# Cigarette smoking substantially alters plasma microRNA profiles in healthy subjects

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**Abstract**

Circulating microRNAs (miRNAs) are receiving attention as potential biomarkers of various diseases, including cancers, chronic obstructive pulmonary disease, and cardiovascular disease. However, it is unknown whether the levels of circulating miRNAs in a healthy subject might vary with external factors in daily life. In this study, we investigated whether cigarette smoking, a habit that has spread throughout the world and is a risk factor for various diseases, affects plasma miRNA profiles. We determined the profiles of 11 smokers and 7 non-smokers by TaqMan MicroRNA array analysis. A larger number of miRNAs were detected in smokers than in non-smokers, and the plasma levels of two thirds of the detected miRNAs (43 miRNAs) were significantly higher in smokers than in non-smokers. A principal component analysis of the plasma miRNA profiles clearly separated smokers and non-smokers. Twenty-four of the miRNAs were previously reported to be potential biomarkers of disease, suggesting the possibility that smoking status might interfere with the diagnosis of disease. Interestingly, we found that quitting smoking altered the plasma miRNA profiles to resemble those of non-smokers. These results suggested that the differences in the plasma miRNA profiles between smokers and non-smokers could be attributed to cigarette smoking. In addition, we found that an acute exposure of ex-smokers to cigarette smoke (smoking one cigarette) did not cause a dramatic change in the plasma miRNA profile. In conclusion, we found that repeated cigarette smoking substantially alters the plasma miRNA profile, interfering with the diagnosis of disease or signaling potential smoking-related diseases.

Keyword: microRNA, smoking, biomarker

## Introduction

MicroRNAs (miRNAs) are a class of endogenous, short non-coding RNAs 19-25 nucleotides in length that negatively regulate gene expression via translational repression or mRNA degradation, primarily by pairing with the 3'-untranslated regions of the target mRNAs (Ambros, 2001). More than 2,000 human miRNAs have been identified (miRBase ver. 19). It is now clear that miRNAs are involved in various important biological processes, including cell differentiation, proliferation, apoptosis, and development (He et al., 2004; Meltzer et al., 2005). Some miRNAs are expressed in a tissue-specific manner, and others are ubiquitously expressed (Liang et al., 2007). It is also known that miRNA expression is significantly altered in several diseases (http://www.miR2disease.org/), and the dysregulation of miRNAs is closely linked with the incidence or progress of these diseases.

It was reported in 2008 that miRNAs exist in plasma or serum (Mitchell et al., 2008). Subsequent reports have revealed the presence of miRNAs in saliva (Park et al., 2009; Michael et al., 2010) and urine (Hanke et al., 2010). These extracellular miRNAs are stable in the RNase-rich environment because they are enclosed in vesicles, including exosomes, microvesicles, apoptotic bodies, and ectosomes (Lee et al., 2012), or are associated with RNA-binding proteins, including nucleophosmin 1 (Wang K et al., 2010) or high-density lipoprotein (Vickers et al., 2011). Following the discovery of extracellular miRNA in body fluids, a significant number of papers reported that the levels of some extracellular miRNAs are linked to different pathophysiological conditions. Examples of this include the associations of miR-141 and miR-375 with prostate cancer (Bryant et al., 2012), miR-29a and miR-92a with colorectal cancer (Huang et al., 2010), miR-499 with myocardial infarction (Olivieri et al., in press), and miR-122 with liver injury (Wang et al., 2009). These findings introduce the possibility of using the levels of specific miRNAs in body fluids as biomarkers for different pathological conditions (Wang K. et al., 2012; Duttagupta et al., 2011). However,

it is unknown whether the levels of extracellular miRNAs in a healthy subject might vary with external factors in daily life.

Cigarette smoking is a habit that has spread all over the world and is a significant risk factor for many diseases, including lung cancer (Shields et al., 1999), chronic obstructive pulmonary disease (Higgins et al., 1984), asthma (Ulrik et al., 2001) and cardiovascular disease (Holbrook et al., 1984). Cigarette smoke contains over 4,800 chemicals, including 69 carcinogens (Hoffmann et al., 2001), which appear to be of crucial importance in causing disease. We questioned whether cigarette smoking alters plasma miRNA profiles in humans, and, if it does, whether the changes may be associated with the pathogenesis of various diseases. In this study, we compared the plasma miRNA profiles of smokers and non-smokers and discussed the biological significance of the different profiles by the smoking status.

**Materials and methods**

*Chemicals and reagents.* The *mir*Vana PARIS Kit, Megaplex pools, TaqMan microRNA Reverse Transcription Kit, TaqMan Universal PCR Master Mix (No AmpErase UNG) and TaqMan Human MicroRNA Array A and B Card v2.0 were from Life Technologies (Carlsbad, CA). Nicotine and cotinine were purchased from Sigma (St. Louis, MO). Acetanilide was purchased from Wako (Osaka, Japan). All other chemicals and solvents were of the highest grade commercially available.

*Study design.* This study was approved by the Ethics Committee of Kanazawa University (Kanazawa, Japan). Written, informed consent was obtained from all the subjects. Eleven smokers (S1 - S11) and seven non-smokers (NS1 - NS7) who were healthy male Japanese taking no medicines or supplements were recruited (Table 1). The smokers smoked their favorite brands of cigarettes as shown in Table 1. The pack year (number of cigarettes per day

× number of years smoked / 20 cigarettes in one pack) ranged from 4 to 35 (18 ± 9). There were no significant differences in the age and body weight of the smokers and non-smokers.

This study consists of three experiments. For the blood collection (5 ml), subjects came at 10 AM without having had breakfast. In experiment I, blood was collected from all subjects to compare the plasma miRNA profiles in smokers and non-smokers. Smoking occurred in the morning or not, according to each smoker's habit. After experiment I, we noticed that four smokers (S1, S2, S4, and S8) had voluntarily quit smoking. Among them, subject S8 had used nicotine patches after experiment I. In experiment II, blood was collected from the four subjects to determine their plasma miRNA profiles after they quit smoking (S1, S2, and S4 were tested one month after quitting; S8 was tested three months after quitting but was using nicotine patches). After that, two subjects, S1 and S4, returned to smoking. We had asked them to contact us when they began smoking again. In experiment III, blood was collected from the two subjects (S1 and S4) 20 min after they smoked the first cigarette (MILD SEVEN, Japan Tobacco, Tokyo, Japan) to determine the plasma miRNA profiles immediately after exposure to cigarette smoke.

