141 predicted targets in mouse endothelial cells**
- **Targets related to oxidative stress response, cytoskeleton remodeling, angiogenic response (*Sfrp1*)**

remodeling, angiogenic response (Sfrp1)

MOUSE CELLULAR SYSTEM to evaluate cellular response in physiological and ischemic condition after exposition of grapevine small RNAs

Immortalized murine coronary endothelial cell line *MCEC-1*

MCEC-1 + 10 % fetal bovine serum (FBS)
MCEC-1 + *grapevine berries small RNAs* (24 h)
MCEC-1 + H_2O_2 (24 h)
MCEC-1 + *grapevine berries small RNAs* + H_2O_2 (24 h)
MCEC-1 + commercial DNA/RNA(24 h)

CELL VIABILITY SUPEROXIDE DETECTION ASSAYS
(MTT and DHE)

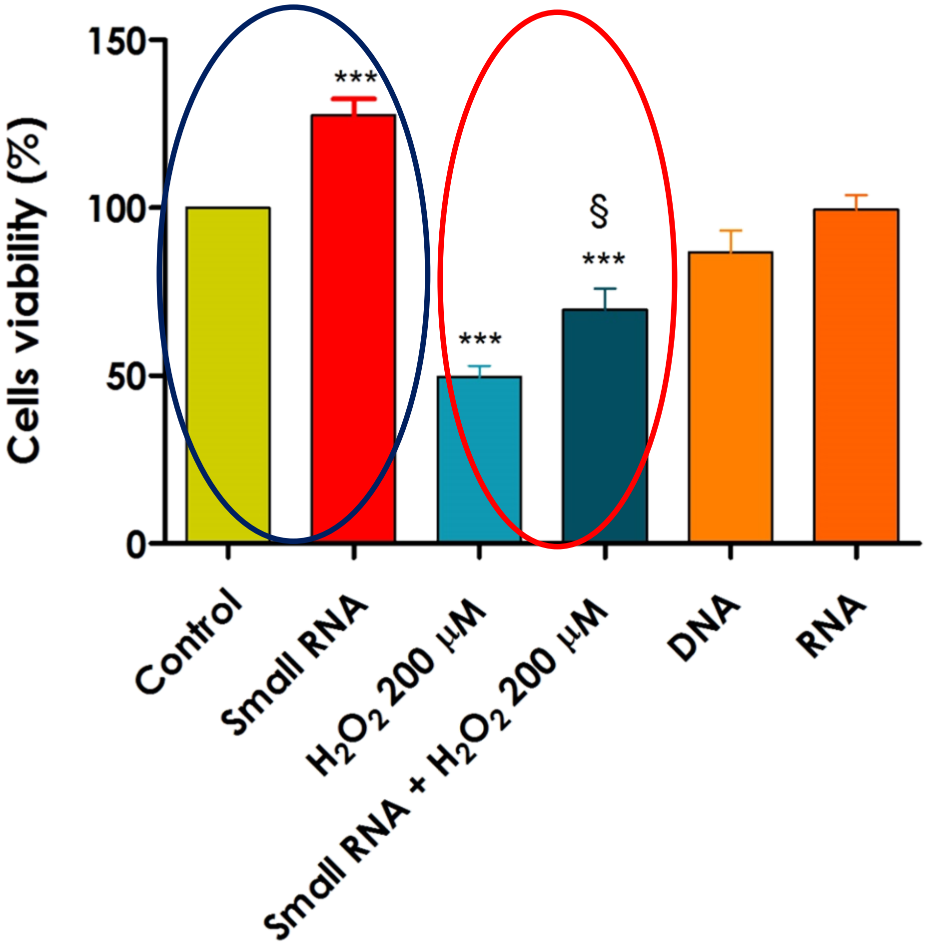
RNA extraction from MCEC-1

Real-Time PCR analysis

Markers of cardiovascular homeostasis

→ Grapevine miRNAs

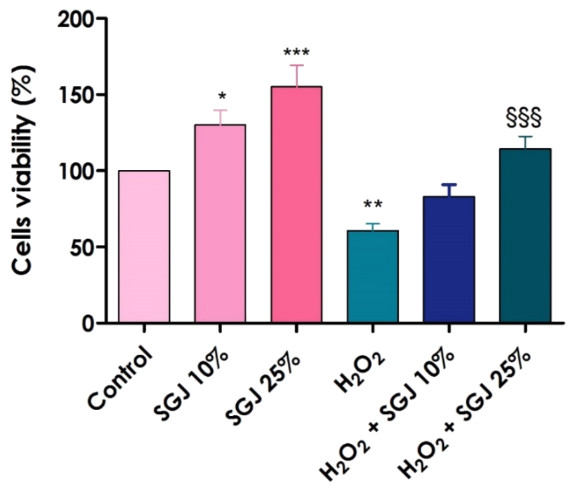
MOUSE CELLULAR SYSTEM to evaluate cellular response in physiological and ischemic condition after exposition of grapevine small RNAs



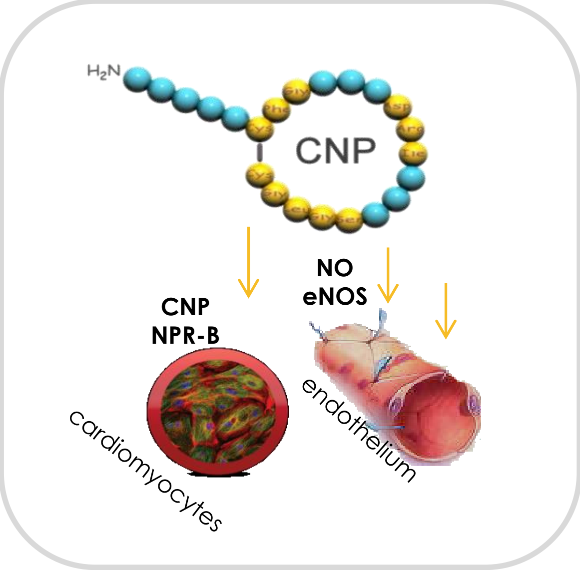
Results

- Treatment with small RNAs increased cell viability also after oxydative stress
- Four grapevine miRNAs identified in the RNA extracted from treated cells

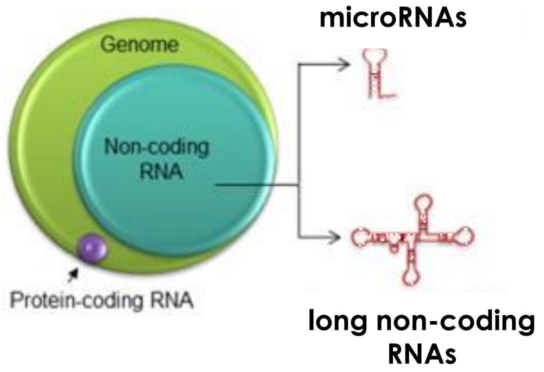
… What about Grape Juice?



Grape juice **improves cell viability and protects endothelial cells by reducing the formation of superoxide anions**



Grape juice **enhances the expression of genes of the endothelial natriuretic peptide system, crucial in cardiovascular homeostasis both at rest and under oxidative stress**

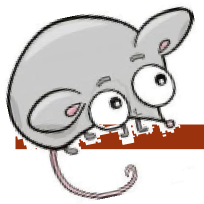


Grape juice **can modulate endothelial non-coding RNAs expression**

Experimental protocol *in vivo*

MURINE MODEL OF CHRONIC MYOCARDIAL INFARCTION EXPOSED TO SANGIOVESE GRAPE JUICE

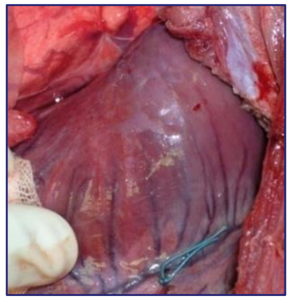
C57BL/6J



1 WEEK — 4 WEEKS — END OF PROTOCOL

Surgery

Ligation of the left anterior descending coronary artery



Different diet condition

- SHAM: control mice + standard diet *(n=7)*
- MI: infarcted mice + standard diet *(n=15)*
- MI + SGJ: infarcted mice + standard diet + *200μl/die of 25% Sangiovese grape juice* *(n=11)*

Cardiac sample collection

- liquid nitrogen
- 5% buffered formalin

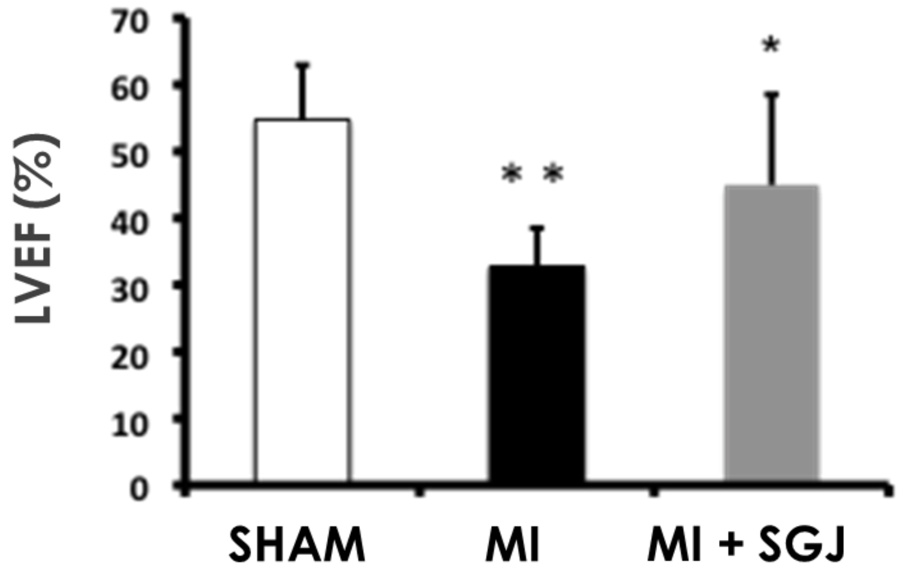
BIOMOLECULAR ANALYSIS

Analysis of genes coding for Components of the Natriuretic peptide System

HISTOLOGICAL ANALYSIS

MOUSE MODEL of myocardial infarction to evaluate the effect of Sangiovese grape juice on infarcted heart

Left ventricular systolic function



Results

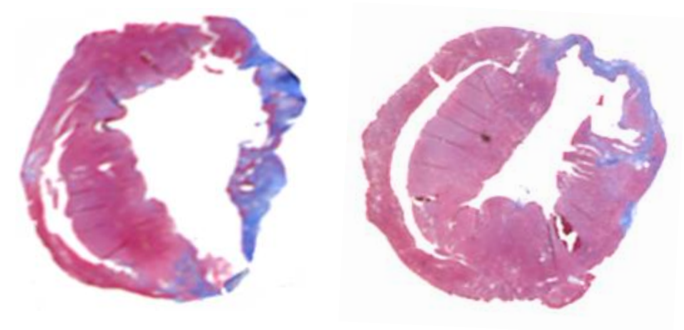
- Treatment with small RNAs increased cell viability also after oxydative stress
- After MI, *grape juice* can restore cardiac function through the activation of the natriuretic peptides system

Hystological analysis

Masson's Trrichrome Stain of the Murine Infarcted Heart

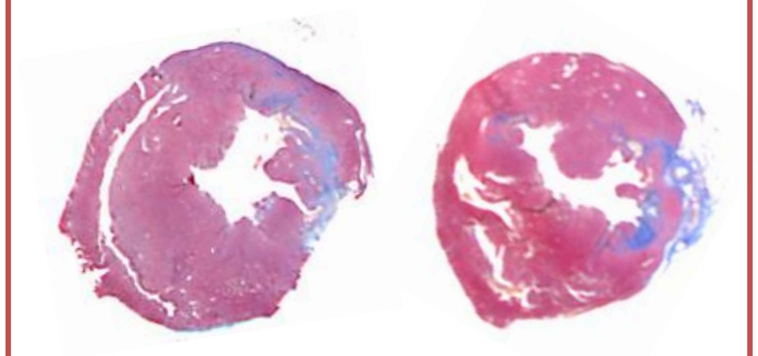
Scanning magnification of the heart section (1x)

