Redox Biology 58 (2022) 102531

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Association between lipid peroxidation biomarkers and microRNA expression profiles

Yingya Zhao [a,1], Marina S. Nogueira [a,1], Ginger L. Milne [b], Xingyi Guo [a], Hui Cai [a], Qing Lan [c], Nathaniel Rothman [c], Qiuyin Cai [a], Yu-Tang Gao [d], Qingxia Chen [e], Xiao-Ou Shu [a], Gong Yang [a,*]

[a] Division of Epidemiology, Department of Medicine, Vanderbilt-Ingram Cancer Center, Vanderbilt Epidemiology Center, Vanderbilt University Medical Center, Nashville, TN, USA
[b] Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA
[c] Division of Cancer Epidemiology and Genetics, Occupational and Environmental Epidemiology Branch, National Cancer Institute, Bethesda, MD, USA
[d] Shanghai Cancer Institute, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China
[e] Department of Biostatistics, Vanderbilt University Medical Center, Nashville, TN, USA

ARTICLE INFO

Keywords:
Biomarker
Oxidative stress
Lipid peroxidation
Isoprostanes
microRNA
β-oxidation

ABSTRACT

Background: In-vitro and animal studies demonstrate that epigenetic regulation may play an important role in lipid peroxidation. No human study to date has directly evaluated microRNAs (miRNAs), as epigenetic modulators, in relation to systemic levels of lipid peroxidation.
Objectives: To evaluate associations between systemic levels of lipid peroxidation and miRNA expression profiles in women.
Methods: Included in the analysis were 92 women aged 40–70 years, a subset of the Shanghai Women's Health Study (SWHS). Lipid peroxidation was assessed by urinary markers F_2-isoprostanes (F_2-IsoPs), the products of free radical-catalyzed peroxidation of arachidonic acid, and its major metabolite after β-oxidation, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (F_2-IsoP-M), with GC/NICI-MS assays. Expression levels of 798 miRNAs were quantified in peripheral plasma with NanoString nCounter assays. A multivariable linear regression model was used to examine the association between lipid peroxidation and miRNA expression.
Results: After adjusting for potential confounders, 29 miRNAs and 213 miRNAs were associated with F_2-IsoPs and F_2-IsoP-M, respectively. When further controlling for multiple comparisons, none of these nominally significant associations with F_2-IsoPs was retained, whereas 71 of 213 miRNAs associated with F_2-IsoP-M remained. The predicted targets of the F_2-IsoP-M associated miRNAs were enriched for several lipid peroxidation-related processes such as PI3K/AKT, MAPK, FOXO and HIF-1 signaling pathways. Moreover, 10 miRNAs (miR-93-5p, miR-761, miR-301b-3p, miR-497-5p, miR-141-3p, miR-186-5p, miR-126-3p, miR-200b-3p, miR-520d-3p, and miR-363-3p) exhibited functional interactions with 50 unique mRNAs targets involved in the regulation of β-oxidation.
Conclusions: To our knowledge, this study, for the first time, provides human data suggesting that miRNA expression may be linked to lipid peroxidation products and their metabolism.

1. Introduction

Oxidative stress reflects an imbalance between systemic manifestation of oxidants and antioxidants in favor of the former, leading to a disturbance of redox homeostasis and molecular damage [1]. One of the major targets of oxidant injury is lipids, which undergo peroxidation. Of concern, over the past few decades, numerous evidence has suggested that lipid peroxidation may play critical roles in several pathologies such as cancer, neurodegenerative diseases, cardiometabolic diseases, and muscular disorders [2,3], while the biological mechanisms that underlie lipid peroxidation formation and regulation remain to be fully

* Corresponding author. 2525 West End Avenue, Suite 600, Nashville, TN, 37203, USA.
E-mail addresses: yingya.zhao@vumc.org (Y. Zhao), marina.s.nogueira@vumc.org (M.S. Nogueira), ginger.milne@vumc.org (G.L. Milne), xingyi.guo@vumc.org (X. Guo), hui.cai@vumc.org (H. Cai), qingl@mail.nih.gov (Q. Lan), nat.rothman@nih.gov (N. Rothman), qiuyin.cai@vumc.org (Q. Cai), ytgao@vip.sina.com (Y.-T. Gao), cindy.chen@vumc.org (Q. Chen), xiao-ou.shu@vumc.org (X.-O. Shu), gong.yang@vumc.org (G. Yang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.redox.2022.102531
Received 7 September 2022; Received in revised form 16 October 2022; Accepted 1 November 2022
Available online 2 November 2022

Abbreviation list:

3'UTR	three prime untranslated region
AMPK	AMP-activated protein kinase
BMI	body mass index
CI	confidence interval
F_2-IsoPs	F_2-isoprostanes
F_2-IsoP-M	2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP
FOXO1	forkhead box protein O1
GC/NICI MS	gas chromatography/negative ion chemical ionization mass spectrometry
IPA	Ingenuity Pathway Analysis
IRS1	insulin receptor substrate 1
ICC	intraclass correlation coefficient
miRNA	microRNA
MDA	malondialdehyde
MAP2K	mitogen-activated protein kinase kinase
MS	mass spectrometry
NSAID	non-steroidal anti-inflammatory drug
PIK3R2	phosphoinositide-3-kinase regulatory subunit 2
PPARγ	peroxisome proliferator-activated receptor gamma
PGC1α	peroxisome proliferator-activated receptor γ coactivator 1α
PTEN	phosphatase and tensin homolog
ROS	reactive oxygen species
SWHS	Shanghai Women's Health Study
SOD	superoxide dismutase
SD	standard deviation

understood.

