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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Effects of HDL-associated miRNAs on HDL functionality

Anke Hermans

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Clinical Molecular Sciences

SUPERVISOR :

Dr. Marjo DONNERS

dr. Jogchum PLAT

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2018

Transnational University Limburg is a unique collaboration of two universities in two countries: the University of Hasselt and Maastricht University.

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Acknowledgements

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Abstract

Introduction: It is generally accepted that high-density lipoprotein (HDL) exerts atheroprotective functions, such as promoting cholesterol efflux from macrophages and modulating inflammation. However, in several diseases, such as chronic kidney disease (CKD), HDL functionality is compromised and the cause for this dysfunction has not yet been fully clarified. Recently, it has been shown that HDL transports and delivers functional miRNAs to recipient cells and that the levels of these HDL-associated miRNAs are altered in several diseases. Therefore, we hypothesized that CKD patients have an altered profile of HDL-associated miRNAs and that these miRNAs are partially responsible for HDL functions, i.e. modulating inflammation.

Material & methods: MiRNA levels (miR-223, miR92a, miR-135a, miR-33a) were quantified in apoB-depleted serum from CKD patients and hypertensive, non-CKD controls using RT-qPCR. The inflammatory responses of J774 macrophages and endothelial cells (HMEC) were determined by quantification of cytokine production (TNFα and IL-10) using ELISA and by gene expression analysis of chemotactic (CCL2) as well as adhesion molecules (ICAM-1 and VCAM-1), respectively.

Results: The HDL-associated miRNA profile was altered in CKD patients compared to the hypertensive controls. MiR-223 showed a marked decrease with progression of the disease. Furthermore, HDL was able to deliver different miRNAs to macrophages and endothelial cells after enrichment, where some miRNAs then affected the inflammatory response.

Conclusion: In conclusion, the HDL-associated miRNA profile of CKD patients is altered compared to hypertensive controls and these miRNAs contribute to HDL functions.

Table of contents

List of abbreviations

Apo	Apolipoprotein
CETP	Cholesteryl ester transfer protein
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DMEM	Dubecco's modified eagle medium
ESRD	End stage renal disease
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
HDL	High density lipoprotein
HDL-C	High density lipoprotein-cholesterol
HMEC	Human microvascular endothelial cell
ICAM	Intercellular adhesion molecule
IL	Interleukin
LCAT	Lecithine:cholesterol acyltransferase
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
miRNA/miR	MicroRNA
oxLDL	Oxidized LDL
PAF-AH	Platelet-activating factor acetylhydrolase
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PON1	Paraoxonase-1
RCT	Reverse cholesterol transport
SAA	Serum amyloid A
SR-BI	Scavenger receptor type B1
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule

Introduction

High-density lipoproteins

High-density lipoproteins (HDL) have been widely considered to be an independent protective factor against cardiovascular disease (CVD)(1). The inverse relationship between HDL cholesterol levels (HDL-C) and CVD, first described in the Framingham Heart study, was later confirmed by multiple clinical and epidemiological studies (2-4), and it was revealed that CVD risk decreases by 2% in men and 3% in women for each increment of 1 mg/dl in blood HDL-C (5). As a result, HDL has attracted enormous interest and is being intensively studied as a therapeutic target for patients with a high CVD risk. In recent years many beneficial and anti-atherogenic properties have been attributed to HDL, including reverse cholesterol transport (RCT), antioxidative and anti-inflammatory effects. Furthermore, interventions that increase HDL-C concentrations were able to inhibit the development and progression of atherosclerosis in several animal models and in human studies, intravenous infusions of reconstituted HDL were able to promote regression of coronary atheroma.

In spite of these findings, the concept of HDL-C as the “good cholesterol” has recently been challenged by pharmacological interventions aimed at increasing HDL-C levels in humans, as they have yet to succeed in reducing clinical cardiovascular (CV) events. Several agents including niacin, PPAR agonists (fibrates), and cholesteryl ester transfer protein (CETP) inhibitors successfully raised concentrations of HDL-C. In clinical trials, however, they failed to significantly reduce CV events. Furthermore, torcetrapib, a CETP inhibitor able to increase HDL-C concentrations by 50%-100%, increased cardiovascular morbidity and all-cause mortality in the ILLUMINATE trail, resulting in the early termination of the trial (6). These contradictory findings are consistent with genetic evidence. In a Mendelian randomization study, they showed that single-nucleotide polymorphisms associated with increased concentrations of HDL-C are not linked with lower CV risk (7). Moreover, although subjects with the apoA-I Milano mutation have lower HDL-C concentrations, this is not accompanied by higher CV risk (8). Together, these studies indicate that simply raising HDL-C levels is not necessarily beneficial. This suggests that HDL-C levels do not reflect the atheroprotective functions of HDL and therefore, are not an appropriate surrogate biomarker for HDL functionality.

It has been proposed that the heterogeneity of HDL functions is intimately linked with alterations in its composition. This led researchers to investigate HDL components other than cholesterol.

Structure of HDL

HDL are a highly heterogeneous class of lipoproteins consisting of a hydrophobic inner core, containing triglycerides and cholesteryl esters, surrounded by an amphipathic outer layer predominantly consisting of phospholipids, free cholesterol, and apolipoproteins. With a mean size of 8-10 nm and density of 1.063-1.21 g/ml, they are the smallest lipoproteins with the highest density and can be subdivided into several subclasses according to their size, density, composition, or charge (9).

The cargo of HDL is composed of a large variety of proteins, lipids, and to a lesser extent other biologically active molecules, including microRNAs (miRNAs). Compared to other lipoprotein classes, HDL carry a large number of proteins (>80 different proteins) (9). These proteins can be

divided in 4 major subgroups: apolipoproteins, enzymes, lipid transfer proteins and minor proteins.

The major protein moieties of HDL are apolipoproteins, including apolipoprotein (apo) A-I, apoA-II, apoC and apoE. The main protein constituent, apoA-I, accounts for approximately 70% of total HDL protein and contributes to many of HDLs functions. ApoA-I is produced and secreted predominantly by the liver (70%) and to a lesser extent by the small intestine (30%). In addition to its role as a structural protein, apoA-I also enables HDL to perform anti-atherosclerotic functions (10). ApoA-I especially plays a crucial part in mediating cholesterol efflux via interaction with ABCA1 and by activating the enzyme lecithin:cholesterol acyltransferase (LCAT), apoA-I promotes the maturation process of HDL. Furthermore, recent findings suggest that apoA-I also contributes to the antioxidative and anti-inflammatory effects of HDL (11).

The minority of the HDL protein moiety (±20%), consist of enzymes and other proteins, such as the acute phase protein SAA. Despite their low concentrations, these molecules are important for the pleiotropic functions of HDL. Enzymes associated with HDL include LCAT, paraoxonase-1 (PON1), platelet-activating factor acetylhydrolase (PAF-AH), and gluthatione peroxidase (GPx) (9). They contribute to the antioxidative and anti-inflammatory effects of HDL. Lipid transfer proteins, such as CETP, are transported by HDL as well. These proteins can exchange cholesteryl esters from HDL for triglycerides from apoB-containing lipoproteins and therefore, are involved in RCT.

Lately, HDL has been shown to carry miRNAs (12). However, the contribution of the miRNA cargo of HDL to HDL functions has not yet been elucidated.

Biological properties of HDL and impaired functionality

HDL have the capacity to exert a broad spectrum of antiatherogenic functions. The exact mechanisms by which HDL protect against CVD, however, are not yet fully characterized.

Reverse cholesterol transport and cholesterol efflux

The most elusively studied and established atheroprotective function of HDL has been attributed to its pivotal role in RCT. RCT is a multistep process by which excess cholesterol is taken up by HDL from peripheral tissues and cells, including lipid-laden macrophages in atherosclerotic plaques, and subsequently transported to the liver for excretion (**Figure 1**) (13).

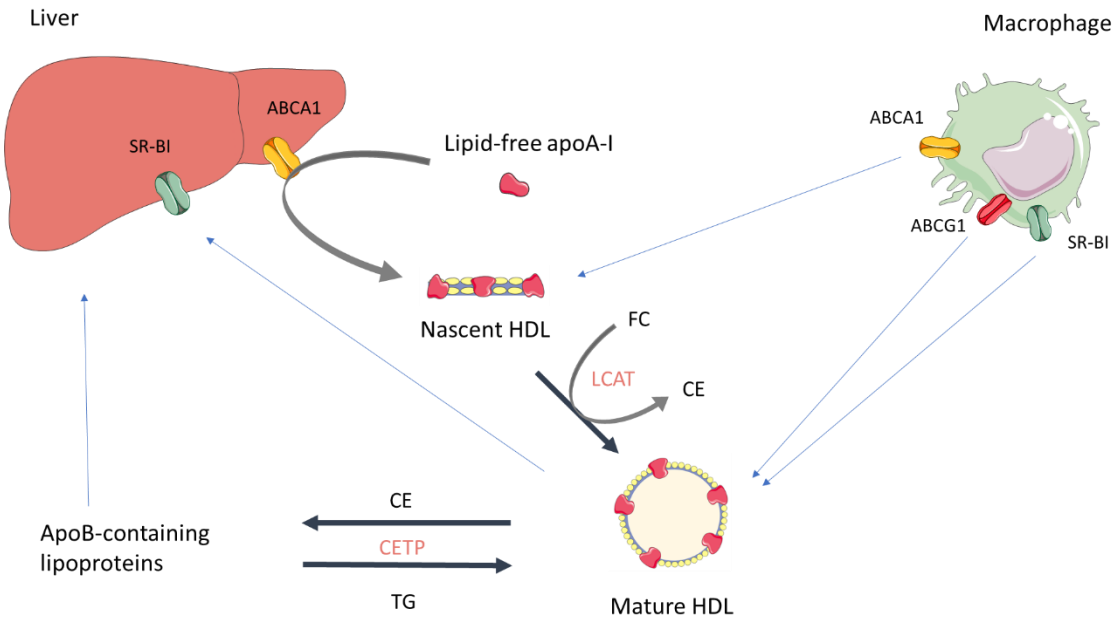


Figure 1 ***Systematic representation of the reverse cholesterol transport by high-density lipoproteins****. ApoA-I is synthesized by the liver and small intestine and released into the circulation. ApoA-1 interacts with ABCA1 to acquire cholesterol and form nascent HDL. Mature HDL is formed after LCAT catalyzes the conversion of free cholesterol into cholesteryl esters that subsequently are sequestered into the hydrophobic core. Cholesteryl esters can be exchanged with triglycerides from apoB-containing lipoproteins through the action of CETP. ABCA1, ATP-binding cassette A1; ABCG1, ATP-binding cassette G1; ApoA-I, apolipoprotein A-1; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; HDL, high density-lipoprotein; SR-BI, scavenger receptor type B1; TG, triglycerides.*

Initially, lipid-poor apoA-I acquires free cholesterol and phospholipids via interaction with ABCA1, resulting in the formation of nascent HDL. These discoid shaped HDL particles consist of a lipid bilayer and are further lipidated by interaction with ABCA1. Subsequently, LCAT catalyzes the conversion of free cholesterol into hydrophobic cholesteryl esters. As a result, the cholesteryl esters migrate towards the core, which converts nascent HDL particles into spherical, mature HDL. Mature HDL then further increases their size by binding to receptors ABCG1 and scavenger receptor class B type I (SR-BI) on peripheral cells. Finally, HDL deliver cholesterol to the liver for excretion in bile. Hepatic uptake of cholesterol can be through direct uptake of cholesteryl esters via SR-BI or indirectly where cholesteryl esters are first exchanged with triglycerides from apoB-containing lipoproteins by CETP and subsequently taken up by the hepatic LDL receptor.

