Circulating ADAM17 Level Reflects Disease Activity in Proteinase-3 ANCA-Associated Vasculitis

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ABSTRACT

ANCA-associated vasculitides are characterized by inflammatory destruction of small vessels accompanied by enhanced cleavage of membrane-bound proteins. One of the main proteases responsible for ectodomain shedding is disintegrin and metalloproteinase domain-containing protein 17 (ADAM17). Given its potential role in aggravating vascular dysfunction, we examined the role of ADAM17 in active proteinase-3 (PR3)-positive ANCA-associated vasculitis (AAV). ADAM17 concentration was significantly increased in plasma samples from patients with active PR3-AAV compared with samples from patients in remission or from other controls with renal nonvascular diseases. Comparably, plasma levels of the ADAM17 substrate syndecan-1 were significantly enhanced in active AAV. We also observed that plasma-derived ADAM17 retained its specific proteolytic activity and was partly located on extracellular microparticles. Transcript levels of ADAM17 were increased in blood samples of patients with active AAV, but those of ADAM10 or tissue inhibitor of metalloproteinases 3, which inhibits ADAMs, were not. We also performed a microRNA (miR) screen and identified miR-634 as significantly upregulated in blood samples from patients with active AAV. In vitro, miR-634 mimics induced a proinflammatory phenotype in monocyte-derived macrophages, with enhanced expression and release of ADAM17 and IL-6. These data suggest that ADAM17 has a prominent role in AAV and might account for the vascular complications associated with this disease.
As a consequence of this excessive amount of inflammatory stimuli in the blood, and through interaction with activated and primed neutrophils, the integrity of the microvascular endothelium is highly disturbed as reflected by increased numbers of detached endothelial cells in patients with active AAV. Another hallmark of active AAV is enhanced proteolytic ectodomain release or shedding of membrane-bound proteins, such as adhesion molecules, members of the TNF-α family, IL-2 receptor, CD30, CD26, and ADAM23. A disintegrin and metalloproteinase domain-containing protein 17 (ADAM17) has emerged as one of the major enzymes responsible for ectodomain shedding. Because one of its first substrates was described as TNF-α, this protease is also known as TNF-α converting enzyme. To date, >70 putative ADAM17-specific substrates have been identified that are involved in inflammatory and regenerative processes of the vasculature (see Dreymueller et al. for a detailed review). Moreover, ADAM17 deficiency resulted in embryonic death, demonstrating the importance of ADAM17-mediated ectodomain shedding during development. The role of ADAM17 in AAV is largely unknown. However, evidence exists that ADAM17 is the putative sheddase that is responsible for many ectodomain cleaving events in AAV. For example, enhanced shedding of vascular endothelial growth factor receptor 1 is implicated in reduced endothelial repair in PR3-AAV, and vascular endothelial growth factor receptor 1 is described to be one of the putative substrates of ADAM17. Also, release of the two ADAM17 substrates, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, is enhanced in active AAV. We, therefore, aimed to characterize the specific role of ADAM17 in PR3-AAV in more detail.

RESULTS
Circulating ADAM17 Protein Levels and Proteolytic Activity in PR3-AAV
One of the characteristics of active AAV is increased serum levels of a number of shed membrane proteins, and evidence exists that the putative sheddase, that is responsible for many of these cleaving events, is ADAM17. Because members of the ADAM superfamily have been found in cell culture supernatants or in human blood samples, we aimed to answer the question of whether we could detect ADAM17 in plasma samples from patients with PR3-AAV and whether plasma-derived ADAM17 levels correlate with disease activity.

