High-density lipoprotein loses its anti-inflammatory capacity by accumulation of pro-inflammatory-serum amyloid A

Markus Tölle†, Tao Huang†, Mirjam Schuchardt†, Vera Jankowski†, Nicole Prüfer†, Joachim Jankowski†, Uwe J.F. Tietge‡, Walter Zidek†, and Markus van der Giet*†

1Med. Klinik mit SP Nephrologie, Charité – Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany; and 2Department of Pediatrics, Center for Liver, Digestive and Metabolic Diseases, University Medical Center Groningen, 9713 Groningen, The Netherlands

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Aims

High-density lipoprotein (HDL) is known to have potent anti-inflammatory properties. Monocyte chemoattractant protein-1 is an important pro-inflammatory cytokine in early atherogenesis. There is evidence that HDL can lose its protective function during inflammatory disease. In patients with end-stage renal disease (ESRD), epidemiological studies have documented that the inverse correlation between HDL-cholesterol and cardiovascular risk is lost. Many structural modifications leading to reduced HDL function have been characterized, but the functional consequences are not fully understood.

Methods and results

We showed that HDL from patients with ESRD has a lower anti-inflammatory potential by reduced inhibition of monocyte chemoattractant protein-1 formation in vascular smooth muscle cells. Via a proteomic approach, we identified proteins in HDL from ESRD patients exerting pro-inflammatory actions. By chromatographic separation of proteins and mass-spectrometric analysis, we found serum amyloid A (SAA) to be one molecule acting as a potent pro-inflammatory protein. SAA is enriched in HDL from ESRD patients, correlating with reduced anti-inflammatory capacity. In SAA signal transduction, activation of formyl-peptide receptor 2 is involved. SAA enrichment in HDL of healthy subjects reduced the anti-inflammatory capacity of HDL and correlated with its decreased function.

Conclusion

These results suggest that SAA enrichment of HDL during disease conditions contributes to the decreased protective function. It is a novel finding that SAA acts as a pro-inflammatory molecule to reduce the anti-inflammatory properties of HDL.

Keywords

High-density lipoprotein • Serum amyloid A • Pro-inflammatory signalling • End-stage renal disease

1. Introduction

In recent decades, numerous epidemiological and clinical studies have documented an inverse relationship between high-density lipoprotein (HDL) levels and the progression of atherosclerosis.1–3 Many studies have demonstrated how HDL acts as a potent anti-atherosclerotic lipid particle with complex pleiotropic effects.1–3 Initially, it was shown that HDL acts as carrier for cholesterol by mediating the reverse cholesterol transport (RCT).4,5 Many other effects have been described, where HDL acts directly as a local anti-inflammatory particle.6,7 Various substances, including apolipoprotein-AI (ApoAI) and sphingosine-1-phosphate, have been identified as potential anti-inflammatory molecules within HDL.8–10 During disease conditions with an acute or chronic inflammatory response, there is evidence that HDL can lose its anti-inflammatory and functional properties,11 e.g. shown for HDL from patients with micro-inflammation in epidemiological studies.12 The reasons for these changes are not fully understood and are mainly attributed to changes in particle composition or modifications of HDL-associated proteins, e.g. modification of ApoAI.13 Patients with end-stage renal disease (ESRD) suffer from chronic micro-inflammation.14 Accelerated atherosclerosis and arteriosclerosis in patients with ESRD contributes to the excessive

† M.T. and T.H. contributed equally to this work.

* Corresponding author. Tel: +49 30 8445 2379; fax: +49 30 8445 3338. Email: markus.vandergiet@charite.de

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risk for cardiovascular morbidity and mortality. Epidemiological studies have demonstrated that the inverse relationship between HDL and cardiovascular risk is lost in patients with ESRD. For more than three decades it has been known that profound changes in HDL composition occur in ESRD patients. Total HDL content is reduced the HDL is enriched with triglycerides, but cholesterol content decreases. Additionally, the amounts of apolipoproteins (Apo) ApoAI/ApoAll in HDL are lower, whereas levels of ApoCII/ApoCIII increase. Decreased paraoxonase activity in renal failure, which normally inhibits HDL and low-density lipoprotein (LDL) oxidation, can impair the structure and function of both HDL and LDL. In ESRD patients, there are hints that HDL might lose its anti-inflammatory properties. Many compositional changes in HDL are observed and have been partly linked to functional modifications, but the precise molecular changes and the signal transduction pathways are only partly understood.