MALE

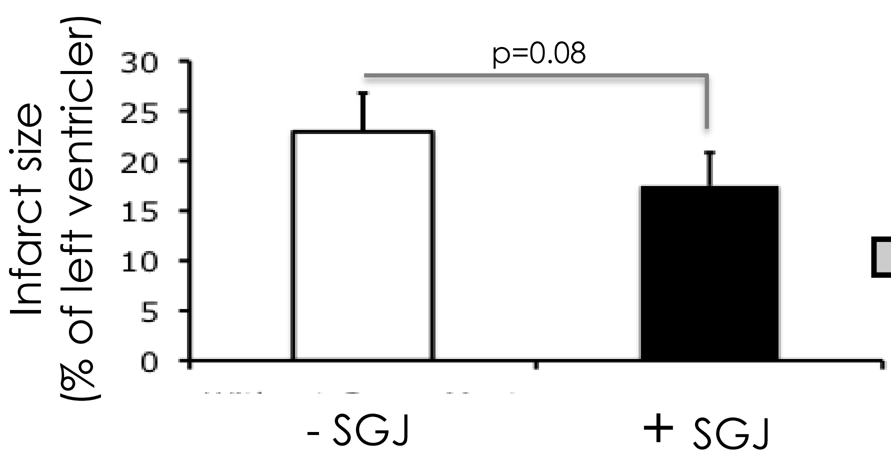


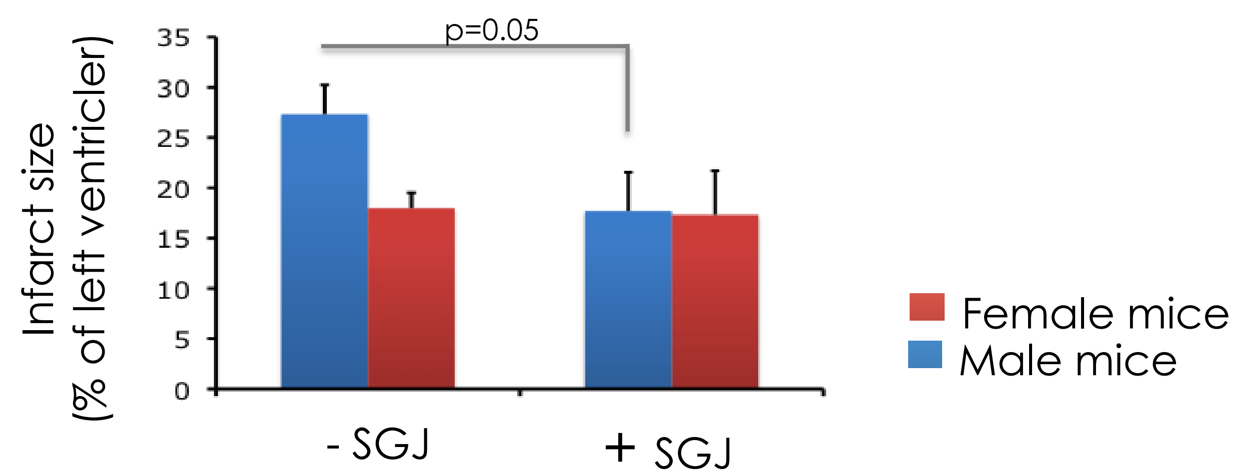
- SGJ **+ SGJ**

FEMALE



- SGJ **+ SGJ**





Recent Literature

Assessing the survival of exogenous plant microRNA in mice

GaoFeng Liang[1,2], YanLiang Zhu[1], Bo Sun[1], YouHua Shao[1], AiHua Jing[2], JunHua Wang[1] & ZhongDang Xiao[1]

Effective detection and quantification of *dietetically* absorbed plant microRNAs in human plasma

Hongwei Liang[1], Suyang Zhang[1], Zheng Fu[1], Yanbo Wang, Nan Wang, Yanqing Liu, Chihao Zhao, Jinhui Wu, Yiqiao Hu, Junfeng Zhang, Xi Chen*, Ke Zen*, Chen-Yu Zhang*

Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses

Zhen Zhou[1,*], Xihan Li[1,*], Jinxiong Liu[2,*], Lei Dong[1,*], Qun Chen[1], Jialing Liu[1], Huihui Kong[2], Qianyi Zhang[2], Xian Qi[3], Dongxia Hou[1], Lin Zhang[1], Guoquan Zhang[2], Yuchen Liu[1], Yujing Zhang[1], Jing Li[1], Jin Wang[1], Xi Chen[1], Hua Wang[3], Junfeng Zhang[1], Hualan Chen[2], Ke Zen[1], Chen-Yu Zhang[1]

Plant microRNAs as novel immunomodulatory agents

Duccio Cavalieri[1,2], Lisa Rizzetto[1], Noemi Tocci[1], Damariz Rivero[3], Elisa Asquini[1], Azeddine Si-Ammour[1], Elena Bonechi[3], Clara Ballerini[3] & Roberto Viola[1]

Detection of dietetically absorbed maize-derived microRNAs in pigs

Yi Luo[1], Pengjun Wang[1], Xun Wang[1], Yuhao Wang[1], Zhiping Mu[1,2], Qingzhi Li[1,3], Yuhua Fu[1,4], Juan Xiao[1], Guojun Li[1], Yao Ma[1], Yiren Gu[5], Long Jin[1], Jideng Ma[1], Qianzi Tang[1], Anan Jiang[1], Xuewei Li[1] & Mingzhou Li[1]

Lack of detectable oral bioavailability of plant microRNAs after feeding in mice

Brent Dickinson, Yuanji Zhang, Jay S Petrick, Gregory Heck, Sergey Ivashuta & William S Marshall

Ineffective delivery of diet-derived microRNAs to recipient animal organisms

Jonathan W. Snow,[1] Andrew E. Hale,[2] Stephanie K. Isaacs,[3] Aaron L. Baggish,[3] and Stephen Y. Chan[2,*]

Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs

Limited evidence for general uptake of dietary plant xenomiRs

Kenneth W. Witwer,* Melissa A. McAlexander, Suzanne E. Queen, and Robert J. Adams

Unsuccessful Detection of Plant MicroRNAs in Beer, Extra Virgin Olive Oil and Human Plasma After an Acute Ingestion of Extra Virgin Olive Oil

Victor Micó[1] · Roberto Martín[1] · Miguel A. Lasunción[2] · Jose M. Ordovás[1,3] · Lidia Daimiel[1]

MIRNAGREEN
health from plants

http://www.mirnagreen.com/

Plant microRNAs: a revolutionary approach to protect people's health

Mirnagreen has unraveled one of nature's best kept secrets: how plants protect us from disease. Using science-based approaches we have discovered the surprisingly effective bioactive capacity of plant microRNAs on our immune system.

We have discovered that anti-inflammatory capacity of fruits and vegetables is attributable to their microRNAs. However, these are poorly assimilated via the diet.

Mirnagreen has developed technologies that increase x 25 fold the bioavailability of dietary microRNAs. The microRNA content of just one portion of fruit or vegetable can be used to provide full protection against inflammatory challenges

A daily dose of Mirnagreen is equivalent to the protective microRNA power of 2Kg of fruits and vegetables.

Acknowledgements

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CNR, Istituto di Fisiologia Clinica

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Dr. Manuela Cabiati

Summary and Conclusions

Bioinformatic tools are necessary to investigate putative mammalian targets of plant miRNAs.
To date, it does not exist an algorithm able to combine plant miRNAs and mammalian transcripts

After MI, ***grape juice*** can restore cardiac function through natriuretic peptides system activation

Grapevine miRNAs increase viability of endothelial cells, in physiological condition and under oxidative stress

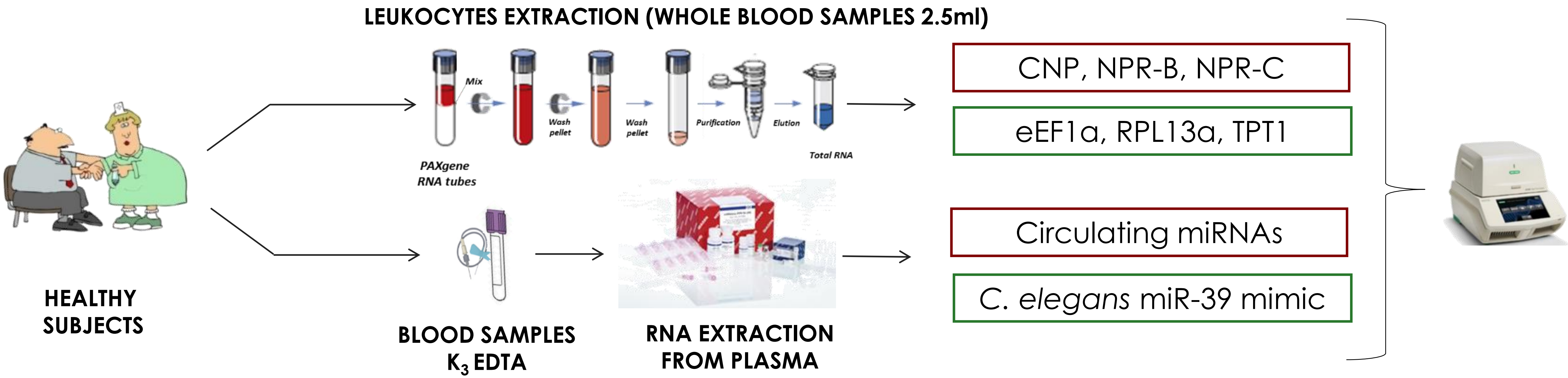
Grapevine miRNAs can modulate endothelial non-coding RNAs

Our results suggest that grapevine miRNAs should be considered as part of the nutraceutical properties of grapes that might have biological effects on human organism

Work in progress

Pre-clinical trial

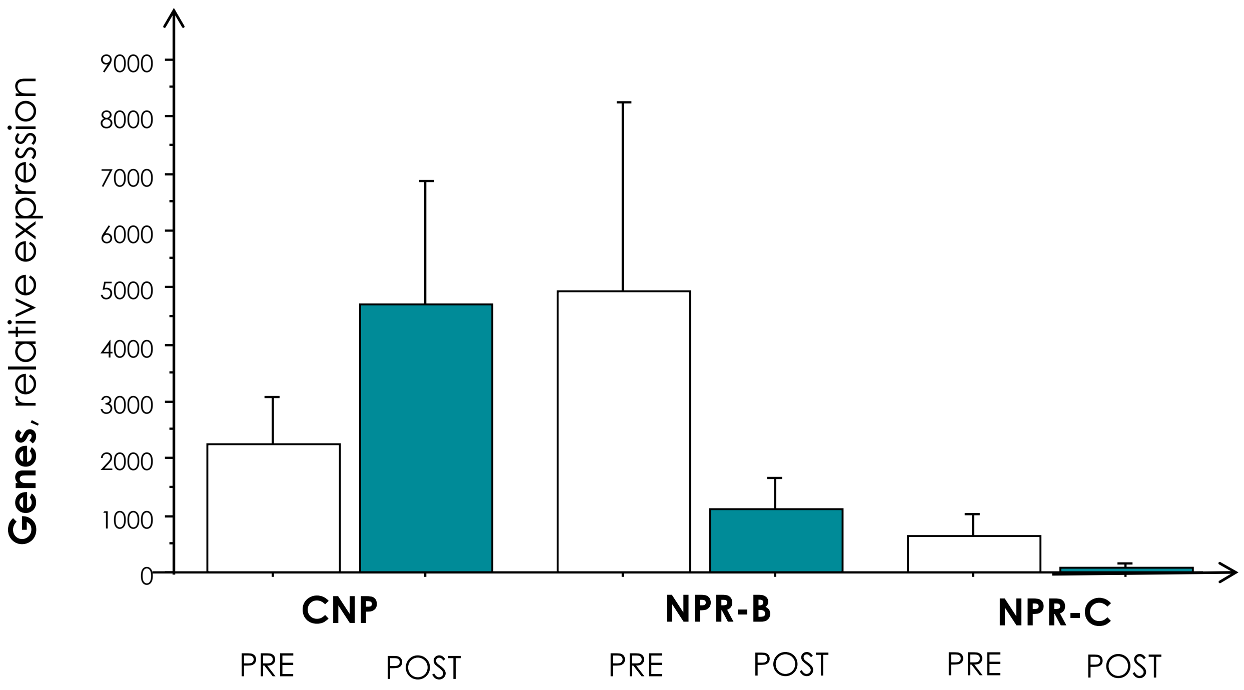
in collaboration with Fondazione Toscana "Gabriele Monasterio"



- Mean age: 34 ± 5 years old
- n = 12 (6 male and 6 female)
- Blood collection at baseline and after 1 L of ***Sangiovese grape juice*** administration ***(1 week)***