microRNAs (miRNAs) are a large class of small, non-coding RNAs that post-transcriptionally regulate gene expression by degrading and/or suppressing multiple mRNA molecules [4,5]. As a main epigenetic regulatory mechanism, miRNAs are involved in several key biological processes related to cell cycle and cell survival [6]. The imbalance of miRNAs has also been shown to be associated with many pathological processes, including oxidative stress [7]. *In-vitro* and animal data have linked oxidative stress, induced by reactive oxygen species (ROS), to certain miRNA profiles implicated in redox signaling and modulation, such as the cellular antioxidant machinery [8,9]. Several miRNAs have been shown to target mediators of lipid metabolism, and their dysregulation results in lipid metabolism disequilibrium and lipid peroxidation [10,11]. Despite the compelling evidence from experimental studies, no human study to date has directly investigated the association between circulating miRNAs and systemic lipid peroxidation.

F_2-isoprostanes (F_2-IsoPs) are non-enzymatic oxidation products of arachidonic acid that are formed via free radical-catalyzed peroxidation. Urinary F_2-IsoPs have been identified as an informative and reliable measure of *in vivo* lipid peroxidation [12,13]. 15-F_{2t}-isoprostane (15-F_{2t}-IsoP), one of the most well-studied F_2-IsoPs, is metabolized via enzymatic β-oxidation and reduction to 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (F_2-IsoP-M) [14]. In certain biological settings, urinary F_2-IsoP-M is a more sensitive biomarker of systemic lipid peroxidation than unmetabolized F_2-IsoPs. Given roles of miRNAs as epigenetic modulators in redox homeostasis, we carefully evaluated the relationship of miRNA expression profiles with urinary concentrations of F_2-IsoPs and F_2-IsoP-M in middle-aged and elderly women, to identify potential biological pathways involved in the regulation of lipid peroxidation and metabolism.

2. Materials and methods

2.1. Study participants

Study participants were drawn from the Shanghai Women's Health Study (SWHS), an ongoing population-based prospective cohort study launched from 1997 to 2000 in Shanghai, China. Details of the design and methods of SWHS have been published elsewhere [15]. Structured questionnaires through in-person interviews were used to collect detailed information on demographic characteristics, lifestyle, and other exposures. Anthropometric measurements were also taken at baseline by trained interviewers. The study protocol was approved by the Institutional Review Boards of Vanderbilt University Medical Center and the Shanghai Cancer Institute. All participants provided written informed consent.

A total of 94 women were selected from a case-control study of colorectal cancer nested within the SWHS [16], in whom urinary lipid peroxidation biomarkers and plasma miRNA expression profiles had been measured. Due to the nature of the prospective study design of the nested case-control study, all participants were apparently healthy and free of cancer at baseline when biological samples were collected. In addition, because urinary biomarkers were examined in this study, none of the participants selected for this study had a history of renal failure, acute or chronic kidney disease.

2.2. Biospecimen collection

For each participant who provided consent at baseline, a 10-ml peripheral blood sample was collected into an EDTA vacuum tube, and a spot urine sample was collected into a sterilized 100-ml container containing 125 mg of ascorbic acid. Blood samples were drawn by experienced medical personnel. Both urine and blood samples were kept at ~4 °C after collection and during transportation and processed within 6 h in a core laboratory. Immediately after processing, all urine and blood aliquots were stored at −80 °C until analyses. More importantly, plasma samples that were visually identified as a pink to red color would not be selected for miRNA profiling.

2.3. Lipid peroxidation biomarker measurement

Urinary F_2-IsoPs and F_2-IsoP-M were measured at the Vanderbilt Eicosanoid Core Laboratory using gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI MS), as previously described [14,17,18]. The lower limit of sensitivity was around 5 pg. The precision of the assays was ±6%, and the accuracy was 96%. After standardization by urinary creatinine concentrations, results were expressed as nanograms per milligram of creatinine (ng/mg Cr).

2.4. miRNA extraction and profiling

Total RNA, including miRNA, was extracted and purified from plasma samples using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Three synthetic spike-in RNA oligo mixture (osa-miR414, cel-miR248, and at-miR159a) was used, to control for variances in the starting material and the efficiency of the downstream total RNA extraction. Three μL of total RNA was used for miRNA expression analysis, representing the RNA from 30 μL of plasma.

Cell-free circulating miRNA levels were profiled using the Human v2 miRNA Expression Assay (NanoString Technologies), which includes probes specific for 798 common human miRNAs. The assays were conducted in Nano String's in-house service laboratory (Seattle, WA) following the standard NanoString protocol [19]. Raw count data were processed for quality assurance and normalization using the R package NanoStringNorm (version 1.1.16) [20]. The background count level,

Table 1
Characteristics of study participants in relation to lipid peroxidation biomarkers.