Recently, our group was able to demonstrate that HDL is a potent inhibitor of pro-inflammatory signalling molecules, such as monococyte chemoattractant protein-1 (MCP-1). MCP-1 is one of the key factors in the initiation and progression of atherosclerotic diseases. This chemokine is secreted by various cell types, including vascular smooth muscle cells (VSMCs), and leads to the recruitment of monocytes in the subendothelial space. In the present study, we wanted to elucidate whether and why HDL might lose its anti-inflammatory capacity in patients with ESRD. As we know from a recent study, HDL can inhibit thrombin-induced MCP-1 formation in VSMCs. We defined a reduced inhibitory capacity of HDL, found in the present study for ESRD patients, as potential accumulation of pro-inflammatory signalling molecules in HDL. We used the bioassay of MCP-1 inhibition in VSMCs to identify molecules that have direct functional pro-inflammatory relevance. Using a proteomic approach, we were able to show a protein accumulation in HDL that might be responsible for this observed effect. Using mass spectrometry, the substance was identified as serum amyloid A (SAA). We can show that SAA levels in HDL significantly correlate with the loss of anti-inflammatory capacity of HDL.

2. Methods

2.1 Reagents

All cell culture reagents were purchased from Biochrom AG (Berlin, Germany). Human SAA was from Peprotech (London, UK). Pertussis toxin (PTX) was purchased from Sigma Aldrich (St Louis, MO, USA). Tryptophan-Arginine-Tryptophan-Tryptophan-Tryptophan-Tryptophan-NH₂ (WRW4) and Leucine-Glutamic acid-Serine-Isoleucine-Phenylalanine-Arginine-Serine-Leucine-Leucine-Phenylalanine-Arginine-Valine-Methionine (MMK-1) were obtained from Tocris (Ellisville, MO, USA), and thombin was from Merck Chemicals (Darmstadt, Germany).

2.2 Patients

Patients were enrolled from dialysis units in Berlin (n = 34), and control subjects (n = 17) were recruited from the outpatient clinic at the Charité, Department of Nephrology, after informed consent. For clinical and biochemical characteristics see Table 1. Owing to material limitations, some experiments were done with subgroups of the complete cohort. For ESRD patients, blood for investigations was sampled prior to dialysis sessions. The studies were approved by the local ethics committee of the Charité in accordance with the Declaration of Helsinki.

2.3 Cell culture

We isolated primary aortic VSMCs from Wistar rats. After the rats had been killed by intraperitoneal injection of pentobarbital (400 mg/kg body weight), thoracic aortas were explanted. VSMCs were isolated by the explant outgrowth method as described previously. Animal experiments conformed to the Guide for Care and Use of Laboratory Animals (Directive 2010/63/EU of the European Parliament) and were approved by local Animal Care Committees at the Landesamt für Gesundheit und Soziales (Berlin, Germany).

The rat VSMCs were cultured in Dulbecco’s modified Eagle’s medium [1 g/L glucose, 10% (v/v) fetal bovine serum and 1% (v/v) penicillin—streptomycin]. Passages three to eight were used for the experiments in this study.

2.4 Lipoprotein analyses

HDL (density = 1.063–1.215 g/mL) from healthy control subjects and ESRD patients was isolated from serum by ultracentrifugation using a SW40Ti rotor (Beckman Coulter, Krefeld, Germany), and dialysed at 4°C against phosphate-buffered saline. Levels of total cholesterol were determined using chromogenic assays (Diasys, Holzheim, Germany). For experiments involving incubation of HDL from healthy control subjects with recombinant SAA, 50 μg of SAA was added to 8 mL of serum from a healthy donor for 2 h; then HDL was isolated as described above. Separation of HDL in the lipid–protein fraction was done by the isopropanol–hexane protocol. Five volumes of hexanes/iso-propanol (3:2) were added to HDL (1 μg/μL). The solution was then mixed for 30 s, and centrifuged at 4°C for 5 min at 4000 g. The upper layer containing HDL lipids was drawn and stored at 4°C. To the aqueous lower layer containing HDL-proteins, four volumes of hexane were added, centrifuged as described above, and the upper lipid-containing fraction was pooled with the first lipid fraction. Both fractions were dried using a Speedvac (Thermo Scientific, Waltham, MA, USA). Protein fractions were resolved in guanidinium hydrochloride (3 mol/L) and separated by high-performance liquid chromatography (HPLC) using a Grace Vydac C18 column (Alltech, Rotterdam, Germany). For detailed information about the fraction scheme see Supplementary material online.

