microRNA expression signatures and parallels between monocyte subsets and atherosclerotic plaque in humans

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Summary
Small non-coding microRNAs (miRNAs) have emerged to play critical roles in cardiovascular biology. Monocytes critically drive atherosclerotic lesion formation, and can be subdivided into a classical and non-classical subset. Here we scrutinised the miRNA signature of human classical and non-classical monocytes, and compared miRNA expression profiles of atherosclerotic plaques from human carotid arteries and healthy arteries. We identified miRNAs to be differentially regulated with a two-fold or higher difference between classical and non-classical monocyte subsets. Moreover, comparing miRNA expression in atherosclerotic plaques compared to healthy arteries, we observed several miRNAs to be aberrantly expressed, with the majority of miRNAs displaying a two-fold or higher increase in plaques and only few miRNAs being decreased. To elucidate similarities in miRNA signatures between monocyte subsets and atherosclerotic plaque, expression of miRNAs highly abundant in monocytes and plaque tissues were compared. Several miRNAs were found in atherosclerotic plaques but not in healthy vessels or either monocyte subset. However, we could identify miRNAs co-expressed in plaque tissue and classical monocytes (miR-99b, miR-152), or non-classical monocytes (miR-422a), or in both monocyte subsets. We thus unravelled candidate miRNAs, which may facilitate our understanding of monocyte recruitment and fate during atherosclerosis, and may serve as therapeutic targets for treating inflammatory vascular diseases.

Keywords
microRNAs, atherosclerosis, classical and non-classical monocytes

Atherosclerotic vascular disease remains the number one cause of death and morbidity in the industrialised world. Small non-coding microRNAs (miRNAs) have emerged to play critical roles in cardiovascular biology, and a growing body of evidence supports a significant contribution of miRNAs in the pathogenesis of neointimal lesion formation, atherosclerosis and coronary artery disease (1–5).

Atherosclerosis is widely recognised as a chronic inflammatory disease of the vessel wall with circulating monocytes critically driving the initiation and progression of atherosclerotic lesions (6). Circulating monocytes can be divided into at least two major populations according to their surface expression of CD14 and CD16, described as ‘classical’ CD14+CD16– monocytes (amounting to 80% of blood monocytes) and ‘non-classical’, macrophage-like CD14+CD16+ monocytes (10% of monocytes). These monocyte subsets differ in their chemokine and receptor expression repertoire and features, reflecting distinct mechanisms of recruitment and specialised functions in atherosclerosis and inflammation (6–9). While gene expression profiles of both monocyte subsets have been elucidated, revealing 270 genes to be differentially expressed between subsets (10), the expression pattern of miRNAs in circulating monocytes has not been investigated. We here scrutinised the miRNA signature of human classical and non-classical monocytes. Given the importance of monocyte accumulation in...
Material and methods

Fluorescence-activated cell sorting (FACS) of monocytes

Human monocytes were isolated fromuffy coats from healthy donors and stained with anti-CD14-PE and anti-CD16-FITC antibodies (both BD Biosciences, Franklin Lakes, NJ, USA). Cell sorting of classical CD14⁺CD16⁻ monocytes and non-classical CD14⁻CD16⁺ monocytes was performed using a FACSAria (BD Biosciences, post-sort sample purity > 95%), and sorted monocyte populations were immediately processed for RNA isolation. Approval was obtained from the ethical committee at University Hospital Aachen.

Plaque tissue

Human advanced atherosclerotic plaques were collected during carotid endarterectomy and were kindly provided by Dr. H. Stövenek (Luisen-Hospital, Aachen, Germany) (11) and plaque tissue from five different patients was pooled. Approval was obtained from the ethical committee at University Hospital Aachen.

Chip array

Total RNA was isolated using the TRIzol kit (Invitrogen, Carlsbad, CA, USA). miRNA was purified using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA). miRNA obtained from 10 μg of total RNA from plaque tissue was labelled using the mirVana™ miRNA Labeling Kit (Ambion) and fluorescent Cy3 (Molecular Probes, Eugene, OR, USA), and hybridised to the Ambion mirVana™ miRNA Bioarray (1566 v.1, www.ambion.com/techlib/prot). Scanning of the slides was performed using a dual-laser scanner Affymetrix 428 (affymetrx, Santa Clara, CA, USA) at various amplification settings (gains) above and below saturation of the most intensely fluorescent spots on each array. ImaGene 5.5.4 (BioDiscovery, El Segundo, CA, USA) software was used to quantify the scanned images. The median pixel intensity of the spot was taken after background correction. Monocyte subsets, pooled from five donors, were assayed by full miRNA chip array service by Biocat. miRNAs with more than two-fold up- or down-regulation were selected. Data have been uploaded to the GEO database (accession: http://www.ncbi.nlm.nih.gov/geo, GPL10860 and GPL3444).