Characteristics	Mean ± SD, or n (%) [a]	F_2-IsoPs		F_2-IsoP-M	
		β (SE) [b] × 10	*P* value	β (SE) [b] × 10	*P* value
Age (years)	60.1 ± 9.0	0.19 (0.11)	0.10	0.09 (0.10)	0.36
Body mass index (kg/m^2)	24.3 ± 2.8	0.07 (0.20)	0.72	0.39 (0.18)	0.04
Charlson comorbidity score	0.5 ± 0.9	0.01 (0.61)	0.98	−0.41 (0.56)	0.46
Cigarette smoking	4 (4.3)	−2.69 (2.57)	0.30	2.02 (2.36)	0.40
Postmenopausal status	71 (77.2)	−3.56 (2.47)	0.15	−2.15 (2.26)	0.34
Use of aspirin and other NSAIDs	6 (6.5)	−3.47 (2.16)	0.11	−0.77 (1.98)	0.70
Use of vitamin supplements	14 (15.2)	−0.56 (1.50)	0.71	0.92 (1.34)	0.49

Note: Total number of participants was 92. Geometric mean (95% CI) for F_2-IsoPs (ng/mg Cr) was 1.68 (1.52, 1.85); for F_2-IsoP-M (ng/mg Cr), 0.55 (0.50, 0.60).

[a] Mean ± SD was presented for continuous variables; and n (%) was presented for categorical variables.

[b] Log-transformed concentrations of biomarkers were treated as dependent variables in the linear regression model, and covariates listed in the table were mutually adjusted for.

estimated as mean value plus 2 standard deviations of the 6 negative controls included in the assay, was subtracted from each sample to correct the level of non-specific binding. To control for variances in the starting material and the miRNA purification efficiency, miRNA counts were normalized using mean counts of the three spike-in synthetic control miRNAs. Further, the resulting counts of each miRNA sample were normalized using the geometric mean of the top 50 miRNAs counts [21], excluding hsa-miR-320e, hsa-miR-16-5p and hsa-miR-451a because these miRNAs may be related to red blood cell contamination or hemolysis [22]. Finally, we performed the principal components analysis (PCA) in the selected sample of 94 participants and dropped 2 samples considered obvious outliers (Supplementary Fig. 1). The exclusion resulted in significantly decreased variances in levels of those erythrocyte-specific miRNAs. After the exclusion, a total of 92 participants were included in this study. Only miRNAs that were expressed in at least 50% of the study participants were retained for downstream analyses.

2.5. Statistical analysis

Descriptive statistics were performed for general characteristics of the study participants. Mean ± SD was used for continuous variables, and n (%) was used for categorical variables. The participant's characteristics in relation to lipid peroxidation biomarkers were evaluated using a linear regression model.

Multivariable linear regression models were applied to examine associations of miRNA expression with lipid peroxidation biomarkers. Both biomarker concentrations and miRNA expression levels were log-transformed to improve normality in the residuals. To avoid zero values in log transformation, one was added to all counts of miRNAs [23]. Covariates that were adjusted for in multivariable models included age, body mass index (BMI), cigarette smoking, and menopausal status. Because various underlying health conditions may result in alterations in both lipid peroxidation and miRNA expression, we further adjusted for the Charlson comorbidity score, constructed based on 19 medical comorbid conditions, such as diabetes, pulmonary and cardiovascular diseases among others [24,25]. We also adjusted for use of vitamin supplements, aspirin, and other non-steroidal anti-inflammatory drugs (NSAIDs) within one week before sample collection.

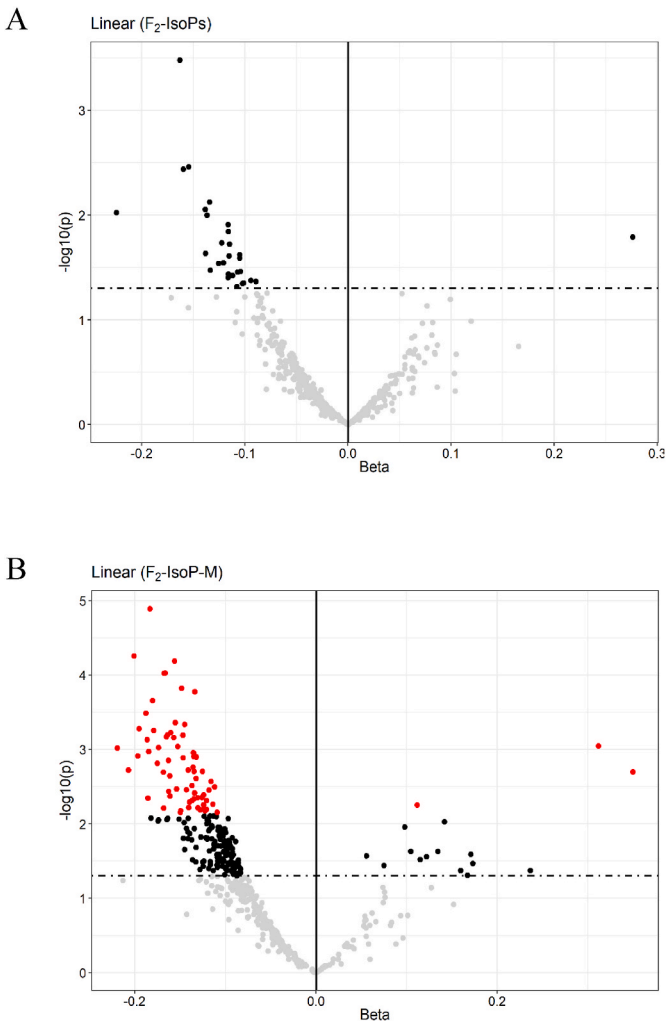

Fig. 1. Volcano plots capturing significant miRNAs identified by our models for lipid peroxidation biomarkers F_2-IsoPs (A) and F_2-IsoP-M (B). The horizontal dotted line represents the threshold with a *P* value of 0.05. Both black and red dots represent miRNAs with $P < 0.05$, while red dots further denote significant miRNAs with FDR-adjusted $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

All analyses were conducted with R software (version 4.0.4). False discovery rate (FDR) with Benjamini and Hochberg correction was performed on the resulting two-sided *P*-values to control for multiple comparisons [26]. We controlled the FDR at 0.05.

