Diploma thesis

Immune dysfunction in chronic kidney disease

submitted by

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Affidavit

I hereby declare that the following diploma thesis has been written only by the undersigned and without any assistance from third parties. I confirm that no sources were used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, 25th of September 2018 *Verena Feuchter eh*

Danksagung

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Abbreviations

AGEs	advanced glycation end products
AGRA	acellular growth retardation assay
AKI	acute kidney injury
AMP	antimicrobial peptide
AOPP	advanced oxidized protein products
BPI	bactericidal permeability increasing factor
CD 14	cluster of differentiation 14
ChE	cholinesterase
CKD	chronic kidney disease
CRP	C-reactive protein
CVD	cardiovascular disease
DMII	diabetes mellitus type 2
DMSO	dimethyl sulfoxide
E. coli	*Escherichia coli*
e.g.	for example
eGFR	estimated glomerular filtration rate
ELISA	enzyme linked immune adsorbent assay
ESRD	end stage renal disease
EU/ml	units per millilitre
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
GALT	gut associated lymphatic tissue
gMFI	geometric mean flourescence intensity
FAE	follicle associated epithelium
h	hour
HC	healthy controls
HCLO	hypo chloric acid
HD	haemodialysis
HD(F)	haemodialysis and hemodiafiltration
HDF	hemodiafiltration
HL-60	human leukaemia cells

HO	hydroxyl radical
IgA	immunoglobulin A
IGF	insulin like growth factor
IL	interleukin
KT	kidney transplanted
LB broth	Luria-Bertani broth
LBP	lipopolysaccharide binding protein
LDL	low density lipoprotein
LMW	low molecular weight protein
Mesh	medical Subject Headings
MPO	myeloperoxidase
NADPH	nicotine adenine dinucleotide phosphate
NF-κB	nuclear factor kappa b
NO	nitric oxide
O_2^-	superoxide anion
PBS	phosphate buffered saline
PAMPS	pathogen associated molecular patterns
PD	peritoneal dialysis
PEW	protein energy wasting
PI	Phagoindex
PMN	polymorphonuclear leukocytes
TLR4	toll like receptor 4
r-HuEPO	recombinant human erythropoietin
ROS	reactive oxidative species
RRT	renal replacement therapies

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Zusammenfassung

Einleitung:

Durch den Verlust der renalen Volumenregulation kommt es in niereninsuffizienten Patientinnen und Patienten zu hämodynamischen Instabilitäten. Eine daraus resultierende Hypo- und Hyperperfusion der Darmbarriere führt zu einer Beeinträchtigung dieser und dem konsekutiven Übertritt von Endotoxin (LPS) ins But. Der Übertritt von im Blut vorhandenen urämischen Toxinen in das Darmlumen, sowie das Einhalten einer strikten Diät verändern das Darmmikrobiom, sodass es nicht mehr zu einer physiologischen Darmbarriere beitragen kann. LPS aktiviert neutrophile Granulozyten, welche konsekutiv vermehrt reaktive Sauerstoffmoleküle (ROS) und Zytokine produzieren. Eine Überaktivierung von Neutrophilen kann zu deren Dysfunktion mit resultierender reduzierter Immunabwehr führen. Zudem oxidiert LPS Albumin. Ein Verlust von Albumin aufgrund des Protein-Energie-Verlust-Syndroms (PEW) in niereninsuffizienten Patientinnen und Patienten sowie der Oxidierung des Restalbumins reduziert die Bindung von ROS und LPS an Albumin. ROS, Zytokine und LPS verursachen Endothelschäden und tragen damit zur Entstehung von Atherosklerose bei. Zusammenfassend sind die Konsequenzen von übertretendem LPS erhöhte Inflammation, verminderte Immunabwehr, sowie ein erhöhtes Risiko für kardiovaskuläre Ereignisse.

Materialen und Methoden:

Veränderte pathophysiologische Prozesse in niereninsuffizienten Patientinnen und Patienten wurden mittels retrospektiver Auswertung von im Zuge der ENARI Studie gesammelten Daten nachverfolgt. Diese Studie wurde von 2010 bis 2013 durchgeführt 238 Patientinnen und Patienten mit Nierenerkrankungen wurden eingeschlossen. Mittels Phagotest® wurde erhoben, in wieweit LPS die Phagozytose von in vitro differenzierten Neutrophil ähnlichen Leukämiezellen (HL-60 Zellen) beeinflusst. Zudem testeten wir in dieser Kohorte einen neu entwickelten Biomarker zur Risikobestimmung für Infektionen in Patientinnen und Patienten mit Leberzirrhose, wobei in vitro die Kapazität des Serums, das Bakterienwachstum zu hemmen, bestimmt wurde.

Ergebnisse:

Wir zeigten signifikant erhöhte Zytokine und LPS bindende Proteine, als Surrogatmarker für erhöhtes LPS, in allen Patientinnen und Patienten. Alle Patientinnen und Patienten wiesen ein erhöhtes Neutrophilen:Lymphozyten Verhältnis, als Biomarker für kardiovaskuläre Ereignisse, auf. Alle Patientinnen und Patienten zeigten erniedrigtes Gesamteiweis und erniedrigte Cholinesterase Werte, welche auf PEW hin deuten. In vitro differenzierte Neutrophil ähnliche HL-60 Zellen zeigten nach Stimulierung mit 500 ng LPS vermehrte Phagozytose. Im Gegensatz dazu zeigten isolierte Neutrophile von Patientinnen und Patienten mit der höchsten LPS Konzentration die niedrigste Phagozytose. Serum von chronisch niereninsuffizienten Patientinnen und Patienten zeigte eine signifikant effizientere Hemmung des Bakterienwachstums.

Conclusio:

Zusammenfassend lässt sich sagen, dass alle untersuchten Patientinnen und Patienten ein erhöhtes Risiko für kardiovaskuläre Ereignisse haben. Den zugrundeliegenden Pathomechanismus konnten wir in unserer Studie anhand von erhöhten Surrogatmarkern für LPS, erhöhten Zytokinen, sowie Biomarker für kardiovaskuläre Ereignisse bestätigen. Im Gegensatz zu unserer Annahme, wiesen alle in vitro untersuchten Seren eine effizientere Hemmung des Bakterienwachstums im Vergleich zu gesunden Kontrollen auf. Weitere Studien zur Anwendung des Biomarkers in Patientinnen und Patienten mit Nierenerkrankungen sind daher notwendig. Unsere Ergebnisse zeigten einen stimulierenden Effekt von LPS auf die Phagozytose von in vitro differenzierten Neutrophil ähnlichen HL-60 Zellen. Weitere Studien zum Einfluss von LPS auf Neutrophile von Patientinnen und Patienten mit Nierenerkrankungen sind jedoch notwendig.

Abstract

Background:

Patients with renal disease experience volume overload and dialysis induced haemodynamic changes leading to alternating splanchnic hyper- and hypoperfusion. Dietary restrictions and the translocation of retained uremic toxins to the gut change the intestinal microbiota in a way which cannot contribute to intestinal barrier integrity anymore. The consequences are intestinal barrier disruption and endotoxin (LPS) translocation to the blood. Systemic LPS stimulates neutrophils to release reactive oxidative species (ROS) and cytokines and thereby induces chronic inflammation and oxidative stress. Cytokines, ROS and LPS itself interact with the endothelial membrane and induce early steps of cardiovascular disease (CVD). An overstimulation of neutrophils might be linked to cellular dysfunction and subsequent reduced pathogen defence. Moreover, patients with renal disease suffer from PEW, which describes an imbalance between protein intake and protein loss. Low albumin levels, because of PEW and oxidation of residual albumin via LPS results in reduced albumin mediated scavenging of ROS. The combination between repeated LPS translocation, oxidative stress, neutrophil activation and dysfunction leads to chronic inflammation, high risk for infections and CVD in patients with renal disease.

Aims:

The aims of this thesis were to test 1) whether patients with renal disease show signs for inflammation, PEW and have elevated biomarkers for CVD, 2) whether the serum induced bacterial growth retardation capacity differs between patients with renal disease and healthy controls, 3) whether LPS influences the phagocytosis of in vitro differentiated neutrophil like leukaemia cells (HL-60 cells).

Materials and methods:

Laboratory parameters were collected during the “endotoxin, neutrophil function and albumin in renal insufficiency” (ENARI) study and retrospectively analysed during this thesis. To assess alterations in the immune system, levels of LPS, LPS binding proteins, immune cells, albumin, cytokines and C-reactive protein were analysed. To determine the risk for CVD, the biomarkers neutrophil:lymphocyte and C-reactive protein:albumin ratio were assessed. To determine the

extent of PEW, total plasma protein and cholinesterase levels were analysed. We used a newly developed biomarker, which predicted infections in patients with liver cirrhosis, in this study cohort. Therefore, we determined the capacity of patient sera to inhibit growth of *Escherichia coli*. Furthermore, we assessed the effect of 250 and 500 ng/ml LPS on the phagocytosis of in vitro differentiated neutrophil like HL-60 cells by Phagotest®.

Results:

All patients had significantly higher blood LPS levels and an elevated inflammatory state, confirmed by elevated cytokine and LPS-binding protein levels. All patients had a significantly higher risk for CVD, as all patients showed higher neutrophil:lymphocyte. All patients had a higher risk for PEW, confirmed by reduced cholinesterase levels, albumin and total plasma protein. All patients had a higher serum mediated bacterial growth retardation compared to healthy controls. The phagocytic capacity and the percentage in phagocytosing neutrophil-like differentiated HL-60 cells was higher at higher levels of LPS, suggesting that LPS at this concentration stimulates phagocytosis.

Conclusion:

We conclude that patients with renal disease have an increased immune activity, systemic LPS concentration and an increased risk for CVD and PEW. We conclude that further studies about the applicability of the newly developed biomarker, which predicted infections in patients with liver cirrhosis, must be done in patients with renal disease. We conclude that LPS at a concentration of 500 ng/ml has a stimulating effect on the phagocytosis of in vitro differentiated neutrophil like HL-60 cells. Nevertheless, further studies about the effect of LPS on neutrophils in patients with renal disease are required.

1. Introduction

Over the last few decades, the identification of underlying causes for the high susceptibility of end-stage renal disease (ESRD) patients to infections and cardiovascular diseases (CVD) has become an important field in scientific research and is still not completed. Cardiac mortality in chronic kidney disease (CKD) and especially in ESRD patients is at least fivefold higher than in the general population and increases up to 100-fold in patients aged 45 or younger. (1) Most patients at CKD 3-4 die from CVD rather than progress to ESRD. (2) The Framingham risk score is used to estimate an individual's chance of developing a cerebrovascular disease within 10 years. Hypertension, hyperlipidaemia, smoking and diabetes mellitus type 2 (DM II) were shown to increase cardiac mortality and worsen clinical outcome.(3) Nevertheless, the chance of CVD in patients with renal disease remained after stratification for age, gender and for Framingham risk factors, still 10-20 times higher than in the general population. 50% of all deaths in ESRD patients are because of CVD. 20% of all deaths in ESRD are because of infections, whereby the annual mortality rate has increased up to 100-fold for sepsis in the dialysis population compared to the general population.(4) Kidney transplanted (KT) patients have a lower risk of CVD compared to patients treated with renal replacement therapies (RRT), yet is still higher compared to the general population. CVD is the most common cause of death in KT patients with a functioning graft.(5) The causative factors for both, CVD and infection, arise from different pathophysiological processes and include dialysis procedure, volume overload, oxidative stress and intestinal barrier disruption with subsequent translocation of intestinal bacteria.(6) Renal failure and insufficient elimination via RRT lead to an accumulation of uremic toxins.(7,8) Retained uremic toxins act as reactive oxidative species (ROS) to induce early steps of atherosclerosis and modify immune cell function.(9) Surplus uremic toxins are secreted to the intestinal lumen as an alternative elimination pathway. Subsequent alterations in the microbiota composition as well as dialysis induced volume changes disrupt the intestinal barrier integrity.(10) Translocated endotoxin (LPS), originated from Gram-negative intestinal bacteria, triggers immune cell alteration, inflammation and oxidative stress.(11) Patients with chronic inflammation and repeated exposure to RRT suffer from a hyper metabolic state and an increased demand of proteins. Protein loss occurs via an insufficient renal barrier or dialysis membrane and via an impaired intestinal barrier. This is exacerbated by a disturbed muscle

protein synthesis due to impaired molecular mechanisms and by insufficient nutritional intake. The imbalance between protein demand and protein supply ends in protein energy wasting (PEW).(12) The following chapters will help to elucidate the vicious cycle comprising RRT induced haemodynamic stress and dysbiosis with consequent intestinal barrier disruption, LPS translocation to the systemic circulation, alterations in the immune system, oxidative stress and PEW. Together, they contribute to increased inflammation and CVD in CKD, ESRD and KT patients. Figure 1 visualises the connections.

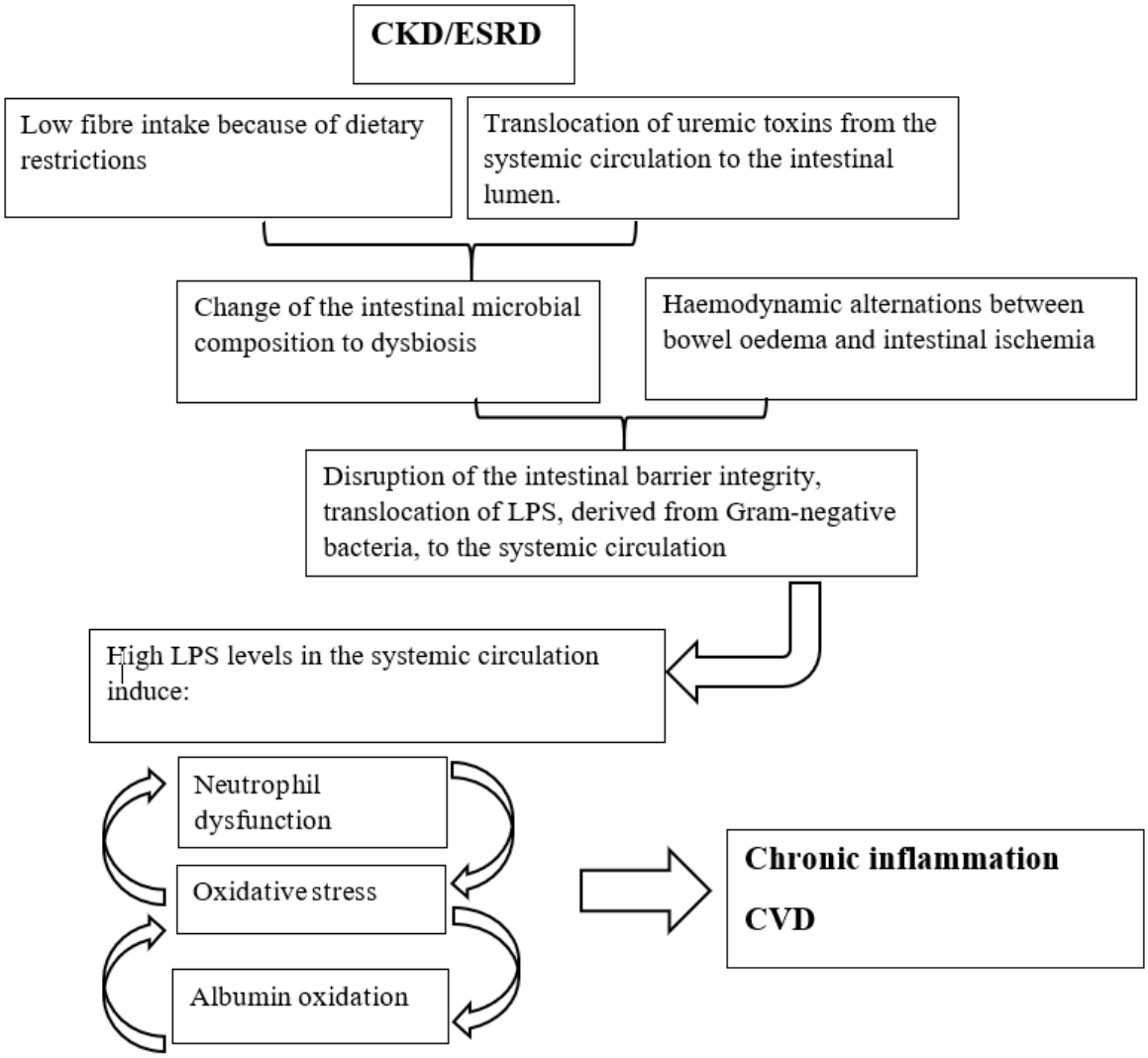

Figure 1 Chronic inflammation and CVD in ESRD: The combination of haemodynamic alternations, low fibre intake and translocation of uremic toxins from the systemic circulation to the intestinal lumen lead to changes in the microbial composition. The resulting dysbiosis cannot contribute to an intestinal barrier integrity anymore. Consequently, LPS, derived from intestinal Gram-negative bacteria translocate to the systemic circulation. High blood LPS levels cause dysfunction, oxidative stress and albumin oxidation, which accelerate each other and end in chronic inflammation and CVD. Alb, albumin; CKD, chronic kidney disease; CVD, cardiovascular disease; ESRD, end stage renal disease; LPS, lipopolysaccharide; adapted from (13,14)

1.2. Chronic kidney disease and end stage renal disease

CKD is defined as the irreversible loss of excretory and incretory kidney function, which is connected to an increase in uremic toxins, which are usually eliminated by the urine. It is characterized by an estimated glomerular filtration rate (eGFR) below 60 ml per minute for more than 3 months. The prevalence of CKD in the general population is about 10% to 20% and has continually grown over the last decades, especially in individuals of age 65 and above.(15) The main reasons for CKD worldwide are DM II, hypertension and glomerulonephritis.(16) Due to a gradual reduction of nephrons, CKD tends to accelerate continually toward a chronic state and finally to ESRD when serum creatinine score increases 1.5 mg/dl.(1) The main reason for premature mortality in patients with renal disease is a chronic inflammatory state, which leads to increased rates in infection and CVD. Figure 2 compares the annual mortality rate between ESRD patients and the general population.

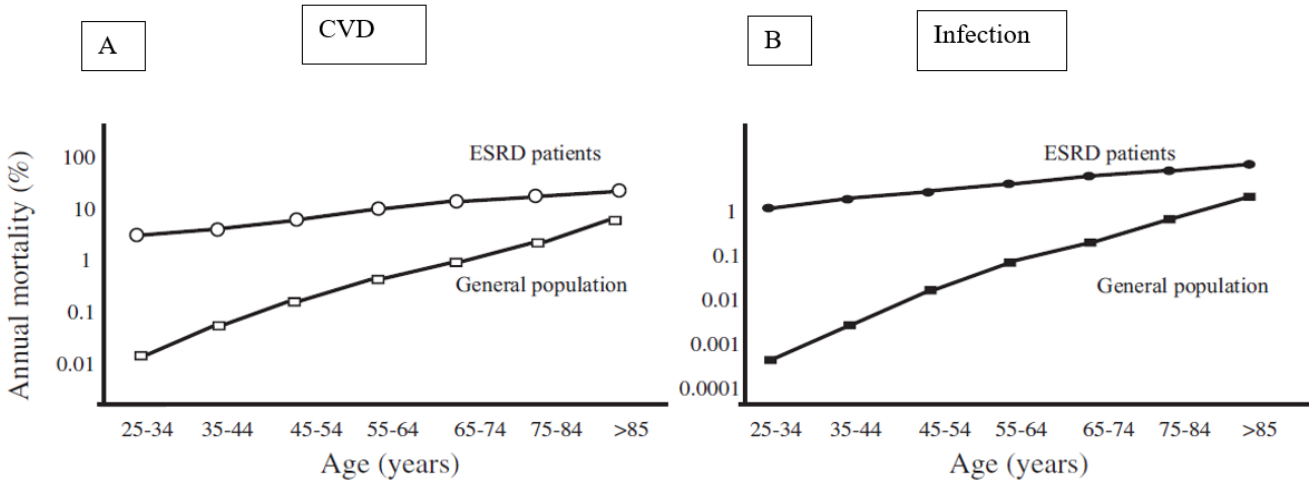

Figure 2 Annual mortality rate in ESRD versus general population:

The figure compares the percentage annual mortality because of CVD (A) and infection (B) between ESRD patients treated by RRT and the general population. CVD, cardiovascular disease, ESRD, end stage renal disease, adapted from (17)

1.3. Renal replacement therapies

RRT are used to replace glomerular filtration as adequately as possible to enable survival of patients without or with insufficient renal excretion. Glomeruli clear solutes approximately up to the molecular weight of albumin (67 kDa).(18) Uremic toxins are smaller than albumin and represent a target for elimination, as they negatively interact with biologic functions. Uremic toxins are classified into low molecular weight (LMW) solutes (e.g. urea, creatinine), middle sized solutes (e.g. β_2 microglobulin) and protein bound solutes (e.g. p-cresol).(19) The capability to provide uremic toxin elimination however retain albumin represents one of the main challenges of dialysis membranes.(20) Indications for RRT should be taken into consideration in patients with ESRD and eGFR <15 ml/min/1.73 m^2 with additional signs of renal failure, such as acidosis or alkalosis, hyperkalaemia, uremic intoxication, or volume overload. In asymptomatic patients, RRT must be started from the point when eGFR falls below 6 ml/min/1.73 m^2.(18,21)

Current worldwide available RRT comprise high flux haemodialysis (HD), chronic ambulatory peritoneal dialysis (PD) and KT. Online hemodiafiltration (HDF) is still very sparsely used, mainly in Europe and Japan.(22) The systematic assessment of the global capacity for kidney care still showed a significant international difference, as most countries in Africa described no facilities for KT or PD.(23) HD is the most widely used therapy and is based on the diffusive transport of solutes across a semipermeable membrane via hydrostatic pressure. The filtered osmosis water is filtered back to the blood side. To balance the volume, a substitute, mainly an electrolytic solution, is reinfused. HD is effective in removing LMW uremic toxins but is poor in removing middle sized uremic toxins.(22) To overcome this deficiency, diffusion-based HD has been extended by convection to HDF. The combination of diffusion and convection provides a clearance of both, middle and LMW uremic toxins. However, the albumin leakage remains high.(19) To maintain fluid balance, a sterile fluid, which is obtained by online filtration of standard dialysate through a series of LPS retaining filters, is directly infused in the patient's blood.(24) In PD, filtration of uremic solutes occurs through the complexly built abdominal layer. Therefore, the dialysate needs to be infused in the abdominal cavity. Exchange of uremic solutes and dialysate occurs between blood capillaries and abdominal cavity. The

entirety of all anatomical layers lain in between and biological processes at the boundary layer determine the dialysis capacity.(25)

1.4 The innate immune system

The innate immune system comprises humoral and cellular mechanisms, as well as the intestinal epithelial barrier and the microbiota to achieve non-specific defence against pathogens.