2.5 Identification of SAA by mass spectrometry

The HPLC fraction with a significant absorption at 214 nm and elution time of about 12 min was analysed by matrix-assisted laser desorption/ ionization tandem time of flight mass spectrometry (MALDI-TOF/TOF mass spectrometry) as recently described. For detailed information see Supplementary material online.

2.6 Cell stimulation and detection of MCP-1

VSMCs were serum starved for 24 h. For measuring MCP-1 expression, cells were stimulated with thrombin (2 IU/mL) ± HDL, with protein/lipid fraction of HDL, or with SAA for 4 h. For measuring MCP-1 secretion, cells were stimulated for 24 h (8 IU/mL), supernatants were collected, and MCP-1 was measured via the Lumines® technique (Millipore, Schwalbach, Germany) and normalized to the protein content of the cells.

2.7 RT-PCR and quantitative real-time PCR

After cell stimulation, RNA was isolated with the RNeasy® Mini kit from Qiagen (Chatsworth, CA, USA) according to the manufacturer’s instructions. One microgram of total RNA from each sample was reverse transcribed using iScript™ cDNA Synthesis kit (Biorad, Munich, Germany). Levels of MCP-1 expression were determined by quantitative real-time PCR using reagents kits from Biorad in the CFX96/384 System (Bio-Rad Laboratories GmbH, Munich, Germany). The primers/probes were synthesized by TibMolBiol (Berlin, Germany). The oligonucleotide

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sequences for MCP-1 and β-actin are as published.\textsuperscript{31} Quantification of gene expression was normalized to β-actin as housekeeping gene.

2.8 Measurement of SAA
Concentrations of SAA in serum and HDL from ESRD patients and healthy control subjects were measured with a specific ELISA kit (Abazyme, Needham, MA, USA).

2.9 Statistical analyses
Data are presented as means ± SEM, and statistic significance was determined using GraphPad Prism software 5.0 (GraphPad Software Inc., La Jolla, CA, USA). We considered a $P$ value <0.05 statistically significant.

3. Results
3.1 HDL from ESRD patients has lower anti-inflammatory capacity than HDL from healthy donors
We have previously shown that HDL can inhibit MCP-1 expression and secretion from VSMCs in a dose-dependent manner.\textsuperscript{23} HDL from healthy control subjects as well as ESRD patients decreases the thrombin-induced MCP-1 expression (Figure 1A) and secretion (Figure 1B). However, the capacity of HDL from ESRD patients to inhibit thrombin-induced MCP-1 production was significantly reduced compared with HDL from healthy donors, with an EC_{50} shift and lower maximal inhibition in expression and secretion studies, respectively (Figure 1A and B).

3.2 HDL from ESRD patients has pro-inflammatory capacity
In the next step, we investigated whether HDL from ESRD patients might stimulate MCP-1 formation and which fraction of HDL (protein/lipid) might be involved. We demonstrated that HDL from ESRD patients stimulated MCP-1 expression (Figure 2A) and secretion (Figure 2B) to a greater extent than HDL from healthy control subjects. To identify the constituent of HDL mediating this biological effect, we first separated HDL from ESRD patients into lipid and protein fractions and tested these for their ability to induce MCP-1. While the lipid fraction showed essentially no stimulatory activity, the protein fraction of HDL from ESRD patients strongly induced MCP-1 formation (Figure 2C and D). Thereafter, the total HDL-proteins were separated in six fractions using reversed-phase HPLC (Figure 2E). Each fraction was analysed for the MCP-1 stimulating effect (Figure 2F). The protein fraction number 2, indicated by an arrow in Figure 2E, showed the strongest MCP-1 inducing activity and was further analysed to identify its protein constituent.

