

1 **DYSREGULATED PLACENTAL MICRORNAS IN EARLY AND LATE ONSET PREECLAMPSIA**

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23 **CONFLICT OF INTEREST**

24 The authors declare that they have no conflict of interest .

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27 **ABSTRACT**

28 *Introduction:* To determine the miRNA expression profile in placentas complicated by Preeclampsia (PE)  
29 and compare it to uncomplicated pregnancies.

30 *Methods:* Sixteen placentas from women with PE, [11 with early onset PE (EOPE) and 5 with late onset PE  
31 (LOPE)], as well as 8 placentas from uncomplicated pregnancies were analyzed using miRNA microarrays.  
32 For statistical analyses the MATLAB® simulation environment was applied. The over-expression of miR-  
33 518a-5p was verified using Quantitative Real-Time Polymerase Chain Reaction.

34 *Results:* Forty four miRNAs were found dysregulated in PE complicated placentas. Statistical analysis  
35 revealed that miR-431, miR-518a-5p and miR-124\* were over-expressed in EOPE complicated placentas as  
36 compared to controls, whereas miR-544 and miR-3942 were down-regulated in EOPE. When comparing the  
37 miRNA expression profile in cases with PE and PE- growth restricted fetuses (FGR), miR-431 and miR-  
38 518a-5p were found over-expressed in pregnancies complicated by FGR.

39 *Discussion:* Since specific miRNAs can differentiate EOPE and LOPE from uncomplicated placentas, they  
40 may be considered as putative PE-specific biomarkers. MiR-518a-5p emerged as a potential diagnostic  
41 indicator for EOPE cases as well as for PE-FGR complicated placentas, indicating a potential link to the  
42 severity of the disease.

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44 **Keywords:** miRNAs, placenta, microarrays, early onset Preeclampsia, late onset Preeclampsia

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47 **ABBREVIATIONS**

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<b>ABBREVIATION</b>	<b>EXPLANATION</b>
<b>ADAM</b>	A Disintegrin And Metalloproteinase
<b>AUC</b>	Area Under the Curve
<b>CDK6</b>	Cyclin Dependent Kinase 6
<b>cDNA</b>	Complementary Deoxyribonucleic Acid
<b>EOPE</b>	Early Onset PE
<b>FDR</b>	False Discovery Rate
<b>FGR</b>	Fetal Growth Restricted Fetuses
<b>IGF1R</b>	Insulin Like Growth Factor 1 Receptor
<b>IUGR</b>	Intrauterine Growth Restriction
<b>LOPE</b>	Late Onset PE
<b>MAPK</b>	Mitogen-Activated Protein Kinases
<b>miRNAs</b>	MicroRNAs
<b>MMP</b>	Matrix Metalloproteinase
<b>PE</b>	Preeclampsia
<b>PTEN</b>	Phosphatase and Tensin homolog
<b>PU</b>	Proteinuria
<b>qRT-PCR</b>	Quantitative Real-Time Polymerase Chain Reaction
<b>ROC curve</b>	Receiver Operating Characteristic curve
<b>RUNX2</b>	Runt-Related Transcription Factor 2
<b>TGF- <math>\beta</math></b>	Transforming Growth Factor beta
<b>TNF</b>	Tumor Necrosis Factor
<b>TP53</b>	Tumor Protein 53
<b>VCAM1</b>	Vascular Cell Adhesion Molecule 1
<b>VEGFA</b>	Vascular Endothelial Growth Factor A

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51 **INTRODUCTION**

52 Preeclampsia (PE) is an obstetric complication affecting 2-8% of all pregnancies responsible for maternal,  
53 fetal and neonatal morbidity and mortality worldwide [1]. Increasing evidence suggests that both women  
54 who develop PE and their neonates have a higher risk of developing cardiovascular disease and metabolic  
55 syndromes including type II diabetes, later in life. Traditionally, PE is characterized by new onset  
56 hypertension and proteinuria after 20 weeks of gestation. Depending on the gestational age by which the  
57 symptoms develop, PE is classified as early-onset (EOPE-before 34 weeks of gestation) or late-onset (LOPE-  
58 at or after 34 weeks of gestation). Despite extensive efforts, the pathogenesis of the disease is not fully  
59 understood. EOPE shows a greater association with impaired trophoblast invasion, ischemia at the placenta  
60 and fetal growth restriction (FGR) as compared to LOPE, whereas LOPE is not often associated with  
61 placenta hypoperfusion and profound FGR [2].