Biological properties independent of cholesterol efflux

Besides promoting cholesterol efflux, HDL is well-known to exert many other potentially atheroprotective actions (**Figure 2**). These include the ability to inhibit vascular inflammation, ameliorate endothelial cell functions, and preventing oxidation of LDL (14).

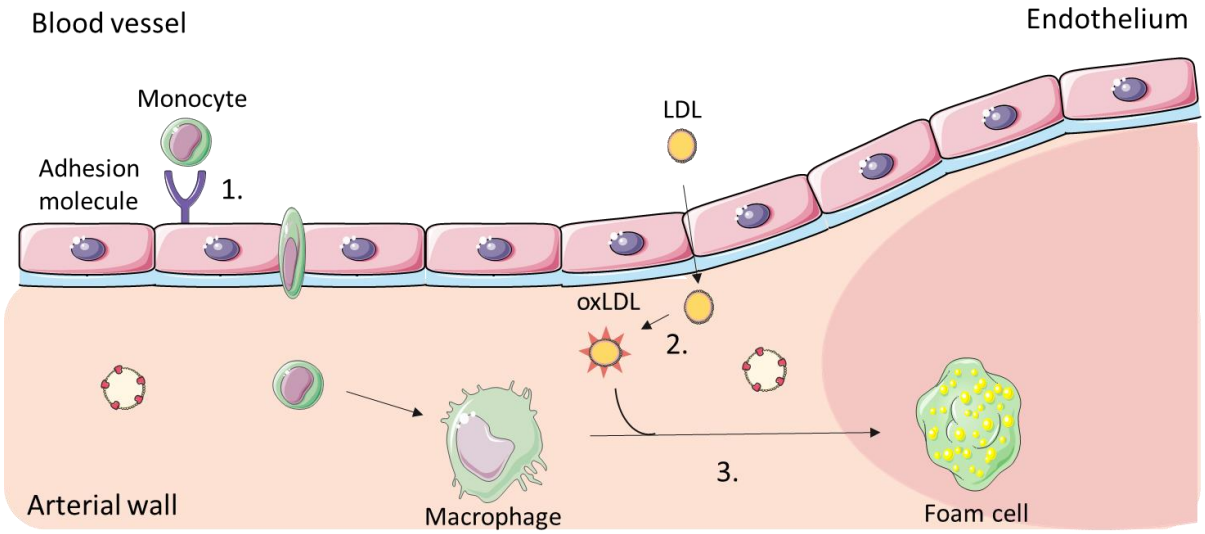


Figure 2 ***Simplified representation of the vasoprotective functions of HDL.*** *In the vascular wall, HDL protects against atherosclerosis by exerting several protective functions. 1) HDL inhibits the expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin on endothelial cells as well as the expression of chemokines CCL2 and CCL5. 2) HDL prevents modification of LDL into oxLDL and prevents uptake by macrophages. 3) HDL slows progression of atherosclerosis by promoting cholesterol efflux of macrophages, therefore reducing the formation of foam cells.*

In contrast to HDL, low-density lipoproteins (LDL) are often referred to as “bad cholesterol”. LDL are easily deposited into the extracellular matrix, where they are prone to oxidation. Oxidated LDL (oxLDL) then induce endothelial damage, stimulating the expression of adhesion molecules. Damaged endothelial cells express intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin. Circulating monocytes bind to these adhesion molecules and migrate towards the subendothelial space following a chemokine gradient, mainly CCL2 and CCL5, also produced by endothelial cells in response to oxLDL. Once in the subendothelial space, monocytes differentiate into macrophages and are able to bind and internalize oxLDL. This leads to the formation of lipid-laden macrophages or foam cells, which is a hallmark of atherosclerosis. The foam cells secrete cytokines that increase the expression of adhesion molecules on the endothelial cells, attracting additional monocytes to the arterial wall (15).

In vitro, HDL has the ability to inhibit inflammation of endothelial cells. In cultured endothelial cells, HDL inhibits binding of monocytes, inhibits the expression of CCL2, and suppresses the expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin after treatment with pro-inflammatory cytokines (e.g. TNF-α) (16, 17). The proposed mechanism by which HDL inhibits adhesion molecule expression is by inhibiting the enzyme sphingosine kinase and therefore, inhibiting the nuclear translocation of NF-κB (18). Furthermore, the anti-inflammatory effects of HDL on endothelial cells have also been demonstrated *in vivo*. Administration of reconstituted HDL to patients with type-2 diabetes, ameliorated the anti-inflammatory effects of HDL (19).

In contrast to the clear anti-inflammatory effects on endothelial cells, HDL have shown contradicting effects on macrophages. Anti-inflammatory effects on macrophages were observed for both native as well as reconstituted HDL (20, 21). Furthermore, pretreatment with

HDL inhibits M1 (pro-inflammatory) macrophage polarization and induces expression of M2 (anti-inflammatory) macrophage markers (22, 23). ABCA1 expressing macrophages pretreated with apoA-I suppress LPS-induced production of inflammatory cytokines TNFα, IL6, and IL-1β (24). In 2015, Song *et al* demonstrated that SR-BI mediates anti-inflammatory effects of HDL on THP-1 macrophages by inhibiting NF-κB activation, a similar mechanism as reported in vascular smooth muscle cells (25, 26). Recently, however, our group reported clear pro-inflammatory effects of HDL on murine macrophages using both native HDL and reconstituted HDL (27).

Dysfunctional HDL and chronic kidney disease

HDL has a variety of functions that are potentially atheroprotective. However, with HDL raising drugs failing to effectively reduce cardiovascular events, it became apparent that HDL-C concentrations are not an optimal therapeutic target as they do not reflect the functionality of HDL. Accumulating evidence indicates that the properties of HDL are highly heterogenous and that HDL may be rendered dysfunctional. The concept of "dysfunctional HDL" was used for HDL that was unable to properly perform its antiatherogenic actions. The term was first used by Van Lenten *et al* when HDL of rabbits and humans showed proinflammatory effects during the acute phase response (28). Since then, dysfunctional HDL has been described in several (chronic) inflammatory diseases, including diabetes mellitus and chronic kidney disease (CKD)(29, 30).

Patients with CKD are a typical population with an elevated risk of cardiovascular mortality due to accelerated atherosclerosis. The increased predisposition of these patients is mostly due to inflammation, oxidative stress, and dyslipidemia. Dyslipidemia in CKD is characterized by elevated triglycerides and decreased levels as well as dysfunctional HDL. Unlike HDL from healthy subjects, HDL from patients suffering from CKD have impaired RCT, anti-inflammatory, and anti-oxidative effects (31). Furthermore, the composition of their HDL particles is altered. ApoA-I levels are reduced as a result of displacement by SAA, leading to impaired cholesterol efflux. Furthermore, the activity of PON1, LCAT and GPx are lowered (31). This results in enhanced expression of adhesion molecules on endothelial cells, upregulation of pro-inflammatory mediators, including CCL2, as well as smaller and denser HDL particles due to delayed maturation.

HDL-associated microRNAs

MicroRNAs (miRNAs) are small, non-coding RNAs of ~18-22 nucleotides in length which can post-transcriptionally regulate gene expression by suppressing translation and/or by promoting mRNA degradation through pairing with partially complementary sites in the 3' untranslated region (UTR) of target mRNAs. MiRNAs can also be released into the circulation. Circulating miRNAs have been shown to be highly stable in body fluids, including blood, due to their association with extracellular vesicles, protein complexes or lipoproteins (e.g. HDL) (32). In 2011, Vickers *et al* proved that HDL not only transports endogenous miRNAs but can also deliver miRNAs to recipient cells with functional gene regulatory consequences (12). In a pilot study, our group determined a panel of HDL-associated miRNAs that consists of miRNA (miR)-223, miR-135a, miR-92a, and miR-33a.

In healthy persons, miR-135a has been identified as the most abundant miRNA in HDL (12). Furthermore, circulating miR-135a has been associated with atheroprotective effects. For example, miR-135a targets KLF4 in senescent vascular smooth muscle cells and thereby

supresses calcification of these cells (33). Additionally, some of its predicted target sites are involved in lipoprotein metabolism, including CETP (34).

In contrast to miR-135a, miR-223 has been shown to be the most abundant miRNA associated with HDL in familial hypercholesterolemia (12). Overexpression of miR-223 is able to repress cholesterol biosynthesis, and cholesterol uptake (by targeting SRBI) as well as indirectly promoting cholesterol efflux through upregulation of ABCA1 (35). By suppressing ICAM-1, it also exerts anti-inflammatory effects on the endothelium (36). Additionally, in macrophages, miR223 inhibits Pknox1, which leads to suppression of their pro-inflammatory effects (37).

MiR-92a is part of the miR17-92 cluster. These miRNAs have shown to exhibit atherogenic properties. It has been previously observed that miR92a is associated with HDL and that its abundance can discriminate between stable and unstable coronary artery disease (38). Furthermore, miR92 was highly expressed in atherosclerotic-prone regions and highly specific for atherosclerotic endothelium. In 2014, Loyer *et al* identified the transcription factors KLF2 and KLF4 as targets of miR92a (39). Through binding to the miR92a recognition elements of mRNA of KLF2 and KLF4, miR92a negatively regulates these transcription factors, resulting in upregulation of cell adhesion molecules or inhibition of the NFκB pathway, respectively, and subsequently resulting in inflammation (39).

