Smoking alters circulating plasma microvesicle pattern and microRNA signatures

Sigrun Badryna; Roland Baumgartner; Alice Assinger
Institute of Physiology, Centre for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria

Summary
Circulating plasma microvesicles (PMVs) and their microRNA content are involved in the development of atherosclerosis and could serve as biomarkers for cardiovascular disease (CVD) progression. However, little is known on how smoking influences the levels of PMVs and microRNA signatures in vivo. Therefore, we aimed to investigate the effects of smoking on circulating PMV levels and CVD-related PMV-derived microRNAs in young, healthy smokers. Twenty young (10 female, 10 male; 25 ± 4 years) healthy smokers (16 ± 6 cigarettes per day for 8 ± 4 years) and age- and sex-matched controls were included in this study. While complete blood count revealed no differences between both groups, smoking significantly enhanced intracellular reactive oxygen species in platelets and leukocytes as well as platelet-leukocyte aggregate formation. Total circulating PMV counts were significantly reduced in smokers, which could be attributed to decreased platelet-derived PMVs. While the number of endothelial PMVs remained unaffected, smoking propagated circulating leukocyte-derived PMVs. Despite reduced total PMVs, PMV-derived microRNA-profiling of six smoker/control pairs revealed a decrease of only a single microRNA, the major platelet-derived microRNA miR-223. Conversely, miR-29b, a microRNA associated with aortic aneurysm and fibrosis, and RNU6–2, a commonly used reference-RNA, were significantly up-regulated. Smoking leads to alterations in the circulating PMV profile and changes in the PMV-derived microRNA signature already in young, healthy adults. These changes may contribute to the development of smoking-related cardiovascular pathologies. Moreover, these smoking-related changes have to be considered when microRNA or PMV profiles are used as disease-specific biomarkers.

Keywords
Blood cells, cardiovascular diseases, cell-derived microvesicles, microRNA, smoking

Introduction
Levels of circulating plasma microvesicles (PMVs) correlate with several biomarkers of cardiovascular disease (CVD) (1). PMVs represent the major carrier of microRNAs in plasma, which are selectively packaged and exported (2–4). These PMV-derived microRNAs, which derive from various blood cells or their precursors, can interfere with messenger RNA and thereby fine-tune the expression of a plethora of proteins. Given the important impact of microRNAs on a variety of biological functions, it is not surprising that changes in the PMV profile and microRNA signature are associated with CVD and could therefore serve as an easy-accessible biomarker.

Smoking is a major risk factor for the development of CVD as smoke and/or tobacco components favour the development of inflammation, oxidative stress and thrombosis, alter leukocyte and endothelial functions and lead to platelet abnormalities such as accelerated platelet turnover and increased platelet activation (5, 6).

It is currently unknown how smoking affects the circulating PMV pattern and their microRNA signature. In vitro studies suggest that tobacco smoke increases the release of pro-coagulant tissue factor (TF) positive microvesicles by human monocytes and macrophages (7), while studies on human subjects rather indicate a decrease in PMVs (8,9). Therefore, we determined the effects of habitual smoking on the circulating PMV profile and alterations in the CVD-related PMV-microRNA signature. Young and healthy smokers who were free of any medication or apparent diseases were chosen for this study to determine the early effects of smoking to gain a better understanding for the underlying processes that result in smoking-related pathologies.

Materials and methods
Study population
Twenty young smokers (male n=10, female n=10) and 20 age- and sex-matched controls were enrolled in this study. In accordance
with the Declaration of Helsinki and the approval of the Human Ethics Committee of the Medical University of Vienna (EK112/2009) informed consent was obtained from all participants. All volunteers were apparently healthy and free of any medication for at least two weeks. Inclusion criteria for smokers were consumption of ≥ 10 cigarettes per day for at least one year.

**PMV isolation**

Blood was collected in the morning (8–10 am) from fasting, matched smoker-non-smoker pairs after at least 8 hours of smoking cessation and a brief rest (10 minutes [min]). Blood was drawn with a 21G needle and anticoagulated with trisodium citrate (3.2%). Blood cell count was determined by a KX-21N haemocytometer (Sysmex, Vienna, Austria). Whole blood was centrifuged at 2000 x g for 20 min at room temperature and resulting cell-free plasma (CFP) immediately stored as 600 µl aliquots at −80°C until use. For collective PMV analysis, frozen CFP samples were thawed on ice and centrifuged at 2,000 x g for 20 min. Five hundred µl of the supernatant were centrifuged at 18,000 x g for 30 min to obtain a microvesicle-rich pellet. The PMV-free supernatant was discarded and the pellet re-suspended in 200 µl sterile filtered (0.1 µm) phosphate-buffered saline (PBS).

