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Uraemia, inflammation and atherosclerosis: molecular and functional contributions of leucocytic Angiotensin Converting Enzymes in chronic kidney disease

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Referat

Die chronische Inflammation, charakteristisch für Patienten mit Nierenversagen, führt zu einer rasch progressiven Atherosklerose mit Organmanifestationen wie Herzinfarkt oder Schlaganfall.
Die Ursachen für die Entstehung der Atherosklerose sind zum Teil noch unbekannt. Bei vielen Patienten findet man klassische Atherosklerose-assoziierte Risikofaktoren wie Diabetes mellitus, Rauchen, Bluthochdruck, erhöhte Blutfettwerte oder eine bekannte Familienanamnese. Häufig ist jedoch kein Risikofaktor zu finden. Dies weist darauf hin, dass es zusätzliche Krankheitsmechanismen geben muss. Insbesondere zirkulierende, inflammatorische Zellen könnten bedeutsame potentielle Trigger der Erkrankung sein. Monozyten, die zur normalen Infektionsabwehr benötigt werden, sind auch bei allen Atherosklerosestadien beteiligt. Beim Nierenkranken scheinen diese Zellen jedoch besondere Eigenschaften zu entwickeln, wodurch sie zur Veränderung der Blutgefäße beitragen.
Das Renin-Angiotensin-System (RAS), inklusive seiner Komponenten Angiotensin-konvertierendes Enzym (ACE) und Angiotensin II, ein potenter Vasokonstriktor, Angiotensin-konvertierendes Enzym 2 (ACE2) und Angiotensin 1-7, ein potenter Vasodilatator, gehört zu den wichtigsten Blutdruck-Regulatoren. Beide ACE-Enzyme sind vor allem auf den Endothelien der Lungen- und Nierengefäße nachweisbar. Aber auch andere Zelltypen, insbesondere immunomodulatorische Zellen wie Monozyten und Makrophagen, exprimieren die RAS-Komponenten.
Experimentelle Untersuchungen weisen darauf hin, dass eine erhöhte Expression des monozytären ACE‘s in Patienten mit Nierenversagen nicht nur mit kardiovaskulären Komplikationen und höheren Mortalitätsraten assoziiert ist, sondern auch als ein Indikator für fortgeschrittene Atherosklerose dienen könnte. Zudem konnte gezeigt werden, dass die Monozyten bei nierenkranken Patienten eine signifikante ACE2 Verminderung sowie eine Hochregulierung von AngII- und Ang1-7-Rezeptoren aufweisen. Induktion von urämischen Bedingungen in vitro konnte diese Ergebnisse ex vivo reproduzieren. Die humanen Monozyten zeigten nicht nur das gleiche Expressionsmuster, sondern reagierten auf urämisches Milieu mit Hochregulierung von Interleukin-6 und Tumor Nekrosis Faktor Alpha, erhöhten Transmigrationsraten und deutlich ausgeprägter Adhäsion an Endothel-Monolayers.
Durch Überexpression von ACE in humanen Monozyten konnte ein pro-atherosklerotisches Verhalten induziert werden. Zudem wiesen diese Zellen auch eine signifikante ACE2 Verminderung auf. Die erhöhte endotheliale Adhäsion von ACE-überexprimierenden oder AngII-vorbehandelten Monozyten konnte durch die Zugabe von ACE-Inhibitor und AngII-Rezeptor-Blocker vermindert werden. Dabei wurden die Adhäsionsmoleküle ICAM-1 und VCAM-1 wie auch der Wachstumsfaktor MCSF signifikant herunterreguliert. Die pharmakologische Blockierung des ACE/AngII Signalwegs in ACE-überexprimierenden Monozyten konnte auch die Expression von ACE2 reinduzieren.
Die Überexpression von anti-atherosklerotischem ACE2 in humanen Monozyten hingegen führte zur Erniedrigung von endothelialer Transmigration und Adhäsion sowie zur Verminderung von ICAM-1, VCAM-1, MCSF und der Rezeptorexpression für AngII.
Im Rahmen einer klinischen Studie konnten wir darüber hinaus belegen, dass die Verwendung von hoch permeablen Dialysemembranen in der Therapie des chronischen Nierenversagens über eine verstärkte Elimination von proentzündlichen Mediatoren zur partiellen Normalisierung der Veränderungen des Angiotensinsystems auf Monozyten führt.
Zusammenfassend deuten die Daten dieser Arbeit darauf hin, dass durch die Urämie eine Induktion von monozytärem ACE und Verminderung von ACE2 getriggert wird, die zur Entstehung und/oder Progression von Atherosklerose beitragen könnte.

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Abstract

Atherosclerosis and vascular obstruction as well as vascular calcification are still among the most important complications leading to morbidity and mortality in patients with chronic renal failure. Angiotensin converting enzymes (ACE1 and ACE2), circulating exopeptidases, belong to the main components of the renin-angiotensin system which participates in the regulation of blood pressure. ACE catalyses the conversion of decapeptide angiotensin I to octapeptide angiotensin II (AngII), a potent vasoconstrictor. ACE2 cleaves AngII into Ang-(1-7), which acts as a vasodilator of the blood vessels. Both ACEs are secreted not only by pulmonary and renal endothelial cells, but are also present on monocytes, which are able to produce AngII, Ang(1-7) and express the receptors for AngII and Ang(1-7).

This work demonstrates that altered relation between monocytic ACE and ACE2 may contribute to the development of atherosclerosis in patients with chronic kidney disease (CKD) via an AngII-dependent mechanism. We found that ACE transcripts were significantly increased in leukocytes, especially monocytes, obtained from haemodialysis (HD) and CKD patients not on dialysis as compared with healthy controls. Correspondingly, ACE2 was downregulated and AngII as well as MAS receptor expression was upregulated in these cells. Human primary or THP-1 monocytes preconditioned with uremic serum reflected the same expressional regulation of ACE/ACE2, MAS and AngII receptors as those observed ex-vivo. These cells demonstrated a highly pro-atherosclerotic phenotype resulting in noticeably elevated endothelial transmigration and adhesion.
Overexpression of monocytic ACE dramatically decreased the levels of ACE2 and induced a pro-atherogenic phenotype characterised not only by increased endothelial transmigration and adhesion but also by an up-regulation of adhesion-related ICAM-1, VCAM-1 and MCSF and pro-inflammatory IL-6 and TNFa.
This pro-atherogenic behaviour of the cells could be partly reversed by AngII-modifying treatments, leading to an increase in ACE2, decrease of monocytic adhesion to endothelial monolayers and down-regulation of previously mentioned adhesion molecules.
Further functional investigations revealed that overexpression of ACE2 in human monocytes led to less differentiated phenotype resulting in decreased transmigration and endothelial adhesion, and downregulation of adhesion-related molecules as well as AngII-receptors.
Furthermore, this study demonstrated that treatment of chronic dialysis patients with high-permeability membranes rather than conventional high flux membranes modulated the local RAS response by altering the relation between ACE2 and ACE transcripts in circulating leucocytes. Increased levels of leucocytic ACE2 over ACE induced by high-cut off and medium-cut off HD treatment may contribute to the anti-inflammatory and anti-atherogenic effects as reported previously.
In conclusion this work demonstrates that the uremic milieu promotes over-activation of monocytic ACE and decrease of ACE2 via an AngII-dependent mechanism, thus contributing to enhanced endothelial adhesion and transmigration. These findings may elucidate an important mechanism contributing relevantly to atherosclerosis progression in patients with chronic renal failure.

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Abbreviations

7-AAD	7-aminoactinomycin D
AAMs	alternatively activated macrophages
ACE	angiotensin converting enzyme
ACEi	angiotensin converting enzyme inhibitor
AGT	angiotensinogen
Ang	angiotensin
ARB	angiotensin receptor blocker
ARG	arginase
AT1R	AngII receptor type 1
AT2R	AngII receptor type 2
CAMs	classically activated macrophages
CCR2	C-C chemokine receptor type 2
CD	cluster of differentiation
CKD	chronic kidney disease
CRP	C-reactive protein
CVD	cardiovascular disease
ESRD	end stage renal disease
GFR	glomerular filtration rate
HCO	high cut-off
HD	haemodialysis
HF	high-flux
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon gamma
IL	interleukin
iNOS	inducible nitric oxide synthase
IS	indoxyl sulphate
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
LPS	lipopolysaccharide
MASR	Mas1 oncogene receptor
MCO	medium cut-off
MCP-1	monocyte chemoattractant protein 1
MCSF	macrophage colony-stimulating factor
MTHFR	methylenetetrahydrofolate reductase

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NKF/KDOQI	National Kidney Foundation-Kidney Disease Outcomes Quality Initiative
NP	healthy individuals
NS	normal serum
Ox-LDL	oxidized low-density lipoprotein
PBMC	peripheral blood mononuclear cell
PD	peritoneal dialysis
PKD1	polycystin-1
PMA	phorbol 12-myristate 13-acetate
RAAS	renin–angiotensin–aldosterone system
RAS	renin-angiotensin system
RRT	renal replacement therapy
sIL-6	soluble form of IL-6
TCF7L2	transcription factor 7-like 2
TNFa	tumor necrosis factor alpha
TNFR1	tumour necrosis factor receptor 1
VCAM-1	vascular cell adhesion protein 1
VSMC	vascular smooth muscle cells

1 Introduction

1.1 Chronic kidney disease

Chronic kidney disease (CKD) involves a spectrum of various pathophysiologic conditions related to abnormal kidney function and progressive decrease in glomerular filtration rate (GFR). According to the National Kidney Foundation-Kidney Disease Outcomes Quality Initiative (NKF/KDOQI), CKD is defined as kidney damage based on structural and/or functional abnormalities for 3 or more months, accompanied with or without declined GFR, or indicated by abnormal composition of the blood and urine parameters, including the values of kidney damage markers. To simplify this description, one can say that CKD is defined as renal failure or GFR below 60ml/min per 1.73 m^2 for 3 or more months regardless of the cause. The actual classification of CKD, including the five stages (CKD1-5) and modified by KDOQI in 2008, subdivides CKD3 into A and B, based on estimated GFR 45-59 ml/min per 1.73 m^2 and 30-44 ml/min per 1.73 m^2, respectively, is presented in Table 1 [1, 2, 3, 4, 5, 6].

Table.1: Classification of CKD based on GFR as proposed by KDOQI guidelines (based on references 1-6)

CKD Stage		Definition
1		Normal or elevated GFR; kidney damage may be reflected by microalbuminuria, proteinuria and/or haematuria; radiologic or histologic changes may occur
2		GFR mildly decreased (89-60 ml/min per 1.73 m^2) kidney damage evidences as reflected by microalbuminuria, proteinuria and/or haematuria; radiologic or histologic changes may occur
3		GFR 59-30 ml/min per 1.73 m^2
	3A	GFR 59 to 45 ml/min per 1.73 m^2
	3B	GFR 44 to 30 ml/min per 1.73 m^2
4		GFR 29-15 ml/min per 1.73 m^2
5		GFR below 15 ml/min per 1.73 m^2; renal replacement therapy if uraemia is present

Staging of CKD is based on the estimation of formula-calculated GFR, incorporating the measured serum creatinine concentration, age, sex, ethnic origin and body weight. There are several equations to estimate GFR, however four of them which are commonly in use, will be presented in this work (Table 2) [7, 8, 9].

Table.2: Recommended equations for estimation of GFR based on serum creatinine (Sct), age (in years), sex, ethnic origin and weight (kg).

Equations for estimating GFR
Cockroft-Gault formula **Male**: Cct (ml/min) = (140 - age) x weight / (72 x Sct(mg/dl)) **or** Cct (ml/min) = (140 - age) x weight /(0.814 x Sct (|µmol/l)) **Female**: Cct (ml/min) = (140 - age) x weight x 0.85 / (72 x Sct(mg/dl)) **or** Cct (ml/min) = (140 - age) x weight x 0.85 / (0.814 x Sct (|µmol/l))
MDRD study equation (four-variable equation) GFR (ml/min/1.73 m^2) = 186 x Sct (mg/dl)$^{-1.154}$ x Age$^{-0.203}$ x 0.742 (if female) x 1.210 (if black) **or** GFR (ml/min/1.73 m^2) = 32,788 x Sct (|µmol/l)-1.154 x Age$^{-0.203}$ x 0.742 (if female) x 1.210 (if black)
MDRD Study Equation for Use with Standardized Serum Creatinine (Four-variable equation) GFR (ml/min/1.73 m^2) = 175 x Standardized Sct(mg/dl)$^{-1154}$ x Age^{-0203} x 0.742 (if female) x 1.210 (if black) or GFR (ml/min/1.73 m^2) = 30,849 x Standardized Sct(|µmol/l)$^{-1.154}$ x Age$^{-0.203}$ x 0.742 (if female) x 1.210 (if black)
CKD-EPI Equation for Use with Standardized Serum Creatinine GFR (ml/min/1.73 m^2) = 141 x min(Sct/K, 1)^a x max(Sct/K, 1)$^{1.209}$ x 0.993^{Age} x 1.018 (if female) x 1.157 (if black) where **K** is 0.7 for females and 0.9 for males, **a** is -0.329 for females and -0.411 for males, min indicates the minimum of Sct/K or 1, and max indicates the maximum of Sct/K or 1. As separate equations for different populations (Sct mg/dl): Female: Sct <0.7; GFR = 144 x (Sct/0.7)$^{-0.329}$ x (0 993)^{Age} Female: Sct >0.7; GFR = 144 x (Sct/0.7)$^{-1.209}$ x (0 993)^{Age} Male: Sct <0.9; GFR = 141 x (Sct/0.9)$^{-0.411}$ x (0 993)^{Age} Male: Sct >0.9; GFR = 141 x (Sct/0.9)$^{-1.209}$ x (0 993)^{Age} x 1.157 (if black)

CKD is prevalent within the global world adult population and the surveys show that a part of this population reveals some stages of CKD. For example, estimations demonstrate that 13.1% and 19.1% of adults in the United States and Japan, respectively, have CKD [10, 11]. The meta-analysis of CKD prevalence of any stage performed on sub-Saharan Africa revealed prevalence within adults of 13.9%, while the survey from Delhi and Chennai, two India's largest cities, found that 1 of 12 individuals

have evidence of CKD [12, 13]. These results indicate clearly that CKD is a common phenomenon in various parts of the world.

The patients achieving CKD5, also known as end stage renal disease (ESRD), commonly require renal replacement therapy (RRT). The incidence of ESRD refers to the number of CKD5 patients beginning RRT in a defined period of time (i.e. a year) in relation to the general population. Usually it is expressed as a number of patients per million population per year. It is worth to note that prevalence is a function of incidence (new cases) and outcomes (renal transplantation or death) rates of ESRD in a distinct population [Table 3; 2].

Table 3: Global incidence and prevalence of RRT (per million population) in 2006

Country	Incidence	Prevalence
United States	360	1,626
Caucasians	279	1,194
African Americans	1,010	5,004
Native Americans	489	2,691
Asians	388	1,831
Hispanics	481	1,991
Australia	115	778
Aboriginal/Torres Strait	441	2,070
Japan	275	1,956
Europe	129	770
United Kingdom	113	725
France	140	957
Germany	213	1,114
Italy	133	1,010
Spain	132	991

It is estimated that 10% of the global population is affected by CKD, and millions of them die each year due to the lack of affordable treatment. More than 2 million people worldwide currently receive treatment with dialysis or a kidney transplant to stay alive. However, the majority of these patients (about 90%) live in the countries with high economical standards. More than 100 countries worldwide do not have any option to provide sufficient RRT. It means that for the people living in the countries with low or middle economies, ESRD is a death sentence [14, 15, 16, 17].

Currently, CKD was ranked 27th (2010 Global Burden of Disease study) in the list of causes of total number of deaths worldwide. For instance, in the year 2005, there were approximately 58 million deaths worldwide, with 35 million due to the chronic diseases, as reported by World Health Organization [17].

Early diagnosis and corresponding treatment are the best options to slow or stop the progression of CKD.

1.1.1 Factors related to initiation and progression of CKD

CKD is a multi-hit process. The progressive nephron loss and decline in GFR, as observed in CKD, are often related with disturbances in electrolyte, water and pH balance, abnormalities in the synthesis and metabolism of erythropoietin and active vitamin D, and excessive accumulation of the waste products, normally excreted by the kidney.

The risk factors for CKD include mainly susceptibility, initiation and progression factors. Susceptibility factors predispose to CKD and involve familial, genetic, ethnic, maternal factors, such as intrauteral malnutrition and low birth weight as well as gender and age. Initiation factors directly trigger the damage of the kidney whereas progression factors affect already established CKD. The main initiation and progression factors are listed below [Table 4; modified from 2].

Table 4: The main risk factors related with initiation and progression of CKD

Initiation Factors	**Progression Factors**
Systemic hypertension	Older age
Diabetes mellitus	Gender (male)
Cardiovascular disease	Race/ethnicity
Dyslipidemia	Genetic predisposition
Obesity/metabolic syndrome	Poor blood pressure control
Hyperuricemia	Poor glycemia control
Smoking	
Low socioeconomic status	
Exposure to nephrotoxins: NSAIDs, analgesics, traditional herbal use, heavy metals exposure (such as lead)	• Dyslipidaemia • Smoking • Obesity/metabolic syndrome • Hyperuricemia • Low socioeconomic status • Alcohol consumption • Nephrotoxins; NSAIDs,RCM, herbal remedies • Acute kidney injury

The risk factors mentioned in table 4 may be categorised as non-modifiable and modifiable.

Non-modifiable risk factors include mainly age, gender, ethnic origin, genetics and loss of renal mass. It has been demonstrated that faster progression of CKD is affected

by the age. For example, the patients with advanced age suffering from glomerulonephritis are characterised by a rapid decrease of GFR. On the other hand, the studies performed on individuals without CKD demonstrated that loss of the nephrons may be a part of normal aging process [18]. Gender is the next important factor related with development and progression of CKD. The data obtained from United States Renal Data System revealed that ESRD due to all causes were observed more frequently in the male population. However this tendency was not so pronounced in Europe [19]. Based on the ethnic origin in the United States, the African Americans revealed the faster progression rates towards ESRD than the Caucasian cohort. Higher incidence and prevalence of hypertensive and diabetic CKD were reported for populations of Hispanic and African Americans as compared with Caucasians [20]. Recently, studies showed that also genetic factors play an important role in initiation and progression of CKD. Genome-wide association studies identified several susceptibility loci for the renal complications as well as for diabetic retinopathy, diabetic cardiovascular disease and mortality [21, 22]. For example Mutation to PKD1 gene (located on chromosome region 16p13.3, encoding protein polycystin-1) is the most common cause of Autosomal-Dominant Polycystic Kidney Disease (~ 86% cases) [23]. Also a single-nucleotide polymorphism in Methylenetetrahydrofolate reductase (MTHFR), an enzyme of homocysteine metabolism, was associated with CKD progression in diabetic nephropathy [25]. Other studies revealed that several genetic variants in the TCF7L2 gene encoding transcription factor 7-like 2, were associated with reduced kidney function or CKD progression among subjects without diabetes [25]. Currently, the episodes of acute kidney injury related with decline in kidney mass and function may contribute to the faster progression of CKD [26].

Modifiable risk factors include mainly hypertension, proteinuria and different metabolic factors. Systemic hypertension is a significant contributor to the progression of CKD and one of the most important causes leading to ESRD worldwide [19]. It is proposed, that the transmission of the systemic hypertension to the glomerulus may result in the glomerular hypertension leading to glomerulosclerosis [27]. Proteinuria is the next crucial factor contributing to CKD progression. Many studies performed on patients with diabetic and nondiabetic glomerular disease or nonglomerular disorders revealed that presence of heavy proteinuria correlated with a faster progression of CKD [28]. Decrease of proteinuria, as a modifiable factor by proper diet or angiotensin II modifying medication predicts a better outcome [29]. It is worth to note that albuminuria is often linked to vascular and endothelial dysfunction as well as with the presence and severity of cardiovascular events [30]. Hermans et al. demonstrated that albuminuria is

associated with increased arterial stiffness, systemic atherosclerosis and maladaptive arterial remodelling [31, 32].
The last group of modifiable risk factors favouring CKD progression includes various endocrine and metabolic factors such as overactivated renin-angiotensin system (RAS), uncontrolled diabetes mellitus, obesity, dyslipidaemia, smoking and hyperuricemia.
Many different studies, including randomized clinical trials, demonstrated that tight glycaemic control may noticeably slow the progression rate of diabetes-related macro- and microvascular disorders such as diabetic nephropathy in type 1 and 2 of diabetes mellitus [33, 34, 35, 36]. Also the body weight reduction positively influences the renal hemodynamic changes and decreases CKD-related proteinuria [37, 38, 39].
The other factors, such lipids, smoking and uric acid have also been associated with progression of CKD. Dyslipidaemia is related with a faster progression rate of CKD [40], as well as smoking, which increases the risk of proteinuria, probably by overactivation of sympathetic nervous system, hypertension or direct toxicity to the tubulus [41].
With regard to hyperuricemia, its relations with CKD, hypertension or CVD (cardiovascular disease) by stimulation of RAS are well known [42]. Recently however, it has been proposed that hyperuricemia in CKD patients is an independent risk factor for all-cause and CVD mortality but not for renal failure [43].
The implications of RAS in the pathogenesis of CKD progression have been linked with proteinuria, systemic hypertension and CVD in different studies [44]. Since the two crucial components of RAS, ACE1 and ACE2, are the subjects of this study, they will be presented separately.

1.1.2 Uraemia

While the CKD stages 1 or 2 are usually not manifested in any symptoms due to the GFR decline, the progression to CKD5 is often associated with the extensive accumulation of toxins, disturbances in patient's life activities, nutritional status and electrolyte balance resulting in uremic syndrome. Uraemia affects virtually all cells in the body and may be manifested clinical, as the following complications: **cardiovascular** (volume overload and systemic hypertension, accelerated atherosclerosis and ischemic heart disease, left ventricular hypertrophy, heart failure, arrhythmia, uremic pericarditis), **neurologic (**cerebrovascular accidents, encephalopathy, seizures, peripheral and autonomic neuropathy), **gastrointestinal (**anorexia, nausea and vomiting, malnutrition, uremic foetor, inflammatory and

ulcerative lesions, gastrointestinal bleeding), **hematologic** (anaemia, leukocyte and immune system dysfunction, platelet dysfunction), **osteologic** (renal osteodystrophy, growth retardation in children, muscle weakness, amyloid arthropathy secondary to ß2-microglobulin deposition), **endocrine** (sexual dysfunction, infertility in women, glucose intolerance due to insulin resistance, hyperlipidaemia), **dermatologic** (paleness, hyperpigmentation, ecchymosis and hematomas, pruritus, skin necrosis, bullous lesions). **Laboratory findings** demonstrate the signs of hyponatremia (if excessive water intake), hyperkalaemia, hyperphosphatemia, hypocalcaemia, hypomagnesemia, hyperuricemia and metabolic acidosis [3].

Hundreds of different toxins, which normally undergo renal excretion, have been implicated in the pathophysiology of uremic syndrome. From the quantitative point of view, urea is the most important compound excreted by the kidney. The other uremic solutes include peptides and small proteins (i.e. b2-microglobulin, poorly dialysed due to its large size), guanidines (i.e. guanidosuccinic acid, extensively produced in uraemia), phenols (i.e. p-cresol sulphate, protein bound), indoles (i.e. indicant, protein bound), aliphatic amines (i.e. dimethylamine, large volume of distribution), furans (i.e. 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, protein bound), polyols (i.e. myoinositol, normally degraded by the kidney), nucleosides (i.e. pseudouridine), dicarboxylic acids (i.e. oxalate, formation of crystal deposits), and carbonyls (i.e. glyoxal, participate in the formation of advanced glycation end products) [45].

Currently, most ESRD patients undergo haemodialysis (HD) three times per week in order to remove about two thirds of the total-body urea content during each treatment [46]. While HD treatment is able to remove the majority of urea as it can relatively freely diffuse throughout body water, elimination of other uremic solutes is limited due to the large molecular size, protein binding or sequestration within body compartments. Since conventional HD with High-flux (HF) membranes eliminates such mediators insufficiently, their accumulation may lead to elevated synthesis of hepatic C-reactive protein (CRP) along with other acute-phase reactants as manifested by progressive systemic inflammation as well as its vascular consequences in form of atherosclerosis [47, 48].

Currently, the HD membranes with increased permeability are able to remove large quantities of inflammation mediators. This fact makes these membranes a promising tool for chronic HD patients with elevated CRP level [49, 50].

The effects of high permeability HD on inflammatory markers and especially leucocytic ACEs are the crucial part of this work and will be presented below.