The human serum mediates humoral innate immune defence. It consists of 90% of water, 7% of Albumin as well as transferrin, lactoferrin, lysozyme, antimicrobial peptides (AMP), pattern recognition receptors and complement factors.(26,27) Albumin binds a wide range of endogenous and exogenous ligands to transport water insoluble molecules, or to dispose of them when they are toxic. It contains 80% thiols, which are potent scavengers for ROS.(28) Serum albumin represents an important part of the humoral innate immune system, as serum concentrations of less than 3.5 g/dl show a risk factor for septicaemia, whereby the normal amount is 3.5 to 5 g/dl. (28) Pattern recognition receptors are dissolved in serum or bound on surfaces of immune cells to detect pathogen associated molecular patterns (PAMPS) on pathogen surfaces. By binding to pathogens surface, they enhance their phagocytosis and induce the complement cascade, whose proteins are suspended in serum and directly attack pathogens and induce immune cell recruitment, but also contribute to inflammation.(29)

Furthermore, cytokines play an important part in the humoral innate immune defence. They are subdivided into pro-inflammatory, anti-inflammatory and regulatory cytokines and mediate innate and adaptive immune response mechanisms, though also induce inflammatory processes and contribute to early steps of atherosclerosis. Anti-inflammatory cytokines downregulate immune activation.(30) Physiologically, cytokines act in a cytokine network and proinflammatory cytokines are counterbalanced by anti-inflammatory cytokines. The proinflammatory cytokines comprise among others IL-6 and tumour necrosis factor alpha (TNF-α). They induce the production of acute phase proteins like CRP, but also inhibit the synthesis of negative acute phase proteins like albumin and transferrin.(31,32) IL-10 reacts as anti-inflammatory cytokine and is secreted delayed to supress proinflammatory cytokines. Patients with higher IL-10 levels have better immune balance. IL-10 downregulates the

atherosclerotic process by interfering with adhesion molecules and inhibiting chemokines which attract leukocytes to the endothelial inflammation.(33)

Cellular innate immune defence is mediated by polymorphonuclear leukocytes (PMNs), monocytes and dendritic cells.(18) Neutrophils represent 40-80% of PMNs and are the most abundant which migrate as first responders to the site of inflammation. They attack pathogens via phagocytosis and ROS release, as well as orchestrate further immune response. Neutrophils express on their surface the pattern recognition receptor toll-like receptor 4 (TLR4), which recognizes LPS on Gram-negative bacteria. The TLR4-LPS interaction activates the intracellular NF-κB pathway, induces phagocytosis and activates the complement cascade.(17) Mediators of the complement cascade induce signs of inflammation and activate further immune cells.(27,34) Neutrophils provide intracellular killing of phagocytosed pathogens by nicotinamide adenine dinucleotide phosphate (NADPH) and myeloperoxidase (MPO). NADPH produces superoxide anion (O_2^-), which converts oxygen to superoxide radicals.(35-37) MPO catalyses the reaction of chloride ion with hydrogen peroxide to generate hypochlorous acid (HClO).(38) Phagocytosis activates a respiratory burst, during which O_2^- and HClO are secreted and react as ROS against pathogens, therefore also contribute to oxidative stress.(35,39) By the release of cytokines, neutrophils attract immune cells to orchestrate further immune defence.(30)

1.5. Histology and physiology of the intestinal epithelial barrier

The gastrointestinal mucosa is highly specialized and differs between different gastrointestinal parts to deal with the local requirements. It is assembled by the epithelial layer, the lamina propria and the muscularis mucosae. The epithelial layer is a single layer of columnar epithelial cells that separates the intestinal lumen from the underlying lamina propria. Intestinal epithelial cells get directly in touch with the chymus and are responsible for secretion, digestion, absorption and immune defence. As the high permeability for nutrients facilitates intestinal bacteria to cross, a specialized intestinal epithelial barrier and intestinal immune system prevent from translocation and infection. The intestinal barrier consists of the intestinal epithelial cells, Paneth cells, goblet cells, the mucosa associated lymphatic tissue and a mucus layer, whose

production is supported by symbiotic gut microbes. Intestinal epithelial cells are bound together to a diffusion barrier by tight junctions. The tight junction complex consists of the adhesion proteins claudin and occludin, the zonula occludens and the prejunctional ring of actin and myosin.(34) Intestinal epithelial cells furthermore secrete cytokines, chemokines and AMP.(40) Paneth cells contribute to antimicrobial defence by secreting AMP.(41) Goblet cells are dispersed in between epithelial cells. They secrete immune protective mucins and lead to a mucous coverage of almost the whole intestinal epithelium.(34) The gut associated lymphatic tissue (GALT) regulates mucosal immunity and summarizes concentrations of lymphfollicles in the submucosa, which are made up by B cell follicles, interfollicular T-cell regions as well as macrophages and dendritic cells. Lymph follicles in the small intestine and next to the appendix are called payer's patch. The intestinal epithelium next to the payer's patch lacks in goblet cells, but still expresses immune active microfold cells. It is called follicle associated epithelium, a single layer of specialized columnar cells interspersed with monocytes, neutrophils, dendritic cells and lymphocytes.(34) Microfold cells envelop antigens to present them to B and T cells and hand them over to dendritic cells.(42,43)

1.5.1 Gut microbiota

A physiological microbial gut composition, called gut homeostasis, maintains as a part of the intestinal immune system protection against infections.(44) The term microbiota summarizes over 100 trillion commensal bacteria from 200 to 500 different species within Bacteroidetes and Firmicutes.(45-47) Humans evolutionary have developed symbiosis with unicellular bacteria, which are harboured in the intestinal lumen to overcome metabolic and genetic deficiencies. These commensals permit functions that are not encoded in the human genome, including nutrient absorption and metabolism, energy provision for colonocytes and contribution to intestinal barrier integrity and immune defence.(48,49) In case of absence of postnatal colonization, immune system cannot be fully educated.(50) The microbial composition adapts to its surrounding environment, which is influenced by hosts nutrition and health.(51) Complex polysaccharides in human diet are recognized as important in terms of enrichment and selection of Firmicutes and Bacteroidetes. A fibre rich diet was shown to be associated with a lower risk

for mortality and inflammation in patients with renal disease.(16,52) Bacteroidetes belong to proteolytic bacteria and comprise among others *Escherichia coli (E. coli)*. Their abundance minimalizes toxin concentration and suppresses intestinal inflammation.(53) Starches and plant derived polysaccharides are sequentially degraded by Bacteroidetes. Resulting products comprise short chain fatty acids and butyrate. Short chain fatty acids serve as energy source for colonocytes.(25) Butyrate has anti-inflammatory properties as it induces mucin, AMP and immunoglobulin A (IgA) production as well as increases the number in intestinal cell connecting tight junctions.(7) IgA affects commensal gene expression and prevents from adhesion of gut microbes to the epithelial surface.(7) Interactions at the intestinal epithelial barrier are displayed in Figure 3.

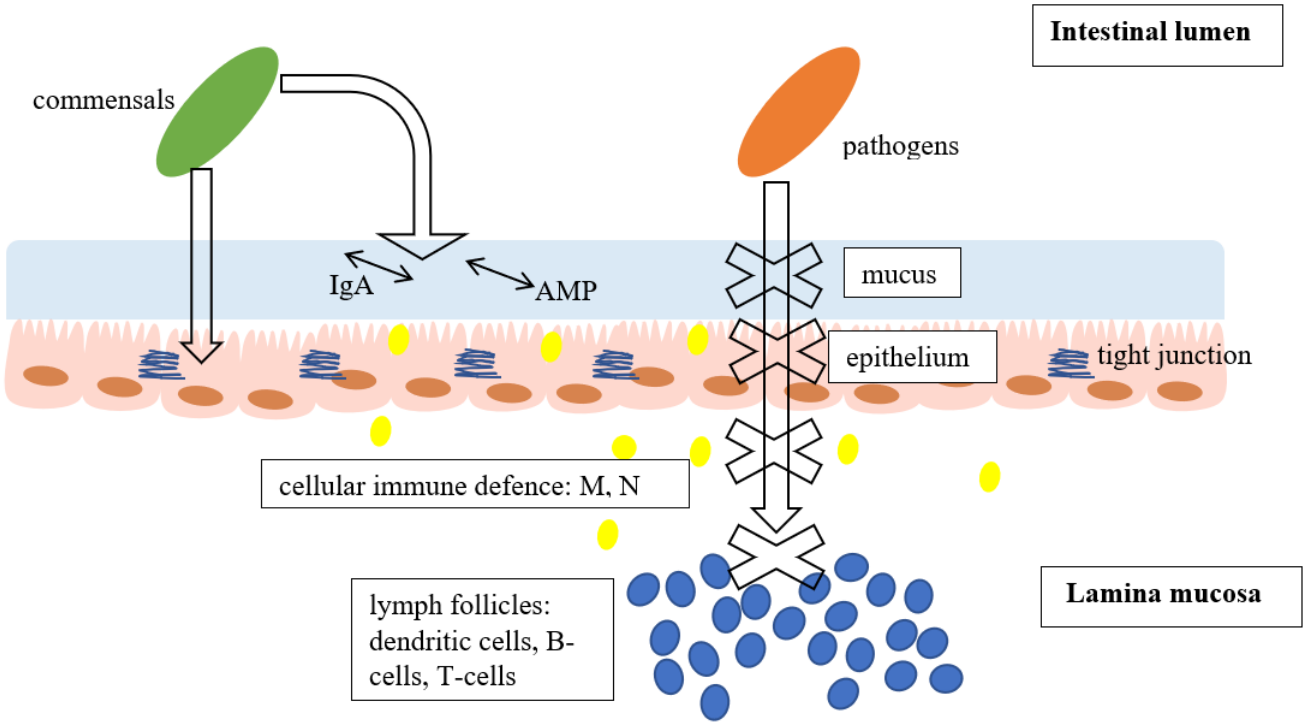

Figure 3 Intestinal immune system: Contributors to a functioning intestinal epithelial barrier are intestinal epithelial cells, mucus, commensals and the lymph follicles. The mucus and in mucus dissolved AMPs ward pathogens. Mucus is produced by goblet cells and commensals. Intestinal epithelial cells are connected by the tight junction complex. neutrophils and monocytes are dispersed in the lamina mucosa and between intestinal epithelial cells. Lymph follicles comprise dendritic cells, B-cells and T-cells and induces adaptive immune processes; AMP, antimicrobial peptides, adapted from (54)

1.6. Translocation of endotoxin

Wang and Vaziri et al. were the first who analysed alterations in the microbiota of patients with kidney disease. They showed that the gut homeostasis in patients with renal disease changes in a way that cannot any longer contribute to the integrity of the intestinal barrier.(55,56) The change of the gut homeostasis is called dysbiosis. Main contributors to dysbiosis in patients with renal disease are dietary restrictions, uraemia and a frequent use of phosphate binders and antibiotics.(44,50) In patients with renal disease, dietary restrictions contradict high potassium intake to avoid hyperkalaemia. Therefore, the intake of fruits and vegetables, which would be important sources for fibres, must be restricted, since they are high in potassium.(55)

Urea allows the kidney to produce hyperosmotic urine. Physiologically, four-fifth of urea produced is excreted by the urine.(57) When the kidney fails, urea accumulates in the blood and is secreted into the gastrointestinal lumen.(58) A diet rich in starches contributes to an immune protective microbial gut composition, whereby an intestinal environment rich in urea and poor in starches is linked to an overgrowth of urease and uricase possessing bacteria, proofed by Vaziri et al., who showed that patients with renal disease had a lack in Bacteroidetes, whereas urease and uricase possessing bacteria were abundant.(55) Metabolization of urease leads to ammoniac and ammonium hydroxide production, which destroys colonic epithelial tight junctions.(59) A breakdown of the immune protective gut homeostasis leads to intestinal inflammation and intestinal barrier disruption. Another important contributor to intestinal barrier disruption is fluid overload in CKD patients and dialysis induced haemodynamic changes in HD, HDF and PD patients. Dialysis procedures induce an alternation between intradialytic splanchnic hypoperfusion and interdialytic splanchnic hyperperfusion. Hyper- and hypoperfusion, as well as the rapid change lead to a disturbance of the barrier integrity.(60,61) Consequently, intestinal Gram-negative bacteria translocate to the systemic circulation. LPS form the outer membranes of Gram-negative bacteria. In case of bacteria breakdown, LPS is released and reacts as free LPS in the systemic circulation, where it is responsible for immune cell alteration and oxidative stress. Figure 4 visualizes the underlying pathophysiological mechanisms.

The hypothesis of intestinal LPS origin could be provided by several observations: 1) CKD patients showed increases in blood LPS levels with the progression of CKD and with an increasing amount of fluid overload. HD and PD patients showed increases in LPS levels in periods between RRT. (31,50) 2) Gut derived microbial DNA was found in plasma from CKD and HD patients, proofing intestinal origin of bacteria.(62) 3) Patients had high blood LPS levels without infection by extraintestinal Gram-negative bacteria, which suggests the colonic origin of LPS.(31) 4) Vaziri et al. analysed the colonic tissue of 5/6 nephrectomised rats to determine the abundance of tight junction proteins. CKD rats showed significantly decreased amounts of zonula occludens proteins, claudin and occludin.(59) 5) Diamino-oxidase (DAO) was used as a surrogate marker to investigate translocation of intestinal bacteria in the ENARI study. (see 4.2 ENARI study) DAO is an intracellular molecule of intestinal cells and serum DAO levels increased when barrier function was impaired. Levels of DAO were elevated in Patients with renal disease.(14) 6) Aronov et al. confirmed the colonic origin of the uremic toxins indoxyl sulphate and p-cresol sulphate, which originate from intestinal protein fermentation.(10,13)

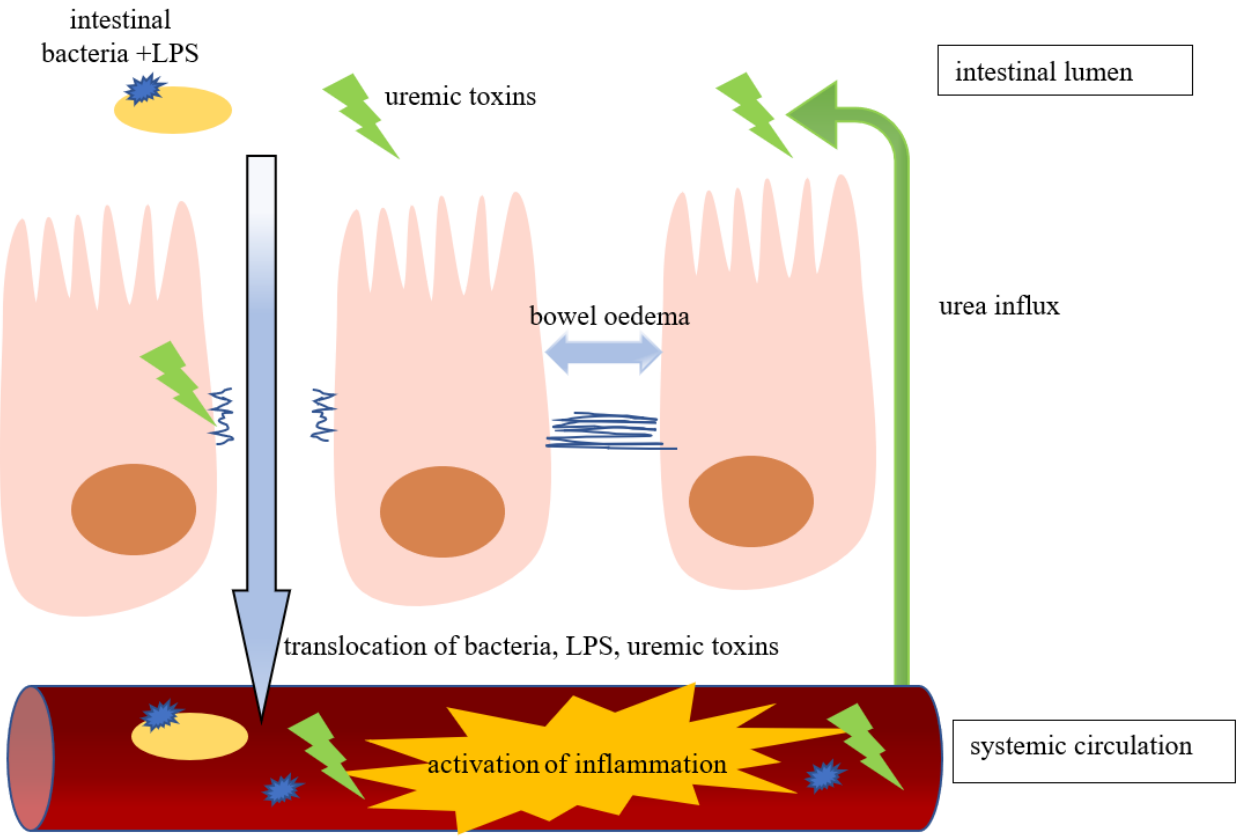

Figure 4 Translocation of LPS: The expansion of urease possessing bacteria changes the gut homeostasis in a way that cannot contribute to intestinal barrier integrity anymore. CKD induced volume overload and RRT induced haemodynamic alternations between bowel oedema and ischemia accelerate intestinal barrier breakdown. The consequence is the translocation of bacteria and bacteria-derived LPS to the systemic circulation. CKD, chronic kidney disease; LPS, lipopolysaccharide; RRT, renal replacement therapy, adapted from (63)

1.7. Chronic inflammation in patients with renal disease

ESRD and CKD patients suffer from persistent immune activation combined with an impaired immune response. Main elicitor is translocated LPS, which on the one hand induces immune cell activation and on the other hand immune cell dysfunction. Impairment of the normal immune cell response is linked to high infection rates and a chronic inflammatory state. Inflammation is accelerated by retained uremic toxins and repeated exposure to RRT. Consequences are atherosclerotic processes with subsequent CVD and cardiovascular mortality.(33)

Anders et al. demonstrated that LPS needs for the reaction with immune cells the serum proteins lipopolysaccharide binding protein (LBP) and cluster of differentiation 14 (CD14).(50) LBP and CD14 play important roles in regulating the LPS induced septic pathway. CD14 is present either in a soluble form (sCD14) or bound on the membrane of macrophages, monocytes or neutrophils (mCD14). s/mCD14 reacts as a co-receptor to achieve contact between LPS and TLR4. LBP belongs to acute phase proteins and transfers LPS to s/mCD14. s/mCD14 can bind LPS only in the presence of LBP.(61,64,65) Blocking either s/mCD14 or LBP would prevent LPS from inducing septic processes. Once bound to the cell surface, LPS activates intracellular mechanisms via NF-κB pathways. Genes responsible for cell growth, metabolism and cytoskeletal rearrangement are modified. Results are, among others, increased expression of TLR4 on cellular surface, increased synthesis of cytokines and ROS, as well as an increase in the phagocytic activity.(66)

Proinflammatory cytokines cause local vascular inflammation and thereby induce early steps of atherosclerosis. By interacting with IGF-1, they contribute to the PEW syndrome. As cytokines are cleared by the kidney, kidney failure contributes by insufficient cytokine elimination to high cytokine levels.(31,32) However, persistent TLR4 activation via LPS induces a refractory state with subsequent tolerance to LPS. Reduced response upon TLR stimulation leads to inappropriate response to LPS, but also to other pathogens and opens the possibility to severe infections.(50) This could be confirmed by Ando et al., who showed reduced TLR4 expression and cytokine release from neutrophils in CKD patients upon stimulation by LPS. ESRD patients had reduced TLR4 expression on monocytes compared to healthy controls, whereby patients with previous bacterial infections had lower TLR4

expression than patients without infection. In addition, the intensity of TLR4 expression correlated to intracellular cytokine production. Cytokine production was significantly reduced in neutrophils and monocytes exposed to LPS compared to cells not exposed to LPS.(67)
The liver represents the major site for LPS clearance. The reasons for high blood LPS levels in ESRD patients, where the liver should have a normal function, remains unclear. One possible explanation could be the amount of LPS that exceeds the physiologic scavenging capacity of the liver.(68)

RRT contribute to persistent high blood LPS levels because of bacterial biofilm formation in tunnelled dialysis catheters. Patients who were dialyzed via tunnelled dialysis catheters showed higher infection rates. Biofilm formation may also occur in fluid pathways of the dialysis machines. Bacterial products that are present in dialysis fluid translocate to the systemic circulation.(60) Rigorous guidelines for water purity have therefore been implemented. Ultrapure dialysis fluid must have <0.03 endotoxin units (EU)/ml LPS according to the European best practice guidelines. This resulted in decreases of blood LPS levels in dialysis patients who were switched to ultrapure dialysate.(25) Nevertheless, in high flux dialysis, LPS may cross the dialysis membrane by convective transfer (back-filtration) or down a concentration gradient. Although full synthetic, non-complement activating dialyzer membranes are used in RRT, immune cells interact with the dialysis membrane, become activated and produce increased amounts of CD14 on their surface, or commit apoptosis.(69) Neutrophils from HD patients showed a decrease in chemotactic response and decreased chemotactic receptors. PD patients additionally suffer from PD induced alterations in the abdominal cavity, such as reduced neutrophils and complement factors.(38)

Another important contributor to the inflammatory state is formed by uremic toxins. Uremic toxins accumulate because of insufficient elimination via the kidney or via RRT and because of increased translocation from the intestine via an impaired intestinal barrier.(25) Neutrophils exposed to a uremic milieu showed a disturbed phagocytosis because of reduced NADPH oxidase. (70,71) Some uremic solutes delay neutrophil's apoptosis and lead to necrosis of neutrophils and release of proinflammatory molecules.(72,73) Lewis et al. showed that MPO activity in neutrophils decreased with advanced stage of CKD.(38) Uraemia also impairs the adaptive immune system, showed by an insufficient priming of antigen specific B/T-cell response upon vaccination.(50)

1.8. Oxidative stress/ cardiovascular disease

Oxidative stress takes place when ROS production exceeds the antioxidant capacity and is a common phenomenon in ESRD patients. Oxidative stress in ESRD patients is provoked by high blood LPS levels, ROS release from activated neutrophils, DM II and by insufficient antioxidative compensation, because of albumin oxidation and insufficient nutritional antioxidant intake due to dietary restrictions.(74,75) Figure 5 visualizes the connections. In inflammatory and oxidative milieu, monocytes and neutrophils express increased levels of TLR4 on their surface through which they are activated via LPS, before overstimulation leads to cellular resistance and immunodeficiency. Increased activation leads via NF-κB pathway to an increased release of ROS.(14,33) This could be confirmed by a study showing that neutrophils exposed to LPS and to the prooxidant paraquat showed increased ROS production and cytokine release.(76) Neutrophil's and monocyte's activation increases intracellular NADPH production, which delivers O_2^- to the extracellular space. neutrophils activation increases intracellular MPO production, which delivers HClO.(64) Both, O_2^- and HClO, react as ROS. O_2^- reacts with nitric oxide (NO) to peroxynitrite. A lack in arterioles dilatating NO contributes to splanchnic hypoperfusion and to an increased vascular permeability.(35) DM II contributes to oxidative stress by forming advanced glycation end-products (AGE) through glycation of proteins. As the kidney is the major site of AGE elimination, impaired kidney function leads to AGE accumulation and reinforces oxidative stress.(77) As the dialysis fluid in PD patients is rich in glucose, PD patients suffer from high amounts of glucose and AGEs.(25) Due to a lack of EPO production because of renal insufficiency, the body's own recruitment of erythrocytes is disturbed, which leads to a high prevalence of anaemia in ESRD patients. In addition, patients with renal disease often suffer from Fe deficiency, as Fe requirements are increased due to chronic blood sequestration in the dialyzer. Fe is necessary for several immune processes, including lymphocyte mitogenic responses as well as phagocytosis, respiratory burst and MPO activity in neutrophils. Therefore, Fe supplementation is important to treat Fe deficiency anaemia and, moreover, patients will not respond to treatments by recombinant human erythropoietin (r-HuEPO) without enough Fe. The application of r-HuEPO and Fe is nowadays considered as the best treatment of anaemia in ESRD patients.(78) Nevertheless, there is a close correlation between high serum Fe concentrations and bacterial virulence. Fe overload

impairs neutrophil's phagocytic function and membrane integrity as well as enhances ROS production.(25) The unpaired electrons Fe^{+2} or Fe^{+3} make redox reactions possible. Hydroxyl free radical is produced by the reaction of Fe^{+2} with hydrogen peroxide. Further Fe^{+2} reacts with the lipid hydroperoxide and initiates lipid peroxidation.(79)

Albumin is one of the most important antioxidants in plasma. It expresses thiol on its surface, which provides the most important scavenger for ROS.(80) However, oxidized thiol groups (carbonyl) lose their antioxidative function.(80,81) A study of Oberg et al. has proofed that plasma carbonyl levels were increased in CKD patients, while albumin thiol content was reduced.(82) The production of albumin by the liver is reduced in patients with renal disease, as the liver focuses on positive acute phase protein production during inflammation. Moreover, blood vessels become leaky during inflammation, which results in serum albumin reduction and oedema formation.(28) The reduction in functioning albumin results in a reduced scavenging of LPS.