Reverse transcription-PCR and real time-PCR

The expression of specific miRNAs in plaque tissue was analysed by quantitative miRNA stem loop RT-PCR technology (Ambion; Applied Biosystems, Foster City, CA, USA). Highly target-specific stem loop structures and reverse-transcription (RT) primer sets (001973 u6 snRNA, 002218 hsa-miR-10b, 000448 hsa-miR-125a, 000407 hsa-miR-26b, 000502 hsa-miR-200a, 000520 hsa-miR-217, 001533 hsa-miR-556, 000509 hsa-miR-205, 002803 hsa-miR-1282, 002870 hsa-miR-1248, 000397 hsa-miR-21, 241070 hsa-miR-196a 000551 hsa-miR-342, 000473 hsa-miR-150, 002248 hsa-miR-142–5p, 000524 hsa-miR-221, 000389 hsa-miR-15a, 000442 hsa-miR-106b, 002863 hsa-miR-1290, 000577 hsa-miR-98, 000421 hsa-miR-30e-5p, 002167 hsa-miR-105, 001119 hsa-miR-520e, 001116 hsa-miR-520b, 000469 hsa-miR-147, 002764 hsa-miR-1225–5P, 001531 hsa-miR-564, 002365 hsa-miR-494, 000488 hsa-miR-189, Applied Biosystems) were used for miRNA amplification, enabling only mature miRNA targets to form RT primer–mature miRNA chimeras to extend the 5’ end of the miRNA. Real-time RT-PCR was performed with real time PCR HT7900 (Applied biosystems).

Statistical analysis

Raw data from both microarrays were normalised by using positive and negative controls on the respective arrays and then transformed into logarithm space before further analysis. Differently expressed miRNAs were identified using LIMMA (12) implemented in statistical language R (13) with a p-value cut-off of 0.05 and log fold change cut-off of 1. Relative expression levels of CD14⁺CD16⁻ or CD14⁻CD16⁺ monocytes and plaque were also estimated and compared by ranking method.

Results

Monocyte subsets were sorted from blood of healthy donors and miRNA expression profiles were analysed in classical CD14⁺CD16⁻ and non-classical CD14⁻CD16⁺ monocytes via Chip Array. Employing the LIMMA package to quantify and analyse microarray data we identified the miRNAs expressed in classi-
Comparison of miRNA expression patterns between both monocyte subsets revealed an exclusive detection of 71 miRNAs in classical and 327 miRNAs in non-classical monocytes (see Suppl. Tables 1 and 2, available online at www.thrombosis-online.com), and 37 miRNAs to be expressed in both monocyte subsets with a differential regulation of two-fold or higher and a p-value greater than 0.05 (see Fig. 1A, B) among all miRNAs analysed (Suppl. Table 3, available online at www.thrombosis-online.com). Expression of randomly selected miRNAs was further verified by RT-PCR technology in both monocyte subsets, with expression levels of U6 snRNA serving as controls (see Suppl. Fig. 1, available online at www.thrombosis-online.com). In addition, the expression of selected miRNAs with the largest and most significant differences between subsets was assessed by quantitative RT-PCR, relative to U6 snRNA. *p<0.05 versus miRNA expression in non-classical monocytes.