1.1.3 Inflammation

Systemic chronic inflammation plays a crucial role in the initiation and progression of atherosclerosis. Patients with CKD or ESRD maintained on intermittent HD develop progressive atherosclerosis leading to cardiovascular events such as myocardial infarction and stroke [51, 52, 53].

Inflammation can be defined as the response of the vasculature or tissues to various injurious stimuli and is initiated by different factors including complement, pro-coagulants, fibrinolytics and cytokines. It proceeds until specific counter-regulatory mechanisms are triggered, such as the activation of leukotrienes, prostaglandins, lipoxins and neutrophils or suppression of macrophages.

Chronic inflammation, similar to above mentioned acute inflammation, is mediated via a cascade of various biological pathways and participation of immune system and the vasculature. Prolonged inflammation leads to the accumulation of pro-inflammatory mediators in the tissue and may result not only in tissue disruption, but continuation of the inflammatory process [54].

Since the kidney is the key organ for elimination of many inflammatory mediators, especially cytokines, the balance between the pro-inflammatory cytokines and their inhibitors is obviously dysregulated in CKD patients [55, 56]. In the context of uraemia and atherosclerosis in patients with renal diseases, it is worth to mention the cytokines related to local and systemic inflammation. The cytokines activated locally and associated with atherogenic behaviour include mainly interferon (IFN)-γ, interleukins (IL)-8, IL-12, IL-18 and tumour necrosis factor alpha (TNFa). To the most important systemic mediators and markers of inflammation belong interleukin-6 (IL-6) and IL-8 [57].

Cytokines are small (ca. 5-20 kDa), soluble proteins that are produced or released in response to an antigen and other signals and function as chemical messengers regulating various aspects of the innate and humoral immune systems. Cytokines are produced by a broad range of cells, including immune cells like monocytes/macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells [58].

Irrespective of the cause of renal disease, there is firm evidence that an acute and chronic pro-inflammatory state exists in CKD and ESRD patients [59]. Elevated levels of CRP, IL-6 and TNFa are well established pro-inflammatory predictors for increased risk of atherosclerotic complications and adverse cardiovascular events related to high mortality rates [60, 61, 62].

This work will show how the uraemia and the fluids obtained from high permeability dialysis in vitro modulate the inflammation profile of THP-1 monocytes.

1.1.4 Atherosclerosis

Non-traditional risk factors, including persistent low-grade inflammation, are crucial factors participating in atherogenesis, vascular calcification, and other causes of CVD and may also contribute to protein-energy wasting and other complications in CKD patients [63]. These patients are at higher risk of all-cause mortality and obviously suffer from both atherosclerosis and arteriosclerosis [64, 65]. Recent data suggest that inflammation is predominantly associated with that plaque forming disease of atherosclerosis rather than vascular stiffening [66].

So called “classical” cardiovascular risk factors such as dyslipidaemia, hypertension, diabetes or smoking promote initiation and progression of atherosclerosis by recruitment of circulating immune cells to infiltrate the injured vascular endothelium [67, 68]. Atherosclerosis can be described as a focal disease process that arises predominantly at sites of disturbed laminar flow, notably, arterial branch points and bifurcations. The morphological and functional studies of the earliest stages of atherogenesis in human and animal models suggest that the key initiating factor is subendothelial accumulation of the proteins with a high apolipoprotein B-content. This leads to the early inflammatory response to retained lipoproteins, activation of overlying endothelial cells and recruitment of blood-borne monocytes. The monocytes infiltrating in the subendothelial space differentiate into macrophages and dendritic cells which trigger the accumulation of lipids, extracellular matrix components and other cells in the vessel wall. Extensive and prolonged accumulation of lipid-carrying apoptotic cells, cell debris and cholesterol crystals leads to the formation of atherosclerotic plaque [69]. Infiltrating monocytes may differentiate into different macrophage subtypes with either protective or pathogenic activities. Recent studies suggest that classically activated macrophages M1 (or CAMs) may possess pro-atherogenic abilities in contrast to alternatively activated athero-protective macrophages M2 (or AAMs) [70, 71]. However in advanced stages of atherosclerosis, secretion of metalloproteinases, typical for M2-like macrophages, may contribute to matrix degradation and plaque rupture, which may trigger a myocardial infarction or stroke [72, 73, 74, 75].

In ESRD patients circulating monocytes are activated and proinflammatory monocytes are expanded. We recently demonstrated that elevated levels of monocytic ACE on those cells in HD patients are associated with increased mortality and cardiovascular morbidity and may also participate in the initial steps of atherosclerosis [66, 76, 77].

1.2 Local RAS and its components

Local RAS refers to cellular or tissue-resident mechanisms of angiotensin peptide formation that act separately or independently of the circulating RAS. The circulating, systemic RAS includes kidney-derived renin (angiotensinogenase) acting on liver-derived angiotensinogen to generate AngI that is further converted to AngII by ACE. Since the tissues are the main site of generation of angiotensin peptides by the systemic RAS, plasma-derived renin acts on plasma-derived angiotensinogen to produce AngI, which is converted to AngII by endothelial ACE [78, 79].
Over-activation of the RAS observed under pathophysiological conditions such as CKD is one of the common factors leading to cardiovascular complications.
Recent data suggest that systemic RAS is not only an important element in the control of blood pressure, but also a modulating factor in the progression of atherosclerosis. Continuous activation and recruitment of circulating leukocytes to the sites of inflammation triggered by overactivated RAS may promote development of atherosclerotic plaques [80]. In addition to these findings circulating leukocytes, especially monocytes, possess their own functional RAS components modulating local immune response and behaviour of the cells. The presence of ACE, angiotensinogen (AGT), AngI, AngII and its receptors AT1R and AT2R on human monocytes indicate that this cell type might have a functionally relevant auto- or paracrine angiotensin system potentially involved in the pathogenesis of vascular disease [81]. Furthermore, the components of the RAS are expressed at strategic sites of human atherosclerotic coronary arteries and colocalize with IL-6 expression in CD68-positive macrophages, indicating that local ACE in these cells is the primary AngII–forming pathway in human atherosclerotic plaques. Since AngII is able to stimulate the synthesis and release of IL-6 in monocytes/macrophages in vitro and ACE is extensively accumulated in human atherosclerotic macrophages, involvement of local RAS and inflammation as proatherosclerotic factors is obvious [82, 83].

Pharmacological blockade of RAS exerts beneficial effects in CKD patients and slows, but does not prevent cardio-renal disease progression, suggesting the involvement of other, non-canonical RAS factors [85, 86, 87]. The actions of the classical RAS are mediated by ACE, which generates AngII, a potent vasoconstrictor and main RAS effector. The pro-inflammatory and pro-proliferative actions of AngII may be counteracted by the recently described ACE2, which degrades AngII to Ang1-7, a potent vasodilator. The actions of Ang1-7 are transmitted through a G protein-coupled receptor, MasR [88, 89, Figure 1].

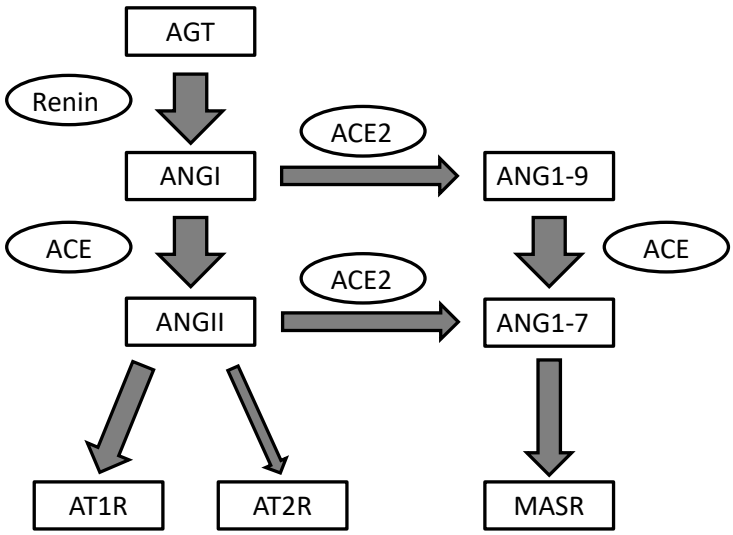

Figure 1: Schematic presentation of RAS. AGT, angiotensinogen; Ang, angiotensin; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; MasR, Mas receptor; modified from [84].

Both ACEs are abundantly expressed in human cardiac, pulmonary and renal tissues, and other organs such as liver, placenta and central nervous system [90, 91, 92, 93, 94]. ACEs are also expressed on the surface of monocytes, macrophages and smooth muscle cells [81, 95].

Recently we demonstrated that elevated levels of monocytic ACE in HD patients are associated with increased mortality and cardiovascular morbidity and may also participate in the initial steps of atherosclerosis [66, 76, 77]. Mouse models demonstrated that ACE deficiency in bone marrow–derived cells decreased hypercholesterolemia-induced atherosclerosis, while macrophages from ACE2-deficient mice promoted atherosclerosis by increased expression and release of inflammatory cytokines and elevated adhesion of these cells to endothelial cells [95, 96]. Conversely adenovirus-mediated ACE2-overexpression in atherogenic animal models was able to decrease pro-atherosclerotic actions and to improve endothelial homeostasis by decreasing monocyte adhesion [97, 98].

2 Results and Discussion

This study will elucidate the molecular and functional contributions of monocytic and leucocytic ACEs in uraemia and their relations with development and progression of atherosclerosis. This study will demonstrate the expression of ACEs and their receptors in leukocytes originating from healthy individuals and CKD patients. The functional behaviour of monocytic ACEs and the expression of the inflammatory and atherosclerotic molecules will be shown in vitro under conditions mimicking those of chronic renal failure and in overexpression studies. Finally, the expressional changes of cellular ACEs and inflammatory markers as a response to high permeable HD and treatment with sera and dialysate fluids will be presented.

2.1 *Pro-atherosclerotic contributions of monocytic ACE under uremic conditions*

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Our studies revealed that either treatment or prolonged monocyte differentiation in the presence of uremic or inflammatory conditions led to up-regulation of ACE. The effects of uremic serum and endotoxic (lipopolysaccharide) LPS on the regulation of ACE were tested on the human primary and THP-1 monocytes in vitro. Treatment of these cells with serum obtained from HD patients resulted in dramatical up-regulation of ACE.
In further experiments we investigated the uremic regulation of ACE during PMA (phorbol 12-myristate 13-acetate)-mediated differentiation into macrophages in the presence of HD or inflammatory conditions, such as supplementation with LPS. The cells differentiated in the presence of uremic conditions revealed significantly increased ACE expression as compared to monocytes treated with normal serum (NS). Furthermore, HD serum potentiated the up-regulation of ACE by LPS.
Similar effects on primary monocytes were also observed with sera originating from non-dialysed CKD patients or patients on peritoneal dialysis (PD). Also these cells reacted with significantly elevated expression of ACE.
Regulation of ACE was demonstrated in different cellular models but was not directly correlated with influences of uraemia or inflammation on human monocytes in vitro in context of ACE expression [127, 99, 100, 101]. Based on the observations of other cell culture systems, it has been demonstrated that uremic toxins may affect oxidative burst activity of the leukocytes and increase their pro-inflammatory effects as resulted in the tendency to vascular damage in CKD patients [102]. Furthermore, Shimizu et al. reported that indoxyl sulphate (IS), one of the most representative uremic toxins, up-regulated AGT expression in proximal tubular cells [103]. In studies by Sun et al. IS and p-cresol sulphate up-regulated in addition to elevated AGT expression, other renin–angiotensin–aldosterone system (RAAS) components such as renin and AT1R [104].
Based on these observations and the fact that HD removes different uremic toxins, we examined the pre- and post-dialysis effects of HD-sera on the regulation of monocytic ACE. Indeed, treatment of the primary monocytes with pre- or post-HD sera revealed significantly up-regulated ACE expression as compared to NS treatments. In addition,

ACE level in post- HD treatments was significantly and noticeably lower as compared to pre-HD.
Up-regulation of the RAS components may subsequently cause increased expression of monocytic ACE and facilitate behavioural and morphological changes of the monocytes under uremic conditions. Previous studies reported a possible link between uremic toxins and cardiovascular diseases. The authors demonstrated that uraemia-mediated increase in leukocyte-endothelial adhesion occurs through elevation of E-selectin in HUVEC cells and is mediated via the JNK (c-Jun N-terminal kinase)- and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)-dependent pathway [105].
Since the uremic stimulus is able to upregulate ACE expression in primary and THP-1 monocytes as demonstrated above, in further investigations we tested whether the cells may respond with the changes in endothelial adhesion and transmigration rates. We were able to demonstrate that primary monocytes reacted to HD stimulus with significantly increased number of the cells adhered to endothelial monolayers.
Similar behaviour of the monocytes was observed under uremic conditions mimicked with PD or CKD5 serum originating from non-dialysed patients. Also here the monocytes transmigrated noticeably faster through endothelial monolayers in the presence of all uremic sera tested as compared with serum obtained from healthy individuals. It worth to note that also in culture conditions uremic stimulus led to development of higher number of more differentiated cells than NS treatment.
Since uraemia-mediated increase in ACE expression coincided with elevated adhesion and transmigration, such a phenotype of the monocytes was studied by stable overexpression of ACE. These results will be discussed below.

The functional correlation between ACE expression and adhesive properties of the monocytes was demonstrated on human primary and especially THP-1 monocytes transfected with a plasmid carrying full coding sequence of human ACE.
Human monocytes overexpressing ACE revealed not only more differentiated, macrophage-like phenotype, but also a higher expression of MCSF (macrophage colony-stimulating factor) as compared with control cells.

These observations correlate with previous findings demonstrating that accumulation of monocyte-derived macrophages at the sites of endothelial dysfunction is a crucial event in atherogenesis [106]. The analysis of macrophage markers in ACE-overexpressing cells demonstrated noticeably elevated expression of Arg1 (arginase 1) and no significant changes for Arg2 (arginase 2) or iNOS (inducible nitric oxide

synthase). Investigations of inflammation markers demonstrated a marked up-regulation of TNFa and IL-6. These observations suggest that ACE mediates an alternative activation of the macrophages, leading to M2-phenotype with pro-inflammatory and pro-atherosclerotic properties. More differentiated phenotype of these cells correlated well with significantly decreased proliferation rates and increased attachment to plastic surface or endothelial monolayers. It has been demonstrated that such M2 macrophages have a higher capacity to accumulate modified lipids than M1 and upon exposure to ox-LDL (oxidized low-density lipoprotein) the pro-inflammatory responses of M2 cells are increased [107]. Furthermore, M2 cells have been detected in plaques where surround the lipid core. It has been suggested that Arg1, typical for these cells, may promote stabilisation of atherosclerotic plaques and enhance the proliferation of vascular smooth muscle cells [108].

Further investigations revealed that uraemia-mimicked conditions add to the ACE-overexpression related effects an additional stimulus leading to elevated endothelial-adhesion. Uremic serum (HD) not only significantly increased the adhesion of control cells, but further amplified the adhesion of ACE-overexpressing monocytes.

In addition to significantly increased endothelial adhesion, ACE-overexpressing cells demonstrated also increased transmigration rates through endothelial barrier. These properties can be explained by the fact that these cells express not only more MCP-1 (monocyte chemoattractant protein 1) but also its ligand CCR2 (C-C chemokine receptor type 2) as compared with corresponding controls. These crucial and novel findings are extremely important because the motility and adhesion of these cells may be boosted in an autocrine manner independently from endothelial function. Furthermore, the fact that MCP-1 is up-regulated in atherosclerotic plaques and arteries of animals fed a high cholesterol diet and that disruption of CCR2 in mouse models is related to anti-atherosclerotic actions, allows us to designate ACE-overexpressing monocytes as highly pro-atherogenic [109, 110, 111, 112, 113]. Animal studies dealing with the infusion of AngII, revealed that this compound led not only to increased plaque size, but also to elevated expression of inflammatory TNFa, IL-6, and migration-related MCP-1, CCR2 in aortic roots. Similar to CCR2 knock-out, as mentioned above, disruption of MCP-1 coincided with decrease in AngII-mediated pro-atherosclerotic actions in this study [114]. Studies by Ishibashi et al. revealed that in CCR2-/- mice, Ang II-induced vascular inflammation and aortic wall thickening and fibrosis were blunted as compared with control mice [115]. In other studies ACE-deficiency in murine bone marrow-derived cells led to diminished level of hypercholesterolemia-induced atherosclerosis and coincided with decreased expression of MCP-1 [95].

Investigations of the genes crucial for monocytic/macrophage endothelial-adhesion, revealed that primary and THP-1 monocytes reacted to ACE-overexpression with increased levels of ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion protein 1). It is worth to note that expression and induction of these molecules has been consistently observed in the initial steps of atherosclerosis and in atherosclerotic plaques [116, 117]. More importantly, ICAM-1 knockout mice are resistant to atherosclerosis [118] and mice with decreased VCAM-1 levels develop less pronounced atherosclerosis [119]. Also disruption or antibody-mediated blockage of ICAM-1 or VCAM-1 proved to exert beneficial effects on atherogenesis [120, 121].

It is well documented that ACE-inhibition and/or anti-AngII-receptor treatment has anti-atherosclerotic effects in experimental models as well as patients with cardiovascular disease [122, 123, 124]. We found in our study that overexpression of ACE into monocytes led to dramatically increased expression of AngII-receptors, AT1R and AT2R.

Investigations whether inhibition of ACE/AngII-receptor signalling could affect the adhesive abilities of ACE-overexpressing monocytes revealed that ACE-inhibitor Captopril or AT1R-blocker Losartan significantly decreased endothelial adhesion of these cells. These observations suggest that elevated endothelial-adhesion of ACE-overexpressing monocytes may be mediated by increased generation of local AngII and its receptor. Verification of these results with ACE-negative THP-1 wild type cells revealed that endothelial adhesion is dramatically increased in the presence of AngII and almost totally abolished by AT1R-blockage.

These important findings further demonstrate that inhibition of local, monocyte-derived AngII-generation might exert anti-atherogenic actions.

With regard to AngII-generation, it has been reported that subcutaneous injections of AngII led to accelerated carotid atherosclerosis in apolipoprotein E-deficient mice. Additionally the authors observed increased expression of adhesion molecules such as E-selectin, ICAM-1, VCAM-1, chemokine MCP-1, and MCSF. Similar to our observations, enalapril, an ACE-inhibitor, decreased these expressions and the number of adhered macrophages and foam cells in the arterial wall [125].

Our results demonstrate that uremic conditions on the one hand up-regulate ACE expression, thus creating pro-atherogenic monocytes. In addition, these conditions serve as an additional amplifier of monocyte-endothelial adhesion even for the cells already overexpressing ACE. This suggests that ACE increase is an important but most likely not the only mechanism by which uraemia enhances monocyte endothelium interactions.

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Uremic Conditions Drive Human Monocytes to Pro-Atherogenic Differentiation via an Angiotensin-Dependent Mechanism

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Abstract

Aims: Elevated expression levels of monocytic-ACE have been found in haemodialysis patients. They are not only epidemiologically linked with increased mortality and cardiovascular disease, but may also directly participate in the initial steps of atherosclerosis. To further address this question we tested the role of monocytic-ACE in promotion of atherosclerotic events in vitro under conditions mimicking those of chronic renal failure.

Methods and Results: Treatment of human primary monocytes or THP-1 cells with uremic serum as well as PMA-induced differentiation led to significantly up-regulated expression of ACE, further increased by additional treatment with LPS. Functionally, these monocytes revealed significantly increased adhesion and transmigration through endothelial monolayers. Overexpression of ACE in transfected monocytes or THP-1 cells led to development of more differentiated, macrophage-like phenotype with up-regulated expression of Arg1, MCSF, MCP-1 and CCR2. Expression of pro-inflammatory cytokines TNFa and IL-6 were also noticeably up-regulated. ACE overexpression resulted in significantly increased adhesion and transmigration properties. Transcriptional screening of ACE-overexpressing monocytes revealed noticeably increased expression of Angiotensin II receptors and adhesion- as well as atherosclerosis-related ICAM-1 and VCAM1. Inhibition of monocyte ACE or AngII-receptor signalling led to decreased adhesion potential of ACE-overexpressing cells.

Conclusions: Taken together, these data demonstrate that uremia induced expression of monocytic-ACE mediates the development of highly pro-atherogenic cells via an AngII-dependent mechanism.

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Introduction

Systemic chronic inflammation plays a crucial role in the initiation and progression of atherosclerosis. Patients with chronic kidney disease (CKD) maintained on intermittent hemodialysis develop progressive atherosclerosis leading to cardiovascular events such as myocardial infarction and stroke [1], [2], [3]. These patients are at higher risk of all-cause mortality [4] and obviously suffer from both atherosclerosis [5] and arteriosclerosis [6]. Recent data suggest that inflammation is predominantly associated with that plaque forming disease of atherosclerosis rather than vascular stiffening [7].

So called "classical" cardiovascular risk factors such as dyslipidemia, hypertension, diabetes or smoking promote initiation and progression of atherosclerosis by recruitment of circulating immune cells to infiltrate the injured vascular endothelium [8], [9]. The monocytes infiltrating in the subendothelial space differentiate into macrophages and dendritic cells which trigger the accumulation of lipids, extracellular matrix components and other cells in the vessel wall. Extensive and prolonged accumulation of lipid-carrying apoptotic cells, cell debris and cholesterol crystals leads to the formation of atherosclerotic plaque [10]. Infiltrating monocytes may differentiate into different macrophage subtypes with either protective or pathogenic activities. Recent studies suggest that classically activated macrophages (M1 or CAMs) may possess pro-atherogenic abilities in contrast to alternatively activated athero-protective macrophages M2 (or AAMs) [11], [12]. However in advanced stages of atherosclerosis, secretion of metalloproteinases, typical for M2-like macrophages, may contribute to matrix degradation and rupture, which may trigger a myocardial infarction or stroke [13], [14], [15], [16].

Angiotensin converting enzyme (ACE) participates in the regulation of blood pressure (arterial vasoconstriction) and sodium and water balance. The circulating exopeptidase catalyzes the conversion of decapeptide angiotensin I (AngI) to octapeptide angiotensin II (AngII), a potent vasoconstrictor. ACE is not only secreted by pulmonary and renal endothelial cells, but is also expressed on the surface of monocytes, macrophages and smooth muscle cells [17]. Furthermore, ACE has also been detected in

human atherosclerotic lesions, where it is associated with a subset of macrophages [18], [19], [20] or cells possessing dendritic-like properties [21].

The presence of angiotensinogen (AGT), AngI, AngII and its receptors on human monocytes indicate that this cell type might have a functionally relevant auto- or paracrine angiotensin system potentially involved in the pathogenesis of vascular disease [22]. Inhibition of ACE and/or employment of AngII receptor 1 (AT1R) antagonists has been shown to be effective in decreasing clinical events in patients with atherosclerosis [23]. Little is known about local cellular effects besides systemic blood pressure control, particularly on monocytic ACE (mACE).

In patients with end stage renal disease circulating monocytes are activated and proinflammatory monocytes are expanded. We recently demonstrated that elevated levels of mACE on those cells in hemodialysis patients are associated with increased mortality and cardiovascular morbidity and may also participate in the initial steps of atherosclerosis [7], [24], [25].

In this study we used the primary human monocytes and myelomonocytic cell line THP-1 to investigate the regulation of mACE under conditions of uremia. Furthermore, a potentially causal involvement of ACE upregulation for atherosclerosis initiation was studied.

Materials and Methods

Human serum and isolation of primary human monocytes

Pooled uremic sera (HD) were obtained from chronic hemodialysis patients (n = 10; both males and females) treated at the Dialysis Ward of the University Clinic Halle. All HD patients were treated three times weekly by standard bicarbonate hemodialysis with ultrapure water quality (by reverse osmosis and sterile filters) using high flux polynephron membranes (Nipro Europe). All HD samples were obtained prior to a regular hemodialysis session from the dialysis access and anti-coagulated with heparin. Pooled uremic sera from peritoneal dialysis (PD) were obtained from 5 patients (both males and females). Additionally pooled uremic sera were isolated from 3 CKD5 patients (males) not on dialysis and 10 HD patients (both males and females) prior to (pre-HD) and after (post-HD) a hemodialysis session. HD, PD, CKD 5, pre- and post-HD patients were aged between 40 and 70 years, they were free of acute infectious complications and were not treated by immunosuppressive medication. Normal sera (NS) were obtained from age matched healthy volunteers (n = 8; age range 50 to 67 years; both males and females). Human primary monocytes were isolated from 3 healthy volunteers by employment of Pan Monocyte Isolation Kit (Miltenyi) and AutoMacs cell separator (Miltenyi) according to manufacturer's instructions. All healthy volunteers were normotensive and none was taking any medication. The isolation of the human sera and primary monocytes were performed according to the declaration of Helsinki. This study was approved by the ethical committee of the Martin Luther University, Faculty of Medicine. All patients and persons involved in this study gave written consent.