*RNA isolation from plasma.* Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid disodium salt was added as an anticoagulant to the blood, which was then kept at room temperature for 30 min. After centrifugation at 3,000 g for 10 min at 4°C, the plasma was collected. Immediately, total RNA was isolated from 600 µl of plasma using the *mir*Vana PARIS kit as described previously (Yamaura et al., 2012).

*Taqman microRNA array analysis.* The expression profiles of miRNAs were assessed using TaqMan Human MicroRNA Array A+B Cards Sets v2.0 containing 377 (A array) or 285 (B array) primer-probe sets for individual miRNAs (total 662 miRNAs). All procedures were performed following the manufacturer's instruction. Briefly, 3 µl of total RNA was reverse

transcribed using Megaplex RT Primer Pool A or B and TaqMan MicroRNA Reverse Transcription Kits. Pre-amplification was carried out using Megaplex PreAmp primers and the TaqMan Preamp Master Mix. The expression of miRNAs was determined by quantitative real-time PCR using the TaqMan Human MicroRNA Array with the 7900HT Fast Real-Time PCR System (Life Technologies) and the manufacturer's recommended cycling conditions. Cycle threshold (Ct) values were calculated using the SDS software v.2.3 with a baseline of 3-15 and an assigned minimum threshold of 0.2. Expression of miRNAs was normalized by global normalization, which implicitly assumes that the mean expression level of all monitored miRNAs is constant. Any miRNA giving 40-Ct < 8, a cutoff value recommended by the manufacturer, in at least one sample was omitted from the data analysis.

*Principal component analysis.* The plasma miRNA expression data were analyzed using the Partek Genomics Suite version 6.12 (Partek, St. Louis, MO). Principal component analysis (PCA) was performed to visualize the difference between groups of the expression profiles of miRNAs that exceeded the cutoff value.

*Measurement of plasma concentrations of nicotine and cotinine.* Plasma concentrations of nicotine and cotinine were measured as described previously (Nakajima et al., 2000) with slight modifications. The plasma sample (0.5 ml) was alkalinized with 25 µl of 10 M NaOH. After the addition of 10 µl of 7 µM acetanilide as an internal standard, the mixture was extracted with 4 ml dichloromethane by shaking for 10 min. After centrifugation at 1,000 g for 10 min, 12 µl of 12 M HCl was added to the organic fraction. The organic fraction was evaporated with a vacuum evaporator at 40°C. The residue was redissolved in 50 µl of the mobile phase, and then a 20 µl portion of the sample was subjected to liquid chromatography/tandem mass spectrometry (LC-MS/MS). The LC-MS/MS condition was described in our previous report (Yamanaka et al., 2004).

*Statistical analysis.* Data are presented as the mean ± standard deviation. Statistical analyses of the differences between two groups were performed by an unpaired two-tailed Student's *t*-test. A value of *P* less than 0.05 was considered statistically significant.

## Results

*Plasma miRNA profiles of non-smokers and smokers*

We determined plasma miRNA expression in 11 smokers and 7 non-smokers by quantitative real-time PCR using the TaqMan MicroRNA Array. The numbers of miRNAs with 40-Ct > 8 in each individual are shown in Table 2. The numbers in smokers (196 ± 18) were significantly ($P < 0.001$) larger than those in non-smokers (143 ± 29). The number of miRNAs that exceeded the cutoff value in all subjects (n = 18) was 66. We compared the levels of 66 miRNAs in smokers and non-smokers. Among them, 44 miRNAs showed a significant difference between the groups (Table 3). Forty-three miRNAs were higher in smokers than in non-smokers, whereas 1 miRNA was lower in smokers than in non-smokers. To visualize the difference in the expression profiles of miRNAs between smokers and non-smokers, PCA was performed using the expression data for the 66 miRNAs. PC1 encompassed a significantly large proportion (63%) of the total variance for each subject, followed by PC2 (10%) and PC3 (6%). As shown in Fig. 1, the profiles of plasma miRNA expression of the smokers were different from those of the non-smokers.

*Quitting smoking altered the plasma miRNA profile*

To investigate whether the difference in the plasma miRNA profiles between smokers and non-smokers was due to cigarette smoking, we examined miRNA expression in the plasma of 4 smokers who had stopped smoking (S1, S2, S4, and S8). To confirm their non-smoking

status, we measured their plasma concentrations of nicotine and cotinine. For comparison, nicotine and cotinine concentrations were also measured when they were still smoking (experiment I); these concentrations were 3.6 - 26.0 ng/ml and 23.4 - 413.0 ng/ml, respectively (Table 4). One month after they stopped smoking, the levels in S1 and S2 had dramatically decreased to values close to the detection limit. In the plasma from S4, low levels of nicotine and cotinine were detected; therefore, he might have been smoking in secret or been exposed to passive smoking. S8 showed substantial nicotine and cotinine levels comparable to those of active smokers. These levels were not surprising because this subject used nicotine patches.

The numbers of miRNAs with 40-Ct > 8 in the 4 subjects who had stopped smoking were 141 ± 13 (129 - 160). Interestingly, the number was very close to that in non-smokers (143 ± 29) and was significantly ($P < 0.01$) lower than that when they were still smoking (209 ± 21) (Table 2). We compared the plasma miRNA expression in the four subjects before and after they stopped smoking. The numbers of miRNAs that exceeded the cutoff value in the four subjects was 93. Among them, 63 miRNAs showed a significant difference between the groups (Table 5). Sixty miRNAs were significantly lower after the subjects had stopped smoking than before they stopped, whereas 3 miRNAs were significantly higher after they stopped than before they stopped. Interestingly, among the 60 miRNAs that were lower after the subjects had stopped smoking, 38 miRNAs (shaded in Table 5) were the ones more highly expressed in smokers than in non-smokers (Table 2). These results suggested that the plasma miRNA expression is unambiguously affected by smoking.