3.3 Identification of SAA in HDL from ESRD patients
To identify which component might be responsible for the pro-inflammatory capacity, we further analysed fraction 2 (indicated by an arrow in Figure 2E). The workflow is demonstrated in Figure 3A. The MALDI-TOF/TOF-fragment mass spectrum of the underlying protein after tryptic digestion is demonstrated in Figure 3B. Each signal was attributable to a fragment of SAA, suggesting that SAA was the protein under investigation (amino acid sequence in Figure 3C).

3.4 SAA induces MCP-1 via activation of G-protein-coupled receptors
We examined whether authentic (recombinant) SAA might have direct effects on MCP-1 formation in VSMCs. SAA induced MCP-1 expression and secretion in VSMCs in a concentration-dependent manner (Figure 4A and B). To exclude a potential contamination of investigated proteins by lipopolysaccharide (LPS) as a mediator of MCP-1 induction, we tested whether endotoxin levels in SAA might mimic the observed effect. It is known that SAA activity is destroyed by boiling, whereas LPS retains its activity.\textsuperscript{32} Indeed, SAA significantly lost the ability to induce MCP-1 formation, whereas there was no change in the LPS activity (see Supplementary material online, Figure S1). In the next step, we investigated whether the MCP-1 inducing effect is regulated by specific receptor activation. Recently, it was shown that the formyl-peptide receptor 2 (FPR2) might be involved in the signalling pathway.\textsuperscript{33} The receptor is expressed in rat VSMCs, as detected by mRNA expression (Figure 4D) and protein expression via western blot (Figure 4D). The FPR2 can be activated by SAA in a Gi-dependent manner. To identify which component might be responsible for the pro-inflammatory capacity, we further analysed fraction 2 (indicated by an arrow in Figure 2E). The protein fraction number 2, indicated by an arrow in Figure 2E, showed the strongest MCP-1 inducing activity and was further analysed to identify its protein constituent.
To prove the hypothesis that SAA in the HDL-protein is sufficient to increase MCP-1 formation, the inhibitory effects of PTX and WRW4 in co-stimulation with HDL-protein from healthy control subjects and ESRD patients were tested. HDL-protein from ESRD patients strongly induced MCP-1 expression/secretion in comparison to HDL-protein from healthy control subjects. Both PTX and WRW4 significantly diminished the MCP-1 formation induced by HDL-protein from ESRD patients, but showed no inhibitory capacity in co-stimulation with HDL-protein from healthy control subjects (Figure 4F and G).

3.5 Enrichment of HDL from healthy control subjects with SAA decreases the anti-inflammatory properties

To investigate whether SAA directly decreases the anti-inflammatory properties of HDL, serum from healthy volunteers was incubated with SAA, followed by the isolation of HDL that results in HDL-SAA concentrations similar to those observed in ESRD patients (Figure 5A and 6A and B). In the next step, we assessed the MCP-1 inhibitory capacity of this SAA-enriched HDL. Consistent with our hypothesis, HDL enrichment with SAA resulted in a significantly reduced MCP-1 inhibition effect compared with non-SAA-substituted HDL from healthy subjects (Figure 5B). The induction of MCP-1 expression and secretion by SAA-enriched HDL-protein could be diminished by PTX and WRW4 treatment, respectively (Figure 5C and D).

3.6 SAA level is increased in serum and HDL from ESRD patients and correlates with its pro-inflammatory capacity

To delineate whether the reduced MCP-1 inhibitory effect of HDL from ESRD patients is associated with pro-inflammatory SAA levels observed in HDL, SAA levels in serum and HDL of healthy control subjects and ESRD patients were quantified. Patients with ESRD had significantly higher levels of SAA in both serum and HDL (Figure 6A and 6B).
There is a positive correlation between the concentration of SAA in HDL and its reduced anti-inflammatory activity.