Cell culture and treatments of primary human monocytes and THP-1 cells

THP-1 monocytes were cultured in RPMI (Sigma) medium, supplemented with 1.125 g/l sodium carbonate, 10% heat inactivated (65°C/30 min) fetal calf serum (FCS) and 100 µg/ml penicillin/streptomycin (Biochrom). THP-1 cells (1×10^6/well) cells were treated with RPMI medium containing 10% HD or NS or 10 ng/ml LPS (Sigma) or combination of LPS and human sera in the presence of 10 ng/ml PMA (Sigma) as differentiating agent for 72 h. Treatment medium was replaced daily.

3×10^5/well primary human monocytes were treated with 10% NS or HD or PD or CKD5 or pre-HD or post-HD sera for 72 h in RPMI medium without FCS. All treatments were performed in hydrophobic 6-well plates (Greiner bio One) in a standard humidified incubator (37 °C, 5% CO2). Total RNA from THP-1 cells and human primary monocytes was isolated at 0 h, 24 h, 48 h and 72 h by employment of Tri Reagent (Sigma) and ZR RNA MiniPrep Kit (Zymo Research), respectively, both according to manufacturer's instructions.

ACE/AngII-receptor inhibition studies were performed with captopril (ACE inhibitor, Santa Cruz Biotechnology) or losartan (Angiotensin II type 1 receptor (AT1R) antagonist, Santa Cruz Biotechnology) or angiotensin II (Sigma). Briefly calcein-labelled (calcein-AM, 1 µM, Cayman Chemical) control or ACE-overexpressing monocytes were tested for their adhesion abilities to endothelial monolayers in the presence or absence of 500 nM captopril or 1 µM losartan for 30 min. Additionally endothelial-adhesion of ACE-negative THP-1 cells was tested in the presence or absence of Angiotensin II (1 µM) or losartan (1 µM). For details see below (adhesion assay) and corresponding figure legends.

HUVEC (Human umbilical vein endothelial cells) cell line was cultured in Medium 200 supplemented with LSGS (Low Serum Growth Supplement, Gibco).

Table 1. Primer sequences employed in this study.

Target	Sequences
RPL37A	S-5'-ATTGAAATCAGCCAGCACGC, AS-5'-AGGAACCACAGTGCCAGATCC
MCSF	S-5'-TAGCCACATGATTGGGAGTG, AS-5'-TATCTCTGAAGCGCATGGTG
AT1R	S-5'-GCACAATGCTTGTAGCCAAA, AS-5'-GGGTTGAATTTTGGGACTCA
AT2R	S-5'-TTCCCTTCCATGTTCTGACC, AS-5'-AAACACACTGCGGAGCTTCT
ICAM-1	S-5'-GGCCTCAGTCAGTGTGA, AS-5'-AACCCCATTCAGCGTCA
VCAM-1	S- 5'-CCGGATTGCTGCTCAGATTGGA, AS- 5'-AGCGTGGAATTGGTCCCCTCA
MCP-1	S-5'-TCGCGAGCTATAGAAGAATCA, AS-5'-TGTTCAAGTCTTCGGAGTTTG
Arg1	S-5'-GGAGACCACAGTTTGGCAAT, AS-5'-CCACTTGTGGTTGTCAGTGG
Arg2	S-5'-TTCCATCCTGAAGAAATCCG, AS-5'-AGAGCCTTTTCATCAAGCCA
iNOS	S-5'-AAAGACCAGGCTGTCGTTGA, AS-5'-ACGGGACCGGTATTCATTCT

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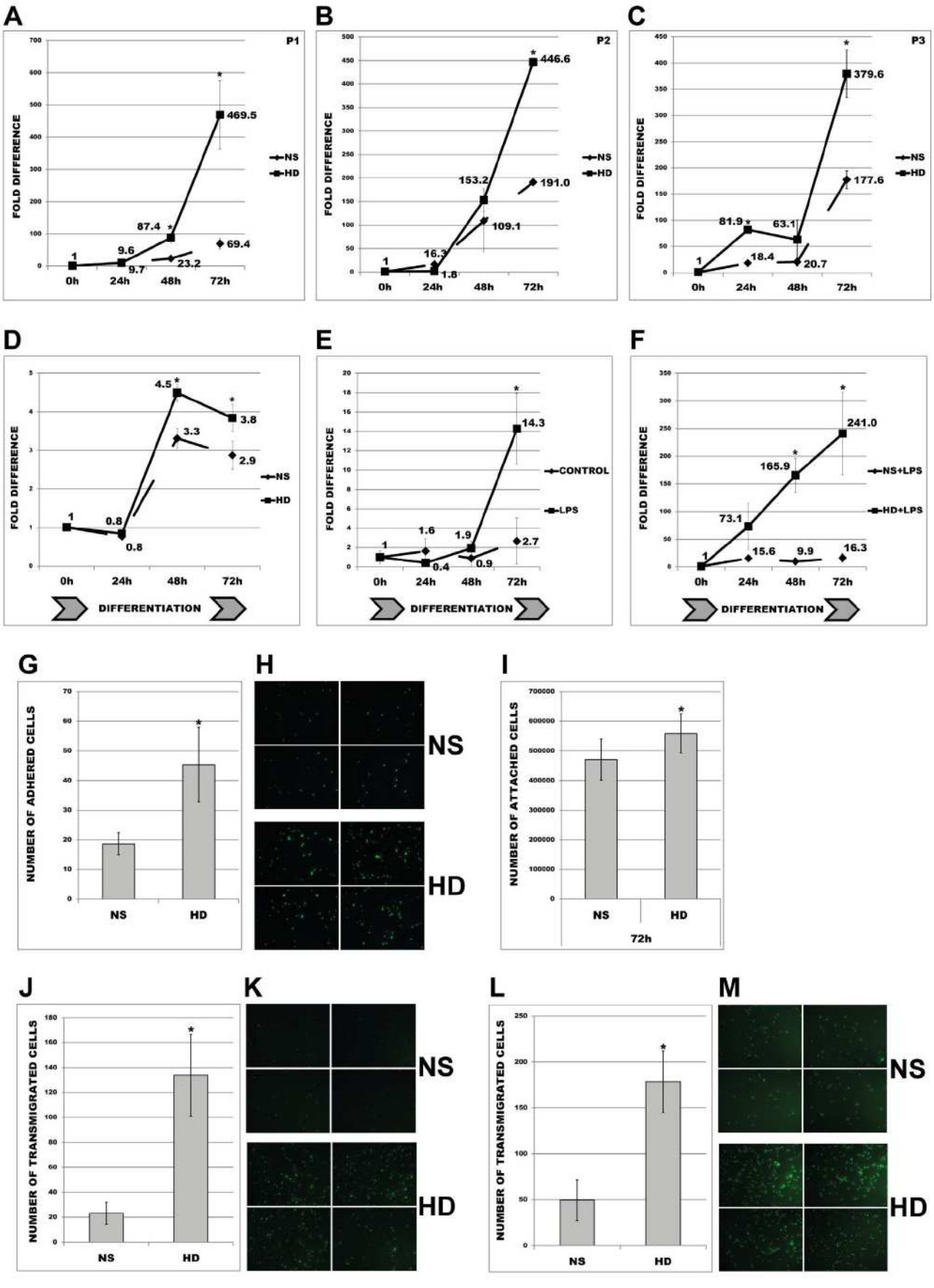

A
P1
FOLD DIFFERENCE
NS
HD
1
9.6
9.7
87.4
23.2
469.5
69.4
0h
24h
48h
72h
B
P2
446.6
153.2
191.0
109.1
16.3
1.8
C
P3
379.6
177.6
81.9
63.1
18.4
20.7
D
4.5
3.8
3.3
2.9
0.8
0.8
DIFFERENTIATION
E
CONTROL
LPS
14.3
1.6
1.9
2.7
0.4
0.9
F
NS+LPS
HD+LPS
241.0
165.9
73.1
15.6
9.9
16.3
G
NUMBER OF ADHERED CELLS
H
I
NUMBER OF ATTACHED CELLS
72h
J
NUMBER OF TRANSMIGRATED CELLS
K
L
M

Figure 1. Expression of ACE and behaviour of human monocytes under uremic status I. (A, B, C) Primary human monocytes obtained from healthy volunteers P1, P2, and P3 were treated with 10% NS or HD sera for 72 h and investigated for ACE-mRNA expression. Means ± SD of three independent experiments. (D, E, F) THP-1 monocytes were PMA-differentiated (10 ng/ml) into macrophages in the presence of (D) 10% NS or HD sera or (E) 10 ng/ml LPS or (F) 10 ng/ml LPS and both of them for 72 h. Means ± SD of three independent experiments. (G, H, I) Attachment and adhesion of THP-1 monocytes and primary monocytes treated with NS or HD. Primary human monocytes were incubated in the presence of 10% NS or HD sera for 30 min and investigated for their endothelial-adhesion. The number (G) and corresponding images of endothelial-adhered monocytes (H) are shown. Means ± SD of cell number in 10 microscopic fields in three independent experiments. (I) THP-1 were incubated in the presence of 10% NS or HD sera for 72 h and investigated for their attachment abilities. The number of attached cells (detached prior to counting) was counted by FACS. Means ± SD of four independent experiments. (J, K, L, M) Transmigration of calcein-labelled primary monocytes (J, K) or THP-1 cells (L, M) through endothelial monolayer towards lower chamber filled with RPMI medium supplemented with 10% NS or HD sera. Transmigrated cells were visualized with fluorescent microscopy (K, M) and counted in 10 random fields (J, L). Representative images are shown. Means ± SD of cell number in 10 microscopic fields in three independent experiments. * $p < 0.05$ indicates statistical significance.
doi:10.1371/journal.pone.0102137.g001

Transfection

For generation of THP-1 cells stably overexpressing human ACE, the cells were transfected with pcDNA3.1(-) carrying the full coding sequence of ACE (a gift from Dr. K. Kohlstedt, Goethe-University, Frankfurt am Main, Germany). Control cells received empty plasmid only. Transfection of the cells was performed in 24-well plates in normal growth medium. 1×10^6 THP-1 cells/well were transfected with 1 µg of ACE or empty plasmid in the presence of Lipofectamine 2000 as a carrier. After 24 h the cells were selected with growth medium supplemented with 1000 µg/ml neomycin G-418 (Biochrom) for next 8 weeks. Thereafter, the concentration of neomycin in growth medium was set up to 500 µg/ml. Transient transfection of primary human monocytes was performed with the same plasmids in 24-well plates in RPMI medium without FCS. 1×10^6/well of pooled primary human monocytes, obtained from volunteers were transfected with 1 µg of ACE or empty plasmid and Lipofectamine 2000 in the presence of RPMI medium. Expression of ACE and corresponding assays were performed 24 h after transfection.

RNA isolation and Real Time PCR

Total RNA was isolated using Tri-Reagent (Sigma) or ZR RNA MiniPrep Kit (Zymo Research), both according to manufacturer's instructions. Depending on the experiment 50 ng-1 µg of total RNA was used as a template for first strand cDNA synthesis employing High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Life Technologies). The samples were stored at -20°C.

Amplifications of ACE, TNFa, IL-6, 18S and RPL37A were performed with TaqMan Gene Expression Assays (Life Technologies) and FastStart Universal Probe Master Mix (Roche) in a StepOne plus System. 18S and RPL37A (Ribosomal Protein 37a) were employed for normalization of target mRNA expression. Thermal cycling conditions were as follows: hold 10 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Amplifications of, MCSF (Macrophage Colony-Stimulating Factor), AT1R (Angiotensin II Type I Receptor), AT2R (Angiotensin II Type II Receptor), ICAM-1 (Intercellular Adhesion Molecule 1), VCAM-1 (Vascular Cell Adhesion Molecule 1), MCP-1/CCL2 (Monocyte Chemotactic Protein-1), Arg1 (Arginase-1), Arg2 (Arginase-2), iNOS (inducible nitric oxide synthase) and RPL37A were performed with sequences presented in Table 1 and Maxima SYBR Green-qPCR Master Mix (Thermo Scientific). RPL37A was employed for normalization. Thermal cycling conditions were as follows: hold 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. Data evaluation was performed with DataAssist Software (Life Technologies).

MTT

In 96-well plates, 3000 THP-1 cells/well were seeded and cultured in growth medium for 24h, 48h and 72 h. For MTT assay, cells were then stained with MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) for 4 h at 37 °C and shortly incubated with dimethyl sulfoxide (DMSO, Roth). Thereby, a coloured formazan salt develops depending on the availability of mitochondrial NADH2 only in living, but not dead cells. Optical density was measured with BioRad ELISA reader at 550 nm. Experiment was repeated at least three times.

Attachment assay

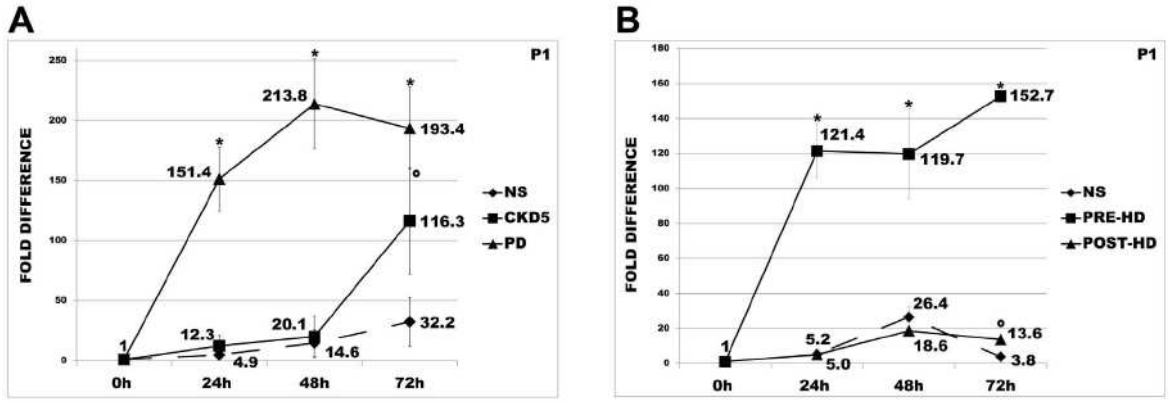
In 96-well plates, 3000 THP-1 cells/well were seeded and cultured in growth medium in the presence of 10 ng/ml PMA for 72 h. Thereafter the cells were fixed with 4% PFA/PBS for 30 min and vigorously washed with PBS. Remaining cells were stained with 0.2% crystal violet (CV, dissolved in water and filtered before use, Roth) for 30 min at RT. The excess of CV was removed by washing with deionized water until no violet colour release from culture plate was observed. Cellular CV was solubilized by addition of 0.1% SDS (Roth). The absorbance was measured after 15 min. incubation at RT at 550 nm. In long time attachment experiments 1×10^6 THP-1 cells were incubated in the presence of 10% NS or HD sera for 72 h. After two times washing with PBS, adhered cells were detached with Accutase and counted with FACS. Experiment was repeated at least three times.

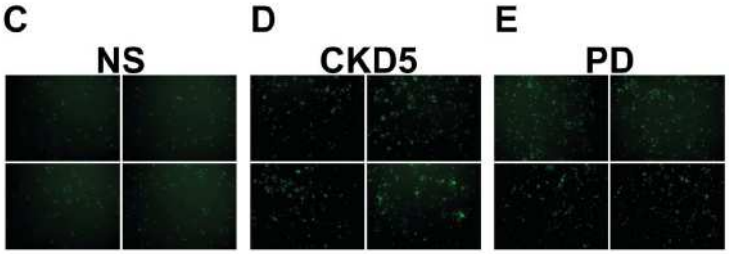
Adhesion assay

Adhesion of primary human monocytes or THP-1 cells to endothelial HUVEC monolayers was investigated in 24-well plates. Briefly, HUVEC cells were cultured in growth medium until they created monolayer on the bottom of 24-well plate. For labelling, primary monocytes or THP-1 cells were incubated in RPMI containing 1 µM calcein-AM at 37 °C for 60 min. The cells were washed with RPMI twice. Depending on the experiment fluorescence-labelled cells were re-suspended in 2 ml RPMI medium or RPMI medium containing 10% NS or HD or PD or CKD5 sera. HUVEC monolayers were washed twice with RPMI medium before addition of 2 ml labelled primary monocytes (3×10^5 cells/well) or THP-1 cells (1×10^6 cells/well) per well. The plates were incubated for 30 min at 37 °C. After incubation, the monolayer was gently washed three times with RPMI. Adherent monocytes were photographed using a Biozero BZ-9000 fluorescence microscope (Keyence). The number of the adherent cells was evaluated in 10 microscopic fields for each situation by employment of ImageJ software (Wayne Rasband, National Institutes of Health, USA). All experiments were repeated at least three times.

Transmigration assay

All transmigration assays were performed by employment of Millicell Cell Culture Inserts (Millipore, 8 µM pore size, 12 mm diameter). Primary monocytes or THP-1 cells were incubated in RPMI medium containing 1 µM calcein-AM at 37 °C for 60 min.


A
P1
FOLD DIFFERENCE
151.4
213.8
193.4
116.3
12.3
4.9
20.1
14.6
32.2
1
NS
CKD5
PD
0h
24h
48h
72h
B
P1
FOLD DIFFERENCE
121.4
119.7
152.7
5.2
5.0
26.4
18.6
13.6
3.8
1
NS
PRE-HD
POST-HD
0h
24h
48h
72h


C
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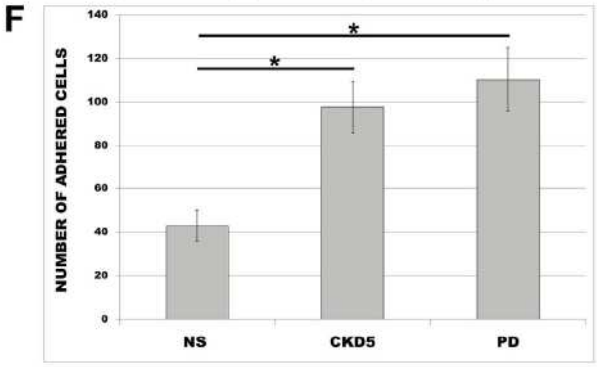
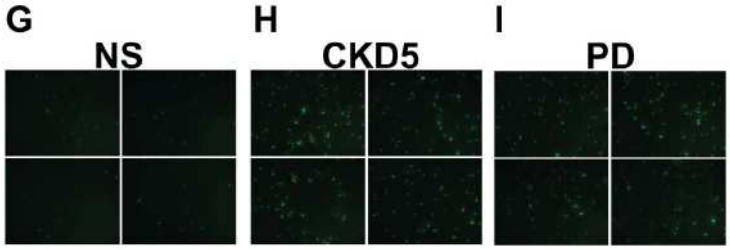

F
NUMBER OF ADHERED CELLS
NS
CKD5
PD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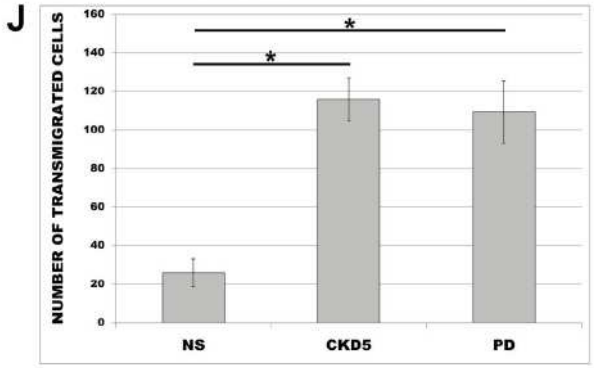

J
NUMBER OF TRANSMIGRATED CELLS
NS
CKD5
PD

Figure 2. Expression of ACE and behaviour of human monocytes under uremic status II. (A, B) Primary human monocytes obtained from healthy volunteer P1 were treated with (A) 10% NS or PD or CKD5 or (B) 10% NS or pre- or post-HD sera for 72 h and investigated for ACE-mRNA expression. Means ± SD of three independent experiments. * $p < 0.05$ (PD vs. NS; pre-HD vs. NS; pre-HD vs. post-HD) and °$p < 0.05$ (CKD5 vs. NS; post-HD vs. NS) indicate statistical significance. (C, D, E, F) Adhesion of primary monocytes treated with NS or PD or CKD5 sera. Primary human monocytes were incubated in the presence of 10% NS or PD or CKD5 sera for 30 min and investigated for their endothelial-adhesion. The number (F) and corresponding images of endothelial-adhered monocytes (C, D, E) are shown. Means ± SD of cell number in 10 microscopic fields in three independent experiments. (G, H, I, J) Transmigration of calcein-labelled primary monocytes through endothelial monolayer towards lower chamber filled with RPMI medium supplemented with 10% NS or PD or CKD5 sera. Transmigrated cells were visualized with fluorescent microscopy (G, H, I) and counted in 10 random fields (J). Representative images are shown. Means ± SD of cell number in 10 microscopic fields in three independent experiments. * $p < 0.05$ indicates statistical significance.
doi:10.1371/journal.pone.0102137.g002

The cells were washed with RPMI twice. 3×10^5 primary monocytes or 5×10^5 calcein-labelled THP-1 cells were re-suspended in 400 µl RPMI medium and seeded into upper transmigration chamber. Depending on the experiment, the cells transmigrated through uncoated or HUVEC monolayers coated membranes towards lower chamber filled with 600 µl RPMI medium supplemented with 50 ng/ml MCP-1 (Miltenyi) or 10% NS or HD or PD or CKD5 sera. In some experiments, THP-1 cells transmigrated towards HUVEC monolayers created on the bottom of lower chamber in the presence of RPMI only in both chambers. All transmigrations were performed at 37 °C for 60 min in the absence of FCS. Transmigrated cells were visualized by fluorescence microscopy (Biozero BZ-9000, Keyence) and counted in 10 microscopic fields for each situation by employment of ImageJ software (Wayne Rasband, National Institutes of Health, USA). In some experiments transmigrated cells were counted by FACS. Experiment was repeated at least three times.

Immunocytochemistry

1×10^5 THP-1 cells were seeded in chamber slides and let grown in normal medium for 24 h. The cells were gently washed with PBS and fixed in 4% PFA/PBS mixture for 10 min at RT. After washing twice with PBS, cells were incubated overnight with FITC-labelled mouse anti-human ACE antibody (AbD Serotec, clone 9B9, diluted 1.100 with PBS) at 4°C in the dark. Negative control was exposed to FITC-labelled IgG2a mouse anti-human antibody (BD Pharmingen) and processed as described above. After 3×10 min washing in PBS, the slides were covered with mounting medium containing a DAPI dye (Vectashield, Vector Laboratories) and dried 1 hour at RT in the dark. Microscopic investigations were performed with fluorescence microscope (Biozero BZ-9000, Keyence). Additionally the living cells were photographed in a phase contrast modus with the same microscope.

FACS

5×10^4 cells were washed twice with MACS buffer (1× PBS supplemented with 0.5%BSA 2 mM EDTA and 0.07% NaN3) and incubated with FITC-conjugated mouse anti-human ACE (AbD Serotec, clone 9B9) and PE-conjugated mouse anti-human CCR2 (R&D, clone 48607) for 30 min at RT in the dark. Measurements were performed with MacsQuant (Miltenyi) flow cytometer. Expression data (Isotype corrected) were presented as mean fluorescence intensity (MFI).

Statistics

Each experiment was repeated at least three times. Data are presented as mean ±SD. Statistical significance was calculated by employment of two-sided Student-t-test. The values of $p<0.05$ were considered statistically significant. Bonferroni correction was applied for multiply comparisons dividing the significance level by the number of tested variables.

Results

Uremic serum or LPS increase the expression of ACE

To test whether uremic serum or inflammatory conditions are able to regulate the expression of ACE we subjected primary human monocytes or undifferentiated THP-1 cells to 72 h treatment with normal (NS) or uremic (HD) serum or LPS. As shown in Fig. 1A–C primary human monocytes (P1, P2, P3) treated with HD revealed dramatically increased expression of ACE resulting after 72 h in an up-regulation range of 379.6–469.5 fold in all investigated samples. In order to investigate the uremic regulation of ACE during PMA-mediated differentiation into macrophages in the presence of HD or inflammatory conditions, THP-1 were incubated with 10 ng/ml PMA and corresponding sera or 10 ng/ml LPS or both of them for 72 h. As demonstrated in Fig. 1D, the cells differentiated in uremic conditions revealed significantly increased ACE after 72 h as compared to monocytes treated with NS. Further, HD serum potentiated the up-regulation of ACE by LPS (Fig. 1E, F).