2.6. Bioinformatics analysis

The Ingenuity Pathway Analysis (IPA) tool (Ingenuity Systems, Redwood City, CA, USA) was used to identify target genes potentially regulated by each miRNA and explore downstream functional enrichments. The putative miRNA-mRNA relationship was constructed using the IPA microRNA Target Filter, based on a knowledgebase of predicted and experimentally validated connections. Only the experimentally validated miRNA-mRNA connections were stringently selected and further used for the enrichment analysis of biological relevance and molecular networks.

3. Results

The mean (SD) age was 60.1 (9.0) years in the study participants. Geometric means (95% CI) of F_2-IsoPs and F_2-IsoP-M were 1.68 (1.52,

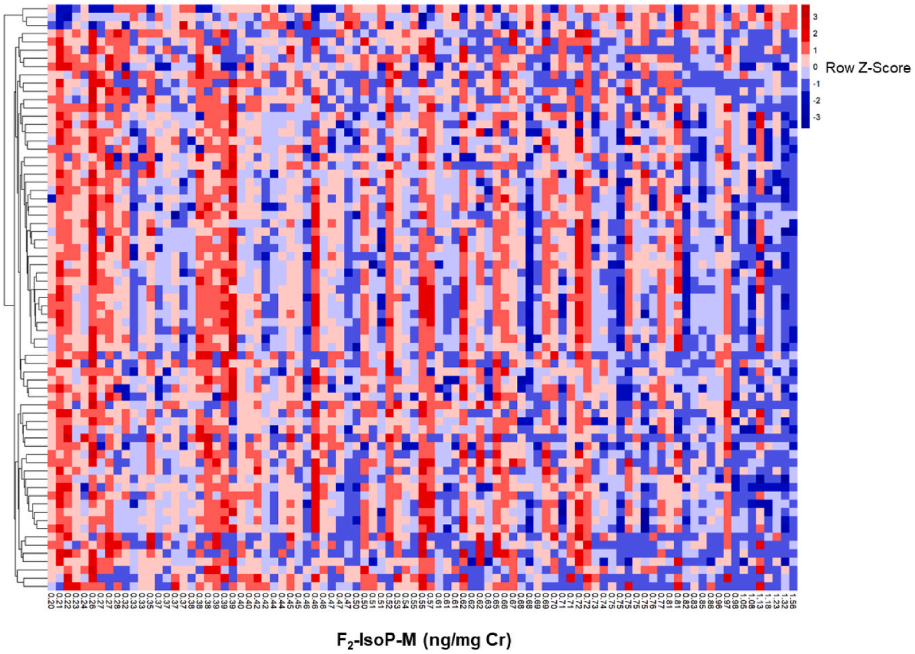

Fig. 2. Heat map representing the expression levels of the 71 miRNAs significantly associated with F_2-IsoP-M concentrations. Row z-scores were calculated and color-coded to display miRNA expression, where red indicates higher expression and blue indicates lower expression. Subjects (n = 92) were ordered from the lowest to highest F_2-IsoP-M concentration (0.20–1.56 ng/mg Cr) on the x-axis, and miRNAs were clustered on the y-axis using a correlation-based distance method. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

1.85) and 0.55 (0.50, 0.60) ng/mg Cr, respectively. Study participants' characteristics in relation to lipid peroxidation biomarkers are presented in Table 1. Women with higher BMI were likely to have a higher concentration of F_2-IsoP-M. In contrast, we did not observe significant associations of F_2-IsoPs with any selected demographic and lifestyle characteristics.

In the miRNA screening panel, 798 miRNAs were detected in at least one sample. In this study, we selected miRNAs detected in at least 50% of samples, resulting in an inclusion of 484 miRNAs. After multivariable adjustment for potential confounding factors, 29 miRNAs (28 negative and 1 positive, $P < 0.05$) were associated with F_2-IsoPs, and 213 miRNAs (210 negative and 3 positive, $P < 0.05$) were associated with F_2-IsoP-M. However, when further controlling for multiple comparisons using FDR correction, none of these nominally significant associations with F_2-IsoPs was retained, whereas the associations with F_2-IsoP-M remained for 71 miRNAs (68 negative and 3 positive, FDR-adjusted $P < 0.05$) (Fig. 1). A heat map of the expression levels of the 71 F_2-IsoP-M-associated miRNAs is shown in Fig. 2. Three miRNAs with a positive association were clustered at the top (blue to red rows with increasing F_2-IsoP-M concentration). Each ln-unit increase in miRNA expression was associated with an increase in F_2-IsoP-M concentrations by 12% for miR-148a-3p, 37% for miR-126-3p, and 42% for miR-93-5p (Table 2). The remaining 68 miRNAs with a negative association were clustered at the bottom (red to blue rows with increasing F_2-IsoP-M concentration, Fig. 2). Among these 68 miRNAs, each ln-unit increase in miRNA expression was associated with a decrease in F_2-IsoP-M concentrations ranging from 10% for miR-3613-5p to 20% for miR-301b-3p.

