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Intra-individual Variation of miRNA Expression Levels in Human Plasma Samples

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Abstract

Background—Circulating miRNAs as potential non-invasive biomarkers for disease risk assessment and cancer early diagnosis have attracted increasing interest. Little information, however, is available regarding the intra-individual variation of circulating miRNA levels.

Methods—We measured expression levels of a panel of 800 miRNAs in repeated plasma samples from 51 healthy individuals that were collected 6 to 12 months apart and evaluated the intra-individual variation by the intra-class correlation coefficient (ICC).

Results—After background correction, a total of 185 miRNAs were detected in at least 10% of the plasma samples, with 69 and 28 miRNAs being detected in 50% and 90% of samples, respectively. The median ICC was 0.46 for these 185 miRNAs. Among them, 41% (75 miRNAs) had an ICC ≥ 0.5 , and 23% (42 miRNAs) had an ICC ≥ 0.6 . The ICC is higher for miRNAs with higher expression levels or higher detection rates, when compared to those with lower expression levels or lower detection rates.

Conclusions—These results suggest that common circulating miRNAs are stable over a relatively long period and can serve as reliable biomarkers for epidemiological and clinical research.

Keywords

circulating miRNA; intra-individual variation; intra-class correlation coefficient (ICC); biomarkers; epidemiology

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INTRODUCTION

MicroRNAs (miRNAs) are a large family of short (19–24 nucleotides in length) non-coding regulatory RNAs that modulate gene expression by targeting mRNAs in a sequence specific manner and causing translational repression or mRNA degradation, depending on the degree of complementarity between miRNAs and their targets (1–3). miRNAs regulate diverse cellular processes including development, proliferation and differentiation, thereby influencing the initiation and progression of various diseases. miRNAs can be exported or released by cells and circulate with the blood in a remarkably stable form by packaging into microparticles, e.g., exosomes, microvesicles, or apoptotic bodies (4, 5), binding to RNA-binding proteins or high-density lipoprotein (6–8). Although the precise role of circulating miRNAs is still largely unknown, dysregulated expression of circulating miRNAs has been found to be associated with the occurrence of a variety of diseases including cancers (8, 9, 9–14), cardiovascular disease (15–20), diabetes mellitus (21), obesity (22, 23), neurodegenerative disease (24, 25), and autoimmune disease (26), as well as altered physiological states such as pregnancy (27). Thus, circulating miRNAs have become of increasing interest as non-invasive biomarkers for predicting disease risk and prognosis, as well as for cancer early diagnosis and monitoring of treatment responses.

However, limited data exist on the normal spectrum and intra-individual variation of circulating miRNAs in apparently healthy individuals (13, 28). Understanding the degree of biological variation in miRNAs is imperative for epidemiological and clinical research, particularly with respect to investigating miRNAs for disease risk assessment. In this study, we measured the circulating miRNA levels in two repeated samples collected over a 6 to 12-month period from 51 healthy individuals who participated in the Shanghai Physical Activity (SPA) study. The inter- and intra-individual variations of circulating miRNA levels were evaluated.

CLINICAL SIGNIFICANCE

Most of the epidemiological and clinical research investigating circulating miRNAs as disease or exposure biomarkers is based on an assessment of blood samples collected at one time point. Knowledge of the variation of miRNA levels in circulation over a relatively long period of time in healthy people is crucial to determining the utility of these biomarkers for epidemiological research and clinical practice. Our study, designed to specifically address intrapersonal variation in the levels of miRNAs over time, provides invaluable information on the utility of circulating miRNAs as biomarkers for disease risk and exposure assessment.