62 MicroRNAs (miRNAs) belong to a highly conserved class of short non-coding molecules that regulate gene  
63 expression at post-transcriptional level [3]. It is estimated that 30% of human genes are transcriptionally  
64 negatively regulated by miRNAs, highlighting their crucial role in several cellular processes including cell  
65 growth, proliferation, differentiation and apoptosis [4]. Given the central role of the placenta in the  
66 development of PE, recent evidence suggests that miRNAs may be implicated in the pathogenesis of the  
67 disease regulating common pathways such as angiogenesis, hypoxia, ischemia and metabolism [5-14].

68 In the present study, we compared the placenta miRNA expression profiles of PE with those of  
69 uncomplicated pregnancies using a microarray hybridization based technology. The obtained miRNA  
70 patterns were correlated with clinical parameters known to be associated with PE including proteinuria,  
71 gestational age of disease onset and the presence of FGR fetus.

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74 **PATIENTS AND METHODS**

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76 **Patient and tissue samples**

77 Placental samples were collected from 16 women with PE ( $n=11$  with EOPE and  $n=5$  diagnosed with LOPE)  
78 and from 8 with uncomplicated term pregnancies at the time of cesarean section in the 3<sup>rd</sup> Department of  
79 Obstetrics and Gynecology at National and Kapodistrian University of Athens. Among the PE complicated  
80 cases, 8 carried a FGR fetus (PE-FGR): in the EOPE group, 6 out of 11 neonates had FGR confirmed by  
81 birth weight percentile below the 10<sup>th</sup> whereas in the LOPE group 1 neonate was confirmed as FGR by birth  
82 weight below the 5<sup>th</sup> percentile and the other was classified as FGR on the basis of a noted reduction of  
83 growth expressed by a static growth of abdominal velocity and abnormal placental Dopplers. Clinical  
84 characteristics of women participating in the study are summarized in **Table 1**.

85 For each case, two pieces of placental tissue approximately 1×1×1cm in size were randomly obtained from  
86 the central part of the placenta maternal phase, as previously described [9]. Tissues were washed with  
87 isotonic saline solution, to remove excess of blood cells and immediately stored at -80C° until further  
88 processing.

89 PE was defined as hypertension (blood pressure  $\geq 140/90$  mmHg) in two determinations, 4 hours apart  
90 associated with proteinuria ( $> 300$  mg/24 h or  $\geq 1+$ , according to a routine urinalysis) after 20 weeks of  
91 gestation in previously normotensive women [15]. In the absence of proteinuria, PE was characterized based  
92 on the new modified guidelines recommended by the task force on Hypertension in pregnancy [16]. In 2 out  
93 of 16 PE cases included in this study, the diagnosis was established on the presence of new onset  
94 hypertension and impaired liver enzymes concentration.

95 FGR was diagnosed when the estimated fetal weight, calculated using the Hadlock formula (Astraia  
96 Software GmbH), was below the 10<sup>th</sup> percentile for the evaluated gestational age [17].

97 Pregnant women with multiple pregnancies, congenital or chromosomal abnormalities of the fetus, renal  
98 disease, chronic hypertension, cardiovascular disease or other pregnancy complications were excluded from  
99 the study.

100 The study was approved by the hospital's ethics committee according to the Helsinki Declaration on ethical  
101 principles for medical research involving human subjects. A written consent form was obtained from all  
102 participants prior to surgery.

103

104 **MicroRNA extraction and expression profiling**

105 For total RNA extraction, 100mg of placental tissue were mechanically homogenized in TRIzol (Life  
106 Technologies, Foster City, CA), treated with DNase I (Ambion, Austin, USA) and enriched for small RNAs  
107 (siRNAs, miRNAs) using the mirVana microRNA Isolation kit (Ambion, Austin, USA). RNA quantity and  
108 purity were measured using a Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, Houston,  
109 TX, USA).

110 Labeling and hybridization were performed using the LabelIT miRNA labeling kit (Mirus Bio LLC, USA)  
111 according to the manufacturer's instructions. Samples were hybridized to an Applied MicroArrays (miRlink

112 Bioarray 300054-3PK) platform containing 1211 human miRNAs (Applied Biosystems, Foster City, CA).  
113 This platform consists of 8 arrays per slide and on each array miRNAs are represented by multiple replicates.  
114 Images were scanned using an Agilent Microarray Scanner (G2565CA).

115

## 116 **Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)**

117 The differential expression of miR-518a-5p in PE complicated placentas, as compared to the control group,  
118 was verified using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Complementary DNA  
119 (cDNA) synthesis and subsequent RT-PCR were performed, using the TaqMan miRNA Reverse  
120 Transcription kit and TaqMan universal PCR master mix (Applied Biosystems, Inc., USA) respectively.  
121 Specific primers and a TaqMan probe for miR-518a-5p (PN4427975, Applied Biosystems, Inc., USA) were  
122 used according to the manufacturer's instructions. All samples were analyzed in triplicate in an LC480  
123 LightCycler system (Roche GmbH, Switzerland). Positive amplification of each sample was considered  
124 when a signal occurred before 40<sup>th</sup> threshold cycle. The miRNA levels were normalized to RNU44 (Applied  
125 Biosystems, Inc., USA) as an internal control and the relative abundance of each miRNA was calculated  
126 using the  $\Delta\Delta C_t$  equation.