We considered the possibility that quitting smoking might alter the plasma miRNA profiles to resemble the profiles of non-smokers. To test this possibility, we compared the plasma miRNA profiles of the 4 subjects before and after they stopped smoking, as well as the profiles of the 7 non-smokers. The number of miRNAs exceeding the cutoff value among the three groups was 66. PCA was performed using the expression data of the 66 miRNAs

(Fig. 2A). PC1 encompassed the largest proportion (60%) of the total variance for each subject, followed by PC2 (10%) and PC3 (6%). These results clearly demonstrated that the plasma miRNA profiles of smokers were altered after they stopped smoking, and the profiles then resembled those of non-smokers.

*Smoking one cigarette does not cause a dramatic change in the expression of plasma miRNAs*

To ascertain how quickly the plasma miRNA profile was changed by smoking, we examined plasma miRNA profiles 20 min after 2 ex-smokers (S1 and S4) had each smoked one cigarette. We chose this time because it is near the Cmax values of nicotine and cotinine after smoking (Yamanaka et al., 2004). The plasma concentrations of nicotine after one cigarette were 13.7 ng/ml and 15.7 ng/ml, and those of cotinine were 5.1 ng/ml and 17.9 ng/ml (Table 4), suggesting that the subjects had inhaled the cigarette smoke. The numbers of miRNAs with 40-Ct > 8 were 177 and 131, slightly larger than the numbers of miRNAs prior to smoking the cigarettes (Table 2). The miRNA expression data were included in the PCA for the 66 miRNAs described above. As shown in Fig. 2B, the miRNA profiles after smoking one cigarette were similar to those of the smokers who had stopped smoking and the non-smokers. These results suggest that changes in the plasma miRNA profiles in smokers might be due to repeated smoking.

**Discussion**

Circulating miRNAs have received considerable attention as potential biomarkers of various diseases (Reid et al., 2011) based on many studies reporting differences in plasma or serum miRNA levels between healthy subjects and patients. However, it remains unclear to what extent the circulating miRNA profiles in healthy subjects vary in daily life due to changes in diet, supplements, alcohol intake, cigarette smoking, exposure to environmental

chemicals, sleeping or circadian rhythm, stress, and exercise or other factors. In the present study, we focused on cigarette smoking because it is a habit spread all over the world and is a significant risk factor for various diseases including cancer. In the present investigation of the plasma miRNA profiles of smokers and non-smokers, the subjects were limited to men because a study had reported subtle sex differences (Ji et al., 2009), although another study reported no large sex differences in plasma miRNA expression (Chen et al., 2008), and because smoking is more prevalent in men than in women in Japan. In addition, considering the potential effects of circadian rhythm (Shende et al., 2011), we standardized the time of blood collection.

We found that a larger number of miRNAs were detected in smokers than in non-smokers, and the plasma levels of two thirds of the detected miRNAs (43 miRNAs) were significantly higher in smokers than in non-smokers (Table 3). Interestingly, we found that quitting smoking changed the plasma miRNA profiles resembling those of the non-smokers (Fig. 2A). These results suggested that the differences in the plasma miRNA profiles of smokers and non-smokers were actually due to cigarette smoking. However, no association was observed between the numbers of detected miRNAs or abundance of miRNAs and plasma nicotine or cotinine levels or smoking history within smokers (data not shown). One subject who stopped smoking, S8, was a nicotine patch user. Because there was no substantial difference between the plasma miRNA profile of this subject and those of the other 3 subjects who stopped smoking, as well as those of the non-smokers, nicotine is unlikely to cause altered plasma miRNA expression. Other chemicals or oxidants in cigarette smoke, or hypoxic stress, might cause changes in plasma miRNA profiles. It would be of interest to investigate whether the miRNAs whose levels were higher in smokers than in non-smokers might be positively correlated with plasma levels of oxidative stress markers such as 8-hydroxydeoxyguanosine or malondialdehyde in smokers. It has been reported that the plasma levels of these markers were approximately 1.5 times higher in smoker than in

non-smokers (Yamaguchi et al., 2005, Bloomer, 2007). Interestingly, Yamaguchi et al (2005) reported that the levels of these oxidative stress markers were further increased within 30 min by smoking one cigarette after quitting smoking at least 10 hr. The results were in contrast to our finding for miRNAs that the exposure to one cigarette smoke did not substantially change the plasma miRNA profile. In addition, our finding is inconsistent with a previous study reporting that urinary genotoxicity was detected 2 hr after smoking one cigarette (De Flora et al., 1996). Although miR-210 and miR-373 are known to be typical miRNAs whose expression in cells was changed in response to hypoxia (Crosby et al., 2009), the levels of these miRNAs in plasma were below the cutoff values in both of smokers and non-smokers in our study. Collectively, there might be a time lag between the changes of intracellular miRNA expression and those of extracellular miRNA levels. Alternatively, consecutive and/or dynamic change of intracellular miRNA expression might be required to be reflected to the change of extracellular miRNA levels. We claim that the differences in the plasma miRNA profiles between smokers and non-smokers could be attributed to repeated smoking. An understanding of the number of cigarettes or the frequency of smoking required to cause changes in plasma miRNA profiles is left for future studies.

It is generally accepted that extracellular miRNAs mirror changes in miRNA expression in cells or tissues (Lee et al., 2012). There are some reports of the effects of cigarette smoke on miRNA expression in tissues (De Flora et al., 2012). Exposure to cigarette smoke caused down-regulation of some miRNAs in mouse and rat lungs (Izzotti et al., 2009a, 2009b), and the down-regulation in mouse lung was reversed by smoking cessation (Izzotti et al., 2011). Similar findings have been reported in humans; the levels of several miRNAs in airway epithelium (Schembri et al., 2009), placenta (Maccani et al., 2010), and alveolar macrophages (Graff et al., 2012) were lower in smokers than in non-smokers. These results lead us to speculate that miRNAs in tissues, including trachea and lung, exposed to cigarette smoke might leak into blood. This might be the reason for higher levels of circulating

miRNAs in smokers. Although it is not known whether extracellular miRNAs are functional, miRNAs undoubtedly function in cells; therefore, changes in miRNA expression in tissues or cells caused by exposure to cigarette smoke may have some pathophysiological significance. Integrated analysis of the expression of circulating miRNAs and the dysregulation of miRNAs and their target genes in tissues could provide insight into the initiation and progression of smoking-related diseases.