It is known that lifestyle and nutrition have a crucial impact on the oxidant defence in patients with renal disease. Ilori et al. showed that patients with higher oxidative defence had lower prevalence of CKD and were associated with lower likelihood of smoking and heavy alcohol intake, with higher income and higher education status as well as higher intake of antioxidants, medication for DM II, hypertension and statins.(83,84) The consequence of oxidative stress and chronic inflammation is atherosclerosis. ROS directly oxidatively modify endothelial cells and initiate inflammation and monocytes attraction.(18) By phagocytosing LDLs, monocytes are converted into foam cells. This local lesion leads to smooth muscle cell invasion in the intima, where they further produce extracellular matrix and form a fibrous cap. Fibrosis cap rupture is triggered by inflammation and leads to thrombus formation or embolism.(2)

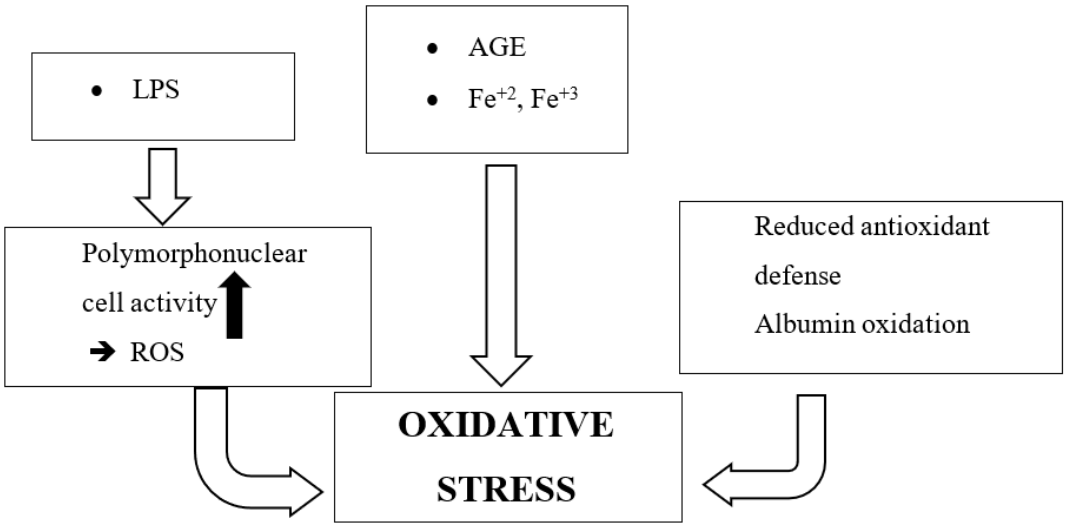

Figure 5 Oxidative stress: Dialysis bio incompatibility, LPS and uremic toxins activate neutrophils to produce ROS. ROS, AGE and LPS induce albumin oxidation and diminish albumin as a potent scavenger for ROS. AGE, advanced glycation end products; ROS, reactive oxidative species; adapted from (85)

1.9. Protein energy wasting

Protein energy wasting (PEW) describes a kidney disease related hypermetabolic condition, which leads to protein and energy loss that cannot be changed by increasing the amount of nutrients. The main reason for weight loss in patients with renal disease represents the chronic subclinical inflammation, followed by the repeated subjection to dialysis procedures.(20,33) In addition, oxidative stress and an impaired insulin like growth factor 1 (IGF-1) receptor signalling pathway on the surface of muscle cells, as well as insufficient protein intake contribute to PEW.(86,87) Due to the proinflammatory capacity of abdominal fat, also patients with an increased waist circumference suffer from PEW.(20) PEW represents a surrogate marker for the chronic morbidity and consequently high CVD in Patients with renal disease. Every 1g/dl decrease in protein was shown to be associated with an 39% increase in the risk of CVD.(88) Figure 6 summarizes the main elicitors for the PEW syndrome.

The PEW-syndrome evolves progressively from an eGFR value below 55 ml/min/1,73 m^2.(89) Diagnostic criteria are low body weight, low muscle mass, low serum albumin levels and low dietary protein intake.(90)The IGF-1 receptor is induced by IGF-1 and insulin and is responsible for cell growth and anabolism of muscle protein and myofibers.(27) Chronic inflammation, uremic toxins and oxidative stress impair the IGF-1 receptor that consequently leads to a disturbed muscle anabolism.(32,91) IL-6 for example inhibits the secretion of IGF-1 and TNF-α increases muscle protein catabolism and disrupts muscle protein differentiation and repair of damaged muscle cells.(33) Low energy intake forces sugar synthesis out of glycogen in the liver.(92,93) ESRD patients who receive RRT suffer from an increased resting energy expenditure, as RRT induce protein loss into the dialysate and into the intestinal lumen and represent a physical stress factor. However, the frequency of dialysis procedures influences the patient's appetite, as several nonrandomized studies suggest a beneficial effect of daily dialysis procedures on protein intake and appetite. The subjective appetite increased when the blood level of uremic toxins declined.(94) In case of severe starvation, glucose is provided through gluconeogenesis in the liver out of lactate, glycerine and amino acids delivered from somatic and visceral muscles (Cori-cycle).(95,96) The combination of IGF-1 receptor impairment and gluconeogenesis out of amino acids results in loss of muscle mass (sarcopenia).(92) In addition, DM II accentuates insulin resistance. Adequate DM II therapy is

therefore necessary, especially in PD who are exposed to high sugar concentrations in the dialysate.(25)

To avoid sarcopenia in CKD patients, dietary protein intake of 0.6-0.8 g protein/kg of ideal body weight, respectively, 30-35 kcal/kg of ideal body weight per day is necessary.(97,98) The minimum requirements for HD or PD patients are 1,2 g protein/kg of ideal body weight per day and 30-35 kcal/kg of ideal body weight per day. At least 50% of protein intake should be of high biological value. Biologic value refers to how well and how fast the body can use the protein.(99) In addition, sufficient amino acid and vitamin intake is required, as they contribute to appropriate immune response.(20,100)

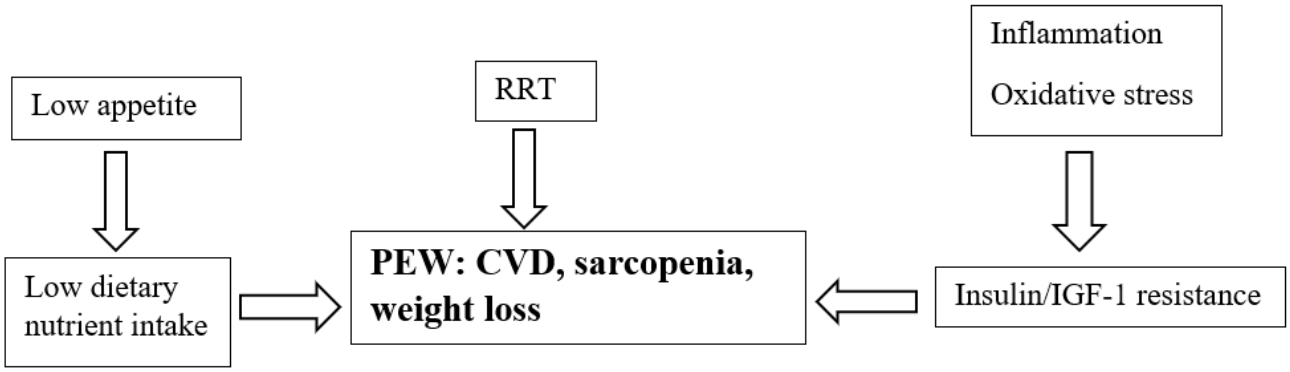

Figure 6 Protein Energy Wasting: Chronic inflammation and dietary restrictions reduce appetite in ESRD patients. RRT induce physical stress and protein loss into the dialysate and the intestinal lumen and thereby enhance the resting energy expenditure. Inflammation and oxidative stress contribute to IGF-1 receptor dysregulation and thereby inhibit protein anabolism in muscle cells. The consequence is PEW which contributes to CVD in Patients with renal disease. Adapted from(85,86)

1.10. Cardiovascular disease in kidney transplanted patients

KT patients are more prone to chronic inflammation and CVD compared to CKD patients and ESRD patients receiving RRT. Underlying causes comprise a long history of ESRD before KT, chronic immunosuppressive therapy and cardiovascular comorbidities.

The ALERT study investigated the effect of serum creatinine levels in KT patients and showed that the risk of CVD increases, when serum creatinine levels increase above 1.5mg/dl and eGFR levels are lower than 45 ml/min/1.73 m^2 one year after KT. In addition, suboptimal graft function and graft loss remains a risk factor for CVD, as it contributes to uremic toxin accumulation and volume overload.(101) Immunosuppressive drugs, including Tacrolimus, Cyclosporin and Steroids, are very controversial in KT patients. They contribute directly to DM II, hyperlipidaemia and hypertension. Smak et al. even showed increased incidence of infection and CVD in association with low dose immune suppression. Immune suppressive therapy is also associated to r-HuEPO resistance.(102) Lopez-Gomez et al. investigated patients on HD after a failed KT. These patients experienced worse r-HuEPO resistance, hypoalbuminemia and had higher levels in CRP and ferritin compared to HD patients without any history of KT. Patients with a failed kidney allograft showed clinical signs of inflammation. Surgical resection of the failed kidney allograft was associated with enhancement in r-HuEPO responsiveness, albumin and reduction inflammatory markers.(103)

1.11. Therapeutic approaches

Although epidemiology links inflammation in Patients with renal disease to a poor outcome, there is no standardized therapy to prevent patients from infections, CVD and PEW. Up to date guidelines request the frequent assessment of inflammation. The presence of elevated inflammatory markers (CRP> 5-10 mg/dl) should prompt a search for immediately treatable causes, such as volume overload, inadequate dialysis treatment, PEW, or the presence of a non-functioning renal allograft. The following interventions are beneficial among patients with CKD or ESRD with detected inflammation(100) :1) Evaluate and treat occult infection, 2) assess for periodontal diseases, 3) screen for tuberculosis, 4) evaluate and treat diabetic foot ulcers, 5)

remove old non-functioning arteriovenous grafts, 6) remove failed kidney grafts, 7) use ultrapure dialysate, 8) treat rheumatologic conditions. Pharmacological interventions are not recommended to treat specifically, however, some medications are undergoing early clinical trials. Statins not only inhibit cholesterol synthesis, but also have anti-inflammatory actions, as studies showed that statins reduce CRP and IL-6 levels.(33) Also, angiotensin-converting-enzyme inhibitors showed anti-inflammatory properties, as they reduce IL-6, TNF-α and CRP levels and furthermore reduce the risk of weight loss.(104) Also the submission of oestrogen and progesterone repressed IL-6 production.(33)

Thalidomide might be useful because of its immune modulatory, anti-inflammatory and antiangiogenetic properties and has been associated with weight gain in patients with tuberculosis or HIV suffering from PEW.(33) Targeted anti-cytokine therapy for Patients with renal disease is highly controversial as TNF-α blocker may induce vascular calcification.(33) Recent studies focus on the treatment of dysbiosis. The administration of probiotics for at least 6 months showed a reduction in p-cresol and cytokine levels in ESRD patients and ω-3 fatty acids ameliorate oxidative stress by increasing glutathione peroxidase activity.(105-107) Insufficient oral nutrient intake should better be compensated by enteral (nasogastric or via PEG tube) application, as studies did not show any beneficial effects of parenteral nutritional interventions. Total parenteral nourishment was even associated with increased infection rates.(108) Inter dialytic supplementation of parenteral nutrition has not been shown to have an improvement on mortality, hospitalization rates, or on the PEW syndrome.(98) Congestive heart failure contributes to chronic inflammation by enhancing volume overload and decreasing kidney function via high blood pressure. Therefore, adequate therapies are required to prevent cardiac induced acceleration of inflammation.(109)

2. Hypothesis

We hypothesize that high blood LPS levels in patients with renal disease lead to an impaired neutrophils phagocytosis and to albumin oxidation. Oxidized albumin is not able to bind LPS and other ROS adequately any more, which leads to a further increase in oxidative stress. LPS and uremic toxin induced chronic inflammation, albumin dysfunction and oxidative stress alter the humoral immune defence in serum.

3. Specific aims

1) Compare LPS levels and levels of LPS-binding proteins between CKD patients, patients who received different forms of RRT, KT patients and patients with acute renal failure (ARF).
2) Compare levels of immune cells and inflammatory parameters between CKD patients, patients who received different forms of RRT, KT patients and patients with ARF, as well as between different dialysis accesses.
3) Characterize whether serum growth retardation capacity of *E. coli* differs between CKD patients, patients who received different forms of RRT, KT patients and patients with ARF.
4) Investigate the influence of LPS on the phagocytosis of in vitro differentiated neutrophil-like leukaemia cells (HL-60 cells)

4. Materials and methods

4.1. Literature research

Literature research was conducted by using online journal databases as well as teaching and professional books from June 2016 until May 2018. To identify eligible information in PubMed, medical Subject Headings (MeSH) were used. The used MeSH were “Inflammation in ESRD patients”, “Impaired intestinal barrier in ESRD patients”, “Neutrophil dysfunction in ESRD”, “Endotoxemia in ESRD” and “serum bactericidal activity”. RefWorks was used for citation.

4.2. ENARI study

ENARI is an abbreviation for “endotoxin, neutrophil function and albumin in renal insufficiency”. The study was performed from 2010 to 2013 at the Department of Internal Medicine, Clinical Division of Nephrology, Medical University of Graz to detect pathophysiological alterations, which lead to an increased inflammatory state and increased PEW in CKD patients, patients who received different types of RRT, KT patients and patients with ARF. The Ethics Committee of the Medical University of Graz approved the study protocols (23-056 ex10/11) and the study was registered under NCT01362569. The study was

conducted according to the Declaration of Helsinki and all participants gave written informed consent prior enrolment. 195 patients with renal dysfunction and 25 healthy controls were included in the study. Patients were subdivided into 6 different groups:

Patients with a CKD either

1a) with an eGFR between 30 and 45 (KDIGO 3B)

1b) with an eGFR between 15 and 30 (KDIGO 4)

1c) with an eGFR under 15 (KDIGO 5)

2a) who received HD for ESRD

2b) who received HDF for ESRD

3a) who received PD without signs of infection

3b) who received PD and suffered from peritonitis (PD+inf.) and showed ≥ 2 out of the 4 criteria (>100 L/50% N, cloudy peritoneal dialysate, typical clinical presentation with fever and abdominal pain, positive culture from the peritoneal dialysate)

4) Patients with ARF, AKIN 3 (defined as an increase in serum creatinine to 300% from baseline or serum creatinine 4.0 mg/dl with an acute rise of at least 0.5 mg/dl or urine output of <0.3 ml/kg/h for 24 h or anuria for 12 h)

5) Stable patients after KT

6) healthy controls

Exclusion criteria were malignancy, pregnancy, chronic inflammatory bowel disease, celiac disease, active alcohol abuse, severe organ dysfunction unrelated to renal dysfunction or clinical evidence of active infection.

4.3. Laboratory parameters

Laboratory parameters were collected during the ENARI study and analysed during the diploma thesis. They include leukocytes, neutrophils, basophils, eosinophils, monocytes, lymphocytes, CRP, cholinesterase, total plasma protein, albumin, haemoglobin (hb), transferrin saturation, IL-6, Interleukin-8 (IL-8), IL-10, TNF-α, LPS, LBP and sCD14. The phagoindex describes the phagocytic capacity of neutrophils isolated from the ENARI cohort. Table 1 summarizes the laboratory parameters.

Table 1 Laboratory parameters

Standard laboratory parameter	**abbreviation**	**unit**	**measurement method**
leukocytes	L	cells/µl	flow cytometry
neutrophils	N	cells/µl	flow cytometry
basophils	Baso	cells/µl	flow cytometry
eosinophils	E	cells/µl	flow cytometry
monocytes	M	cells/µl	flow cytometry
lymphocytes	Lym	cells/µl	flow cytometry
C-reactive protein	CRP	mg/dl	immunoturbidimetry
total plasma protein	TPP	g/l	photometry
serum albumin	Alb	g/l	photometry
cholinesterase	ChE	U/l	photometry
haemoglobin	hb	g/dl	photometry
transferrin saturation	TSAT	%	photometry
Additional parameters			
endotoxin	LPS	HEK EU/ml	LAL assay
lipopolysaccharide binding protein	LBP	µg/ml	ELISA
soluble form of cluster of differentiation 14	sCD14	µg/ml	ELISA
Interleukin 6	IL-6	pg/ml	ProcartaPlex
Interleukin 8	IL-8	pg/ml	ProcartaPlex
Interleukin 10	IL-10	pg/ml	ProcartaPlex
tumour necrosis factor alpha	TNF-α	pg/ml	ProcartaPlex
Phagoindex	PI	% of controls	Flow cytometry

Laboratory parameters were collected during the ENARI study and not measured during this thesis. Routine laboratory parameters were analysed at the Clinical Institute of Medical and Chemical Laboratory Diagnostics of the Medical University of Graz.

4.4. Volume overload

Volume overload was estimated by assessing the extent of oedema clinically. Patients of each study group were subdivided into patients with oedema and without oedema to determine the prevalence of volume overload in each study group. The assessment was done during the ENARI study, results were compared during this thesis.

4.5. Acellular growth retardation assay

The acellular growth retardation assay (AGRA) was developed to measure serum induced growth retardation of *E. coli*. Our aim was to determine the extent of growth retardation in patient sera collected during the ENARI study. Results were compared between predefined study groups: CKD, HD, HDF, PD, PD+inf., KT, ARF and healthy controls. Results from healthy controls were taken from a previously conducted study and were not measured during this thesis. Table 2 summarizes the used materials.

Table 2 AGRA materials

6 Thermo Scientific™ 96-Well Microtiter™ Microplates, Thermo Fischer scientific
patient sera (stored at -80°C, prewarmed to 22°C before experiment)
XL1-blue *E. coli*
sterile Luria-Bertani broth (LB broth) Sigma-Aldrich®

Procedure:

Sera were collected with sterile equipment and were kept frozen at -80°C until assay. XL1-blue *E. coli* was prepared in an overnight culture the day before the experiment. XL1-blue *E. coli* were resuspended in 5 ml LB broth and incubated at 37°C at 130-180 rpm overnight. Bacterial working solution was gained by diluting the overnight culture 1:20 in 100 ml fresh LB broth and vortexed vigorously. Two wells per microtiter plate were dedicated to "blanc control" and "*positive control*", respectively. Serum samples of 195 patients were analysed in duplicate. 100 µl of bacterial working solution was pipetted into all wells except to "blanc control". Fresh,

sterile LB broth was used for "blanc control". Plates were read at 600 nm (OD_{600}) by spectrophotometry SPECTROstar® (timepoint T0) and then incubated at 37°C for two hours. Plates were read once more at 600 nm (timepoint 2h). The OD_{600} was measured at T0 and T2h to see bacterial growth without serum.

Prewarmed patient sera were diluted separately 1:10 in LB broth and 100 µl were added to the corresponding wells. 100 µl fresh LB broth was added to "*E. coli*" and "blanc control". Figure 7 summarizes the experimental setup. Plates were read immediately at 600 nm (timepoint base) and then incubated for 5 hours at 37°C and read every h. "Blanc control" wells were left free from *E. coli* and patient serum to measure OD_{600} of LB broth. "*E. coli*" wells were left free from serum to measure OD_{600} of *E. coli* growth without patient serum. Growth curves were analysed to determine acellular growth retardation capacity. A patent has been filed for this assay with the European Patent Office (EP 17 181 908).

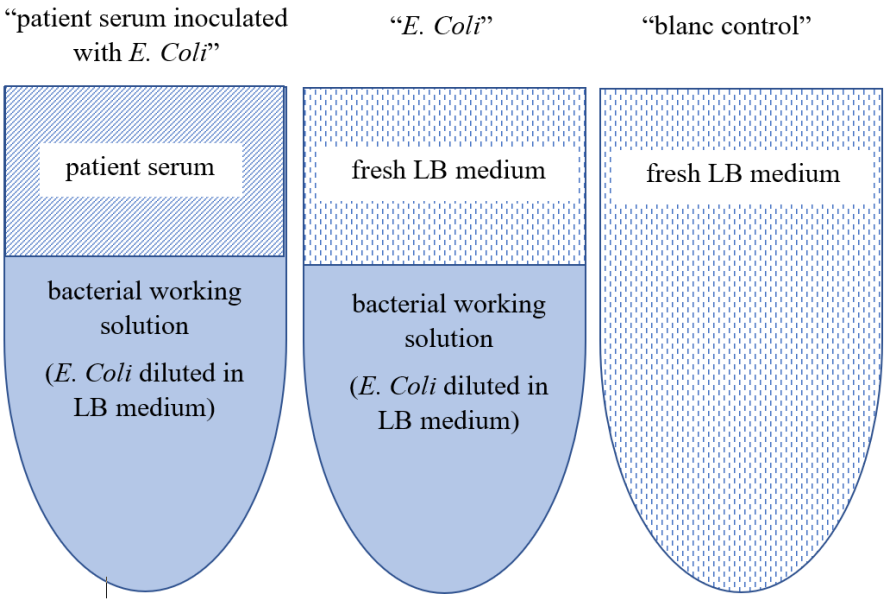

Figure 7 AGRA, experimental setup: Patient serum inoculated with *E. coli* contains bacterial working solution and patient serum. *E. coli* contains bacterial working solution and LB broth. Blanc control contains fresh, sterile LB broth.

4.6. Determination of phagocytic capacity of in vitro cultivated neutrophil-like differentiated human leukaemia cells by Phagotest®

Our aim was to compare the effect of 250 ng and 500 ng on the phagocytic capacity of in vitro cultivated N-like differentiated HL-60 cells compared to LPS-free controls. Table 3 summarizes the used materials.

Table 3 Materials used in the Phagotest®

human leukaemia 60 (HL-60) cells ATCC® CCL-240™
Roswell Park Memorial Institute (RPMI) 1640 cell culture medium supplemented with 10% foetal bovine serum (FBS) and 1% Pen/Strep, ThermoFischer Scientific
dimethyl sulfoxide (DMSO), Sigma-Aldrich®
trypan blue, Sigma-Aldrich®
phosphate buffered saline (PBS), Sigma-Aldrich®
LPS *E. coli* O111:B4, Sigma-Aldrich®
Phagotest™ kit, Glycotope Biotechnology

Procedure:

HL-60 cells were seeded at $5x10^5$ cells/ml in RPMI cell culture medium in a 25 cm^2 cell culture flask with 0.2 µm vent cap. The culture medium was stored at 4°C and prewarmed to 37°C before experiment. All cell work was performed under a biologic safety cabinet. For one Phagotest®, HL-60 cells were cultivated up to the required cell amount of $6x10^6$ living cells. Cell culture medium was changed every other day. Cell count was determined by TC20™ automated cell counter. Therefore, 10 µl of cell suspension needed to be mixed with 10 µl trypan blue. When cells reached the required cell count, $3x10^6$ HL-60 cells were passaged in each of two 25 cm^2 cell culture flasks with 0.2 µm vent cap.

Prewarmed culture medium was added to each flask to split cells to a concentration of $5x10^5$ HL-60 cells/ml. To induce differentiation of HL-60 cells to N-like differentiated HL-60 cells, one flask was inoculated with 1.25% DMSO. Both cell culture flasks were incubated for 5 days at humified atmosphere at 37°C. Cell culture medium was changed every other day. After

5-6 days of incubation, DMSO induced cells were transferred to 6 FACS tubes. Each tube contains $5x10^5$ cells. During the first test, not induced cells were also transferred to 6 FACS tubes. Afterwards, cells were spun down for 5 min at 350 g and cell culture medium was discarded. Cells were resuspended in 1 ml PBS and inoculated with either 0 ng, 250 ng or 500 ng LPS. All tubes were incubated for 2 h in water bath at 37°C to activate cells.

Cells were spun down for 5 min at 350 g and all tubes were incubated with 100 µl foetal bovine serum and 20 µl of fluorescein isothiocyanate labelled opsonized *E. coli* (FITC-labelled *E. coli*) Tubes were either incubated for 10 min in water bath at 37°C (test) or remained on ice (control). During this process, cells in the test samples phagocytosed FITC-labelled *E. coli,* whereby cells in the control samples did not phagocytose. Afterwards, 100 µl of quenching solution was added to every tube to quench fluorescence of not phagocytosed FITC-labelled *E. coli*. Two washing steps were performed, whereby 3 ml wash solution was added to the cells and then discarded. 2 ml of lysing solution was added to fix neutrophils.(110)

After incubation for 20 min at room temperature, cells were spun down for 5 min at 350 g. 200 µl of DNA staining solution was added to each sample ten minutes prior to flow cytometric measurement for discrimination of bacteria during leukocyte analysis. For flow cytometric analysis BD™ LSR II flow cytometer was used. 10000 N-like differentiated HL-60 cells of each tube were recorded. Data were analysed with FACSDiva software version 6.0. A forward-side scatter gate was set on the recorded cells to select N-like differentiated HL-60 cells. (see Figure 8). The selection was done for cells in test samples as well as for cells in control samples. For further analysis, all selected cells of control samples and test samples of the same LPS concentration were displayed in a green fluorescence histogram. The fluorescence intensity of cells from the control sample shows the autofluorescence of cells having not performed phagocytosis and was used to set a fluorescence cut-off on the x-axis. The percentage of phagocytosing cells in the test sample could be determined by counting the number of cells above the set fluorescence cut-off. (see Figure 9).