Uremia-mediated increase in ACE expression coincided with elevated adhesion and transmigration

In further investigations we tested whether uremic stimulus is able to affect the adhesion and transmigration of primary human monocytes and undifferentiated THP-1 cells. As shown in Fig. 1G, H primary monocytes reacted on HD stimulus with significantly increased number of the cells adhered to endothelial monolayers. Similarly, THP-1 treatment with HD for 72 h led to significantly stronger attachment abilities when compared to NS (Fig. 1I). Additionally HD-stimulus led to development of higher number of more differentiated cells than NS treatment (data not shown). Also under HD both cell-types transmigrated significantly faster through endothelial monolayers than corresponding controls (Fig. 1J, K, L, M).

In order to test whether observed effects on primary monocytes may also be induced with sera coming from non-dialysed CKD patients or patients on other type of dialysis, we subjected P1-monocytes to the treatment with NS or PD or CKD5 sera. Additionally, to examine the pre- and post-dialysis effects of HD-sera on the regulation of ACE, the same cells were preconditioned in the presence of NS or sera obtained prior to (pre-HD) and after (post-HD) dialysis sessions. As demonstrated in Fig. 2A, treatment with PD or CKD5 sera for 72 h, exerted significantly up-regulated ACE expression pattern, similar to those observed under HD treatment. Furthermore, PD or CKD5 conditions led to significantly increased transmigration and endothelial adhesion of the monocytes when compared to corresponding controls (Fig. 2C, D, E, F, G, H, I, J). Treatment of the primary monocytes with pre- or post-HD sera for 72 h revealed significantly up-regulated ACE expression as compared to NS treatments. However, increase in ACE level in pre- HD treatments was significantly and noticeably higher as compared to post-HD or NS (Fig. 2B).

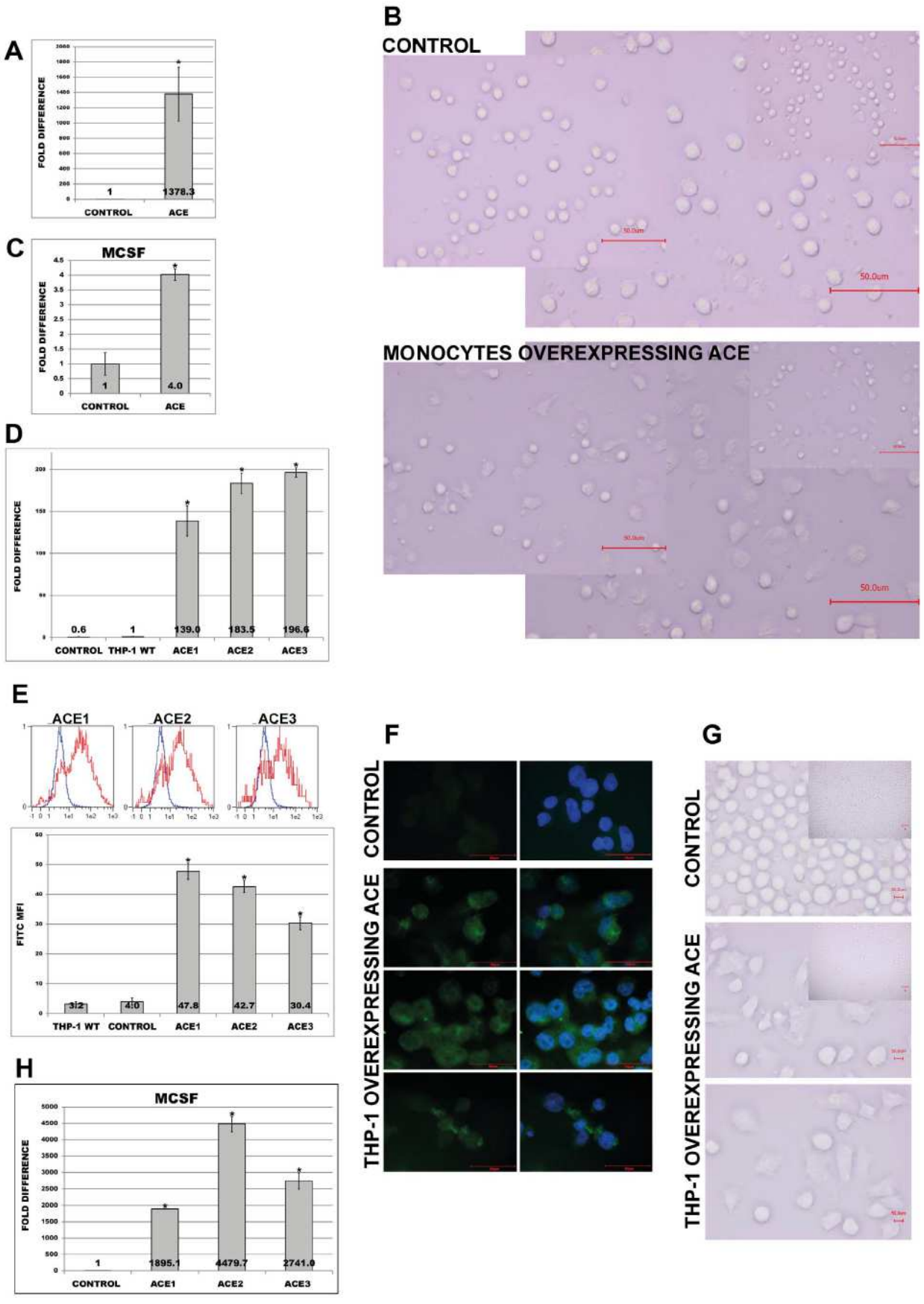

A
FOLD DIFFERENCE
1
1378.3
CONTROL
ACE
B
CONTROL
50.0um
MONOCYTES OVEREXPRESSING ACE
50.0um
C
MCSF
FOLD DIFFERENCE
1
4.0
CONTROL
ACE
D
FOLD DIFFERENCE
0.6
1
139.0
183.5
196.6
CONTROL
THP-1 WT
ACE1
ACE2
ACE3
E
ACE1
ACE2
ACE3
FITC MFI
3.2
4.0
47.8
42.7
30.4
THP-1 WT
CONTROL
ACE1
ACE2
ACE3
F
CONTROL
THP-1 OVEREXPRESSING ACE
G
CONTROL
THP-1 OVEREXPRESSING ACE
H
MCSF
FOLD DIFFERENCE
1
1895.1
4479.7
2741.0
CONTROL
ACE1
ACE2
ACE3

Figure 3. Expression of ACE and morphology of human monocytes overexpressing ACE. (A, B, C) Human primary monocytes were transiently transfected with empty or pcDNA3.1(-) plasmid carrying full coding sequence of ACE. Investigations of (A) ACE-expression, (B) cell morphology and (C) MCSF-expression were performed 24 h after transfection. Note differentiated, macrophage-like phenotype of primary monocytes overexpressing ACE. (D, E, F, G, H) Wild type (THP-1 WT), empty plasmid (Control) and ACE-overexpressing cells (ACE1, ACE2, ACE3) were investigated for (D) ACE transcript or (E) protein levels by employment of specific TaqMan probes or FACS analysis. (F) Representative immunofluorescence of control and ACE1 cells stained with FITC-ACE antibody (green) and DAPI staining (blue, nuclear). Note that left panel represents ACE staining only; right panel- ACE expression merged with DAPI; note mostly membrane-cytoplasmic localization of ACE. (G) Representative microscopic analysis of control and ACE1 cells under different magnifications. Note differentiated, macrophage-like phenotype of ACE1 cells. (H) RT-PCR analysis of MCSF expression in control and ACE-overexpressing cells (ACE1, ACE2, ACE3). Means ± SD of three independent experiments. * $p < 0.05$ indicates statistical significance.
doi:10.1371/journal.pone.0102137.g003

Phenotype, attachment and adhesion of monocytes are affected by ACE

To demonstrate a functional correlation between ACE expression and adhesive properties of the monocytes, we transfected primary human monocytes or THP-1 cells with plasmid carrying full coding sequence of human ACE. As demonstrated in Fig. 3A, transient transfection of primary monocytes resulted not only in significantly increased overexpression of ACE, but also led to development of more differentiated, macrophage-like phenotype (Fig. 3B). In addition these cells showed higher expression of MCSF (Fig. 3C).

Due to the limited nature of transient transfection and very short life-span of primary monocytes, we generated THP-1 monocytes stably overexpressing ACE. For further experiments we selected three clones demonstrating the highest ACE levels (designated as ACE1, ACE2 and ACE3). Control cells were transfected with empty plasmid. The overexpression of ACE in THP-1 was verified by RT-PCR, FACS analysis and immunofluorescence microscopy (Fig. 3D, E, F). Similar to primary monocytes, ACE-clones revealed also a macrophage-like phenotype and significantly increased expression of MCSF (Fig. 3G, H). Further analysis of macrophage markers demonstrated noticeably elevated expression of Arg1; however no significant changes for Arg2 or iNOS were observed (Fig. 4A, B, C). Investigations of inflammation markers demonstrated a marked up-regulation of TNFa and IL-6 (Fig. 4D, E). These observations suggest that ACE may promote an alternative activation of the macrophages, leading to M2-phenotype with pro-inflammatory and pro-atherosclerotic properties.

Fig. 5A shows that these more differentiated, ACE-overexpressing cells revealed significantly decreased proliferation rates than corresponding controls. Furthermore, these cells demonstrated not only increased attachment to plastic surface (Fig. 5B), but also to endothelial human monolayers as compared to corresponding controls (Fig. 5C, D, E). To test whether uremia-mimicked conditions add to the transfection related effects we subjected wild type, empty plasmid and ACE1 cells to adhesion assays in the presence of HD or NS. As demonstrated in Fig. 5F, G treatment with HD not only led to significantly increased adhesion of control THP-1, but further amplified the adhesion of ACE1 cells.

Overexpression of ACE led to increased transmigratory potential

We observed that ACE-overexpressing cells possess stronger adhesion properties than corresponding controls. In order to investigate whether these cells are able to transmigrate faster through the endothelial barrier, we subjected wild type, control and ACE clones to transmigration assays. As demonstrated in Fig. 6A, the number of cells transmigrating through the membrane upon MCP-1 was significantly higher with ACE transfectants than corresponding controls. Significantly increased transmigratory potential of ACE clones was also demonstrated when the cells were transmigrated without MCP-1 towards an endothelial-monolayer seeded on the bottom of the chamber (Fig. 6B) or through an endothelial-barrier in the presence of MCP-1 (Fig. 6C, D, E).

We speculated that increased transmigration potential may be due to altered chemotactic signalling towards these cells. In order to prove this hypothesis, we investigated ACE-clones and control cells for the expression of MCP-1/CCL2 and its receptor CCR2. As demonstrated in Fig. 6F expression of MCP-1/CCL2 was noticeably increased in ACE-overexpressing cells than in empty plasmid cells and resulted in an up-regulation range of 13.7–15.4 fold. Similar results were obtained for CCR2 where FACS analysis revealed a 7.8–9.0 times increase in MFI as compared to corresponding control (Fig. 6G).

Increased ACE levels affect the expression of adhesion-related genes and the status of Angiotensin II receptors

The effect of ACE-overexpression on adhesion-related genes was tested by qPCR. As demonstrated in Fig. 7A, expression of ICAM-1 and VCAM-1 was noticeably increased in primary monocytes transiently overexpressing ACE (4.3 and 16.5 fold increase respectively). ACE overexpression also led to a strong increase in the expression of AT1R (increase by factor 11.8) and AT2R (13.3). Investigations on THP-1 monocytes stably overexpressing ACE revealed similar expression patterns to those observed in primary monocytes (Fig. 7B). Expression of ICAM-1 and VCAM-1 was strongly up-regulated in all ACE-clones when compared to control cells (increase 189.9–712.6 fold and 17.8–53.5 fold respectively). Further ACE-mediated up-regulations were also observed for two AngII-receptors AT1R (increase by factor 184.8–809.2) and AT2R (86.9–257.0).

Inhibition of ACE/AngII-receptor signalling and AT1R-blockage in AngII-treated THP-1 monocytes led to decreased adhesion to endothelial monolayers

To test whether inhibition of ACE/AngII-receptor signalling could affect the adhesive abilities of ACE-monocytes, the effects of ACE-inhibitor Captopril or AT1R-blocker Losartan were tested. As demonstrated in Fig. 8A, B, both drugs were effective in decreasing the endothelial adhesion of ACE-overexpressing monocytes significantly. These results suggest that higher endothelial-adhesion of ACE-overexpressing monocytes may be mediated by increased generation of local AngII and its receptor. To finally confirm these observations we subjected ACE-negative THP-1 wild type cells to adhesion assay with AngII and/or losartan. As demonstrated in Fig. 8C, adhesion of THP-1 is dramatically increased in the presence of AngII and almost totally abolished by AT1R-blockage (co-incubation with losartan).

Discussion

This study clearly demonstrates a role of mACE for the uremia-associated atherogenic potential of monocytes and suggests an

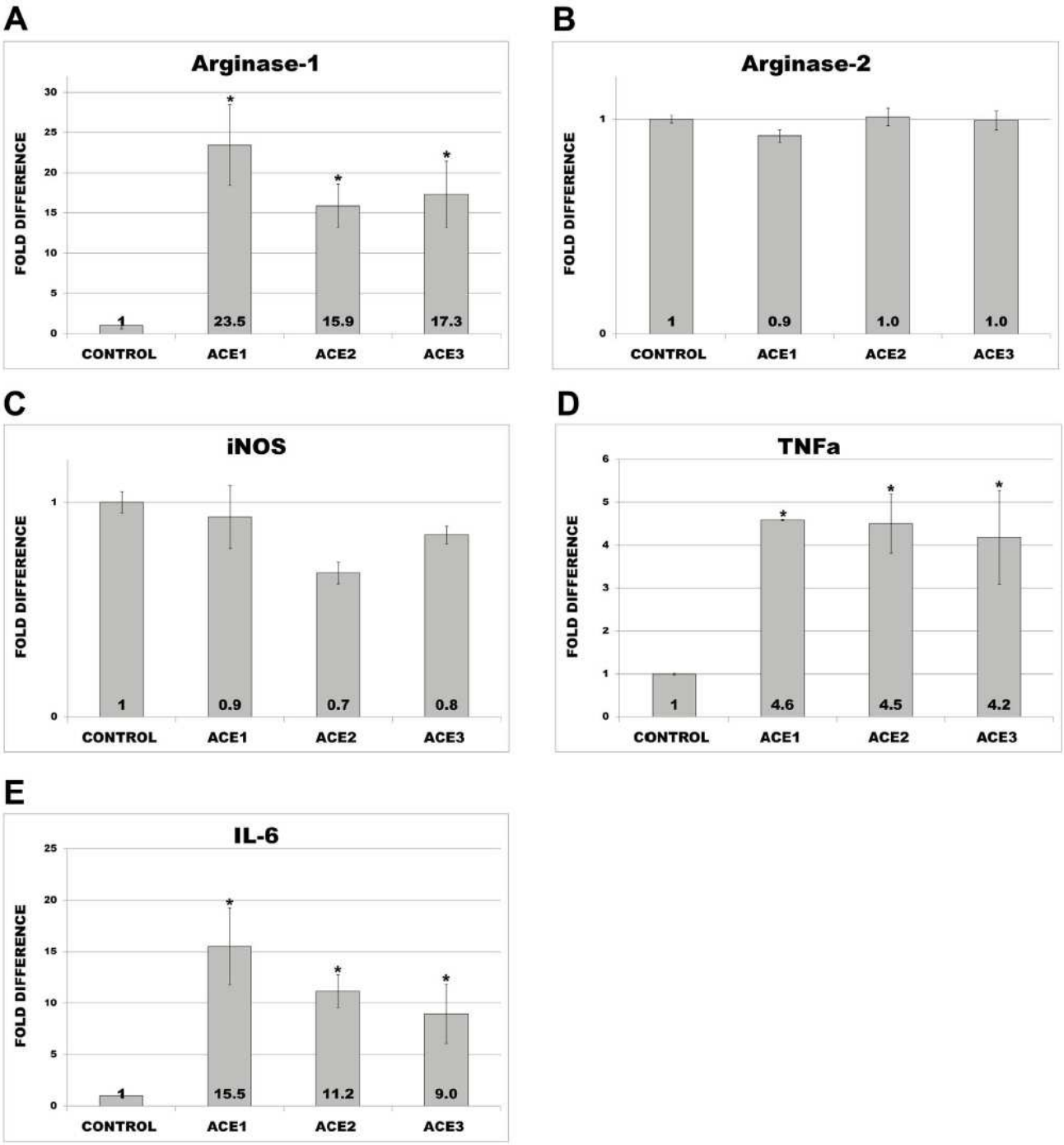


Figure 4. RT-PCR analysis of macrophage markers in control and THP-1 cells overexpressing ACE (ACE1, ACE2 and ACE3). Analyses were performed with primers specific for Arg1 (A), Arg2 (B), and iNOS (C), and TaqMan probes for TNFa (D) and IL6 (E). * $p < 0.05$ indicates statistical significance. Means ± SD of three independent experiments.
doi:10.1371/journal.pone.0102137.g004

autocrine/paracrine mechanism of activation of these pro-atherogenic cells.

We found that both uremic and inflammatory conditions affect and regulate the expression of mACE. Our studies revealed that either treatment or prolonged monocyte differentiation in the presence of uremic or inflammatory conditions led to up-regulation of ACE. Furthermore, we were able to show that elevation of mACE coincided with increased transmigration and adhesion of these cells. To our knowledge, this is the first report demonstrating uremic elevation of mACE-expression in relation to pro-atherogenic behaviour of primary human monocytes or THP-1 cells. Such a potential of ACE was previously reported in animal and cell culture models but was not directly correlated with influences of uremia on human monocytes in vitro [26], [27], [28],

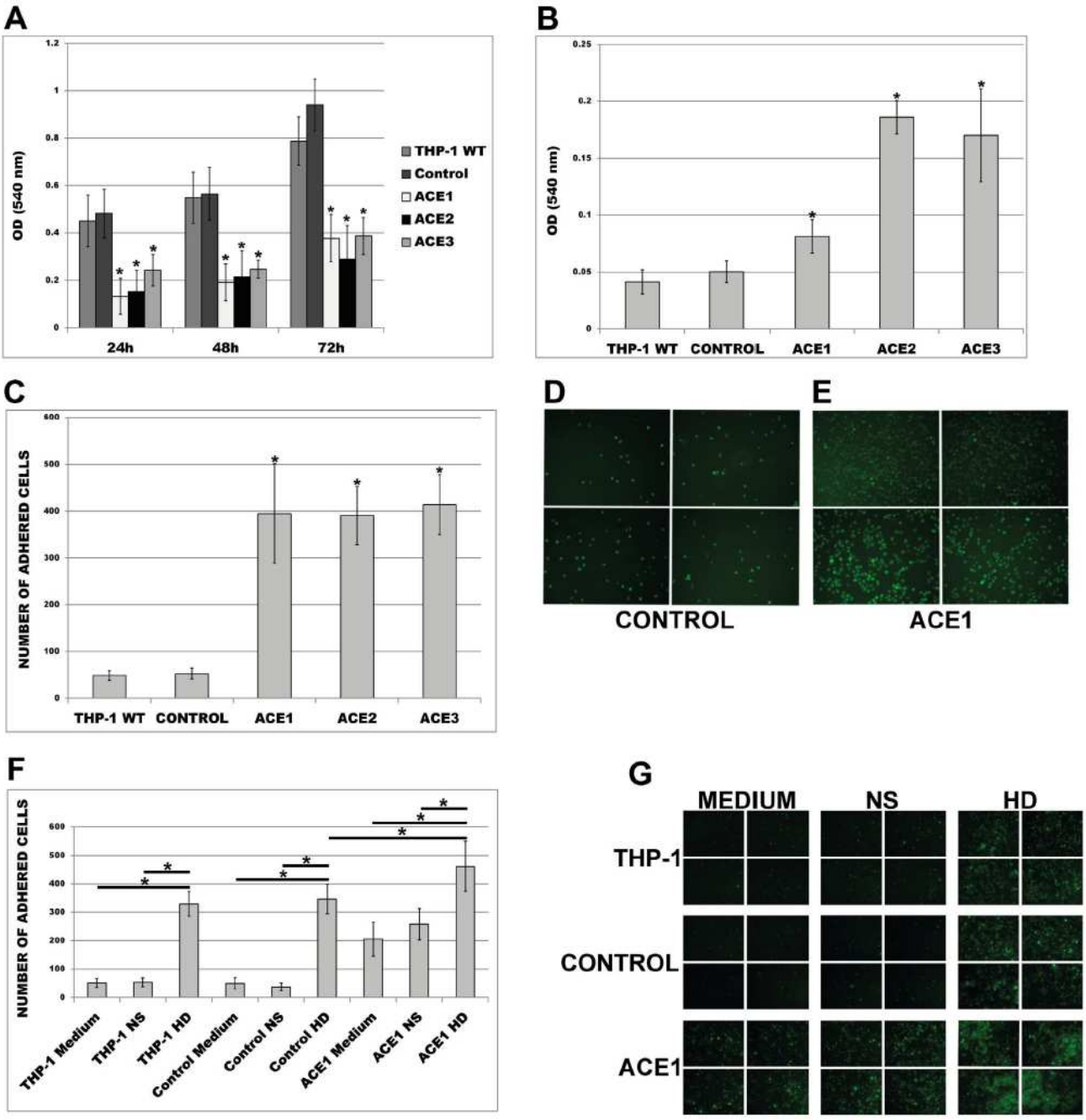


Figure 5. Proliferation, attachment and adhesion of ACE-overexpressing THP-1 monocytes investigated on wild type (THP-1 WT), empty plasmid (Control) and ACE-overexpressing cells (ACE1, ACE2, ACE3). (A) MTT-proliferation and (B) attachment of the cells. The cells for (B) were treated with 10 ng/ml PMA for 72 h prior to assay. (C, D, E, F, G) Adhesion of THP-1 to HUVEC endothelial monolayers. Calcein–labelled cells were incubated for 30 min in the presence of endothelial monolayers at the chamber bottom. Means ± SD of three independent experiments. (C) Adhesion under normal conditions (medium only, no FCS). See representative images (D, E). * $p < 0.05$ vs. control indicates statistical significance. (F) Adhesion under normal conditions or medium supplemented with 10% NS or HD sera. See representative images (G). * $p < 0.05$ indicates statistical significance. Note that for F and G one representative ACE-overexpressing clone (ACE1) was used. Analyses of (C, D, E, F, G) were performed in 10 random microscopic fields each. Means ± SD of cell number in 10 microscopic fields in three independent experiments.
doi:10.1371/journal.pone.0102137.g005

[29]. With regards to other cell culture systems, it has been demonstrated that uremic toxins may affect oxidative burst activity of the leukocytes and increase their pro-inflammatory effects. This may contribute to the tendency to vascular damage in CKD [30]. Furthermore, Shimizu et al. reported that indoxyl sulphate (IS), one of the most representative uremic toxins, up-regulated AGT expression in proximal tubular cells [31]. In studies by Sun et al. IS and p-cresol sulphate up-regulated in addition to elevated AGT expression, other renin–angiotensin–aldosterone system (RAAS) components such as renin and AT1R [32]. Up-regulation of these