Lastly, miR-33a is an important miRNA in atherosclerosis. Inhibition has been associated with increased circulating HDL levels, and reduced plaque size and anti-inflammatory effects (40-42). However, the presence of this miRNA in HDL has not been reported.

Since it was reported that HDL transport and deliver miRNAs to recipient cells, several studies have investigated the differences in miRNA profiles in several cardiovascular disorders, including familial hypercholesterolemia, CAD, and ACS. However, hardly any studies have tried to examine the effect of these miRNAs on HDL functionality. In an attempt to further elucidate the causes of HDL dysfunction and possibly provide new biomarkers or therapeutic targets, we determined the HDL-associated miRNA profile of CKD patients and investigated the role of HDL-transported miR-223, miR-92a, miR-135a, and miR-33a on modulation of inflammation in macrophages and endothelial cells.

Material and methods

Study design

Samples were acquired through collaboration with the "Institute for Molecular Cardiovascular Research" (IMCAR) from the University Hospital RWTH Aachen. Serum was collected from a cohort consisting of 27 individuals with varying stages of CKD 5 CKD2, 7 CKD3a, 7 CKD3b, 6 CKD4, 2 CKD5), classified based on their glomerular filtration rate (GFR) (43), and 7 hypertensive, but otherwise healthy control subjects.

ApoB depletion

Serum from patients with CKD and hypertensive subjects was depleted of apoB-containing lipoproteins, including LDL and VLDL, using polyethylene glycol (PEG) precipitation as previously described (44). Briefly, 1 ml (whole) serum was incubated with 400 µl of a 20% v/v PEG solution (ChemCruz, molecular weight 6,000) for 20 minutes. Then, the mixture was centrifuged at 10,000 rpm for 30 min and the supernatant, which contains HDL, was recovered while the pellet containing apoB lipoproteins was discarded. The concentration of apoA-I was measured using ABX Pentra 400 (Horiba ABX).

MicroRNA analysis

RNA isolation

To determine miRNA expression in HDL, total RNA was isolated from apoB-depleted serum using the the mirVana™ PARIS™ Kit (Ambion). In brief, to 200 µl serum, an equal volume of denaturing solution was added. An exogeneous spike-in control, *Caenorhabditis elegans* (cel)-miR39 (1.6 µl of 5nM, RNA oligonucleotide, Sigma-Aldrich), was added for unbiased normalization and the mixture was incubated on ice for 5 minutes. Then, the lysate was combined with an equal volume of acid-phenol:chloroform. After thorough mixing, the samples were centrifuged for 15 min at 14,000 g at room temperature. Then, RNA was recovered and washed with 1.25 volumes of 100% ethanol prior to passing the lysate/ethanol mixture through a filter cartridge. Following 3 wash steps with the provided wash solutions, RNA was eluted from the filter with 50 µl preheated (95°C) nuclease-free water. The concentration and purity of the RNA were determined spectrophotometrically using Nanodrop (ND-1000 spectrophotometer) before storing at -80°C.

Reverse Transcription qPCR (RT-qPCR) analysis

To quantify mature miRNAs in apoB-depleted plasma, total RNA was reverse transcribed into cDNA using the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems). In brief, to 6 µl of total RNA, 9 µl of the corresponding Mix solution (1,5 µl 10X RT buffer, 0.15 µl 100mM DNTPs, 3 µl RT primer miRNA of interest (5X), 3 µl 5X RT primer cel-miR-39, 0.19 µl RNase inhibitor, 1 µl Multiscribe RT enzyme, and 0.16 µl nuclease-free water) was added.

The reverse transcription product was used for the detection of miRNA expression by real-time PCR using individual Taqman MicroRNA Assays (Applied Biosystems) and the Applied Biosystems 7300 Real-Time PCR System. For this, 10 µl of the Taqman Universal PCR Master Mix II (2x) no AmpErase UNG (Applied Biosystems), 1 µl of primer for the miRNA of interest and cel-miR-39 (20X), and 5 µl water were added to 3 µl of the reverse transcription product. Relative expression levels of miRNAs were calculated after normalization against the exogeneous spike-in control, cel-miR-39, using the comparative threshold (Ct) method ($2^{-\Delta\Delta Ct}$) (45).

HDL enrichment with miRNAs

For analyzing the effect of HDL-associated miRNAs in non-pathological conditions, commercial HDL (cHDL, Genway) was loaded with mature miRNAs. Here, 80 µg cHDL was incubated with mature, single stranded miRNAs (200 or 400 pmol hsa-miR-223-3p, hsa-miR-92a-3p, hsa-miR-135a-3p, or hsa-miR-33a-3p) overnight at 37°C while rotating. Prior to applying the HDL-miRNA complexes to the cells, unbound miRNAs were removed using an Amicon® Ultra-0.5 centrifugal filter with 10k cut-off.

Table 1. List of miRNA sequences.

miRNA	Sequence
hsa-miR-223-3p	5'-UGUCAGUUUGUCAAAUACCCCA-3'
hsa-miR-92a-3p	5'-UAUUGCACUUGUCCCGGCCUGU-3'
hsa-miR-135a-3p	5'-UAUAGGGAUUGGAGCCGUGGCG-3'
hsa-miR-33a-3p	5'-CAAUGUUUCCACAGUGCAUCAC-3'

Effects on endothelial inflammation

Cell culture and treatments

A human microvascular endothelial cell line, HMEC-1, was used to study the effect of HDL on endothelial inflammation. HMEC-1 were cultured on gelatin-coated culture flasks in MCDB 131 medium (Thermo Fisher Scientific) supplemented with 10% FBS, 1% v/v penicillin/streptomycin, 1 µg/ml hydrocortisone (Sigma), 50 µg/ml ECGF (Reliatech), and glutamine at 37°C with 5% CO_2. In order to examine the effect of patient HDL on endothelial inflammation, HMEC were seeded in 48-well plates (50,000 cells/well) and allowed to adhere for 24h prior to treatment. Next, the cells were incubated in the absence or in the presence of HDL^{HC}, HDL^{HT} or HDL^{CKD} (300 µg/ml) for 24h. After 20h, inflammation was induced by adding TNFα (2.5 ng/ml, R&D systems).

Gene expression

RNA extraction from HMEC cells was performed using TRIzol (Thermo Fisher Scientific) followed by alcohol precipitation. Briefly, cells were lysed and homogenized using TRIzol and chloroform. Then, the lysate was incubated for 15 minutes prior to centrifugation (15 minutes, 12,000 g, 4°C) to separate the phases. The upper aqueous phase, containing RNA, was collected and precipitated with isopropanol overnight at -20°C. Then, the mixture was centrifuged for 15 minutes at 12,000g at 4°C. The RNA pellet was washed with 70% cold ethanol followed by resuspension in 25 µl nuclease-free water. RNA concentrations were determined by Nanodrop 2000 (Thermo Fisher Scientific).

Reverse transcription was carried out on 250 ng total RNA using the iScript™ cDNA synthesis kit (Bio-Rad) according to manufacturer's guidelines. SYBR Green chemistry-based qPCR was performed according to manufacturer's guidelines using the CFX96 Real-time system (Bio-Rad). Gene expression data were calculated relative to the GAPDH following the $2^{-\Delta\Delta CT}$ method.

Table 2: List of primer sequences

	Forward primer	Reverse primer
ICAM-1	5'-CCTTCCTCACCGTGTACTGG-3'	5'-AGCGTAGGGTAAGGTTCTTGC-3'
VCAM-1	5'-TGAATCTAGGAAATTGGAAAAAGG-3'	5'-TGAATCTCTGGATCCTTAGGAAA-3'
CCL2	5'-AGTCTCTGCCGCCCTTCT-3'	5'-GTGACTGGGGCATTGATTG-3'
GAPDH	5'-AGCCACATCGCTCAGACAC-3'	5'-GCCCAATACGACCAAATCC-3'

ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Effect on macrophages

Cell culture and treatments

For analysis of cytokine secretion, murine J774.A1 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, cat# 31966021) supplemented with 10% fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin. Cells were seeded in 24-well plates with a density 250,000 cells/well. Then, cells were incubated with apoB-depleted serum from a healthy volunteer (HDL^{HC}), hypertensive controls (HDL^{HT}), or CKD patients (HDL^{CKD}) (300 µg/ml) or with cHDL (300 µg/ml) enriched with 0, 200, or 400 pmol miRNAs for 24h, washed and stimulated with LPS (10 ng/ml) for 6h before collecting the conditioned medium. All cells were maintained at 37°C with 5% CO_2.

ELISA

Secreted levels of IL-10 (Invitrogen) and TNFα (Invitrogen) in conditioned medium from J774 cells were measured by enzyme-linked immunosorbent assays (ELISA) according to manufacturer's guidelines. Fluorescence was read at 450 nm on an iMark™ Microplate Absorbance Reader (Bio-Rad).

Statistical analysis

Results are expressed as mean ± standard error of mean. Statistical differences between groups were assessed using Kruskal-Wallis unless stated otherwise. To assess the correlation between HDL-associated miRNAs and their effects on gene expression or cytokine production, Spearman correlation was used. Statistical analyses were performed using Graphpad Prism 6 and SPSS. P values < 0.05 were considered as statistically significant

Results

Characteristics of the study population

The study population characteristics are summarized in **Table 3**. There were no significant differences in the ages of the subjects. For CKD3a and CKD5, there were significantly more males than females. Serum of patients with CKD2-4 had slightly lower apoA-I concentrations compared to the hypertensive subjects and the apoA-I levels of patients with CKD5 were significantly lower than those of the hypertensive subjects.