**Determination of platelet and leukocyte function parameters**

Immediately after blood collection, platelet-leukocyte aggregates (PLA), intracellular reactive oxygen species (ROS) and leukocyte CD11b activation were determined in whole blood using anti-CD61-Alexa647, anti-CD45-FITC, anti-CD11b activated-PE and anti-CD45-Alexa647 (BioLegend; San Diego, CA, USA) or respective isotype antibodies. Moreover, resuspended CD142-FITC (CLB/TF5; Thermo Scientific Pierce, Rockford, IL, USA) staining with 5 µl of either anti-CD61-Alexa647, anti-CD45-Alexa647 or -FITC, anti-CD11b activated-APC (CBRM1/5; BioLegend), anti-CD144-PerCP-Cy5.5 (BD Biosciences), anti-CD142-FITC (CLB/TF5; Thermo Scientific Pierce, Rockford, IL, USA) or respective isotype antibodies. Moreover, re-suspended PMVs were stained with 5 µl Annexin V-FITC in Annexin V binding buffer (BioLegend) or PBS without calcium as control. After 20 min of incubation in the dark, samples were fixed with 0.5% formaldehyde (at final concentration) and analysed in a total volume of 500 µl by flow cytometry. To evaluate cell membrane damages resulting from storage or thawing, which is reflected by f-actin exposure, every batch of samples was incubated for 20 min with phalloidin-FITC (16 µg/ml; Sigma-Aldrich, Vienna, Austria). Freshly isolated and frozen/thawed platelet-rich plasma (PRP) served as references for phalloidin staining (see Suppl. Figure 1, available online at www.thrombosis-online.com) (11). For size control and calibration Megamix beads (0.5, 0.9, 3.0 µm; Biocytex, Marseille, France) were used to set the PMV gate (12). PMVs were defined as events between 0.5 and 0.9 µm and analysed for 60 seconds by flow cytometry. PMV count was calculated according to following formula (13;14): PMV/mL = (event-count in PMV gate/volume sample analysed) * (total volume of the sample/amount of CFP).

**Quantification of TF and TFPI antigens by ELISA**

Plasma antigen levels of tissue factor (TF) and tissue factor pathway inhibitor (TFPI) of 20 smokers and 20 non-smokers were determined by ELISA human coagulation Factor III/TF or human TFPI (DY2339 or DY2974; R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. Assay sensitivity was 7.81 pg/ml for TF and 15.6 pg/ml for TFPI. Plasma concentrations is expressed as pg/ml or ng/ml.

**Analysis of microRNA profile**

Six smokers and six non-smokers were randomly chosen, and PMV isolation was performed as mentioned above. To increase the microRNA yield of each subject, four CFP- aliquots were pooled and lysed with 700 µl QIAzol lysis reagent (Qiagen, Hilden, Germany). All subsequent steps were performed according to the manufacturer’s protocol. Prior to RNA isolation using miRNeasy Microkit (Qiagen), cel-miR-39 (Qiagen) was added to each sample. 10 µL of eluted RNA was subjected to reverse transcription for 1 hour at 37°C using miScript II RT kit (Qiagen) and stored at −20°C. For Real-time PCR, cDNA was thawed on ice and diluted with 90 µl high-grade ddH2O. miScript miRNA PCR Array Human Cardiovascular disease (MIHS-113Z) plates, loaded with primers for 84 CVD-relevant microRNAs, and several controls (cel-miR-39; snoRNAs, snRNAs, RT-control, PCR-control) were used on a StepOnePlus Cycler (Applied Biosystems, Foster City, CA, USA). Raw data were uploaded to Qiagen’s online quantification tool (http://pcrdataanalysis.sabiosciences.com/mirna) and normalised as recommended by the manufacturer on the basis of plate-mean (if CT<30) and spiked cel-miR-39. Using alternative normalisation strategies such as cel-miR-39 in combination with either RNU6-2, mirR-16 or mirR-30e (as suggested in [15]) did not alter the outcome of the results. MicroRNAs with CT>35 were excluded from the analysis.