Interestingly, we noticed that 24 of the 44 miRNAs that showed a significantly different expression between smokers and non-smokers were previously reported as potential biomarkers of diseases (Table 6). Our observations show that smoking status might lead to incorrect conclusions when circulating miRNAs are used as biomarkers of diseases. Smoking status should therefore be considered when using circulating miRNAs as biomarkers of disease.

In conclusion, we found that cigarette smoking unambiguously alters plasma miRNA profiles. A larger number of miRNAs were detected and their expression levels were higher in smokers than in non-smokers. Because more than half of the miRNAs were reported to be potential biomarkers of diseases, we suggest the possibility that smoking status might complicate diagnosis. The plasma miRNA profiles that mirror changes in miRNA expression in tissues might signal smoking-related diseases. The information presented here provides new insight into an area of future research on circulating miRNAs.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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Table 1. Characteristics of subjects.

| Subject | Age (years) | Height (cm) | Body weight (kg) | Brand of cigarette | Nicotine (mg) | Tar (mg) | Age at starting smoking (years) | Number of cigarettes per day | Number of years smoked (years) | Pack-year |
|---|---|---|---|---|---|---|---|---|---|---|
| S1 | 36 | 168 | 50 | MILD SEVEN | 0.7 | 8 | 20 | 15 | 16 | 12 |
| S2 | 41 | 170 | 64 | MILD SEVEN LIGHTS | 0.7 | 8 | 20 | 20 | 21 | 21 |
| S3 | 35 | 169 | 65 | MILD SEVEN | 0.8 | 10 | 20 | 30 | 15 | 23 |
| S4 | 55 | 177 | 68 | Marlboro | 1.0 | 12 | 20 | 20 | 35 | 35 |
| S5 | 21 | 172 | 80 | Hi-Lite menthol | 0.7 | 10 | 16 | 15 | 5 | 4 |
| S6 | 46 | 165 | 53 | Hi-Lite | 1.4 | 17 | 20 | 20 | 26 | 26 |
| S7 | 38 | 167 | 57 | MILD SEVEN AQUA menthol | 0.1 | 1 | 20 | 10 | 18 | 9 |
| S8 | 37 | 165 | 58 | LARK menthol | 0.1 | 1 | 20 | 20 | 17 | 17 |
| S9 | 40 | 156 | 53 | CABIN | 0.4 | 5 | 18 | 20 | 22 | 22 |
| S10 | 36 | 172 | 55 | MILD SEVEN super LIGHTS | 0.5 | 6 | 20 | 20 | 16 | 16 |
| S11 | 39 | 168 | 65 | KOOL mild | 0.7 | 8 | 16 | 10 | 23 | 12 |
| Mean ± SD | 38.5 ± 8.2 | 168 ± 5.0 | 61.7 ± 8.7 | | 0.6 ± 0.4 | 8 ± 5 | | 18 ± 6 | 19 ± 8 | 18 ± 9 |
| NS1 | 26 | 163 | 65 | | | | | | | |
| NS2 | 39 | 177 | 84 | | | | | | | |
| NS3 | 50 | 164 | 63 | | | | | | | |
| NS4 | 29 | 169 | 62 | | | | | | | |
| NS5 | 41 | 173 | 80 | | | | | | | |
| NS6 | 43 | 161 | 57 | | | | | | | |
| NS7 | 28 | 170 | 67 | | | | | | | |
| Mean ± SD | 36.6 ± 9.0 | 168.1 ± 5.8 | 68.3 ± 9.9 | | | | | | | |

Table 2. The numbers of plasma miRNAs with 40-Ct > 8 in non-smokers (NS) and smokers (S).

| Non-smokers | | Smokers | | Status | | |
|---|---|---|---|---|---|---|
| | | | | Smoking | Quit | Acute exposure |
| NS1 | 174 | S1 | 198 | 198 | 160 | 177 |
| NS2 | 191 | S2 | 216 | 216 | 138 | |
| NS3 | 138 | S3 | 208 | | | |
| NS4 | 138 | S4 | 234 | 234 | 129 | 131 |
| NS5 | 110 | S5 | 189 | | | |
| NS6 | 121 | S6 | 189 | | | |
| NS7 | 129 | S7 | 185 | | | |
| | | S8 | 187 | 187 | 137 | |
| | | S9 | 177 | | | |
| | | S10 | 170 | | | |
| | | S11 | 200 | | | |
| Mean ± SD | 143 ± 29 | Mean ± SD | 196 ± 18*** | 209 ± 21 | 141 ± 13## | |

***$P < 0.001$, compared with non-smokers.

##$P < 0.01$, compared with smoking status in 4 smokers (S1, S2, S4, and S8).

Table 3. Forty-four plasma miRNAs are differently ($P < 0.05$) expressed in 7 non-smokers (NS) compared to 11 smokers (S).