SAA is an amphipathic, α-helical protein that is mainly produced in the liver. It has apolipoprotein properties and is transported in the circulation primarily in association with HDL. The human SAA gene family encodes SAA1–SAA4. SAA1 and SAA2 share approximately 95% sequence homology and are acute-phase proteins. SAA4 is the major form of SAA in normal HDL. SAA3 is a pseudogene. SAA levels (SAA1 and SAA2) rapidly increase up to 1000-fold in the blood of humans with acute inflammation, and are elevated in patients with ESRD and the associated micro-inflammation. For a long time, SAA has been recognized as an acute-phase reaction protein with biomarker function, for instance in infection and allograft rejection. There are several lines of evidence that SAA elevation is associated with an increased cardiovascular risk. Furthermore, elevated SAA levels were predictive for short-term mortality in patients with peripheral artery disease. In recent years, there have been some reports that SAA itself could act as a pro-inflammatory, atherogenic mediator. SAA-enriched HDL has reduced capacity to inhibit the oxidation of low-density lipoprotein. Lee and coworkers demonstrated that SAA can induce production of the chemokine MCP-1 in human monocytes, leading to migration and tissue infiltration of monocytes into atherosclerotic plaques. Recently, it has been shown that SAA-enriched HDL binds biglycan, which reduces the athero-protective function of HDL. In contrast, others have reported that only lipid-free SAA exerts any biological function, whereas HDL binding seems to affect its activity. In the present study, we clearly demonstrated that the MCP-1 protective capacity of HDL is reduced by SAA enrichment during disease conditions, i.e. ESRD. Compared with the increase of up to 1000-fold during the acute response, the SAA level in ESRD patients is modest. This has also been found for obesity. However, SAA alone seems to be much more potent on MCP-1 activation than the same amount of SAA in HDL. Lipid association of SAA might alter its biological activity, so it might be possible that pro-inflammatory signalling by SAA is attenuated by HDL binding. Nevertheless, HDL then loses part of its anti-inflammatory capacity. SAA is a molecule that is also thought to have other effects on HDL composition and function. There is evidence that SAA displaces Apo AI from HDL₃ particles during the acute-phase response.

In the present study, we investigated the Apo AI content in HDL and found no significant differences between HDL from healthy control subjects and patients with ESRD. This might be conflicting, but we did not differentiate between Apo AI content in HDL₂ and HDL₃. In addition, level of SAA can rise higher during the acute-phase response compared with the chronic increase observed for ESRD patients, for example. There are reports that patients with ESRD have significantly elevated SAA levels, potentially as a sign of chronic micro-inflammation. Furthermore, LCAT activity, which is essential for RCT, seems to be affected during disease conditions, and there is evidence for both a positive and a negative correlation with atherogenesis. We found decreased LCAT activity in serum from ESRD patients compared with healthy control subjects. Reduced LCAT in ESRD patients and chronic uraemic status has been known for more than three decades. Recently, Holzer and coworkers demonstrated a reduced RCT activity in HDL from patients with ESRD.

Different studies suggest that SAA can interact with several receptors: scavenger receptor B type I, CD36, Toll-like receptor 2/4, P2X₉, or FPR2. It seems likely that SAA exerts its

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<th>Figure 3 MALDI analysis identifies SAA as the protein constituent within HDL fraction 2. (A) Work scheme for analysis of the protein in HDL from ESRD patients. (B) MALDI-TOF/TOF fragmentation mass spectrum after tryptic digestion of fraction 2, indicated by an arrow in Figure 2E. (C) Analysis of the fragment mass signals, leading to the identification of SAA protein.</th>
<th>A</th>
<th>B</th>
<th>C</th>
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4. Discussion

In the present study, we showed that HDL from patients with ESRD loses part of its anti-inflammatory capacity. This loss of function is partially based on enrichment of pro-inflammatory substances in HDL. Using a proteomic approach, we identified the protein SAA, which has potent G-protein receptor-mediated pro-inflammatory properties. Artificial accumulation of recombinant SAA in HDL from healthy subjects leads to an increase in pro-inflammatory activity of HDL and a significant reduction in anti-inflammatory properties.
effects via activation of different receptors in different cell types. For MCP-1 production, FPR2-mediated signalling has already been shown in human monocytes. Although the FPRs are mainly expressed in mammalian phagocytic leukocytes, they have also been detected in VSMCs and are known to be important in host defense and the regulation of inflammation. We showed that the MCP-1 expression induced by HDL-associated SAA is in part mediated via FPR2, because WRW4 is inhibitory at this receptor. Using MMK-1, which acts as an agonist at the FPR2, we demonstrated that MCP-1 expression can be controlled by FPR2 activation. This is in line with findings by Lee and coworkers, who demonstrated the FPR2-mediated MCP-1 production in monocytes. However, the activation of other receptors could not be excluded. Interestingly, the MCP-1 inducing effect of SAA was only partly inhibited during co-stimulation with WRW4, whereas the effect of HDL-proteins was diminished by WRW4 to a greater extent. This can be attributed to the fact that HDL has multiple components affecting MCP-1 expression. Furthermore, serum SAA and HDL-associated SAA may have different biological functions via activation of diverse receptors.