MATERIALS AND METHODS

Study population

Study subjects were drawn from participants of the Shanghai Physical Activity (SPA) study, an observational study designed to evaluate the instrument of measuring physical activity. The SPA study was nested in two population-based cohort studies: the Shanghai Women's Health Study (SWHS; N=74,941; enrollment 1997–2000) and the Shanghai Men's Health Study (SMHS; N=61,480; enrollment 2002–2006). Details of the study design and methods

for the SPA and its parent studies have been reported previously (29–31). Briefly, the SPA study was conducted between December 2005, and September 2008. Of 1101 people contacted for the study, 619 agreed to participate. Participants completed 4 physical activity assessments and provided two blood samples during the one year study period after completion of the study survey interviews (29). The two samples were collected 6 to 12 months apart, with a mean distance in time of 9 months. Among participants in the SPA study with two blood samples, 27 males and 26 females were randomly selected for the current study. Exclusion criteria for this study are: age >70, BMI<18.5 or >35, CRP>10 ng/ml, antibiotics and NSAID use in the past 2 weeks prior to sample collection, or reporting any sign of a common cold during the 24 hours before biospecimen collection. We excluded individuals with age older than 70 to minimize the influence of senile diseases. This study was approved by the Institutional Review Boards of the participating research institutions.

Biospecimen collection and miRNA isolation

Ten milliliters of blood sample were drawn from each participant into an EDTA vacutainer tube. The samples were kept in a portable Styrofoam box with ice packs (0–4°C), processed within 6 hours, and stored at –80°C until RNA isolation was conducted.

Total RNA, including miRNA, was isolated and purified from plasma samples using Qiagen's miRNeasy Serum/Plasma Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. To minimize cellular contamination, the plasma samples were centrifuged at 16,000×g at 4°C for 10 min and 200 µl supernatant was used for total RNA extraction. To control for variations in the starting material as well as the efficiency of the downstream total RNA extraction, 5 µl each of three synthetic spike-in RNA oligos (osa-miR414, cel-miR248, and ath-miR159a) were added at concentrations of 1 pg/µl for cel-miR248, 2 pg/µl for osa-miR414, and 4 pg/µl for ath-miR159a. Five microliters of total RNA from 25 µl total yield was used for miRNA expression analysis, which represented the RNA from 40 µl of plasma.

miRNA expression assays

The Human v2 miRNA Expression Assay (NanoString, Seattle, WA), which includes CodeSets of probes specific for 800 common human miRNAs, was used for the miRNA assays. Six positive and 6 negative miRNA assay controls were included in the assays code set. In addition, 5 non-mammalian spikes in miRNA probes (ath-miR159a, cel-miR248, cel-miR254, osa-miR414, and osa-miR442) were also included. The assays were conducted in NanoString's in-house service lab (Seattle, WA) following their standard protocol (32). In brief, after hybridization of the Human v2 miRNA CodeSet with the tagged miRNA preparation, samples were transferred to the nCounter Prep Station where excess probes were removed and probe/target complexes were aligned and immobilized in the nCounter Cartridge. Cartridges were then placed in the nCounter Digital Analyzer for data collection. Each miRNA of interest was identified by the "color code" generated by six ordered fluorescent spots present on the Reporter Probe. The Reporter Probes on the surface of the cartridge were then counted and tabulated for each miRNA species. Based on a published TechNote from NanoString, un-normalized data from 5 replicate plasma samples for hsa-

miR-451 expression exhibit a coefficient of variation (CV) of 20%, and spike-ins normalized data showed a CV of 5%.

To minimize technical variation, the paired plasma samples from each subject were processed in the same batch for RNA isolation and the obtained RNA samples were assayed on the two lanes next to each other in the same cartridge on the miRNA expression assay.

Data analysis and normalization

Sample quality assurance and data normalization were performed using NanoString nSolver (version 2.0). Briefly, the specific miRNA counts were first corrected by subtracting the background (mean value plus 2 standard deviations of the 6 negative controls). Values < 0 were assigned 1 to facilitate downstream statistics. Second, to normalize the RNA content variation, the resulting counts of each sample were multiplied by the ratio of average signals obtained from three spike-in oligos across all samples over that of the individual sample, i.e., $\frac{\sum(Y_i)/N}{Y_i}$, where Y_i = mean count of three spike-in oligos of a given sample i and N is the

sample size. Finally, the derived counts were further normalized by using the average signals obtained from the top fifty miRNAs that gave the strongest hybridization signals following the same procedures as for spike-in oligos.