127

## 128 **Data analyses and statistics**

### 129 *Microarray Data Extraction and Analysis*

130 Microarray raw data were extracted, as previously described, using the Imagen 6.0 software (Biodiscovery  
131 Inc., USA) [18]. The MATLAB® simulation environment (The Mathworks, Inc, Natick, MA) was applied  
132 for multi-parameter analyses. Background correction was performed by subtracting the median local  
133 background from the signal intensity. Normalization was performed using the quantile normalization  
134 algorithm. The two-tailed student t-test was used to assess the mean differences between the two groups.  
135 Relative expression was estimated as the  $\log_2$ -transformed ratio of the individual miRNA expression levels  
136 over the mean expression level of all control samples for the respective miRNA under investigation. Final  
137 miRNA values have been calculated as the mean of each specific miRNA replicates on each array.  
138 Continuous variables were expressed as median  $\pm$  standard deviation, unless differently indicated. MiRNAs  
139 were considered significant if they had a  $p$ -value $<0.05$  and False Discovery Rate (FDR) $\leq 0.05$ . Microarray  
140 platform as well as raw data have been uploaded on the Gene Expression Omnibus (GEO) database  
141 (<https://www.ncbi.nlm.nih.gov/geo/>) with reference numbers [GPL23980](#) and [GSE103542](#) respectively.

142

### 143 *Group-wise Comparison of miRNA expression and clinical data*

144 MiRNA expression values were compared with clinical parameters, including PE (EOPE and LOPE), as well  
145 as the presence of proteinuria and FGR with the *Kruskal-Wallis* test. *Bonferroni* correction was used in order  
146 to adjust  $p$ -values for the statistical test performed and reduce the chances of obtaining false-positive results  
147 (type I errors) with the multiple pair-wise tests performed on the present dataset. Differences were  
148 considered significant at  $p<0.02$ .

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151 *ROC Analysis*

152 Receiver Operating Characteristic (ROC) curves were performed with the MATLAB® simulation  
153 environment (The Mathworks, Inc, Natick, MA) to evaluate the diagnostic potential of differentially  
154 expressed miRNAs with 95% Standard Error (SE) and 95% Confidence Intervals (CI). ROC curves were  
155 considered significant when an Area Under the Curve (AUC) value  $> 0.8$  at a  $p < 0.05$  was obtained.

156

157 *MiRNA Enrichment, Gene Ontology and Pathway Analysis*

158 Differentially expressed miRNAs were further enriched and analyzed for known functions and pathway  
159 participation. Functional annotation was performed using Webgestalt web-tool [19]. RNA target prediction  
160 for selected miRNAs was performed with TargetScan v.7.0 [20], DIANA tools [21-23] and the miROB  
161 annotation web tool (<http://mirob.interactome.ru/>). Further on, we have used the GOMiR [24] multi-compare  
162 tool for a more in-depth functional annotation of investigated microRNAs.

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164



165 **RESULTS**

166

167 **MicroRNA expression profiles between PE and the control group**

168 Overall, 44 miRNAs were dysregulated in PE-complicated placentas, as compared to the control cohort,  
169 with a two-fold change and statistical significance ( $p < 0.05$ ) (**Figure 1**). The vast majority was up-regulated  
170 with a total of 27 miRNAs (61.4%) showing over-expression and 17 (38.6%) decreased expression.

171

172 **MicroRNA expression in early-onset and late-onset PE**

173 When comparing miRNA expression in samples obtained from women with EOPE ( $n=11$ ) and LOPE ( $n=5$ )  
174 with controls ( $n=8$ ), 9 miRNAs were found associated with the early or the late type of the disease (**Figure**  
175 **2**). Specifically, miR-431 ( $p=0.003$ ), miR-518a-5p ( $p=0.002$ ) and miR-124\* ( $p=0.001$ ) were significantly  
176 over-expressed in EOPE complicated placentas, as compared to controls, whereas miR-544b ( $p=0.001$ ) was  
177 significantly down-regulated and miR-3942 was marginally down-regulated ( $p=0.02$ ) in EOPE when  
178 compared to controls. Further on, miR-130b ( $p=0.004$ ) and miR-423-3p ( $p=0.001$ ) were significantly up-  
179 regulated in EOPE placentas as compared to controls, while miR-383 was marginally up-regulated in EOPE  
180 ( $p=0.02$ ) and significantly up-regulated ( $p=0.01$ ) in LOPE samples. **MiR-1183 was marginally up-regulated**  
181 **( $p=0.02$ ) in LOPE samples, as compared to controls. In the EOPE placentas, following *Bonferroni***  
182 **correction, the over-expression of miR-1183 was not considered significant ( $p=0.03$ ).**

183

184 **MicroRNA expression in pregnancies complicated by FGR**

185 The miRNA expression profile was also compared in cases of PE and PE-FGR. Two miRNAs were found  
186 overexpressed in PE-FGR complicated placentas, including miR-431 and miR-518a-5p (**Figure 3**).