Table 3. Characteristics of the study population

	Hypertensive control (n = 7)	CKD2 (n = 5)	CKD3a (n = 7)	CKD3b (n = 7)	CKD4 (n = 6)	CKD5 (n = 2)	*P value*
Age (year)	63 ± 14	67 ± 8	71 ± 10	73 ± 11	62 ± 10	72 ± 10	*> 0.05*
Gender (% male)	57	20	86*	43	67	100*	
Statin medication (%)	57	60	71	100	100	100	
ApoA-I (g/l)	1.48 ± 0.09	1.42 ± 0.16	1.32 ± 0.06	1.35 ± 0.12	1.32 ± 0.08	0.68* ± 0.02	*0.03**
GFR (ml/min/1.73 m²)	>90	74 ± 9.11	51 ± 4.04	36 ± 3.14	25 ± 3.77	<15	
CRP (mg/ml)	2.83 ±1.67	5.3 ± 3.02	2.58 ± 1.13	5.24 ± 2.33	5.46 ± 1.22	5.95 ± 3.55	*> 0.05*

GFR, glomerular filtration rate; CRP, C-reactive protein; Values are presented as mean ± SEM or %. Kruskal-Wallis test was performed for Age, ApoA-I, and CRP levels, followed by a post hoc test (Dunnett t-test). * $p < 0.05$

HDL-associated miRNA levels are decreased in HDL from CKD patients compared to hypertensive controls

Previously, it was reported that HDL contains miRNAs and that the HDL-miRNA signature is altered in familial hypercholesterolemia and coronary artery disease (12, 38). To determine whether the HDL-associated miRNA profile of CKD patients is also altered, miRNA levels were quantified in apoB-depleted serum acquired from 7 hypertensive controls and 27 individuals with varying stages of CKD using RT-qPCR (**Figure 3**). After adjustment for apoA-I concentrations, miRNA-223 shows a gradual decrease in abundancy with disease progression (**Figure 3a**), indicating that miRNA-223 is negatively correlated with CKD. MiR-92a and miR-135a, on the other hand, do not appear to be correlated with the progression of CKD (**Figure 3b, 3c**). Levels of miRNA-33a were not detectable in apoB-depleted serum for both the controls or CKD patients (data not shown).

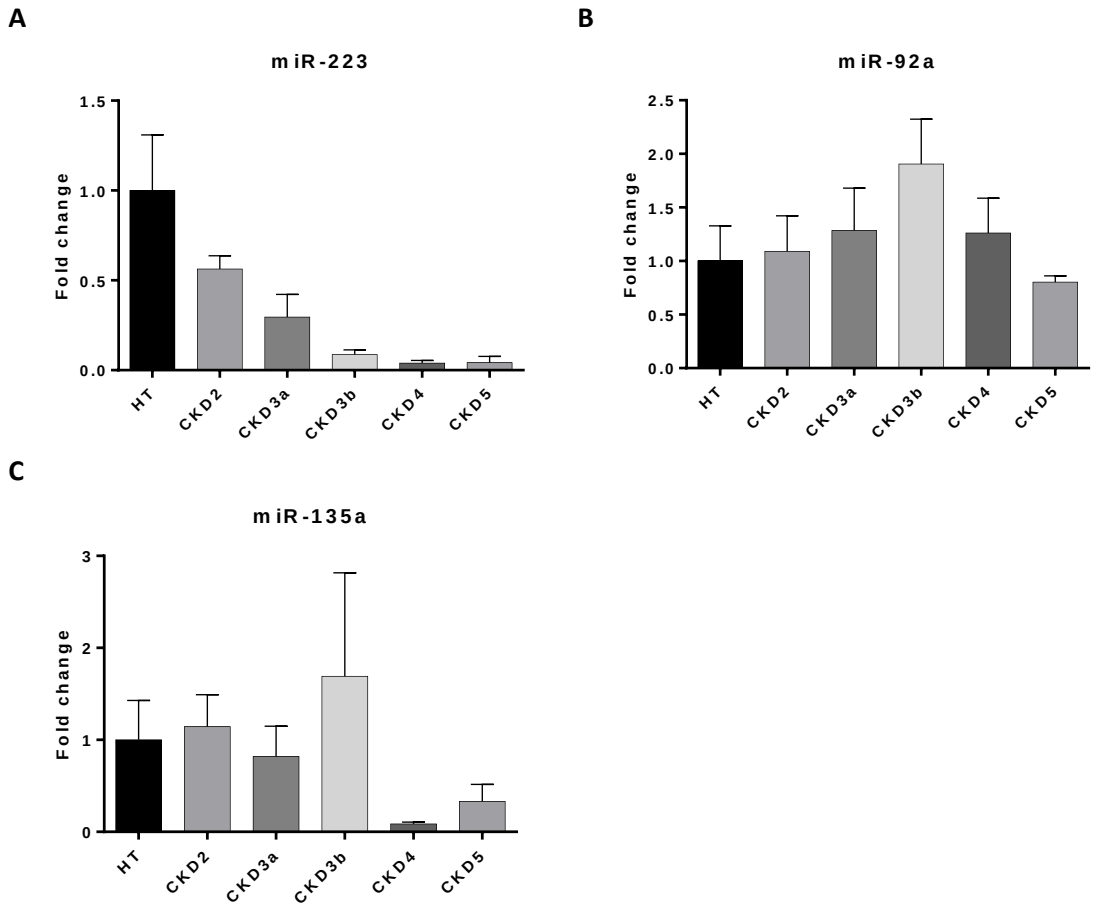


Figure 3. ***HDL-transported miRNA levels in CKD****. Expression of miRNA-223 (A), miRNA-92a (B), and miRNA-135a (C) in apoB-depleted serum from hypertensive subjects (n=7), CKD2 (n=5), CKD3 (n=7), CKD3b (n=7), CKD4 (n=6), and CKD5 (n=2) as quantified by RT-qPCR. The fold changes are presented as mean ± SEM.*

HDL diminishes TNFα-induced inflammation in human microvascular endothelial cells

To confirm that HDL has anti-inflammatory effects on endothelial cells, HMECs were pre-incubated with cHDL (**Figure 4**). Subsequently, the mRNA expression of adhesion molecules ICAM-1 and VCAM-1 as well as chemokine CCL2 were measured after TNFα-induced inflammation and expressed relative to untreated cells.

While there was a small increase in gene expression in cells pretreated with HDL without the addition of TNFα, in TNFα-treated endothelial cells cHDL indeed diminished gene expression of the adhesion molecules ICAM-1 (19.75-fold ± 2.33 compared to 28.81-fold ± 0.04) and VCAM-1 (20.20-fold ± 1.36 compared to 32.29-fold ± 1.46), and the chemokine CCL2 (14.59-fold ± 0.41 compared to 47.59-fold ± 6.11) compared to cells without HDL treatment. However, there was an increase in gene expression in cells pretreated with HDL without the addition of TNFα. Therefore, cHDL was filtered prior to addition to the cells. When filtering the cHDL with a 0.20 µm filter prior to addition to the cells, there was no longer an increase in gene expression of ICAM-1, VCAM-1, or CCL2 without addition of TNF-α (**Figure 4b**). However, the anti-inflammatory effect of cHDL was attenuated after filtration.

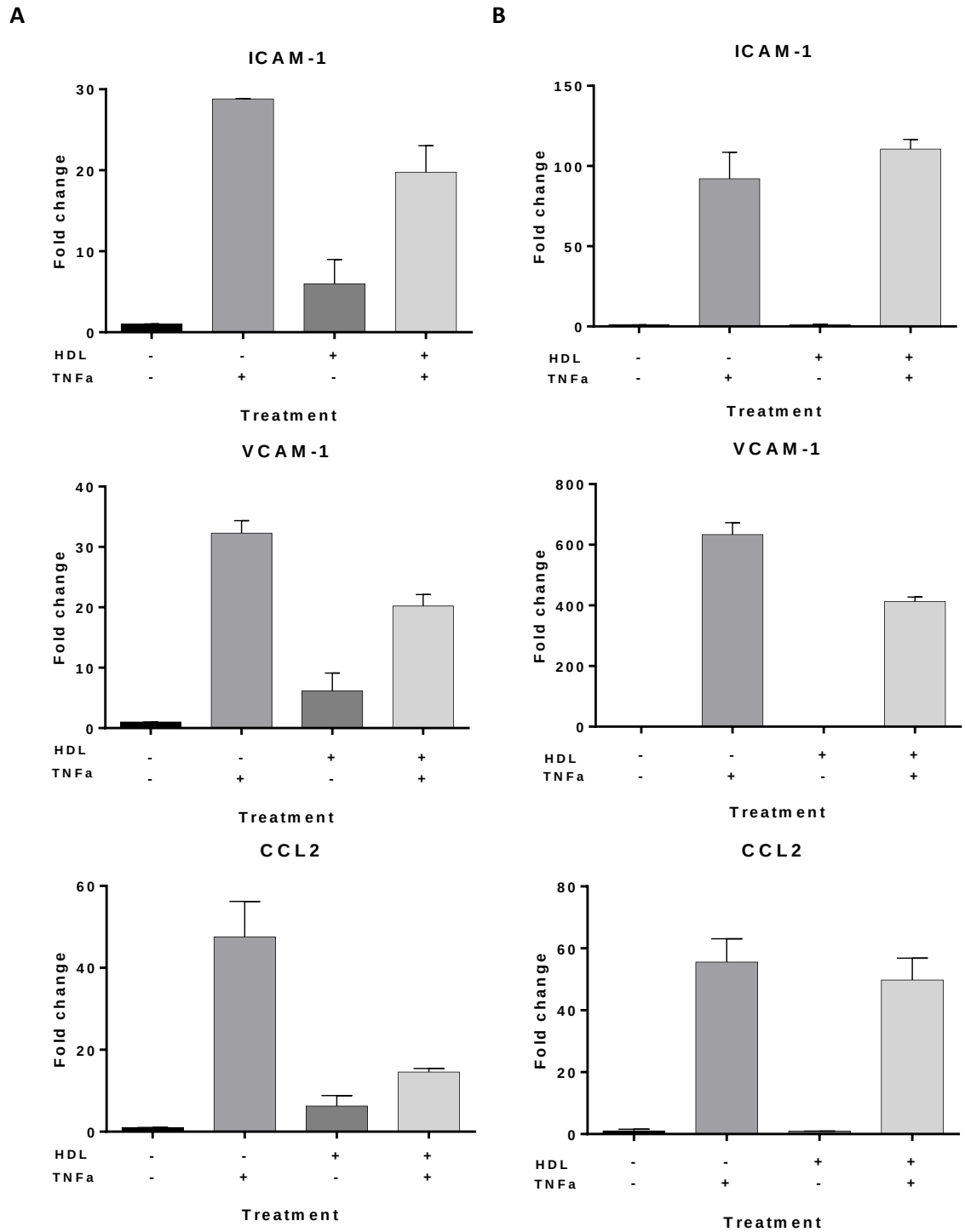


Figure 4 ***Ability of commercial HDL to inhibit expression of markers of endothelial inflammation.*** *HMEC were pre-incubated in the absence or presence of unfiltered (A) or filtered (B) commercial HDL (300 µg/ml, 20h) before inflammation was induced by adding TNFα (2.5 ng/ml, 4h). The fold changes are reported as mean ± SEM.*

The anti-inflammatory effects of HDL on endothelial cells are impaired in hypertensive subjects and CKD patients

In order to examine the differences in the effects of HDL on endothelial adhesion molecule and chemokine expression between healthy and diseased HDL, HMECs were pretreated with apoB-depleted serum from either CKD patients, hypertensive subjects, or a healthy volunteer prior to inducing inflammation.