A total of 102 samples from 51 individuals were included in the analysis after exclusion of two pairs of samples with either high background (>3 standard deviations of the mean of all background levels) or unusual RNA content normalization factor (>3 standard deviations of the mean of all normalization factors) for one of the paired samples. We excluded miRNAs with more than 90% of their samples having normalized counts below 10 because infrequently expressed and low abundance miRNAs are especially vulnerable to random errors and our study does not have adequate statistical power to evaluate uncommon miRNAs. This process left 185 miRNAs for the current analysis.

Statistical analyses

Selected characteristics, lifestyles, and indexes from anthropometric measurements were compared between males and females. Log-transformation was applied to normalize the distribution of miRNAs. ANOVA was used for comparing continuous variables, and the chi square test was used for comparing dichotomous variables between male and female study participants. The intra-individual reproducibility of miRNA expression over time was evaluated by the intra-class correlation coefficient (ICC). The ICC is the ratio of inter-individual variance (between-person difference) to the total variance (inter- and intra-individual [within-person] variance). The value of an ICC ranges from 0 to 1. The ICC was calculated by using the one-way random effect model: $ICC = \sigma_B^2 / (\sigma_B^2 + \sigma_W^2)$ = [inter-individual variance/(inter-individual variance + intra-individual variance)] (33). The inter-person coefficient of variation (interCV%) was also used to further estimate the inter-individual variation. The two measurements from each person were averaged and the means were log transformed because the distributions of mean miRNAs were skewed. The interCV % was calculated by (standard deviation/mean) × 100%. Two-sided *P* values less than 0.05

were considered to be statistically significant. All statistical analyses were carried out using SAS software, version 9.4 (SAS Institute, Inc., Cary, North Carolina).

RESULTS

The basic demographic characteristics and selected lifestyle factors of the participants are shown in Table 1. The mean age was 54 years for all participants. No significant differences were found between females and males regarding age at recruitment, exercise participation and body mass index (BMI), but some lifestyle factors, including smoking status, alcohol and tea consumption, and total energy intake differed significantly between females and males, as expected.

Among a total of 185 miRNAs that were detected in more than 10% of the plasma samples, 69 (37%) were commonly expressed in 50% samples and 28 (15%) were detected in 90% samples. Of the 185 miRNAs, 11 miRNAs showed differential expression levels between females and males ($p < 0.05$) after adjusting for smoking status, alcohol consumption, tea consumption, energy intake, and WHR (Table 1). All of these 11 differentially-expressed miRNAs showed the higher expression (1.2 to 4.8 fold increases) in females compared to males, with let-7a-5p showing the largest gender difference in expression level.

Table 2 reports the ICCs, expression levels, and detection rates for 69 miRNAs that were detected in 50% plasma samples, categorized in the order of their ICCs from high (> 0.5), medium ($0.4 \sim 0.5$), to low (< 0.4). To estimate the inter-individual variation for the miRNAs in relation to their ICCs, we averaged the expression levels per person and calculated the coefficient of variation across 51 individuals (CV, standard deviation/mean $\times 100\%$) for each miRNA, then further sorted these miRNAs within ICC categories by their inter-individual CVs from high to low. As shown in Table 2, among 24 miRNAs that had ICCs > 0.5 , 16 had an inter-individual CV $> 20\%$. The miRNAs miR-16-5p, miR-376a-3p, miR-576-5p, miR-590-5p, miR-769-5p, miR-146a-5p and miR-199a-5p had both high ICCs (> 0.6) and high inter-individual variation (interCV $> 30\%$). The miRNAs miR-520f, miR-302d-3p, miR-25-3p, miR-378e, and miR-222-3p had high to medium ICCs (0.42 to 0.61) but the lowest levels of inter-individual variation (interCV $< 10\%$). The average expression levels of these five miRNAs were relatively high and they were all detected in 100% of samples, indicating a relatively constant and abundant expression across all samples. The miRNAs miR-520f, miR-451a, and miR-223-3p were the top three most abundant miRNAs, with a 26-, 12-, and 8-fold higher abundance in reference to the 20th most common miRNA, miR-222-3p, respectively.