ADAM17 was significantly increased in patients with active PR3-AAV (mean, 13,025 pg/mL; range, 261–59,935 pg/mL) compared with patients with PR3-AAV in disease remission (mean, 572 pg/mL; range, 26–3195 pg/mL), patients with membranous glomerulonephritis (mGN) or IgA nephropathy (IgAN) (mean, 518 pg/mL; range, 54–3122 pg/mL), or healthy individuals (mean, 349 pg/mL; range, 56–1114 pg/mL) (Figure 1A, Tables 1 and 2). Western blot analysis revealed that mature ADAM17 (approximately 85 kD) was in the plasma samples and that this ADAM17-specific band attenuated in samples from patients that entered the remission phase (Figure 1A). Paired ADAM17 measurements in 25 patients with PR3-AAV revealed a significant decrease in ADAM17 levels in remission samples taken 11–13 months after the active disease (P=0.004) (Figure 1B). The same kinetics were observed for the ADAM17 substrate syndecan-1. Soluble syndecan-1 in these 25 patients was significantly decreased during the remission phase (P=0.004) (Figure 1C). ADAM17 is described as membrane-associated protease, and membrane localization seems to be mandatory for its proteolytic activity. We therefore wondered whether plasma-derived ADAM17 maintained its specific proteolytic activity. Consistent ADAM17 activity was detected in patients with active AAV. Concordant with the ELISA data, ADAM17 activity was significantly increased in plasma from active patients compared with samples from patients during remission or disease controls (Figure 1D).

ADAM17 Is Located on Extracellular Microparticles
To explain this specific activity we hypothesized that ADAM17 might be located on extracellular microparticles. Flow cytometry demonstrated that stimulation of human dermal microvascular endothelial cells (HDMECs) with TNF-α led to the generation of ADAM17+/ulex europaeus agglutinin 1-positive microparticles in vitro (Figure 2A). Also, cultured monocyte-derived macrophages (MDMs) released ADAM17+/CD14+ microparticles in vitro as shown by flow cytometry and western blot analysis (Figure 2B). ELISA analysis revealed that ADAM17 protein levels are increased in microparticle preparations from patients with AAV compared with samples collected during remission (Figure 2C). In blood samples from patients with AAV we detected predominantly ADAM17+/CD31+/CD42b+/CD14+ microparticles, suggesting that these microparticles probably originate from platelets. We also detected very few ADAM17+/CD31+/CD42b− particles that derived from endothelial cells (Figure 2D).

Cellular and Tissue Localization of ADAM17
The staining pattern for ADAM17 in human PMN revealed a dot-like structure resembling the pattern of the ANCA autoantigen PR3 that is stored in azurophilic granules. In MDMs, ADAM17 could be located in the plasma membrane and also in the intracellular granule-like structures (Figure 3A). Endothelial ADAM17 revealed a very prominent staining in secretory Weibel–Palade bodies of HDMECs as shown by costainings for ADAM17 and vWF or angiopoietin-2, which are both known to be found in Weibel–Palade bodies (Figure 3B). ADAM17 immunostainings in kidney biopsies from patients with IgAN or mGN showed a strong endothelial expression pattern in both the glomerular capillary tuft and in peritubular capillaries. In contrast, the glomerular and peritubular endothelium of patients with PR3-AAV were almost completely devoid of ADAM17. Instead, a strong ADAM17-specific staining in the interstitium could be observed (Figure 3C).
ADAM17 mRNA Expression in Whole-Blood Samples

ADAM17 transcript levels were significantly increased in whole-blood samples from ten patients with active PR3-AAV compared with patients during remission or controls. Western blot analysis revealed mature ADAM17 in plasma samples. Paired ADAM17 measurements in 25 patients at active disease and during remission (1-year follow-up). Soluble syndecan-1 level in the same patients at active disease and during remission. ADAM17 activity is increased in plasma samples from patients with active PR3-AAV compared with patients during remission or disease controls. Cell lysates from MDM or PMN served as positive controls. a, active AAV; DC, disease controls; M, protein ladder; r, remission phase; RFU, relative fluorescence units; SDC-1, soluble syndecan-1. **P<0.01; ***P<0.001.

Table 1. Intra- and interassay variations of ADAM17 ELISA

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<tr>
<th>Sample (pg/ml)</th>
<th>Intra-Assay Precision</th>
<th>Interassay Precision</th>
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<tr>
<td></td>
<td>1250</td>
<td>2500</td>
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<tr>
<td>n</td>
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<tr>
<td>SD (%)</td>
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ADAM17 mRNA Expression in Whole-Blood Samples

ADAM17 transcript levels were significantly increased in whole-blood samples from ten patients with active PR3-AAV compared with the disease control group (P<0.05) or healthy individuals (P<0.01). In contrast, mRNA levels of the closely related ADAM10 were increased in both the PR3-AAV and disease control groups (P<0.01). Analysis of tissue inhibitor of metalloproteinases 3 (TIMP3) revealed no statistically significant regulation between all three groups; however, because of the small number of patient samples used for this analysis, the power of the statistical analysis is low (Figure 4).