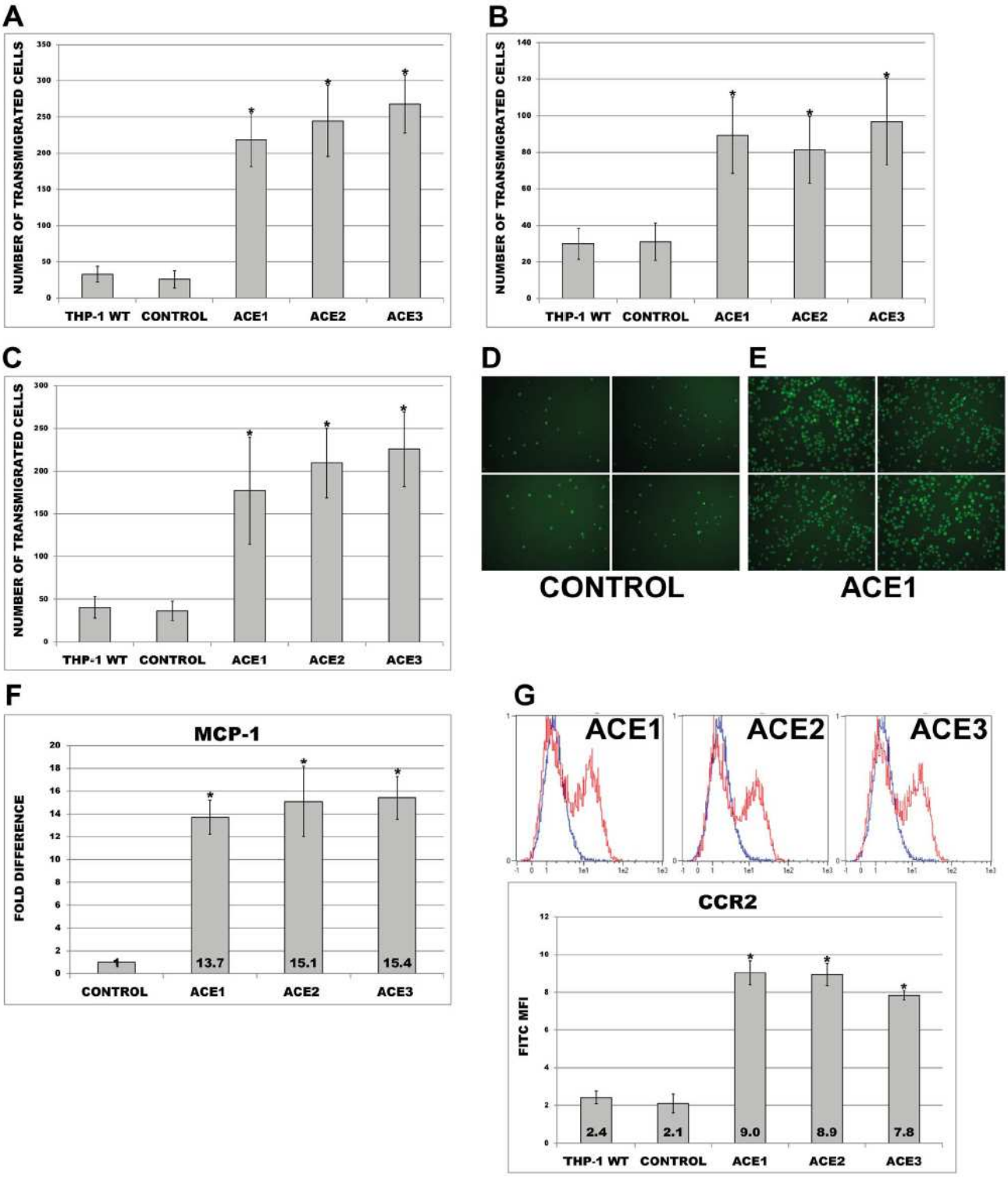


Figure 6. Transmigration of wild type (THP-1 WT), empty plasmid (Control) and ACE-overexpressing THP-1 monocytes (ACE1, ACE2, ACE3). (A) Transmigration of calcein-labelled cells through membrane towards (A) medium supplemented with MCP-1 or (B) HUVEC monolayers in the presence of medium only. (C, D, E) Transmigration of the cells through endothelial monolayers under MCP-1. See representative images (D, E). Analyses were performed in 10 random microscopic fields each. Means ± SD of cell number in 10 microscopic fields in three independent experiments. (F, G) Expression of MCP1 and CCR2 by RT-PCR and FACS analysis respectively. * $p < 0.05$ vs. control indicates statistical significance. Means ± SD of three independent experiments.
doi:10.1371/journal.pone.0102137.g006

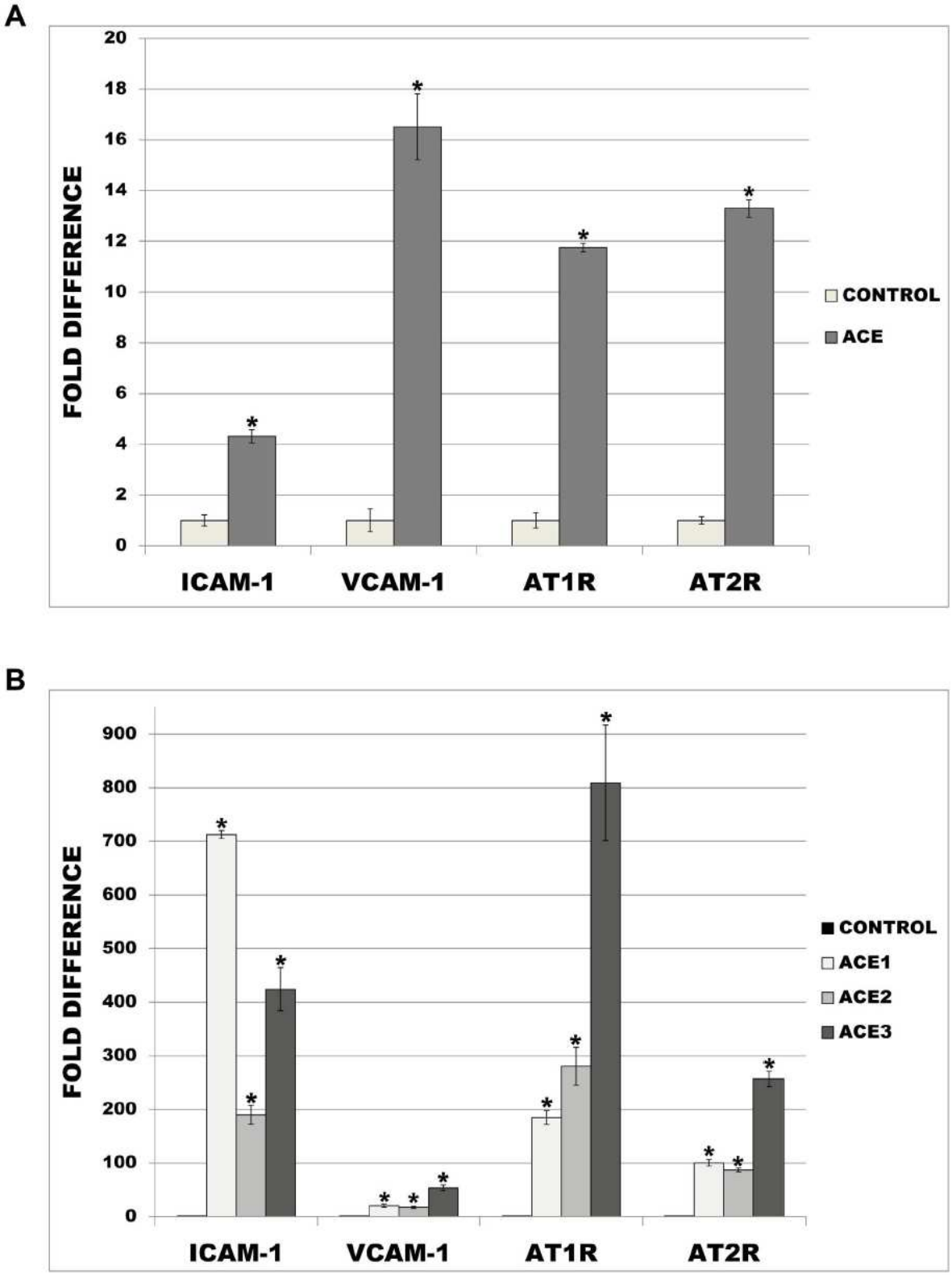
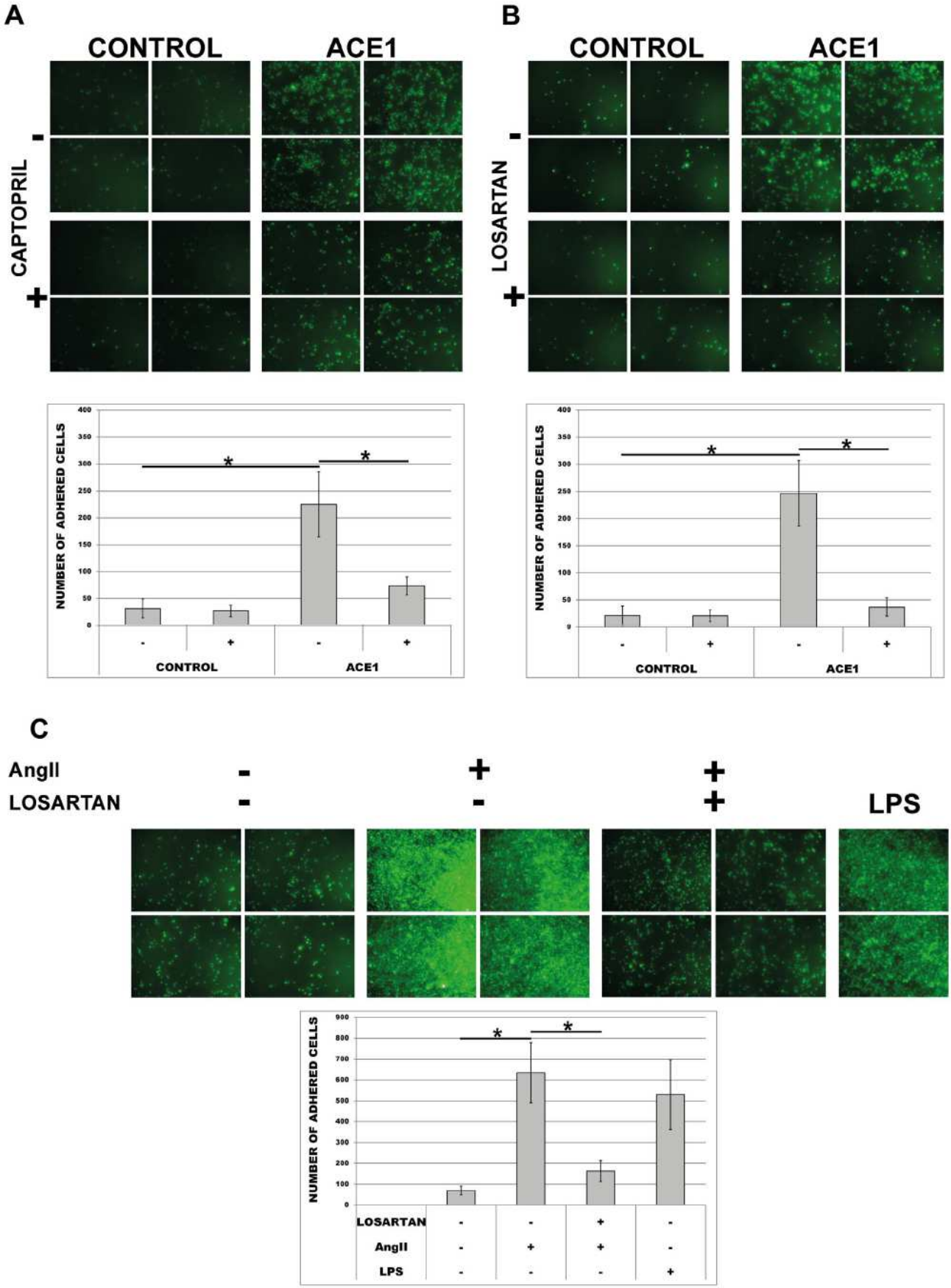


Figure 7. RT-PCR analysis of human primary monocytes and THP-1 cells overexpressing ACE. (A) Primary monocytes were transiently transfected with empty (Control) or ACE-plasmid and subjected to qPCR with primers specific for ICAM-1, VCAM-1, AT1R and AT2R. (B) Analysis of empty plasmid (Control) and ACE-overexpressing THP-1 monocytes (ACE1, ACE2, ACE3) were performed for the same transcripts. * $p < 0.05$ indicates statistical significance. Means ± SD of three independent experiments.
doi:10.1371/journal.pone.0102137.g007


A
CONTROL
ACE1
CAPTOPRIL
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+
NUMBER OF ADHERED CELLS
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B
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ACE1
LOSARTAN
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+
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CONTROL
ACE1
C
AngII
LOSARTAN
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NUMBER OF ADHERED CELLS
LOSARTAN
AngII
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Figure 8. Effect of the ACE-inhibitor Captopril, the AngII-receptor blocker Losartan and AngII on adhesion of wild type (THP-1 WT), empty plasmid (Control) and ACE-overexpressing cells (ACE1). Calcein-labelled cells were incubated in the presence or absence of (A) 500 nM captopril or (B) 1 μM losartan for 30 min and tested for their adhesion abilities to endothelial HUVEC monolayers. (C) Endothelial-adhesion of ACE-negative wild type THP-1 cells in the presence of 1 μM AngII only or co-incubation with 1 μM losartan investigated for 30 min. Representative images for (A, B, C) are shown. Analyses for (A, B, C) were performed in 10 random microscopic fields each. * $p < 0.05$ indicates statistical significance. Means ± SD of cell number in 10 microscopic fields in three independent experiments.
doi:10.1371/journal.pone.0102137.g008

RAAS components may subsequently exert, as observed in our study, increased expression of mACE and facilitate behavioural and morphological changes of the monocytes under uremia. Indeed, previous studies reported a possible link between uremic toxins and cardiovascular diseases. The authors demonstrated that uremia-mediated increase in leukocyte-endothelial adhesion occurs through elevation of E-selectin in HUVEC cells and is mediated via the JNK- and NF-κB-dependent pathway [33]. Furthermore, the studies by Vanholder et al. and Pletinck et al. showed clearly that proinflammatory effects exerted by protein-bound uremic toxins contribute to vascular damage and renal disease progression by stimulating crosstalk between leukocytes and vessels [34,35]. On the other hand the existence of lipid or smooth muscle-derived serum factor which could be responsible for such alterations had been previously speculated [16], [36]. The levels of oxidized LDL are generally increased in hemodialysis patients and previous reports demonstrated that local AngII production increases as macrophages become activated by ox-LDL [37], [38]. Also the levels of MCP-1 and its receptor in uremic serum and atherosclerotic plaques were reported to be significantly higher than in healthy controls and could be the reason of increased transmigration [39].

What are the consequences of ACE-overexpression in human monocytes? Microscopic investigations revealed noticeable alterations in cell morphology. Introduction of ACE into monocytes changed not only their structure towards macrophage-like cells, but also dramatically elevated the expression of MCSF. These observations correlate with previous findings demonstrating that accumulation of monocyte-derived macrophages at the sites of endothelial dysfunction is a crucial event in atherogenesis [40]. Our data suggest that ACE mediates an alternative activation of macrophages and may promote M2-phenotype with pro-inflammatory and pro-atherosclerotic properties. ACE-overexpressing cells revealed not only significantly elevated levels of Arg1, but also pro-inflammatory cytokines TNFa and IL-6. It has been demonstrated that such M2 macrophages have a higher capacity to accumulate modified lipids than M1 and upon exposure to ox-LDL the pro-inflammatory responses of M2 cells are increased [41]. Furthermore, M2 cells are present in plaques where surround the lipid core. Arg1, typical for these cells, may promote stabilisation of atherosclerotic plaques and enhance the proliferation of vascular smooth muscle cells [42].

We demonstrated that ACE-overexpressing monocytes transmigrate through endothelial barrier significantly faster than corresponding controls as they express not only more MCP-1 but also its ligand CCR2. These novel findings are extremely important because the motility of these cells may be boosted in an autocrine manner independently from endothelial function. The fact that MCP-1 is up-regulated in atherosclerotic plaques and arteries of animals fed a high cholesterol diet and that disruption of CCR2 in mouse models is related to anti-atherosclerotic actions [43], [44], [45], [46], [47], allows us to designate ACE-overexpressing monocytes as highly pro-atherogenic.

Studies in mice revealed that infusion of AngII led not only to increased plaque size, but also induced the expression of inflammatory TNFa, IL-6, and migration-related MCP-1, CCR2 in aortic roots. In that study disruption of MCP-1 led to decrease in AngII-mediated pro-atherosclerotic events [48]. Interestingly, in CCR2 knock-out mice or mice bearing specific leukocyte CCR2-deficiency, AngII was not able to induce previously described vascular remodelling but promoted the development of left ventricular hypertrophy instead [49]. In studies by Chen at al. the authors demonstrated that ACE deficiency in bone marrow–derived cells decreased hypercholesterolemia-induced atherosclerosis and correlated with reduced levels of MCP-1[17].

ACE overexpression led to a marked up-regulation of ICAM-1 and VCAM-1 in monocytes. Expression and induction of these molecules has been consistently observed in the initial steps of atherosclerosis and in atherosclerotic plaques [50], [51]. Disruption or antibody-mediated blockage of these molecular targets proved to exert beneficial effects on atherogenesis [52], [53].

It is well documented that ACE-inhibition and/or anti-AngII-receptor treatment has anti-atherosclerotic effects in experimental models as well as patients with cardiovascular disease [54], [55], [56]. We found in our study that introduction of ACE into monocytes led to dramatically increased expression of AngII-receptors, AT1R and AT2R. ACE-inhibition or AngII-receptor blockage significantly decreased the adhesion of these monocytes to endothelial cells. These novel findings suggest that inhibition of local, monocyte-derived AngII-generation might exert anti-atherogenic actions.

Da Cunha et al. reported that subcutaneous infusions of AngII led to accelerated carotid atherosclerosis in apolipoprotein E-deficient mice. Additionally increased expression of adhesion molecules such as E-selectin, ICAM-1, VCAM-1, chemokine MCP-1, and MCSF was demonstrated. Enalapril, an ACE-inhibitor, reduced these expressions and the number of adhered macrophages and foam cells in the arterial wall [57].

We found that uremic serum on the one hand induces ACE overexpression, thus creating pro-atherogenic monocytes. In addition, uremic serum serves as an additional amplifier of monocyte-endothelial adhesion even for the cells already overexpressing ACE. This suggests that ACE induction is an important but most likely not the only mechanism by which uremia enhances monocyte endothelium interactions.

In summary we demonstrate uremia-induced elevation of ACE expression paralleled by a pro-atherogenic nature of ACE-overexpressing monocytes, partially mediated by enhancement of migratory and adhesion potential to endothelial monolayers. Inhibition of local, monocyte-derived AngII-generation exerts anti-atherosclerotic actions in vitro. These findings justify further investigation and verification in animal models.

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Author Contributions

Conceived and designed the experiments: BT CU MG. Performed the experiments: BT CU. Analyzed the data: BT CU. Contributed reagents/materials/analysis tools: ES RF. Contributed to the writing of the manuscript: BT MG.

References

1. Foley RN, Parfrey PS, Sarnak MJ (1998). Clinical epidemiology of cardiovascular disease in chronic renal disease. Am J Kidney 32: 112–119.
2. Cheung AK, Sarnak MJ, Yan G, Dwyer JT, Heyka RJ, et al. (2000) Atherosclerotic cardiovascular disease risks in chronic hemodialysis patients. Kidney Int 58: 353–362.
3. Vanholder R, Massy Z, Argiles A, Spasovski G, Verbeke F, et al. (2005) Chronic kidney disease as cause of cardiovascular morbidity and mortality. Nephrol Dial Transplant 20:1048–1056.
4. Chronic Kidney Disease Prognosis Consortium, Matsushita K, van der Velde M, Astor BC, Woodward M, et al. (2010) Association of estimated glomerular filtration rate and albuminuria with all-cause and cardiovascular mortality in general population cohorts: a collaborative meta-analysis. Lancet 12: 2073–2081.
5. Tyralla K, Amann K (2003) Morphology of the heart and arteries in renal failure. Kidney Int Suppl 84: 80–83.
6. Guerin AP, Blacher J, Pannier B, Marchais SJ, Safar ME, et al. (2001) Impact of aortic stiffness attenuation on survival of patients in end-stage renal failure. Circulation 103: 987–992.
7. Ulrich C, Seibert E, Heine GH, Fliser D, Girndt M (2011) Monocyte angiotensin converting enzyme expression may be associated with atherosclerosis rather than arteriosclerosis in hemodialysis patients. Clin J Am Soc Nephrol 6: 505–511.
8. Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362: 801–809.
9. Steinberg D (2002) Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. Nature Med 8: 1211–1217.
10. Moore KJ, Tabas I (2011) Macrophages in the pathogenesis of atherosclerosis. Cell 145: 341–355.
11. Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, et al. (2010) Macrophage plasticity in experimental atherosclerosis. PLoS One 5: e8852.
12. Pello OM, Silvestre C, De Pizzol M, Andres V (2012) A glimpse on the phenomenon of macrophage polarization during atherosclerosis. Immunobiology 216: 1172–1176.
13. Martinez FO, Helming L, Gordon S (2009) Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 27: 451–483.
14. Gratchev A, Kzhyshkowska J, Utikal J, Goerdt S (2005) Interleukin-4 and dexamethasone counterregulate extracellular matrix remodelling and phagocytosis in type-2 macrophages. Scand J Immunol 61: 10–17.
15. Xu W, Roos A, Schlagwein N, Woltman AM, Daha MR, et al. (2006) IL-10-producing macrophages preferentially clear early apoptotic cells. Blood 107: 4930–4937.
16. Kohlstedt K, Trouvain C, Namgaladze D, Fleming I (2011) Adipocyte-derived lipids increase angiotensin-converting enzyme (ACE) expression and modulate macrophage phenotype. Basic Res Cardiol 106: 205–215.
17. Chen X, Lu H, Zhao M, Tashiro K, Cassis LA, et al. (2013) Contributions of leukocyte angiotensin-converting enzyme to development of atherosclerosis. Arterioscler Thromb Vasc Biol 33: 2075–2080.
18. Schieffer B, Schieffer E, Hilfiker-Kleiner D, Hilfiker A, Kovanen PT, et al. (2000) Expression of angiotensin II and interleukin 6 in human coronary atherosclerotic plaques: potential implications for inflammation and plaque instability. Circulation 101: 1372–1378.
19. O'Brien KD, Shavelle DM, Caulfield MT, McDonald TO, Olin-Lewis K, et al. (2002) Association of angiotensin-converting enzyme with low-density lipoprotein in aortic valvular lesions and in human plasma. Circulation 106: 2224–2230.
20. Ouimet T, Lancelot E, Hyafil F, Rienzo M, Deux F, et al. (2012) Molecular and cellular targets of the MRI contrast agent P947 for atherosclerosis imaging. Mol Pharm 9: 850–861.
21. Shen XZ, Billet S, Lin C, Okwan-Duodu D, Chen X, et al. (2011) The carboxypeptidase ACE shapes the MHC class I peptide repertoire. Nat Immunol 12: 1078–1085.
22. Kitazono T, Padgett RC, Armstrong ML, Tompkins PK, Heistad DD (1995) Evidence that angiotensin II is present in human monocytes. Circulation 91: 1129–1134.
23. Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, et al. (2000) Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators. N Engl J Med 342: 145–153.
24. Ulrich C, Heine GH, Seibert E, Fliser D, Girndt M (2010) Circulating monocyte subpopulations with high expression of angiotensin-converting enzyme predict mortality in patients with end-stage renal disease. Nephrol Dial Transplant 25: 2265–2272.
25. Ulrich C, Heine GH, Garcia P, Reichart B, Georg T, et al. (2006) Increased expression of monocytic angiotensin-converting enzyme in dialysis patients with cardiovascular disease. Nephrol Dial Transplant 21: 1596–1602.
26. Simolin MA, Pedersen TX, Bro S, Mäyränpää MI, Helske S, et al. (2009) ACE inhibition attenuates uremia-induced aortic valve thickening in a novel mouse model. BMC Cardiovasc Disord 9: 10.
27. Petrov MN, Shilo VY, Tarasov AV, Schwartz DE, Garcia JG, et al. (2012) Conformational changes of blood ACE in chronic uremia. PLoS One 7: e49290.
28. Finch JL, Suarez EB, Husain K, Ferder L, Cardema MC, et al. (2012) Effect of combining an ACE inhibitor and a VDR activator on glomerulosclerosis, proteinuria, and renal oxidative stress in uremic rats. Am J Physiol Renal Physiol 302: 141–149.
29. Mizobuchi M, Ogata H, Hosaka N, Kumata C, Nakazawa A, et al. (2011) Effects of calcimimetic combined with an angiotensin-converting enzyme inhibitor on uremic cardiomyopathy progression. Am J Nephrol 34: 256–260.
30. Schepers E, Meert N, Glorieux G, Goeman J, Van der Eycken J, et al. (2007) P-cresylsulphate, the main in vivo metabolite of p-cresol, activates leucocyte free radical production. Nephrol Dial Transplant 22: 592–596.
31. Shimizu H, Saito S, Higashiyama Y, Nishijima F, Niwa T (2013) CREB, NF-κB, and NADPH oxidase coordinately upregulate indoxyl sulfate-induced angiotensinogen expression in proximal tubular cells. Am J Physiol Cell Physiol 304: 685–692.
32. Sun CY, Chang SC, Wu MS (2012) Uremic toxins induce kidney fibrosis by activating intrarenal renin-angiotensin-aldosterone system associated epithelial-to-mesenchymal transition. PLoS One. 2012;7: e34026.
33. Ito S, Osaka M, Higuchi Y, Nishijima F, Ishii H, et al. (2010) Indoxyl sulfate induces leukocyte-endothelial interactions through up-regulation of E-selectin. J Biol Chem 285: 38869-38875.
34. Vanholder R, Schepers E, Pletinck A, Nagler EV, Glorieux G. (2014) The Uremic Toxicity of Indoxyl Sulfate and p-Cresyl Sulfate: A Systematic Review. J Am Soc Nephrol.
35. Pletinck A, Glorieux G, Schepers E, Cohen G, Gondouin B, et al. (2013) Protein-bound uremic toxins stimulate crosstalk between leukocytes and vessel wall. J Am Soc Nephrol 24: 1981–1994.
36. Metzger R, Franke FE, Bohle RM, Alhenc-Gelas F, Danilov SM (2011) Heterogeneous distribution of angiotensin I-converting enzyme (CD143) in the human and rat vascular systems: vessel, organ and species specificity. Microvasc Res 81: 206–215.
37. Diet F, Pratt RE, Berry GJ, Momose N, Gibbons GH, et al. (1996) Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. Circulation 94: 2756–2767.
38. Rafatian N, Milne RW, Leenen FH, Whitman SC (2013) Role of Renin Angiotensin System in Activation of Macrophages by modified Lipoproteins. Am J Physiol Heart Circ Physiol 305: 1309–1320.
39. Okumoto S, Taniguchi Y, Nakashima A, Masaki T, Ito T, et al. (2009) C–C chemokine receptor 2 expression by circulating monocytes influences atherosclerosis in patients on chronic hemodialysis. Ther Apher Dial 13: 205–212.
40. Stöger JL, Gijbels MJ, van der Velden S, Manca M, van der Loos CM, et al. (2012) Distribution of macrophage polarization markers in human atherosclerosis. Atherosclerosis 2012;225:461–468.
41. van Tits LJ, Stienstra R, van Lent PL, Netea MG, Joosten LA, et al. (2011) Oxidized LDL enhances pro-inflammatory responses of alternatively activated M2 macrophages: a crucial role for Krüppel-like factor 2. Atherosclerosis 214: 345–349.
42. Pourcet B, Pineda-Torra I (2013) Transcriptional regulation of macrophage arginase 1 expression and its role in atherosclerosis. Trends Cardiovasc Med 23: 143–152.
43. Libby P, Geng YJ, Aikawa M, Schoenbeck U, Mach F, et al. (1996) Macrophages and atherosclerotic plaque stability. Curr Opin Lipidol 7: 330–335.
44. Nelken NA, Coughlin SR, Gordon D, Wilcox JN (1991) Monocyte chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 88: 1121–1127.
45. Wiesner P, Tafelmeier M, Chittka D, Choi SH, Zhang L, et al. (2013) MCP-1 binds to oxidized LDL and is carried by lipoprotein(a) in human plasma. J Lipid Res 54: 1877–1883.
46. Boring L, Gosling J, Cleary M, Charo IF (1998) Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 394: 894–897.
47. Dawson TC, Kuziel WA, Osahar TA, Maeda N (1999) Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 143:205–211.
48. Ni W, Kitamoto S, Ishibashi M, Usui M, Inoue S, et al. (2004) Monocyte chemoattractant protein-1 is an essential inflammatory mediator in angiotensin II-induced progression of established atherosclerosis in hypercholesterolemic mice. Arterioscler Thromb Vasc Biol 24: 534–539.
49. Ishibashi M, Hiasa K, Zhao Q, Inoue S, Ohtani K, et al. (2004) Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling. Circ Res 94: 1203–1210.
50. Blankenberg S, Barbaux S, Tiret L (2003) Adhesion molecules and atherosclerosis. Atherosclerosis 170: 191–203.
51. Tuttolomondo A, Di Raimondo D, Pecoraro R, Arnao V, Pinto A, et al. (2012) Atherosclerosis as an inflammatory disease: Curr Pharm Des 18: 4266–4288.
52. De Meyer I, Martinet W, De Meyer GR (2012) Therapeutic strategies to deplete macrophages in atherosclerotic plaques. Br J Clin Pharmacol 74: 246–263.