A total of 428 experimentally validated mRNA targets were identified for the 71 distinctive miRNAs using the IPA tool (Fig. 3). The functional enrichment analysis of these target genes suggested that several important metabolic and signaling pathways were likely to be involved in modulation of systemic lipid peroxidation, including PI3K/AKT, MAPK, FOXO, and HIF-1 signaling pathways among others, with the PI3K/AKT signaling pathway being the most implicated.

The miRNAs and mRNA targets were further mapped to molecular networks to depict connectivity of various genes in some important pathways. We found interactions between 10 miRNAs (miR-93-5p, miR-761, miR-301b-3p, miR-497-5p, miR-141-3p, miR-186-5p, miR-126-3p, miR-200b-3p, miR-520d-3p, and miR-363-3p) and 50 unique, experimentally validated mRNA targets (Fig. 4). These interactions were enriched in β-oxidation related pathways such as AMPK signaling, PPAR signaling, and mitochondrial dysfunction.

4. Discussion

In this study of middle-aged and elderly women, we found that expression profiles of 71 miRNAs were associated with urinary levels of F_2-IsoP-M. The predicted targets of these miRNAs were enriched for molecules implicated in several lipid peroxidation-related processes, with the PI3K/AKT signaling pathway being most significant. Further pathway network analyses identified that 10 miRNAs were specifically involved in the regulation of β-oxidation.

To the best of our knowledge, this was the first study to investigate the association of lipid peroxidation biomarkers isoprostanes with miRNA expression in humans. In this study, we found that 29 miRNAs (28 negative and 1 positive) were associated with F_2-IsoPs, although none of them survived correction for multiple testing. However, we found that miRNA profiles were associated with urinary levels of F_2-IsoP-M, an F_2-IsoP metabolite formed from a single step of β-oxidation and reduction of the Δ^5 double bond. The differences noted herein between F_2-IsoPs and F_2-IsoP-M appear biologically plausible, i.e., miRNAs, acting as epigenetic modulators, are likely involved in regulation of enzymatic products such as F_2-IsoP-M, but less likely to be involved in those non-enzymatic products such as F_2-IsoPs. In addition, an increasing body of evidence has demonstrated that F_2-IsoP-M may be a more sensitive biomarker of endogenous lipid peroxidation than unmetabolized F_2-IsoPs [27]. F_2-IsoP-M is unaffected by local renal or bladder production, thus this biomarker may better represent systemic lipid peroxidation [14]. To this end, we have previously demonstrated that F_2-IsoP-M, but not F_2-IsoPs, is positively associated with aging and obesity, conditions well-known to be associated with oxidative stress [28,29]. Further, some environmental exposures to phthalates and herbicides were found to be only positively associated with F_2-IsoP-M but not the unmetabolized F_2-IsoPs [30,31].

Some miRNAs identified in our study have been already implicated in the pathophysiologic mechanisms of lipid peroxidation in previous *in vitro* and/or animal studies. For instance, Wu et al. demonstrated in endothelial progenitor cells that treatment with hydrogen peroxide resulted in reduction of miR-126 expression in a dose-dependent manner, while miR-126 overexpression decreased levels of

Table 2
miRNAs significantly associated with urinary concentrations of F_2-IsoP-M (FDR-adjusted $P < 0.05$).