The ICCs by percentage of detection and expression abundance are presented in Table 3. The median ICC was 0.46 for all 185 miRNAs. Among them, 117 miRNAs (63%) had ICCs > 0.4 . 75 miRNAs (41%) had ICCs > 0.5 and 42 (23%) had ICCs > 0.6 . When categorizing miRNAs by detected rate and expression levels, the higher ICCs (lower intra-personal variations) were found in miRNAs with higher expression levels or higher detection rate. The percentage of miRNAs with ICCs > 0.4 increased from 63.2% to 71.4% when the detected rate increased from 10% to 90%. A similar pattern was seen when miRNA expression levels increased. The percentages of miRNAs with ICC > 0.4 increased from

65.4% to 76.5% when the median of normalized counts increased from 5 to 100. The median ICC was 0.47 for the 17 miRNAs with normalized counts >100.

DISCUSSION

Despite the potential for circulating miRNAs to act as non-invasive biomarkers, little information is available regarding the intra-individual variability of common circulating miRNAs in healthy individuals. In this study, we evaluated the intra-individual variability of circulating miRNAs by measuring a panel of 800 miRNAs in 51 pairs of repeated plasma samples collected from 51 healthy individuals 6–12 months apart. A total of 185 miRNAs were detected in at least 10% of samples, with 69 miRNAs being detected in >50% of samples. We used ICC to estimate the intra-individual variability over time. A higher ICC indicates a large inter-individual variation or a small intra-individual variation. Biomarkers with a high ICC are highly desirable for epidemiology research because they not only improve exposure or disease assessment but also reduce the sample size need for the study. The median ICC was 0.46 for all of the 185 detected miRNAs; 75 and 42 miRNAs had ICCs of 0.5 and 0.6, respectively. The ICCs increased with rate of detection and miRNA abundance level. Our findings suggest that the expression levels of several common circulating miRNAs are stable over 6 to 12 months and a single measurement may be adequate to identify larger effects in future clinical and epidemiological studies investigating the biological functions of these miRNAs. To the best of our knowledge, only one study has previously evaluated the intra-individual variability of circulating miRNAs over time (34). In that study, Bertoia *et al.* used Real-Time PCR to measure 61 pre-selected miRNAs in two plasma samples from 80 participants donated 9 months apart. They detected 41 miRNAs in at least 50% of samples and 10 of them had an ICC above 0.40. Of these 41 miRNAs, 13 were detected in >50% of samples in our study and 77% (10 of 13) had an ICC >0.40.

Among the 69 miRNAs detected in >50% of samples in our study, 24 had ICCs 0.5, indicating acceptable intra-individual variation for epidemiologic applications. The miRNAs miR-16-5p, miR-376a-3p, miR-576-5p, miR-590-5p, miR-769-5p, miR-146a-5p and miR-199a-5p had both high ICCs and high inter-individual variation, suggesting that they have the potential to be used as markers for risk assessment. In fact, many of these miRNAs have been previously reported as potential predictive and distinguishable biomarkers for a variety of diseases. For example, miR-16-5p was found to be associated with cancers (35), cardiovascular disease (36), and rheumatoid arthritis (37). The expression of the miR-16, miR-21 and miR-199a-5p was reported significantly lower in the plasma of patients with triple negative breast cancer (TNBC) and was restored in post-operative plasma (38). In a systematic review of the literature followed by bioinformatics analysis, Assmann *et al.* reported that miR-146a-5p and seven other circulating miRNAs were consistently dysregulated in type 1 diabetes mellitus patients compared to controls (39). We anticipate that, with the accumulation of knowledge and the improvement of analysis technology, more miRNAs will be uncovered as informative biomarkers for a range of diseases in the near future. For the 23 miRNAs with ICCs <0.4, especially the 12 miRNAs with ICCs <0.3, multiple assessments across a time period may be needed when considering them as biomarkers for risk assessment.

Careful selection of so-called “invariant” miRNAs as endogenous controls is extremely important in miRNA expression analysis. An ideal endogenous control generally demonstrates gene expression that is relatively constant and highly abundant across samples. In our study, we found five miRNAs (miR-520f, miR-302d-3p, miR-25-3p, miR-378e, and miR-222-3p) had higher ICCs (0.42 to 0.61) and the lowest inter-individual variation ($\text{interCV} < 10\%$) with relatively high expression levels and 100% detection rates, suggesting more constant and abundant expression across all samples, making them useful candidates to serve as endogenous controls for the study of circulating miRNAs.