The mean fluorescence intensity was determined in phagocytosing cells and in cells from the control sample by calculating the geometric mean fluorescence intensity (gMFI). The fluorescence shift of the gMFI from cells in the control sample to phagocytosing cells shows the mean number of phagocytosed FITC labelled *E. coli*.

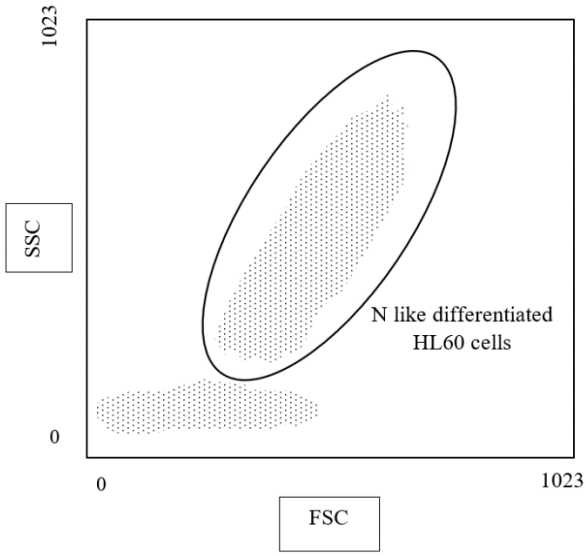

Figure 8 Forward side scatter gate set on recorded cells: The FSC shows the cell size, SSC shows the cell granularity. N-like differentiated HL-60 cells were selected for further analyzation. FSC, forward scatter; SSC, side scatter; N, neutrophil granulocytes.

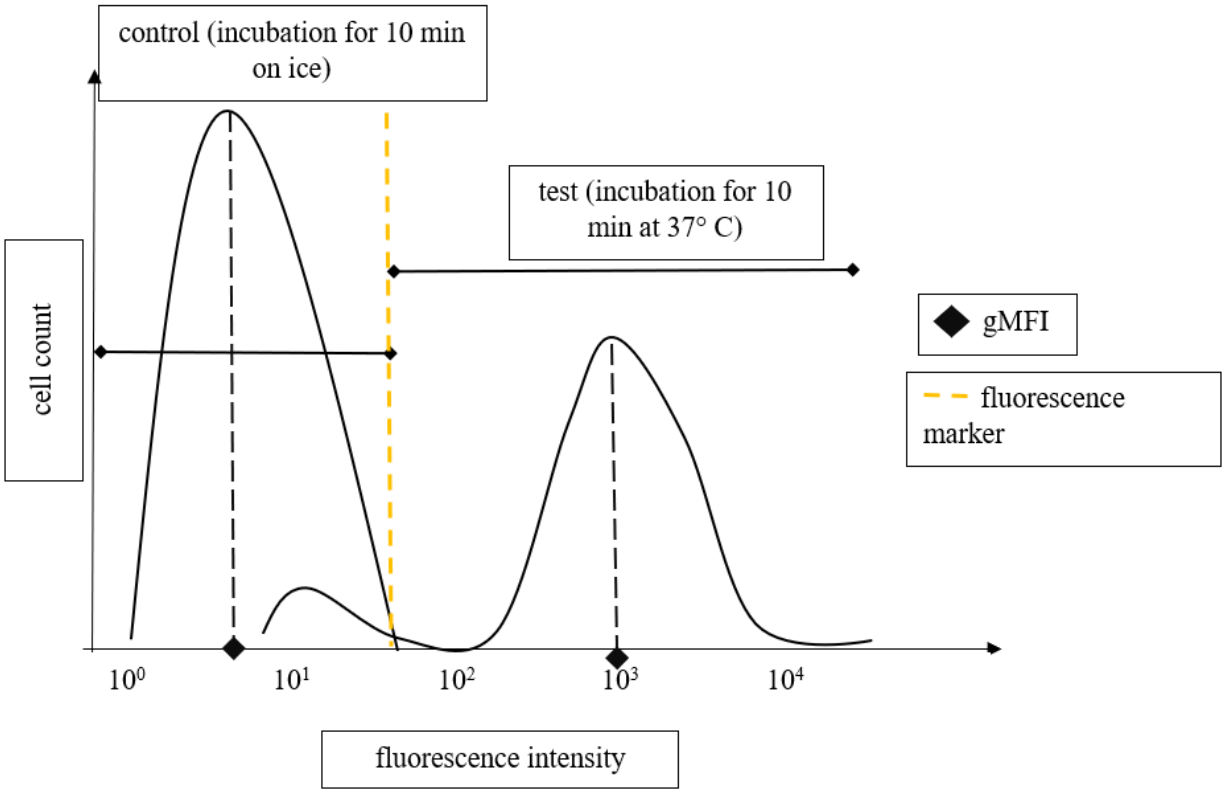

Figure 9 Schematic histogram for the fluorescence gating of cells: The x-axis displays the fluorescence intensity; the y-axis displays the cell count. Cells, N-like differentiated HL-60 cells; gMFI, geometric mean fluorescence intensity, adapted from (111)

4.7. Statistics

All statistical analyses were performed by using SPSS version 23.0 (SPSS Inc., Chicago, IL). Significant differences between groups were assessed by ANOVA (normally distributed levels), Kruskal Wallis and Man Whitney U (not normally distributed levels). Parametric distributed parameters were tested for homogeneity (Levene's test) and accordingly compared by ANOVA using Bonferroni correction. Metrical data correlation was measured by two-tailed Pearson or Spearman test. Nominal and ordinal scaled data comparison was done by Chi-square test using Pearson test or Spearman test. All statistical tests were 2-sided and p-levels < 0.05 were considered statistically significant. Laboratory parameters were considered significantly different at a p-value≤ 0.002 (0.05/21 possible combinations) when compared between 7

different study-groups, significantly different at a p-value ≤ 0.016 (0.05/3 possible combinations) when compared between CKD subgroups and significantly different at a p-value ≤ 0.005 (0.05/10 possible combinations) when compared between dialysis accesses. Data are represented by box plot.

5. Results

5.1. Demographics

238 patients were enrolled in the ENARI study. 154 (65%) were male and 84 (35%) were female. Patients were between 18 and 85 years old. The mean age was 57 years. 55 age and sex matched healthy controls were included in the study. These subjects had no evidence of renal disease and did not take any medication. Patients with an eGFR>30 ml/min/1.73 m^2 were younger than patients with an eGFR<29 ml/min/1.73 m^2 and patients with an eGFR<15 ml/min/1.73 m^2. Table 4 displays the detailed demographic characteristics of the study population. The frequency distribution of dialysis access types used for RRT is displayed in Table 5. The frequency distribution of the comorbidities CVD and DM II is displayed in Table 6

Table 4 Characteristics

	total		female		male		age	BMI
	n	(%)	n	(%)	n	(%)	mean (range)	kg/m^2 mean (SD)
patients total	**238**	(100%)	**154**	**(65%)**	**84**	**(35%)**	**57 (18-85)**	**27 (6)**
HD	12	(5%)	9	(75%)	3	(25%)	60 (18-81)	26 (6)
HDF	20	(8%)	6	(30%)	14	(70%)	60 (18-81)	28 (5)
PD	28	(12%)	8	(29%)	20	(71%)	52 (23-80)	28 (5)
PD+inf.	13	(6%)	5	(39%)	8	(62%)	59 (33-77)	26 (5)
ARF	25	(11%)	5	(20%)	20	(80%)	59 (18-85)	26 (5)
KT	67	(28%)	25	(37%)	42	(63%)	55 (20-75)	29 (6)
CKD	73	(31%)	26	(36%)	47	(64%)	60 (26-85)	29 (6)
eGFR 30-45	26	(11%)	8	(31%)	18	(38%)	51 (26-76)	27 (5)
15-30	36	(15%)	15	(58%)	21	(45%)	65 (29-85)	31 (7)
<15	11	(5%)	3	(12%)	8	(17%)	64 (41-82)	29 (6)
HC	55		27	(49%)	28	(51%)	50 (21-73)	not assessed

n, number; SD, standard deviation; BMI, body mass index; CKD, chronic kidney disease, eGFR<45; HD, patients received haemodialysis, HDF, patients received hemodiafiltration; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate.

Table 5 Dialysis access

	no	fistula	graft	catheter	PD catheter
CKD	69 (97%)	2 (3%)			
HD(F)		5 (25%)	6 (30%)	9 (45%)	
PD					27 (100%)
PD+inf.					4 (100%)
ARF	1 (100%)				
NTX	49 (82%)	8 (13%)	2 (3%)	1 (2%)	2 (3%)

CKD, chronic kidney disease, eGFR<45; HD(F), patients received either haemodialysis or hemodiafiltration; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate. Percentage levels describe percentages within study groups.

Table 6 Comorbidities

	DMII	CVD
HD	6 (50%)	9 (75%)
HDF	6 (30%)	10 (50%)
PD	5 (18%)	11 (39%)
PD+inf.	3 (23%)	7 (54%)
ARF	5 (20%)	7 (28%)
KT	17 (25%)	43 (64%)
CKD	27 (37%)	47 (67%)

DMII, diabetes mellitus type 2; CVD, cardiovascular disease; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either haemodialysis or hemodiafiltration; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate. Percentage levels describe percentages within study groups.

5.2. Endotoxin and endotoxin-binding proteins

All patients had significantly higher sCD14 and LBP levels compared to healthy controls ($p<0.001$). HD(F) patients had significantly higher LPS and sCD14 levels compared to CKD ($p=0.001$), KT patients ($p<0.001$) and healthy controls ($p<0.001$). PD+inf. patients had significantly higher sCD14 and LBP levels compared to CKD, HD(F), PD and KT patients ($p<0.001$). Patients with ARF had significantly higher sCD14 and LBP levels compared to CKD patients ($p<0.001$, $p=0.001$), HD(F), PD and KT patients ($p<0.001$). LPS, LBP and sCD14 did not differ significantly between ARF and PD+inf. patients. sCD14, LBP and LPS levels did not differ significantly between patients who receive different forms of RRT. Within the CKD group, patients with CKD 4 and CKD 5 had significantly higher LBP levels compared to patients with CKD 3 ($p=0.001$). Patients with and without oedema did not differ significantly in LPS levels. There was no correlation between sCD14 and LBP as well as between LBP and LPS. Results are displayed in Figure 10 and Figure 11.

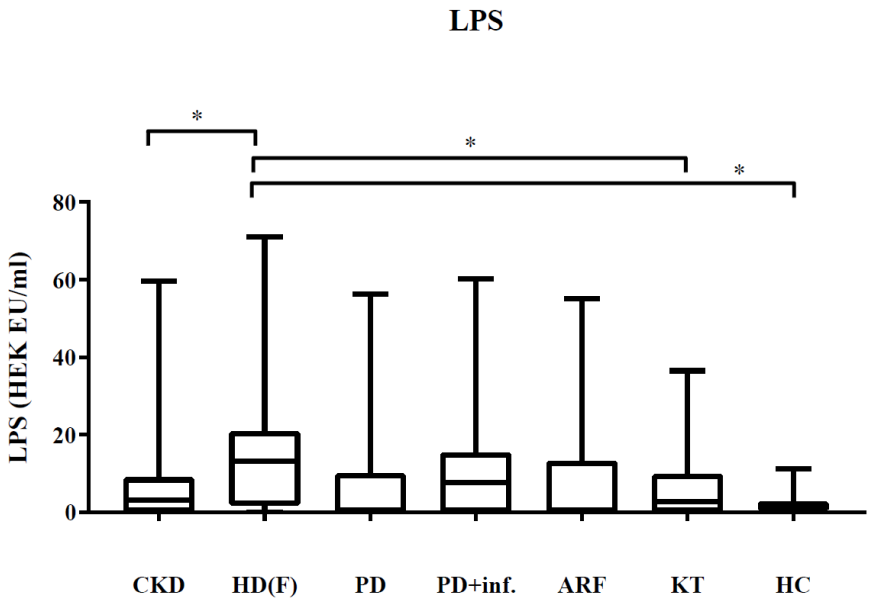

Figure 10 LPS: CKD, chronic kidney disease, eGFR<45; HD(F), patients received either haemodialysis or hemodiafiltration; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate; *, significant difference ($p\leq0.002$)

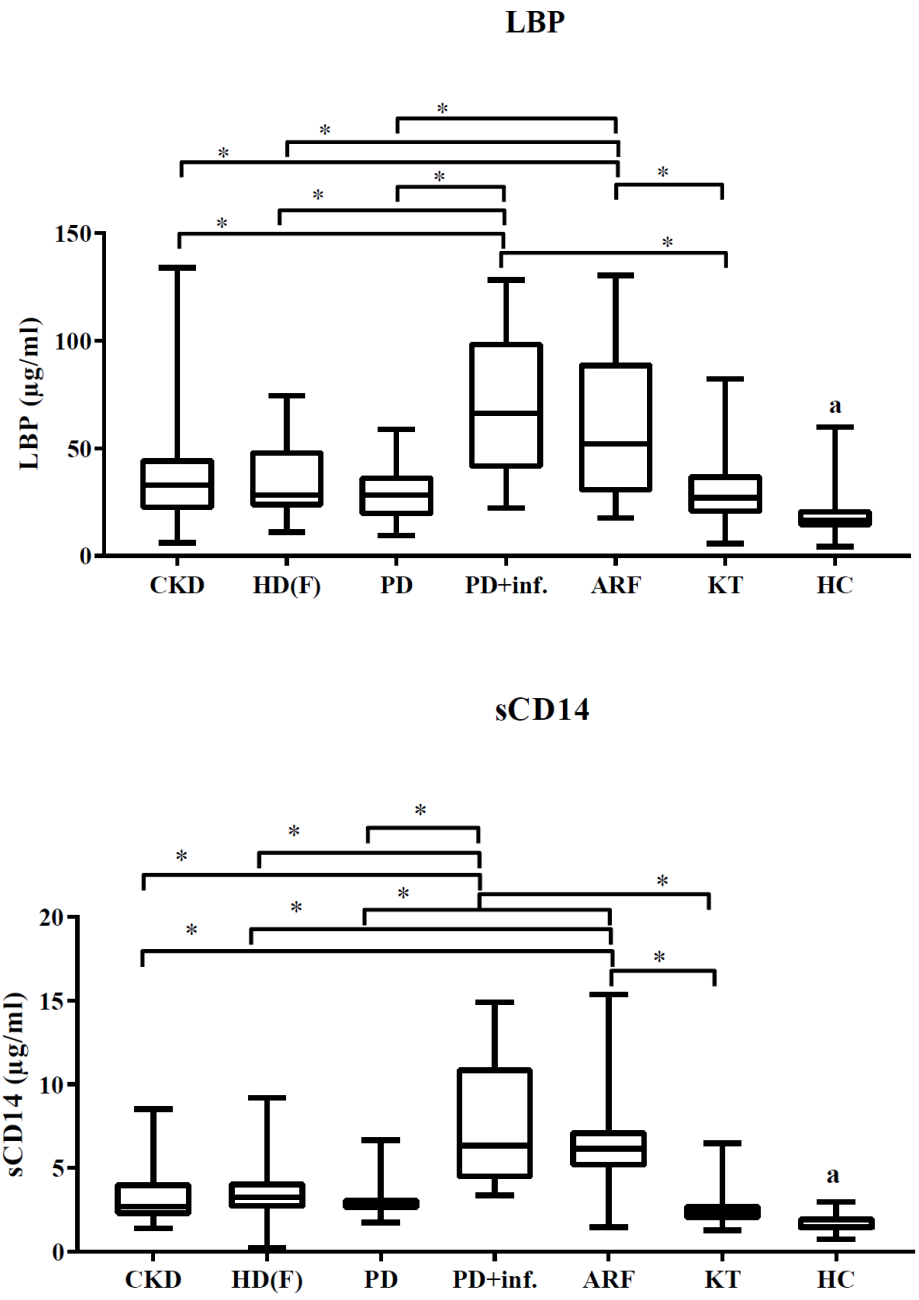

Figure 11 LPS binding proteins: LBP, lipopolysaccharide binding protein; sCD14, soluble factor of cluster of differentiation 14; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either haemodialysis or hemodiafiltration; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate; *, significant difference ($p \leq 0.002$); a, significantly different to all displayed patient groups ($p \leq 0.002$)

5.3. Inflammation

To compare the extent of prevalent inflammation between study groups, significant differences between leukocytes, neutrophils, basophils, eosinophil, lymphocytes and monocytes, as well as CRP, IL-6, IL-8, IL-10 and TNF-α levels were tested. All patients had significantly lower lymphocytes, higher IL-6 and IL-10 levels compared to healthy controls ($p<0.001$).
PD+inf. had significantly higher leukocytes compared to CKD ($p=0.001$), HD(F) ($p=0.002$), KT ($p=0.002$) and healthy controls ($p<0.001$).

All groups, except PD had significantly higher neutrophils compared to healthy controls ($p<0.001$). PD+inf. had significantly higher neutrophils compared to PD patients ($p=0.002$). Patients with ARF had significantly higher neutrophils compared to PD and KT patients ($p<0.001$). Patients with ARF had significantly lower lymphocytes compared to CKD and PD patients ($p=0.001$). KT patients had significantly higher monocytes compared to CKD patients ($p=0.001$). Neutrophils correlated negatively with lymphocytes ($p<0.001$, $r=0.93$). All patients had significantly higher neutrophil/lymphocyte ratios compared to healthy controls ($p\leq0.002$). ARF patients had significantly higher neutrophil/lymphocyte ratios compared to CKD and PD patients ($p<0.001$, $p=0.002$). Patients with CVD had a significantly higher neutrophil/lymphocyte ratio compared to patients without CVD ($p<0.015$).

HD(F) had significantly higher basophils compared to KT patients ($p=0.001$). PD+inf. patients had significantly higher basophils compared to HD(F) ($p=0.001$). CKD and PD patients had significantly higher eosinophils compared to healthy controls ($p<0.001$, $p=0.001$). CKD, HD(F) and PD patients had significantly higher eosinophils compared to KT patients ($p<0.001$, $p=0.001$, $p=0.001$). PD patients had significantly higher eosinophils compared to PD+inf. patients ($p=0.001$) and patients with ARF ($p<0.001$).

HD(F) patients had significantly higher CRP levels compared to healthy controls ($p=0.002$). PD+inf. had significantly higher CRP levels compared to CKD, PD, KT and healthy controls ($p<0.001$). Patients with ARF had significantly higher CRP levels compared to CKD, HD(F), PD, KT and healthy controls ($p<0.001$). HD(F) patients had significantly higher IL-6 levels compared to CKD and KT patients ($p=0.001$). PD+inf. had significantly higher IL-6 levels compared to CKD ($p<0.001$), HD(F) ($p=0.002$), PD and KT patients ($p<0.001$).

PD patients had significantly higher IL-10 levels compared to CKD ($p<0.001$), HD(F) ($p=0.001$) and KT patients ($p<0.001$). Patients with ARF had significantly higher IL-10 levels compared to PD patients ($p=0.002$). HD(F), PD and PD+inf. patients had significantly higher IL-8 levels compared to healthy controls ($p<0.001$). PD patients had significantly higher TNF-α levels compared to healthy controls ($p<0.001$). Patients with ARF had significantly higher TNF-α levels compared to CKD ($p<0.001$) and to PD patients ($p=0.002$). PD+inf. and ARF patients did not differ significantly in inflammatory parameters.

All patients had significantly lower albumin levels compared to healthy controls ($p<0.001$). CKD, HD(F), PD, PD+inf. and ARF patients had significantly lower albumin levels compared to KT patients ($p<0.001$, $p<0.001$, $p=0.001$, $p<0.001$, $p<0.001$). PD+inf. patients had significantly lower albumin levels compared to CKD, HD(F) and PD patients ($p<0.001$). Patients with ARF had significantly lower albumin levels compared to HD(F) ($p<0.001$). Albumin did not correlate with IL-6, IL-10 and TNFα.

All patients, except KT patients had a significantly higher CRP:albumin ratio compared to healthy controls ($p\leq0.001$). PD+inf. patients had a significantly higher CRP:albumin ratio compared to CKD ($p<0.001$), HD(F) ($p=0.002$), PD ($p<0.001$) and KT ($p<0.001$) patients. Patients with ARF had a significantly higher CRP:albumin ratio compared to CKD ($p<0.001$), HD(F) ($p<0.001$), PD ($p<0.001$) and KT ($p<0.001$) patients. Results are displayed in Figure 12-Figure 16.

Patients at different CKD levels did not differ significantly in CRP, IL-6 and TNF-α. Patients with CKD 5 had significantly higher IL-8 levels compared to CKD 4 and CKD 3 ($p=0.003$). CKD patients at different CKD stages did not show differences in leukocytes, neutrophils, monocytes, eosinophils and lymphocytes.