**Statistical analysis**

Flow cytometric and ELISA data are presented as means ± standard deviation (SD) or depicted as scatter or box plots indicating the median, 1st and 3rd Quartiles. Statistical calculations and graphics were performed with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA) using D’Agostino and Pearson omnibus normality test, paired t-test for normally distributed data or Wilcoxon matched-pairs signed rank test for skewed data. * p-values < 0.05 indicate statistical significance; ** p-values < 0.01 indicate high statistical significance; ns indicates no statistical significance.
Table 1: Study cohort characteristics. Means ± SD; P < 0.01 indicates high statistical significance. ns, no statistical significance; WBC, white blood cell; RBC, red blood cell; PLA, platelet-leukocyte aggregates; ROS, reactive oxygen species.

<table>
<thead>
<tr>
<th></th>
<th>Non-Smoker</th>
<th>Smoker</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25 ± 4</td>
<td>24 ± 4</td>
<td>ns</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.8 ± 4.1</td>
<td>22.4 ± 2.2</td>
<td>ns</td>
</tr>
<tr>
<td>Cigarettes (no./day)</td>
<td>0</td>
<td>16 ± 6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Years of smoking</td>
<td>0</td>
<td>8 ± 4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WBC count (x10⁹/µl)</td>
<td>5.0 ± 1.6</td>
<td>5.3 ± 1.3</td>
<td>ns</td>
</tr>
<tr>
<td>RBC count (x10⁹/µl)</td>
<td>4.1 ± 0.5</td>
<td>4.2 ± 0.4</td>
<td>ns</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>12.6 ± 1.2</td>
<td>13.2 ± 1.3</td>
<td>ns</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>35.6 ± 4.2</td>
<td>36.9 ± 3.4</td>
<td>ns</td>
</tr>
<tr>
<td>Mean RBC volume (fl)</td>
<td>87.7 ± 2.8</td>
<td>87.8 ± 3.2</td>
<td>ns</td>
</tr>
<tr>
<td>Mean RBC haemoglobin (pg)</td>
<td>31.0 ± 2.0</td>
<td>31.5 ± 2.2</td>
<td>ns</td>
</tr>
<tr>
<td>Mean RBC haemoglobin conc. (g/dl)</td>
<td>35.3 ± 1.7</td>
<td>35.9 ± 1.8</td>
<td>ns</td>
</tr>
<tr>
<td>Platelet count (x10⁹/µl)</td>
<td>193.0 ± 67.0</td>
<td>174.0 ± 41.0</td>
<td>ns</td>
</tr>
<tr>
<td>Lymphocyte count (x10⁹/µl)</td>
<td>1.8 ± 0.4</td>
<td>2.1 ± 0.5</td>
<td>ns</td>
</tr>
<tr>
<td>Monocyte count (x10⁹/µl)</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>Neutrophil count (x10⁹/µl)</td>
<td>2.8 ± 1.2</td>
<td>2.8 ± 1.2</td>
<td>ns</td>
</tr>
<tr>
<td>Platelet distribution width (fl)</td>
<td>11.1 ± 1.8</td>
<td>12.1 ± 1.7</td>
<td>ns</td>
</tr>
<tr>
<td>Mean platelet volume (fl)</td>
<td>9.0 ± 1.2</td>
<td>9.5 ± 0.8</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma microvesicle (x10⁶/ml)</td>
<td>1.5 ± 0.6</td>
<td>1.0 ± 0.5</td>
<td>0.0028</td>
</tr>
<tr>
<td>PLA (%)</td>
<td>20.5 ± 1.5</td>
<td>26.4 ± 3.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Intraplatelet ROS (%)</td>
<td>4.3 ± 0.6</td>
<td>7.9 ± 2.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Neutrophil ROS (%)</td>
<td>4.2 ± 0.7</td>
<td>7.9 ± 2.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Monocyte ROS (%)</td>
<td>4.0 ± 0.6</td>
<td>8.2 ± 3.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Neutrophil CD11b activated (%)</td>
<td>9.9 ± 0.7</td>
<td>15.9 ± 3.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Monocyte CD11b activated (%)</td>
<td>10.3 ± 1.1</td>
<td>15.9 ± 3.4</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Results

Alteration in PMV count, platelet and leukocyte activation in healthy smokers

As listed in Table 1, smokers and matched non-smoking controls showed similar blood cell counts, while total PMVs were significantly reduced in smokers, with an effect more pronounced in female subjects (Table 1, Suppl. Figure 2A, available online at www.thrombosis-online.com). The amount of intracellular ROS in platelets and leukocytes and the number of circulating PLA were significantly increased in smokers with a mean percentage of platelet positive leukocytes of 26.4 ± 3.8% compared to 20.5 ± 1.5% in non-smokers (Table 1). Analysis of PLA subpopulations revealed that smoking significantly up-regulated platelet interaction with neutrophils, monocytes as well as lymphocytes (Figure 1A-D).