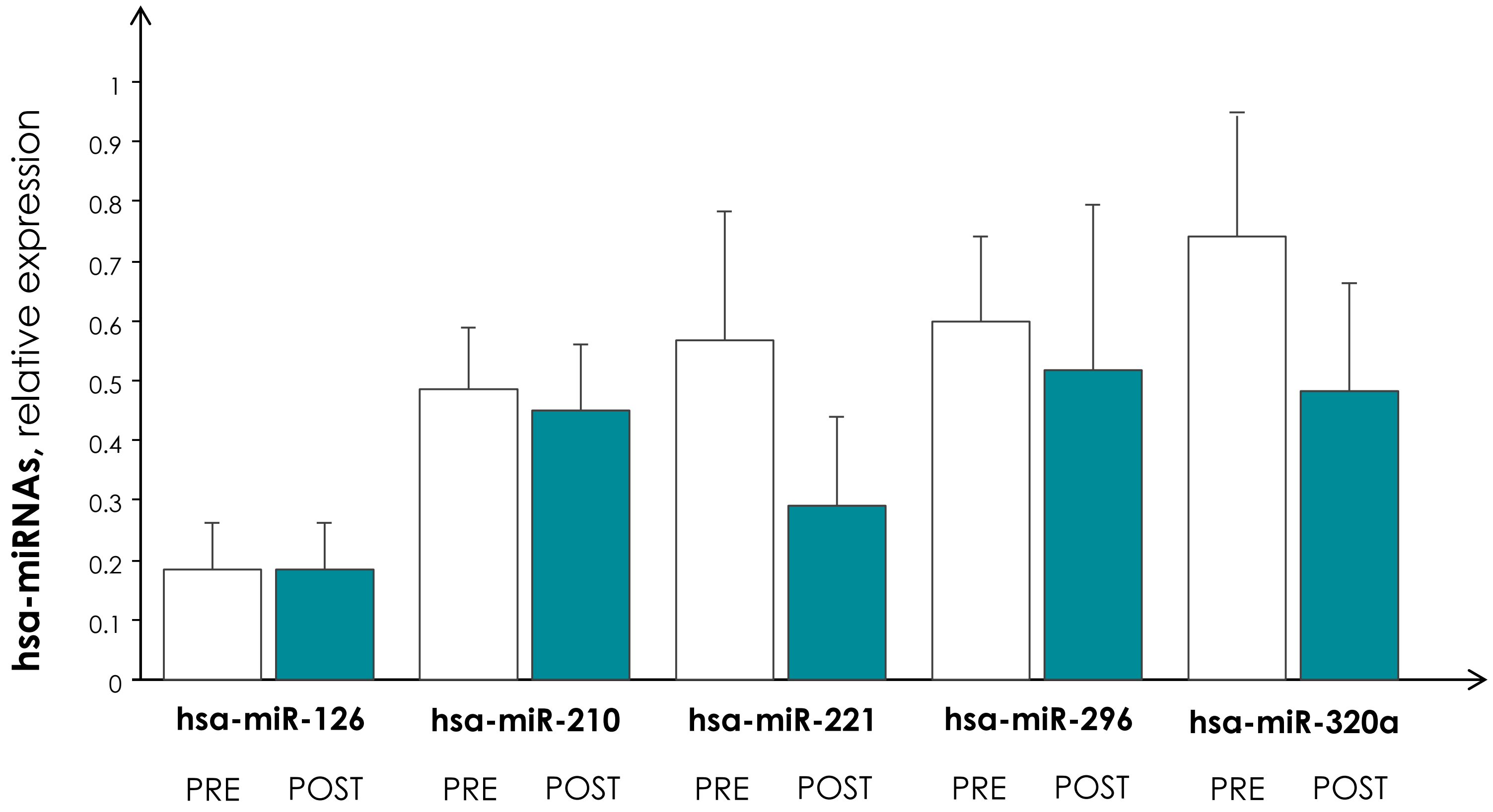
Leukocytes transcriptomic profile

mRNA relative expression of ***CNP***, ***NPR-B*** and ***NPR-C*** in peripheral leukocytes normalized on the three most stable reference genes: *eEF1a*, *RPL13a*, *TPT1*



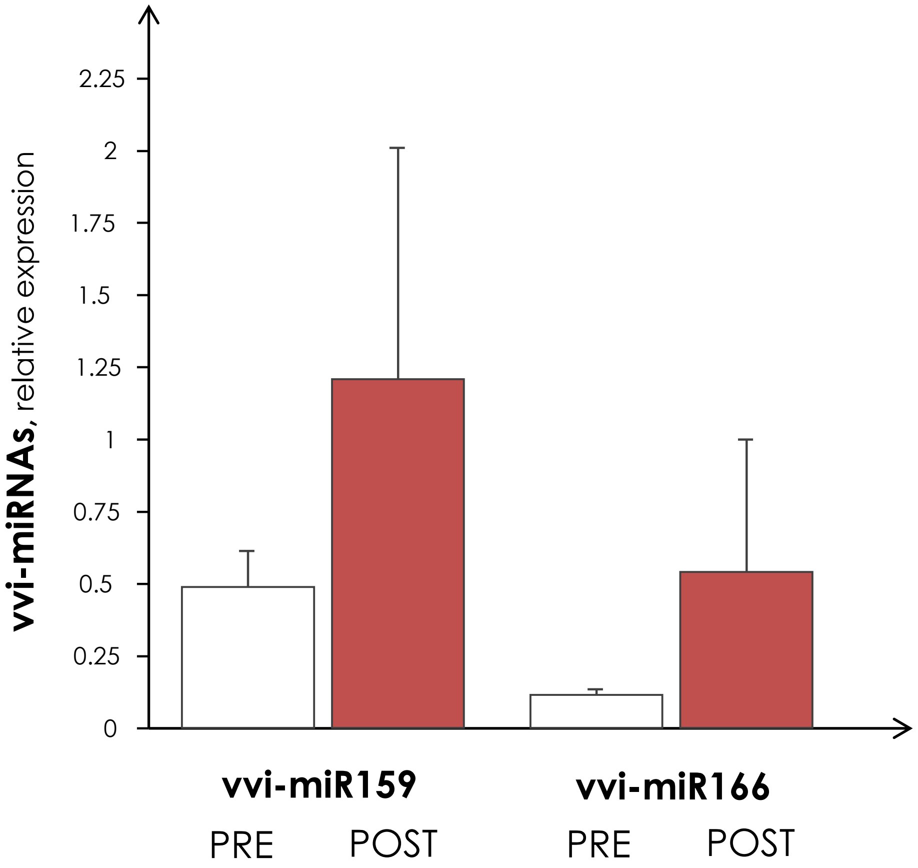
Circulating miRNAs profile

Relative expression of ***miR-126***, ***miR-210***, ***miR-221***, ***miR-296***, ***miR-320a*** in human plasma normalized on *miR-39*



Circulating miRNAs profile

Expression level of plant ***miR159-3p*** and ***miR166-3p*** in human plasma at baseline and after grape juice administration, normalized on *miR-39*

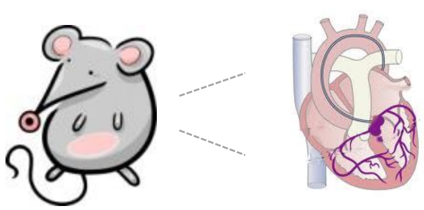


Clinical data on dietary supplementation of grape juice suggests that *CNP/NPR-B/NPR-C* pathway might be activated as well as absorption of *plant miRNAs*

Future perspective

Apply ***high throughput sequencing*** approach to identify grapevine miRNAs target in mammals and also differently expressed non-coding RNAs

Clarify whether the effect of ***grapevine small RNAs*** are cellular specific, using different cell lines and small RNAs doses

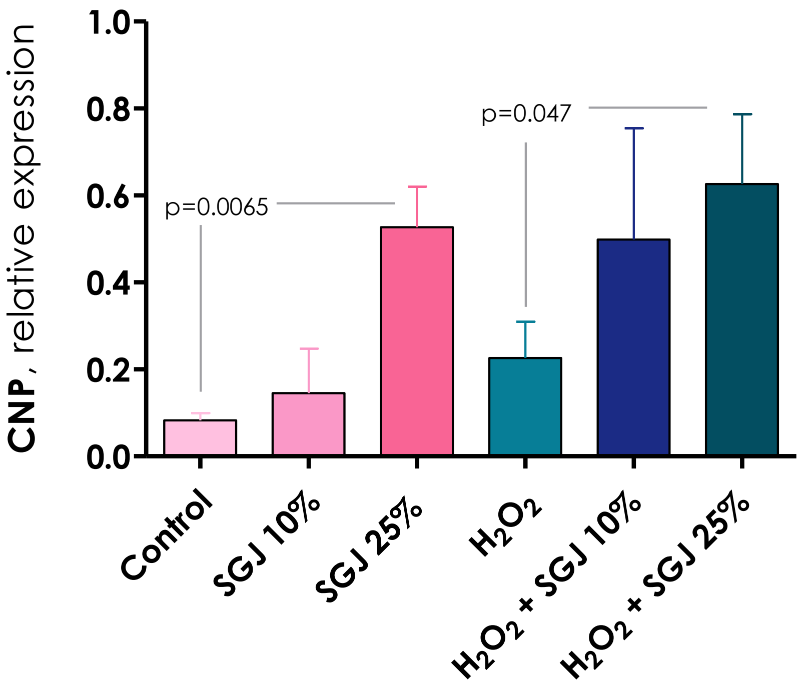


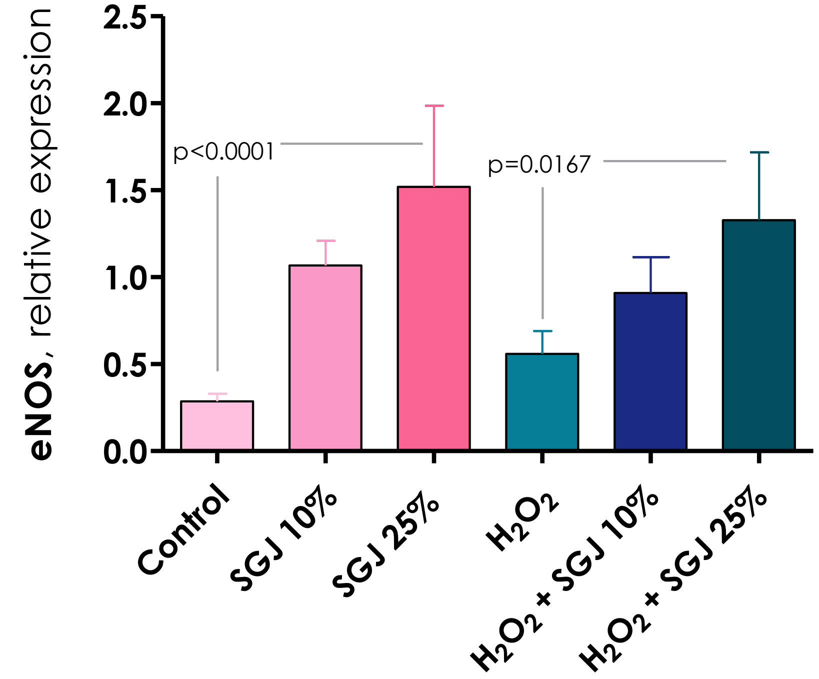
Perform a ***murine model*** of myocardial infarction ***feed with grapevine small RNAs***, in order to detect plant miRNAs in serum and tissues

Increase the number of ***healthy subjects*** for the pre-clinical study. This would be preliminary to the recruitment of ***patients who have suffered from infarction***

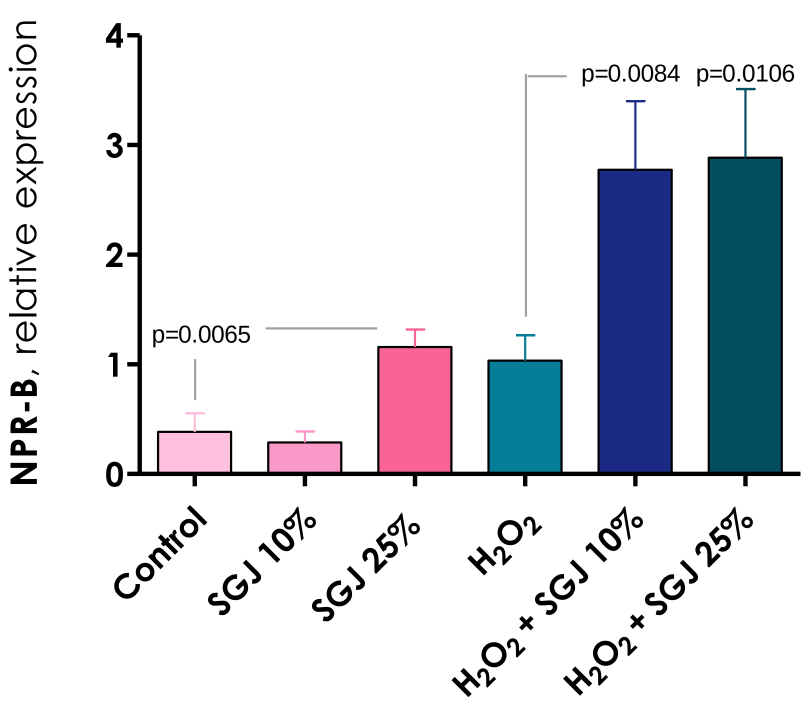
Real time PCR: transcriptomic profile in Sangiovese-treated MCEC-1 cells

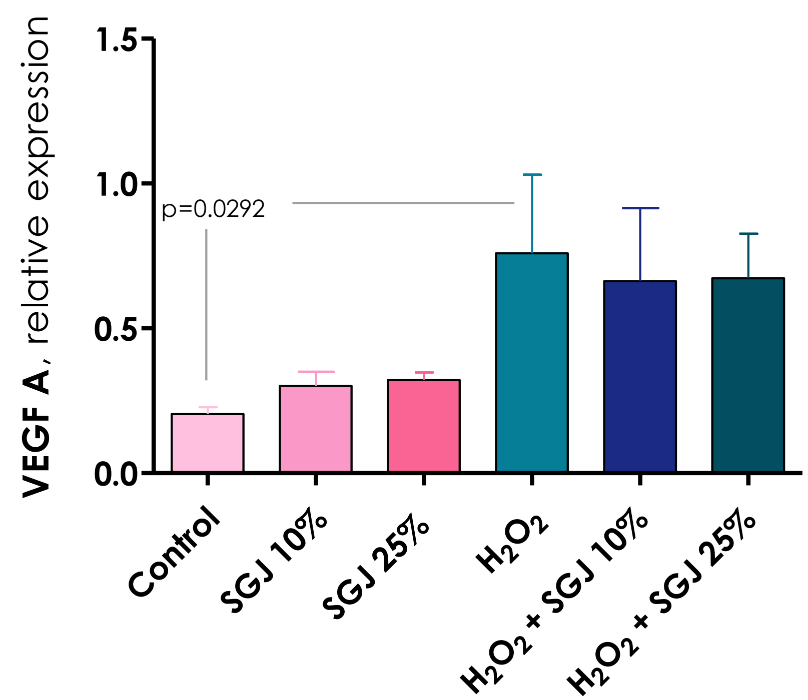
- mRNA expression of C-type natriuretic peptide (***CNP***) and endothelial nitric oxide synthase (***eNOS***)