microRNA name	Effect estimate [a]		FDR-adjusted *P* value [c]
	Fold change (95% CI) [b]	*P* value	
miR-450b-5p	0.83 (0.77, 0.90)	1.28E-05	0.006
miR-10a-5p	0.85 (0.78, 0.92)	9.48E-05	0.009
miR-1268a	0.85 (0.78, 0.92)	9.38E-05	0.009
miR-302e	0.86 (0.80, 0.92)	6.45E-05	0.009
miR-548 m	0.82 (0.75, 0.90)	5.53E-05	0.009
miR-1183	0.87 (0.82, 0.93)	1.67E-04	0.01
miR-200b-3p	0.86 (0.80, 0.93)	1.51E-04	0.01
miR-1972	0.83 (0.76, 0.91)	2.20E-04	0.01
miR-4443	0.83 (0.75, 0.91)	3.25E-04	0.02
miR-1973	0.85 (0.78, 0.93)	6.40E-04	0.02
miR-3136-5p	0.86 (0.80, 0.94)	6.41E-04	0.02
miR-495-3p	0.82 (0.74, 0.91)	5.23E-04	0.02
miR-511-5p	0.86 (0.79, 0.93)	4.36E-04	0.02
miR-521	0.85 (0.78, 0.93)	6.93E-04	0.02
miR-548al	0.85 (0.78, 0.93)	5.91E-04	0.02
miR-551a	0.85 (0.77, 0.93)	6.77E-04	0.02
miR-571	0.86 (0.80, 0.94)	4.63E-04	0.02
miR-6721-5p	0.84 (0.76, 0.92)	5.55E-04	0.02
miR-597-5p	0.83 (0.75, 0.92)	7.36E-04	0.02
miR-126-3p	1.37 (1.14, 1.63)	8.95E-04	0.02
miR-301b-3p	0.80 (0.71, 0.91)	9.57E-04	0.02
miR-363-3p	0.84 (0.76, 0.93)	9.45E-04	0.02
miR-572	0.86 (0.79, 0.94)	9.13E-04	0.02
miR-1269a	0.87 (0.81, 0.95)	1.13E-03	0.02
miR-3202	0.86 (0.79, 0.94)	1.29E-03	0.02
miR-384	0.88 (0.81, 0.95)	1.26E-03	0.02
miR-598-3p	0.83 (0.75, 0.92)	1.07E-03	0.02
miR-650	0.87 (0.81, 0.94)	1.10E-03	0.02
miR-665	0.87 (0.81, 0.95)	1.24E-03	0.02
miR-922	0.82 (0.73, 0.92)	1.22E-03	0.02
miR-548ah-5p	0.85 (0.77, 0.94)	1.40E-03	0.02
miR-548v	0.84 (0.76, 0.93)	1.54E-03	0.02
miR-1289	0.88 (0.82, 0.95)	1.98E-03	0.03
miR-186-5p	0.81 (0.72, 0.92)	1.90E-03	0.03
miR-493-3p	0.87 (0.80, 0.95)	1.72E-03	0.03
miR-5196-5p	0.87 (0.81, 0.95)	1.96E-03	0.03
miR-522-3p	0.85 (0.76, 0.94)	2.02E-03	0.03
miR-873-3p	0.87 (0.80, 0.95)	1.90E-03	0.03
miR-93-5p	1.42 (1.14, 1.76)	2.01E-03	0.03
miR-497-5p	0.85 (0.77, 0.94)	2.26E-03	0.03
miR-606	0.88 (0.81, 0.95)	2.46E-03	0.03
miR-1271-3p	0.89 (0.83, 0.96)	2.69E-03	0.03
miR-3928-3p	0.87 (0.80, 0.95)	3.06E-03	0.03
miR-499a-3p	0.89 (0.83, 0.96)	3.17E-03	0.03
miR-2110	0.86 (0.78, 0.95)	3.38E-03	0.04
miR-508-5p	0.87 (0.79, 0.95)	3.46E-03	0.04
miR-520d-3p	0.89 (0.82, 0.96)	3.51E-03	0.04
miR-197-5p	0.85 (0.76, 0.95)	3.65E-03	0.04
miR-640	0.87 (0.80, 0.96)	3.84E-03	0.04
miR-208b-3p	0.88 (0.81, 0.96)	4.10E-03	0.04
miR-802	0.85 (0.76, 0.95)	4.23E-03	0.04
miR-1264	0.88 (0.81, 0.96)	4.41E-03	0.04
miR-200a-3p	0.83 (0.73, 0.94)	4.50E-03	0.04
miR-323b-3p	0.88 (0.80, 0.96)	4.43E-03	0.04
miR-132-3p	0.87 (0.80, 0.96)	4.60E-03	0.04
miR-3192-5p	0.89 (0.82, 0.96)	4.84E-03	0.04
miR-4792	0.87 (0.79, 0.96)	4.85E-03	0.04
miR-769-5p	0.87 (0.79, 0.96)	5.11E-03	0.04
miR-148a-3p	1.12 (1.04, 1.21)	5.56E-03	0.04
miR-3065-3p	0.88 (0.81, 0.96)	5.51E-03	0.04
miR-6724-5p	0.89 (0.83, 0.96)	5.44E-03	0.04
miR-1197	0.85 (0.75, 0.95)	6.16E-03	0.05
miR-141-3p	0.89 (0.81, 0.96)	6.41E-03	0.05
miR-513c-3p	0.88 (0.80, 0.96)	6.07E-03	0.05
miR-548g-3p	0.87 (0.79, 0.96)	6.03E-03	0.05
miR-577	0.88 (0.81, 0.96)	6.36E-03	0.05
miR-761	0.88 (0.80, 0.96)	6.44E-03	0.05
miR-1269b	0.88 (0.81, 0.96)	6.67E-03	0.05
miR-629-5p	0.86 (0.78, 0.96)	6.68E-03	0.05
miR-3613-5p	0.90 (0.83, 0.97)	6.97E-03	0.05
miR-933	0.86 (0.77, 0.96)	7.00E-03	0.05

[a] Covariates adjusted for included age, BMI, Charlson comorbidity score, cigarette smoking, and menopausal status, as well as use of vitamin supplements, aspirin, and other non-steroidal anti-inflammatory drugs (NSAIDs) within one week before sample collection.
[b] Estimated fold change (exponential function of regression coefficient) of the concentrations of F_2-IsoP-M per one ln-unit increase in miRNAs levels.
[c] FDR *p*-values adjusted for multiple testing for 484 miRNAs.

malondialdehyde (MDA), a by-product of lipid peroxidation [32], and stimulated superoxide dismutase (SOD) activity by regulating PI3K/AKT and ERK1/2 pathways [33]. Moreover, Nong et al. showed that upregulation of miR-126 was related to increased catalase (CAT) and SOD activities and decreased MDA levels in the brain tissue of septic rats [34]. In this study, we found that miR-126-3p expression was positively associated with F_2-IsoP-M. miR-126 inhibits insulin receptor substrate 1 (IRS1) and phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) by binding target sequences in the three prime untranslated region (3'UTR), which results in downregulation of IRS1 and PIK3R2 expression and activation of AMP-activated protein kinase (AMPK) and consequently alters fatty acid β-oxidation [35,36].