Several previous studies have investigated the normal spectrum of circulating miRNAs in healthy individuals using different platforms. Consistent with the data from previous reports on circulating miRNAs in plasma (10, 12, 13, 28, 34), the detected numbers and expression levels of circulating miRNAs were generally low in the plasma samples from the healthy individuals in our study. Chen et al. reported of detection of 101 miRNAs in the serum of pooled male and female healthy subjects using a sequencing approach (13). Mitchell et al. reported a finding of 130 miRNAs in a normal individual using a RT-qPCR array (12). Tanaka (40) and Wang (41) detected 148 and 170 miRNAs in 7 and 4 plasma samples from healthy controls in the studies of acute leukemia and acute myocardial infarction based on microarray analysis. In our study, we detected 185 miRNAs with a normalized count >10 in at least 10% of samples using NanoString technology and stringent background correction criteria; 69 of them were detected in $>50\%$ samples and 28 were detected in $>90\%$ samples. Of note, there is a considerable overlap of the circulating miRNA content found in our study with that found in previous reports. Of the 20 miRNAs identified in at least 4 of the previous studies (42) that investigated blood samples from normal individuals, 17 were detected in our study and 10 of them were on our top 50 most abundant miRNA list.

The number of detected miRNAs in plasma is influenced by the background correction criteria. We used mean plus 2 standard deviations of the 6 negative controls as background value and subtracted it from real count for each miRNA. We detected 185 miRNAs with normalized count >10 in at least 10% of samples. This detection rate could increase to 512 or 303 if we use mean value only or mean plus 1 standard deviation of the 6 negative controls, respectively, for background correction (data not shown). In terms of the relative abundance of the most commonly detected miRNAs, the second most abundant in our study, miR-451, was found to be the most abundant in both the Chen (13) and Wang studies (41). miR-223 was the third most abundant in our study and was identified as the most abundant miRNA in Hunter's (28) and Mitchell's studies (12). Some miRNAs that are commonly found in the circulation of cancer patients, including miR-21, miR-155 and miR-29a, were not detected in healthy individuals in our study, supporting that they are disease specific biomarkers.

With regard to the functional relevance of circulating miRNAs, many authors have proposed a role for maintaining homeostasis of the circulatory system and involving in hematopoiesis based on the known function of the miRNAs commonly found in normal healthy circulation. The miRNA profiles of serum and those of blood cells showed extensive overlap, suggesting that the majority of circulating miRNAs in normal individuals may be released into the circulation by these cells (13). Merkerova *et al.* observed that miR-451 robustly expressed in

erythrocytes, and miR-223 strongly expressed in platelets, granulocytes and monocytes (43). Both of these miRNAs are among the most abundant plasma miRNAs in our study. We found miR-520f to be the most abundant miRNA, with 26-fold abundance relative to the 20th most common miRNA, miR-222-3p. The role of miR-520f in the circulation remains unclear and needs to be investigated.

Differences in miRNA expression between males and females have been observed in animal models and cell studies (44–46), but limited data are available in humans (22, 47). Ameling *et. al.* (47) evaluated the associations of circulating plasma microRNAs with age, body mass index and sex in a population-based study, the Study of Health in Pomerania (SHIP). 35 miRNAs were found to be significantly associated with sex and 7 remained significant after adjustment for age, BMI, and blood cell parameters. In our study, we found 11 miRNAs that showed higher expression (1.2 to 4.8 fold increases) in females compared to males ($p<0.05$) after adjustment for smoking, alcohol consumption, tea consumption, energy intake, and WHR. However, none of these 11 miRNAs overlapped with the 7 miRNAs that showed sex differences in Ameling's study. Wang *et. al.* discovered that miR-221 and let-7g were expressed more prominently in the plasma of women than men manifesting metabolic syndrome (22). These two miRNAs also showed higher expression in females compared to males in our study. The sex differences in miRNA expression can be partly attributed to sex chromosomes, sex hormones, sex-specific environmental exposures, and other sex-related factors. To minimize the impact of sex hormones on the differences in miRNA expression, we conducted a sub-group analysis in men and postmenopausal women only, and found that 11 miRNAs (let-7a-5p, let-7g-5p, miR-126-3p, miR-185-5p, miR-197-3p, miR-199a&b-3p, miR-221-3p, miR-26a-5p, miR-376a-3p, miR-382-5p, and miR-520h) continued to show significant differences between men and women at $p<0.05$ except for miR-221-3p, which showed borderline significance at $p=0.06$ (data not shown). Our findings suggest that sex hormones may not be the major determinants for the differences in expression by sex that we observed in these 11 circulating miRNAs. Further studies are needed to replicate this observation, and functional studies exploring the sex-specific roles of microRNAs are warranted.