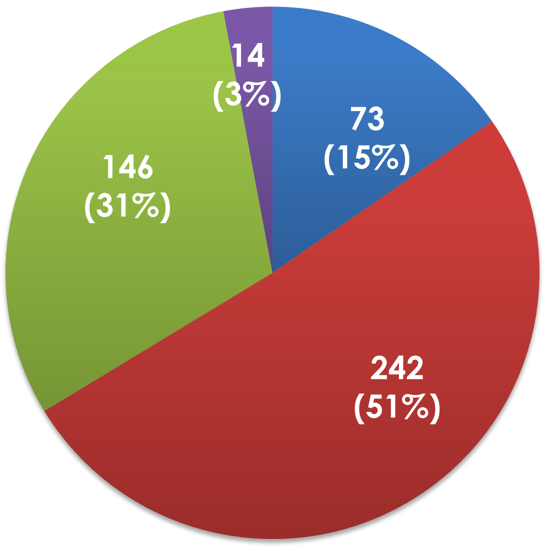
- mRNA expression of natriuretic peptide receptor B (***NPR-B***) and vascular endothelial growth factor (***VEGF A***)





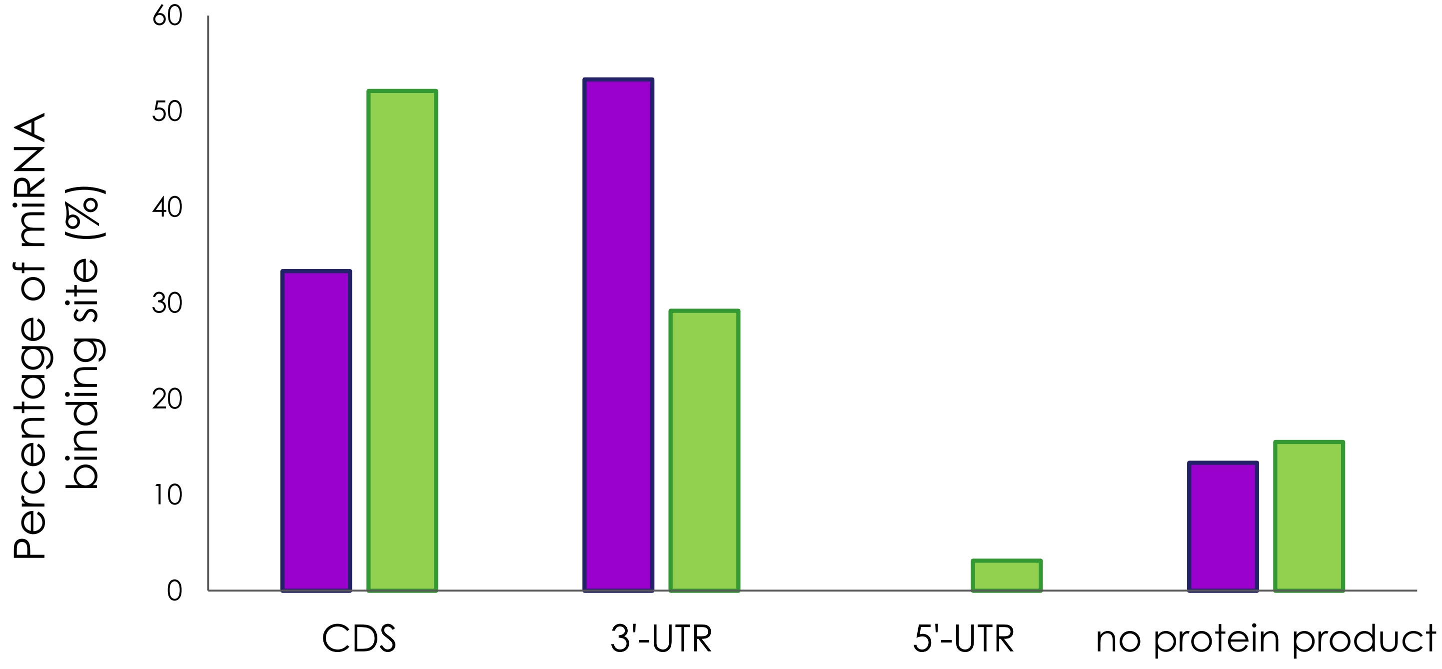
Reference genes:
Rpl13a, Ppia, Tbp

Grapevine miRNAs target site in *Mus musculus*



Grapevine miRNAs Target sites distribution on murine transcripts.

- CDS
- 3'-UTR
- 5'- UTR
- No protein products

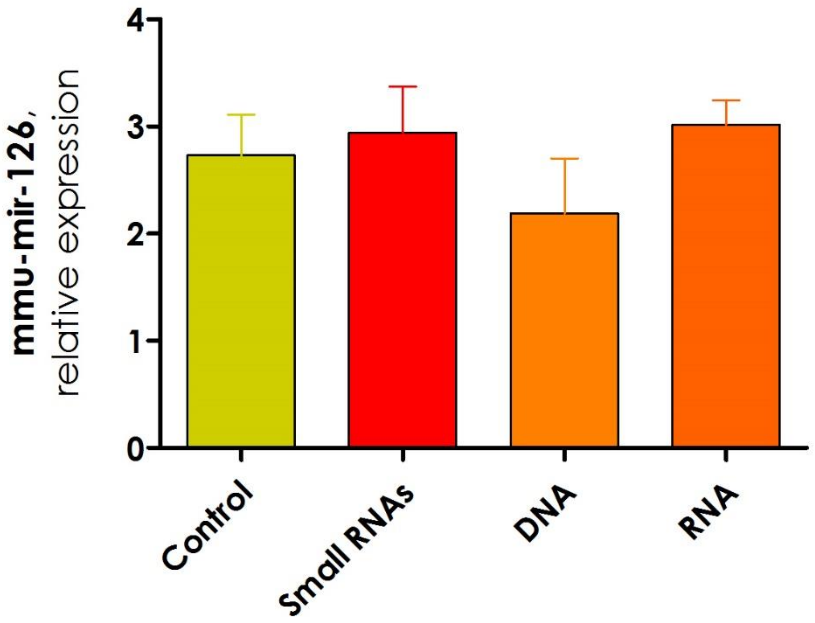


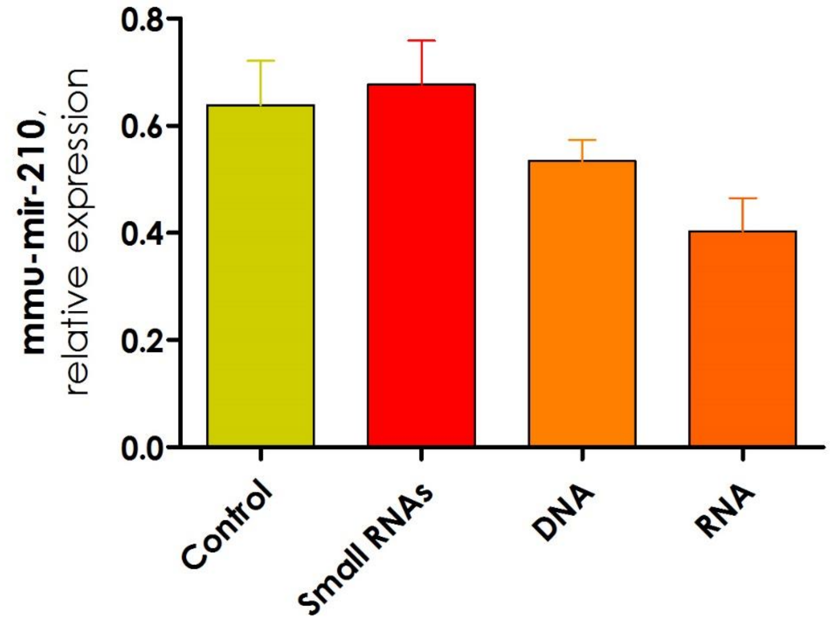
Comparison of miRNA binding sites between conserved and grapevine specific miRNA families.

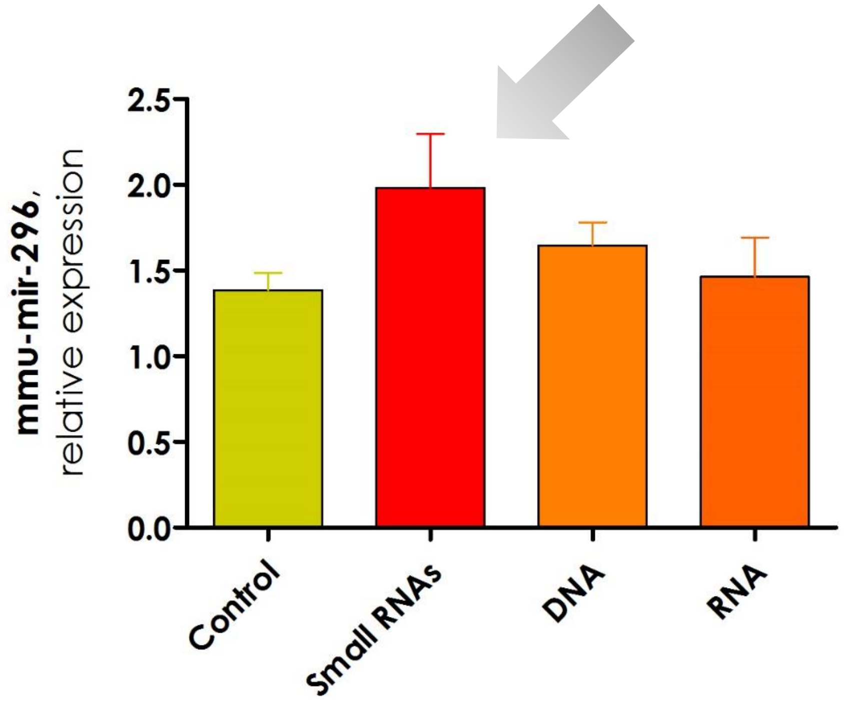
- Plant conserved miRNAs
- Grapevine specific miRNAs

Real time PCR: angiomiR detection in transfected MCEC-1

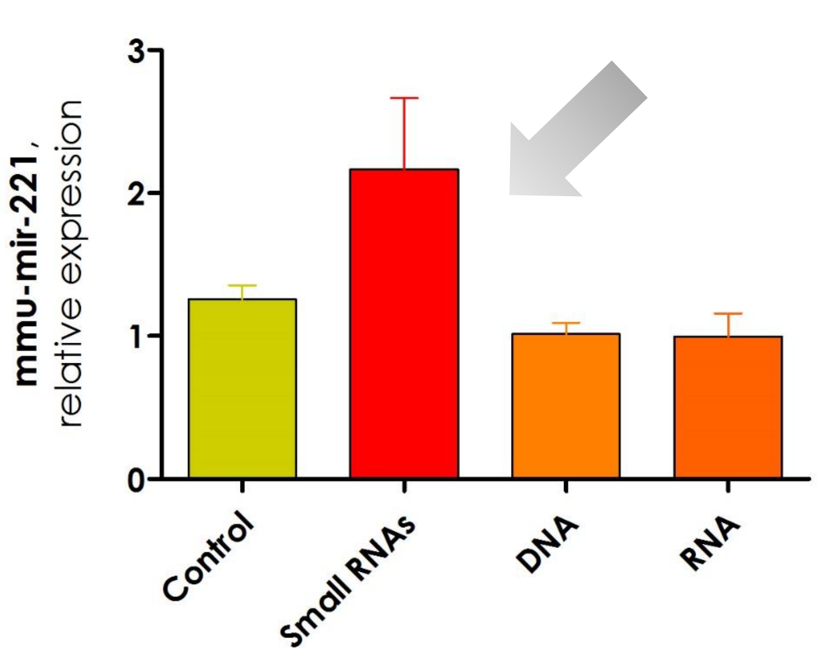
- Pro-angiomiRs: ***miR-126-3p***, ***miR-210-3p*** and ***miR-296-5p***