MicroRNA Analysis in Whole-Blood Samples of Patients with PR3-AAV

We performed a global microRNA (miRNA) screen from whole-blood samples collected from ten patients with active PR3-AAV and ten patients with mGN or IgAN. The patients with AAV did not receive any immunosuppression therapy at the time point of blood sampling. Significantly deregulated miRNA is listed in Table 3. One miRNA that was differentially expressed in active AAV was miR-634. Quantitative PCR analysis confirmed the miRNA array data (Figure 5A). In vitro, exposure of MDMs or monocytic U937 cells to LPS or TNF-α induced expression of miR-634 (data not shown). Transfection of synthetic miR-634 mimics into MDMs led to enhanced expression or ADAM17 (Figure 5B). Also, release and activity of ADAM17 increased on exposure to miR-634 mimics, whereas inhibition of miR-634 did not have any obvious effect of ADAM17 release or enzymatic activity (Figure 5C). Likewise, production and secretion of the proinflammatory cytokine IL-6 is increased after transfection with miR-634 mimics suggesting that expression of this miRNA is sufficient to induce a proinflammatory phenotype (Figure 5D).

DISCUSSION

One of the hallmarks of AAV is the vast numbers of soluble proteins detected in the blood of active patients. This plethora of soluble proteins includes chemokines and cytokines, growth factors, adhesion molecules, and receptors and significantly contributes to the aggravation of the disease. Although many of these proteins were directly secreted by endothelial cells, platelets, or leukocytes, others resulted from ectodomain cleavage of originally membrane-located receptors and growth factors. Ectodomain release not only serves to regulate the expression of
Table 2. Patient demographics

<table>
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<tr>
<th>Demographic</th>
<th>PR3-AAV Active</th>
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<td>14*</td>
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<td>50.1±12.3</td>
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<td>Birmingham vasculitis activity score</td>
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<tr>
<td>Creatinine (µmol/L)</td>
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<td>105.0 (33–608)</td>
<td>106 (61–415)</td>
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<td>C-reactive protein (mg/L)</td>
<td>37 (1–439)</td>
<td>1 (0.03–121)</td>
<td>3 (1–68)</td>
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<td>White blood cells (1/µl)</td>
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<td>Hemoglobin (g/dl)</td>
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<td>Thrombocytes (1/µl)</td>
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<td>Joint</td>
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<td>n.a.</td>
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<tr>
<td>Ear, nose, and throat</td>
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<td>n.a.</td>
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<td>Lung</td>
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<td>0</td>
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<td>Central nervous system</td>
<td>11</td>
<td>0</td>
<td>n.a.</td>
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</table>

Data are presented as n, mean±SD, or median (range). n.a., not applicable.
*mgN=12; IgAN=2.

cell surface proteins, but it also generates functionally active
soluble proteins that exert diverse biologic functions in several
biologic and pathologic processes (e.g., inflammation, vascular
development, immunity, cell fate determination, cell migration,
wound healing or angiogenesis25,31). In this context, the met-
alloprotease ADAM17 has emerged to be one of the key sheddases
controlling ectodomain release of a large number of substrates
under inflammatory conditions.32

With this work we demonstrated that ADAM17 plays a
significant role in active AAV. This is in line with other findings
assigning ADAM17 an essential role in inflammatory or auto-
immune diseases, such as sepsis, inflammatory bowel diseases,
rheumatoid arthritis, or multiple sclerosis.33 We also showed that
ADAM17 could be detected in plasma samples and, most im-
portantly, that plasma-derived ADAM17 retained its specific ac-

tivity. However, our data suggest that plasma ADAM17 levels
show high variations among the individual patients. Also, cor-
relation analyses revealed no significant correlation between the
plasma ADAM17 level and any clinical parameter. This prompted
us to compare ADAM17 levels in patients at the time point of
active disease and during remission. Remarkably, ADAM17 de-
creased in the 1-year follow-up samples in all patients analyzed.
Soluble syndecan-1, one of the substrates of ADAM17, showed
an almost identical regulation in these patients. Given that other
known substrates of ADAM17, such as soluble CD40 ligand
(CD154) or CD30, also positively correlated with disease activity,
it seems assumable that the plasma ADAM17 level reflects dis-

ease progression and proteolytic activity of ADAM17 in the in-
dividual patient with AAV.10,21

These findings have some pathophysiologic implications.
On the one hand, free circulating active ADAM17 might act on
vascular sites far distal from its place of generation, implying
that a local, closely restricted focus of infl-

amation may radiate and aggravate vas-
cular activation. On the other hand, detecting
plasma ADAM17 levels and activity may help to monitor disease progress in diseases
with vascular involvement, such as sepsis or diabetes.