Our study has several strengths, including using repeated samples collected from the same subjects 6 to 12 months apart using the same sample collection and processing protocol; application of NanoString's Human v2 miRNA Expression Assay which offers a high specificity and sensitivity on measured miRNAs; and inclusion of male and female participants. This study was limited by its modest sample size of 26 men and 25 women, which limited our ability to evaluate the associations between plasma miRNA levels with lifestyle factors and sex. However, our study has adequate statistical power (at least 80%) to detect miRNAs with an ICC of 0.40 and above. Another limitation of this study is the lack of our study specific information on assay reliability and sample processing variability. Furthermore, the lack of subjects from other ethnicities prohibited us from evaluation of ethnic variations in miRNA levels. Nevertheless, to the best of our knowledge, our study is among the first to comprehensively investigate the reproducibility of circulating miRNAs among healthy individuals over a 6 to 12 month period.

Several assay platforms, including RT-qPCR, hybridization and sequencing, have been developed and successfully applied to quantify miRNA expression. Each technology and platform has specific strengths and weaknesses. While RT-qPCR is considered the gold standard method to measure miRNA, its utilization in epidemiological research is limited by low throughput. NanoString nCounter miRNA expression assay is a high throughput assay with proven reliability and accuracy in measuring miRNA levels (48), and for these reasons we used it in our study. Its utilization in epidemiological research has been increasing. However, ICCs for miRNAs may differ by which assay is used. Therefore, a further validation study using other technologies is needed.

CONCLUSION

We found that 185 miRNAs were expressed in at least 10% of plasma samples, and 69 miRNAs in >50% of samples from healthy Chinese men and women. Of them, 75 miRNAs had an ICC ≥ 0.5 based on the paired samples collected from the same subjects at two time points 6 to 12 months apart. The ICC increased with abundance of miRNA expression. Our findings indicate that the expression levels of common circulating miRNAs are stable over a relatively long period, supporting the premise of using miRNAs as reliable biomarkers for epidemiological and clinical research. Our study provides valuable information regarding the utility of using circulating miRNAs in epidemiological investigations of disease risk assessment.

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Figure 1.
Flow diagram for the selection of study population

Table 1

Characteristics of participants for the current study by gender

Characteristics	Total subjects (n = 51)	Male (n = 26)	Female (n=25)	P value
Demographic factors				
Age (years) at recruitment, mean (SD)	54.06 (9.34)	53.93 (9.46)	54.19 (9.41)	0.9233
Exercise regularly (%)	31.4	38.5	24	0.2659
Current smoking (%)	35.3	65.4	4	<0.0001
Current alcohol consumption (%)	17.6	34.6	0	0.0012
Tea consumption (%)	47.1	65.4	28	0.0075
Total energy intake (kcal/day)	1686.9 (377.4)	1857. 8 (339.5)	1509.3 (334. 8)	0.0006
Body mass index (BMI), mean (SD)	23.52 (3.51)	23.39 (2.23)	23.65 (4.52)	0.7978
Waist-to-hip ratio (WHR), mean (SD)	0.87 (0.08)	0.90 (0.06)	0.83 (0.08)	0.0004
miRNAs [*] , GM (95% CI)				
let-7a-5p	5.4 (3.9 – 7.6)	2.5 (1.5 – 4.2)	12.1 (5.9 – 24.7)	0.0196
let-7g-5p	68.7 (50.3 – 93.8)	34.5 (17.2 – 69.2)	140.8 (95.1 – 208.4)	0.0202
miR-126-3p	101.3 (78.4 – 131.0)	60.8 (34.0 – 109.0)	172.4 (122.9 – 241.9)	0.0457
miR-185-5p	21. 8 (17.8 – 26.6)	15.8 (9.9 – 25.2)	30.6 (23.3 – 40.2)	0.0209
miR-197-3p	20.0 (15.0 – 26.7)	11.5 (6.4 – 20.5)	35.8 (21.8 – 58.5)	0.0158
miR-199a-3p+miR-199b-3p	12.8 (9.4 – 17.5)	8.3 (4.5 – 15.3)	20.3 (11.1 – 37.2)	0.0228
miR-221-3p	9.6 (7.1 – 13.2)	6.0 (3.3 – 10.9)	15.8 (8.6 – 29.1)	0.0271
miR-26a-5p	7.8 (5.8 – 10.4)	5.2 (3.0 – 9.1)	11.8 (6.8 – 20.7)	0.0031
miR-376a-3p	9.0 (6.6 – 12.2)	7.1 (3.8 – 13.3)	11.6 (6.5 – 20.8)	0.0027
miR-382-5p	4.0 (3.1 – 5.0)	3.7 (2.2 – 6.1)	4.3 (2.8 – 6. 8)	0.0428
miR-520h	5.7 (4.3 – 7.6)	4.7 (2.5 – 8.6)	7.0 (4.0 – 12.3)	0.0071