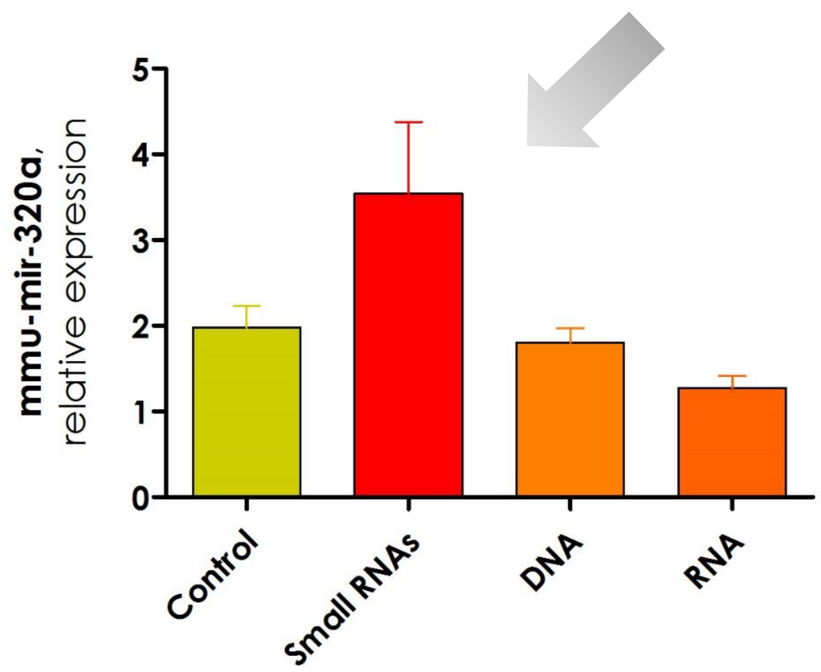






- Anti-angiomiRs: ***miR-221-3p*** and ***miR-320a***

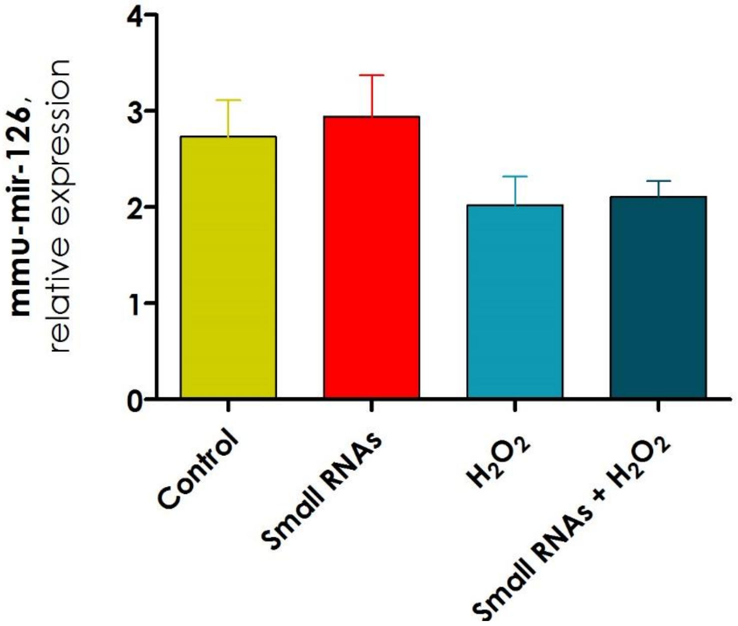


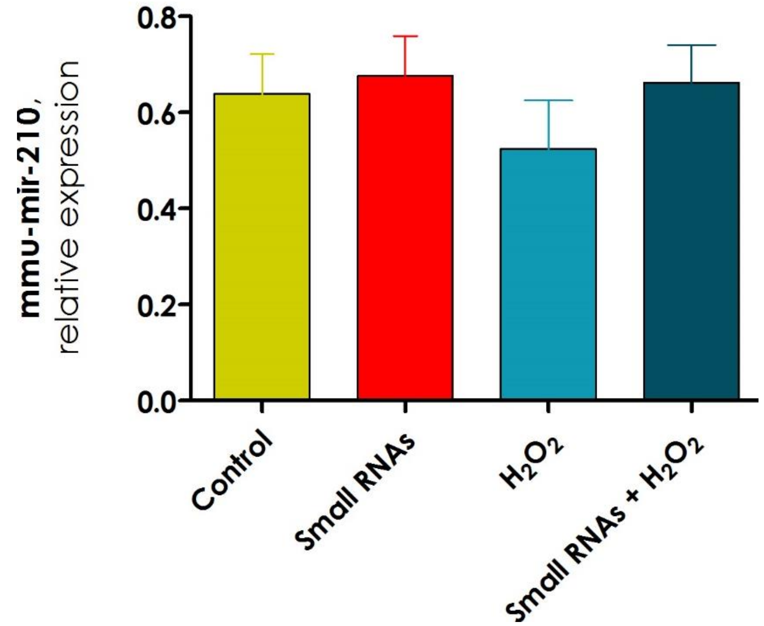


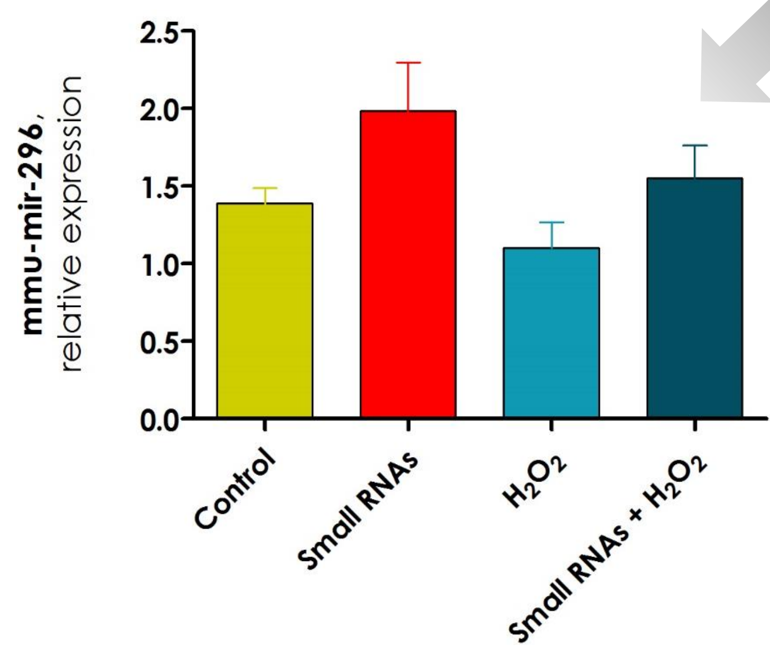
Reference gene:
Rnu6

Real time PCR: angiomiR detection in transfected MCEC-1 (*STRESS CONDITION*)

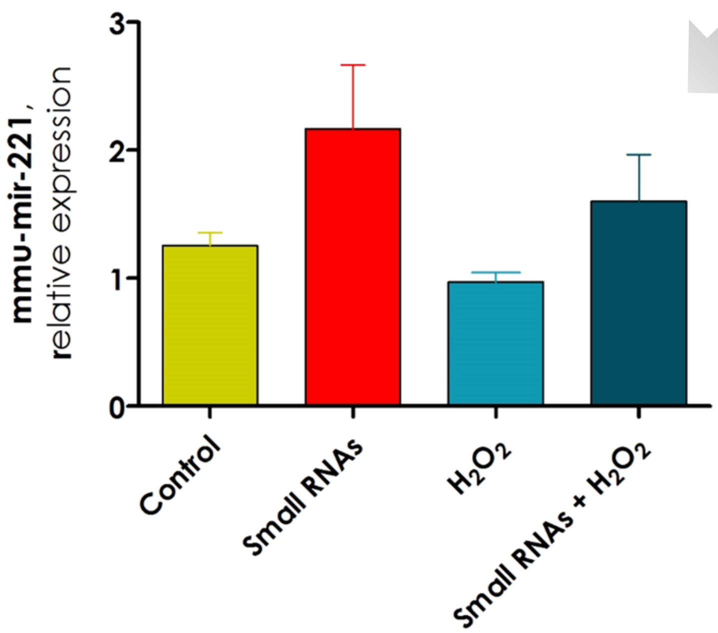
- Pro-angiomiRs: ***miR-126-3p***, ***miR-210-3p*** and ***miR-296-5p***

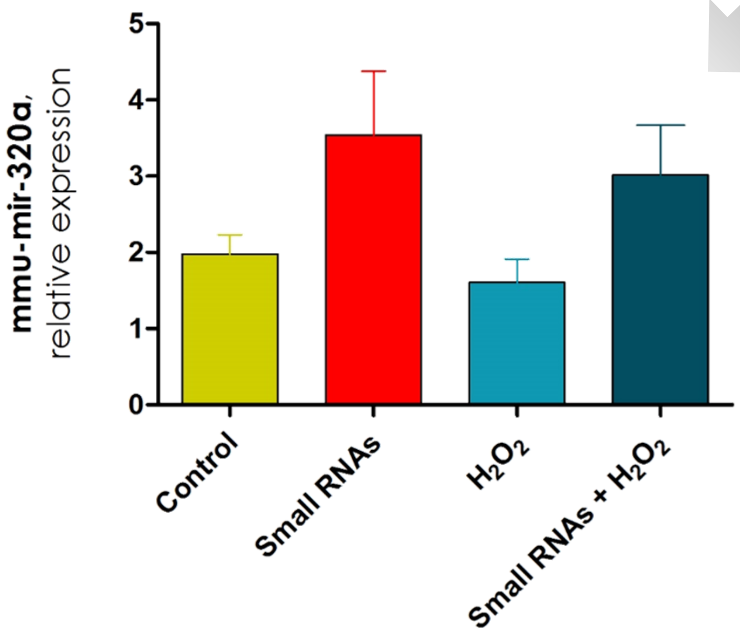






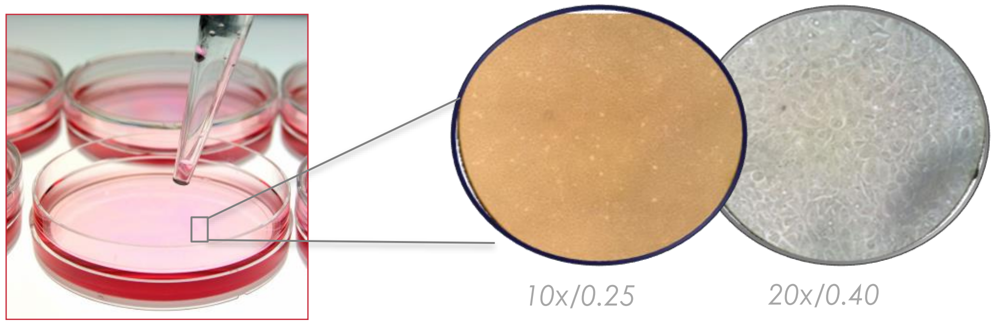
- Anti-angiomiRs: ***miR-221-3p*** and ***miR-320a***





Reference genes:
Rnu6

Experimental protocol *in vitro*: grape juice

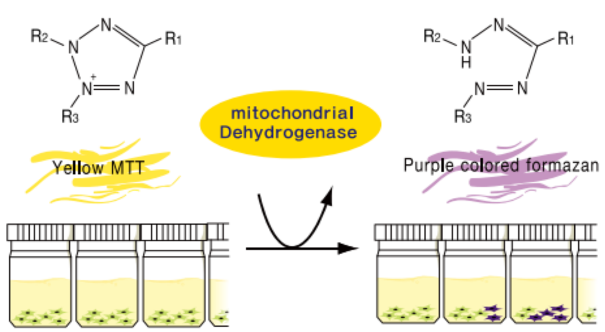


Immortalized murine coronary endothelial cell line (***MCEC-1***)

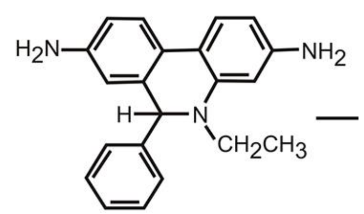
- MCEC-1 + 10% FBS
- MCEC-1 + ***Sangiovese grape juice (SGJ, 24 h)***
- MCEC-1 + 200 μM H_2O_2 (24 h)
- MCEC-1 + 200 μM H_2O_2 + ***Sangiovese grape juice (SGJ, 24 h)***

MTT ASSAY

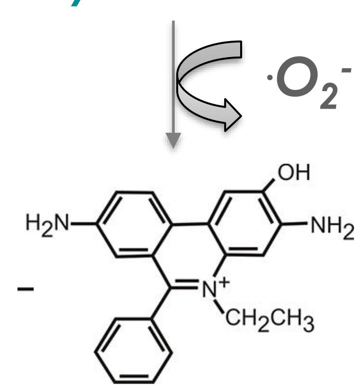
Cell growth determination kit



DHE ASSAY



dihydroethidium



2-hydroxyethidium

RNA extraction from MCEC-1

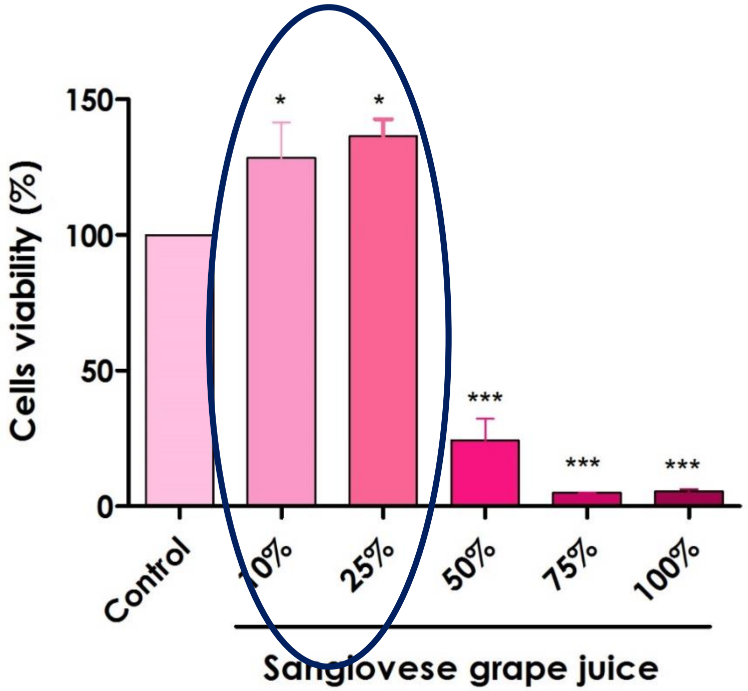
Acid guanidinium thiocyanate-phenol-chloroform method