We here demonstrated that soluble
syndecan-1 is increased in active AAV. Syndecan-1 is a member of the syndecan
family of transmembrane proteoglycans
that is part of the glycocalyx; because of
their interaction with integrins and the
cytoskeleton, they are assigned an im-
portant role in transducing extracellular
signals to the cell.34,35 A recently pub-
lished study demonstrated that endothelial
syndecan-1, because of its mechanosensing
properties, is essential for flow-mediated
endothelial homeostasis and that loss of
syndecan-1 results in an inflammatory
phenotype. Therefore, increased shed-
ing of the syndecan-1 ectodomain by

ADAM17 may aggravate the vascular phenotype in active
AAV.36

Membrane localization of ADAM17 is a prerequisite for its
proper function.37,38 Therefore, we speculated that ADAM17-
specific activity in the blood samples of patients with AAV
depends on its localization on cell-derived microparticles. Mi-
croparticles contain high amounts of lipid rafts, and these
cholesterol-rich membrane microdomains are known to
harbor a huge variety of signaling molecules, receptors, and
proteases, including the mature form of ADAM17.39–41 Lee
and coworkers demonstrated that ADAM17, on its activation
by paxillin, the HIV Nef protein, and the polycomb protein
Eed, is recruited to extracellular particles. After fusion of these
microparticles with blood mononuclear cells, ADAM17-
induced TNF-α release from the target cells.42 It is conceivable
that similar mechanisms of ADAM17 activation and release
take place in active AAV. So far, we do not know which cell
type is mainly responsible for enhanced release of ADAM17-
containing microparticles into the vasculature in patients
with AAV. Fluorescence-activated cell sorting analysis of blood
from patients with AAV revealed that mostly platelet-derived
microparticles, and to a much fewer content endothelial micro-
particles, were positive for ADAM17. In vitro, we observed gen-
eration of ADAM17-bearing microparticles from primary
microvascular endothelial cells and freshly isolated granulocytes
or cultured primary monocytes. In endothelial cells ADAM17
could be assigned to secretory Weibel–Palade bodies, and the
staining pattern of ADAM17 granulocytes and monocytes
resembles that of proteins stored in secretory granules. It is
therefore tempting to speculate that these granules facilitate
the release of ADAM17. Patients with active AAV displayed en-
riched numbers of endothelial microparticles in their
circulation, and Canault and colleagues showed that ADAM17-containing microparticles of atherosclerotic plaques are partly of endothelial origin. On the other hand, expression of ADAM17 is increased in activated leukocytes, and release of neutrophil microparticles is also strongly enhanced in AAV. Moreover, expression of ADAM17, but not of ADAM10, is strongly enhanced in blood cells of patients with active AAV. It is therefore likely that ADAM17-bearing microparticles originated from both endothelial cells and leukocytes.

Figure 2. ADAM17 is located on extracellular microparticles. (A) In vitro TNF-α induced generation of ADAM17-positive microparticles in HDMEC (upper panel). Note that ADAM17+/ulex europaeus agglutinin 1-positive microparticles are only in part positive for Annexin V (lower panel). (B) Similarly, TNF-α induced ADAM17+/CD14+ microparticles in MDM (upper panel). Western blot analysis revealed that microparticles induced on stimulation with TNF-α or LPS carry the mature form of ADAM17. Distribution of the membrane protein Annexin 3 served as control for successful microparticle preparations (lower panel). (C) Microparticles prepared from platelet-poor plasma from patients with active AAV contained enhanced amounts of ADAM17 compared with patients with AAV during remission (p<0.01). (D) Flow cytometric analysis of microparticles prepared from plasma obtained from patients with AAV showing predominantly ADAM17+/CD31+/CD42B+/CD14− microparticles originated from platelets and few ADAM17+/CD31+ positive endothelial cell-derived microparticles. FSC-A, forward scatter; M, protein size ladder; SSC-A, side scatter; Tace, ADAM17. **P<0.01.