187

188 **ROC analysis**

189 ROC analysis revealed that 8 miRNAs up-regulated in PE complicated placentas (miR-500a, miR-383, miR-  
190 518a-5p, miR-431, miR-423-3p, miR-124\*, miR-1183 and miR-130b) and 2 down-regulated (miR-3942,  
191 miR-544b) were associated with PE and miRNAs with  $p < 0.05$  and  $AUC > 0.8$  were considered as potential  
192 biomarkers. The ROC curves yielded the following AUCs: miR-500a ( $AUC=0.8$ ), miR-3942 ( $AUC=0.8$ ),  
193 miR-544b ( $AUC=0.84$ ), miR-383 ( $AUC \approx 0.82$ ), miR-518a-5p ( $AUC=0.83$ ), miR-431 ( $AUC=0.82$ ), miR-423-  
194 3p ( $AUC \approx 0.90$ ), miR-124\* ( $AUC=0.88$ ), miR-1183 ( $AUC=0.80$ ) and miR-130b ( $AUC=0.82$ ) (**Figure 4**).

195

196 **qRT PCR verification**

197 The over-expression of miR-518a-5p in PE complicated placentas as compared to controls was verified using  
198 qRT-PCR in all samples. Consistent with microarray results the change of dysregulated miRNA was  
199 approximately two fold above (**Figure 1 suppl.**).

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203 **MiRNA Enrichment, Gene Ontology and Pathway Analysis**

204 Several genes have been reported as predicted or verified targets of the aforementioned miRNAs.  
205 Specifically, miR-126\*, miR-885, miR-130B, miR-1915, miR-1204, miR-3928, miR-518a-5p, miR-631,  
206 miR-383, miR-19a, miR-302c, miR-155, miR-30a, miR-542 and miR-1248 manifested significant  
207 associations with genes including *SMAD1/2/4/5*, *ADAM9*, *VCAM1*, *TGF $\beta$ 2*, *PTEN*, *TP53* and *RUNX2*  
208 **(Figure 2 suppl.)**. Pathway analysis of significantly annotated miRNAs and their respective targets revealed  
209 that they participate in biological pathways associated with PE including MAPK, TNF signaling, T-cell  
210 receptor, B-cell receptor and TGF- $\beta$  pathways **(Figure 5)**. Further on, the GOmiR tool searches in the  
211 MiRanda, TargetScan, PicTar, Sanger, RNAhybrid databases unraveled common gene and functional targets  
212 for all the aforementioned miRNAs. In particular, GOmiR confirmed the results obtained by the other tools,  
213 revealing that miR-126\* and miR-130B target genes participate in TNF-signaling. It also revealed *PPARG*,  
214 another target gene, which participates in obstetric diseases such as Polycystic Ovary Syndrome, Ovarian  
215 Cysts, Ovarian Diseases and Metabolic Syndrome. In addition, GOmiR confirmed that miR-518a-5p  
216 participates in MAPK signaling. Further on, miR-631 was found to target *TBX2*, *HDAC1* and *AXIN1* which  
217 participate in ectodermal placode formation. Target genes of the miR-631 were shown to participate in  
218 metabolic pathways and the insulin signaling pathway, whereas miR-30a target genes are involved in  
219 MAPK, JAK-STAT signaling pathways as well as in metabolic pathways.

220

221

## 222 **DISCUSSION**

223 Several studies have demonstrated dysregulation of placenta miRNAs in PE, implying their involvement in  
224 the pathogenesis of the disease by targeting a variety of genes and regulating crucial biological pathways [5-  
225 7, 10, 11, 14, 25, 26]. Despite extensive efforts, however, conflicting results exist which may be attributed to  
226 the small number of samples included in each study, the increased number of molecules analyzed, the  
227 position of the placenta from which samples are obtained, the gestational age at sampling and the differences  
228 in the platforms used for analysis [7].

229 In the present study, 44 miRNAs were found dysregulated in PE complicated placentas. The results obtained  
230 verified the differential expression of several miRNAs previously reported, including the over-expression of  
231 miR-155 and miR-130b, as well as the down-regulation of miR-126\* [11,25, 27-35].