Monocyte accumulation within the vessel wall is critical in the development of atherosclerosis (6). While a number of circulating miRNAs have been identified in patients with coronary artery disease and stroke (14, 15) and miRNA expression was studied in neointimal lesion formation (2), miRNA expression profiles within atherosclerotic lesions have not been investigated. We therefore analysed miRNA expression in advanced atherosclerotic plaques obtained from patients during endarterectomy, and in healthy control arteries (A. mammaria interna). We could identify 255 miRNAs to be expressed in atherosclerotic plaques and healthy vessels (Suppl. Table 4, available online at www.thrombosis-online.com). Among these, a total of 23 miRNAs could be observed to be aberrantly expressed in atherosclerotic plaques compared to healthy arteries (Fig. 2A, B); while 20 miRNAs displayed a two-fold of higher increase in expression in plaques compared to healthy arteries, expression levels of only three miRNAs were significantly lower in atherosclerotic plaques compared to healthy arteries. The expression of randomly selected miRNAs in plaque tissue was verified by qRT-PCR, with expression levels of U6 snRNA serving as controls (see Suppl. Fig. 2c, available online at www.thrombosis-online.com). In addition, the expression of selected miRNAs was further verified by quantitative miRNA stem loop RT-PCR technology in both monocyte subsets, with expression levels of U6 snRNA serving as controls (see Suppl. Fig. 1, available online at www.thrombosis-online.com).
and highly regulated miRNAs were analysed by qPCR, confirming lower expression of miR-520b, and miR-105, but substantially up-regulated expression of miR-30e-5p, miR-26b, and miR-125a in plaque tissue compared to tissue from healthy *A. mammaria interna* (Fig. 2C).

Monocytes recruited to the vessel wall carry their miRNAs into the plaque, which may alter the miRNA profile of the inflamed vessel. In order to elucidate parallels between the miRNA profiles of CD14+CD16- and CD14+CD16+ monocyte populations and atherosclerotic plaques, miRNAs, which show positive expression in plaque sample, were hierarchically clustered according to their ranked expression levels (Fig. 3A). Notably, several miRNAs were expressed in healthy vessels and/or atherosclerotic plaques but could not be detected in either monocyte subset (Suppl. Table 5, available online at www.thrombosis-online.com), indicating a predominant expression by vascular cells or non-monocytic leukocytes. Interestingly, among the miRNAs displaying an increased expression in plaque tissue, only miR-422a was concordantly expressed also in classical monocytes, and miR-99b and miR-152 were co-expressed in non-classical monocytes, while 22 miRNAs shared expression with both classical and non-classical monocytes (Fig. 3B and Suppl. Tables 4, 5, available online at www.thrombosis-online.com). These miRNAs within plaques may thus originate from monocytes.

**Discussion**

In the cardiovascular system, miRNAs are expressed in a tightly regulated fashion, and emerging studies have epitomised their importance in cardiovascular pathology and atherosclerosis (3, 16, 17). For instance, miRNAs have been implicated in arterial hyperplasia through the regulation of proliferation and phenotype switching of vascular smooth muscle cells (2, 18) or to confer vascular protection by modulating chemokine expression (19). In addition, a number of circulating miRNAs have been identified in patients with coronary artery disease and stroke (14, 15), and postulated to serve as biomarkers for prognosis or treatment stratifications. The comparative miRNA expression profile of atherosclerotic plaque tissue and monocytes, known to be critical in atherosclerotic lesions formation, however, had not been previously investigated.

Here we surveyed and compared miRNA expression profiles of atherosclerotic plaque tissue and healthy arteries. Notably, only miR-520b, miR-105 and miR-520e displayed significantly higher expression in healthy vessels than in atherosclerotic plaques, in contrast to 20 miRNAs highly expressed in plaque tissue compared to healthy arteries. Differences in miRNA expression may stem from up- or down-regulation in cells of the vessel wall, but may also reflect the inflammatory cell infiltrate of vascular lesions. The
Figure 3: miRNA expression parallels between monocyte subsets and atherosclerotic plaques. A) miRNA expression and unsupervised hierarchical clustering of miRNAs expressed in human non-classical and classical monocytes, and atherosclerotic plaques. A partial heat map depicts distinct patterns of miRNA expression, ranging from lowly (green) to highly ranked expression (red). B) miRNAs expressed in atherosclerotic plaques as well as in non-classical and/or classical monocytes, but not in healthy vessels.
profile specific for healthy arteries may unveil miRNAs involved in the homeostasis of a physiological steady-state that may be therapeutically enforced.

For comparison with plaque material from the carotid artery, specimen from the A. mammaria interna were used as control tissue. Being derived from a smaller, more muscular, and atherosclerosis-resistant vessel, this control may constitute a limitation of our study. As for carotid endarterectomies, however, these specimens were obtained immediately during surgery, thus avoiding post-mortem changes of miRNA expression and deterioration of RNA preparations.