Molecular mechanisms that explain the specific inflammatory response in AAV are largely unknown. Hence, we performed a global miRNA screen from whole-blood samples from patients with active AAV. One of the most deregulated miRNA in active AAV, miR-634, unexpectedly induced an inflammatory phenotype in MDM with increased expression and release of the proinflammatory cytokine IL-6 and ADAM17. The exact regulatory mechanisms of this miRNA are still under investigation. Recently, miRNA-145 was described to negatively control ADAM17 expression in patients with renal cell carcinoma. However, we found no differential expression pattern of this miRNA in our miRNA screen.

Strikingly, immunohistologic analysis of ADAM17 distribution in kidney biopsies drew a very complex picture. In biopsy samples of patients with IgAN and mGN, ADAM17 showed a strong expression pattern in peritubular capillaries and capillary loops of the glomerulus. These ADAM17-specific staining patterns were in line with a study by Melenhorst et al., who analyzed ADAM17 mRNA distribution in renal diseases. The staining
patterns of biopsies from patients with active AAV draw a completely different picture. So far, we could only speculate about the nature of these changes. It seems unrealistic that endothelial ADAM17 expression was completely downregulated, and the strong staining of interstitial lesions demonstrated high renal expression level of ADAM17. Nevertheless, the exact mechanisms why the glomerular and peritubular endothelium seems to be completely devoid of ADAM17 are still under investigation.

In conclusion, we have described the specific regulation of ADAM17 expression, release, and activity in active AAV. We suggest a model in which increased plasma ADAM17 correlates with enhanced proteolytic activity and disease progression. We also identified proinflammatory miRNA, which might contribute to enhanced activity of ADAM17 and other proinflammatory cytokines in AAV. Although we are still far from understanding the molecular pathophysiology of AAV, our results might pave the way for a deeper understanding of the molecular processes that are responsible for the massive vascular impairment in AAV.

**CONCISE METHODS**

**Patients**

For determination of plasma ADAM17, a total of 47 patients with active PR3-AAV were enrolled. From 25 patients we obtained paired samples drawn at disease onset and at 11–13 month follow-up (labeled as 1-year follow-up). Disease activity was classified according to the Birmingham vasculitis activity score. Patients with systemic infections were excluded. For analysis of miRNA and mRNA expression, whole-blood samples of ten patients with active PR3-AAV were collected directly after patients were admitted to the intensive care unit or the vasculitis outpatient clinic of Hannover Medical School. Ten patients with either IgAN or mGN or healthy individuals were recruited as controls. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Hannover Medical School. Written informed consent was obtained from all participants. Clinical data are summarized in Table 2.
Blood Sampling and Processing
Peripheral blood was collected in pyrogen-free tubes containing ethylenediaminetetraacetic acid as anticoagulant (Sarstedt, Nümbrecht, Germany). Tubes were immediately inversed and centrifuged at 2,500 g for 10 minutes, and plasma samples were stored at –80°C. For enrichment of microparticles samples were centrifuged at 9,900 g for 10 minutes at 10°C to obtain platelet-free plasma. Microparticles were pelleted by two additional centrifugation steps at 19,900 g for 10 minutes, and pellets were dissolved in Annexin V Binding Buffer (BD Biosciences, Heidelberg, Germany). For determination of whole-blood miRNA or mRNA expression, peripheral blood was collected using PAXgene Blood RNATubes (Preanalytix, Hombrechtikon, Switzerland). The RNA tubes were subsequently inverted several times and stored at –20°C until further processing.

Cell Culture
HDMECs were obtained from PromoCell (Heidelberg, Germany) and cultured in EGM-MV2 medium (PromoCell). Human primary mononuclear and PMN cells were isolated from peripheral blood from healthy volunteers by density-gradient centrifugation over isotonic Biocoll (Biochrom, Berlin, Germany). After hypertonic lysis of the erythrocytes with ice-cold lysis buffer (155 mM NH4Cl, 10 mM NaHCO3, 0.5 mM EDTA, pH 7.4), monocytes were cultured in RPMI 1680 medium supplemented with 10% FCS and gentamycin. After 1 hour, nonadherent cells were removed, and monocytes were cultured for 6–10 days in RPMI 1680 medium supplemented with macrophage colony-stimulating factor. PMN were incubated in HBSS and processed immediately.