* The miRNAs show differential expression between males and females ($p<0.05$) after adjusting for smoking status, alcohol consumption, tea consumption, energy intake, and WHR.

Table 2

miRNAs detected in 50% of plasma samples and their intra-individual variation

Name of miRNA	Median (Q1-Q3)	ICC	InterCV (%)	Detected (%)
<i>ICC 0.5</i>				
miR-376a-3p	13 (2.1 – 33.9)	0.72	54.3	55
miR-107	11.7 (1.9 – 26)	0.54	53.5	52
miR-590-5p	15.3 (3.3 – 41.4)	0.71	50.5	61
miR-769-5p	12.8 (2 – 30.8)	0.65	50.4	58
miR-612	16.6 (2.1 – 37.4)	0.55	49.0	61
miR-146a-5p	44.7 (4.3 – 82.6)	0.61	39.9	72
miR-761	25.1 (10.7 – 51.6)	0.58	37.4	75
miR-106a-5p+miR-17-5p	34.1 (6.8 – 73.6)	0.53	35.9	71
miR-576-5p	43.3 (21.1 – 93.6)	0.71	33.3	83
miR-148b-3p	19.7 (4.6 – 37.9)	0.53	31.8	68
miR-16-5p	66.7 (21.6 – 162.1)	0.74	31.5	83
miR-1183	49 (23.8 – 85.9)	0.57	31.4	86
miR-199a-5p	27 (14.4 – 39.4)	0.60	31.0	79
let-7g-5p	88.2 (41.8 – 195.9)	0.65	28.7	84
miR-185-5p	32.9 (15.9 – 45.6)	0.52	23.3	81
miR-598	74.3 (39.1 – 114)	0.63	21.3	94
miR-495	42.5 (28.8 – 71.1)	0.63	19.7	94
miR-1976	356.1 (157.1 – 588.8)	0.58	18.4	98
miR-548aa	84.3 (56.4 – 122.6)	0.53	16.2	98
miR-22-3p	157.7 (89.4 – 235.2)	0.57	14.8	95
miR-93-5p	102.1 (56.4 – 133.3)	0.50	14.3	94
miR-4454	440.8 (266.9 – 661)	0.62	12.3	100
miR-302d-3p	120.3 (90.7 – 191.8)	0.57	9.8	100
miR-520f	2031.2 (1408.3 – 2705.2)	0.61	5.5	100
<i>ICC 0.4 ~ 0.5</i>				
miR-26a-5p	9.9 (2 – 29.8)	0.44	54.2	50