232 MiR-155 and miR-130b have been implicated in the pathogenesis of PE through their involvement in  
233 pathways regulating trophoblast cells such as TGF- $\beta$  signaling [11, 27-30]. Recently, miR130b was reported  
234 as differentially expressed in relation to pre-pregnancy BMI and adipogenesis, both of which have been  
235 associated with increased risk for the development of pregnancy complications including PE [31-33].

236 Although conflicting results exist regarding the role of mir-126 in the pathogenesis of PE, miR-126 and its  
237 isoform 126\* regulate many target genes mainly related to angiogenesis [25, 34, 35]. Notably, *in vitro*  
238 experiments, using endothelial cells, revealed that miR-126 positively regulates their migration and  
239 proliferation, by enhancing fibroblast growth during arterial development [35]. Lack of miR126 has a  
240 negative angiogenic response, through VEGFA, bFGF and MAP kinase [34, 36]. In the current study, *in*  
241 *silico* analysis also revealed that miR126\* can potentially modulate *ADAM9*, *MMP7*, *MMP13*, *VEGFA* and  
242 *VCAM1*. Proteins encoded by these genes are currently under investigation as potential biomarkers for the  
243 identification of pregnant women destined to develop PE.

244 Over-expression of miR-423-3p, miR-124\*, miR-500a, miR-431 and miR-1305 and down-regulation of  
245 miR-544 and miR-3942 were observed in PE complicated placentas, as compared to controls. These  
246 miRNAs have not been previously associated with the pathogenesis of PE. MiR-423-3p, has been linked to  
247 renal function, acute kidney injury and cell proliferation [37, 38]. MiR-124 has been implicated in  
248 angiogenesis through the renin-angiotensin system and is hence linked with the regulation of blood pressure  
249 [39]. MiR-1305 has been recently implicated in cell cycle regulation and apoptosis in pluripotent stem cells  
250 by targeting *CDK6*, *CYCLIND2* and *RUNX2* [40]. Mir-431, a member of C14MC placenta cluster, directly  
251 regulates the expression of *SMAD4* [41]. Over-expression may negatively regulate *IGF1R* and consequently  
252 inhibit cell proliferation through MAPK pathway, which plays an important role in the development of  
253 vascular lesions that are observed in diabetes and hypertension [42].

254 Results were also analyzed with respect to the gestational age of disease onset. In the literature, a limited  
255 number of studies have focused on comparisons between miRNA expression pattern in EOPE and LOPE. In  
256 the current study, when correlations between EOPE, LOPE and the control cohort were performed, 9  
257 miRNAs were found aberrantly expressed. Specifically, miR-383 and miR-1183 were over-expressed in PE,  
258 higher in LOPE cases as compared to controls, possibly reflecting an association with a milder form of the  
259 disease. Additionally, higher levels of expression for miR-518a-5p, miR-423, miR-124\* and miR-431 were

260 detected in EOPE as compared to controls. It seems likely that all four miRNAs are associated with the  
261 severity of the disease and the severity of the clinical symptoms. On the contrary, miR-544 and miR-3942  
262 were down-regulated in the EOPE cases, indicating a putative PE-specific diagnostic marker. Mir-130b was  
263 found over-expressed in the EOPE cases when compared to the LOPE, signifying its potential use as a  
264 biomarker for severe PE.

265 When examining miRNA expression profiles in PE complicated by FGR, miR-518-5p and miR-431 were up-  
266 regulated in all PE-FGR cases, indicating their potential as unfavorable prognostic markers. It is noticeable  
267 that overexpression of both miRNAs was also observed in previous correlations, reinforcing the prediction  
268 about their possible linkage to poor PE outcomes when up-regulated.

269 Although the properties of miR-518a-5p remain to be elucidated, statistically significant over-expression was  
270 found in all associations performed in the present study, including PE diagnosis, EOPE, LOPE and PE-FGR  
271 following ROC analysis. Vashukova *et al.*, reported up-regulation of miR-518a-5p in superimposed PE on  
272 chronic hypertension [43]. The aforementioned observation, along with the data obtained in the present  
273 study, possibly indicate a common pathway in the pathogenesis of EOPE, superimposed PE and PE-FGR.  
274 Since miR-518a-5p is also expressed in maternal peripheral blood during pregnancy, it represents a potential  
275 noninvasive diagnostic biomarker for all types of PE [10]. These findings however, should be validated in  
276 larger PE cohorts.

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403 FIGURE LEGENDS

404 **Figure 1.** Histogram of the 44 dysregulated miRNAs in PE complicated placentas as compared to controls ( $p < 0.05$  and  $FDR \leq 0.05$ ). Over-expressed miRNAs are  
405 indicated with upward bars and down-regulated with downward bars. Y axis values represent miRNA fold change (ratios presented include Early Onset  
406 Preeclampsia (EOPE) ( $n=11$ ) and Late Onset Preeclampsia (LOPE) ( $n=5$ ).