ADAM17 Immunoassay
For determination of ADAM17 in serum samples, we established an in-house ELISA using the human TACE/ADAM17 DuoSet ELISA Development kit from R&D Systems (Wiesbaden, Germany). Briefly, PolySorb Immuno Modules (Thermo Fisher Scientific) were coated with an ADAM17 capture antibody at 4°C overnight. After blocking unspecific binding with 1% BSA in PBS for 2 hours, samples were applied to the wells for additional 2 hours at room temperature followed by incubation with the biotinylated detection antibody for another 2 hours. Signals were visualized by adding a streptavidin horseradish conjugate for 20 minutes followed by adding substrate solution for approximately 15–20 minutes. Reaction was stopped by adding 50 μl 2 N H2SO4, and optical density was measured at 450 nm with wavelength correction set to 570 nm (Tecan, Crailsheim, Germany). Inter- and intra-assay variations of this ELISA were between 8.1% and 10.8% (Table 1).

ADAM17 Activity Assay
Activity of serum-derived ADAM17 was analyzed by applying diluted serum samples to the InnoZyme TACE Activity Kit (EMD Millipore, Darmstadt, Germany). The activity assay was performed according to the manufacturer’s protocol, with the only exception being that the incubation time for binding of serum ADAM17 to the immobilized antibody was prolonged to 2 hours.

Flow Cytometry
A detailed protocol for flow cytometry analysis of microparticles can be found elsewhere. Antibodies used for fluorescence-activated cell sorting analysis were a monoclonal mouse anti-human ADAM17 conjugated to phycoerythrin (clone 111633; R&D Systems), mouse anti-human CD42B conjugated to PerCP-Cy-5.5 (clone HIP1; BD Biosciences), mouse anti-human CD14 conjugated to APC (clone MCP9), mouse anti-human CD31 conjugated to Alexa Fluor 647 (clone WM 59), Annexin V conjugated to APC (all from BD Biosciences), and ulex europaeus agglutinin 1 coupled to fluorescein (Vector Laboratories, Burlingame, CA).

Immunocytochemistry
HMVEC or primary human monocytes were seeded onto collagen-coated glass coverslips or on ibidi μ-slides (ibidi, Planegg-Martinsried, Germany). PMNs were cultured on Cytocapture slides (Zell-Kontakt, Nörten-Hardenberg, Germany). Immunostaining and confocal microscopy were performed as previously described. Antibodies used in this study were a polyclonal rabbit anti-human ADAM17 antibody (Biozol), polyclonal goat anti-human ADAM17 antibodies (R&D Systems), a polyclonal goat anti-angiopoietin-2 antibody (R&D Systems), and polyclonal rabbit and sheep anti-human vWF antibodies (Dako, Hamburg, Germany; Abd Serotec, Dusseldorf, Germany).

Immunohistochemistry
One-μm-thick sections of 4% formalin-fixed paraffin-embedded human kidney biopsies were deparaffinized and treated with...
3% hydrogen peroxide in methyl alcohol for 10 minutes to block endogenous peroxidase activity. Staining was performed using a goat anti-human ADAM17 antibody (R&D Systems) and the Vector ABC Elite Kit (Vector Laboratories) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin, mounted, and analyzed using a Zeiss Axioplan 2 (Carl Zeiss Microscopy, Göttingen, Germany).

**Western Blot**

Western blot analysis was performed as previously described. For detection of ADAM17 a polyclonal rabbit anti-human ADAM17 antibody was used (LifeSpan BioSciences, Seattle, WA). As loading control, expression of pan 14–3-3 or annexin A3 as marker for microparticles was determined (Santa Cruz Biotechnology, Heidelberg, Germany).

**miRNA Transfection**

Primary MDMs were transfected with miR-634–specific miScript miRNA Mimics (Qiagen) or a miR-634 miScript miRNA Inhibitor (Qiagen) using HiPerFect Transfection Reagent (Qiagen). miRIDIAN microRNA Mimic Negative Controls were used as controls (Fisher Scientific, Schwerte, Germany).

