Oxidative stress and inflammation as a response to glucose exposure and dialysis

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Title: Oxidative stress and inflammation as a response to glucose exposure and dialysis

Abstract: The main player of this thesis is glucose, both on a cellular level and with a clinical approach. Too much or wrongly handled glucose contributes to increased inflammation and oxidative stress, which is reinforced by the negative influences of uraemia and dialysis treatment. In addition, trace element status is also affected in dialysis patients. Hyperglycaemia contributes to glucose degradation products (GDP) and advanced glycation end product (AGE), inducing inflammation, oxidative stress and cell death through activation of several pathways.

We investigated GDP content in commercially available infusion fluids and compared patients receiving those with a control group, by looking at GDPs and AGE levels, and inflammatory response. We also investigated hyperglycaemia and GDPs impact with or without citrate addition on protein kinase C (PKC) and adhesion molecule expression, cell death and secretion of cytokines. A transwell model was used to analyse neutrophil migration across endothelial cell layer. This thesis also had a clinical approach, looking at inflammation, oxidative stress and AGE formation, in combination with trace elements in diabetic- and non-diabetic dialysis patients.

All investigated infusion fluids contained GDPs in varying concentrations, some similar to LC50 values of neutrophils *in vitro*. Both GDPs and AGE could be found in patients' blood and urine after infusion. Furthermore, GDPs and hyperglycaemia increased cell death of both neutrophils and endothelial cells. They also increased endothelial expression of PKC, adhesion molecules and cytokines, reduced by the addition of citrate. There was a significant lack of the trace elements selenium and rubidium generally in dialysis patients compared with healthy subjects and a significant correlation between low plasma selenium and high markers of oxidative stress in diabetic dialysis patients. Other trace elements, which can contribute to increased oxidative stress, such as chromium and copper were increased in hemodialysis patients compared with healthy subjects.

In conclusion, a therapeutic aspect is necessary, looking at the possibilities of using citrate and taking control over trace element reduction and supplementation. Further work improving dialysis fluids, might be a way of controlling these substances and administrate them where they might have an immediate effect, i.e. on the blood cells and the endothelial cells.

Key words: Glucose, glucose degradation products, dialysis, diabetes, oxidative stress, inflammation, endothelial dysfunction

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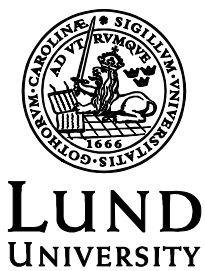

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To Tilda

Contents

ABBREVIATIONS

AGE- Advanced glycation end product

CIC- Citrate carrier

CKD- Chronic kidney disease

CRP- C-reactive protein

CRRT- Continuous renal replacement therapy

CVD- Cardiovascular disease

CXCL8- IL-8, interleukin-8

DAG- Diacylglycerol

ESRD- End stage renal disease

GDP- Glucose degradation product

GFR- Glomerular filtration rate

DISC- Death-inducing signalling complex

GSH/GSSG- Reduced/Oxidized glutathione

HD- Hemodialysis

HUVEC- Human umbilical vein endothelial cells

ICAM-1- Intercellular adhesion molecule-1

ICU- Intensive care unit

IL-1- Interleukin-1

IL-6- Interleukin-6

LC50- Lethal concentration when 50% of the population is dead

LDL- Low-density lipoprotein

MAPK- Mitogen-activated protein kinase

MICS- Malnutrition-inflammation complex syndrome

MTT- (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NGAL- Neutrophil gelatinase-associated lipocalin

NADPH- Nicotinamide adenine dinucleotide phosphate-oxidase

NF-κB- Nuclear factor-kappaB

PD- Peritoneal dialysis

PKC- Protein kinase C

RAGE- Receptor-AGE

ROS- Reactive oxygen species

PTX-3- Pentraxin-3

SOD- Super dismutase oxide

TNF-α- Tumor necrosis factor-alpha

UDP-GlcNAc- Uridine diphosphate N-acetylglucosamine

VCAM-1- Vascular cell adhesion molecule-1

VEGF- Vascular endothelial growth factor

3-DG- 3-deoxyglucosone

3,4-DGE- 3,4-Dideoxyglucosone-3-ene

5-HMF- 5-hydroxymethylfurfural

LIST OF PAPERS

This thesis is based on the following papers and manuscript, which are referred to in the text by their roman numerals *I-III*

Paper I. Bryland A, Broman M, Erixon M, Klarin B, Lindén T, Friberg H, Wieslander A, Kjellstrand P, Ronco C, Carlsson O, Godaly G.

Infusion fluids contain harmful glucose degradation products.

Intensive Care Med. 2010 36(7):1213-20. doi: 10.1007/s00134-010-1873-x. Epub 2010 Apr 16.