| microRNA | 40 - Ct value (Mean ± SD) | | Fold (S/NS) | *P*-value |
|---|---|---|---|---|
| | NS | S | | |
| miR-374b | 12.75 ± 2.89 | 15.76 ± 1.03 | 8.11 | 0.006 |
| miR-331-3p | 11.72 ± 2.85 | 14.61 ± 1.26 | 7.39 | 0.009 |
| miR-221 | 12.84 ± 2.46 | 15.61 ± 1.57 | 6.83 | 0.010 |
| let-7g | 12.85 ± 1.78 | 15.40 ± 0.89 | 5.89 | 0.001 |
| miR-301a | 10.66 ± 1.79 | 13.09 ± 0.78 | 5.39 | 0.001 |
| let-7e | 15.47 ± 1.07 | 17.74 ± 1.13 | 4.83 | 0.001 |
| miR-335 | 11.21 ± 1.94 | 13.48 ± 0.80 | 4.83 | 0.003 |
| miR-26a | 14.91 ± 1.45 | 17.09 ± 0.91 | 4.51 | 0.001 |
| miR-30c | 15.21 ± 1.13 | 17.35 ± 0.82 | 4.40 | 0.000 |
| miR-185 | 10.65 ± 2.06 | 12.77 ± 0.67 | 4.36 | 0.005 |
| miR-374a | 13.71 ± 1.50 | 15.83 ± 0.96 | 4.36 | 0.002 |
| miR-30b | 15.24 ± 1.20 | 17.36 ± 0.76 | 4.33 | 0.000 |
| let-7b | 14.33 ± 1.06 | 16.43 ± 0.94 | 4.30 | 0.000 |
| miR-451 | 15.62 ± 1.75 | 17.70 ± 1.61 | 4.22 | 0.020 |
| miR-27a | 12.36 ± 2.02 | 14.26 ± 0.48 | 3.74 | 0.008 |
| miR-29a | 12.78 ± 1.32 | 14.64 ± 0.43 | 3.63 | 0.000 |
| miR-191 | 18.18 ± 1.79 | 20.04 ± 0.87 | 3.63 | 0.009 |
| miR-26b | 14.15 ± 1.33 | 15.99 ± 0.75 | 3.59 | 0.002 |
| miR-199a-3p | 14.49 ± 1.54 | 16.30 ± 0.82 | 3.52 | 0.005 |
| miR-425 | 12.11 ± 1.50 | 13.92 ± 0.78 | 3.51 | 0.004 |
| miR-223 | 21.95 ± 1.59 | 23.70 ± 0.45 | 3.36 | 0.003 |
| miR-328 | 13.18 ± 1.91 | 14.87 ± 0.94 | 3.23 | 0.023 |
| miR-21 | 14.41 ± 1.42 | 16.06 ± 0.38 | 3.14 | 0.002 |
| let-7d | 13.32 ± 0.94 | 14.97 ± 0.91 | 3.12 | 0.002 |
| miR-19b | 18.36 ± 1.39 | 20.00 ± 0.56 | 3.12 | 0.003 |
| miR-106b | 13.34 ± 1.71 | 14.92 ± 0.82 | 2.99 | 0.017 |
| miR-19a | 14.53 ± 1.29 | 16.10 ± 0.53 | 2.96 | 0.002 |
| miR-186 | 15.79 ± 1.55 | 17.33 ± 0.60 | 2.92 | 0.008 |
| miR-93 | 14.99 ± 1.67 | 16.44 ± 0.73 | 2.72 | 0.021 |
| miR-454 | 14.12 ± 1.00 | 15.52 ± 0.88 | 2.64 | 0.007 |
| miR-345 | 11.46 ± 1.29 | 12.86 ± 0.71 | 2.63 | 0.009 |
| miR-20b | 15.24 ± 1.44 | 16.60 ± 0.36 | 2.57 | 0.008 |
| miR-17 | 18.67 ± 1.31 | 19.89 ± 0.50 | 2.33 | 0.013 |
| miR-20a | 18.08 ± 1.31 | 19.29 ± 0.58 | 2.32 | 0.015 |
| miR-24 | 18.26 ± 1.58 | 19.43 ± 0.74 | 2.25 | 0.048 |
| miR-106a | 18.58 ± 1.43 | 19.75 ± 0.56 | 2.25 | 0.026 |
| miR-126 | 19.24 ± 1.06 | 20.41 ± 0.75 | 2.24 | 0.015 |
| miR-16 | 20.12 ± 1.09 | 21.26 ± 0.78 | 2.19 | 0.020 |
| miR-25 | 13.92 ± 1.18 | 15.04 ± 0.68 | 2.18 | 0.020 |
| miR-923 | 13.19 ± 1.59 | 14.31 ± 0.58 | 2.16 | 0.016 |
| miR-195 | 14.69 ± 1.10 | 15.79 ± 0.91 | 2.14 | 0.035 |
| miR-126* | 16.28 ± 1.02 | 17.33 ± 0.58 | 2.07 | 0.013 |
| miR-92a | 17.36 ± 0.81 | 18.12 ± 0.64 | 1.70 | 0.039 |
| miR-188-5p | 12.90 ± 0.95 | 12.05 ± 0.74 | 0.55 | 0.047 |

Plasma miRNAs shaded with gray have been reported to be candidate biomarkers of diseases (Table 6).

Table 4. Plasma concentrations of nicotine and cotinine in 4 smokers who quit smoking for at least one month and then smoked again.

| Smoker | Nicotine (ng/ml) | | | Cotinine (ng/ml) | | |
|---|---|---|---|---|---|---|
| | Status | | | Status | | |
| | Smoking | Quit | Acute exposure | Smoking | Quit | Acute exposure |
| S1 | 3.6 | 0.1 | 13.7 | 282.1 | 1.1 | 5.1 |
| S2 | 16.3 | 1.0 | | 23.4 | 0.6 | |
| S4 | 16.7 | 3.3 | 15.7 | 413.0 | 9.5 | 17.9 |
| S8 | 26.0 | 25.9 | | 243.5 | 364.3 | |

Table 5. Sixty-three plasma miRNAs differently ($P < 0.05$) expressed in 4 subjects (S1, S2, S4, and S8) who smoked (S) and then quit smoking (Q).