408 **Figure 2.** Differences in miRNA expression in EOPE, LOPE and control placentas. Significant differential expression was identified for (A) miR-3942 between  
409 EOPE and controls ( $p=0.02$ ), (B) miR-544b between EOPE and controls ( $p=0.001$ ), (C) miR-383 between EOPE and controls as well as LOPE and controls ( $p=0.02$   
410 and  $p=0.01$  respectively), (D) miR-518a-5p between EOPE and controls ( $p=0.002$ ), (E) miR-431 between EOPE and controls ( $p=0.003$ ), (F) miR-423-3p between  
411 EOPE and controls as well as LOPE and controls ( $p=0.001$  and  $p=0.007$  respectively), (G) miR-124\* between EOPE and controls ( $p=0.001$ ), (H) miR-1183 between  
412 EOPE and controls as well as LOPE and controls ( $p=0.03$  and  $p=0.02$  respectively) and (I) miR-130b between EOPE and controls as well as LOPE and controls  
413 ( $p=0.004$  and  $0.04$  respectively) (EOPE: Early Onset Preeclampsia, LOPE: Late Onset Preeclampsia) (Expression levels concern the natural,  $\log_2$  untransformed  
414 miRNA expression values. \* depicts a significance at the  $p < 0.05$  level and \*\* depicts a significance at the  $p < 0.01$  level).

416 **Figure 3.** Over-expression of (A) miR-518a-5p and (B) miR-431 in PE complicated placentas with FGR fetuses as compared to those with unaffected fetal growth  
417 (PE: Preeclampsia, FGR: Fetal Growth Restriction) (Expression levels correspond to the natural  $\log_2$  untransformed expression values. Differences were significant  
418 at  $p < 0.05$ ).

420 **Figure 4.** Receiver Operating Characteristic (ROC) curves of miRNAs differentiating controls from Preeclampsia complicated placentas. Ten miRNAs can  
421 differentiate controls from Preeclampsia complicated placentas: (A) miR-500a, (B) miR-03942, (C) miR-544b, (D) miR-383, (E) miR-518a-5p, (F) miR-431, (G)  
422 miR-423-3p, (H) miR-124\*, (I) miR-1183 and (J) miR-130b manifested an Area Under the Curve (AUC) value greater than 0.8 ( $AUC > 0.8$ ) and  $p < 0.05$ , suggesting  
423 that they are possible candidates for Preeclampsia.

424 **Figure 5.** Selected significant signaling pathways associated with annotated differentially expressed miRNAs (miR-125b, miR-155-5p, miR-21-5p and miR-145).  
425 MiRNA annotation was performed with the DIANA miRNA web annotation tool <http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath/index>).



**Table 1.** Maternal and neonatal characteristics of Preeclampsia cases and controls included in the study.

	<b>Controls (n=8)</b>	<b>EOPE* (n=11)</b>	<b>LOPE** (n=5)</b>	<b>p-value<sup>1</sup></b>	<b>p-value<sup>2</sup></b>	<b>p-value<sup>3</sup></b>	<b>p-value<sup>4</sup></b>
<b>Maternal Age (y)</b>	35.7 [35-39]	35.1 [28-45]	28.4 [20-35]	0.051	0.820	0.036	0.055
<b>Pre-pregnancy BMI (kg/m<sup>2</sup>)</b>	29.2 [22.96-31.99]	29.9 [19.83-41.77]	28.6[24.02 –36.57]	0.942	0.835	0.867	0.778
<b>Blood Pressure (mmHg)</b>							
<b>Systolic</b>	121 [122-118]	197.75 [160-250]	172.25 [152-197]	0.702	0.889	0.612	0.412
<b>Diastolic</b>	75 [72-85]	113.5 [90-150]	107.75 [94-135]	0.966	0.962	0.923	0.798
<b>Proteinuria (n)</b>							
<b>Yes</b>	0	10 (91%)	4 (80%)	0.0002 <sup>‡</sup>			
<b>No</b>	8 (100%)	1 (9%)	1 (20%)				
<b>Fetal Growth Restriction (FGR)</b>	None	6 (54.5%)	2 (40%)				
<b>Gestational Age at delivery (wk)</b>	36.3 [34.2 – 38.5]	31.2 [28 – 33.5]	35.6 [34.4 – 37.3]	0.051	0.0003	0.29	0.78
<b>Fetal Birth Weight (grams)</b>	2792.6 [2215 - 3560]	1335 [1025 - 1540]	2454 [1900 - 3020]	0.0003	0.0002	0.336	0.002
<b>&gt;10th percentile (n)</b>	8 (100%)	5 (45.4%)	3 (60%)				
<b>&lt;10th percentile (n)</b>	0	4 (36.4%)	1 (20%)				
<b>&lt;5th percentile (n)</b>	0	2 (18.2%)	1 (20%)				