Quality assurance and determination of apoptotic, phosphatidylserine positive PMVs

To exclude false positive results caused by contaminating cell fragments in frozen/thawed samples, we analysed the percentage of phalloidin-FITC binding to f-actin exposed on particle fragments by flow cytometry. None of the sample preparations exceeded the recommended threshold of 10% phalloidin positive events (11, 16), therefore all samples were included in the study and subjected to further analysis (Figure 2A). To further verify the quality of PMV samples, we determined phalloidin positive events in fresh and frozen PMV and PRP samples (Suppl. Figure 1, available online at www.thrombosis-online.com). In line with Mobarrez et al. (17) we observed that freeze/thawing highly affected platelets, as phalloidin positivity significantly increased in PRP samples from less than 1% to over 30% after one freeze/thaw cycle. However, freeze/thawing had no effect on PMV's (phalloidin positivity of 2.5 ± 2.2% vs 3.0 ± 1.8%) (Suppl. Figure 1, available online at www.thrombosis-online.com). Binding of Annexin V to phosphatidylserine exposed on the surface of PMVs was significantly decreased in smokers (Figure 2B) with on average 35.33 ± 16.91% Annexin V-positive PMVs in non-smokers and 24.57 ± 14.78% in smokers, along with reduced total PMV count (Table 1).

Smoking induced alterations in the PMV pattern

Determination of the cellular origin of PMVs showed that platelet-derived PMVs (pPMVs), the major PMV subpopulation, were significantly reduced in smokers (Figure 3A) and correlated with Annexin V positivity (r = 0.38, p = 0.016), while the level of endothelial-derived PMVs (ePMVs) remained unchanged (Figure 3B). Leukocyte-derived PMVs (lPMVs) were significantly up-regulated by smoking (Figure 3C), with more pronounced effects in female smokers (Suppl. Figure 2C, available online at www.thrombosis-online.com). Moreover, smokers showed an increase in PMVs positive for activated CD11b (Figure 3D).

Plasma levels of tissue factor (TF) and TF pathway inhibitor (TFPI) antigens

In smokers we found a slight, however not significant, increase of TF-bearing PMVs (Figure 4A). When we analysed plasma TF levels of smokers and non-smokers, TF was only detectable in 25% of non-smokers and 45% of smokers (Figure 4B). Moreover, plasma levels of TFPI were also slightly increased in smokers compared to non-smokers (Figure 4C). Plotting plasma levels of
TF versus TFPI revealed that increased TF-levels in smokers were not counterbalanced by higher TFPI levels (▶Figure 4D).

**Altered microRNA signature in smokers**

While most of the 84 CVD-relevant microRNAs in PMVs were unaffected by smoking (Suppl. Figure 3B, available online at www.thrombosis-online.com), miR-29b and RNU6–2 were significantly increased in smokers (▶Figure 5C), and miR223–3p was significantly decreased (▶Figure 5B). Levels of other microRNAs (miR-99, miR-125b, miR-144, miR-24, miR-1, miR-22) were changed more than two-fold, but did not reach statistical significance (▶Figure 5C), independent of normalisation strategy. Notably, miR-149, miR-133a and -b, miR-206, miR-208a and -b, miR302a and –b, and snoRNA could not be detected in PMVs (Suppl. Figure 3, available online at www.thrombosis-online.com).

**Discussion**

Compared to non-smoking controls, young and apparently healthy smokers showed similar blood cell counts, while platelet and leukocyte activation was increased as reflected by elevated numbers of PLA and increased CD11b activation on leukocytes.
Figure 3: Characterisation of PMV subpopulations according to their cellular origin. PMVs derived from platelets (A), endothelial cells (B), leukocytes (C), with surface expression of activated CD11b (D) of non-smokers (n=20) and smokers (n=20). * P < 0.05; ** P < 0.01.