| microRNA | 40 - Ct values (Mean ± SD) | | Fold (Q/S) | *P*-value |
|---|---|---|---|---|
| | S | Q | | |
| hsa-miR-766 | 15.07 ± 1.48 | 10.44 ± 0.92 | 0.04 | 0.002 |
| hsa-miR-374b | 16.30 ± 0.47 | 11.73 ± 0.87 | 0.04 | 0.000 |
| hsa-miR-340 | 13.41 ± 0.32 | 9.64 ± 1.05 | 0.07 | 0.000 |
| hsa-let-7e | 18.30 ± 1.06 | 14.57 ± 0.50 | 0.08 | 0.001 |
| hsa-miR-191 | 20.53 ± 0.80 | 16.85 ± 0.65 | 0.08 | 0.000 |
| hsa-miR-103 | 13.99 ± 1.22 | 10.34 ± 1.98 | 0.08 | 0.020 |
| hsa-miR-199a-3p | 16.67 ± 0.91 | 13.13 ± 0.46 | 0.09 | 0.000 |
| hsa-miR-625* | 14.33 ± 1.50 | 10.83 ± 0.53 | 0.09 | 0.005 |
| hsa-miR-186 | 17.45 ± 0.47 | 14.01 ± 0.96 | 0.09 | 0.001 |
| hsa-let-7g | 15.68 ± 0.86 | 12.25 ± 1.41 | 0.09 | 0.006 |
| hsa-miR-185 | 12.85 ± 0.54 | 9.43 ± 0.84 | 0.09 | 0.000 |
| hsa-miR-197 | 15.88 ± 0.97 | 12.52 ± 1.36 | 0.10 | 0.007 |
| hsa-miR-331-3p | 15.43 ± 0.91 | 12.19 ± 0.76 | 0.11 | 0.002 |
| hsa-miR-454 | 15.71 ± 0.91 | 12.50 ± 0.33 | 0.11 | 0.001 |
| hsa-miR-374a | 16.15 ± 0.49 | 12.95 ± 0.84 | 0.11 | 0.001 |
| hsa-let-7b | 16.62 ± 1.04 | 13.52 ± 0.70 | 0.12 | 0.003 |
| hsa-miR-495 | 12.63 ± 0.42 | 9.54 ± 0.76 | 0.12 | 0.000 |
| hsa-let-7d | 15.36 ± 0.78 | 12.44 ± 0.70 | 0.13 | 0.001 |
| hsa-miR-484 | 19.64 ± 0.80 | 16.77 ± 0.94 | 0.14 | 0.004 |
| hsa-miR-19a | 16.28 ± 0.18 | 13.51 ± 0.99 | 0.15 | 0.001 |
| hsa-miR-301a | 13.38 ± 0.62 | 10.65 ± 0.76 | 0.15 | 0.001 |
| hsa-miR-26a | 17.63 ± 0.70 | 14.93 ± 0.95 | 0.15 | 0.004 |
| hsa-miR-151-3p | 15.05 ± 1.23 | 12.35 ± 0.30 | 0.15 | 0.005 |
| hsa-miR-335 | 13.88 ± 0.77 | 11.21 ± 0.54 | 0.16 | 0.001 |
| hsa-miR-181a | 17.87 ± 1.20 | 9.21 ± 0.84 | 0.16 | 0.011 |
| hsa-miR-222 | 19.08 ± 0.74 | 16.44 ± 1.15 | 0.16 | 0.008 |
| hsa-miR-28-3p | 14.60 ± 0.79 | 11.98 ± 1.10 | 0.16 | 0.008 |
| hsa-miR-590-5p | 14.43 ± 0.24 | 11.82 ± 0.26 | 0.16 | 0.000 |
| hsa-miR-140-5p | 15.85 ± 0.76 | 13.28 ± 0.67 | 0.17 | 0.002 |
| hsa-miR-30b | 17.77 ± 0.51 | 15.20 ± 0.83 | 0.17 | 0.002 |
| hsa-miR-30d | 12.03 ± 1.26 | 9.47 ± 0.98 | 0.17 | 0.018 |
| hsa-miR-15b | 15.56 ± 0.58 | 13.03 ± 0.78 | 0.17 | 0.002 |
| hsa-miR-20b | 16.63 ± 0.30 | 14.14 ± 0.82 | 0.18 | 0.001 |
| hsa-miR-93 | 16.81 ± 0.69 | 14.38 ± 0.92 | 0.19 | 0.006 |
| hsa-miR-223 | 23.87 ± 0.27 | 21.45 ± 0.45 | 0.19 | 0.000 |
| hsa-miR-342-3p | 17.36 ± 0.42 | 15.00 ± 0.10 | 0.19 | 0.007 |
| hsa-miR-320 | 18.79 ± 0.88 | 16.45 ± 0.71 | 0.20 | 0.006 |
| hsa-miR-28-5p | 12.55 ± 0.73 | 10.23 ± 1.06 | 0.20 | 0.011 |
| hsa-miR-328 | 15.56 ± 0.91 | 13.25 ± 0.08 | 0.20 | 0.002 |
| hsa-miR-24 | 19.90 ± 0.65 | 17.63 ± 0.52 | 0.21 | 0.002 |
| hsa-miR-126 | 20.70 ± 0.57 | 18.57 ± 0.49 | 0.23 | 0.001 |
| hsa-miR-26b | 16.19 ± 0.33 | 14.07 ± 0.80 | 0.23 | 0.003 |
| hsa-miR-152 | 12.97 ± 0.65 | 10.89 ± 0.19 | 0.24 | 0.022 |
| hsa-miR-27a | 14.50 ± 0.37 | 12.51 ± 0.29 | 0.25 | 0.000 |
| hsa-miR-17 | 20.04 ± 0.45 | 18.05 ± 0.65 | 0.25 | 0.002 |
| hsa-miR-30c | 17.78 ± 0.66 | 15.82 ± 0.90 | 0.26 | 0.013 |
| hsa-miR-19b | 20.02 ± 0.28 | 18.21 ± 0.72 | 0.28 | 0.003 |
| hsa-miR-106a | 19.90 ± 0.63 | 18.09 ± 0.39 | 0.29 | 0.003 |
| hsa-miR-20a | 19.41 ± 0.36 | 17.61 ± 0.48 | 0.29 | 0.001 |
| hsa-miR-923 | 14.40 ± 0.85 | 12.66 ± 0.88 | 0.30 | 0.030 |
| hsa-miR-29a | 14.62 ± 0.48 | 12.93 ± 0.33 | 0.31 | 0.001 |
| hsa-miR-345 | 13.14 ± 0.88 | 11.46 ± 0.61 | 0.31 | 0.020 |
| hsa-miR-18a | 13.32 ± 0.53 | 11.64 ± 0.23 | 0.31 | 0.001 |
| hsa-miR-106b | 14.88 ± 0.22 | 16.42 ± 0.43 | 0.36 | 0.001 |
| hsa-miR-16 | 21.06 ± 0.45 | 19.60 ± 0.65 | 0.36 | 0.010 |
| hsa-miR-126* | 17.04 ± 0.35 | 15.60 ± 0.95 | 0.37 | 0.029 |
| hsa-miR-532-5p | 12.25 ± 0.76 | 10.83 ± 0.53 | 0.37 | 0.022 |
| hsa-miR-148a | 12.15 ± 0.66 | 10.90 ± 0.78 | 0.42 | 0.050 |
| hsa-miR-92a | 18.31 ± 0.42 | 17.07 ± 0.65 | 0.42 | 0.019 |
| hsa-miR-21 | 15.98 ± 0.36 | 14.81 ± 0.65 | 0.45 | 0.020 |
| hsa-miR-135a* | 15.80 ± 0.88 | 17.06 ± 0.39 | 2.39 | 0.039 |
| hsa-miR-188-5p | 11.68 ± 0.45 | 13.20 ± 0.12 | 2.86 | 0.001 |
| hsa-miR-138-1* | 11.61 ± 1.31 | 14.36 ± 0.21 | 6.73 | 0.006 |