Real-Time PCR analysis

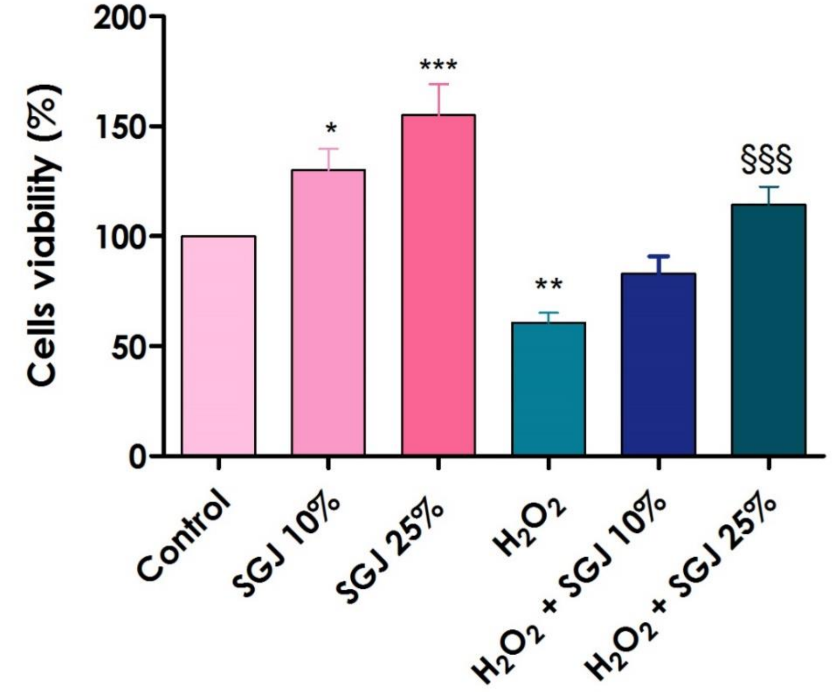
- CNP, NPR-B, eNOS, VEGF A
- AngiomiRs
- Long non-coding RNAs
- Rpl13a, Ppia, Tbp; Rnu6

Results

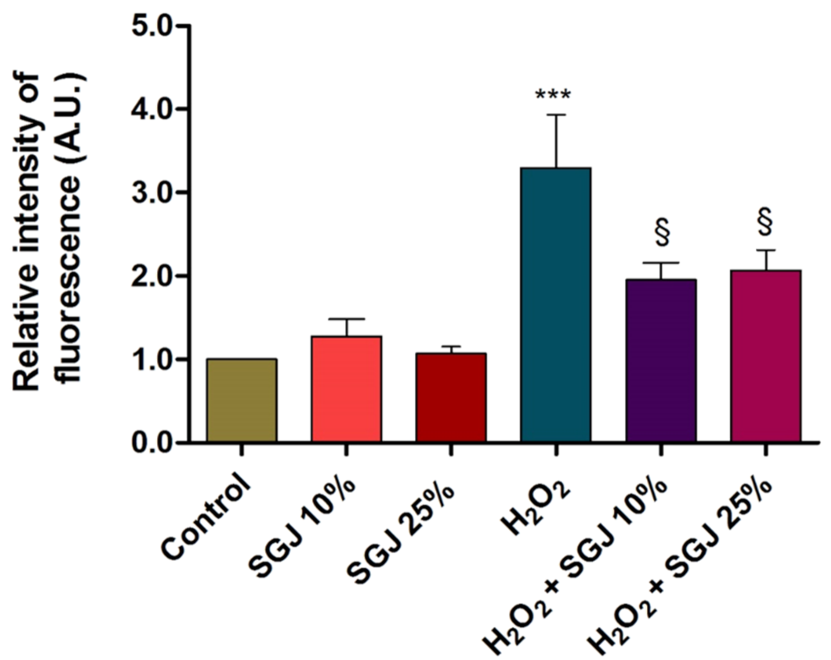
MTT assay : assessment of MCEC-1 viability after 24 h of Sangiovese exposition



+ 200 μM H_2O_2
(24 h)



DHE assay: superoxide anion detection in MCEC-1 after 8 h of Sangiovese exposition

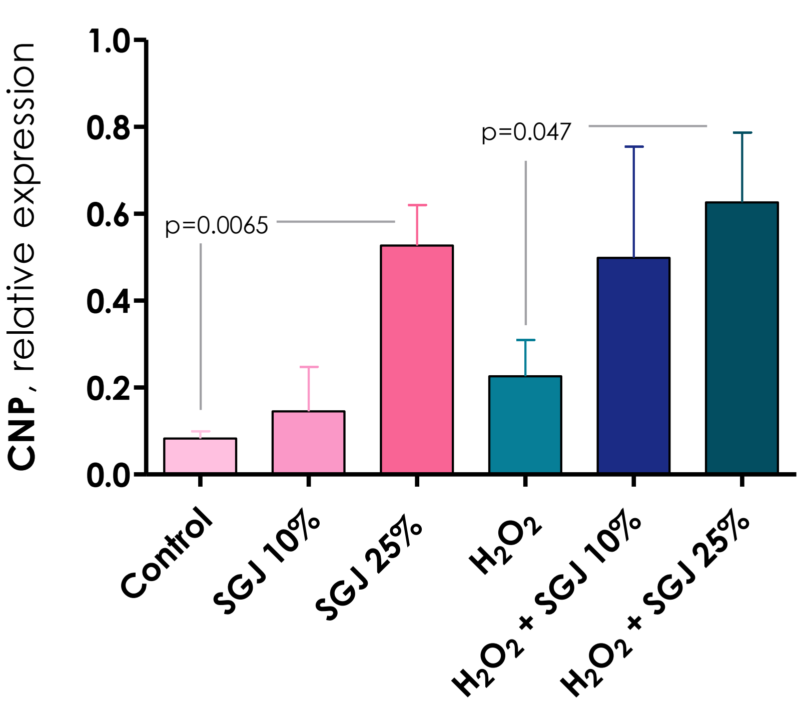


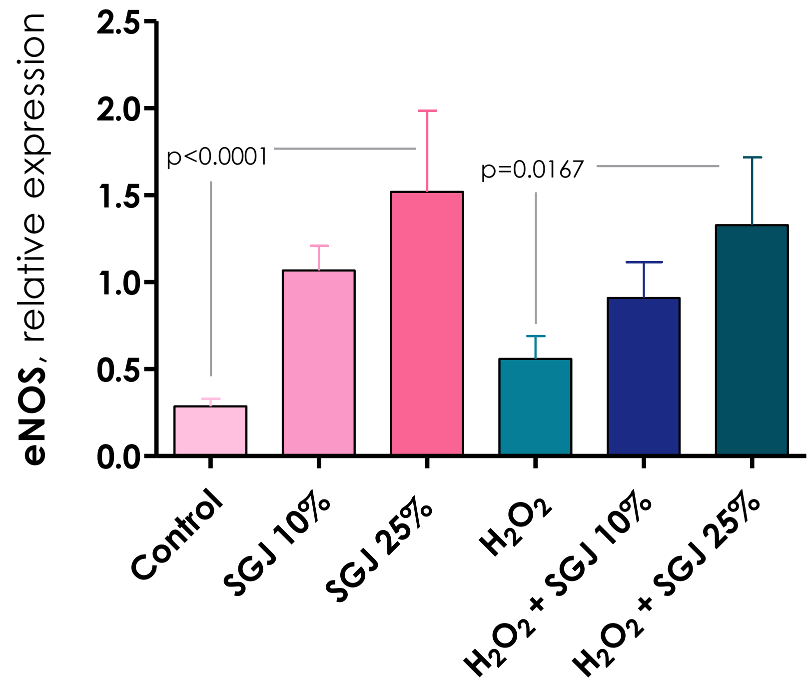
200 μM H_2O_2
(8 h)

* $p < 0.05$ versus Control
** $p < 0.01$ versus Control
*** $p < 0.001$ versus Control
§ $p < 0.05$ versus H_2O_2
§ § § $p < 0.001$ versus H_2O_2

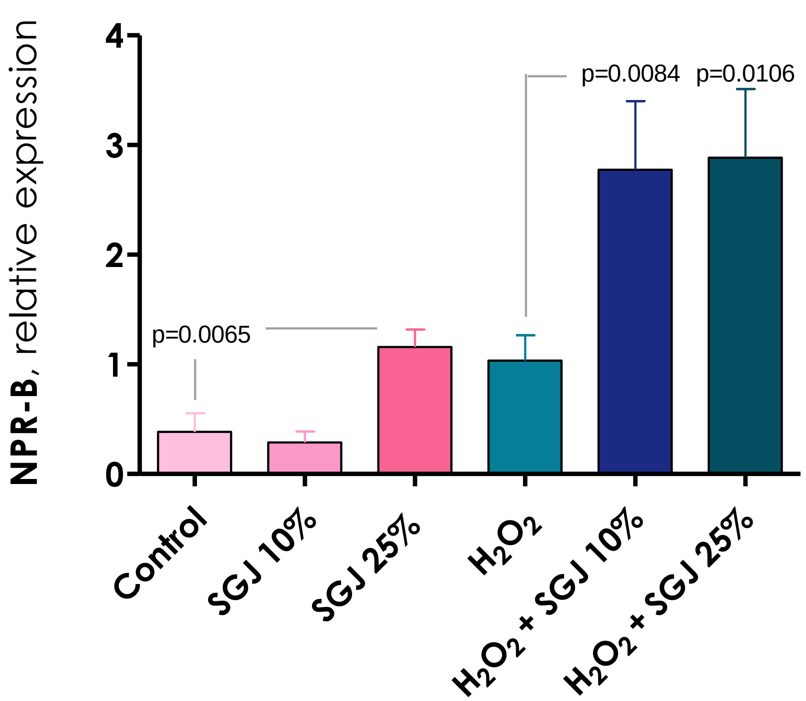
Real time PCR: transcriptomic profile in Sangiovese-treated MCEC-1 cells

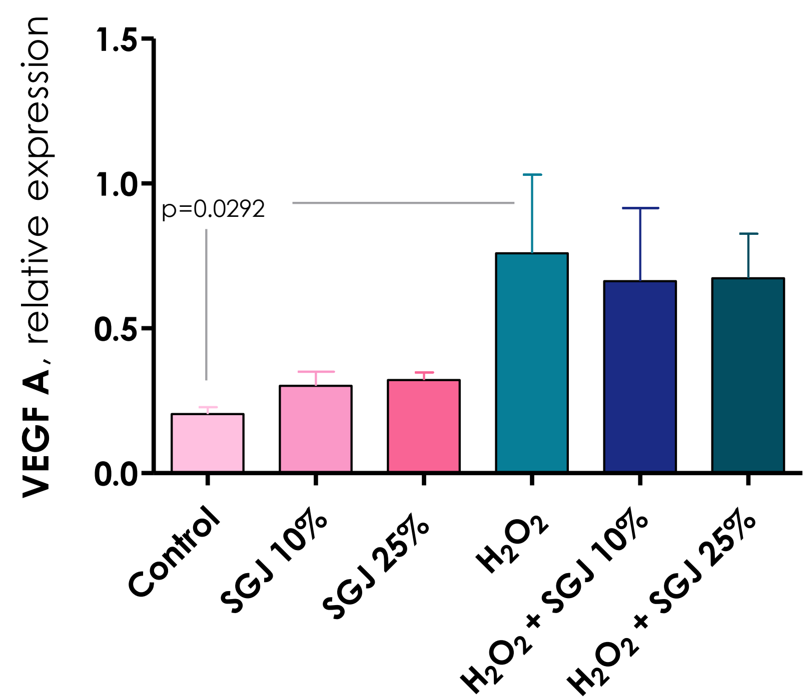
- mRNA expression of C-type natriuretic peptide (***CNP***) and endothelial nitric oxide synthase (***eNOS***)





- mRNA expression of natriuretic peptide receptor B (***NPR-B***) and vascular endothelial growth factor (***VEGF A***)