\*EOPE: Early onset Preeclampsia \*\* LOPE: Late onset Preeclampsia

<sup>1</sup>Significance level between all groups<sup>2</sup>Significance level between Control and EOPE placentas<sup>3</sup>Significance level between Control and LOPE placentas<sup>4</sup>Significance level between EOPE and LOPE placentas<sup>‡</sup>p-value estimated with *chi-square* test for independence between proteinuria and early and late onset Preeclampsia.

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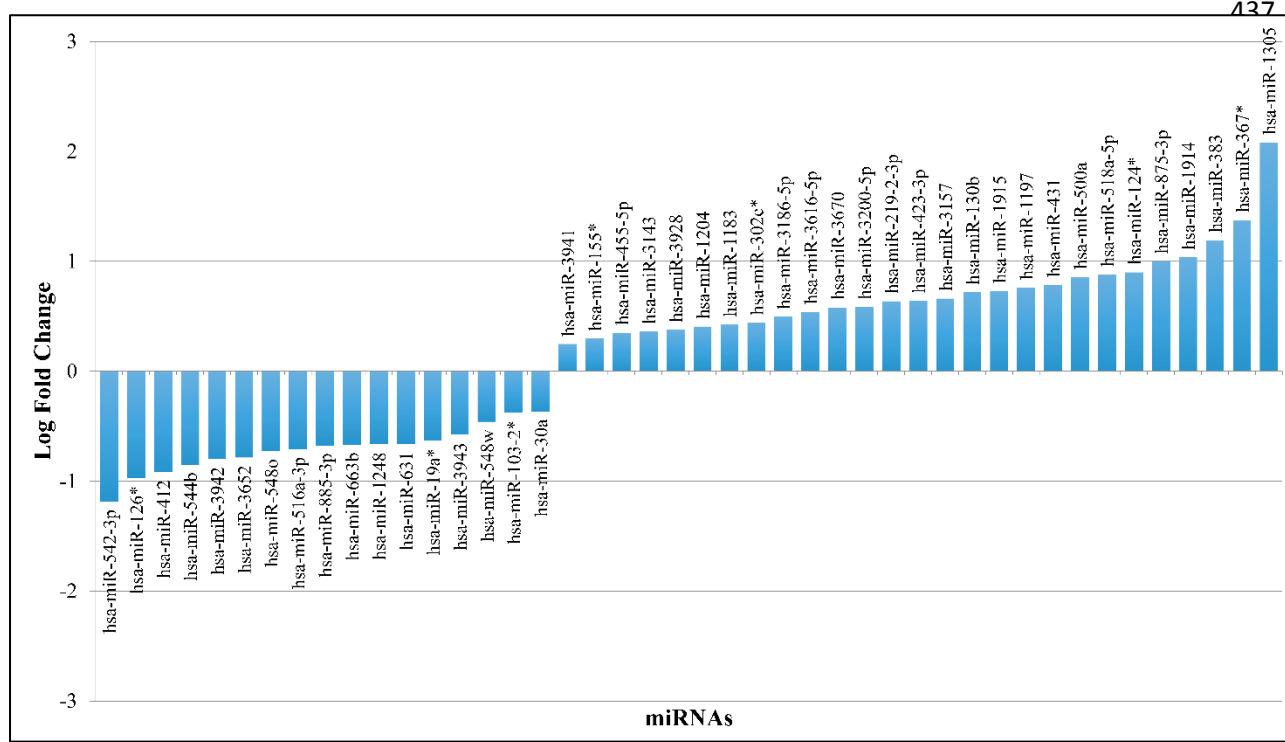
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435 **Figure 1.**  
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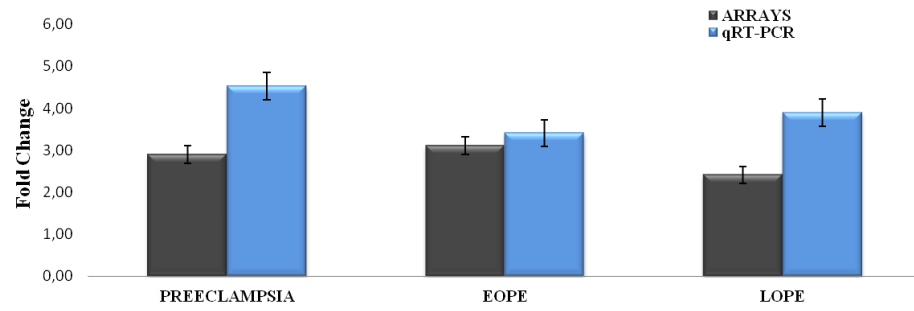
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461 SUPPLEMENTARY FIGURES