Three miRNAs (miR-141-3p, miR-497-5p and miR-363-3p), which were inversely associated with F_2-IsoP-M in our study, have been linked to mitogen-activated protein kinase kinase (MAP2K) as their common target mRNA [37–39]. MAP2K functions as a tumor suppressor and is well known for decreasing the expression of peroxisome proliferator-activated receptor gamma (PPARγ) [40]. By binding to fatty acids or their derivatives, PPARs are able to regulate fatty acid oxidation, in particular β-oxidation of long chain fatty acids [41]. Similarly, miR-186-5p also showed an inverse association with F_2-IsoP-M in this study, and its main target mRNA was forkhead box protein O1 (FOXO1) [42]. FOXO acts in response to starvation and plays a role in the protection of cells from oxidative stress and lipid abnormalities [43]. FOXO activation increases the expression of antioxidant enzymes SOD and CAT, responsible for redox homeostasis, and activates peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), which interacts with and regulate the activity of PPARγ [43].

Through the functional enrichment analysis, we identified several important biological pathways related to lipid peroxidation, which share miRNA targets, including the PI3K/AKT, MAPK, FOXO, HIF-1, and AMPK signaling pathways. The PI3K/AKT signaling pathway is of great relevance as it plays a central role in cellular physiology by mediating growth factor signals, glucose homeostasis, lipid metabolism, protein synthesis, and cell proliferation and survival [44]. PI3K/AKT has been associated with lipid metabolism to promote lipid biosynthesis and inhibit lipolysis, a dynamic and tightly controlled process accompanied by lipid peroxidation [44]. Aberrant PI3K/AKT signaling has been shown to contribute to increased ROS levels [45,46], and excessive oxidative stress can also activate the PI3K/AKT pathway by inhibiting the activity of its negative regulator phosphatase and tensin homolog (PTEN) [47,48]. Moreover, the Pl3K/AKT pathway can activate the MAPK, FOXO and HIF-1 pathways, all of which are involved in the regulation of lipid peroxidation. For example, deletion of *HIF-1α* genes in HepG2 cells could mimic hypoxia-induced lipid accumulation via reducing PGC1α-mediated fatty acid β-oxidation [49]. It is of interest that PI3/AKT pathway has also been directly linked to β-oxidation of fatty acids to generate acetyl-CoA. Metabolic stress stimulates β-oxidation via the Cpt1A enzyme, and PI3K/AKT regulates its expression to inhibit β-oxidation during anabolic growth [50].

Our study has several notable strengths that provide valuable contributions to the literature. Urinary F_2-IsoPs are sensitive and specific biomarkers that reflect time- and dose-dependent lipid peroxidation in both animal and human models of oxidative stress [12,51]. Urine samples used in this study are considered an ideal biological material for the measurement of F_2-IsoPs and their metabolites because, unlike plasma, urine does not contain high lipid contents; thus, there is less concern about artifactual generation of F_2-IsoPs by lipid autoxidation during

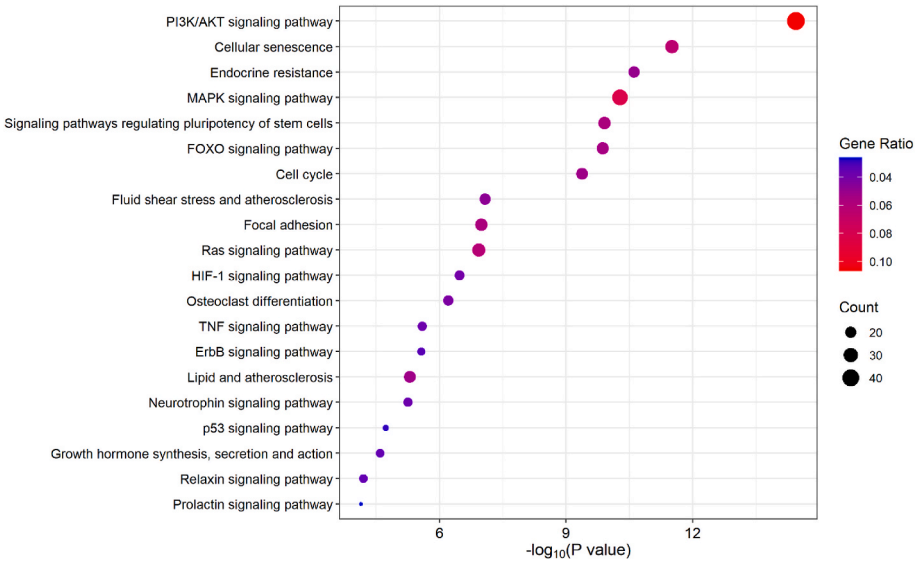

Fig. 3. Functional enrichment analysis. Biological metabolic and signaling pathways enriched for the gene targets of miRNAs significantly associated with urinary concentrations of F_2-IsoP-M.