TNFα effectively induced inflammation, indicated by upregulation of ICAM-1, VCAM-1, and CCL2 mRNA levels (50.25-fold ± 8.07, 241.68-fold ± 48.09, and 58.06-fold ± 3.66, respectively) (**Figure 5**). As expected, HDL from the healthy volunteer diminished the expression of the adhesion molecules and chemokines after TNFα-induced inflammation (ICAM-1 19.53-fold ± 1.43, VCAM-1 30.18-fold ± 9.26, CCL2 4.21-fold± 0.40, **Figure 5**). The anti-inflammatory effects of HDL^{CKD} were impaired compared to healthy HDL, as indicated by higher values for ICAM-1, VCAM-1 and CCL2 expression. Expression of ICAM-1 and CCL2 in cells incubated with HDL^{CKD} shows a gradual increase with progression of the CKD stages, even becoming slightly upregulated in CKD4 (CCL2) and CKD5 patients (ICAM-1 and CCL2) (**Figure 5a, 5c**). Surprisingly, VCAM expression in HMEC cells incubated with HDL^{CKD} decreases after CKD3b, implying that HDL from CKD stages 4-5 is more effective in inhibiting VCAM expression (**Figure 5b**), although the sample numbers of these end-stage renal disease patients is too low to draw firm conclusions.

Interestingly, the anti-inflammatory effects of HDL were impaired in hypertensive subjects as well compared to healthy HDL. Moreover, pretreatment with HDL of hypertensive subjects shows no effect on CCL2 expression (**Figure 5c**).

Overall, these results indicate that HDL of CKD patients as well as hypertensive subjects have lower anti-inflammatory capacity on HMEC cells.

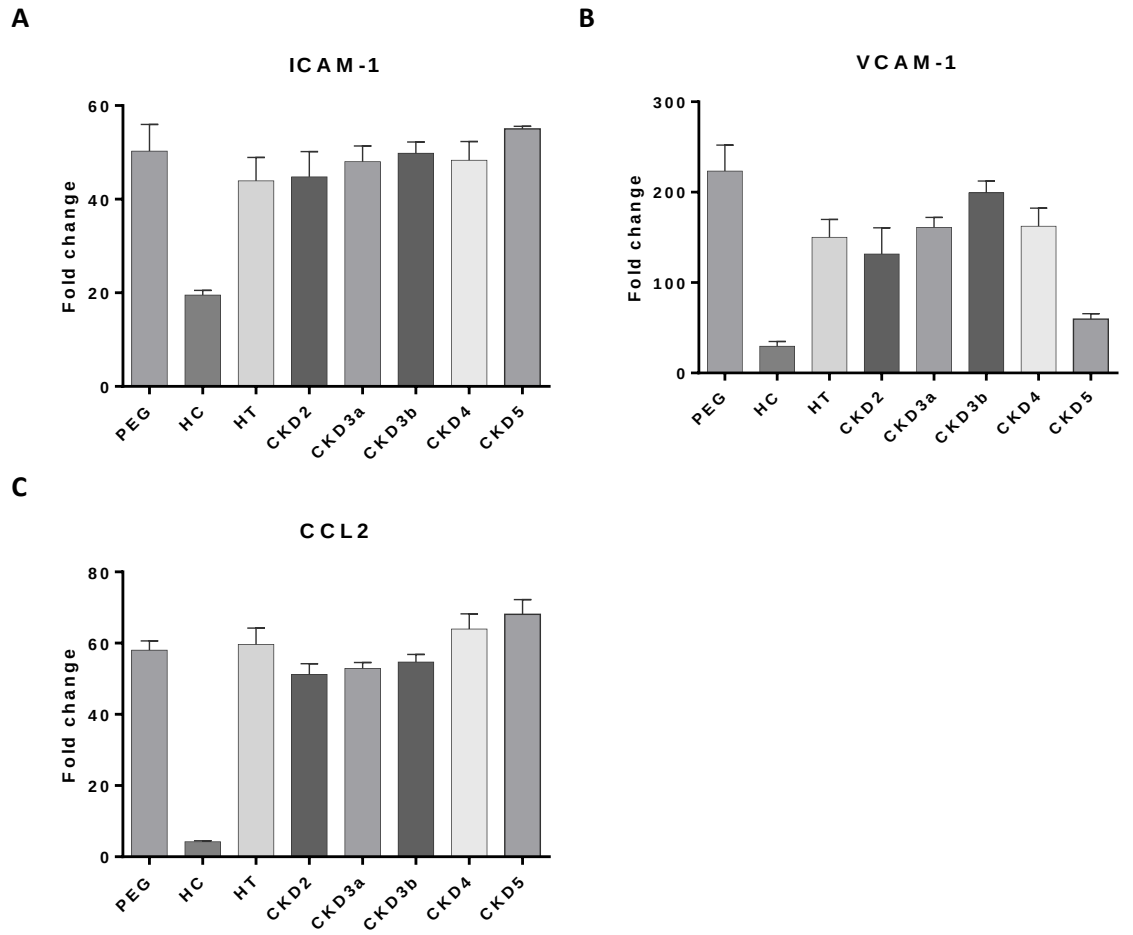


Figure 5 ***The ability of HDL to inhibit TNFα-induced expression of ICAM-1 (A), VCAM-1 (B), and CCL2 (C) in HMEC cells****. HMEC were pre-incubated in the absence (PEG) or presence of apoB-depleted serum (300 µg apoA-1/ml) from a healthy volunteer (HC), hypertensive subjects (HT), or apoB-depleted serum from CKD patients (CKD2-5) for 20h before inflammation was induced by adding TNFα (2.5 ng/ml, 4h). The fold changes are reported as mean ± SEM. (n=2-7)*

Effect of HDL on cytokine production by macrophages

In order to examine the effect of HDL on macrophages, secretion of the pro-inflammatory cytokine TNFα and the anti-inflammatory cytokine IL-10 were measured in the medium by ELISA. For this, murine J774 cells were pretreated with apoB-depleted serum from a healthy volunteer, hypertensive subjects, or patients suffering from varying stages of CKD for 24h prior to inducing inflammation by addition of LPS.

Treatment with HDL^{HC} resulted in an increase in production of IL-10 (592.26 pg/ml ± 54.05, **Figure 6b**) and a slight increase in TNFα production (32687.24 pg/ml ± 3448.38, **Figure 6a**) compared to the controls without addition of HDL (IL-10 133.52 pg/ml ± 9.54 and TNFα 29316 pg/ml ± 240.24). However, it is noteworthy that after HDL^{HC} treatment, many cells detached and were washed away, most likely due to the presence of EDTA in the apoB-depleted serum. Therefore, fewer cells contributed to the production and secretion of cytokines compared to the other conditions. Treatment with HDL^{HT} and HDL^{CKD} from stages 2 to 4 also resulted in an increase in the production of the anti-inflammatory cytokine IL-10 but shows a gradual decline with disease severity (**Figure 6b**). Production of the pro-inflammatory cytokine TNFα on the other hand, slightly increases with disease severity. However, HDL^{CKD} of stages 4 and 5 impairs TNFα production.

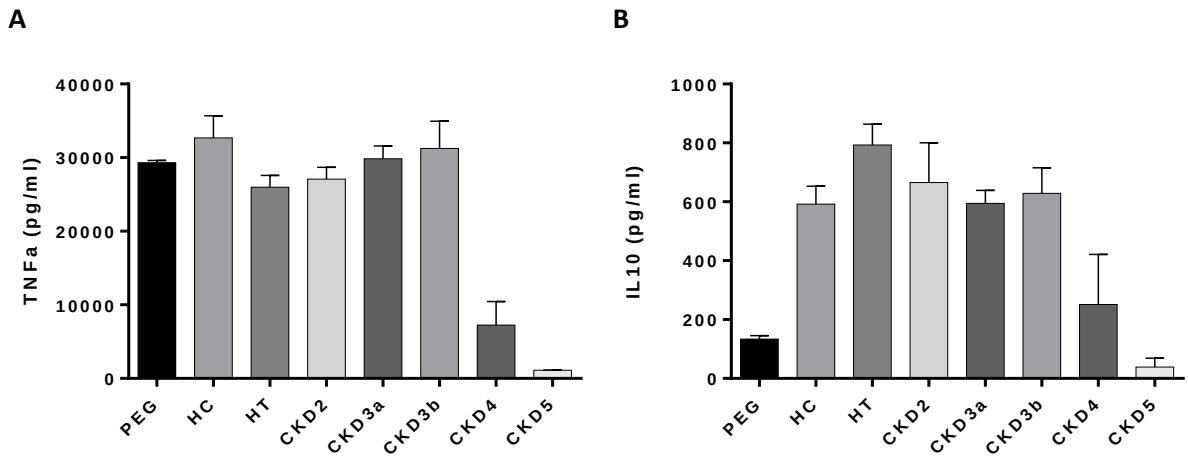


Figure 6 ***Effect of HDL on cytokine secretion in macrophages****. TNFα (A) and IL-10 (B) secretion of J774 cells incubated for 24h in the absence (PEG) or presence of apoB-depleted serum from a healthy volunteer (HC), hypertensive subjects (HT), or patients with varying stages of CKD (CKD2-5). All results are expressed as mean ± SEM.*

Correlation of HDL-associated miRNAs and HDL functionality

Next, we wanted to elucidate the connection between the HDL-associated miRNAs and HDL functionality. Therefore, the quantity/expression of the miRNAs measured in the apoB-depleted serum of hypertensive subjects and CKD patients stages 2-5 was compared to their effects on both endothelial expression of adhesion molecules and chemokines as well as the production of cytokines by macrophages.

There were no significant correlations between HDL-associated miRNA expression levels and the expression of ICAM-1, VCAM-1, and CCL2 in endothelial cells (**Supplementary Figure 1**). In contrast, miR-135a was positively correlated with TNFα (Spearman correlation coefficient 0.41, p = 0.025) and IL-10 (Spearman correlation coefficient 0.45, p = 0.012) production in macrophages (**Figure 7, Supplementary Figure 2**). IL-10 production was also positively

correlated with expression levels of miR-223 (Spearman correlation coefficient 0.45, p = 0.015) (**Figure 7a**).