Plasma miRNAs shaded with gray were more highly expressed in smokers than in non-smokers (Table 3).

Table 6. Circulating miRNAs reported to be potential biomarkers of diseases in humans.

| miRNA | Disease |
|---|---|
| hsa-miR-221 | Colorectal cancer ↑ (Pu et al., 2010), Malignant melanoma ↑ (Kanemaru et al., 2011) |
| let-7g | Breast cancer ↑ (Cookson et al., 2012) |
| hsa-let-7e | Papillary thyroid carcinomas ↑ (Yu et al., 2012) |
| hsa-miR-26a | Pancreatic cancer ↑ (Mahn et al., 2011), Type 1 diabetes ↑ (Nielsen et al., in press) |
| hsa-miR-30c | Acute myocardial infarction ↑ (Meder et al., 2011) |
| hsa-let-7b | Acute myocardial infarction ↑ (Long et al., 2012b) |
| hsa-miR-451 | Systemic lupus erythematosus ↑ (Wang H. et al., 2012), Renal cell carcinoma ↓ (Redova et al., 2012) |
| hsa-miR-27a | Type 1 diabetes ↑ (Nielsen et al., in press) |
| hsa-miR-29a | Type 1 diabetes ↑ (Nielsen et al., in press), Active pulmonary tuberculosis ↑ (Fu et al., 2011), Colorectal cancer ↑ (Huang et al., 2010) |
| hsa-miR-191 | Type 2 diabetes ↓ (Zampetaki et al., 2010) |
| hsa-miR-223 | Nasopharyngeal carcinoma ↑ (Zeng et al., 2012), Systemic lupus erythematosus ↑ (Wang H. et al., 2012), Gastric cancer ↑ (Li et al., 2010) |
| | Hepatocellular carcinoma ↑ (Qi et al., 2011; Xu et al., 2011), Type 2 diabetes ↓ (Zampetaki et al., 2010), Sepsis ↓ (Wang J.F. et al., 2010) |
| hsa-miR-328 | Acute myocardial infarction ↓ (Wang et al., 2011) |
| hsa-miR-21 | Breast cancer ↑ (Si et al., in press), Aortic stenos ↑ (Villar et al., in press), Esophageal squamous cell carcinoma ↑ (Komatsu et al., 2011) |
| | Non-small cell lung cancer ↑ (Wei et al., 2011), Gastric cancer ↑ (Li et al., 2010; Zheng et al., 2011-2012) |
| hsa-miR-106b | Gastric cancer ↑ (Tsujiura et al., 2010) |
| hsa-miR-20b | Non-small cell lung cancer ↓ (Silva et al., 2011) |
| hsa-miR-17 | Nasopharyngeal carcinoma ↓ (Zeng et al., 2012) |
| hsa-miR-20a | Non-small cell lung cancer ↓ (Silva et al., 2011) |
| hsa-miR-24 | Type 1 diabetes ↑ (Nielsen et al., in press), Type 2 diabetes ↓ (Zampetaki et al., 2010) |
| hsa-miR-106a | Gastric cancer ↑ (Tsujiura et al., 2010) |
| hsa-miR-126 | Acute myocardial infarction ↑ (Long et al., 2012a), Type 2 diabetes ↓ (Zampetaki et al., 2010) |
| hsa-miR-16 | Hepatocellular carcinoma ↓ (Qu et al., 2011) |
| hsa-miR-25 | Type 1 diabetes ↑ (Nielsen et al., in press) |
| hsa-miR-195 | Acute myocardial infarction ↑ (Long et al., 2012b), Breast cancer ↑ (Heneghan et al., 2010) |
| hsa-miR-92a | Breast cancer ↓ (Si et al., 2012), Colorectal cancer ↑ (Huang et al., 2010) |