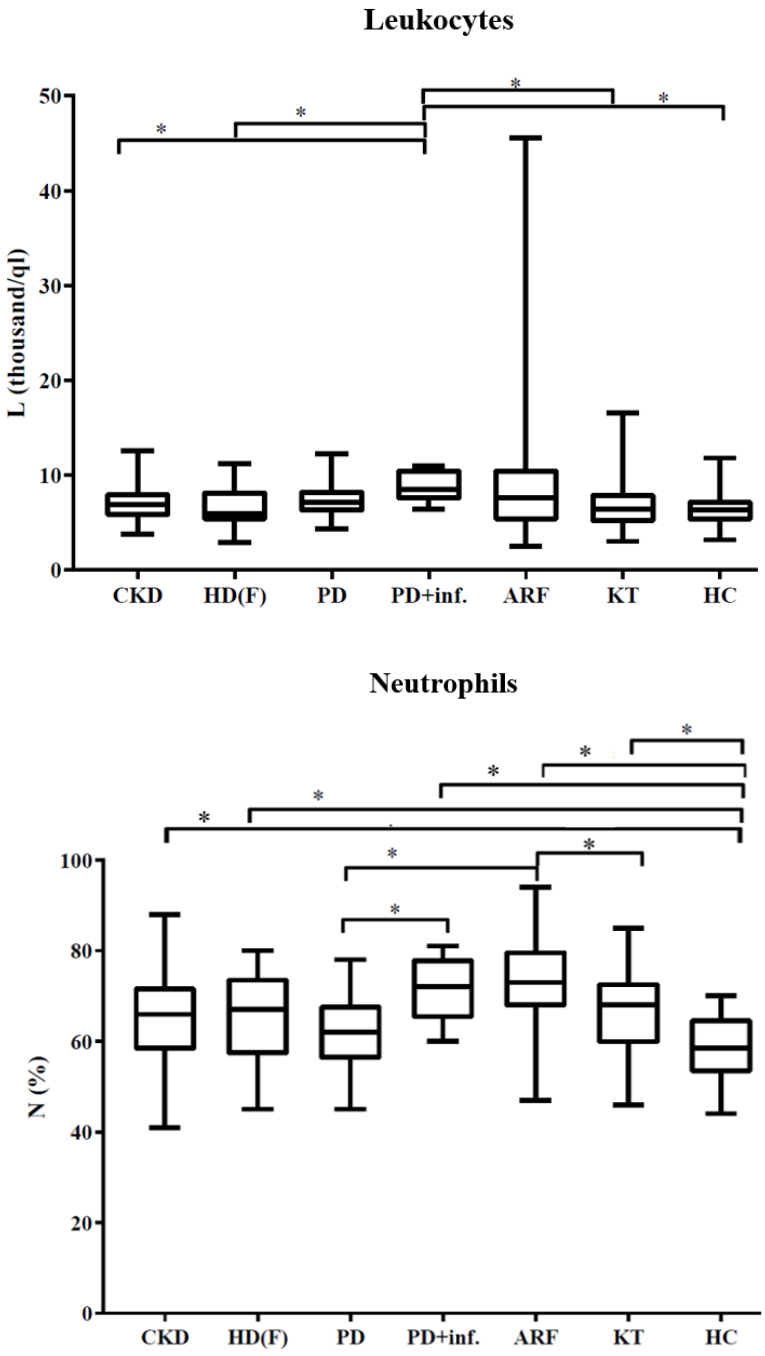

Figure 12 Leukocytes and neutrophils: L, leukocytes; neutrophils, neutrophils; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either HD or HDF; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate; HC, healthy controls *, significant difference ($p \leq 0.002$)

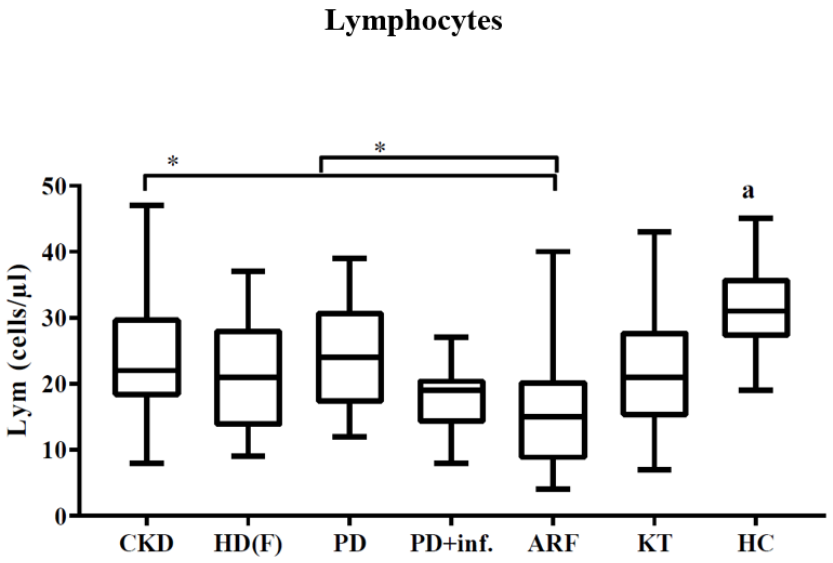

Figure 13 Lymphocytes: Lym, lymphocytes; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either HD or HDF; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate; HC, healthy controls; *, significant difference ($p \leq 0.002$); a, significantly different to all displayed patient groups ($p \leq 0.002$)

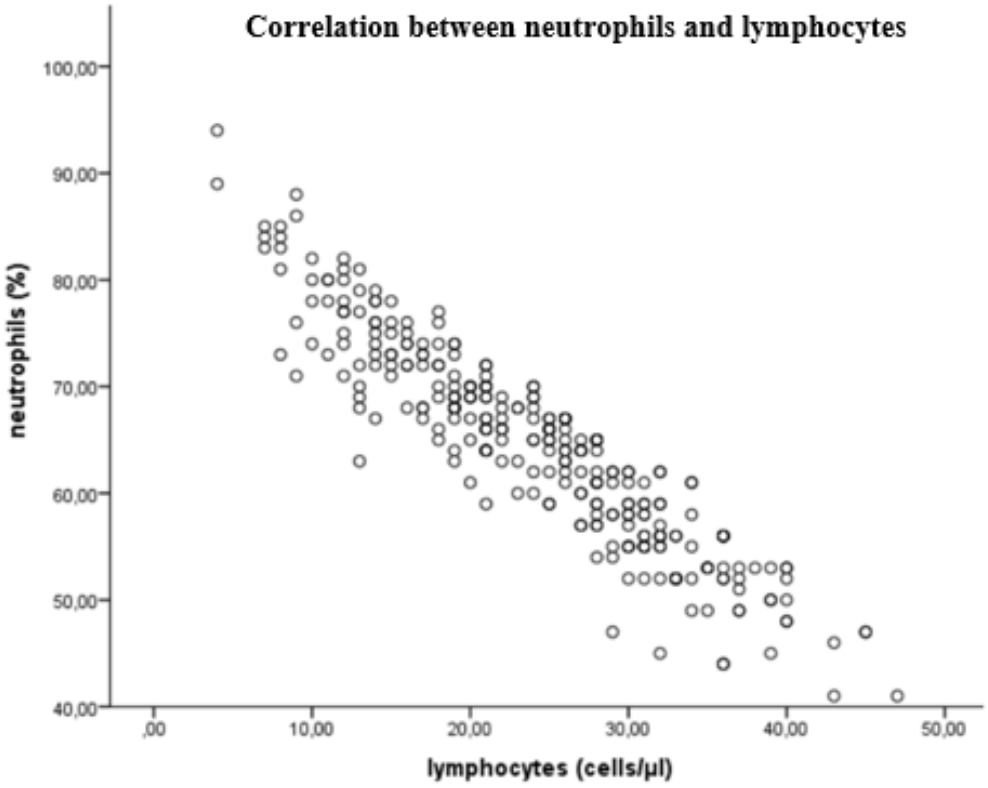

Figure 14 Correlation between neutrophils and lymphocytes: Neutrophils significantly negatively correlated to lymphocytes ($p<0.001$, $r=0.93$).

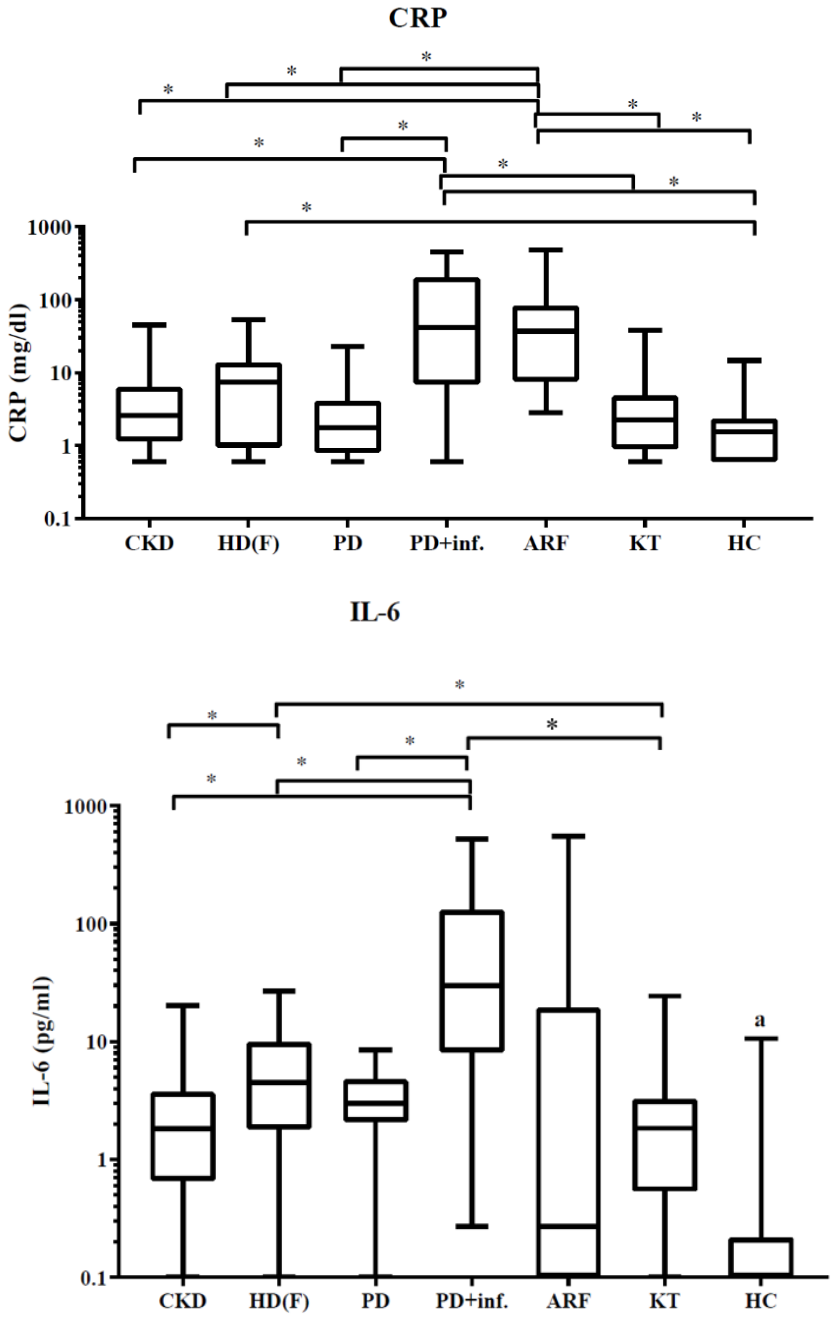

Figure 15 Proinflammatory mediators: CRP, C-reactive protein; IL-6, interleukin 6; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either HD or HDF; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate; HC, healthy controls; *, significant difference ($p \leq 0.002$); a, significantly different to all displayed patient groups ($p \leq 0.002$)

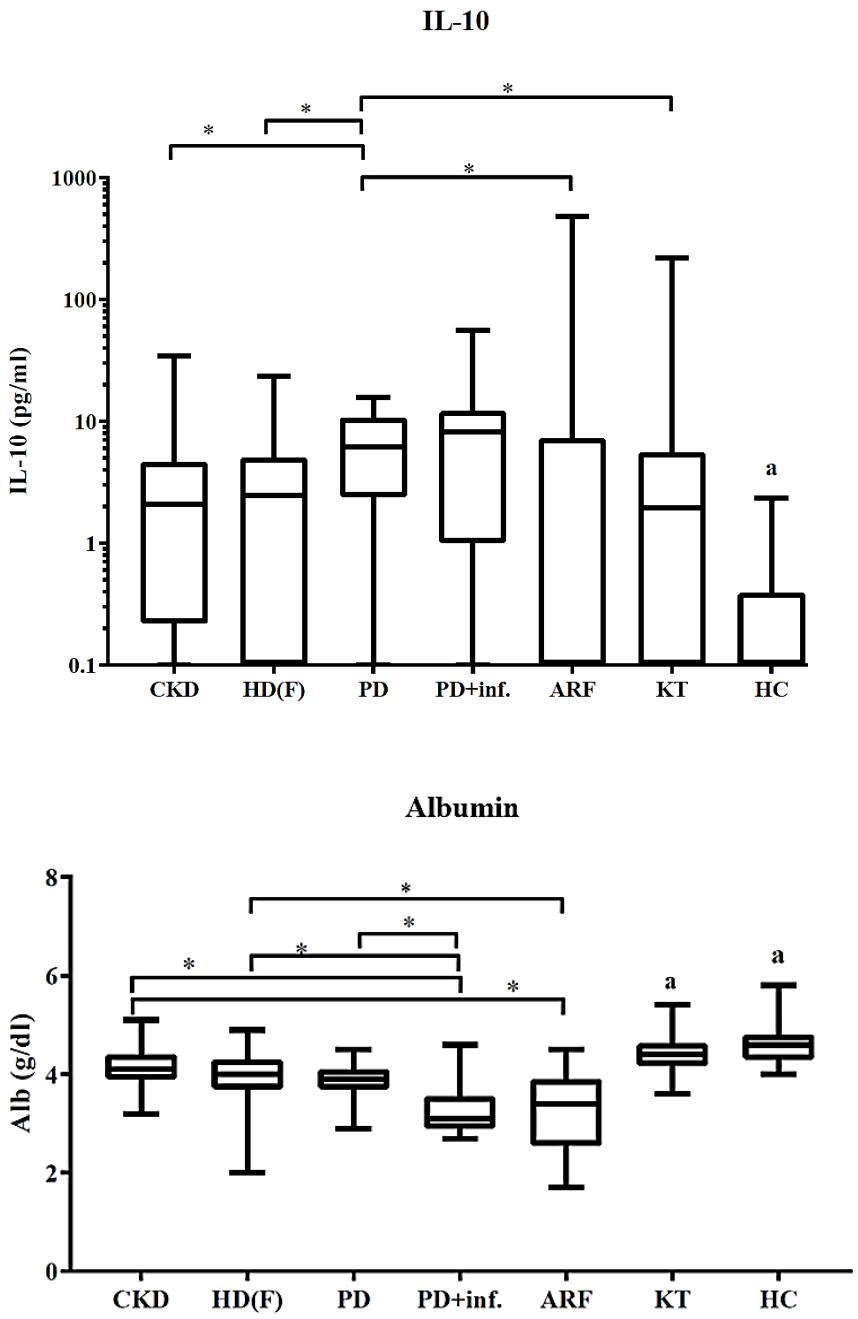

Figure 16 Anti-inflammatory cytokine IL-10 and albumin: IL-10, interleukin 10; alb, albumin; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either HD or HDF; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate; HC, healthy controls; *, significant difference ($p \leq 0.002$); a, significantly different to all displayed patient groups ($p \leq 0.002$)

In addition, the influence of different dialysis access types on inflammation was assessed: Patients with a catheter had significantly higher CRP levels compared to patients with an AV fistula ($p=0.003$), with a PD access ($p=0.002$) and to patients without any access ($p<0.001$). Patients with a catheter and patients with a PD access had significantly higher IL-6 levels compared to patients without any access ($p<0.001$). Patients with a PD access had significantly higher IL-10 levels compared to patients with a catheter and to patients without any access ($p<0.001$). Patients with a PD access had significantly higher eosinophils compared to patients with an AV fistula ($p=0.004$) and to patients without any access ($p<0.001$). Patients with an AV graft did not differ significantly from patients without any access and from patients with an AV fistula in CRP, IL-6, IL-8 and IL-10 levels. Neutrophils, leukocytes, monocytes and lymphocytes did not differ significantly between patients with a dialysis access of any kind compared to patients without any access. Results are displayed in Figure 17 and Figure 18.

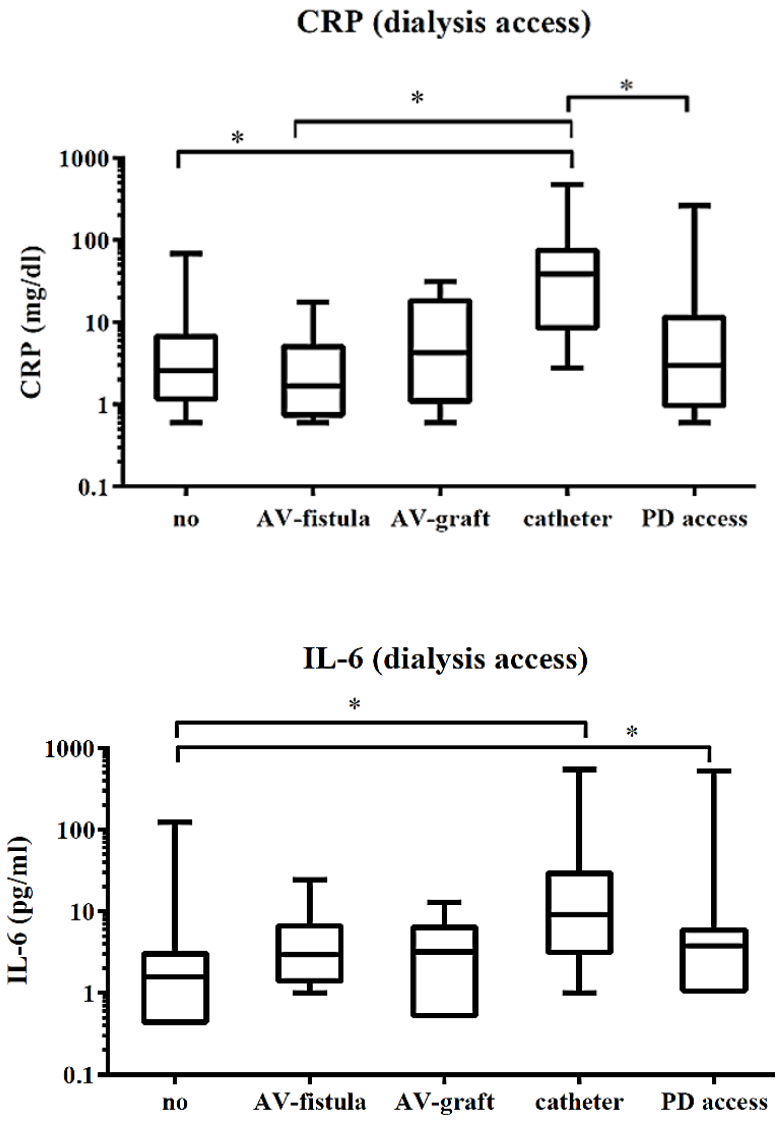

Figure 17 Proinflammatory mediators in patients with different dialysis accesses: CRP, C-reactive protein; IL-6, interleukin 6; AV, arterio-venous; PD, peritoneal dialysis; *, significant difference ($p<0.016$) a, significantly different to all displayed patient groups ($p<0.016$)

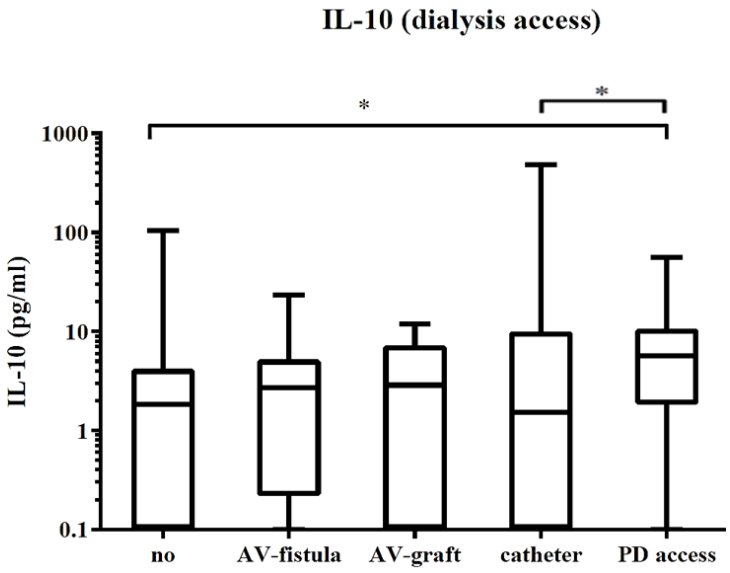

Figure 18 Anti-inflammatory cytokine IL-10 in patients with different dialysis accesses: IL-10, interleukin 10; AV, arterio-venous; PD, peritoneal dialysis; *, significant difference ($p<0.016$)

5.4. Phagoindex

Neutrophils from HD(F) patients and ARF showed a significantly lower phagocytic capacity compared to CKD patients ($p<0.001$), KT patients and healthy controls ($p\leq0.001$). CKD patients did not differ significantly between different CKD stages.

5.5. Protein energy wasting, volume overload

To investigate the prevalence of the PEW syndrome significant differences in total plasma protein and cholinesterase were tested. All patients had significantly lower total plasma protein ($p<0.001$) and cholinesterase ($p=0.002$) levels compared to healthy controls. HD(F) patients had significantly lower total plasma protein levels compared to KT patients ($p=0.001$) and significantly lower cholinesterase levels compared to CKD ($p<0.001$) and KT patients ($p=0.001$). Patients with ARF had significantly lower total plasma protein levels compared to CKD and KT patients ($p<0.001$) and significantly lower cholinesterase levels compared to CKD ($p<0.001$), PD ($p=0.001$) and KT patients ($p<0.001$). Albumin and cholinesterase correlated positively ($p<0.001$, $r=0.55$). Total plasma protein, albumin and cholinesterase levels did not differ significantly between different CKD stages, between HD, HDF and PD and between different dialysis access types. Results are displayed in Figure 19 and Figure 20.

The prevalence of oedema was assessed to estimate the extent of volume overload. CKD patients had a significantly higher frequency of oedema compared to HD(F) patients ($p=0.001$) and PD patients ($p<0.001$). CKD patients did not differ significantly in the frequency of oedema. Results are displayed in Table 7.

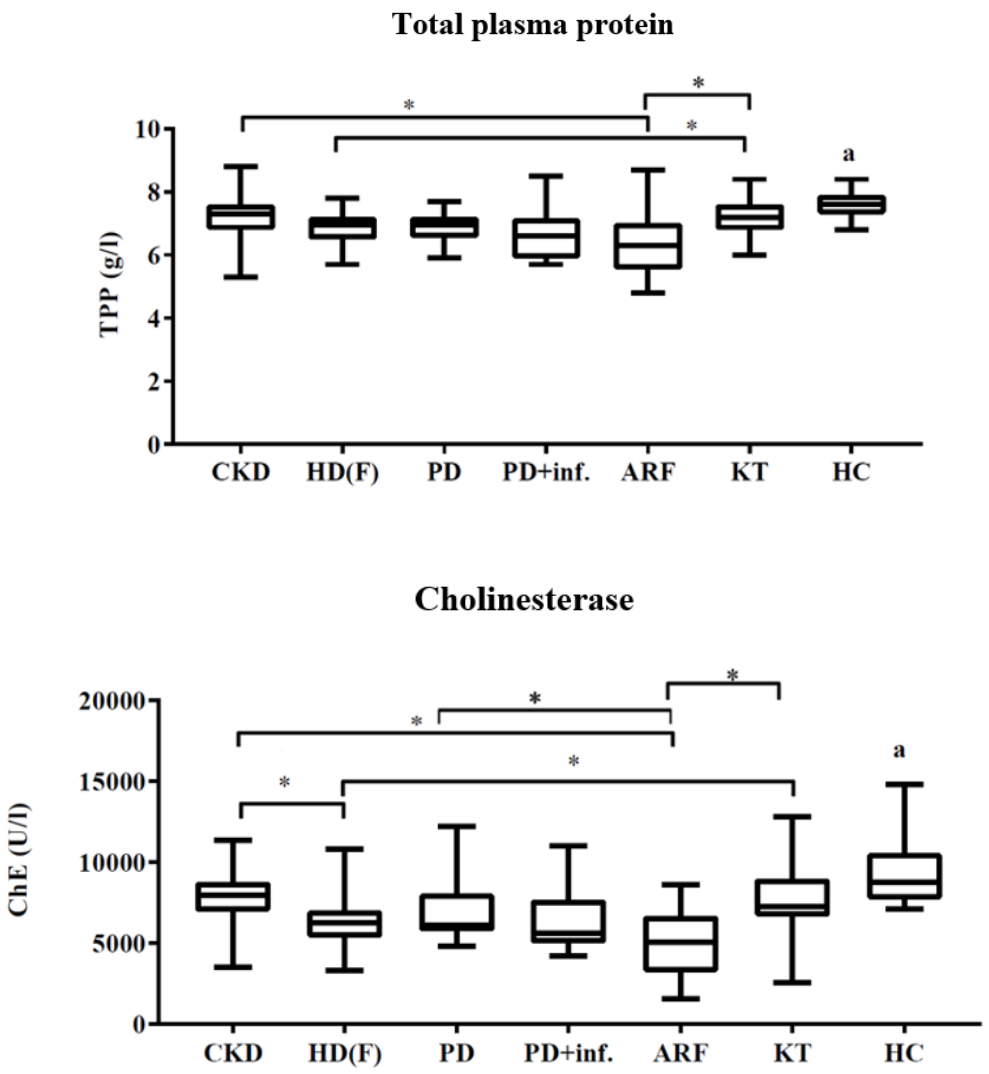

Figure 19 Total plasma protein and cholinesterase: TPP, total plasma protein; ChE, cholinesterase; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either HD or HDF; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate; HC, healthy controls; *, significant difference ($p \leq 0.002$); a, significantly different to all displayed patient groups ($p \leq 0.002$)

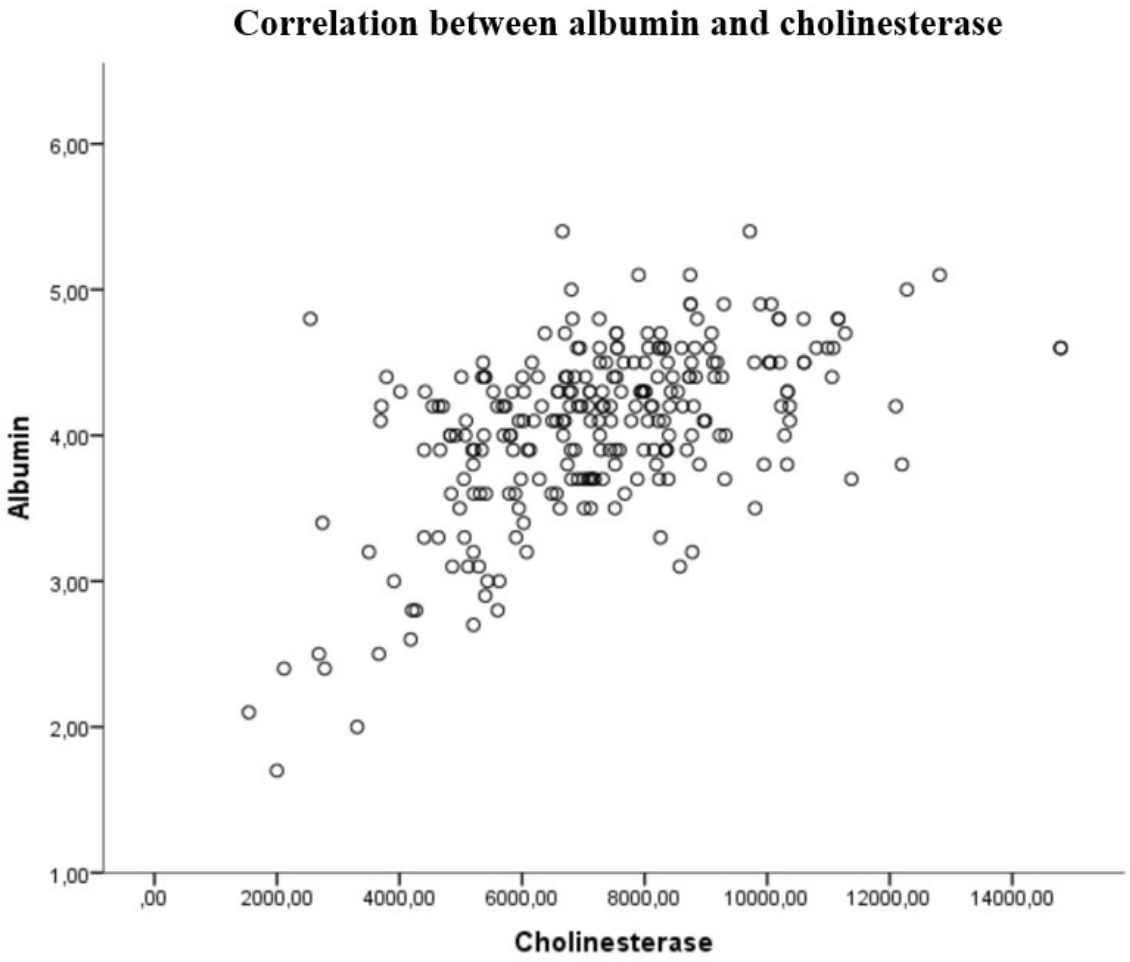

Figure 20 Correlation between albumin and cholinesterase: albumin significantly positively correlated to cholinesterase ($p<0.001$, $r=0.55$)

Table 7 Oedema

	no oedema		oedema	
HD(F)	20	(91%)	2	(9%)
PD	25	(93%)	2	(7%)
PD+inf.	7	(78%)	2	(22%)
ARF	13	(57%)	10	(43%)
KT	48	(73%)	18	(27%)
CKD	36	(51%)	34	(49%)
eGFR 30-45	18	(72%)	7	(27%)
15-30	15	(43%)	20	(57%)
<15	3	(30%)	7	(70%)

CKD, chronic kidney disease, eGFR<45; HD(F), patients received either HD or HDF; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate, percentages describe the percentage distribution within study groups.