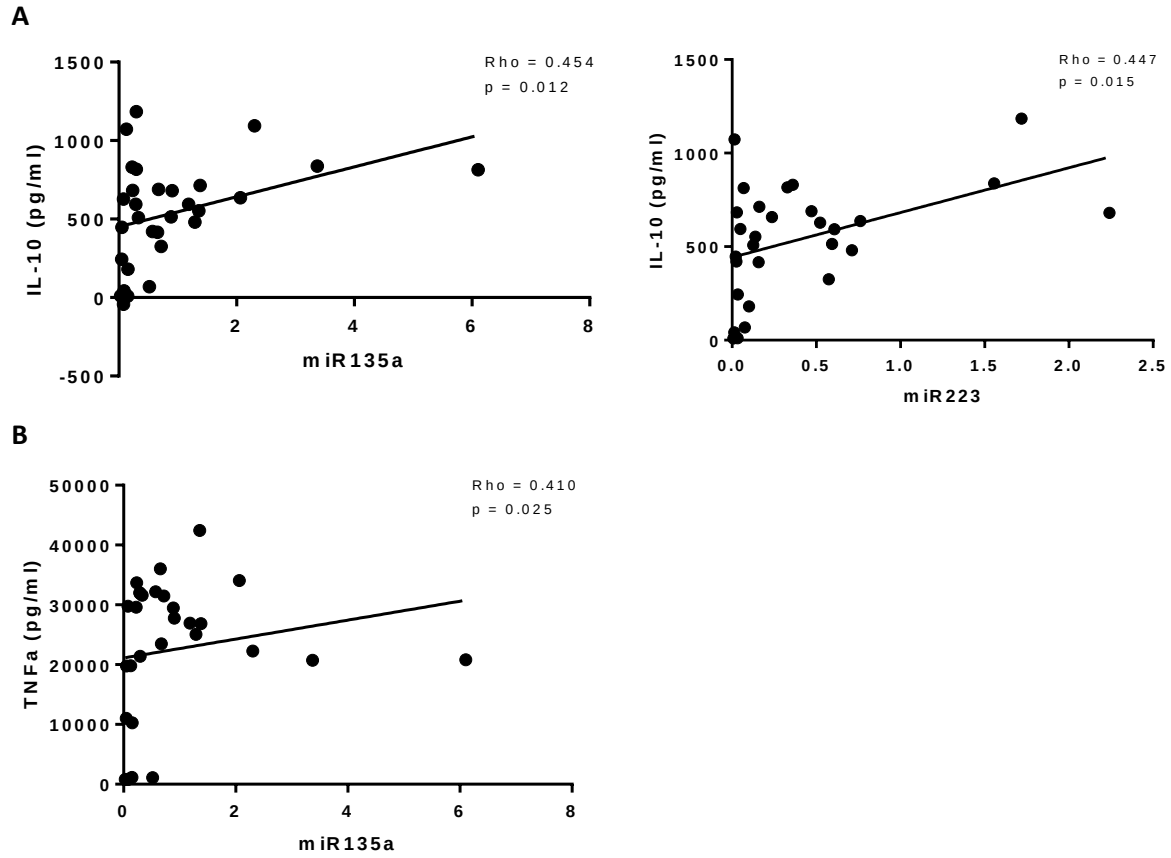


Figure 7 ***Spearman correlation between anti-inflammatory cytokine, IL-10 (A), and pro-inflammatory cytokine, TNFα (B), production and miRNA expression in apoB-depleted serum.***

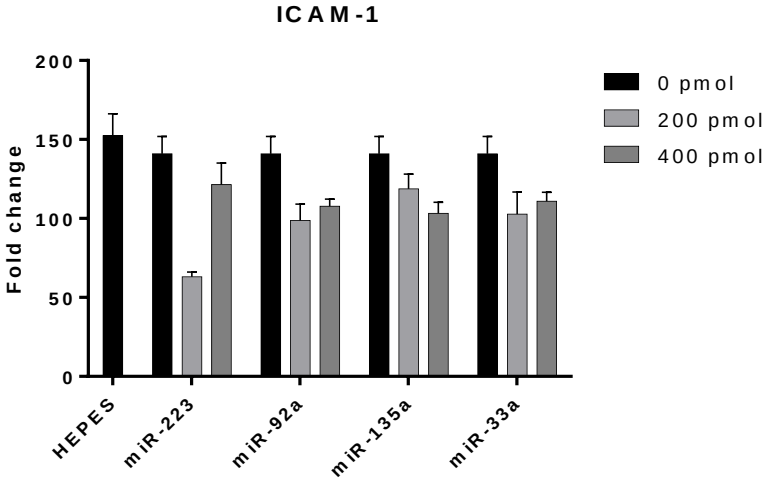
Enrichment of healthy HDL with miRNAs

As mentioned before, HDL is a complex heterogeneous particle, which especially in diseased conditions can vary in composition, i.e. not only miRNA content, but also a variety of proteins that affect HDL functionality. To investigate whether HDL-associated miRNAs influence HDL functionality in non-pathological conditions, commercial HDL was enriched with single stranded, mature miRNAs and subsequently added to macrophages and endothelial cells. Next, the effects of the miRNA-enriched HDL on macrophages and endothelial inflammation were assessed using ELISA and qPCR, respectively.

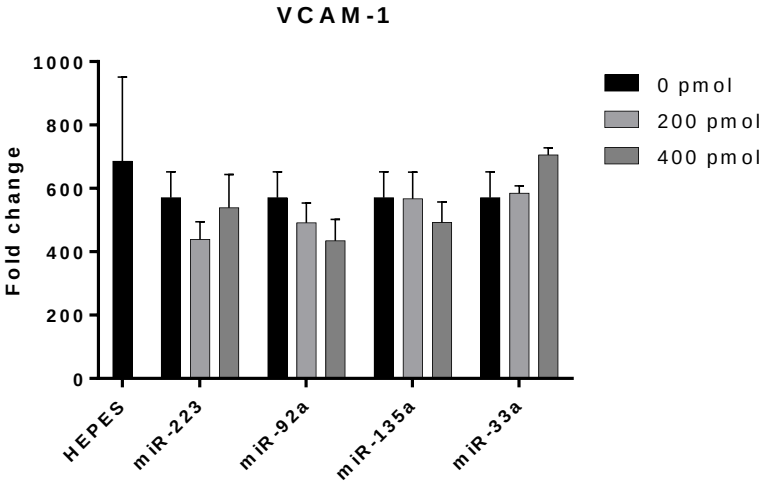
Treatment of TNFα-treated HMECs with cHDL without added miRNAs showed no apparent effect on mRNA levels of ICAM-1 and VCAM-1 and only a small reduction in CCL2 mRNA levels (**Figure 8**), which is consistent with earlier results with filtered cHDL (**Figure 4**). However, enrichment of HDL with miRNAs affected the anti-inflammatory effects of cHDL on endothelial cells. Addition of 200 pmol miR-223 to cHDL prior to application to the cells resulted in a reduction of ICAM-1 expression (63.15-fold ± 1.7 compared to 140.91-fold ± 8.96). Interestingly, this effect is attenuated in cells treated with cHDL enriched with 400 pmol miR-223 (121.48-fold ± 11.06). Similarly, 200 pmol miR-223 also slightly reduced VCAM-1 expression (438.72-fold ± 45.75 compared to 570.45-fold ± 81.51), whereas 400 pmol seemingly did not affect VCAM-1 expression compared to cHDL (538.82-fold ± 85.35 compared to 570.45-fold ± 81.51). Treatment

with HDL enriched with miR-92a results in diminished ICAM-1 and VCAM-1 levels. MiR-33a-enriched HDL diminished ICAM-1 expression, whereas it resulted in an increase in VCAM-1 and CCL2 expression (**Figure 8**). Lastly, similar to the other analyzed miRNAs, miR-135a reduces ICAM-1 expression. However, it does not affect VCAM-1 or CCL2 (**Figure 8**).

A



B



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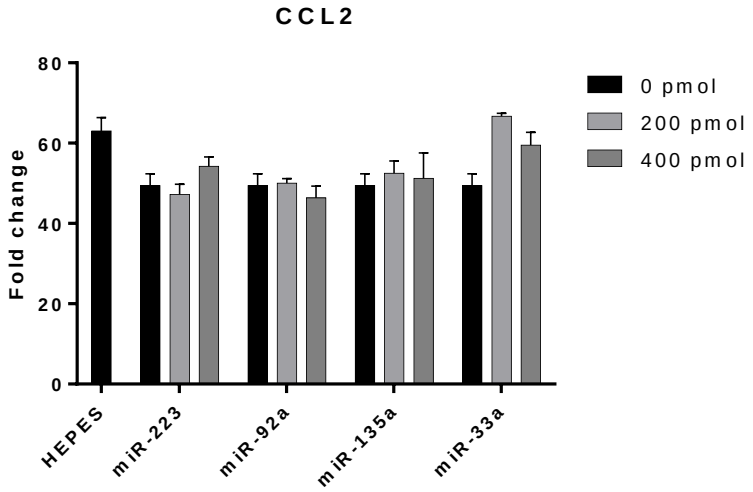

Figure 8 ***The effect of miRNAs on the ability of HDL to inhibit TNFα-induced expression of ICAM-1 (A), VCAM-1 (B), and CCL2 (C) in HMEC cells****. HMEC were pre-incubated in the absence (HEPES) or presence of commercial HDL (300 µg/ml) enriched with miR-223, miR-92a, miR-135a, or miR-33a for 20h before inflammation was induced by adding TNFα (2.5 ng/ml, 4h). The fold changes are reported as mean ± SEM.*

As shown in **Figure 9a**, there is no difference in TNFα production in J774 macrophages treated with miRNA-enriched HDL compared to not enriched cHDL. IL-10 production, on the other hand, appears to be impaired by miR-135a and miR-33a when compared to not enriched cHDL (**Figure 9b**).