Paper II. Bryland A, Wieslander A, Carlsson O, Hellmark T, Godaly G.

Citrate treatment reduces endothelial death and inflammation under hyperglycaemic conditions.

Diab Vasc Dis Res. 2012 9(1):42-51. doi: 10.1177/1479164111424297. Epub 2011 Nov 1.

Paper III. Bryland A, Carlsson O, Hellmark T, Godaly G

The complexity of inflammation, oxidative stress and trace element status in non-diabetic and diabetic hemodialysis patients

Manuscript

SUMMARY

The main player of this thesis is glucose, both on a cellular level and with a clinical approach. Too much or wrongly handled glucose contributes to increased inflammation and oxidative stress, which is reinforced by the negative influences of uraemia and dialysis treatment.

Under normal conditions glucose is a substance metabolised through the glycolysis in order to generate energy. It is also a common substance in the medical field, used both as an energy source and as an osmotic agent. The degradation of glucose to glucose degradation products (GDPs) during unfavourable conditions is dependent on temperature, pH and time [1]. Some GDPs have been proven highly reactive and cytotoxic, contributing to increased cell death, inflammation and oxidative stress [2-5]. The amount of GDPs in serum of a healthy human is low, but increases at least twofold with diseases such as diabetes and threefold with uraemia [6, 7].

Nephropathy affects 35% of the diabetic patients, and diabetic nephropathy is one of the primary sources of renal failure, contributing to increased complications of cardiovascular diseases (CVD). Approximately 70 million people worldwide suffer from chronic kidney disease (CKD) and 2 to 3 million of these are treated for end stage renal disease (ESRD). Diabetic patients on dialysis have a higher risk of hyperglycaemia since the glycemic control in these patients is more complex, due to the balance between dialysis, clearance and modified insulin resistance [8-10]. Severely hyperglycaemic patients typically suffer from complications such as infections and decreased wound healing [9-12].

The inner layer of cells lining our blood vessels is called the endothelium. Endothelial cells have important functions in our immune system by expression and secretion of inflammatory mediators, they are also important regulators of blood flow and blood pressure by expression and secretion of substances that are able to relax or constrict the vessels. In addition, the endothelial cells work as a semi-permeable barrier between the circulating blood in the vessels and surrounding tissue [13]. Hyperglycaemia can cause endothelial dysfunction by several pathways presented later in this thesis, resulting in altered vessel formation, oxidative stress, inflammation and cell death. In addition atherosclerosis is often the final result, contributing to forthcoming cardiovascular problems [9, 10]. The use of antioxidants as a strategy to decrease oxidative stress has been proposed with varying results, both *in vitro* and *in vivo* [14-17]. Other key players in the oxidative burden are the trace elements that can work both as parts of antioxidants and by inducing oxidative stress by their chemical properties. Scientific journals vary in information regarding trace element status in dialysis and diabetic patients, as for their connection to inflammation and oxidative stress in these patient groups [18, 19].

Paper I

In the first part of this thesis, we examined GDP content in commercially available glucose-containing infusion fluids. LC50 values of various GDPs on leukocytes were identified and we studied the effects of GDPs on inflammatory markers. In addition, blood samples were analysed from post-operative patients receiving glucose-containing fluids and compared with a control group. GDPs and advanced glycation end products (AGEs), which are the result of GDPs reacting further with protein in circulation, were also measured over time.

We found GDPs in different concentrations in all the fluids examined. Moreover, increased concentrations of GDPs were found in the blood circulation of critically ill patients receiving standard postoperative fluid therapy. The concentrations of GPDs in infusion fluids were in some cases similar to LC50 concentrations of leukocytes. The commercial fluids also induced more cell death and increased inflammatory markers, and we found GDPs and AGE in patient blood and urine up to 9 hours after infusion of GDP-containing fluids [20].

Paper II

In the second part of the thesis, we look deeper into the signalling pathways involved in hyperglycaemia-induced damage of endothelial cells. Several different harmful glucose-based pathways are proposed in literature, all of which ultimately leads to increased oxidative stress, inflammation, various forms of cell death and atherosclerosis.

Furthermore, we added citrate to the cells in combination with glucose and GDPs to investigate the proposed anti-inflammatory effects of citrate. Citrate is a part of the Krebs cycle and produce cellular energy in the form of adenosine tri-phosphate (ATP). Citrate is used as an anticoagulant and has been shown to have anti-inflammatory and anti-oxidative properties, often by binding calcium. In this work, we proved that the addition of glucose and GDPs induces cell death in endothelial cells, both via apoptosis, which is the controlled form of cell death, and necrosis leading to damage in the surrounding tissue. We also observed that the part of neutrophils migrating through the endothelial cell layer increased after glucose or GDP treatment. Furthermore, an up-regulation of adhesion molecules and inflammatory cytokines that helps the white blood cells to locate and migrate through the endothelial layer was observed [21].