Figure 4: Analysis of tissue factor (TF) and tissue factor pathway inhibitor (TFPI) in smokers and non-smokers. Flow cytometric analysis of TF (CD142)-positive PMVs (A). Scatter plots of plasma antigen levels of TF (B) and TFPI (C) and scatter diagram of TF/TFPI (D) of non-smokers (n=20) and smokers (n=20) quantified by ELISA.
Figure 5: MicroRNA analysis of PMVs from six randomly chosen smoker-non-smoker pairs. Total PMVs/ml of selected smokers (open squares) and non-smokers (open circles) (A). MicroRNAs significantly or at least two-fold decreased (B) or increased (C) and expression panel of detectable PMV-derived microRNAs (D). * P < 0.05.
Smoking is a major risk factor for the development of cardiovascular events. The number of circulating PMVs, usually associated with cellular activation and apoptosis, was decreased in smokers, indicating that smoking somehow interferes with PMV budding and/or release. Annexin V binding to phosphatidylserine is a common marker for early apoptosis but also frequently used as a characteristic marker to define and quantify PMVs. Here, a significant decrease in Annexin V positive PMVs was observed in smokers. However, there is emerging evidence that only a subset of PMVs are positive for Annexin V, while the majority of PMVs, depending on the agonist and mechanism of release, is Annexin V negative (20, 21). Indeed, less than 50% of PMVs were positive for Annexin V in smokers and non-smokers. Furthermore, in vitro studies suggested that nicotine as well as calpeptin – both inhibitors of apoptosis (22) – resulted in a reduced release and Annexin V binding of PMVs in smokers (8). The reduced number of PMVs could be explained by elevated PMV clearance, which is phosphatidylserine-dependent and would be in line with the decrease of Annexin V PMVs in smokers. In general, platelet and megakaryocyte-derived microvesicles are the most abundant subpopulation of PMVs, with IPMs and ePMVs representing only minor fractions (23). In accordance, we found the majority of PMVs in smokers as well as non-smokers derived from megakaryocytes and platelets. While previous studies already indicated a decreased number of PMVs in smokers (8, 9), the microvesicle pattern has never been analysed for its cellular origin. Here, we show that only pPMVs were significantly reduced in smokers, and correlated with Annexin V positive PMVs. Interestingly, pPMVs, which are highly pro-coagulant stimulators of immune and endothelial responses, are increased in various pathologic states (24). It is currently unknown if smoking or (smoking-associated) accelerated platelet turnover has an impact on vesicle budding from megakaryocytes, the major source of pPMVs in healthy individuals (24), which could explain the observed pPMV-decrease in smokers. While we detected no changes in ePMV levels in young smokers, older smokers (>40 years, >19 pack-years) are reported to have slightly elevated levels of ePMVs from pulmonary capillary origin with apoptotic characteristics which correlate with early lung destruction (25), making ePMVs an interesting prognostic marker for early development of emphysema.

Strikingly, we found IPMs significantly elevated in young smokers. LPMVs, but not ePMVs or pPMVs, are reported to be elevated in symptom-free subjects in subclinical stages of atherosclerosis and in patients at high atherothrombotic risk (26, 27), suggesting IPM levels as an independent predictive marker for future cardiovascular events.

Together with increased IPMs, we found a slight increase in PMVs positive for activated CD11b and also TF. TF activity of PMVs is of central importance for the progression of various diseases and it was previously shown that the vast majority of TF activity in atherosclerotic plaques is associated with microvesicles derived from apoptotic leukocytes (28). Moreover, TF immunoreactivity and activity in carotid plaques are increased in smokers (29). In line with previous reports, which showed an increase of circulating TF directly after smoking (30), we found more circulating TF in plasma samples of smokers compared to non-smokers. The effects were not very pronounced, which might be explained by the fact that young, healthy volunteers after at least eight hours of smoking-cessation were investigated. Moreover, we found plasma levels of soluble TFPI slightly elevated in smokers. Increased levels of TFPI reflect either the extent of endothelial perturbation and platelet activation, or a compensatory mechanism for the increased pro-coagulant state observed in cardiovascular diseases (31).