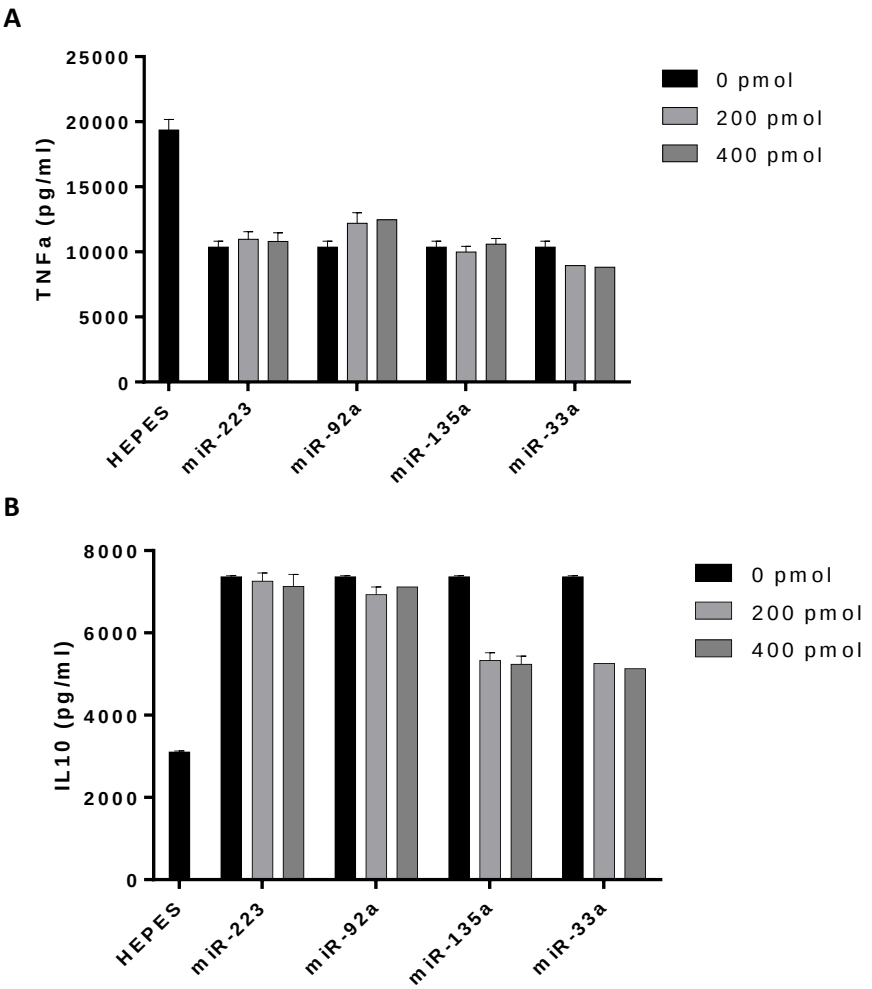

Figure 9 ***Enrichment of HDL with miRNAs affects cytokine secretion in macrophages.*** *Secretion of TNFα (A) and IL-10 (B) by cHDL-treated (300 µg/ml, 24h) and LPS-stimulated (10 ng/ml, 6h) J774 macrophages. Commercial HDL was enriched with 0, 200, or 400 pmol miR-223, miR-92a, miR-135a, or miR-33a prior to incubation with the cells. All results are expressed as mean ± SEM.*

Discussion

HDL have been shown to become dysfunctional in several inflammatory diseases, including CKD. However, what causes these particles to lose their beneficial properties remains to be further elucidated. Therefore, the purpose of this study was to identify HDL associated miR expression profiles in CKD patients and assess the effect of potential differences in HDL-associated miRNAs on the functional properties of HDL (i.e. anti-/pro-inflammatory).

It has been previously reported that the profile of HDL-transported miRNAs is altered in disease (e.g. familial hypercholesterolemia) (12). In our study, we show that the profile of miRNAs transported by HDL differs in patients with CKD compared to hypertensive subjects. Most notably, miR-223 gradually decreases with disease progression. This finding is consistent with findings in murine models of CKD where serum levels of miR-223 were lower in mice with experimentally induced CKD when compared with SHAM operated mice (46). Expression of other HDL-associated miRNAs (miR-92a and miR-135a) were also altered; however, they showed no correlation with CKD progression.

As one of the most abundant miRNAs in macrophages, miR-223 is also one of the most elusively studied HDL-associated miRNA (46). First described in the haematopoeietic system, miR-223 has been associated with many inflammatory disorders, including rheumatoid arthritis, type 2 diabetes, and more recently, cardiovascular diseases (47). In inflammatory disorders, cellular miR-223 expression is often upregulated and believed to play a role in attenuating inflammation. In CKD, expression of miR-223 in smooth muscle cells increases, whereas serum levels decrease (46). Furthermore, overexpression of miR-223 is associated with anti-inflammatory effects. For example, by targeting Pknox1, miR-223 directs monocytes towards the alternative M2 phenotype (37). Moreover, Tabet *et al* showed that miR-223 represses endothelial inflammation by targeting ICAM-1 (36). We were unable to demonstrate an association between miR-223 expression in HDL^{CKD} and the effect of HDL on endothelial inflammation, despite the clear decrease in HDL-associated miR-223 expression with disease progression. However, when adding miR-223 to cHDL, ICAM-1 expression was diminished. The latter result is in line with that of Vickers *et al* who showed that miR-223 gets taken up by endothelial cells (HCAEC and HUVEC), where it diminishes endothelial inflammation by targeting ICAM-1 (12, 36). By contrast, Wagner argued that the number of miRNAs transferred from HDL to HUVECs was too small to reduce endothelial inflammation (48). Therefore, when enriching HDL with miRNAs, possibly more miRNAs are transferred to the recipient cells to elicit a larger effect. Additionally, it should be noted that due to the heterogeneity of HDL, other alterations in HDL composition also contribute to HDL dysfunction.

Several reports have demonstrated that HDL of CKD patients loses its protective properties and becomes dysfunctional. HDL^{CKD} has been shown to gradually increase VCAM-1 expression in TNFα stimulated HCAECs (49). In HMECs, we confirmed that apoB-depleted serum from CKD patients is less effective in inhibiting TNFα-induced inflammation than healthy HDL. HDL^{CKD} becomes progressively less effective in diminishing endothelial expression of the adhesion molecules ICAM-1, VCAM-1, and the chemokine CCL2, which play a crucial role in the development of atherosclerosis (49, 50). Surprisingly, VCAM-1 expression is diminished in cells treated with HDL^{CKD} stage 5. This inconsistency could be due to the low apoA-1 concentrations measured in the serum. CKD stage 5 was the only group in which the apoA-1 concentration was

significantly lower compared to the controls. In CKD, apoA-1 gets progressively displaced by SAA. Therefore, when adding the same concentration apoA-1 to the cells, more HDL particles are added, resulting in a larger effect. If this were the case, however, ICAM-1 and CCL2 expression would be diminished as well. However, VCAM-1 expression has larger interindividual differences than ICAM-1 and CCL2 (51), therefore, due to the small sample size, this effect must be interpreted with caution.

In addition to becoming more pro-inflammatory in endothelial cells, HDL from patients with end stage renal disease was reported to increase macrophage cytokine production, including TNFα (30). In our study, we demonstrated that TNFα production in J774 macrophages gradually increases after incubation with apoB-depleted serum from hypertensive subjects to apoB-depleted serum from CKD stage 3b patients. Contrary to expectations, apoB-depleted serum from CKD stage 4-5 patients impaired TNFα production. This finding is in contrast with the previous findings by Yamamoto in THP-1 macrophages (30). Furthermore, our group recently demonstrated that HDL exerts pro-inflammatory effects on macrophages (27). Here, native as well as reconstituted HDL upregulated production of the pro-inflammatory cytokine TNFα and downregulated anti-inflammatory cytokine IL-10. In the current study, however, commercial native HDL displayed anti-inflammatory effects by diminishing TNFα production and increasing IL-10 production. ApoB-depleted serum from a healthy volunteer, on the other hand, induced a minor increase in TNFα production despite having fewer cells. However, IL-10 production was increased after incubation with apoB-depleted serum from the healthy volunteer. ApoB-depleted serum from CKD patients decreased IL-10 production with disease progression, further supporting the idea that HDL from CKD patients becomes progressively less effective in inhibiting inflammation and therefore, more dysfunctional.

HDL enriched with miR-135a or miR-33a both impaired IL-10 production in J774 macrophages. Previously, inhibition of miR-33a increased the plasma levels of HDL in both mice and primates, and reduced plaque size and inflammation in mouse models of atherosclerosis (40-42). The fact that we showed that miR-33a-enriched HDL is able to impair IL-10 production further supports the idea that miR-33a has atherogenic effects. So far, there is only very limited knowledge about miR-135a functions in cardiovascular diseases. Recently, miR-135a demonstrated to target TLR4 in RAW264.7 cells and thus, were able to inhibit atherosclerosis progression (34). Therefore, our finding that enrichment of HDL with miR-135a resulted in an impairment of IL-10 is surprising.

Taken together, the findings of this study provide further support of the hypothesis that miRNAs transported in HDL are taken up by cells where they affect gene expression. Since many of the HDL-associated miRNA have (predicted) target sites that are involved in cholesterol metabolism and the RCT, assessing their effects on cholesterol efflux might give more insight in their contribution to HDL functions. Further studies focusing on the effect of these HDL-transported miRNAs on cholesterol efflux are therefore suggested.

Conclusion

The aim of the present study was to assess the effect of HDL-associated miRNAs on the anti-atherogenic effects of HDL and to determine the miRNA expression profile in HDL of CKD patients. It was confirmed that the expression levels of HDL-associated miRNAs were altered in disease, more specifically in CKD. Especially miR-223 showed a marked decrease with progression of the disease. Furthermore, HDL was able to deliver different miRNAs to macrophages or endothelial cells after enrichment, where some miRNAs then affected the inflammatory response. Since we did not assess the effect of the miRNAs on cholesterol efflux, further research regarding this aspect is needed to fully determine the contribution of HDL-transported miRNAs on HDL functionality.

Valorization

During this project, we established the HDL-associated miRNA profile of CKD patients as well as elucidated the role of these HDL-associated miRNAs on HDL functionality. This study contributes to the knowledge about miRNAs, a field of research that has been gaining interest over the last few years. In the future, miRNA profiles might provide new non-invasive biomarkers for several diseases as well as new potential targets for innovative therapies. We demonstrate that miR-223 might be a suitable biomarker for CKD progression and increasing the number of miR-223 in endothelial cells diminishes ICAM-1 expression. Furthermore, we confirmed that HDL can deliver miRNAs to different cell types (endothelial cells and macrophages) and that these miRNAs then can modulate the inflammatory response of these cells, therefore, HDL could be an interesting new carrier for miRNAs as a therapy.