5.6. Anaemia

All patients had significantly lower hb (p=0.001) levels compared to healthy controls. HD(F) patients had significantly lower hb levels compared to CKD and KT patients (p<0.001). PD+inf. patients had significantly lower hb levels compared to CKD (p<0.001), PD (p<0.001) and KT patients (p<0.001) and a significantly lower TRFSAT compared to CKD (p<0.001), HD(F) (p<0.001), PD (p<0.001), ARF (p=0.002), KT patients (p<0.001) and healthy controls (p<0.001). Patients with ARF had significantly lower hb levels compared to CKD and KT patients (p<0.001). Hb and TRFSAT did not differ significantly between different CKD stages, between HD, HDF and PD and between different dialysis access types. Results are displayed in Figure 21.

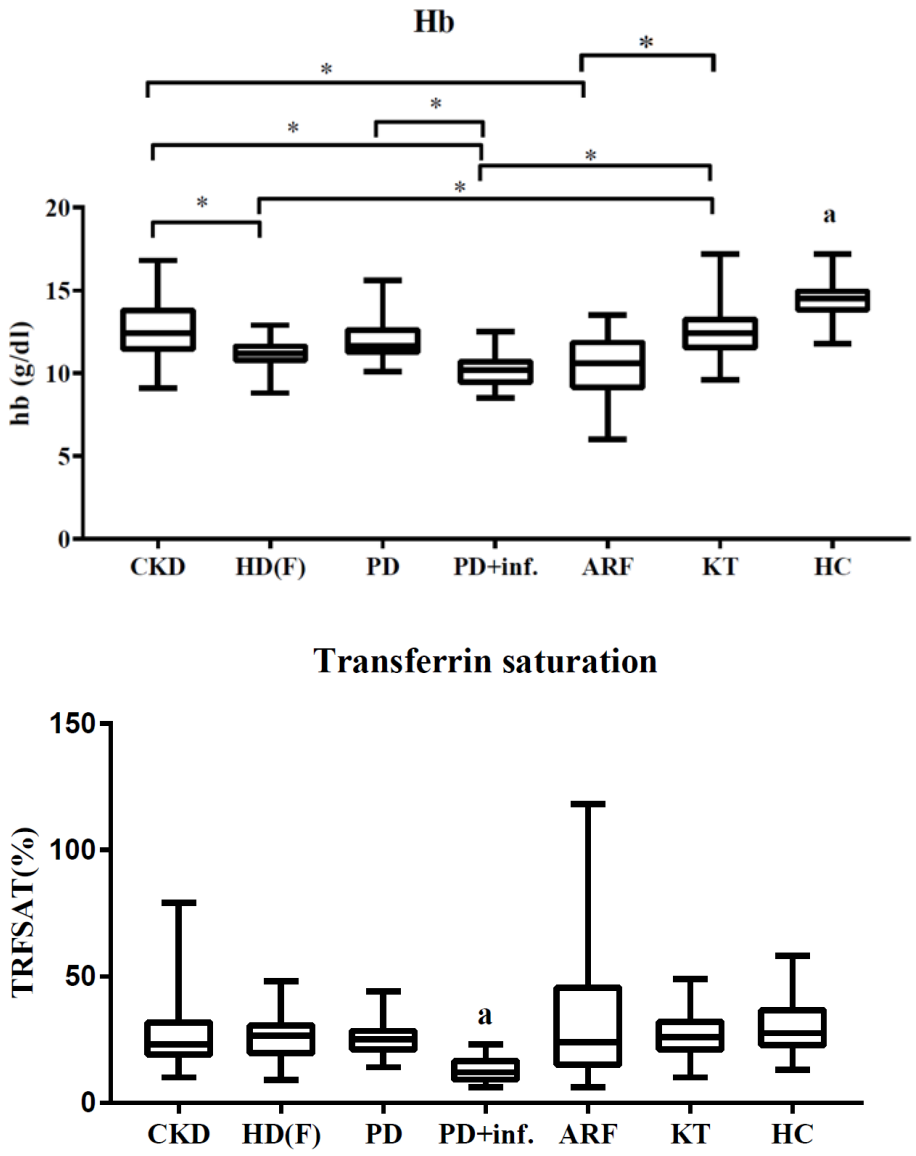

Figure 21 Anaemia: Hb, haemoglobin; TRFSAT, transferrin saturation; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either HD or HDF; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate; HC, healthy controls; *, significant difference ($p \leq 0.002$); a, significantly different to all displayed patient groups ($p \leq 0.002$)

PD patients were significantly more often treated with r-HuEPO compared to CKD patients ($p<0.001$), HDF ($p<0.001$), ARF ($p<0.001$) and KT patients ($p<0.001$). PD+inf were significantly more often treated with r-HuEPO compared to CKD ($p=0.002$), HD(F) ($p<0.001$) and KT patients ($p=0.001$). **Fehler! Ungültiger Eigenverweis auf Textmarke.** visualizes the results.

Table 8 r-HuEPO supplementation

	no r-HuEPO supplementation		r-HuEPO supplementation	
CKD	58	(80%)	15	(21%)
HD(F)	29	(94%)	2	(7%)
PD	11	(39%)	17	(61%)
PD+inf.	5	(39%)	8	(62%)
ARF	23	(96%)	1	(4%)
KT	56	(84%)	11	(16%)

r-HuEPO, recombinant human erythropoietin; CKD, chronic kidney disease, eGFR<45; HD(F), patients received either HD or HDF; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; eGFR, estimated glomerular filtration rate

5.7. Acellular growth retardation assay

All patients, except PD patients, showed significantly higher growth retardation capacities of their sera compared to healthy controls ($p<0.001$). CKD, HD(F), PD+inf. and ARF patients showed significantly higher growth retardation capacities of their sera compared to PD patients ($p=0.001$, $p=0.001$, $p<0.001$, $p=0.002$). CKD subgroups and patients with different dialysis access types did not differ significantly in the growth retardation capacities of their sera. The

serum induced growth retardation capacity was neither correlated to CRP levels, nor to albumin, nor to IL-6 or IL-10. Results are displayed in Figure 22-Figure 24.

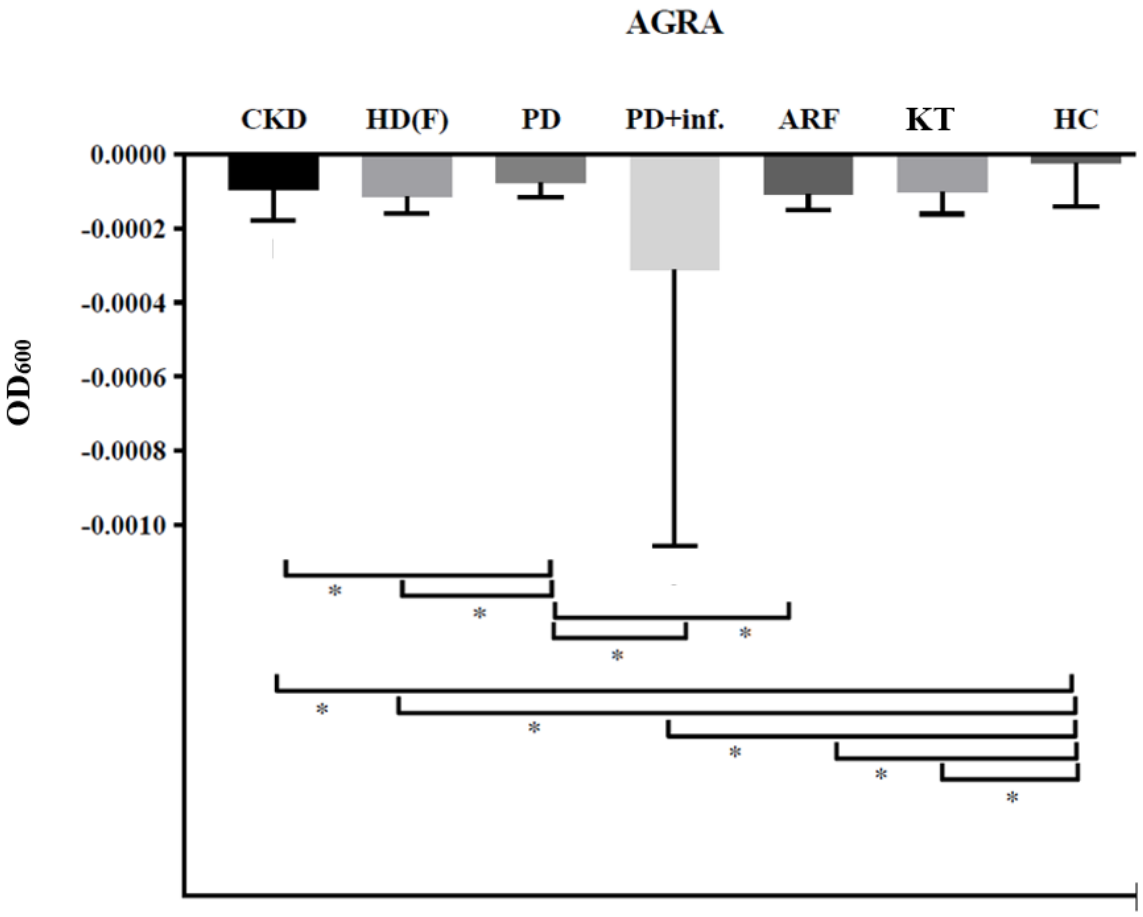

Figure 22 Serum induced growth retardation: CKD, eGFR<45; HD(F), patients received either haemodialysis or hemodiafiltration; PD, patients received peritoneal dialysis; PD+inf., patients received peritoneal dialysis and had peritonitis; ARF, patients with acute renal failure; KT, kidney transplanted patients; HC, healthy controls; eGFR, estimated glomerular filtration rate. OD_{600} levels of healthy controls were taken from a previously conducted study and not measured during this thesis.

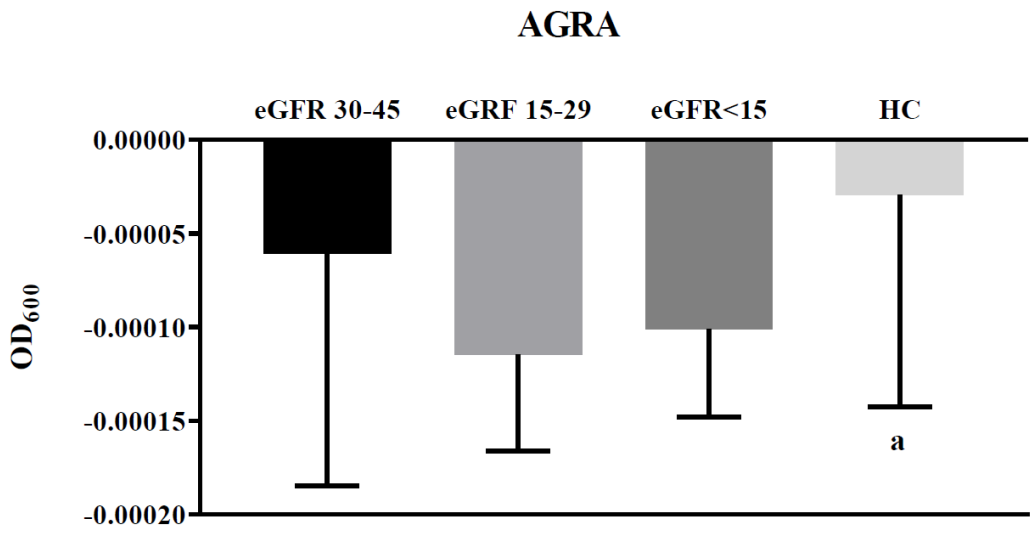

Figure 23 Serum induced growth retardation in CKD subgroups: eGFR, estimated glomerular filtration rate; HC, healthy controls; a, significantly different to all displayed patient groups ($p \leq 0.005$), OD_{600} levels of healthy controls were taken from a previously conducted study and not measured during this thesis.

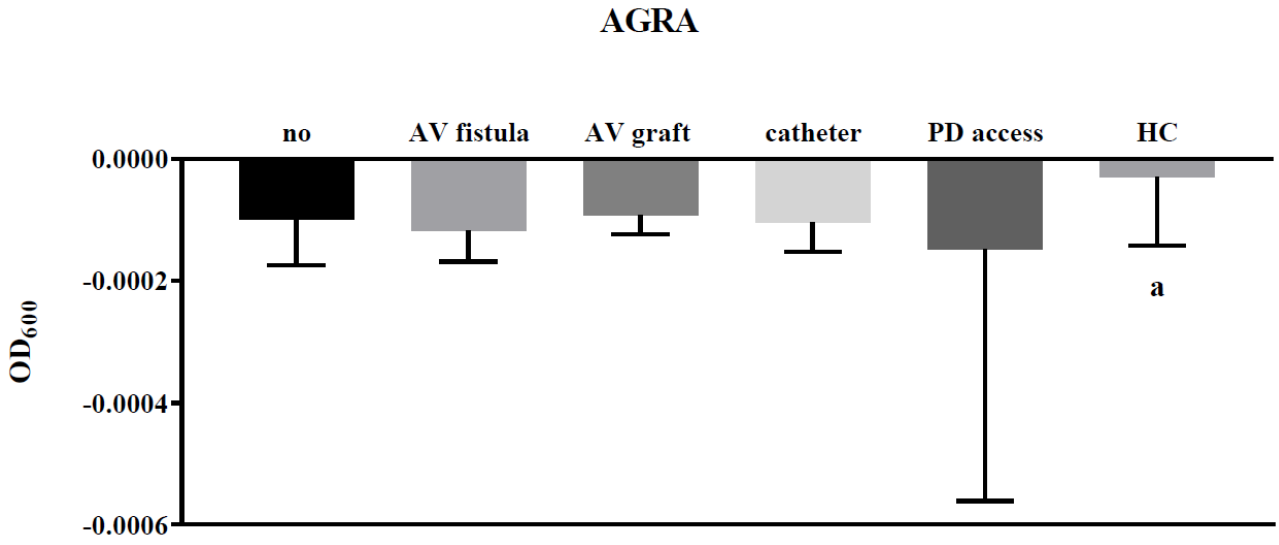

Figure 24 Serum induced growth retardation in different dialysis accesses: eGFR, estimated glomerular filtration rate. HC, healthy controls; a, significantly different to all displayed patient groups ($p \leq 0.016$), OD_{600} levels of healthy controls were taken from a previously conducted study and not measured during this thesis

5.8. Phagotest®

Since only two experiments were performed, no statistical analysis is possible. The percentage of phagocytosing N-like differentiated HL-60 cells as well as the number of phagocytosed bacteria was higher when incubated with higher LPS concentrations in both performed Phagotests®. Results are displayed in Table 9 and Figure 25.

Table 9 Percentage of phagocytosing N-like differentiated HL-60 cells

Phagocytosing cells			
Phagotest® 1		**Phagotest® 2**	
Control (no LPS)	35%	Control (no LPS)	23%
250ng/ml LPS	39%	250ng/ml LPS	27%
500ng/ml LPS	44%	500ng/ml LPS	42%

N, neutrophils; Phagotest® 1, first performed experiment; Phagotest® 2, second performed experiment. LPS, Lipopolysaccharide**;**

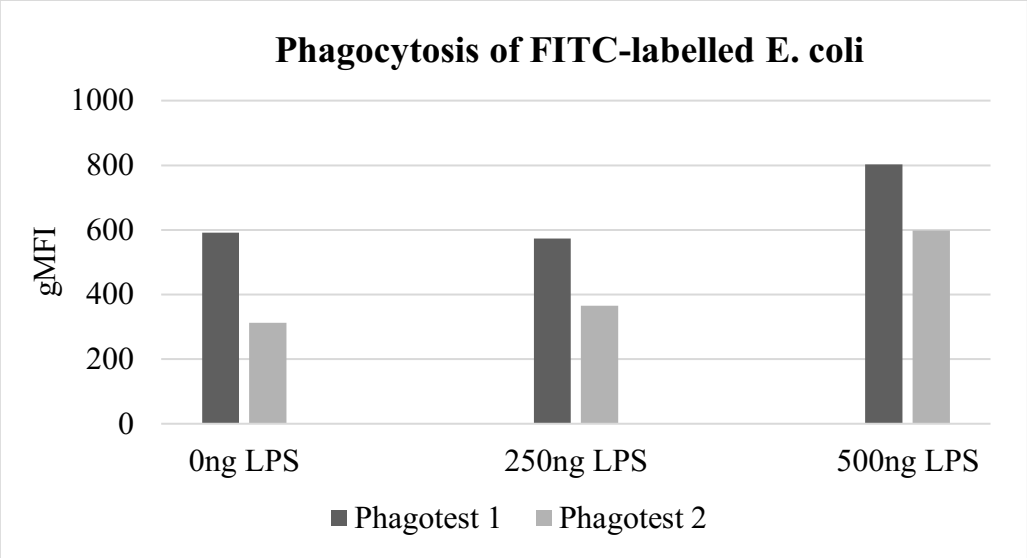

Figure 25 Phagocytic capacity: gMFI, geometric mean fluorescence intensity; LPS, lipopolysaccharide; Phagotest® 1, first performed experiment; Phagotest® 2, second performed experiment.

6. Discussion

The aim of this thesis was to investigate inflammation, immune defence as well as protein energy wasting, anaemia and complications in patients with renal disease. Clinical data and biomarker of inflammation, immune defence, energy wasting and anaemia were analysed from an existing patient cohort (ENARI cohort). Data from this study have already been published(14). For this diploma thesis, parts of the data have been reanalysed considering different aspects. Furthermore, patient sera were analysed in vitro for their capacity to retard growth of *E. coli*. We also evaluated whether neutrophil's phagocytosis is influenced by LPS in a model of in vitro differentiated neutrophil-like HL-60 cells.

We showed that all patients had significantly higher LPS binding protein levels and thereby confirmed the results of Nakamura et al., Kotera et al. and Kaden et al.(112-114) Nakamura et al. showed that patients with renal disease with and without sepsis had elevated LPS binding protein levels compared to healthy controls.(112) Kotera et al. showed significantly elevated sCD14 levels in ARF patients with or without infection.(113) Kaden at al. showed that KT patients had significantly elevated LBP levels.(114) We showed that HD(F) patients did not differ from CKD patients in LPS and LPS binding protein levels and that patients with a lower kidney function showed higher LBP levels. We thereby confirmed the study of Schindler et al., who showed that LBP levels did not rise in patients who received 4 h of HD compared to CKD patients.(115) Moreover, we confirmed the study of Pereira et al., who showed that LPS binding protein levels did not differ between different types of RRT and between patients receiving RRT and CKD patients. In addition, they showed that patients with lower kidney function had higher LBP levels, although LBP did not correlate to serum creatinine.(116) We showed that HD patients had significantly higher LPS levels and thereby confirmed the study of McIntry et al., who noticed that HD initiation increases systemic LPS concentration and that patients, who experienced haemodynamic changes, had the highest LPS levels.(60) We conclude that all patients with renal disease need to deal with translocated LPS, as all patients showed significantly elevated LPS binding protein levels. (112,113). LPS binding protein levels are regarded as surrogate markers for LPS levels lower than the detectable concentration.(14) In our studies, blood samples were collected before dialysis procedure, where patients were in a volume overloaded state. Therefore, we can hypothesize from our data that splanchnic hyper

perfusion contributes to LPS translocation. The reason why CKD and HD(F) patients have equal LPS binding protein levels, but HD(F) patients have higher LPS levels compared to CKD patients, might be due to frequent procedure related haemodynamic changes in HD(F) patients, which may enhance LPS translocation.(60) Another cause for high LPS levels in HD(F) patients might be the formation of biofilms in tunnelled catheters and in the fluid pathway of the dialysis machine (25) although others have shown that LPS from biofilms is only detectable at nanogram amounts at which a further cell activation could not be measured.(117) The reasons for higher LBP levels in patients with lower kidney function are unclear. The normal kidney might fulfil a role in the metabolism of this protein.(116) The consequence of increased LBP and sCD14 levels is increased LPS binding to the cellular surface and activation of neutrophils. Activated neutrophils release ROS and cytokines, which induce oxidative stress and inflammation. LBP is therefore regarded as an acute phase protein and sCD14 is considered as an early marker for the diagnosis of sepsis.(118)

We showed that all patients suffer from chronic inflammation, as all patients had significantly higher IL-6 and IL-10 levels. We therefore confirmed and extended the results of Santarpia et al., Goicoechea et al. and Xu et al., who showed that IL-6 levels were significantly elevated in patients with renal disease compared to healthy controls.(119-121). IL-6 is a proinflammatory cytokine and is released by neutrophils when they are activated via LPS. Therefore, it seems plausible that high systemic LPS concentrations induce high IL-6 production. Goldstein et al. showed that proinflammatory cytokines increased significantly after the onset of HD and PD, whereas anti-inflammatory cytokines did not differ significantly from healthy controls. PD and HD(F) patients did not differ significantly in IL-6, but PD patients had significantly higher IL-10 levels compared to HD(F) patients.(122) We confirmed the results of Goldstein et al. in our studies, although in our study both pro- and anti-inflammatory cytokines were elevated in patients receiving RRT. The reasons why HD(F) patients have a reduced IL-10 production compared to PD patients are unclear and might rely on an imbalance of cytokine production favouring the proinflammatory response.(122) We showed that patients with a catheter access had significantly higher CRP levels. Thereby, we support the results of the study of Powe et al., who reported that the use of tunnelled catheters is a leading risk factor for infections.(123) We conclude that the stimulation of neutrophils in patients with renal disease is linked to a release of pro- and anti-inflammatory cytokines. Cytokines initiate early steps of

atherosclerosis and thereby contribute to CVD. Previous studies showed that IL-6 is the best marker for CVD and mortality in patients with renal disease.(121,124,125) In addition, cytokines impair the IGF-1 receptor, which consequently leads to a disturbed muscle anabolism resulting in PEW. We conclude that patients with a catheter suffer from a higher inflammatory state and therefore may have an increased risk for CVD and PEW-syndrome.(121,124,125)