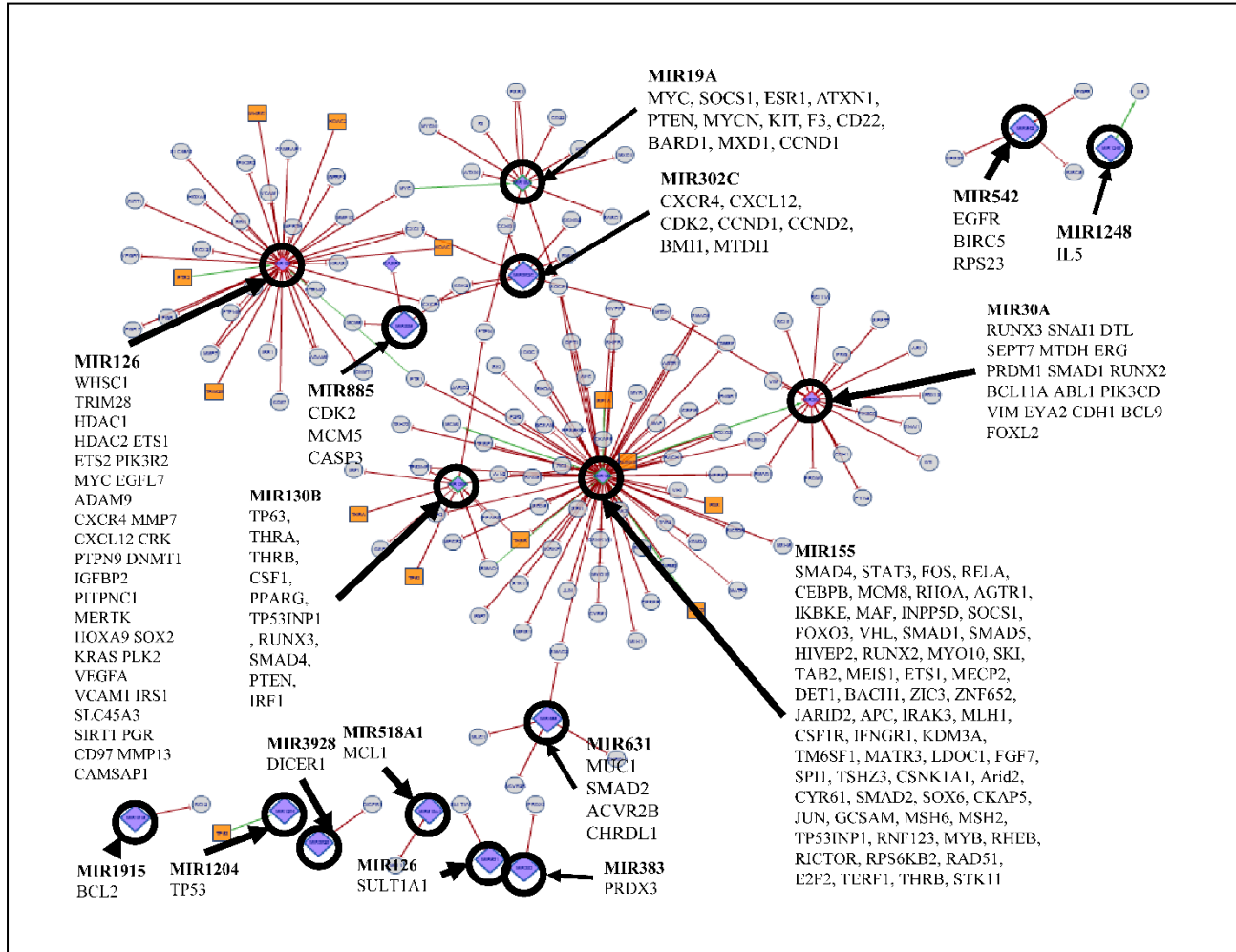
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463 **Supplementary Figure 1.**

Comparative diagram of miRNA expression between qRT-PCR and microarrays



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**Supplementary Figure 2.** Networks of miRNAs and their association with target genes. In particular, we have found that miR-126, miR-885, miR-130b, miR-1915, miR-1204, miR-3928, miR-518a1, miR-126, miR-631, miR-383, miR-19a, miR-302c, miR-155, miR-30a, miR-542, miR-1248, manifested significant associations with gene groups. miRNA annotation was performed with the miROB web tool (<http://mirob.interactome.ru/>).

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