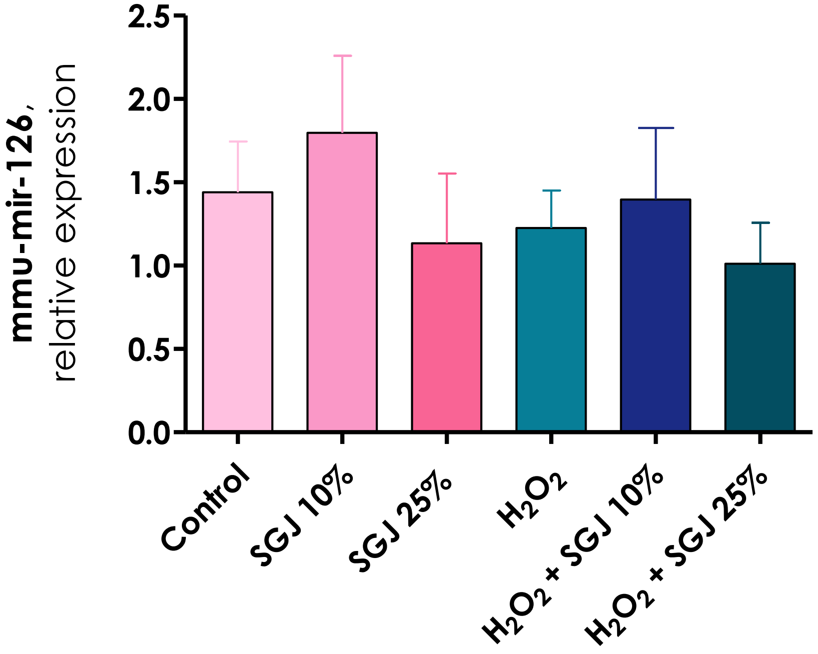


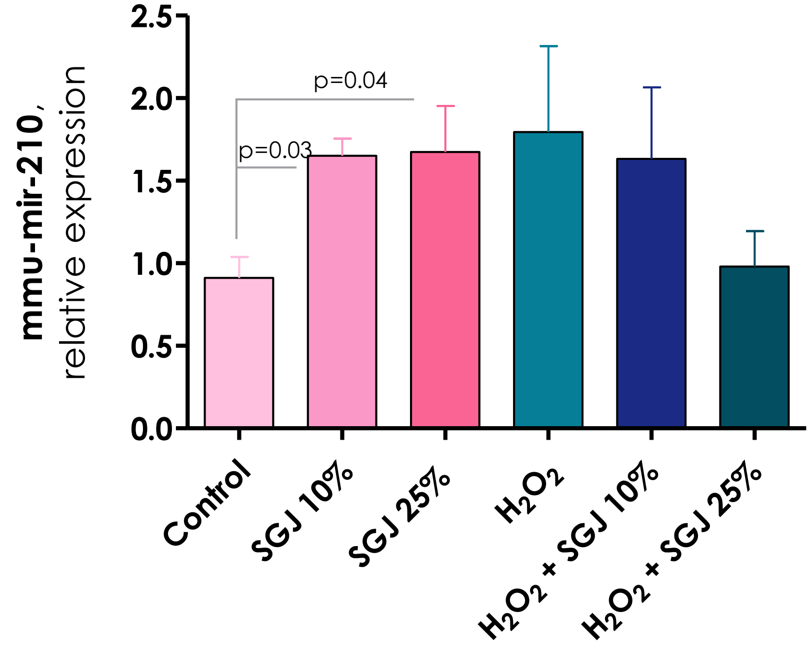


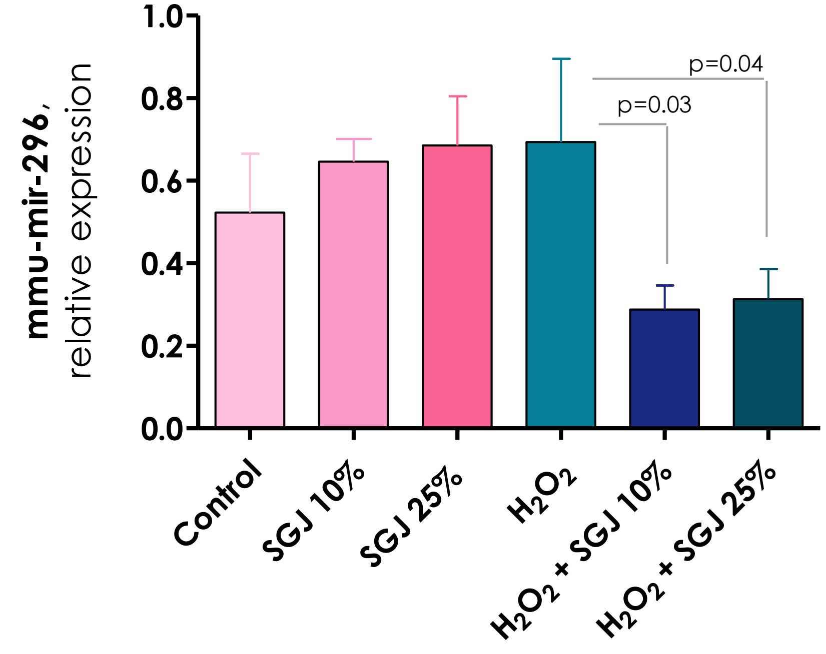
Reference genes:
Rpl13a, Ppia, Tbp

Real time PCR: angiomiRs detection in Sangiovese-treated MCEC-1

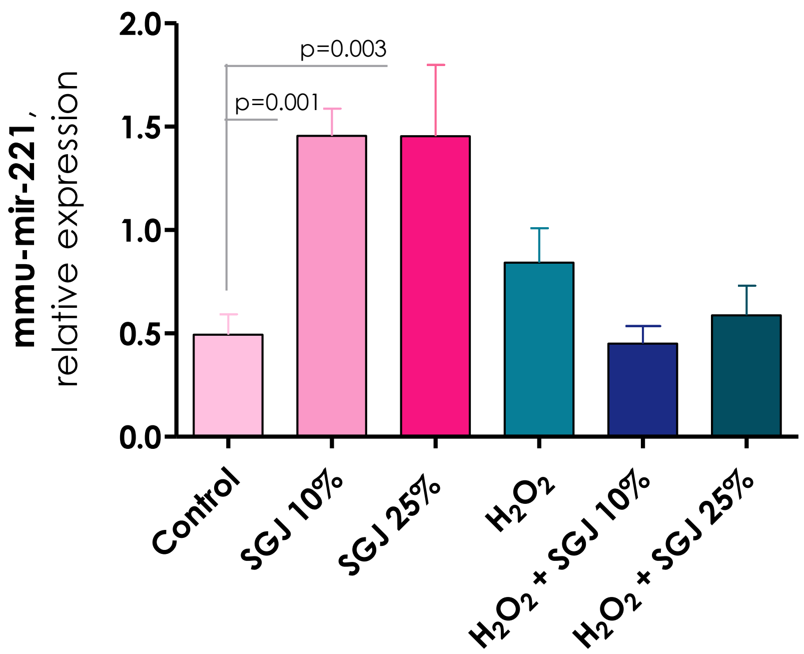
- Pro-angiomiRs: ***miR-126-3p***, ***miR-210-3p*** and ***miR-296-5p***

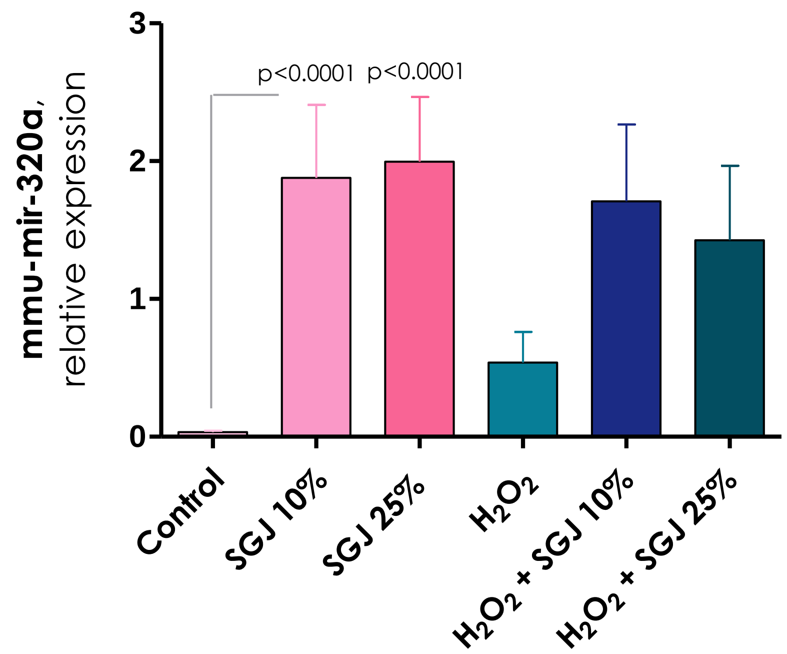






- Anti-angiomiRs: ***miR-221-3p*** and ***miR-320a***





Reference genes:

Rnu6

Real time PCR: long non-coding RNAs detection in Sangiovese-treated MCEC-1

- Long non-coding RNAs (lncRNAs) are a class of single stranded non-coding RNAs >200 nuclotides long, that are poorly conserved between species.
- LncRNAs are involved in ***transcriptional***, ***post-transcriptional*** and ***post-translational*** processes.

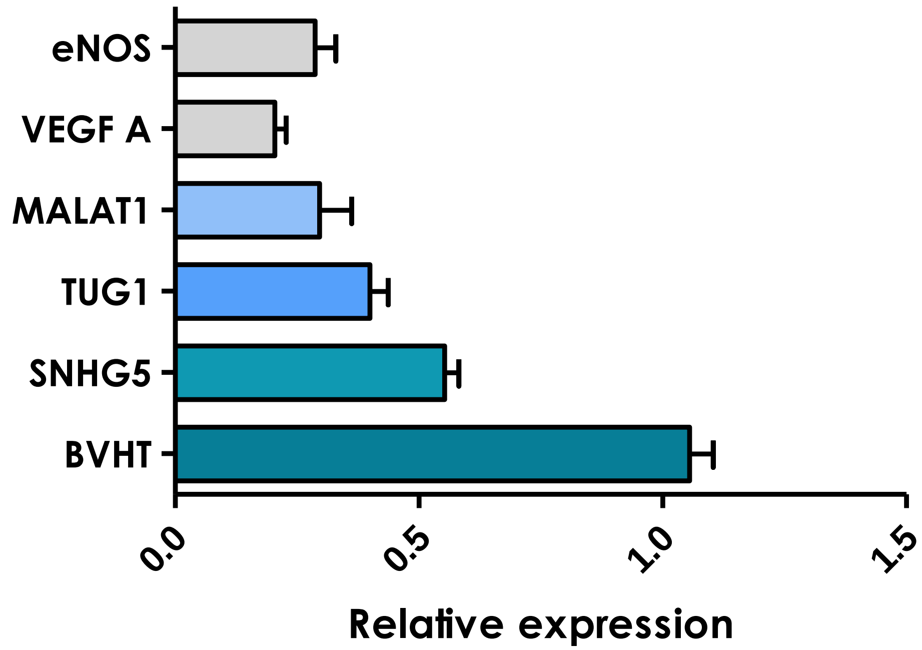
*LncRNAs in **HUVEC** cell line (Michalik et al., 2014)*

Human lncRNA	RPKM[1]	Orthologous in mouse[2]
RP11-291L15.2	86.59115	no (locus conservation)
SNHG5	79.13517	Snhg5
LINC00657	78.23839	no
AC021224.1	77.99524	no
ZNFX1-AS1	67.96695	Zfas1
CTD-2139B15.2	62.96667	no
RP11-203M5.8	59.63135	no (locus conservation)
CTD-2207P18.1	57.50935	no (locus conservation)
RP11-488C13.7	39.83597	no
TUG1	35.65244	Tug1
MEG3	35.28998	Meg3
RP11-638I2.6	34.43343	no (locus conservation)
MALAT1	34.07055	Malat1
ANKRD62P1	29.30348	no
RP3-523C21.1	25.55078	no (locus conservation)

[1]Reads per kilobase per million mapped reads.

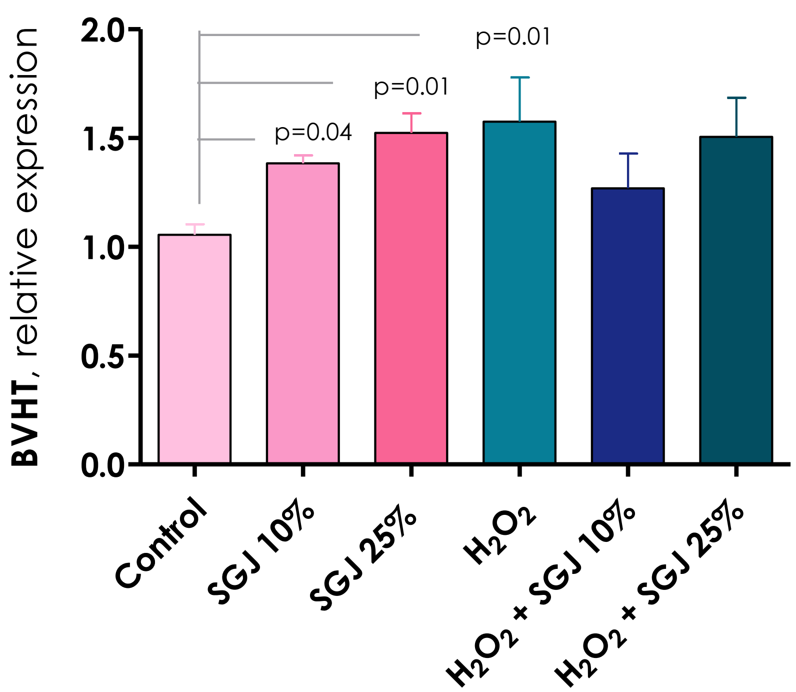
[2] Orthologous mouse gene were checked on LNCipedia (http://www.lncipedia.org/) and Mouse Genome Informatics (http://www.informatics.jax.org/) websites.