Given the importance of monocytes in the development of atherosclerosis (6), we further elucidated the miRNA signature of non-classical and classical monocyte populations sorted from peripheral blood. While 70 miRNAs were exclusively expressed in classical monocytes and nine miRNAs were identified to be significantly higher expressed in classical compared to classical monocytes, 327 miRNAs could be detected in non-classical monocytes only, and 28 miRNAs were significantly higher expressed in non-classical monocytes compared to classical monocytes.

These data are partially in line with previous findings revealing a high expression of miRNA-16, miR-106b and miR-142–5p (here identified in classical monocytes), low expression of miR-150 and miR-155 (here identified in non-classical monocytes) in blood monocytes (20). Studies emerge to address functions of individual miRNAs. For example, expression of miR-15a and miR-16 has been recently unveiled to decrease considerably during monocyte-macrophage differentiation and to prevent macrophage hyper-activation (21). In addition, miR-15a, miR-16, miR-106b and miR-142–5p were shown to decrease whereas miR-155 increased during differentiation into dendritic cells (22, 23) and during macrophage inflammatory responses (24). Together with the differential expression unraveled in monocyte subsets, these findings imply that these miRNAs may contribute to the maintenance of the circulating monocyte phenotype and to control their differentiation into macrophages or dendritic cells (6). As most of the miRNAs identified in our study, however, have not been investigated, further studies are warranted to elucidate the interrelation and differentiation of monocyte subsets, and their differential functions in inflammation and atherosclerosis. Interestingly, miR-15, miR-16a and miR-106b (preferentially expressed in classical monocytes) and miR-222 (expressed in non-classical monocytes) have been found to be elevated in peripheral blood of young patients after cerebral ischaemic stroke (15), which may be consistent with a monocytic origin. Conversely, miR-155 (preferentially expressed in non-classical monocytes) was significantly down-regulated in the circulation of patients with coronary artery disease (14). This may be attributable to a relative decrease in non-classical monocytes and a shift towards higher classical monocyte counts in the circulation in hyperlipidaemia and atherosclerosis (25).

In order to further elucidate potential parallels in the miRNA expression profiles between monocyte subsets and atherosclerotic plaques, we used an algorithm for comparing miRNA expression. We thereby identified miRNAs that showed an increased expression in plaque tissue compared to healthy vessels, which were not co-expressed in either monocyte subset, indicating their predominant expression in vascular cells or other leukocyte populations. For instance, miRNA-1 has been described to be expressed by smooth muscle cells (26, 27), while miR-10b and miR-218 were allocated to endothelial cells (28, 29). However, differentiation processes of emigrated monocytes may entail alterations with a gain or loss of certain miRNAs, enabling the occurrence of miRNAs not observed in blood monocytes.

Nevertheless, we could identify common miRNAs that were expressed in non-classical and/or classical monocytes with an increased expression in plaque tissue, which may mirror monocyte extravasation into the plaque. Among these, miR-99b and miR-152 were exclusively detected in non-classical monocytes. Interestingly, expression of these miRNAs were previously demonstrated in monocyte-derived dendritic cells (30, 31), which may be in line with the differentiation of non-classical monocytes into dendritic cells within plaques after extravasation (32). Little is known about functions of miR-422a, upregulated in plaque and detected in classical monocytes only. In hepatocytes, this miRNA was involved in controlling cholesterol metabolism (33). Many of the miRNAs commonly detectable in both monocyte subsets and plaque tissue have not been characterised previously. Among these, however, miR-27a was demonstrated to regulate the development of myeloid cells (34), mir-98 functioned to repress cytokine production in macrophages (35), and miR-214 was shown to protect monocytes from apoptosis (36). Interestingly, miR-34a was recently described to be upregulated in human atherosclerotic plaques (37), which, together with our findings, may suggest its monocytic origin.

Notably, most of the miRNAs found to be increased in plaque tissue were co-expressed in classical and non-classical monocytes. Given the predominant recruitment of classical monocytes in atherosclerosis (25, 32), this may reflect the close relationship of both monocyte subsets, or common differentiation fates within lesions.

In conclusion, our study identifies miRNA signatures and candidate miRNAs, which may be of paramount importance in understanding the circuits of regulation controlling monocyte fate, differentiation and recruitment driving atherosclerosis and which may open interesting avenues for the development of therapeutic targeting strategies to treat inflammatory vascular diseases.

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Conflicts of interest
None declared.

References