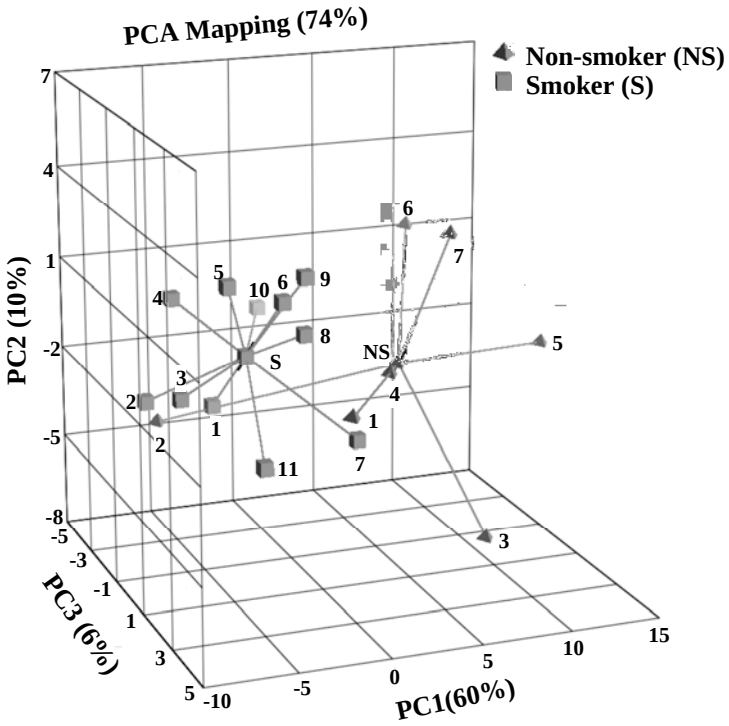
**Figure legends**

Fig. 1. PCA of plasma miRNA expression in 7 non-smokers (NS) and 11 smokers (S). PCA was performed using 66 miRNAs that exceeded the cutoff value in all subjects (n = 18). A three-component model was developed that explained a total of 79% (PC1, 63%; PC2, 10%; PC3, 6%) of the variability of the data. Each ball representing an individual is connected to the centroid (marked as NS or S) of each group. The numbers near the balls represent the subject number. This plot illustrates the level of spread between individuals and groups using three principal components.

Fig. 2. The effects of quitting smoking and of subsequently smoking one cigarette on plasma miRNA profiles. (A) PCA was performed using 66 miRNAs that exceeded the cutoff value in 4 subjects who smoked and who quit smoking, as well as in 7 non-smokers. A three-component model was developed explaining a total of 76% (PC1, 60%; PC2, 10%; PC3, 6%) of the variability of the data. Each ball representing an individual is connected to the centroid (marked as NS, S, or Q) of each group. The numbers near the balls represent the subject number. (B) PCA for the expression of 66 miRNAs in 4 groups: 4 subjects who smoked and who quit smoking, 7 non-smokers and 2 subjects who quit smoking but then smoked one cigarette. A three-component model was developed explaining a total of 74% (PC1, 58%; PC2, 10%; PC3, 6%) of the variability of the data. Each ball representing an individual is connected to the centroid (marked as NS, S, Q, or A) of each group.

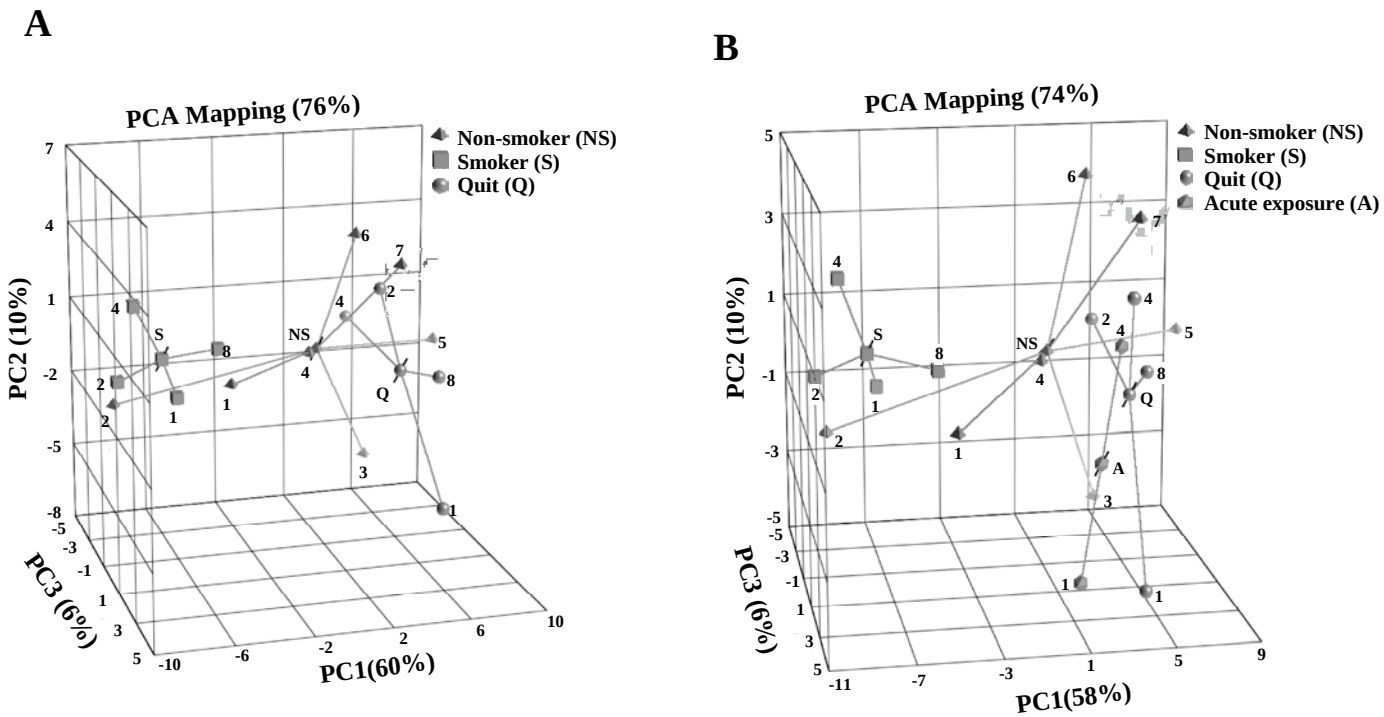
Figure

Fig. 1


PCA Mapping (74%)
Non-smoker (NS)
Smoker (S)
PC2 (10%)
PC3 (6%)
PC1(60%)
NS
S
7
4
1
-2
-5
-8
-5
-3
-1
1
3
5
-10
-5
0
5
10
15

Figure

Fig. 2


A
PCA Mapping (76%)
Non-smoker (NS)
Smoker (S)
Quit (Q)
PC2 (10%)
PC3 (6%)
PC1(60%)
B
PCA Mapping (74%)
Non-smoker (NS)
Smoker (S)
Quit (Q)
Acute exposure (A)
PC2 (10%)
PC3 (6%)
PC1(58%)