53. Lal H, Guleria RS, Foster DM, Lu G, Watson LE, et al. (2007) Integrins: novel therapeutic targets for cardiovascular diseases. Cardiovasc Hematol Agents Med Chem 5: 109–132.
54. Kojima C, Kawakami A, Takei T, Nitta K, Yoshida M (2007) Angiotensin-converting enzyme inhibitor attenuates monocyte adhesion to vascular endothelium through modulation of intracellular zinc. J Pharmacol Exp Ther 323: 855–860.
55. Bernardi S, Candido R, Toffoli B, Carretta R, Fabris B (2011) Prevention of accelerated atherosclerosis by AT1 receptor blockade in experimental renal failure. Nephrol Dial Transplant 26: 832–838.
56. Koga J, Egashira K, Matoba T, Kubo M, Ihara Y, et al (2008) Essential role of angiotensin II type 1a receptors in the host vascular wall, but not the bone marrow, in the pathogenesis of angiotensin II-induced atherosclerosis. Hypertens Res 31: 1791–1800.
57. da Cunha V, Tham DM, Martin-McNulty B, Deng G, Ho JJ, et al (2005) Enalapril attenuates angiotensin II-induced atherosclerosis and vascular inflammation. Atherosclerosis 178: 9–17.

2.2 *Relations of monocytic ACE2 with atherosclerosis in CKD*

Trojanowicz B, Ulrich C, Kohler F, Bode V, Seibert E, Fiedler R, Girndt M. **Monocytic angiotensin-converting enzyme 2 relates to atherosclerosis in patients with chronic kidney disease**. Nephrol Dial Transplant. 2017 Feb 1;32(2):287-298.

Since uremic conditions up-regulate ACE expression in vitro, thus creating pro-atherogenic monocytes, this study investigated the next important player in the CKD-related atherogenesis, ACE2, and its relations with ACE.
Investigations of the expression pattern of ACE and AngII receptors, AT1R and AT2R in the cells circulating in CKD patients were performed on the leukocytes obtained from HD and CKD3-5 patients not on dialysis. Expression of ACE was significantly up-regulated in HD and CKD3-5 patients. AngII-receptor I, AT1R, showed a significantly elevated expression in HD and CKD3-5 patients as compared to healthy individuals (NP), while the expression of AT2R did not differ between those groups.
The leucocytic expression of ACE did not differ between HD patients taking Angiotensin Receptor Blockers (ARB), ACE Inhibitors (ACEi) or those not treated with Angiotensin modifying medication. It worth to note that ARB therapy in HD patients reduced the elevated expression of AT1R and AT2R towards the levels in NP, however the difference was not significant when compared with patients not treated with Angiotensin modifying medication. Investigations of diabetic and non-diabetic HD patients revealed no differences in the expression of ACE and AngII-receptors within and between any examined groups.
Such elevated expression of ACE may contribute to the ubiquitous arterial hypertension in dialysis patients. In addition, the receptors for AngII, AT1R and AT2R were also up-regulated. Previous reports revealed that receptors for AngII are detectable on all major leukocyte subsets, show the highest expression on granulocytes and monocytes, and correlate with pathophysiological conditions [127]. In agreement with our data, high level expression of AT1R has been shown in CKD patients before [127]. Furthermore, in individuals with high risk for vascular events or hyperlipidemic patients, expression of AT1R in circulating leukocytes was noticeably elevated as compared to a healthy low-risk control group [127]. Pharmacological intervention by either RAS blockers or statins led to decreased expression of AT1R and by interfering with AngII exerted anti-inflammatory actions in these patients [127, 129, 130]. Recently, Chon et al. [128] studied non-dialysed CKD patients and found that despite a relevant antihypertensive effect, angiotensin blockade did not consistently

affect AT1R leucocytic expression. The authors postulated that factors involved in uraemia are more dominant modulators of leucocytic AT1R expression in these patients. In contrast to these findings, therapy with AngII receptor blockers in our HD patients led to some degree of reduction of leucocytic AT1R and AT2R expression.

Investigations of leucocytic ACE2 and MasR were performed on the same patients' collective as ACE study. ACE2 expression in the HD group was significantly decreased as compared to healthy controls. In contrast the levels of MASR were noticeably increased in the HD group. Similar to leucocytic ACE, expression of ACE2 in HD group was not affected with Angiotensin modifying medication.
In further investigations we studied whether HD itself may affect the ACE2 expression in patients' leukocytes. This hypothesis was tested on the leukocytes originating from CKD3-5 group not on dialysis. We found that ACE2 in CKD3-5 patients revealed similar expression pattern to those observed in HD group and differed significantly as compared to NP. Expression of MASR in CKD3-5 patients was significantly higher as compared to NP and its levels were noticeably up-regulated in CKD3-5 than in HD group.
Investigations of leucocytic balance between both ACEs demonstrated that the expression ratio ACE/ACE2 was significantly elevated in the HD and CKD3-5 groups as compared to NP

Since ACE mediates the generation of the vasoconstrictive AngII and ACE2 degrades AngII to Ang 1-7, a vasodilator, the altered relation between ACE and ACE2 may contribute to the ubiquitous arterial hypertension observed in HD or CKD patients. These data suggest that the uremic milieu is able to induce a specific dysregulation of cellular ACEs, which in turn is responsible for the formation of a proinflammatory and adhesive monocyte phenotype. These observations are in agreement with our earlier data demonstrating high expression of ACE on monocytes in HD patients in association with subclinical atherosclerosis [66] or cardiovascular mortality [76].
As compared to healthy population with significantly lower leucocytic MASR and up-regulated ACE2 levels, it seems that uraemia and especially uremic toxins may disturb the proper functioning of local ACE2/MASR-axis as a counter-regulatory mechanism or affect the crucial RAS components [102, 103, 104].
Our results cannot rule out that dysregulation of both ACEs, and especially decreased levels of leucocytic ACE2 are due to the additional participation of the systemic RAS. Kovarik et al. demonstrated elevated plasma levels of Ang 1-7 in HD patients as compared to healthy controls [131]. This finding cannot be explained by the expression

pattern of ACE2 on leukocytes or monocytes, as demonstrated in our studies, thus other sources of Ang 1-7 in patients with renal failure are possible. Such elevated levels of plasma Ang 1-7 may also strongly up-regulate the leucocytic MASR as observed in HD and CKD patients.
Recent findings suggest that micro RNAs may trigger the silencing of particular transcripts and contribute to the development and/or progression of renal disease [132]. It has been demonstrated that miR-421 is able to bind and regulate ACE2 mRNA in primary human cardiac myofibroblasts [133]. Based on these observations we cannot rule out the possibility that uremic milieu may affect the expression of particular micro RNAs and in this way participate in the regulation of target transcripts.

Data in vitro revealed that the uremic milieu leads to up-regulated expression of monocytic ACE, increased transmigration and endothelial adhesion as compared to conditions mimicking healthy environment. The same ACE-expression pattern was found in leukocytes obtained from renal failure patients and corresponding healthy controls. Also the expression of ACE2 is decreased on circulating leukocytes obtained from uremic patients.
Investigations performed on human primary monocytes treated with serum originating from healthy individuals, HD patients or CKD patients not on dialyses, demonstrated that uremic milieu led to strong up-regulation of ACE with subsequently diminished levels of monocytic ACE2 and up-regulation of corresponding receptors. This effect can at least in part be reversed by the ACEi captopril and to a lesser degree by the ARB losartan. This indicates that local formation of AngII by the monocytic ACE is important for the effect on ACE2 expression. Likely, the AT2R, which is not blocked by losartan, plays a role in ACE2 regulation.
As compared with experimental overexpression of ACE, induction of ACE2 in THP-1 monocytes led to the opposite effects resulting in a smaller size of the cells. Functionally, the cells overexpressing ACE2 demonstrated decreased endothelial adhesion, diminished transmigration rates and down-regulated expression of MCP-1. These findings correlate well with earlier data demonstrating that ACE and ACE2 act in a counter-regulatory manner not only within the kidney, heart or astrocytes, but also within the circulating leukocytes [134, 135, 136].
In summary the data presented here indicate that uremic milieu promote over-activation of monocytic ACE and inhibition of ACE2 thus contributing to increased endothelial adhesion and transmigration. Such an imbalance between both ACEs may elucidate an important mechanism contributing relevantly to atherosclerosis progression in patients with chronic renal failure.

Monocytic angiotensin-converting enzyme 2 relates to atherosclerosis in patients with chronic kidney disease.

Trojanowicz B, Ulrich C, Kohler F, Bode V, Seibert E, Fiedler R, Girndt M.

Nephrol Dial Transplant. 2017 Feb 1;32(2):287-298. doi: 10.1093/ndt/gfw206.

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Abstract
Background: Increased levels of monocytic angiotensin-converting enzyme (ACE) found in haemodialysis (HD) patients may directly participate in the pathogenesis of atherosclerosis. We demonstrated recently that uremia triggers the development of highly pro-atherogenic monocytes via an angiotensin II (AngII)–dependent mechanism. Opposing actions of the AngII-degrading ACE2 remain largely unknown. We examined the status of both ACEs and related receptors in circulating leukocytes of HD, not-dialyzed CKD and healthy individuals. Furthermore, we tested the possible impact of monocytic ACEs on atherogenesis and behaviour of the cells under conditions mimicking chronic renal failure.

Methods: Expression of ACE, ACE2, AT1R, AT2R and MASR was investigated on circulating leukocytes from 71 HD (62 ± 14 years), 24 CKD stage 3–5 (74 ± 10 years) patients and 37 healthy control subjects (53 ± 6 years) and isolated healthy monocytes treated with normal and uremic serum. Analyses of ACE, ACE2, ICAM-1, VCAM-1, MCSF and endothelial adhesion were tested on ACE-overexpressing THP-1 monocytes treated with captopril or losartan. ACE2-overexpressing monocytes were subjected to transmigration and adhesion assays and investigated for MCP-1, ICAM-1, VCAM-1, MCSF, AT1R and AT2R expression.

Results: The ACE mRNA level was significantly increased in HD and CKD stage 3–5 leukocytes. Correspondingly, ACE2 was downregulated and AngII as well as MAS receptor expression was upregulated in these cells. Healthy monocytes preconditioned with uremic serum reflected the same expressional regulation of ACE/ACE2, MAS and AngII receptors as those observed in HD and CKD stage 3–5 leukocytes. Overexpression of monocytic ACE dramatically decreased levels of ACE2 and induced a pro-atherogenic phenotype, partly reversed by AngII-modifying treatments, leading to an increase in ACE2. Overexpression of ACE2 in monocytes led to reduced endothelial adhesion, transmigration and downregulation of adhesion-related molecules.

Conclusions: HD and not-dialyzed CKD stage 3–5 patients show enhanced ACE and decreased ACE2 expression on monocytes. This constellation renders the cells endothelial adhesive and likely supports the development of atherosclerosis.

2.3 *Fluids from high-permeability dialysis and inflammation profile in THP-1 monocytes*

Trojanowicz B, Ulrich C, Fiedler R, Storr M, Boehler T, Martus P, Pawlak M, Glomb MA, Henning C, Templin M, Werner K, Zickler D, Willy K, Schindler R, Girndt M. **Impact of serum and dialysates obtained from chronic haemodialysis patients maintained on high cut-off membranes on inflammation profile in human THP-1 monocytes.** Hemodial Int. 2017 Jul;21(3):348-358.

Systemic chronic inflammation and overactivation of RAS are highly associated with CKD as revealed in about one third of the patients maintained on regular HD.
Increased levels of inflammation mediators such as CRP, IL-6 and TNFa [137, 138] are well established predictors of atherosclerotic complications and adverse cardiovascular events related to high mortality rates [139, 140, 141].
Conventional HD with HF membranes may eliminate the proinflammatory mediators insufficiently [142, 143]; their accumulation may lead to the modulation of the components of local, leucocytic RAS, especially both ACEs.
Recently, employment of the membranes with increased permeability, High cut-off (HCO) and newly developed Medium cut-off (MCO) [144], for the treatment of chronic HD patients with elevated CRP led to decreased expression of multiple pro-inflammatory transcripts in leukocytes [145, 146, 147, 148].
In this study we tested the effects of serum and dialysates obtained from patients treated either with HCO or HF membranes within a randomized crossover pilot trial on THP-1 monocytes [147]. We applied these fluids to the cells in vitro and investigated whether HCO treatment could remove proinflammatory substances from serum to the dialysate.
Global investigation of the inflammation profile upon HCO- and HF serum treatment led to noticeable decrease of 72 pro-inflammation transcripts while only 12 were up-regulated. Out of 72 decreased target genes, 48 were ≤ 5 and 24 were ≥ 5 fold down-regulated as compared to HF-serum treatment.
Among 84 pro-inflammatory transcripts tested, the expression of two crucial cytokines, TNFa and IL-6, was significantly lower in THP-1 monocytes upon HCO serum as compared to HF treatment.
Previous reports demonstrated that these two cytokines are increased in plasma from CKD patients and strongly associated with frequently observed complications such as wasting, progressive atherosclerosis, vascular calcification, and cardiovascular events such as myocardial infarction and stroke [149, 150, 151, 152, 153, 154, 155, 156].

Higher levels of TNFa and IL-6 were also reported to be associated with anaemia in CKD patients [157].

Investigations of osteopontin and osteocalcin, two mediators associated with calcium and bone metabolism, revealed similarly reduced expression pattern as that observed for TNFa and IL-6. Serum osteocalcin levels are high in CKD patients and their accumulation can be due to the decreased renal clearance, higher bone metabolism, or a combination of both [158]. Also the levels of osteopontin were significantly correlated with decreased renal function [159] and positively correlated with the aortic calcification index in CKD patients [160, 161]. It is worth to note that both proteins are detectable in calcified arterial wall, further revealing their crucial roles in the process of active vascular ossification [162]. Furthermore, as approximately the half of the deaths in HD patients are related to vascular disease including calcification, the therapeutic influence of calcium-metabolism associated markers is of predominant relevance [163].

Investigations performed with HCO-dialysates revealed that in addition to anti-inflammatory effects of HCO on serum, these fluids decrease cellular viability significantly stronger than corresponding HF- or unused dialysates. We suggest that these anti-proliferative effects of the high permeability dialysates may be due to the elimination of substances within the 15-45 kDa spectrum that can be removed by HCO HD.

Kneis et al. demonstrated previously that HCO-treatment by dialysis patients removed soluble TNFR1 (tumour necrosis factor receptor 1), complement factor D, IL-6 and sIL-6 receptor significantly better than conventional HF membranes [164]. Similar to our observations Bordoni et al. reported that ultrafiltrates obtained from laboratory-based dialysis of endotoxin-spiked blood with a super-permeable membrane (SHF/ HCO) induced in U937 monocytes a noticeable increase in caspase-3 and caspase-8 activity [165]. Morgera et al. demonstrated that P2SH membranes with a nominal cut-off point of 60 kDa in septic patients restored significantly proliferation of mononuclear cells. Inversely, treatment of PBMCs (peripheral blood mononuclear cell) with HCO-ultrafiltrates decreased proliferation of the cells and significantly induced the release of TNFa. Similar to our findings, the authors speculated that observed functional effects were due to the elimination of immunomodulatory mediators [166].

In summary we demonstrated that HCO-fluids affect the immunomodulation of cellular apoptosis and expression of inflammation associated genes. Anti-inflammatory effects of HCO-serum and efficient removal of mediators decreasing cellular viability by HCO-haemodialysis create a solid base for future improvements in development of membranes with increased nominal cut-off point.

Impact of serum and dialysates obtained from chronic hemodialysis patients maintained on high cut-off membranes on inflammation profile in human THP-1 monocytes.

Trojanowicz B, Ulrich C, Fiedler R, Storr M, Boehler T, Martus P, Pawlak M, Glomb MA, Henning C, Templin M, Werner K, Zickler D, Willy K, Schindler R, Girndt M.

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Abstract
Introduction: Patients with chronic kidney disease maintained on intermittent hemodialysis suffer from systemic chronic inflammation which is causally associated with high mortality. Inflammation mediators of 15-45 kDa range cannot be effectively removed by conventional dialysis membranes. In this study, we tested the influence of serum and dialysates obtained from patients maintained on High cut-off or High flux membranes on the inflammation profile of THP-1 monocytes.

Methods: THP-1 monocytes were treated with serum or dialysates obtained from patients maintained on High cut-off and High flux membranes within a randomized crossover pilot trial. Serum-treated cells were subjected to qPCR analyses with TaqMan probes specific for IL6, TNFa, osteopontin and osteocalcin, and transcriptional screening with Inflammatory Array. Apoptosis assay was performed flow cytometrically with 7-AAD and Annexin V staining.

Findings: Treatment of the cells with High cut-off serum led to significant reduction of TNFa and IL-6 expression as well as inflammation-related osteopontin and osteocalcin as compared to High flux membrane treatment. As a complementary finding, treatment with High cut-off dialysates induced a pro-apoptotic phenotype in the cells as demonstrated by a significantly increased percentage of 7-AAD and Annexin V positivity. Global screening of serum-treated cells revealed noticeably decreased inflammation profile under High cut-off serum as compared to High flux treatment.

Discussion: Taken together, these data demonstrate that High cut-off -membranes eliminate a spectrum of mediators from serum into the dialysate that possess proinflammatory properties and may impair cellular viability.

2.4 *Leucocytic ACEs in patients maintained on high permeability HD and dialysis in vitro*

Trojanowicz B, Ulrich C, Fiedler R, Martus P, Storr M, Boehler T, Werner K, Hulko M, Zickler D, Willy K, Schindler R, Girndt M. **Modulation of leucocytic angiotensin-converting enzymes expression in patients maintained on high-permeable haemodialysis.** Nephrol Dial Transplant. 2018 Jan 1;33(1):34-43.

Initial investigations of the high permeable membranes were performed in an artificial in vitro dialysis system. Plasma obtained from endotoxin-spiked whole healthy blood was subjected to in vitro dialysis with HF, HCO and MCO membranes. Plasma and dialysate fluids were further employed for assays in vitro.

We demonstrated that THP-1 monocytes treated with HCO- or MCO-plasma reacted with significantly declined levels of pro-inflammatory TNFa and IL-6 transcripts as compared to the incubation with HF-plasma. With regard to monocytic ACEs, the same treatments led to decreased expression of ACE transcripts and significant upregulation of ACE2. These data are better presented as the proportion between both ACEs demonstrating that the expression ratio ACE2/ACE was significantly elevated in the HCO and MCO serum treatments as compared to HF. This pattern could also be demonstrated on protein level, immunocytochemically, as the THP-1 monocytes revealed noticeably stronger ACE2 staining under treatment with HCO and MCO patients' sera as compared to HF. It is worth to note, that ACE protein tended to decrease under HCO and MCO sera; however the differences were not so pronounced.

Employment of the in vitro dialysate fluids to the cells revealed complementary effects to those observed with plasma incubation. THP-1 monocytes reacted with up-regulated levels of TNFa and IL-6 transcripts under HCO or MCO dialysates as compared to HF. In relation to monocytic ACEs, incubation of the cells with HCO or MCO dialysates led to ACE increase, while ACE2 transcript expression was down-regulated. Contrary to plasma incubations, the expression ratio ACE2/ACE was significantly decreased in the HCO and MCO dialysate-treatments as compared to HF.

We demonstrated recently that ACE2 on leukocytes obtained from healthy individuals is noticeably stronger as compared to patients with renal failure. As the uremic milieu triggers a specific disbalance of ACEs, favouring decrease of ACE2 and the formation of a proinflammatory and adhesive leukocyte phenotype, employment of high permeable membranes may reverse this phenotype towards more favourable one.

ACE expression in the patients groups treated with HCO and MCO membranes revealed the falling tendency as compared to HF treatments. In contrast the levels of ACE2 transcripts were significantly increased in the HCO and MCO groups. Furthermore, the leucocytic balance between both ACEs demonstrated that the expression ratio ACE2/ACE was significantly elevated in the HCO and MCO groups as compared to HF treatment.