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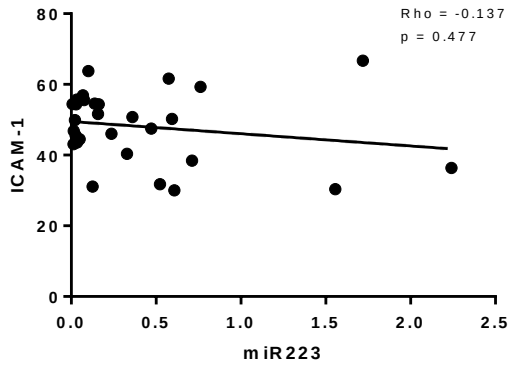
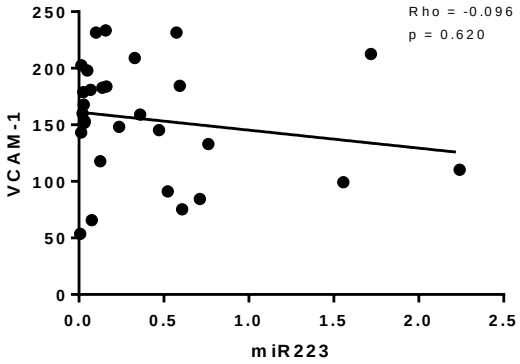
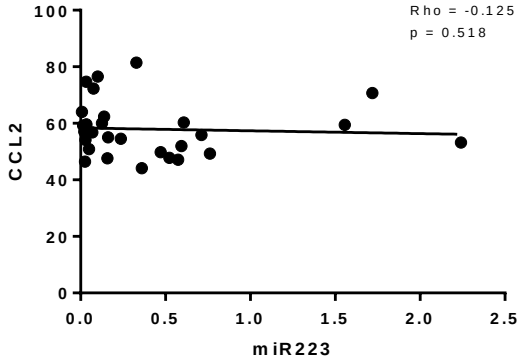
Supplemental figures

A







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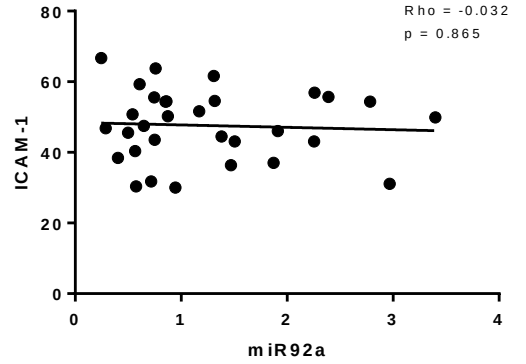

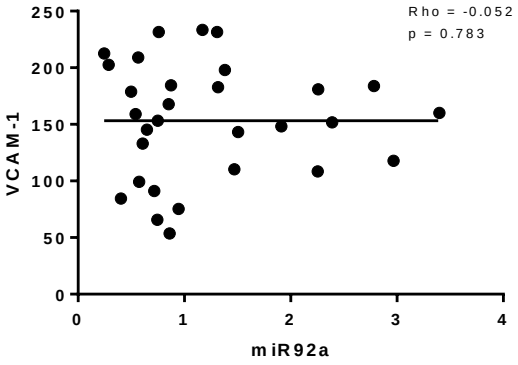

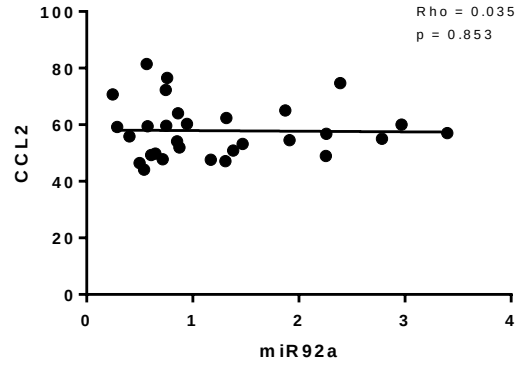

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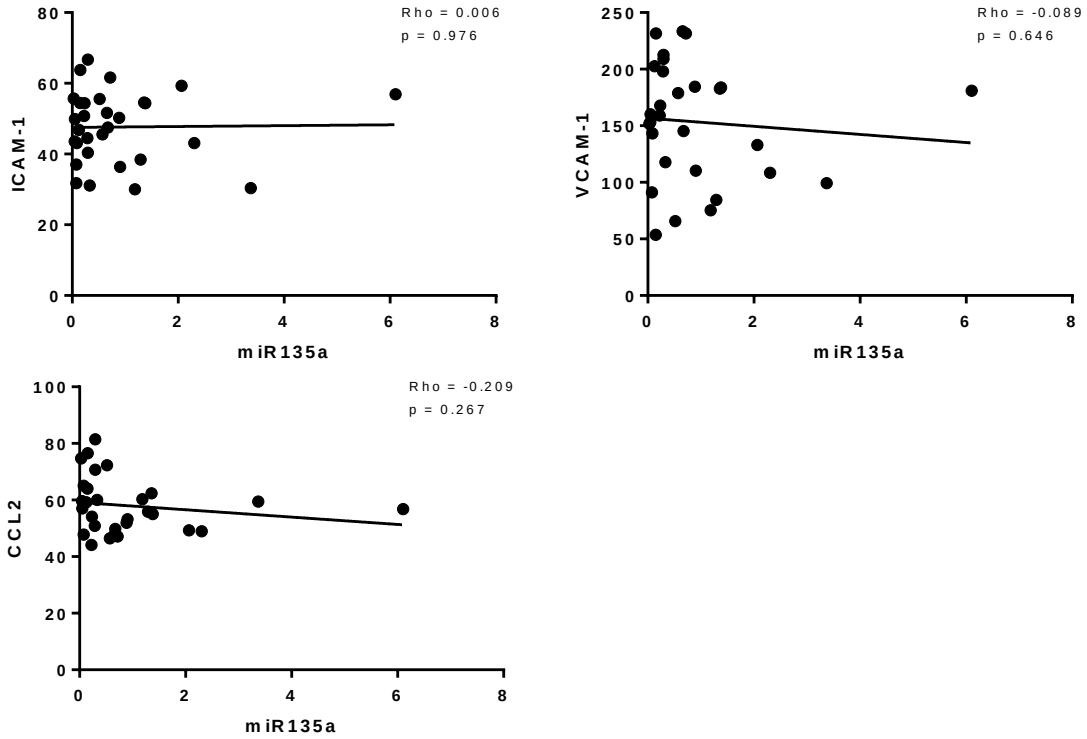
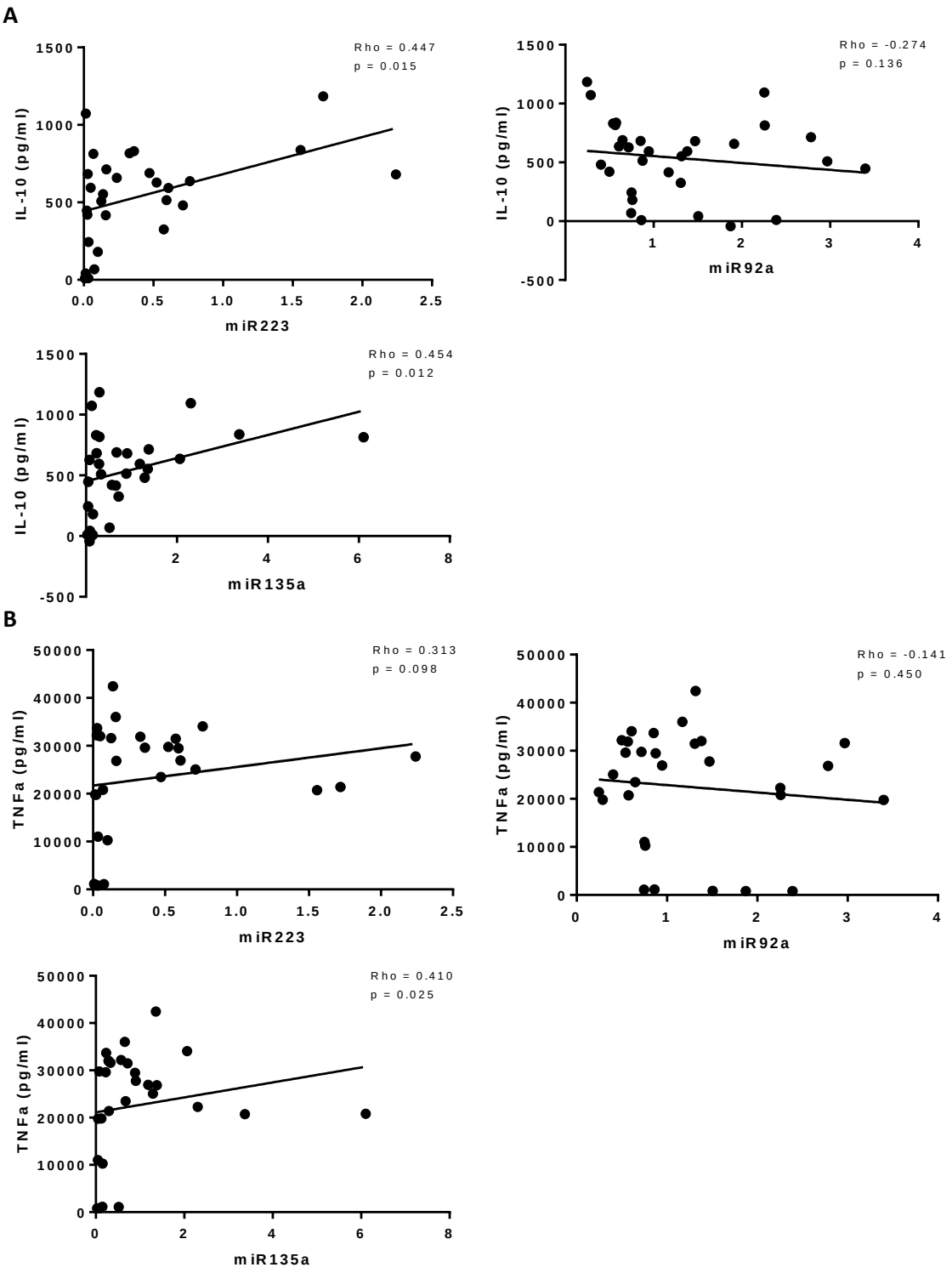


Supplemental figure 1. ***Spearman correlation between gene expression of adhesion molecules and miRNA expression of miRNA-223 (A), miRNA-92a (B) and miRNA-135a (C) in apoB-depleted serum.***



Supplementary figure 2. ***Spearman correlation between anti-inflammatory cytokine IL-10 (A) and pro-inflammatory cytokine TNFα (B) production and miRNA expression in apoB-depleted serum.***

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Datum: **20/08/2018**