As PMVs are important carriers for microRNAs (2, 4), we investigated in a cohort of six randomly chosen smoker-non-smoker pairs, how smoking affects the microRNA signature in PMVs. Despite a significantly decreased total PMV count in smokers, only miR-223 was significantly reduced. MiR-223 is the most abundant microRNA in pPMVs (32, 33) and the level of miR-223 inversely correlates with the risk of myocardial infarction (17). As miR-223 targets ADP receptor P2Y12, a decrease in miR-223 is associated with a higher on-clopidogrel reactivity (34), which could explain up-regulated expression of P2Y12 on platelets in smokers (35). Platelet microparticle-derived miR-223 can be transferred to endothelial cells, which then targets and down-regulates endothelial FBXW7 and EFNA1 (36), proteins involved in cell division, survival, angiogenesis and adhesion.

In contrast, miR-29b was significantly increased in PMVs of smokers, which might be attributed to the observed increase of IPMs, as miR-29b is reported to be concentrated up to six-fold in microvesicles released by leukocytes compared to their cells of origin (37). MiR-29b is shown to be expressed in atherosclerotic plaque-tissue (38) and up-regulated upon macrophage polarisation (39). Moreover, miR-29b is involved in fibrosis and aortic aneurysm development (40, 41) and positively correlates with matrix-metalloprotease (MMP) 2 and 9 activity (42, 43). In line with these observations, smoking is found to be associated with an increase in MMP2 and MMP9 expression (44), which contributes to plaque progression and instability. Moreover, nuclear radiation from radioactive elements, also found in tobacco and tobacco...
smoke (45), or smoking-induced oxidised lipoproteins, have been identified as inducers of miR-29b (46).

Notably, RNU6–2, commonly used for data-normalisation, was significantly increased in smokers. The up-regulation of RNU6–2 was still observed using different normalisation strategies (as suggested in [15]). Moreover, changes in RNU6–2 levels have been reported previously in healthy men, taking prasugrel and aspirin (33). Furthermore, snoRNAs, included by the manufacturer as alternative reference RNAs, were found to be underrepresented in PMVs by us and others (47).

This study was the first to show that smoking alters the PMV-profile and their microRNA cargo, which might contribute to atherogenesis and could serve as an early biomarker in the context of smoking-related disease. However, the results of our study are limited by the relatively small sample size, which might underestimate the smoking-induced changes of circulating PMVs and microRNAs. Further studies are warranted to elucidate if our results can also be transferred to populations with other risk factors for CVD, or already diseased patients, and to further validate PMV borne microRNAs as diagnostic tool. Notably, one report already found a smoking-related increase of plasma miR-29b in patients with diastolic dysfunction (48), indicating that our observations on smoking-related changes in the microRNA pattern are not limited to healthy populations.

Moreover, our results indicate that smoking might be a considerable confounding factor when PMV profiles or microRNAs are used as disease-specific biomarkers and that normalisation candidates have to be chosen with care when smokers are included in a study population.

Previous studies have already shown that changes in PMV pattern can modulate disease progression and affect protein expression levels (36, 49). However, to date it is not clear if the observed changes in the PMV pattern and microRNA profile are sufficient to induce (patho-)physiological changes, which might contribute to atherogenesis and could serve as an early biomarker in the context of smoking-related disease.

This study provides first evidence that smoking influences the circulating PMV profile and induces changes in the PMV-derived microRNA signature already in young, healthy adults. Smokers showed increased levels of circulating IPMV and miR29b accompanied by decreased pPMVs and miR-223. Further studies are warranted to elucidate the functional impact of smoking-induced changes on PMV amount and composition. For example, it is currently unclear if the observed smoking-induced changes in circulating PMVs and microRNA content are sufficient to modulate protein expression and functions of recipient cells, such as endothelial cells or leukocytes. It has been previously shown that endothelial cells take up PMVs from patients with CVD less efficiently compared to that of healthy individuals (50). It is not known if this can also be attributed to PMVs from smoking individuals. These questions need to be addressed to be able to interpret the smoking-induced changes in PMV pattern and microRNA content and to validate if the observed changes contribute to atherogenesis and could serve as an easy accessible, early biomarker for smoking-related disease.

**Acknowledgements**

The authors wish to thank Dr. Ivo Volf for critical review of the manuscript and Dr. Stefan Heber for technical support.

**Conflicts of interest**

None declared.

**References**

21. Connor DE, Exner T, Ma DD, et al. The majority of circulating platelet-derived microparticles fail to bind annexin V, lack phospholipid-dependent procoagu-