The protective role for ACE2 and the involvement of ACE2/Ang 1-7/Mas receptor axis in the modulation of oxidative stress, renal fibrosis and inflammation was demonstrated in different studies [167, 168, 169, 170, 171, 172]. Based on the results obtained in our study that HD with high permeable membranes increases the leucocytic ACE2, the clear advantages of the ACE2 activation for the treatment of kidney and cardiovascular disorders are obvious. In agreement with our findings it has been reported that administration of xanthenone and diminazene aceturate, two ACE2 activators, to hypertensive rats led to decreased blood pressure, reversed myocardial and perivascular fibrosis, and improved myocardial function [173, 174, 175, 176]. Infusion of the recombinant ACE2 led to diminished progression of diabetic nephropathy and decreased AngII-induced tubulointerstitial fibrosis [177]. Similarly, injection of Ang 1-7, a product of AngII conversion mediated by ACE2, attenuated in vivo diabetes-induced leukocyte adhesion and extravasation in Wistar rats [178]. Experimental induction of ACE2 in THP-1 monocytes, as presented in our previous studies, contributed to diminished endothelial adhesion and transmigration of these cells as verified by downregulation of MCP-1, ICAM-1 and VCAM-1. As the serum originating from patients maintained on HCO membranes contains less VCAM-1 and VCAM-1 itself is able to increase calcification of vascular smooth muscle cells (VSMC) in vitro, the beneficial effects of HCO dialysis should be taken into consideration [179].

We have shown that RAS modifying treatments and the increased permeability of HD membranes are not the reason for direct ACE2 regulation. Thus, the leucocytic increase in ACE2 expression cannot be explained by HCO- or MCO-mediated elimination. We postulate instead, that a decrease of other inflammation modulators related to secondary ACE2 upregulation is a conceivable explanation. We demonstrated previously that treatment of human THP-1 monocytes with sera obtained from HCO dialysis led to diminished expression profile of many transcripts related to inflammation, including proinflammatory TNFa and IL-6 [147].

In summary this study demonstrated that high permeable HD is able to modulate the transcript expression of local RAS components. Increased levels of leucocytic ACE2 over ACE induced by HCO- and MCO-HD treatment, may contribute to the anti-inflammatory and anti-atherogenic effects as reported previously.

Modulation of leucocytic angiotensin-converting enzymes expression in patients maintained on high-permeable haemodialysis.

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Abstract
Background: High mortality of haemodialysis patients is associated with systemic chronic inflammation and overactivation of the renin-angiotensin system (RAS). Insufficient elimination of pro-inflammatory immune mediators, especially in the molecular weight range of 15-45 kDa, may be one of the reasons for this. Employment of haemodialysis membranes with increased permeability was shown to ameliorate the inflammatory response and might modulate the effects of local RAS. In this study, we tested the impact of high cut-off (HCO), medium cut-off (MCO) and high-flux (HF) dialysis on leucocytic transcripts of angiotensin-converting enzymes (ACE and ACE2). Additionally, the impact of HCO, MCO and HF sera and dialysates on local ACEs and inflammation markers was tested in THP-1 monocytes.

Methods: Patients' leucocytes were obtained from our recent clinical studies comparing HCO and MCO dialysers with HF. The cells were subjected to quantitaive polymerase chain reaction (qPCR) analyses with TaqMan probes specific for ACE, ACE2 and angiotensin II (AngII) and Ang1-7 receptors. Sera and dialysates from the clinical trials as well as samples from in vitro dialysis were tested on THP-1 monocytic cells. The cells were subjected to qPCR analyses with TaqMan probes specific for ACE, ACE2, interleukin-6 and tumour necrosis factor α and immunocytochemistry with ACE and ACE2 antibodies.

Results: Leucocytes obtained from patients treated with HCO or MCO demonstrated decreased transcript expression of ACE, while ACE2 was significantly upregulated as compared with HF. Receptors for AngII and Ang1-7 remained unchanged. THP-1 monocytes preconditioned with HCO and MCO patients' or in vitro dialysis sera reflected the same expressional regulation of ACE and ACE2 as those observed in HCO and MCO leucocytes. As a complementary finding, treatment with HCO and MCO in vitro dialysates induced a pro-inflammatory response of the cells as demonstrated by elevated messenger RNA expression of tumour necrosis factor α and interleukin-6, as well as upregulation of ACE and decreased levels of ACE2.

Conclusions: Taken together, these data demonstrate that employment of membranes with high permeability eliminates a spectrum of mediators from circulation that affect the RAS components in leucocytes, especially ACE/ACE2.

3 Summary

In conclusion this work demonstrates that uremic milieu promotes over-activation of monocytic ACE and decrease of ACE2 via an AngII-dependent mechanism, thus contributing to enhanced endothelial adhesion and transmigration. These findings may elucidate an important mechanism contributing relevantly to atherosclerosis progression in patients with chronic renal failure. The altered relation between locally expressed ACE and ACE2 may contribute to the ubiquitous arterial hypertension frequently observed in patients with renal failure.

Experimental overexpression of ACE on monocytes or uremic conditions leads to downregulation of ACE2, more differentiated pro-atherogenic phenotype of the cells and increased expression of adhesion-related molecules. Experimental overexpression of ACE2 on monocytes or employment of AngII-modifying compounds to ACE-overexpressing monocytes can at least in part reverse this pro-atherogenic pattern of the cells in vitro.

HD with highly permeable membranes is able to modulate the transcript expression of local RAS components and inflammatory markers. Elevated levels of leucocytic ACE2 over ACE induced by HCO- and MCO-HD treatment, may contribute to the anti-inflammatory and anti-atherogenic effects.

Data presented in this work may help to optimize the ACE-inhibition based therapy in patients with end stage renal disease and provide insight in new molecular mechanisms involving both ACEs in the processes of inflammation and atherosclerosis.

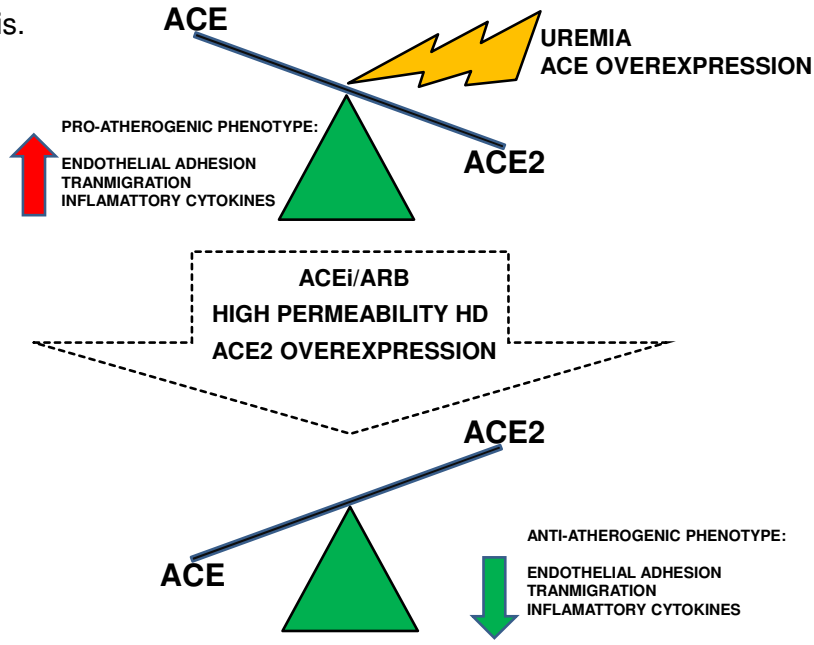

Figure 2: Schematic presentation of the results of this work: uraemia or ACE-overexpression promote a pro-atherogenic balance between both ACEs in human leukocytes/monocytes that can be reversed by employment of high permeability HD, ACE2-overexpression or at least in part with AngII-modifying treatments; ACEi, ACE inhibition; ARB, AngII receptor blocker.

4 Literature

1. J. Larry Jameson, Joseph Loscalzo: Harrison's Nephrology and Acid-Base Disorders. ISBN: 978-0-07-166340-3. MHID: 0-07-166340-1.
2. Jurgen Floege Richard Johnson John Feehally: Comprehensive Clinical Nephrology 4th Edition. ISBN: 9780323077668
3. Edgar V. Lerma, Jeffrey S. Berns, Allen R. Nissenson: CURRENT Diagnosis & Treatment: Nephrology & Hypertension. ISBN: 978-0-07-164108-1, MHID: 0-07-164108-4
4. Noordzij M, Korevaar JC, Boeschoten EW, Dekker FW, Bos WJ, Krediet RT; Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD) Study Group. The Kidney Disease Outcomes Quality Initiative (K/DOQI) Guideline for Bone Metabolism and Disease in CKD: association with mortality in dialysis patients. Am J Kidney Dis. 2005 Nov;46(5):925-32.
5. Glassock RJ, Winearls C. An epidemic of chronic kidney disease: fact or fiction? Nephrol Dial Transplant. 2008 Apr;23(4):1117-21.
6. Thomas B, Matsushita K, Abate KH, Al-Aly Z, Ärnlöv J, et al. Global Cardiovascular and Renal Outcomes of Reduced GFR. J Am Soc Nephrol. 2017 Jul;28(7):2167-2179
7. Stevens LA, Levey AS. Measurement of kidney function. Med Clin North Am. 2005 May;89(3):457-73.
8. Miller WG, Myers GL, Ashwood ER, Killeen AA, Wang E, Thienpont LM, Siekmann L. Creatinine measurement: state of the art in accuracy and interlaboratory harmonization. Arch Pathol Lab Med. 2005 Mar;129(3):297-304.
9. Levey AS, Schoolwerth AC, Burrows NR, Williams DE, Stith KR, McClellan W; Centers for Disease Control and Prevention Expert Panel. Comprehensive public health strategies for preventing the development, progression, and complications of CKD: report of an expert panel convened by the Centers for Disease Control and Prevention. Am J Kidney Dis. 2009 Mar;53(3):522-35.
10. Coresh J, Selvin E, Stevens LA, Manzi J, Kusek JW, Eggers P, Van Lente F, Levey AS: Prevalence of chronic kidney disease in the United States. JAMA 298: 2038–2047, 2007.
11. Imai E, Horio M, Iseki K, Yamagata K, Watanabe T, Hara S, Ura N, Kiyohara Y, Hirakata H, Moriyama T, Ando Y, Nitta K, Inaguma D, Narita I, Iso H, Wakai K, Yasuda Y, Tsukamoto Y, Ito S, Makino H, Hishida A, Matsuo S: Prevalence of chronic kidney disease (CKD) in the Japanese general population predicted by the MDRD equation modified by a Japanese coefficient. Clin Exp Nephrol 11: 156–163, 2007.
12. Stanifer JW, Jing B, Tolan S, Helmke N, Mukerjee R, Naicker S, Patel U: The epidemiology of chronic kidney disease in sub-Saharan Africa: A systematic review and meta-analysis. Lancet Glob Health 2: e174–e181, 2014.
13. Anand S, Shivashankar R, Ali MK, Kondal D, Binukumar B, Montez-Rath ME, Ajay VS, Pradeepa R, Deepa M, Gupta R, Mohan V, Narayan KM, Tandon N, Chertow GM, Prabhakaran D; CARRS Investigators: Prevalence of chronic kidney disease in two major Indian cities and projections for associated cardiovascular disease. Kidney Int 88: 178–185, 2015.
14. World Kidney Day: Chronic Kidney Disease. 2015; http://www.worldkidneyday.org/faqs/chronic-kidney-disease/ .
15. Jha V, Garcia-Garcia G, Iseki K, et al. Chronic kidney disease: global dimension and perspectives. Lancet. Jul 20 2013;382(9888):260-272.

16. Couser WG, Remuzzi G, Mendis S, Tonelli M. The contribution of chronic kidney disease to the global burden of major noncommunicable diseases. Kidney Int. Dec 2011;80(12):1258-1270.
17. Levey AS, Atkins R, Coresh J, et al. Chronic kidney disease as a global public health problem: approaches and initiatives - a position statement from Kidney Disease Improving Global Outcomes. Kidney Int. Aug 2007;72(3):247-259.
18. Eriksen BO, Ingebretsen OC. The progression of chronic kidney disease: a 10-year population-based study of the effects of gender and age. Kidney Int. 2006 Jan;69(2):375-82.
19. U.S. Renal Data System. USRDS 2007 Annual Data Report: Atlas of End Stage Renal Disease in the United States. Bethesda, Md: National Institutes of Health, National Institutes of Diabetes and Digestive and Kidney Diseases; 2007. Available at: www.usrds.org.
20. Tarver-Carr ME, Powe NR, Eberhardt MS, LaVeist TA, Kington RS, Coresh J, Brancati FL. Excess risk of chronic kidney disease among African-American versus white subjects in the United States: a population-based study of potential explanatory factors. J Am Soc Nephrol. 2002 Sep;13(9):2363-70.
21. Sandholm N, Groop PH. Genetic basis of diabetic kidney disease and other diabetic complications. Curr Opin Genet Dev. 2018 Feb 13;50:17-24.
22. Franceschini N, Shara NM, Wang H, Voruganti VS, Laston S, Haack K, Lee ET, Best LG, Maccluer JW, Cochran BJ, Dyer TD, Howard BV, Cole SA, North KE, Umans JG. The association of genetic variants of type 2 diabetes with kidney function. Kidney Int. 2012 Jul;82(2):220-5.
23. Ong AC, Harris PC. Molecular pathogenesis of ADPKD: the polycystin complex gets complex. Kidney Int. 2005 Apr;67(4):1234-47.
24. Ramanathan G, Harichandana B, Kannan S, Elumalai R, Paul S. Association between end-stage diabetic nephropathy and MTHFR (C677T and A1298C) gene polymorphisms. Nephrology (Carlton). 2017 Dec 11.
25. Köttgen A, Hwang SJ, Rampersaud E, Coresh J, North KE, Pankow JS, Meigs JB, Florez JC, Parsa A, Levy D, Boerwinkle E, Shuldiner AR, Fox CS, Kao WH. TCF7L2 variants associate with CKD progression and renal function in population-based cohorts. J Am Soc Nephrol. 2008 Oct;19(10):1989-99.
26. Polichnowski AJ, Lan R, Geng H, Griffin KA, Venkatachalam MA, Bidani AK. Severe renal mass reduction impairs recovery and promotes fibrosis after AKI. J Am Soc Nephrol. 2014 Jul;25(7):1496-507.
27. Locatelli F, Marcelli D, Comelli M, Alberti D, Graziani G, Buccianti G, Redaelli B, Giangrande A. Proteinuria and blood pressure as causal components of progression to end-stage renal failure. Northern Italian Cooperative Study Group. Nephrol Dial Transplant. 1996 Mar;11(3):461-7.
28. Jafar TH, Stark PC, Schmid CH, Landa M, Maschio G, Marcantoni C, de Jong PE, de Zeeuw D, Shahinfar S, Ruggenenti P, Remuzzi G, Levey AS; AIPRD Study Group. Angiotensin-Converting Enzymne Inhibition and Progression of Renal Disease. Proteinuria as a modifiable risk factor for the progression of non-diabetic renal disease. Kidney Int. 2001 Sep;60(3):1131-40.
29. Jafar TH, Stark PC, Schmid CH, Landa M, Maschio G, de Jong PE, de Zeeuw D, Shahinfar S, Toto R, Levey AS; AIPRD Study Group. Progression of chronic kidney disease: the role of blood pressure control, proteinuria, and angiotensin-converting enzyme inhibition: a patient-level meta-analysis. Ann Intern Med. 2003 Aug 19;139(4):244-52.
30. Arnlöv J, Evans JC, Meigs JB, Wang TJ, Fox CS, Levy D, Benjamin EJ, D'Agostino RB, Vasan RS. Low-grade albuminuria and incidence of cardiovascular disease events in nonhypertensive

and nondiabetic individuals: the Framingham Heart Study. Circulation. 2005 Aug 16;112(7):969-75.

31. Hermans MM, Henry R, Dekker JM, Kooman JP, Kostense PJ, Nijpels G, Heine RJ, Stehouwer CD. Estimated glomerular filtration rate and urinary albumin excretion are independently associated with greater arterial stiffness: the Hoorn Study. J Am Soc Nephrol. 2007 Jun;18(6):1942-52.
32. Hermans MM, Henry RM, Dekker JM, Nijpels G, Heine RJ, Stehouwer CD. Albuminuria, but not estimated glomerular filtration rate, is associated with maladaptive arterial remodeling: the Hoorn Study. J Hypertens. 2008 Apr;26(4):791-7.
33. Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group, Lachin JM, Genuth S, Cleary P, Davis MD, Nathan DM. Retinopathy and nephropathy in patients with type 1 diabetes four years after a trial of intensive therapy. N Engl J Med. 2000 Feb 10;342(6):381-9.
34. Saenz A, Fernandez-Esteban I, Mataix A, Ausejo M, Roque M, Moher D. Metformin monotherapy for type 2 diabetes mellitus. Cochrane Database Syst Rev. 2005 Jul 20;(3):CD002966.
35. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). Lancet. 1998 Sep 12;352(9131):854-65.
36. UK Prospective Diabetes Study (UKPDS) Group. ntensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet. 1998 Sep 12;352(9131):837-53.
37. Bello AK, de Zeeuw D, El Nahas M, Brantsma AH, Bakker SJ, de Jong PE, Gansevoort RT. Impact of weight change on albuminuria in the general population. Nephrol Dial Transplant. 2007 Jun;22(6):1619-27.
38. Abdelaal M, le Roux CW, Docherty NG. Morbidity and mortality associated with obesity. Ann Transl Med. 2017 Apr;5(7):161.
39. Stenvinkel P, Zoccali C, Ikizler TA. Obesity in CKD--what should nephrologists know? J Am Soc Nephrol. 2013 Nov;24(11):1727-36.
40. Ritz E, Wanner C. Lipid changes and statins in chronic renal insufficiency. J Am Soc Nephrol. 2006 Dec;17(12 Suppl 3):S226-30.
41. Orth SR, Hallan SI. Smoking: a risk factor for progression of chronic kidney disease and for cardiovascular morbidity and mortality in renal patients--absence of evidence or evidence of absence? Clin J Am Soc Nephrol. 2008 Jan;3(1):226-36.
42. Johnson RJ, Kivlighn SD, Kim YG, Suga S, Fogo AB. Reappraisal of the pathogenesis and consequences of hyperuricemia in hypertension, cardiovascular disease, and renal disease. Am J Kidney Dis. 1999 Feb;33(2):225-34.
43. Madero M, Sarnak MJ, Wang X, Greene T, Beck GJ, Kusek JW, Collins AJ, Levey AS, Menon V. Uric acid and long-term outcomes in CKD. Am J Kidney Dis. 2009 May;53(5):796-803.
44. Remuzzi G, Perico N, Macia M, Ruggenenti P. The role of renin-angiotensin-aldosterone system in the progression of chronic kidney disease. Kidney Int Suppl. 2005 Dec;(99):S57-65.
45. Meyer TW, Hostetter TH. Uremia. N Engl J Med. 2007 Sep 27;357(13):1316-25.
46. Depner TA. Uremic toxicity: urea and beyond. Semin Dial 2001;14:246-251.
47. Ramirez R, Carracedo J, Merino A, et al. Microinflammation induces endothelial damage in hemodialysis patients: the role of convective transport. Kidney Int. 2007; 72:108-113.

48. Vanholder R, Baurmeister U, Brunet P, et al. A bench to bedside view of uremic toxins. J Am Soc Nephrol. 2008; 19:863-870.
49. Fiedler R, Neugebauer F, Ulrich C, et al. Randomized controlled pilot study of 2 weeks' treatment with high cutoff membrane for hemodialysis patients with elevated C-reactive protein. Artif Organs 2012; 36:886-893.
50. Villa G, Zaragoza JJ, Sharma A, Chelazzi C, Ronco C, De Gaudio AR. High Cutoff Membrane to Reduce Systemic Inflammation Due to Differentiation Syndrome: A Case Report. Blood Purif 2014; 38:234-238.
51. Foley RN, Parfrey PS, Sarnak MJ (1998). Clinical epidemiology of cardiovascular disease in chronic renal disease. Am J Kidney 32: 112-119.
52. Cheung AK, Sarnak MJ, Yan G, Dwyer JT, Heyka RJ, et al. (2000) Atherosclerotic cardiovascular disease risks in chronic hemodialysis patients. Kidney Int 58: 353-362.
53. Vanholder R, Massy Z, Argiles A, Spasovski G, Verbeke F, et al. (2005) Chronic kidney disease as cause of cardiovascular morbidity and mortality. Nephrol Dial Transplant. 20:1048-1056.
54. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, Li Y, Wang X, Zhao L. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2017 Dec 14;9(6):7204-7218.
55. Descamps-Latscha B, Herbelin A, Nguyen AT, Zingraff J, Jungers P, Chatenoud L: Immune system dysregulation in uremia. Semin Nephrol 1994;14:253-260.
56. Descamps-Latscha B, Jungers P, Witko-Sar- sat V: Immune system dysregulation in ure- mia: role of oxidative stress. Blood Purif 2002;20:481-484.
57. Elenkov IJ, Iezzoni DG, Daly A, Harris AG, Chrousos GP: Cytokine dysregulation, in¬flammation and well-being. Neuroimmuno- modulation 2005;12:255-269.
58. Carrero JJ, Yilmaz MI, Lindholm B, Stenvinkel P. Cytokine dysregulation in chronic kidney disease: how can we treat it? Blood Purif 2008; 26: 291-299.
59. Silverstein DM. Inflammation in chronic kidney disease: role in the progression of renal and cardiovascular disease. Pediatr Nephrol. 2009 Aug;24(8):1445-52.
60. Meuwese CL, Stenvinkel P, Dekker FW, Carrero JJ. Monitoring of inflammation in patients on dialysis: forewarned is forearmed. Nat Rev Nephrol 2011; 7:166-176.
61. Carrero JJ, Stenvinkel P. Persistent inflammation as a catalyst for other risk factors in chronic kidney disease: a hypothesis proposal. Clin J Am Soc Nephrol 2009; 4 Suppl 1:S49-S55.
62. Miyamoto T, Carrero JJ, Stenvinkel P. Inflammation as a risk factor and target for therapy in chronic kidney disease. Curr Opin Nephrol Hypertens 2011; 20:662-668.
63. Dai L, Golembiewska E, Lindholm B, Stenvinkel P. End-Stage Renal Disease, Inflammation and Cardiovascular Outcomes. Contrib Nephrol. 2017;191:32-43.
64. Tyralla K, Amann K (2003) Morphology of the heart and arteries in renal failure. Kidney Int Suppl 84: 80-83.
65. Guerin AP, Blacher J, Pannier B, Marchais SJ, Safar ME, et al. (2001) Impact of aortic stiffness attenuation on survival of patients in end-stage renal failure. Circulation 103: 987-992.
66. Ulrich C, Seibert E, Heine GH, Fliser D, Girndt M (2011) Monocyte angiotensin converting enzyme expression may be associated with atherosclerosis rather than arteriosclerosis in hemodialysis patients. Clin J Am Soc Nephrol 6: 505-511.
67. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s (1993). Nature 362: 801-809.
68. Steinberg D (2002) Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. Nature Med 8: 1211-1217.

69. Moore KJ, Tabas I (2011) Macrophages in the pathogenesis of atherosclerosis. Cell 145: 341-355.
70. Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, et al. (2010) Macrophage plasticity in experimental atherosclerosis. PLoS One 5: e8852.
71. Pello OM, Silvestre C, De Pizzol M, Andres V (2012) A glimpse on the phenomenon of macrophage polarization during atherosclerosis. Immunobiology 216: 1172-1176.
72. Martinez FO, Helming L, Gordon S (2009) Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 27: 451-483.
73. Gratchev A, Kzhyshkowska J, Utikal J, Goerdt S (2005) Interleukin-4 and dexamethasone counterregulate extracellular matrix remodelling and phagocytosis in type-2 macrophages. Scand J Immunol 61: 10-17.
74. Xu W, Roos A, Schlagwein N, Woltman AM, Daha MR, et al. (2006) IL-10-producing macrophages preferentially clear early apoptotic cells. Blood 107: 4930-4937.
75. Kohlstedt K, Trouvain C, Namgaladze D, Fleming I (2011) Adipocyte-derived lipids increase angiotensin-converting enzyme (ACE) expression and modulate macrophage phenotype. Basic Res Cardiol 106: 205-215.
76. Ulrich C, Heine GH, Seibert E, Fliser D, Girndt M (2010) Circulating monocyte subpopulations with high expression of angiotensin-converting enzyme predict mortality in patients with end-stage renal disease. Nephrol Dial Transplant 25: 2265-2272.
77. Ulrich C, Heine GH, Garcia P, Reichart B, Georg T, et al. (2006) Increased expression of monocytic angiotensin-converting enzyme in dialysis patients with cardiovascular disease. Nephrol Dial Transplant 21: 1596-1602.
78. Campbell DJ. The site of angiotensin production. J Hypertens (1985) 3:199–20710.
79. Campbell DJ. Circulating and tissue angiotensin systems. J Clin Invest (1987) 79:1–610.
80. Yasojima K, Schwab C, McGeer EG, McGeer PL. Generation of Creactive protein and complement components in atherosclerotic plaques. Am J Pathol 2001;158:1039-1051.
81. Kitazono T, Padgett RC, Armstrong ML, Tompkins PK, Heistad DD (1995) Evidence that angiotensin II is present in human monocytes. Circulation 91: 1129-1134.
82. Schieffer B, Schieffer E, Hilfiker-Kleiner D, Hilfiker A, Kovanen PT, Kaartinen M, Nussberger J, Harringer W, Drexler H. Expression of angiotensin II and interleukin 6 in human coronary atherosclerotic plaques: potential implications for inflammation and plaque instability. Circulation 2000; 101:1372-1378.
83. Frank Diet, Richard E. Pratt, Gerald J. Berry, Naoko Momose, Gary H. Gibbons, Victor J. Dzau. Increased Accumulation of Tissue ACE in Human Atherosclerotic Coronary Artery Disease. Circulation. 1996;94:2756-2767.
84. Sara Conti, Paola Cassis, Ariela Benigni. Aging and the Renin-Angiotensin System. Hypertension. 2012;60:878-883
85. Khan UA, Garg AX, Parikh CR, et al. Prevention of chronic kidney disease and subsequent effect on mortality: a systematic review and meta-analysis. PLoS One 2013; 8: e71784.
86. Parving HH, Lehnert H, Bröchner-Mortensen J, et al. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria Study Group. N Engl J Med 2001; 345: 870-878.
87. Mann JF, Anderson C, Gao P, et al. Dual inhibition of the renin-angiotensin system in high-risk diabetes and risk for stroke and other outcomes: results of the ONTARGET trial. J Hypertens 2013; 31: 414-421.