Name of miRNA	Median (Q1-Q3)	ICC	InterCV (%)	Detected (%)
miR-221-3p	11.9 (2 – 41.4)	0.47	52.7	53
miR-2116-5p	14.4 (2.1 – 29.3)	0.41	43.6	57
miR-216b	13.8 (2.4 – 24.7)	0.45	41.8	60
miR-20a-5p + miR-20b-5p	14.1 (2.2 – 38.1)	0.44	41.8	57
miR-1827	18.3 (4.3 – 30.8)	0.47	35.9	63
miR-297	25.6 (9.2 – 48)	0.44	35.4	72
miR-30e-5p	30.8 (11.2 – 51.8)	0.47	32.5	80
miR-151a-3p	37.6 (17.2 – 59.7)	0.45	29.2	80
let-7b-5p	54.5 (26.4 – 98)	0.45	24.7	87
miR-126-3p	150.7 (61 – 253.2)	0.45	21.2	92
miR-371a-3p	55.3 (29.1 – 83.8)	0.42	20.5	88
miR-188-5p	48.7 (27.7 – 77.4)	0.48	19.4	93
miR-361-3p	55.7 (24.9 – 82.3)	0.49	16.5	94
miR-191-5p	140.6 (85 – 222.1)	0.43	14.9	92
miR-579	47.9 (30.6 – 77.8)	0.41	14.3	96
miR-451a	989 (557.7 – 1623.7)	0.47	12.2	99
miR-574-5p	104.9 (66.2 – 149.8)	0.46	11.4	100
miR-631	158.6 (108.7 – 215.4)	0.47	10.3	100
miR-222-3p	78.3 (54.3 – 104.6)	0.43	8.2	100
miR-378e	271.3 (188.1 – 377.3)	0.42	7.6	100
miR-25-3p	326.4 (252 – 391.7)	0.49	5.4	100
ICC < 0.4				
miR-570-3p	146.5 (113.6 – 233.7)	0.39	9.8	100
miR-1202	20.1 (9.5 – 37.3)	0.37	30.1	74
miR-514b-5p	83 (55.8 – 131.4)	0.37	11.3	99
miR-92a-3p	220.5 (138.9 – 263.4)	0.37	6.8	100
miR-720	75.5 (28.1 – 147.4)	0.36	35.9	83
miR-199a-3p + miR-199b-3p	22.1 (2.2 – 56)	0.35	41.4	59
miR-494	12.2 (2.6 – 22)	0.35	41.1	55
miR-197-3p	25.8 (9.1 – 67.9)	0.34	36.3	72

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Name of miRNA	Median (Q1-Q3)	ICC	InterCV (%)	Detected (%)
miR-142-3p	37 (23.8 – 58.8)	0.34	23.0	87
miR-15b-5p	16.9 (2.1 – 45.5)	0.33	49.7	55
miR-489	34.9 (19.7 – 56.2)	0.30	17.3	87
miR-425-5p	20.7 (2.2 – 46.8)	0.29	42.5	57
miR-504	11.3 (3 – 23.8)	0.29	39.4	55
miR-337-3p	14.5 (4.7 – 28.6)	0.29	34.5	64
miR-655	14.3 (2.9 – 25.3)	0.28	38.7	63
miR-888-5p	56.1 (38.2 – 72)	0.26	10.3	98
miR-223-3p	860.1 (581 – 1164.9)	0.25	7.9	98
miR-548a-5p	36.2 (23.4 – 50.4)	0.24	14.0	91
miR-23a-3p	155.3 (102.2 – 210.2)	0.22	9.9	93
miR-320e	70.1 (41.2 – 107.3)	0.17	14.6	98
miR-106b-5p	11.1 (2.1 – 21.4)	0.16	45.5	51
miR-484	41.2 (18.3 – 70.7)	0.15	22.7	80
miR-423-5p	73.6 (45.5 – 109.3)	0.10	16.7	88

Table 3

The number of miRNAs detected in plasma and the distribution of variations by detected rate and expression levels

	Detected Rate (count 10, n=102)			
	10%	50%	75%	90%
Number of miRNAs	185	69	44	28
ICC_Median	0.46	0.45	0.465	0.465
ICC 0.4, %	63.2%	66.7%	70.5%	71.4%
	Expression levels (Median of normalized counts)			
	>5	>10	>50	>100
Number of miRNAs	81	68	30	17
ICC_Median	0.45	0.45	0.455	0.47
ICC 0.4, %	65.4%	66.2%	70.0%	76.5%