Albumin is the main serum protein and fulfils an important role in the serum mediated innate immune defence. It reacts as a scavenger for ROS as well as for toxic products.(28,123) The PEW-syndrome is the main reason for reduced albumin levels and will be discussed later. The combination of albumin oxidation and reduced albumin levels leads to reduced albumin mediated immune defence. Low functioning albumin levels might not achieve appropriate binding of ROS, LPS and toxins, which may result in a higher risk for infections, oxidative stress and CVD.(80,81) CRP production in the liver rises in acute infection, inflammation as well as upon stimulation by LPS. CRP induces early steps of atherosclerosis and thereby contributes to an increased risk for CVD.(2,18) We showed that all patients with renal disease had significantly lower albumin levels and all patients, except KT patients, showed a significantly higher CRP:albumin ratio. Thereby, we confirmed the results of previous studies. Santarpia and Xu et al. showed that patients with renal disease had significantly lower serum albumin levels compared to healthy controls.(119,120) Kaysen et al. showed that low albumin concentrations in HD patients were associated to high CRP levels.(126) Xu and Cachofeiro et al. showed that patients with renal disease had significantly higher CRP levels compared to healthy controls.(2,120) We showed that PD+inf. patients and HD(F) patients had significantly higher CRP levels and thereby confirmed the study of Gonclaves and Stenvinkel et al., who showed that CRP levels correlated directly with the duration of HD and demonstrated elevated plasma CRP levels during dialysis sessions.(31,127) High CRP levels in HD(F) patients derive from increased LPS translocation from the gut, which consequently triggers CRP production in the liver. PD+inf. patients have higher CRP levels because of the acute infection, which triggers CRP release. We conclude that all patients with renal disease have an increased risk for CVD, because of a significantly elevated CRP:albumin ratio. Wong et al. showed that CRP:albumin ratio is an independent risk factor for mortality in patients with sepsis (128) and Zimmermann et al. observed that a high CRP:albumin ratio predicts mortality in HD patients.(129)

In our studies all patients had significantly lower lymphocyte counts and all patients, except PD patients, had significantly higher neutrophil counts compared to healthy controls. All patients had a significantly higher neutrophil:lymphocyte ratio compared to healthy controls. We thereby confirmed the results of previous studies, which showed that patients with renal disease had significantly higher neutrophils and lower lymphocytes compared to healthy controls.(130,131) Okyay et al. showed that HD patients had higher neutrophil:lymphocyte ratios compared to healthy controls as well as patients with CVD compared to patients without CVD. Furthermore, they showed that the neutrophil:lymphocyte ratio is positively correlated to IL-6 and CRP and negatively to albumin and hb.(132) The most common cause for lymphopenia is infection and inflammation, which induce a percental shift of leukocytes to neutrophils and reduces the percentage of lymphocytes. In addition, protein-calorie malnutrition induces lymphopenia.(133) A higher neutrophil:lymphocyte ratio is associated with a higher risk for oxidative stress, inflammation and CVD, as an increased number in neutrophils is linked to an increased production in ROS and cytokines. Yilmaz et al. showed that patients with a higher neutrophil:lymphocyte ratio had a poorer outcome and a higher risk for CVD. Neutrophil:lymphocyte ratio is superior to CRP and leukocytes a predictor for ARF in septic patients.(134)

The PEW syndrome describes a hypermetabolic condition in patients with renal disease. Elicitors are repeated dialysis procedures, inflammation, reduced protein intake and protein loss via the dialysate. The consequences are weight loss and a reduction in total plasma protein, which comprises albumin. Therefore, the PEW syndrome results in a reduced albumin mediated immune defence and an increased oxidative stress. Cholinesterase is physiologically produced in the liver and reflects the availability of amino acids for protein production. Reduced cholinesterase levels are linked to reduced protein levels.(119) This could be confirmed in our study, as we showed a positive correlation between albumin and cholinesterase. We hypothesize that all patients suffer from PEW as all patients had significantly lower total plasma protein, albumin and cholinesterase levels. Stojanov et al. showed that cholinesterase is a surrogate marker for CVD and mortality and positively correlated to albumin and negatively to CRP.(135) Santarpia et al. showed that inflammation reduces cholinesterase levels, as IL-6 levels are inversely correlated to cholinesterase.(119) Waterlow et al. showed that cholinesterase levels are reduced in patients with renal disease.(95) The fact that patients with renal disease have

lower albumin levels has been confirmed above. Our data suggest that the type of RRT and the stage of CKD does not affect the extent of PEW, as there was no significant difference in total plasma protein levels, albumin and cholinesterase between different RRT and between different CKD stages. We did not use the BMI value to estimate the extent of PEW, as BMI levels were normal or even higher in investigated patients with renal disease. Previous literature showed that neither low BMI nor a loss of BMI predicts mortality in ESRD patients.(136) Causes for high BMI levels in our study population might be on the one hand that the weight measurement before RRT is biased by volume overload. On the other hand, also overweight patients might suffer from obese sarcopenia because of the proinflammatory properties of abdominal fat.(137) We have proven the prevalence of anaemia in all patients with renal disease and thereby confirmed the studies of Drueke et al., who showed that patients with renal disease suffer from anaemia.(78) TRFSAT levels were significantly reduced in PD+inf. patients compared to all other study groups. PD+inf. patients have therefore been significantly more often treated with rHuEPO. We showed in our studies that HD patients suffer more often from CVD and DMII compared to HDF patients. Thereby, we confirmed results of Jia et al., who showed that HD patients had significantly higher markers of endothelial cell damage than HDF patients did. A single 2-week HDF session decreased markers of endothelial cell injury. (138) Therefore, we conclude, that HD patients have a higher risk for CVD compared to HDF patients.

We also studied a newly developed biomarker of cell free immune response in this cohort, that has been shown to predict infections in liver cirrhosis (EP 17 181 908).(139) We hypothesized that also patients with renal disease will show a reduced ability of their serum to kill pathogens. Controversially to our hypothesis that the increased susceptibility to infections is caused by a reduced ability of the serum to kill pathogens, our studies showed that all patients, except PD patients, had a significantly higher growth retardation compared to healthy controls. PD+inf. patients had the highest growth retardation, although not significantly. There are a few studies that previously investigated other markers of serum mediated growth retardation of bacteria in patients with renal disease. Bertazzoni et al. investigated the growth retardation of *proteus rettgeri* in sera from patients with renal disease. They showed that in the presence of serum, either from healthy controls or patients with renal disease, bacterial growth was delayed. All investigated patients had a reduced growth retardation compared to healthy controls, whereby CKD patients showed a slight decrease in growth retardation and HD and PD patients

showed a significant reduction in growth retardation compared to healthy controls. Although they found a correlation between creatinine and growth retardation, patients with a severe uraemia showed only a slight decrease in the growth retardation. In addition, they investigated heat inactivated sera (at 56°C, 30 min) and found out that sera from healthy controls and PD patients lost their growth retardation capacity, whereas heat inactivated sera of CKD and HD patients had a residual growth retardation.(140) Montgomery et al. examined the ability of uremic sera to kill *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis*. They showed that uremic sera killed *Bacillus subtilis* more readily than sera from healthy controls. They showed that uremic sera and normal sera did not differ significantly in killing *E. coli*, *Staphylococcus aureus* and *Streptococcus faecalis*.(141) Kays et al. investigated serum of HD patients for their bactericidal effect towards *E. coli* and *Pseudomonas aeruginosa* and found out that neither sera from HD nor sera from healthy controls could kill the bacteria.(142) Taylor et al. showed that sera supplemented with additional antibodies and complement factors had a higher bacterial growth retardation.(29) Crokaert et al. reported that serum mediated bacterial killing or growth retardation is highly dependent upon in vitro conditions, such as growth medium, serum concentration, buffer, temperature, metabolic state of bacteria and experiment duration.(143)

Our results are not directly comparable to previous studies, as we used a newly developed biomarker for the prediction of infections in patients with renal disease. Further studies are necessary to see if the newly developed biomarker predicts infections in patients with renal disease. Reasons for divergent results derive from different analysed parameters, different investigated bacteria and different investigated serum concentrations. Bertazzoni et al. used 10% serum, whereas we used 5% serum. Taylor et al. reported that bacterial growth retardation in sera from patients with inflammation is insignificant at a dilution of serum greater than 1:16 (lower than 6%). Serum concentrations between 20% and 100% are suitable.(29) The time of incubation might have influenced the result, as Bertazzoni et al. incubated samples for 36h and we did for 5h. In addition, the number of bacteria influenced the result. Taylor et al reported that many studies use bacteria from an overnight culture at an undefined metabolic state and an undefined count. They showed that when bacterial growth reaches the logarithmic phase, they had the highest susceptibility to serum induced growth retardation. In our studies, results from healthy controls were measured previously and not during this thesis. Although the same experimental setup was used, results might differ between two laboratory tests. For example, in

both tests bacteria were cultivated in overnight culture and used in an undefined metabolic state. One cause for the higher growth retardation in all patients with renal disease and especially in PD+inf. patients could be the prevalent inflammatory state. Taylor et al. showed that immune system activation, accompanied with higher amounts of antibodies and complement factors in serum, might be linked to a higher serum mediated bacterial killing.(29) Another cause for the high serum growth retardation capacities in our study might have been bacterial agglutination. The growth retarding has then been overestimated.(29) We conclude that further experiments are necessary to show whether results are reproducible. Moreover, we need to modify technical conditions, including type of bacteria, time of incubation and serum concentrations, to see if results change. (29,141,143) The reasons why sera from PD patients and healthy controls did not differ significantly remain unclear and need further investigations.

We showed in a preliminary experiment that neutrophil-like differentiated HL-60 cells had a higher percentage in phagocytosing cells as well as a higher number in phagocytosed bacteria when exposed to higher LPS concentrations. We confirmed in our studies the results of previous studies, which showed that LPS up to a concentration of 1µg/ml has a stimulating effect on neutrophil phagocytosis.(144,145) Results about the effect of LPS concentrations higher than 1µg/ml were divergent. Prokhorenko et al. showed that phagocytic activity was higher in neutrophils pre-exposed to 100 ng/ml LPS (from *E. coli* O55:B5 or *E. coli* JM103), for 30 min at 37°C. Böhmer et al. reported that neutrophils had a higher phagocytosis at LPS (LPS from *E. coli* O55:B5) levels from 0.1 to 100 ng/ml compared to cells not exposed to LPS. The priming effect was lost above LPS concentrations of 1 µg/ml, at which no higher or even a reduced phagocytosis rate was observed.(144) Cohn and Morse et al. investigated the concentration dependent effect of LPS (AE1688S4 and AE1298S4) on leukocyte's phagocytosis of *Staphylococcus albus*. They showed that LPS concentrations of 0.1-1µg/ml had a more marked influence on leukocyte's phagocytosis than concentrations of 10-50 µg/ml.(145) Conversely to Cohn and Morse et al., Proctor et al. showed that the percentage in phagocytosing neutrophils and the number of phagocytosed *Staphylococcus aureus* and *E. coli* was not affected in the presence of 10-100 µg/ml LPS (O111:B4).(146) Previous studies reported that the stimulating effect of LPS on neutrophil's phagocytosis could just be shown in serum containing test tubes, as serum constituents, including complement factors, sCD14 and LBP, are necessary for LPS to be able to react with the neutrophil.(29) Prokherenko et al. investigated whether the pre-

treatment with 1µg anti-human CD14 and anti TLR4 for 30 min at 37°C influences neutrophil's phagocytosis. They showed that the administration of anti-human CD14 and TLR4 resulted in a decreased phagocytic activity, concluding that serum constituents CD14 and TLR4 are required for phagocytosis.(147) Cohn and Morse et al. showed that leukocytes had a higher phagocytosis in the presence of LPS and 10% serum than at lower serum concentrations or in the absence of serum.(145) We conclude that LPS up to at least 500 ng/ml has a stimulating effect on neutrophil's phagocytosis. The reasons why neutrophils show higher phagocytosis after being exposed to serum and LPS are still not fully understood and need further investigation. We used the same serum concentration as previous studies. A lower serum concentration would have probably changed the result, as 10% of serum are required.(145) The type of LPS used seemed not to have any effect, as results did not differ between studies using different LPS types. Although we used a model of in vitro cultivated neutrophil like HL-60 cells, our results were comparable to neutrophils derived from whole blood.(146) Further experiments at LPS concentrations of 1-100 µg/ml are required to prove results of Cohn and Morse et al. and show whether LPS at this concentration has a stimulating or inhibiting effect on neutrophil's phagocytosis. To investigate the effect of in vivo conditions on neutrophil's phagocytosis, we evaluated neutrophil phagocytosis in the ENARI study population. We showed that ARF and HD(F) patients had a significantly lower phagocytosis capacity compared to CKD, KT patients and healthy controls and thereby we confirmed previous studies. Wierusz-Wysocka et al. showed that neutrophils derived from patients with renal disease had a reduced bactericidal capacity. They reported that additional complications in patients with renal disease impair neutrophils and reduce phagocytic capacity.(148) Ando et al. and Anders et al. showed that the repeated exposure to high LPS concentrations shifts neutrophils to a refractory state with subsequent reduced TLR4 expression.(50,149) Therefore, it seems to be plausible that in our study HD(F) patients, who had the highest LPS concentrations, had a significantly lower phagoindex. Another cause for cellular exhaustion might be the repeated interaction with the dialysis membrane.(14) Wierusz-Wysocka et al. showed that PD patients had a significantly lower phagocytosis compared to HD patients.(148) Dysfunctional neutrophils in patients with renal disease may not be able to achieve immune defence any longer and show an increased release of ROS, which contribute to the oxidation of albumin. Oxidized albumin cannot achieve

scavenging of pathogens, ROS and LPS, which reactivate neutrophils and induce neutrophil dysfunction.

7. Summary and conclusion

The aim of this study was 1) to investigate whether patients with renal disease show signs for elevated LPS levels, inflammation and PEW, 2) whether patients with renal disease differ in their serum induced growth retardation capacity from healthy controls and 3) whether neutrophils phagocytosis is influenced by LPS. Previous studies showed that LPS activates neutrophils to produce ROS and cytokines. It reacts itself as an ROS and together with ROS released from neutrophils induces early steps of atherosclerosis and oxidatively modifies albumin. Reduced serum albumin levels because of PEW and oxidation of residual albumin leads to insufficient albumin mediated ROS scavenging and oxidant defence, which consequently might result in a reduced immune defence and oxidative stress. Cytokines induce a chronic inflammatory state and contribute to the development of PEW and atherosclerosis. The abundance of LPS, ROS and cytokines triggers frequent cardiovascular events in patients with renal disease.

We conclude that all investigated patients with renal disease need to deal with high systemic LPS concentrations, as the surrogate markers for LPS, LPS binding protein levels sCD14 and LBP, were significantly elevated. We suggest that dialysis induced volume changes enhance LPS translocation from the gut, as HD(F) patients have significantly higher LPS concentrations and CRP levels compared to healthy controls. We conclude that all patients have an elevated state of chronic inflammation, verified by elevated levels of pro and anti-inflammatory cytokines and neutrophils. We conclude that all patients with renal disease have a higher risk for CVD, confirmed by elevated biomarkers for CVD, neutrophil:lymphocyte ratio and CRP:albumin ratio. We suggest that patients with a catheter have an increased inflammatory state and consequent complications, as they showed higher markers of CRP and IL-6.

We confirmed the presence of the PEW in the ENARI cohort, as our data showed significantly lower cholinesterase, albumin and total plasma protein levels in all patients. We investigated a newly developed biomarker, which describes the serum induced bacterial growth

retardation and which was shown to predict infections in patients with liver cirrhosis. Controversially to our hypothesis, our data showed that patients with renal disease have a higher serum induced bacterial growth retardation compared to healthy controls. Therefore, we conclude that further studies are necessary to see if the biomarker predicts infection in patients with renal disease. We conclude that in vitro differentiated neutrophil like HL-60 cells show a higher phagocytosis when exposed to higher levels of LPS. However, neutrophils derived from HD(F) patients from the ENARI study population, who had the highest LPS concentration, showed the lowest phagocytosis. Therefore, we conclude that further studies about the influence of LPS on the phagocytosis of neutrophils are necessary. Regarding the expanding population suffering from renal disease and the associated high inflammation, consequent CVD and mortality rates, further research on risk factors, preventive strategies and therapies is required to reduce this burden to patients, families and the health care system.

1. References

(1) Karl Skorecki, Glenn M. Chertow, Philip A. Marsden, Maarten W. Taal, Alan S. L. Yu. Brenner and Rector's The Kidney. 10th ed.; 2015.

(2) Cachofeiro V, Goicochea M, de Vinuesa SG, Oubina P, Lahera V, Luno J. Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease. Kidney Int Suppl 2008 Dec;(111):S4-9. doi(111):S4-9.

(3) Goncalves S, Pecoits-Filho R, Perreto S, Barberato SH, Stinghen AE, Lima EG, et al. Associations between renal function, volume status and endotoxaemia in chronic kidney disease patients. Nephrol Dial Transplant 2006 Oct;21(10):2788-2794.

(4) Kato S, Chmielewski M, Honda H, Pecoits-Filho R, Matsuo S, Yuzawa Y, et al. Aspects of immune dysfunction in end-stage renal disease. Clin J Am Soc Nephrol 2008 Sep;3(5):1526-1533.

(5) Lentine KL, Brennan DC, Schnitzler MA. Incidence and predictors of myocardial infarction after kidney transplantation. J Am Soc Nephrol 2005 Feb;16(2):496-506.

(6) Foley RN, Parfrey PS, Sarnak MJ. Epidemiology of cardiovascular disease in chronic renal disease. J Am Soc Nephrol 1998 Dec;9(12 Suppl):S16-23.

(7) Ramezani A, Raj DS. The gut microbiome, kidney disease and targeted interventions. J Am Soc Nephrol 2014 Apr;25(4):657-670.

(8) Poesen R, Viaene L, Verbeke K, Claes K, Bammens B, Sprangers B, et al. Renal clearance and intestinal generation of p-cresyl sulfate and indoxyl sulfate in CKD. Clin J Am Soc Nephrol 2013 Sep;8(9):1508-1514.

(9) Cohen G, Horl WH. Immune dysfunction in uremia—an update. Toxins (Basel) 2012 Oct 24;4(11):962-990.

(10) Aronov PA, Luo FJG, Plummer NS, Quan Z, Holmes S, Hostetter TH, et al. Colonic Contribution to Uremic Solutes. J Am Soc Nephrol 2011 Sep;22(9):1769-1776.

(11) Lau WL, Kalantar-Zadeh K, Vaziri ND. The Gut as a Source of Inflammation in Chronic Kidney Disease. Nephron 2015;130(2):92-98.

(12) Kalantar-Zadeh K, Ikizler TA, Block G, Avram MM, Kopple JD. Malnutrition-inflammation complex syndrome in dialysis patients: causes and consequences. Am J Kidney Dis 2003 Nov;42(5):864-881.

(13) Sabatino A, Regolisti G, Brusasco I, Cabassi A, Morabito S, Fiaccadori E. Alterations of intestinal barrier and microbiota in chronic kidney disease. Nephrol Dial Transplant 2015 Jun;30(6):924-933.

(14) Lemesch S, Ribitsch W, Schilcher G, Spindelbock W, Hafner-Giessauf H, Marsche G, et al. Mode of renal replacement therapy determines endotoxemia and neutrophil dysfunction in chronic kidney disease. Sci Rep 2016 Oct 4;6:34534.

(15) Sureshbabu A, Ryter SW, Choi ME. Oxidative stress and autophagy: crucial modulators of kidney injury. Redox Biol 2015;4:208-214.

(16) Felizardo RJ, Castoldi A, Andrade-Oliveira V, Camara NO. The microbiota and chronic kidney diseases: a double-edged sword. Clin Transl Immunology 2016 Jun 10;5(6):e86.

(17) Kato S, Chmielewski M, Honda H, Pecoits-Filho R, Matsuo S, Yuzawa Y, et al. Aspects of immune dysfunction in end-stage renal disease. Clin J Am Soc Nephrol 2008 Sep;3(5):1526-1533.

(18) Arasthéh K, Baenkler HW, Bieber C, Brandt R, Chatterjee T. Innere Medizin. 3rd ed. Stuttgard: Thieme Duale Reihe; 2013.

(19) Susantitaphong P, Siribamrungwong M, Jaber BL. Convective therapies versus low-flux hemodialysis for chronic kidney failure: a meta-analysis of randomized controlled trials. Nephrol Dial Transplant 2013 Nov;28(11):2859-2874.

(20) Meert N, Eloot S, Schepers E, Lemke HD, Dhondt A, Glorieux G, et al. Comparison of removal capacity of two consecutive generations of high-flux dialysers during different treatment modalities. Nephrol Dial Transplant 2011 Aug;26(8):2624-2630.

(21) Arici M. 'Ideal criteria' for starting chronic hemodialysis: numbers, symptoms or an alerting 'traffic light' system? Nephron Clin Pract 2012;120(1):c17-24.

(22) Canaud B, Vienken J, Ash S, Ward RA, Kidney Health Initiative HDF Workgroup. Hemodiafiltration to Address Unmet Medical Needs ESKD Patients. Clin J Am Soc Nephrol 2018 Mar 6.

(23) Bello AK, Levin A, Tonelli M, Okpechi IG, Feehally J, Harris D, et al. Assessment of Global Kidney Health Care Status. JAMA 2017 May 9;317(18):1864-1881.

(24) Tattersall JE, Ward RA, EUDIAL group. Online haemodiafiltration: definition, dose quantification and safety revisited. Nephrol Dial Transplant 2013 Mar;28(3):542-550.

(25) Girndt M, Kuhlmann M, Köhler H. Aktuelle Behandlungsstrategien in der Hämodialyse. 1st ed.: UNI-MED; 2005.

(26) Giles S, Czuprynski C. Novel role for albumin in innate immunity: serum albumin inhibits the growth of Blastomyces dermatitidis yeast form in vitro. Infect Immun 2003 Nov;71(11):6648-6652.

(27) Behrends JC, Bischofberger J, Deutzmann R. Physiologie. 2nd ed. Stuttgard: Thieme Duale Reihe; 2012.

(28) Arroyo V, Garcia-Martinez R, Salvatella X. Human serum albumin, systemic inflammation and cirrhosis. J Hepatol 2014 Aug;61(2):396-407.

(29) Taylor PW, Kroll HP. Interaction of human complement proteins with serum-sensitive and serum-resistant strains of Escherichia coli. Mol Immunol 1984 Jul;21(7):609-620.

(30) Tecchio C, Micheletti A, Cassatella MA. Neutrophil-derived cytokines: facts beyond expression. Front Immunol 2014 Oct 21;5:508.

(31) Goncalves S, Pecoits-Filho R, Perreto S, Barberato SH, Stinghen AE, Lima EG, et al. Associations between renal function, volume status and endotoxaemia in chronic kidney disease patients. Nephrol Dial Transplant 2006 Oct;21(10):2788-2794.

(32) Stenvinkel P, Ketteler M, Johnson RJ, Lindholm B, Pecoits-Filho R, Riella M, 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 Apr;67(4):1216-1233.

(33) Stenvinkel P. Inflammatory and atherosclerotic interactions in the depleted uremic patient. Blood Purif 2001;19(1):53-61.

(34) Lüllmann-Rauch R, Paulsen F. Taschenlehrbuch Histologie. 4th ed.: Thieme; 2009.

(35) Segal AW. How neutrophils kill microbes. Annu Rev Immunol 2005;23:197-223.

(36) Portaluppi F, Boari B, Manfredini R. Oxidative stress in essential hypertension. Curr Pharm Des 2004;10(14):1695-1698.

(37) Dou L, Jourde-Chiche N, Faure V, Cerini C, Berland Y, Dignat-George F, et al. The uremic solute indoxyl sulfate induces oxidative stress in endothelial cells. J Thromb Haemost 2007 Jun;5(6):1302-1308.

(38) Lewis SL, Van Epps DE. Neutrophil and monocyte alterations in chronic dialysis patients. Am J Kidney Dis 1987 May;9(5):381-395.