HDL-associated SAA also stimulates MCP-1 production in VSMCs. We have previously reported that HDL isolated from healthy subjects has anti-atherogenic potential by reducing MCP-1 formation in VSMCs. In that study, we were able to demonstrate that sphingosine-1-phosphate in HDL acts as a potent anti-inflammatory molecule by inhibiting MCP-1 formation. Most of the observed effects were attributed to conformational changes of HDL and changes in particle composition leading to a reduction of protective proteins, such as...
Figure 5 Enrichment of HDL with SAA decreases the anti-inflammatory properties of the particle. (A) Levels of SAA in HDL from healthy control subjects with or without addition of SAA to serum \( (n = 6) \) prior to HDL isolation by ultracentrifugation; for details, see Methods. (B) VSMCs were stimulated with thrombin (2 IU/mL) in the presence of increasing amounts of HDL from healthy control subjects specifically enriched in SAA as detailed in the Methods vs. control HDL. The gene expression of MCP-1 was measured using quantitative real-time PCR (B and C), and secretion of MCP-1 was measured using the Luminex™ technique (D). \( * P < 0.05 \) significant change vs. non-enriched HDL. (C and D) VSMCs were stimulated with protein fractions of HDL from healthy donors with or without enrichment of SAA in the presence or absence of PTX and WRW4. \( * P < 0.05 \) significant change vs. induction by proteins from HDL + SAA; Mann–Whitney U test.

Figure 6 SAA level in serum and HDL, and correlation with anti-inflammatory capacity. Quantification of SAA levels in serum (A) and HDL (B) from healthy control subjects \( (n = 17) \) and patients with ESRD \( (n = 29) \). \( * P < 0.05 \) significant change vs. controls, Mann–Whitney U test. (C) Influence of HDL \( (n = 30) \) from ESRD patients and healthy control subjects on thrombin-induced MCP-1 expression in VSMCs in correlation to SAA levels within HDL. Owing to the complex investigations, only some of the patients from A and B were analysed functionally.
ApoAI. SAA is one of the acute-phase molecules associated with HDL that significantly disturbs HDL composition. HDL that is enriched with SAA is larger, denser, and ApoAI depleted. Recently, we have added a further piece of the puzzle concerning the complex changes of HDL composition during disease conditions, correlated with reduced vascular-protective effects of HDL. SAA seems to be a relevant factor in changes of HDL function. We could demonstrate that: (i) in ESRD patients, HDL shows diminished anti-atherogenic and increased pro-atherogenic actions; (ii) patients with ESRD have significantly higher SAA levels, documenting the micro-inflammation that resembles a kind of ‘chronic’ acute-phase response; (iii) the pro-atherogenic effects of HDL in ESRD are largely mediated by SAA leading to dysfunctional HDL, which is present in HDL from ESRD patients, but at significant low levels in healthy subjects; and (iv) the decreased anti-inflammatory properties of HDL may substantially contribute to the excessive cardiovascular morbidity and mortality in ESRD, but also in every pro-inflammatory vascular disease condition.

Table I Clinical and biochemical characteristics of healthy subjects and patients with end-stage renal disease (ESRD)

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<th>Healthy</th>
<th>ESRD</th>
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<tr>
<td>Age (years)</td>
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<td>19 (9/10)</td>
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<td>Body weight (kg)</td>
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<td>Duration of dialysis (months)</td>
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<td>Systolic blood pressure (mmHg)</td>
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<td>Diastolic blood pressure (mmHg)</td>
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References