**miRNA Array Profiling**

miRNA from PAXgene-stabilized whole-blood samples was extracted using the miRNeasy Kit (Qiagen, Hilden, Germany). Quality control of the RNA preparations was done with the Agilent 2100 Bioanalyzer, using the RNA 6000 Nano Kit according to the manufacturer’s instructions (Agilent, Waldbronn, Germany). MiRNA microarray analysis was performed at febit (febit is now CBC Comprehensive Biomarker Center, Heidelberg, Germany) using Geniom Biochip MPEA homosapiens (on the basis of the Sanger miRBase release v16.0 from September 2011, see also http://www.mirbase.org/index.shtml). In brief, RNA was hybridized to the miRNA arrays for 16 hours at 42°C using the Geniom RT Analyzer. After stringent washes the hybridized miRNA was labeled using a microfluidic-based primer extension assay with biotinylated nucleotides. Biotin incorporation was detected with phycoerythrin-labeled streptavidin and febit’s consecutive signal enhancement procedure. Signal recognition (using Cy3 filter set) and signal calculation were done automatically. After background correction array data were normalized using variance stabilizing normalization. These background-subtracted and normalized intensity values were used for all further analyses. For identification of miRNA patterns, clusters were identified by applying miRNA Array Proﬁling.

### Table 3. Differentially regulated miRNAs in active PR3-AAV

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Median AAV</th>
<th>Median DC</th>
<th>qmedian</th>
<th>logqmedian</th>
<th>ttest rawp</th>
<th>ttest adjp</th>
<th>limma rawp</th>
<th>limma adjp</th>
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DC, disease controls; qmedian, median fold change; logqmedian, log median fold change; ttest rawp, raw P value of t test; ttest adjp, adjusted P value of t test; limma rawp, raw P value using empirical Bayes statistic; limma adjp, adjusted P value using empirical Bayes statistic.
hierarchical clustering analysis. First, a similarity matrix was generated containing all pairwise similarities of probes or samples. For similarity measurement, the Euclidean distance was applied. Then a hierarchy of clusters was built and a heat map was generated displaying colored representation of samples and probes, ordered by their similarity with a dendrogram on top (clustering of samples) and on the right side (clustering of probes). Differentially regulated miRNA was calculated using unpaired parametric t test, nonparametric Wilcoxon–Mann–Whitney test or empirical Bayes statistics. All P values were adjusted for multiple testing.

RNA Isolation and Real-Time Quantitative PCR
Isolation of total RNA from primary cells or tissue and real-time quantitative PCR was performed as previously described.50 Total RNA and miRNA from whole blood stabilized in PAXgene Blood RNA tubes were purified using the PAXgene Blood miRNA Kit according to the manufacturer’s instructions (Qiagen). Gene-specific oligonucleotides for ADAM17, ADAM10, and TIMP3 and miScript primers for miRNA-643 were obtained from Qiagen (QuantiTect Primer Assay) with the following corresponding ordering numbers: ADAM17 (QT00055580), ADAM10 (QT00032641), TIMP3 (QT00046382),...
and miRNA-634 (MS0005201). PCR results were normalized to the expression of ribosomal protein 13 A. The sequences of the ribosomal protein 13 A oligonucleotides were 5’-GTGTTTGACCGCATC-CAC-3’ and 5’-CTTCAGGCAAGACCTGA-3’.

**Statistical Analyses**

For comparison of more than two groups of individuals, one-way ANOVA or the nonparametric Kruskal–Wallis test were used depending on data distribution. If significant differences between the groups were found, the Mann–Whitney U test, Bonferroni multiple comparison test, or Dunn multiple comparison test were used to calculate the difference between each pair of groups. Coefficients of correlations were calculated using Pearson or Spearman rank test, depending on the distribution of the data. Two-sided P values were considered statistically significant at $P<0.05$. Statistical analysis was performed using SPSS Statistics 19 (SPSS, Inc., Chicago, IL).

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**Authorship**

T.K. designed the study; T.K. and A.B. wrote the manuscript; S.L. and J.K.P. analyzed the data; H.H. edited the manuscript; M.H., T.K., S.L., and A.B. interpreted data and edited the manuscript; and T.K. supervised the study.

**DISCLOSURES**

None.

**REFERENCES**