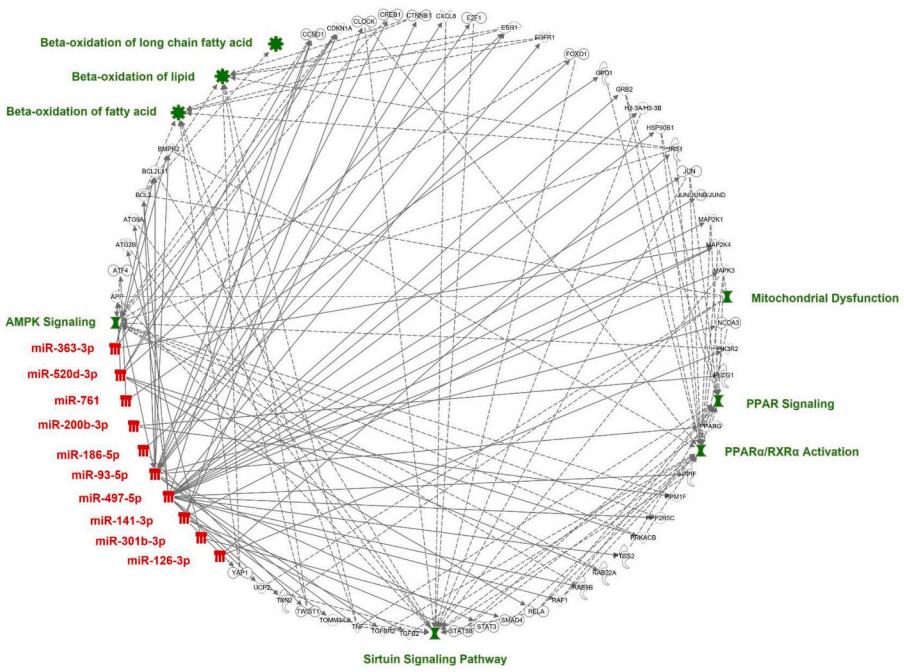

Fig. 4. Pathway network analysis illustrating the link between the significant miRNAs and biological pathways related to β-oxidation. Ten miRNAs (in red) associated with F_2-IsoP-M concentrations and their experimentally validated mRNA targets as identified by the Ingenuity Pathway Analysis software. The biological pathways involved in β-oxidation for miRNA-mRNA interactions are shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sampling and storage. Moreover, MS-based methods were used to assess systemic lipid peroxidation status accurately and reliably [13,27]. Another distinguished feature of our study is the use of NanoString nCounter assay, a state-of-the-art technology, to screen for 798 miRNAs. This allows a systematic and hypothesis-free assessment of changes in miRNA expression.

There are also some limitations or concerns in our study. First, samples at a single time point were collected and used in this study. However, our previous data have indicated that F_2-IsoPs and F_2-IsoP-M measurements based on a single spot urine sample can reliably represent long-term exposure reasonably well, with intraclass correlation coefficient (ICC) being 0.69 for F_2-IsoPs and 0.76 for F_2-IsoP-M over a long period of time [52]. Moreover, we have carefully evaluated the degree of biological variations in miRNAs and found that circulating miRNA levels remained rather stable over a relatively long period, with ICC increasing with abundance of miRNA expression and being greater than 0.50 for most common miRNAs [21].

Another concern is that we could not eliminate the possibility of residual confounding due to unmeasured or inaccurately measured covariates. However, the availability of data on a wide range of covariates, including demographic and lifestyle factors and inflammation- and oxidative stress-related exposures/conditions, allowed us to comprehensively control for potential confounding factors. On the other hand, all participants were middle-aged and elderly Chinese women. Such distribution virtually confined to a single ethnic population is likely to reduce potential confounding and strengthen the internal validity of the study finding, although its generalizability to other populations may be limited. Additionally, because of the lack of mRNA expression data, we were unable to determine transcript levels of the related candidate genes; thus, the relationship between miRNA and gene

expression could not be directly assessed. We are planning a phase-2 analysis, a multiomics-based analysis, to further explore biological functions of identified miRNAs as epigenetic modulators in redox homeostasis.

In summary, our study provides first human evidence linking miRNA expression with systemic lipid peroxidation and related metabolisms in women. The predicted targets of identified miRNAs were enriched for several lipid peroxidation-related processes such as PI3K/AKT, MAPK, FOXO, HIF-1, and AMPK signaling pathways. The finding is biologically plausible and may help uncover targets for preventive and therapeutic interventions. Further investigation into the role of the identified miRNAs in modulation of systemic lipid peroxidation is warranted.

Funding statement

This work was supported by the National Institutes of Health [grant numbers R01CA237895, R01CA122364, R01CA227133 and UM1CA182910]. GLM is in part supported by the Vanderbilt Diabetes Research and Training Center [P30DK020593]. The funders had no role in the design of the study; the collection, analysis, and interpretation of the data; the writing of the manuscript; and the decision to submit the manuscript for publication.

Authors' responsibilities

GY: designed research; YZ, MN, GM, XG, HC, QL, NR, QYC, YTG, QXC, XOS and GY: conducted research; YZ: analyzed data; YZ and MN: wrote paper; GY had primary responsibility for the final content; and all authors: read and approved the final manuscript.

None of the authors reported a conflict of interest related to the study.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Acknowledgments

We are grateful to the participants and research staff of the Shanghai Women's Health Study for their contributions to the study. We thank Jie Wu, Regina Courtney and Rodica Cal-Chris for contributions to sample preparation at the Survey and Biospecimen Shared Resources, which is supported in part by the Vanderbilt-Ingram Cancer Center (P30CA068485).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102531.

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