88. Donoghue M, Hsieh F, Baronas E, et al. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ Res 2000; 87: E1-9.
89. Santos RA, Simoes e Silva AC, Maric C, et al. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci U S A 2003; 100:8258-8263.
90. Ye M, Wysocki J, William J, et al. Glomerular localization and expression of Angiotensin-converting enzyme 2 and Angiotensin-converting enzyme: implications for albuminuria in diabetes. J Am Soc Nephrol 2006; 17: 3067-3075.
91. Soler MJ, Wysocki J, Batlle D. ACE2 alterations in kidney disease. Nephrol Dial Transplant 2013; 28: 2687-2697.
92. Hamming I, Timens W, Bulthuis ML, et al. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. J Pathol 2004; 203: 631-637.
93. Imai Y, Kuba K, Rao S, et al. Angiotensin-converting enzyme 2 protects from severe acute lung failure. Nature 2005; 436:112-116.
94. Xiao L, Haack KK, Zucker IH. Angiotensin II regulates ACE and ACE2 in neurons through p38 mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 signaling. Am J Physiol Cell Physiol 2013; 304: C1073-1079.
95. Chen X, Lu H, Zhao M, et al. Contributions of leukocyte angiotensin-converting enzyme to development of atherosclerosis. Arterioscler Thromb Vasc Biol 2013; 33: 2075-2080.
96. Thatcher SE, Zhang X, Howatt DA, et al. Angiotensin-converting enzyme 2 deficiency in whole body or bone marrow-derived cells increases atherosclerosis in low-density lipoprotein receptor-/- mice. Arterioscler Thromb Vasc Biol 2011; 31: 758-765.
97. Dong B, Zhang C, Feng JB, et al. Overexpression of ACE2 enhances plaque stability in a rabbit model of atherosclerosis. Arterioscler Thromb Vasc Biol 2008; 28: 1270-1276.
98. Lovren F, Pan Y, Quan A, et al. Angiotensin converting enzyme-2 confers endothelial protection and attenuates atherosclerosis. Am J Physiol Heart Circ Physiol 2008; 295: 1377-1384.
99. Petrov MN, Shilo VY, Tarasov AV, Schwartz DE, Garcia JG, et al. (2012) Conformational changes of blood ACE in chronic uremia. PLoS One 7: e49290.
100. Finch JL, Suarez EB, Husain K, Ferder L, Cardema MC, et al. (2012) Effect of combining an ACE inhibitor and a VDR activator on glomerulosclerosis, proteinuria, and renal oxidative stress in uremic rats. Am J Physiol Renal Physiol 302: 141-149.
101. Mizobuchi M, Ogata H, Hosaka N, Kumata C, Nakazawa A, et al. (2011) Effects of calcimimetic combined with an angiotensin-converting enzyme inhibitor on uremic cardiomyopathy progression. Am J Nephrol 34: 256-260.
102. Schepers E, Meert N, Glorieux G, Goeman J, Van der Eycken J et al. (2007) P-cresylsulphate, the main in vivo metabolite of p-cresol, activates leucocyte free radical production. Nephrol Dial Transplant. 22: 592-596.
103. Shimizu H, Saito S, Higashiyama Y, Nishijima F, Niwa T (2013) CREB, NF-?B, and NADPH oxidase coordinately upregulate indoxyl sulfate-induced angiotensinogen expression in proximal tubular cells. Am J Physiol Cell Physiol. 304: 685-692.
104. Sun CY, Chang SC, Wu MS. Uremic toxins induce kidney fibrosis by activating intrarenal renin-angiotensin-aldosterone system associated epithelial-to-mesenchymal transition. PLoS One. 2012;7: e34026.
105. Ito S, Osaka M, Higuchi Y, Nishijima F, Ishii H, et al. (2010) Indoxyl sulfate induces leukocyte-endothelial interactions through up-regulation of E-selectin. J Biol Chem. 285: 38869-38875.

106. Stöger JL, Gijbels MJ, van der Velden S, Manca M, van der Loos CM, et al. (2012) Distribution of macrophage polarization markers in human atherosclerosis. Atherosclerosis 2012;225:461-468.
107. van Tits LJ, Stienstra R, van Lent PL, Netea MG, Joosten LA, et al. (2011) Oxidized LDL enhances pro-inflammatory responses of alternatively activated M2 macrophages: a crucial role for Krüppel-like factor 2. Atherosclerosis. 214: 345-349.
108. Pourcet B, Pineda-Torra I (2013) Transcriptional regulation of macrophage arginase 1 expression and its role in atherosclerosis. Trends Cardiovasc Med. 23: 143-152.
109. Libby P, Geng YJ, Aikawa M, Schoenbeck U, Mach F, et al. (1996) Macrophages and atherosclerotic plaque stability. Curr Opin Lipidol 7: 330-335.
110. Nelken NA, Coughlin SR, Gordon D, Wilcox JN (1991) Monocyte chemoattractant protein-1 in human atheromatous plaques. J Clin Invest 88: 1121-1127.
111. Wiesner P, Tafelmeier M, Chittka D, Choi SH, Zhang L, et al. (2013) MCP-1 binds to oxidized LDL and is carried by lipoprotein(a) in human plasma. J Lipid Res 54: 1877-1883.
112. Boring L, Gosling J, Cleary M, Charo IF (1998) Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 394: 894-897.
113. Dawson TC, Kuziel WA, Osahar TA, Maeda N (1999) Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 143:205-211.
114. Ni W, Kitamoto S, Ishibashi M, Usui M, Inoue S, et al. (2004) Monocyte chemoattractant protein-1 is an essential inflammatory mediator in angiotensin II-induced progression of established atherosclerosis in hypercholesterolemic mice. Arterioscler Thromb Vasc Biol 24: 534-539.
115. Ishibashi M, Hiasa K, Zhao Q, Inoue S, Ohtani K, et al. (2004) Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling. Circ Res 94: 1203-1210.
116. Blankenberg S, Barbaux S, Tiret L (2003) Adhesion molecules and atherosclerosis. Atherosclerosis 170: 191-203.
117. Tuttolomondo A, Di Raimondo D, Pecoraro R, Arnao V, Pinto A, et al. (2012) Atherosclerosis as an inflammatory disease: Curr Pharm Des 18: 4266-4288.
118. Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L and Beaudet AL. P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. J Exp Med 2000; 191(1): 189-194.
119. Dansky HM, Barlow CB, Lominska C, Sikes JL, Kao C, Weinsaft J, et al. Adhesion of monocytes to arterial endothelium and initiation of atherosclerosis are critically dependent on vascular cell adhesion molecule-1 gene dosage. Arterioscler Thromb Vasc Biol 2001; 21(10): 1662-1667.
120. De Meyer I, Martinet W, De Meyer GR (2012) Therapeutic strategies to deplete macrophages in atherosclerotic plaques. Br J Clin Pharmacol 74: 246-263.
121. Lal H, Guleria RS, Foster DM, Lu G, Watson LE, et al. (2007) Integrins: novel therapeutic targets for cardiovascular diseases. Cardiovasc Hematol Agents Med Chem 5: 109-132.
122. Kojima C, Kawakami A, Takei T, Nitta K, Yoshida M (2007) Angiotensin-converting enzyme inhibitor attenuates monocyte adhesion to vascular endothelium through modulation of intracellular zinc. J Pharmacol Exp Ther 323: 855-860.
123. Bernardi S, Candido R, Toffoli B, Carretta R, Fabris B (2011) Prevention of accelerated atherosclerosis by AT1 receptor blockade in experimental renal failure. Nephrol Dial Transplant 26: 832-838.

124. Koga J, Egashira K, Matoba T, Kubo M, Ihara Y, et al (2008) Essential role of angiotensin II type 1a receptors in the host vascular wall, but not the bone marrow, in the pathogenesis of angiotensin II-induced atherosclerosis. Hypertens Res 31: 1791-1800.
125. da Cunha V, Tham DM, Martin-McNulty B, Deng G, Ho JJ, et al (2005) Enalapril attenuates angiotensin II-induced atherosclerosis and vascular inflammation. Atherosclerosis 178: 9-17.
126. Rasini E, Cosentino M, Marino F, et al. Angiotensin II type 1 receptor expression on human leukocyte subsets: a flow cytometric and RT-PCR study. Regul Pept 2006; 134: 69-74.
127. Simolin MA, Pedersen TX, Bro S, Mäyränpää MI, Helske S, et al. (2009) ACE inhibition attenuates uremia-induced aortic valve thickening in a novel mouse model. BMC Cardiovasc Disord 9: 10.
128. Chon H, Neumann J, Boer P, et al. Enhanced Angiotensin II type 1 receptor expression in leukocytes of patients with chronic kidney disease. Eur J Pharmacol 2011; 666: 205-210.
129. Guasti L, Marino F, Cosentino M, et al. Prolonged statin-associated reduction in neutrophil reactive oxygen species and angiotensin II type 1 receptor expression: 1-year follow-up Eur. Heart J 2008; 29: 1118-1126.
130. Chon H, Gaillard CA, van der Meijden BB, et al. Broadly altered gene expression in blood leukocytes in essential hypertension is absent during treatment. Hypertension 2004; 43: 947-951.
131. Kovarik JJ, Antlanger M, Domenig O, et al. Molecular regulation of the renin-angiotensin system in haemodialysis patients. Nephrol Dial Transplant 2015; 30:115-123.
132. Dwivedi RS, Herman JG, McCaffrey TA, Raj DS. Beyond genetics: epigenetic code in chronic kidney disease. Kidney Int. 2011 Jan;79(1):23-32.
133. Lambert DW, Lambert LA, Clarke NE, Hooper NM, Porter KE, Turner AJ. Angiotensin-converting enzyme 2 is subject to post-transcriptional regulation by miR-421. Clin Sci (Lond). 2014 Aug;127(4):243-9.
134. Ferrario CM, Jessup J, Gallagher PE, et al. Effects of renin-angiotensin system blockade on renal angiotensin-(1-7) forming enzymes and receptors. Kidney Int 2005; 68:2189-2196.
135. Gallagher PE, Chappell MC, Ferrario CM, et al. Distinct roles for ANG II and ANG-(1-7) in the regulation of angiotensin-converting enzyme 2 in rat astrocytes. Am J Physiol Cell Physiol 2006; 290: 420-426.
136. Ferrario CM, Jessup J, Chappell MC, et al. Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. Circulation 2005; 111: 2605-2610.
137. Carrero JJ, Yilmaz MI, Lindholm B, Stenvinkel P. Cytokine dysregulation in chronic kidney disease: how can we treat it? Blood Purif 2008; 26: 291-299.
138. Zimmermann J, Herrlinger S, Pruy A, Metzger T, Wanner C: Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. Kidney Int 1999; 55:648-658.
139. Meuwese CL, Stenvinkel P, Dekker FW, Carrero JJ. Monitoring of inflammation in patients on dialysis: forewarned is forearmed. Nat Rev Nephrol 2011; 7:166-176.
140. Carrero JJ, Stenvinkel P. Persistent inflammation as a catalyst for other risk factors in chronic kidney disease: a hypothesis proposal. Clin J Am Soc Nephrol 2009; 4 Suppl 1:S49-S55.
141. Miyamoto T, Carrero JJ, Stenvinkel P. Inflammation as a risk factor and target for therapy in chronic kidney disease. Curr Opin Nephrol Hypertens 2011; 20:662-668.
142. Morgera S, Slowinski T, Melzer C, et al. Renal replacement therapy with high-cut off hemofilters: impact of convection and diffusion on cytokine clearances and protein status. Am J Kidney Dis 2004; 43:444-453.

143. Gondouin B, Hutchison CA. High cut-off dialysis membranes: current uses and future potential. Adv Chronic Kidney Dis 2011; 18:180-187.
144. Boschetti-de-Fierro A, Voigt M, Storr M, Krause B. MCO Membranes: Enhanced Selectivity in High-Flux Class. Sci Rep 2015; 5:18448.
145. Fiedler R, Neugebauer F, Ulrich C, et al. Randomized controlled pilot study of 2 weeks' treatment with high cutoff membrane for hemodialysis patients with elevated C-reactive protein. Artif Organs 2012; 36:886-893.
146. Villa G, Zaragoza JJ, Sharma A, Chelazzi C, Ronco C, De Gaudio AR. High Cutoff Membrane to Reduce Systemic Inflammation Due to Differentiation Syndrome: A Case Report. Blood Purif 2014; 38:234-238.
147. Girndt M, Fiedler R, Martus P, et al. High cut-off dialysis in chronic hemodialysis patients. Eur J Clin Invest 2015; 45:1333-1340.
148. Zickler D, Schindler R, Willy K, et al. Membranes Reduce Inflammation in Chronic Dialysis Patients-A Randomized Controlled Clinical Trial. PLoS One 2017; 12:e0169024.
149. Stenvinkel P, Ketteler M, Johnson RJ, et al. IL-10, IL-6, and TNF-alpha: central factors in the altered cytokine network of uremia--the good, the bad, and the ugly. Kidney Int 2005; 67:1216-1233.
150. Gupta J, Mitra N, Kanetsky PA, et al. Association between albuminuria, kidney function, and inflammatory biomarker profile in CKD in CRIC. Clin J Am Soc Nephrol 2012; 7:1938-1946.
151. Pruijm M, Ponte B, Vollenweider P, et al. Not all inflammatory markers are linked to kidney function: results from a population-based study. Am J Nephrol. 2012; 35:288-294.
152. Arici M, Walls J. End-stage renal disease, atherosclerosis, and cardiovascular mortality: is C-reactive protein the missing link? Kidney Int 2001; 59:407-414.
153. Stenvinkel P, Barany P, Heimbürger O, Pecoits-Filho R, Lindholm B. Mortality, malnutrition, and atherosclerosis in ESRD: what is the role of interleukin-6? Kidney Int Suppl 2002; 80:103-108.
154. Avesani CM, Carrero JJ, Axelsson J, Qureshi AR, Lindholm B, Stenvinkel P. Inflammation and wasting in chronic kidney disease: Partners in crime. Kidney Int 2006; 70:S8-S13.
155. Carrero JJ, Park SH, Axelsson J, Lindholm B, Stenvinkel P. Cytokines, atherogenesis, and hypercatabolism in chronic kidney disease: a dreadful triad. Semin Dial 2009; 22:381-386.
156. Barreto DV, Barreto FC, Liabeuf S, et al. Plasma interleukin-6 is independently associated with mortality in both hemodialysis and pre-dialysis patients with chronic kidney disease. Kidney Int 2010; 77:550-556.
157. Keithi-Reddy SR, Addabbo F, Patel TV, Mittal BV, Goligorsky MS, Singh AK. Association of anemia and erythropoiesis stimulating agents with inflammatory biomarkers in chronic kidney disease. Kidney Int 2008; 74:782-790.
158. Delmas PD, Wilson DM, Mann KG, Riggs BL. Effect of renal function on plasma levels of bone Gla-protein. J Clin Endocrinol Metab 1983; 57:1028-1030.
159. Xu TY, Zhang Y, Li Y, Zhu DL, Gao PJ. The association of serum inflammatory biomarkers with chronic kidney disease in hypertensive patients. Ren Fail 2014; 36:666-672
160. Nitta K, Ishizuka T, Horita S, et al. Soluble osteopontin and vascular calcification in hemodialysis patients. Nephron. 2001; 89:455-458.
161. Ohmori R, Momiyama Y, Taniguchi H, et al. Plasma osteopontin levels are associated with the presence and extent of coronary artery disease. Atherosclerosis 2003; 170:333-337.
162. Moe SM, O'Neill KD, Duan D, et al. Medial artery calcification in ESRD patients is associated with deposition of bone matrix proteins. Kidney Int 2002; 61:638-647.

163. Mizobuchi M, Towler D, Slatopolsky E. Vascular calcification: the killer of patients with chronic kidney disease. J Am Soc Nephrol 2009; 20:1453-1464.
164. Kneis C, Beck W, Boenisch O, et al. Elimination of middle-sized uremic solutes with high-flux and high-cut-off membranes: a randomized in vivo study. Blood Purif 2013; 36:287-294.
165. Bordoni V, Bolgan I, Brendolan A, et al. Caspase-3 and -8 activation and cytokine removal with a novel cellulose triacetate super-permeable membrane in an in vitro sepsis model. Int J Artif Organs 2003; 26:897-905.
166. Morgera S, Haase M, Rocktaschel J, et al. High permeability haemofiltration improves peripheral blood mononuclear cell proliferation in septic patients with acute renal failure. Nephrol Dial Transplant 2003; 18:2570-2576.
167. Oudit GY, Liu GC, Zhong J, et al. Human recombinant ACE2 reduces the progression of diabetic nephropathy. Diabetes 2010; 592:529-538.
168. Oudit GY, Herzenberg AM, Kassiri Z, et al. Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. Am J Pathol 2006; 168:1808-1820.
169. Wong DW, Oudit GY, Reich H, et al. Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. Am J Pathol 2007; 171:438-451.
170. Soler MJ, Wysocki J, Ye M, Lloveras J, Kanwar Y, Batlle D. ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. Kidney Int 2007; 725:614-623.
171. Dilauro M, Zimpelmann J, Robertson SJ, Genest D, Burns KD. Effect of ACE2 and angiotensin-(1-7) in a mouse model of early chronic kidney disease. Am J Physiol Renal Physiol 2010; 298:1523-1532.
172. Liu Z, Huang XR, Chen HY, Penninger JM, Lan HY. Loss of angiotensin-converting enzyme 2 enhances TGF-β/Smad-mediated renal fibrosis and NF-κB-driven renal inflammation in a mouse model of obstructive nephropathy. Lab Invest 2012; 92:650-661.
173. Marques FD, Ferreira AJ, Sinisterra RD, et al. An oral formulation of angiotensin-(1-7) produces cardioprotective effects in infarcted and isoproterenol-treated rats. Hypertension 2011; 57:477-483.
174. Kulemina LV, Ostrov DA. Prediction of off-target effects on angiotensin-converting enzyme 2. J Biomol Screen 2011; 16:878-885.
175. Velkoska E, Patel SK, Griggs K, Pickering RJ, Tikellis C, Burrell LM. Short-term treatment with diminazene aceturate ameliorates the reduction in kidney ACE2 activity in rats with subtotal nephrectomy. PLoS One 2015; 10:e0118758.
176. Haber PK, Ye M, Wysocki J, Maier C, Haque SK, Batlle D. Angiotensin-converting enzyme 2-independent action of presumed angiotensin-converting enzyme 2 activators: studies in vivo, ex vivo, and in vitro. Hypertension 2014; 63:774-782.
177. Treml B, Neu N, Kleinsasser A, et al. Recombinant angiotensin-converting enzyme 2 improves pulmonary blood flow and oxygenation in lipopolysaccharide-induced lung injury in piglets. Crit Care Med 2010; 38:596-601.
178. Bossi F, Bernardi S, De Nardo D, et al. Angiotensin 1-7 significantly reduces diabetes-induced leukocyte recruitment both in vivo and in vitro. Atherosclerosis. 2016; 244:121-130.
179. Zickler D, Willy K, Girndt M, et al. High cut-off dialysis in chronic haemodialysis patients reduces serum procalcific activity. Nephrol Dial Transplant 2016; 31:1706-12.

5 Thesen

1. Angiotensin-konvertierendes Enzym (ACE) als Dipeptidylcarboxy-Peptidase spaltet das Decapeptide Angiotensin I (AngI) zu Octapeptide Angiotensin II (AngII), ein potenter Vasokonstriktor. ACE2 als Monocarboxy-Peptidase spaltet das AngII zu Ang 1-7, ein potenter Vasodilatator.

2. Beide ACE-Enzyme sind vor allem auf den Endothelien der Lungen- und Nierengefäße, sowie Monozyten und Makrophagen nachweisbar. Monozyten, die zur normalen Infektionsabwehr benötigt werden sind auch bei allen Atherosklerosestadien beteiligt.

3. Erhöhte Expression des monozytären ACE‘s in Patienten mit chronischem Nierenversagen (CKD) ist nicht nur mit kardiovaskulären Komplikationen und höheren Mortalitätsraten assoziiert, sondern könnte auch als ein Indikator für fortgeschrittene Atherosklerose dienen. Dabei ist die Expression des monozytären ACE2 in diesen Patienten signifikant erniedrigt.

4. Leukozytärer AngII-Rezeptor Type I und MAS-Rezeptor für Ang 1-7 sind in Patienten mit CKD signifikant erhöht. In CKD Patienten lässt sich die Expression von diesen Rezeptoren durch die Anwendung von Angiotensin-II-Rezeptor-Antagonisten und ACE-Hemmer nicht beeinflussen.

5. Urämische Bedingungen verursachen die pro-atherogene Differenzierung humaner Monozyten über einen Angiotensin-abhängigen Mechanismus. Dabei wird das monozytäres ACE hoch und ACE2 herunterreguliert.

6. Überexpression des ACEs in humanen Monozyten reguliert das ACE2 runter, induziert eine Hochregulierung von Zelladhäsionsmolekülen und verstärkte endotheliale Adhäsion. ACE-überexpriemierende Zellen verfügen über einen stark differenzierten Phänotyp.

7. Pro-atherogene Effekte der ACE-überexpriemierenden Monozyten lassen sich teilweise durch die Behandlung mit Angiotensin-II-Rezeptor-Antagonisten und ACE-Hemmer vermindern.

8. Überexpression des ACE2s in humanen THP-1 Monozyten induziert eine Herunterregulierung von Zelladhäsionsmolekülen und verminderte endotheliale Adhäsion. ACE2-überexprimierende Zellen verfügen über einen weniger differenzierten Phänotyp im Vergleich zu Kontrollzellen.

9. Expression des leukozytären ACE2s ist in Hämodialyse-Patienten nach Behandlung mit Medium- und High-Cut-Off Membranen signifikant erhöht. Expression des leukozytären AngII-Rezeptor Type I und MAS-Rezeptor wird durch Behandlung mit hochpermeablen Membranen nicht geändert.

10. In THP-1 Monozyten ist die Expression von ACE nach Behandlung mit Serum von Patienten, die mit hochpermeablen Membranen behandelt wurden, herunterreguliert. In besonderem Maße ist die monozytäre Expression des ACE2s nach Behandlung mit diesen Seren signifikant erhöht. Die Expression von beiden ACEs zeigte nach Behandlung mit hochpermeablen Dialysaten ein umgekehrtes Verhältnis im Vergleich zu den Serum-Untersuchungen.

11. Das Expressionsmuster von beiden ACEs in Patienten auf hochpermeablen Membranen lässt sich in vitro reproduzieren. Des Weiteren konnte gezeigt werden, dass das Inflammationsprofil in THP-1 Monozyten nach Behandlung mit HCO-Serum deutlich vermindert ist.

12. Die Daten dieser Arbeit deuten darauf hin, dass durch die Urämie getriggerte Induktion von monozytärem ACE und Verminderung von ACE2 zur Entstehung und/oder Progression von Atherosklerose beitragen könnten.

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