(39) Dahlgren C, Karlsson A. Respiratory burst in human neutrophils. J Immunol Methods 1999 Dec 17;232(1-2):3-14.

(40) Dahan S, Roth-Walter F, Arnaboldi P, Agarwal S, Mayer L. Epithelia: lymphocyte interactions in the gut. Immunol Rev 2007 Feb;215:243-253.

(41) Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol 2010 Mar;10(3):159-169.

(42) Neutra MR, Mantis NJ, Kraehenbuhl JP. Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat Immunol 2001 Nov;2(11):1004-1009.

(43) Mabbott NA, Donaldson DS, Ohno H, Williams IR, Mahajan A. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. Mucosal Immunol 2013 Jul;6(4):666-677.

(44) Wing MR, Patel SS, Ramezani A, Raj DS. Gut microbiome in chronic kidney disease. Exp Physiol 2016 Apr 1;101(4):471-477.

(45) Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature 2012 Sep 13;489(7415):242-249.

(46) Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science 2005 Jun 10;308(5728):1635-1638.

(47) Vaziri ND. CKD impairs barrier function and alters microbial flora of the intestine: a major link to inflammation and uremic toxicity. Curr Opin Nephrol Hypertens 2012 Nov;21(6):587-592.

(48) Allen JM, Light JE, Perotti MA, Braig HR, Reed DL. Mutational meltdown in primary endosymbionts: selection limits Muller's ratchet. PLoS One 2009;4(3):e4969.

(49) Martin FP, Sprenger N, Yap IK, Wang Y, Bibiloni R, Rochat F, et al. Panorganismal gut microbiome-host metabolic crosstalk. J Proteome Res 2009 Apr;8(4):2090-2105.

(50) Anders HJ, Andersen K, Stecher B. The intestinal microbiota, a leaky gut and abnormal immunity in kidney disease. Kidney Int 2013 Jun;83(6):1010-1016.

(51) Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature 2012 Jul 5;487(7405):104-108.

(52) Krishnamurthy VM, Wei G, Baird BC, Murtaugh M, Chonchol MB, Raphael KL, et al. High dietary fiber intake is associated with decreased inflammation and all-cause mortality in patients with chronic kidney disease. Kidney Int 2012 Feb;81(3):300-306.

(53) Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature 2007 Oct 18;449(7164):804-810.

(54) Iwase T, Uehara Y, Shinji H, Tajima A, Seo H, Takada K, et al. Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization. Nature 2010 May 20;465(7296):346-349.

(55) Vaziri ND, Wong J, Pahl M, Piceno YM, Yuan J, DeSantis TZ, et al. Chronic kidney disease alters intestinal microbial flora. Kidney Int 2013 Feb;83(2):308-315.

(56) Strid H, Simren M, Stotzer PO, Ringstrom G, Abrahamsson H, Bjornsson ES. Patients with chronic renal failure have abnormal small intestinal motility and a high prevalence of small intestinal bacterial overgrowth. Digestion 2003;67(3):129-137.

(57) Long CL, Jeevanandam M, Kinney JM. Metabolism and recycling of urea in man. Am J Clin Nutr 1978 Aug;31(8):1367-1382.

(58) Vaziri ND, Freel RW, Hatch M. Effect of chronic experimental renal insufficiency on urate metabolism. J Am Soc Nephrol 1995 Oct;6(4):1313-1317.

(59) Vaziri ND, Yuan J, Rahimi A, Ni Z, Said H, Subramanian VS. Disintegration of colonic epithelial tight junction in uremia: a likely cause of CKD-associated inflammation. Nephrol Dial Transplant 2012 Jul;27(7):2686-2693.

(60) McIntyre CW, Harrison LE, Eldehni MT, Jefferies HJ, Szeto CC, John SG, et al. Circulating endotoxemia: a novel factor in systemic inflammation and cardiovascular disease in chronic kidney disease. Clin J Am Soc Nephrol 2011 Jan;6(1):133-141.

(61) Yu AW, Nawab ZM, Barnes WE, Lai KN, Ing TS, Daugirdas JT. Splanchnic erythrocyte content decreases during hemodialysis: a new compensatory mechanism for hypovolemia. Kidney Int 1997 Jun;51(6):1986-1990.

(62) Bossola M, Sanguinetti M, Scribano D, Zuppi C, Giungi S, Luciani G, et al. Circulating bacterial-derived DNA fragments and markers of inflammation in chronic hemodialysis patients. Clin J Am Soc Nephrol 2009 Feb;4(2):379-385.

(63) Vaziri ND, Zhao YY, Pahl MV. Altered intestinal microbial flora and impaired epithelial barrier structure and function in CKD: the nature, mechanisms, consequences and potential treatment. Nephrol Dial Transplant 2015 Apr 16.

(64) Anderton JG, Thomas TH, Wilkinson R. Increased susceptibility to membrane lipid peroxidation in renal failure. Nephron 1996;74(2):373-377.

(65) Jiang Q, Akashi S, Miyake K, Petty HR. Lipopolysaccharide induces physical proximity between CD14 and toll-like receptor 4 (TLR4) prior to nuclear translocation of NF-kappa B. J Immunol 2000 Oct 1;165(7):3541-3544.

(66) Malcolm KC, Arndt PG, Manos EJ, Jones DA, Worthen GS. Microarray analysis of lipopolysaccharide-treated human neutrophils. Am J Physiol Lung Cell Mol Physiol 2003 Apr;284(4):L663-70.

(67) Ando M, Shibuya A, Tsuchiya K, Akiba T, Nitta K. Reduced expression of Toll-like receptor 4 contributes to impaired cytokine response of monocytes in uremic patients. Kidney Int 2006 Jul;70(2):358-362.

(68) Nolan JP. The role of intestinal endotoxin in liver injury: a long and evolving history. Hepatology 2010 Nov;52(5):1829-1835.

(69) Carracedo J, Ramirez R, Madueno JA, Soriano S, Rodriguez-Benot A, Rodriguez M, et al. Cell apoptosis and hemodialysis-induced inflammation. Kidney Int Suppl 2002 May;(80):89-93. doi(80):89-93.

(70) Vanholder R, Van Biesen W, Ringoir S. Contributing factors to the inhibition of phagocytosis in hemodialyzed patients. Kidney Int 1993 Jul;44(1):208-214.

(71) Kimmel PL, Phillips TM, Simmens SJ, Peterson RA, Weihs KL, Alleyne S, et al. Immunologic function and survival in hemodialysis patients. Kidney Int 1998 Jul;54(1):236-244.

(72) Glorieux G, Vanholder R, Lameire N. Uraemic retention and apoptosis: what is the balance for the inflammatory status in uraemia? Eur J Clin Invest 2003 Aug;33(8):631-634.

(73) Kettritz R, Falk RJ, Jennette JC, Gaido ML. Neutrophil superoxide release is required for spontaneous and FMLP-mediated but not for TNF alpha-mediated apoptosis. J Am Soc Nephrol 1997 Jul;8(7):1091-1100.

(74) Canaud B, Cristol J, Morena M, Leray-Moragues H, Bosc J, Vaussenat F. Imbalance of oxidants and antioxidants in haemodialysis patients. Blood Purif 1999;17(2-3):99-106.

(75) Himmelfarb J, Stenvinkel P, Ikizler TA, Hakim RM. The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. Kidney Int 2002 Nov;62(5):1524-1538.

(76) Mitra S, Abraham E. Participation of superoxide in neutrophil activation and cytokine production. Biochim Biophys Acta 2006 Aug;1762(8):732-741.

(77) Goh SY, Cooper ME. Clinical review: The role of advanced glycation end products in progression and complications of diabetes. J Clin Endocrinol Metab 2008 Apr;93(4):1143-1152.

(78) Drueke T, Witko-Sarsat V, Massy Z, Descamps-Latscha B, Guerin AP, Marchais SJ, et al. Iron therapy, advanced oxidation protein products and carotid artery intima-media thickness in end-stage renal disease. Circulation 2002 Oct 22;106(17):2212-2217.

(79) Zhuang T, Han H, Yang Z. Iron, oxidative stress and gestational diabetes. Nutrients 2014 Sep 25;6(9):3968-3980.

(80) Kisic B, Miric D, Dragojevic I, Rasic J, Popovic L. Role of Myeloperoxidase in Patients with Chronic Kidney Disease. Oxid Med Cell Longev 2016;2016:1069743.

(81) Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. Arch Biochem Biophys 1990 Jul;280(1):1-8.

(82) Oberg BP, McMenamin E, Lucas FL, McMonagle E, Morrow J, Ikizler TA, et al. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. Kidney Int 2004 Mar;65(3):1009-1016.

(83) Wang S, Eide TC, Sogn EM, Berg KJ, Sund RB. Plasma ascorbic acid in patients undergoing chronic haemodialysis. Eur J Clin Pharmacol 1999 Sep;55(7):527-532.

(84) Ilori TO, Sun Ro Y, Kong SY, Gutierrez OM, Ojo AO, Judd SE, et al. Oxidative Balance Score and Chronic Kidney Disease. Am J Nephrol 2015;42(4):320-327.

(85) Libetta C, Sepe V, Esposito P, Galli F, Dal Canton A. Oxidative stress and inflammation: Implications in uremia and hemodialysis. Clin Biochem 2011 Oct;44(14-15):1189-1198.

(86) Anderstam B, Mamoun AH, Sodersten P, Bergstrom J. Middle-sized molecule fractions isolated from uremic ultrafiltrate and normal urine inhibit ingestive behavior in the rat. J Am Soc Nephrol 1996 Nov;7(11):2453-2460.

(87) McCarthy DO. Tumor necrosis factor alpha and interleukin-6 have differential effects on food intake and gastric emptying in fasted rats. Res Nurs Health 2000 Jun;23(3):222-228.

(88) Jadeja YP, Kher V. Protein energy wasting in chronic kidney disease: An update with focus on nutritional interventions to improve outcomes. Indian J Endocrinol Metab 2012 Mar;16(2):246-251.

(89) Fouque D, Kalantar-Zadeh K, Kopple J, Cano N, Chauveau P, Cuppari L, et al. A proposed nomenclature and diagnostic criteria for protein-energy wasting in acute and chronic kidney disease. Kidney Int 2008 Feb;73(4):391-398.

(90) Kalantar-Zadeh K, Kilpatrick RD, Kuwae N, McAllister CJ, Alcorn H,Jr, Kopple JD, et al. Revisiting mortality predictability of serum albumin in the dialysis population: time dependency, longitudinal changes and population-attributable fraction. Nephrol Dial Transplant 2005 Sep;20(9):1880-1888.

(91) Kalantar-Zadeh K, Block G, McAllister CJ, Humphreys MH, Kopple JD. Appetite and inflammation, nutrition, anemia and clinical outcome in hemodialysis patients. Am J Clin Nutr 2004 Aug;80(2):299-307.

(92) Carrero JJ, Stenvinkel P, Cuppari L, Ikizler TA, Kalantar-Zadeh K, Kaysen G, et al. Etiology of the protein-energy wasting syndrome in chronic kidney disease: a consensus statement from the International Society of Renal Nutrition and Metabolism (ISRNM). J Ren Nutr 2013 Mar;23(2):77-90.

(93) Shetty PS. Adaptation to low energy intakes: the responses and limits to low intakes in infants, children and adults. Eur J Clin Nutr 1999 Apr;53 Suppl 1:S14-33.

(94) Pierratos A, McFarlane P, Chan CT, Kwok S, Nesrallah G. Daily hemodialysis 2006. State of the art. Minerva Urol Nefrol 2006 Jun;58(2):99-115.

(95) Waterlow JC. Protein turnover in the whole animal. Invest Cell Pathol 1980 Apr-Jun;3(2):107-119.

(96) Horn F, Moc I. Biochemie des Menschen. 5th ed. Stuttgard: Thieme; 2012.

(97) Lim VS, Kopple JD. Protein metabolism in patients with chronic renal failure: role of uremia and dialysis. Kidney Int 2000 Jul;58(1):1-10.

(98) Cano NJ, Aparicio M, Brunori G, Carrero JJ, Cianciaruso B, Fiaccadori E, et al. ESPEN Guidelines on Parenteral Nutrition: adult renal failure. Clin Nutr 2009 Aug;28(4):401-414.

(99) Ikizler TA, Cano NJ, Franch H, Fouque D, Himmelfarb J, Kalantar-Zadeh K, et al. Prevention and treatment of protein energy wasting in chronic kidney disease patients: a consensus statement by the International Society of Renal Nutrition and Metabolism. Kidney Int 2013 Dec;84(6):1096-1107.

(100) Kovesdy C,P., Kopple J,D., Kalantar-Zadeh K. Inflammation in renal insufficiency. 2018; Available at: https://www.uptodate.com/contents/inflammation-in-renal-insufficiency?search=inflammation%20in%20esrd&source=search_result&selectedTitle=1~150&usage_type=default&display_rank=1#H109221274. Accessed 06/21, 2018.

(101) Vella J, Lentine KL. Risk factors for cardiovascular disease in the renal transplant recipient. 2017; Available at: https://www.uptodate.com/contents/risk-factors-for-cardiovascular-disease-in-the-renal-transplant-recipient?topicRef=14039&source=see_link. Accessed 06/22, 2018.

(102) Smak Gregoor PJ, Zietse R, van Saase JL, op de Hoek CT, IJzermans JN, Lavrijssen AT, et al. Immunosuppression should be stopped in patients with renal allograft failure. Clin Transplant 2001 Dec;15(6):397-401.

(103) Lopez-Gomez JM, Perez-Flores I, Jofre R, Carretero D, Rodriguez-Benitez P, Villaverde M, et al. Presence of a failed kidney transplant in patients who are on hemodialysis is associated with chronic inflammatory state and erythropoietin resistance. J Am Soc Nephrol 2004 Sep;15(9):2494-2501.

(104) Goicoechea M, de Vinuesa SG, Lahera V, Cachofeiro V, Gomez-Campdera F, Vega A, et al. Effects of atorvastatin on inflammatory and fibrinolytic parameters in patients with chronic kidney disease. J Am Soc Nephrol 2006 Dec;17(12 Suppl 3):S231-5.

(105) Shi K, Wang F, Jiang H, Liu H, Wei M, Wang Z, et al. Gut bacterial translocation may aggravate microinflammation in hemodialysis patients. Dig Dis Sci 2014 Sep;59(9):2109-2117.

(106) Wang IK, Wu YY, Yang YF, Ting IW, Lin CC, Yen TH, et al. The effect of probiotics on serum levels of cytokine and endotoxin in peritoneal dialysis patients: a randomised, double-blind, placebo-controlled trial. Benef Microbes 2015;6(4):423-430.

(107) Bouzidi N, Mekki K, Boukaddoum A, Dida N, Kaddous A, Bouchenak M. Effects of omega-3 polyunsaturated fatty-acid supplementation on redox status in chronic renal failure patients with dyslipidemia. J Ren Nutr 2010 Sep;20(5):321-328.

(108) Kalantar-Zadeh K, Cano NJ, Budde K, Chazot C, Kovesdy CP, Mak RH, et al. Diets and enteral supplements for improving outcomes in chronic kidney disease. Nat Rev Nephrol 2011 May 31;7(7):369-384.

(109) Carrero JJ, Axelsson J, Avesani CM, Heimburger O, Lindholm B, Stenvinkel P. Being an inflamed peritoneal dialysis patient - a Dante's journey. Contrib Nephrol 2006;150:144-151.

(110) Becton, Dickinson and Company BD Biosciences. BD FACS™ lysing solution, 2015.

(111) Glycotope Biotechnology GMBH. Instructions PHAGOTEST Version 05/12.

(112) Nakamura Y, Ishikura H, Nishida T, Kawano Y, Yuge R, Ichiki R, et al. Usefulness of presepsin in the diagnosis of sepsis in patients with or without acute kidney injury. BMC Anesthesiology 2014.

(113) Kotera A, Sagishima K, Tashiro T, Niimori D, Kamohara H, Kinoshita Y. A validation of presepsin levels in kidney dysfunction patients: four case reports.. Journal of Intensive Care 2014.

(114) Kaden J, Zwerenz P, Lambrecht HG, Dostatni R. Lipopolysaccharide-binding protein as a new and reliable infection marker after kidney transplantation. Transpl Int 2002 Apr;15(4):163-172.

(115) Schindler R, Marra MN, McKelligon BM, Lonnemann G, Schulzeck P, Schulze M. Plasma levels of bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP) during hemodialysis.. Clinical Nephrology 1993.

(116) Pereira S, Sundaram B, Snodgrass P, Hogan AJ. Plasma lipopolysaccharide binding protein and bactericidal/permeability increasing factor in CRF and HD patients.. Journal of the American Society of Nephrology : JASN AID - 2010121220 pii] AID - 10.1681/ASN.2010121220 doi] 1996.

(117) Pearson FC, Dubczak J, Weary M, Anderson J. Determination of endotoxin levels and their impact on interleukin-1 generation in continuous ambulatory peritoneal dialysis and hemodialysis.. Blood purification 1988.

(118) Behnes M, Bertsch T, Lepiorz D, Lang S, Trinkmann F, Brueckmann M, et al. Diagnostic and prognostic utility of soluble CD 14 subtype (presepsin) for severe sepsis and septic shock during the first week of intensive care treatment.. Critical Care 2014.

(119) Santarpia L, Grandone I, Contaldo F, Pasanisi F. Butyrylcholinesterase as a prognostic marker: a review of the literature. J Cachexia Sarcopenia Muscle 2013 Mar;4(1):31-39.

(120) Xu G, Luo K, Liu H, Huang T, Fang X, Tu W. The progress of inflammation and oxidative stress in patients with chronic kidney disease. Ren Fail 2015 Feb;37(1):45-49.

(121) Goicoechea M, Quiroga B, Garcia de Vinuesa S, Verdalles U, Reque J, Panizo N, et al. Intraindividual interleukin-6 variations on the cardiovascular prognosis of patients with chronic renal disease. Ren Fail 2012;34(8):1002-1009.

(122) Goldstein SL, Leung JC, Silverstein DM. Pro- and anti-inflammatory cytokines in chronic pediatric dialysis patients: effect of aspirin. Clin J Am Soc Nephrol 2006 Sep;1(5):979-986.

(123) Powe NR, Jaar B, Furth SL, Hermann J, Briggs W. Septicemia in dialysis patients: incidence, risk factors and prognosis. Kidney Int 1999 Mar;55(3):1081-1090.

(124) Rao M, Guo D, Perianayagam MC, Tighiouart H, Jaber BL, Pereira BJ, et al. Plasma interleukin-6 predicts cardiovascular mortality in hemodialysis patients. Am J Kidney Dis 2005 Feb;45(2):324-333.

(125) Cho Y, Johnson DW, Vesey DA, Hawley CM, Pascoe EM, Clarke M, et al. Baseline serum interleukin-6 predicts cardiovascular events in incident peritoneal dialysis patients. Perit Dial Int 2015 Jan-Feb;35(1):35-42.

(126) Kaysen GA, Greene T, Daugirdas JT, Kimmel PL, Schulman GW, Toto RD, et al. Longitudinal and cross-sectional effects of C-reactive protein, equilibrated normalized protein catabolic rate and serum bicarbonate on creatinine and albumin levels in dialysis patients. Am J Kidney Dis 2003 Dec;42(6):1200-1211.

(127) Stenvinkel P. Inflammation in end-stage renal disease--a fire that burns within. Contrib Nephrol 2005;149:185-199.

(128) Wong TC, Su HY, Chen YT, Wu PY, Chen HH, Chen TH, et al. Ratio of C-Reactive Protein to Albumin Predicts Muscle Mass in Adult Patients Undergoing Hemodialysis. PLoS One 2016 Oct 21;11(10):e0165403.

(129) Zimmermann J, Herrlinger S, Pruy A, Metzger T, Wanner C. Inflammation enhances cardiovascular risk and mortality in hemodialysis patients. Kidney Int 1999 Feb;55(2):648-658.

(130) Bhaskaran M, Ranjan R, Shah H, Siu J, Colvin R, Radhakrishnan N, et al. Lymphopenia in dialysis patients: a preliminary study indicating a possible role of apoptosis. Clin Nephrol 2002 Mar;57(3):221-229.

(131) Agarwal R, Light RP. Patterns and prognostic value of total and differential leukocyte count in chronic kidney disease. Clin J Am Soc Nephrol 2011 Jun;6(6):1393-1399.

(132) Okyay GU, Inal S, Onec K, Er RE, Pasaoglu O, Pasaoglu H, et al. Neutrophil to lymphocyte ratio in evaluation of inflammation in patients with chronic kidney disease. Ren Fail 2013;35(1):29-36.

(133) Bradley JS, Long SS. 289 - Principles of Anti-Infective Therapy. 2012 2012:1412-1421.e2.

(134) Yilmaz H, Cakmak M, Inan O, Darcin T, Akcay A. Can neutrophil-lymphocyte ratio be independent risk factor for predicting acute kidney injury in patients with severe sepsis? Ren Fail 2015 Mar;37(2):225-229.

(135) Stojanov MD, Jovicic DM, Djuric SP, Konjevic MM, Todorovic ZM, Prostran MS. Butyrylcholinesterase activity and mortality risk in hemodialysis patients: comparison to hsCRP and albumin. Clin Biochem 2009 Jan;42(1-2):22-26.

(136) Fiedler R, Jehle PM, Osten B, Dorligschaw O, Girndt M. Clinical nutrition scores are superior for the prognosis of haemodialysis patients compared to lab markers and bioelectrical impedance. Nephrol Dial Transplant 2009 Dec;24(12):3812-3817.

(137) Choi KM. Sarcopenia and sarcopenic obesity. Korean J Intern Med 2016 Nov;31(6):1054-1060.

(138) Jia P, Jin W, Teng J, Zhang H, Zou J, Liu Z, et al. Acute Effects of Hemodiafiltration Versus Conventional Hemodialysis on Endothelial Function and Inflammation: A Randomized Crossover Study. Medicine (Baltimore) 2016 Apr;95(16):e3440.

(139) Horvath A, Rainer F, Leber B, Schmerböck B, Spindelboeck W, Stauber R, et al. AGRA â€“ a functional biomarker for humoral immune function predicts the development of severe bacterial infections in cirrhosis. ; 2018.

(140) Minelli Bertazzoni E, Panzetta G. Effects of different forms of dialytic treatment on serum antibacterial activity in patients with chronic renal failure. Nephron 1984;36(4):224-229.

(141) Montgomerie JZ, Kalmanson GM, Guze LB. Leukocyte phagocytosis and serum bactericidal activity in chronic renal failure. Am J Med Sci 1972 Nov;264(5):385-393.

(142) Kays MB, White RL, Friedrich LV. Effect of serum from different patient populations on the serum bactericidal test. J Antimicrob Chemother 2001 Sep;48(3):417-420.

(143) Crokaert F, Lismont MJ, van der Linden MP, Yourassowsky E. Determination of serum bactericidal activity against Escherichia coli by an automated photometric method. J Clin Microbiol 1988 Oct;26(10):2069-2076.

(144) Bohmer RH, Trinkle LS, Staneck JL. Dose effects of LPS on neutrophils in a whole blood flow cytometric assay of phagocytosis and oxidative burst. Cytometry 1992;13(5):525-531.

(145) COHN ZA, MORSE SI. Functional and metabolic properties of polymorphonuclear leucocytes. II. The influence of a lipopolysaccharide endotoxin. J Exp Med 1960 May 1;111:689-704.

(146) Proctor RA. Endotoxin in vitro interactions with human neutrophils: depression of chemiluminescence, oxygen consumption, superoxide production and killing. Infect Immun 1979 Sep;25(3):912-921.

(147) Prokhorenko I, Zubova S, Kabanov D, Voloshina E, Grachev S. Toll-like receptor 4 in phagocytosis of *Escherichia coli* by endotoxin-activated human neutrophils in whole blood. Published online 2012 Nov 14.

(148) Wierusz-Wysocka B, Wysocki H, Michta G, Wykretowicz A, Czarnecki R, Baczyk K. Phagocytosis and neutrophil bactericidal capacity in patients with uremia. Folia Haematol Int Mag Klin Morphol Blutforsch 1984;111(5):589-594.

(149) Ando M, Shibuya A, Yasuda M, Azuma N, Tsuchiya K, Akiba T, et al. Impairment of innate cellular response to in vitro stimuli in patients on continuous ambulatory peritoneal dialysis. Nephrol Dial Transplant 2005 Nov;20(11):2497-2503.