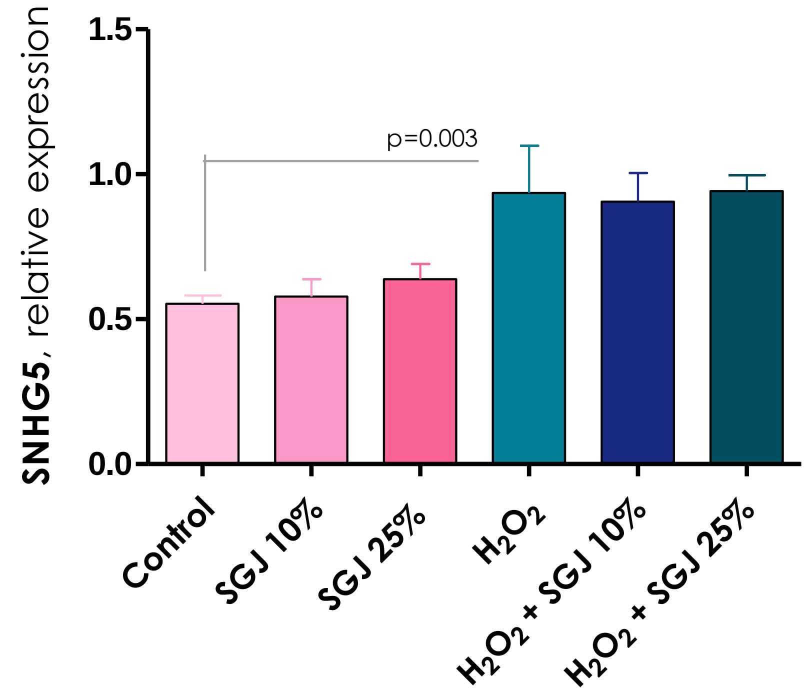
*LncRNA in **MCEC-1** cell line compare to endothelial genes (eNOS, VEGF A)*

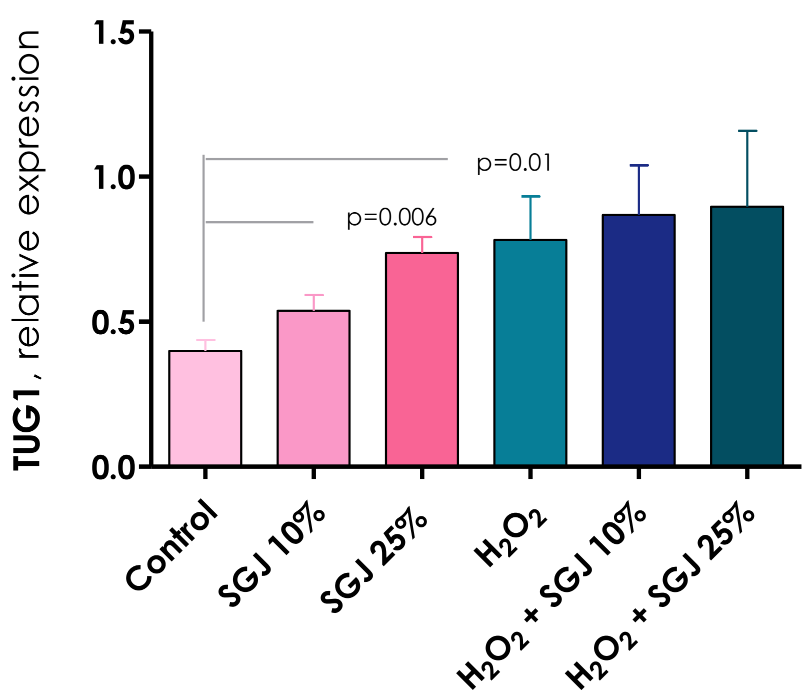


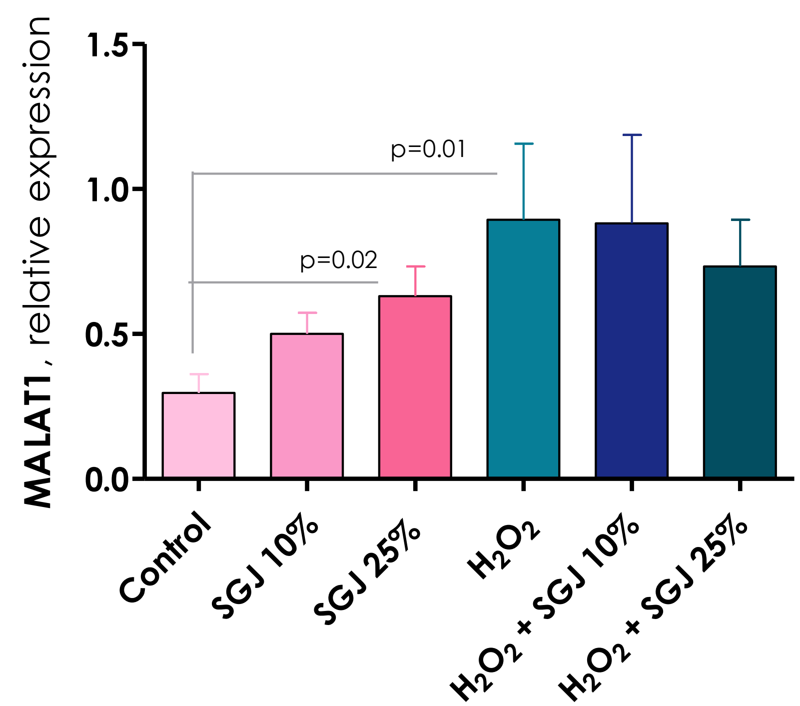
Reference genes: Rpl13a, Ppia, Tbp

- mRNA expression of Braveheart (***BVHT***, *murine specific lncRNA*), *Small Nucleolar Host Gene 5* (***SNHG5***), Taurine up-regulated gene (***TUG1***) and Metastasis associated lung adenocarcinoma transcript 1 (***MALAT1***)









Reference genes:
Rpl13a, Ppia, Tbp

Recent Literature

Assessing the survival of exogenous plant microRNA in mice

GaoFeng Liang[1,2], YanLiang Zhu[1], Bo Sun[1], YouHua Shao[1], AiHua Jing[2], JunHua Wang[1] & ZhongDang Xiao[1]

Effective detection and quantification of *dietetically* absorbed plant microRNAs in human plasma

Hongwei Liang[1], Suyang Zhang[1], Zheng Fu[1], Yanbo Wang, Nan Wang, Yanqing Liu, Chihao Zhao, Jinhui Wu, Yiqiao Hu, Junfeng Zhang, Xi Chen*, Ke Zen*, Chen-Yu Zhang*

Honeysuckle-encoded atypical microRNA2911 directly targets influenza A viruses

Zhen Zhou[1,*], Xihan Li[1,*], Jinxiong Liu[2,*], Lei Dong[1,*], Qun Chen[1], Jialing Liu[1], Huihui Kong[2], Qianyi Zhang[2], Xian Qi[3], Dongxia Hou[1], Lin Zhang[1], Guoquan Zhang[2], Yuchen Liu[1], Yujing Zhang[1], Jing Li[1], Jin Wang[1], Xi Chen[1], Hua Wang[3], Junfeng Zhang[1], Hualan Chen[2], Ke Zen[1], Chen-Yu Zhang[1]

Plant microRNAs as novel immunomodulatory agents

Duccio Cavalieri[1,2], Lisa Rizzetto[1], Noemi Tocci[1], Damariz Rivero[3], Elisa Asquini[1], Azeddine Si-Ammour[1], Elena Bonechi[3], Clara Ballerini[3] & Roberto Viola[1]

Detection of dietetically absorbed maize-derived microRNAs in pigs

Yi Luo[1], Pengjun Wang[1], Xun Wang[1], Yuhao Wang[1], Zhiping Mu[1,2], Qingzhi Li[1,3], Yuhua Fu[1,4], Juan Xiao[1], Guojun Li[1], Yao Ma[1], Yiren Gu[5], Long Jin[1], Jideng Ma[1], Qianzi Tang[1], Anan Jiang[1], Xuewei Li[1] & Mingzhou Li[1]

Lack of detectable oral bioavailability of plant microRNAs after feeding in mice

Brent Dickinson, Yuanji Zhang, Jay S Petrick, Gregory Heck, Sergey Ivashuta & William S Marshall

Ineffective delivery of diet-derived microRNAs to recipient animal organisms

Jonathan W. Snow,[1] Andrew E. Hale,[2] Stephanie K. Isaacs,[3] Aaron L. Baggish,[3] and Stephen Y. Chan[2,*]

Real-time quantitative PCR and droplet digital PCR for plant miRNAs in mammalian blood provide little evidence for general uptake of dietary miRNAs

Limited evidence for general uptake of dietary plant xenomiRs

Kenneth W. Witwer,* Melissa A. McAlexander, Suzanne E. Queen, and Robert J. Adams

Unsuccessful Detection of Plant MicroRNAs in Beer, Extra Virgin Olive Oil and Human Plasma After an Acute Ingestion of Extra Virgin Olive Oil

Victor Micó[1] · Roberto Martín[1] · Miguel A. Lasunción[2] · Jose M. Ordovás[1,3] · Lidia Daimiel[1]

Presupposti della Ricerca

- The availability of *Vitis vinifera genome sequence* made possible to characterize grapevine miRNAs and to describe miRNAs abundance in several tissues, including berries

- *Diet-derived miRNAs* could be novel bioactive compounds able to exert their action in mammals. It is conceivable that miRNAs expressed in *grapevine berries* may play a modulatory role in human physiology

Obiettivi della Ricerca

To evaluate whether *miRNAs* in *grapevine berries* could be involved in the *French Paradox* by protecting cardiac cells and improving heart performance

Analyses of murine endothelial cells transcriptome

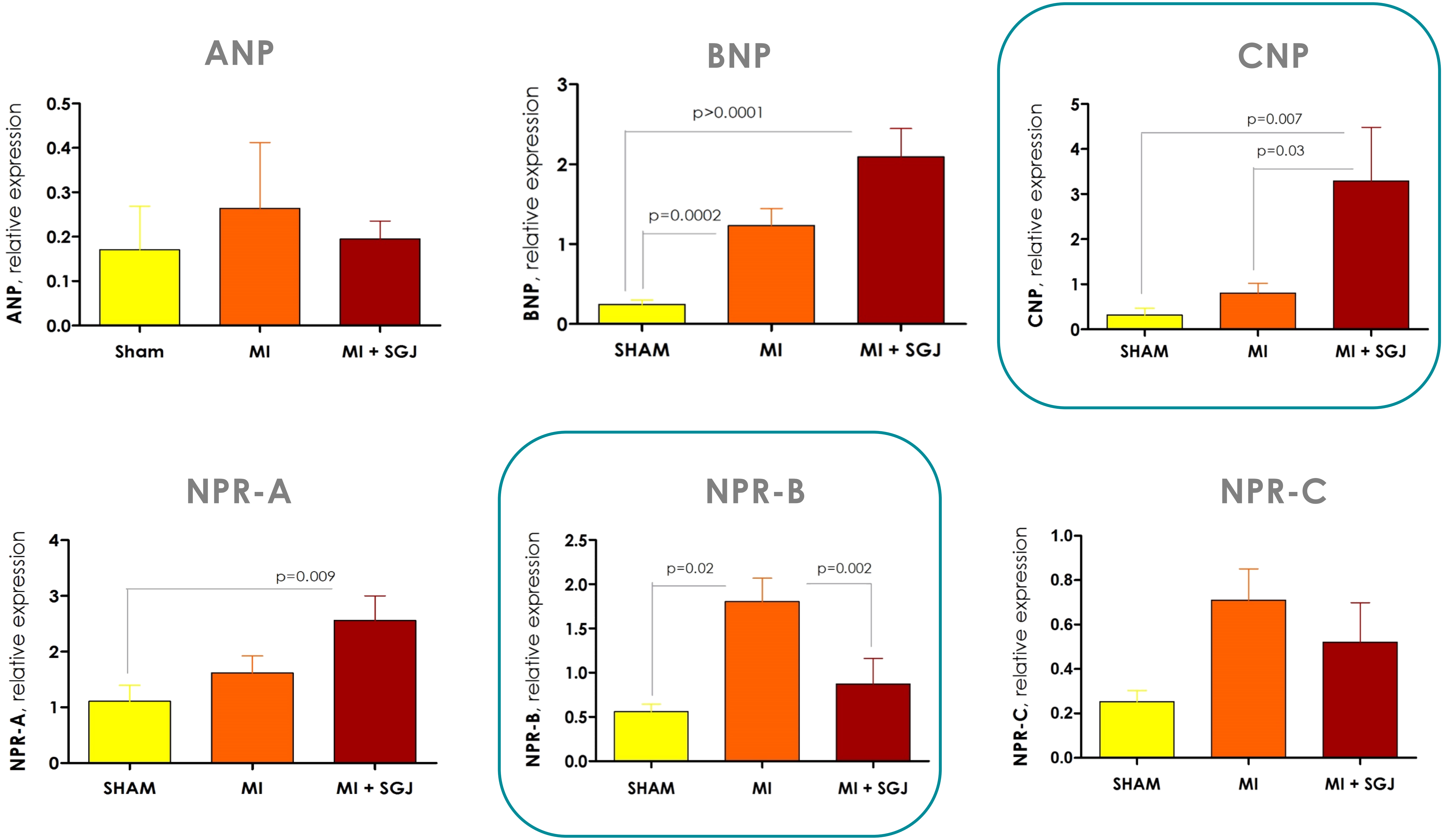
- **RNA-seq data** from 3 libraries *(Kartalei et al., 2015)*
- Reads were mapped to the mouse genome **NCBI build37/mm9**

289 putative targets in murine endothelial cells → **141** expressed genes

Interesting putative vvi-miRNA targets in mouse

vvi-miRNAs	Putative murine target
vvi-miRC482a-5p	Flna (Filamin A)
vvi-miR319e	Jarid 2 (jumonji, AT rich interactive domain 2)
vvi-miR482-5p	Kdm7a (lisyne K-specific demethylase 7A)
vvi-miRC482a-3p	Romo 1 (reactive oxygen species modulator 1)
vvi-miRC482b-3p	Sfrp1 (secreted frizzled related-protein 1)
vvi-miRC477j-3p	Trim28 (tripartite motif-containing protein 28)
vvi-miRC3624a	Vegf-B (vascular endothelial growth factor B)

Real-Time PCR : Natriuretic peptides system gene expression in cardiac tissue



Real-Time PCR : *BNP, NPR-A, NPR-B* mRNAs expression between FEMALE and MALE