Protein kinase C (PKC) is a family of enzymes that upon activation leads to oxidative stress, inflammation, changes in vessel formation and cell death. A specific form of PKC, PKC-β was previously shown to be activated by hyperglycaemia and we could confirm this in our study [22]. The addition of citrate showed positive results in all experimental settings mentioned above and may therefore have therapeutic potential [21].

Paper III

Trace elements are micronutrients that are needed in very small quantities in our biological systems for everything to work properly. Some serve as antioxidants, as part of, or activating important enzymes. But some of them act in the opposite direction when being present in excess, inducing oxidative stress by chemical reactions. During hemodialysis, several small molecules are removed, including some trace elements; at the same time as certain trace elements instead seem to accumulate. To complicate it further, these substances can exist as protein bound, intra- or extracellular, and whether they accumulate or are removed in hemodialysis patients is linked to uraemia and diabetes [18, 19, 23].

In the final part of this thesis, *paper III*, we took a clinical approach with the purpose to measure the levels of trace elements in plasma and blood, before and after hemodialysis. In addition, we also measured markers for inflammation, oxidative stress and AGE formation. The results were compared with results from a group of healthy volunteers. Furthermore, we analysed a specific subgroup of dialysis patients with diabetes and compared them with dialysis patients without diabetes. The aim was to investigate if there is a correlation between specific trace elements, AGE formation, inflammation, oxidative stress, dialysis and diabetes.

Although the results in this study were challenging to interpret, significant lack of the trace elements selenium and rubidium generally in dialysis patients compared with healthy subjects was observed. In addition, we noticed a significant correlation between low plasma selenium and high markers of oxidative stress in diabetic dialysis patients. Other trace elements, which can contribute to increased oxidative stress, such as chromium and copper were increased in hemodialysis patients compared with healthy subjects.

All together this thesis shows the complexity of glucose and its subsequent degradation product when exceeding physiological concentrations, especially in vulnerable populations, such as dialysis- and diabetic patients. Exposing patients with these conditions to a hyperglycaemic environment might cause a lot of harm, leaving us with the remaining question, what can we do about it? Are possible antioxidants, such as citrate, the answer, or perhaps trace element supplementation or removal?

In conclusion, a therapeutic aspect is necessary – to look at the possibilities of using citrate and take control over trace element reduction and supplementation. Further work improving dialysis fluids, might be a way of controlling these substances and administrate them where they may have an immediate effect, i.e. on the blood cells and the endothelial cells.

SVENSK SAMMANFATTNING

För mycket eller felaktigt hanterad glukos (socker), bidrar till ökad inflammation och oxidativ stress, både på cellulär nivå och kliniskt. Detta kan förstärkas ytterligare av de negativa effekterna från uremi och dialysbehandling.

Glukos är ett livsviktigt ämne för alla levande organismer för att generera energi. Det används ofta inom sjukvården, framförallt som energitillskott i droppbaserade lösningar. Förutom att vara en viktig energikälla kan glukos brytas ner till olika glukos nedbrytningsprodukter (GDP) under vissa omständigheter, som exempelvis upphettning, för lång lagringstid och felaktigt pH. Detta sker också i kroppen när vi har för högt blodsocker. GDPer är en grupp av molekyler där vissa är mer reaktiva och giftiga än andra, och dess reaktivitet avgör om de är mätbara eller inte, men samtliga existerar i någon form av förskjutbar och reversibel korrelation till varandra. Friska individer har förhållandevis låga koncentrationer av GDPer i blodet, att jämföra med diabetespatienter som på grund av sin sjukdom ofta lider av för högt blodsocker eftersom de har svårt att reglera koncentrationen glukos i blodet.

Diabetes leder ofta till njursvikt och en kombination av diabetes och njursvikt gör att patienten har ännu svårare att reglera blodsocker-nivåerna. Detta på grund av att de då lider av uremi, som i sig inducerar inflammation och oxidativ stress som påverkar glukosupptag och insulinutsöndring. Dessutom behandlas de ofta med någon form av dialys, där eventuell medicinering påverkas, samt behandlingen i sig är en ytterligare bidragande faktor till inflammation och oxidativ stress. Under kronisk- och intensivvårdsdialys, renas blodet utanför kroppen via ett semi-permeabelt membran, där blodet möter dialysvätska. Överflödig

vätska och uremiska toxiner avlägsnas med hjälp av tryckskillnad och flödeshastighet, samt via diffusion.

För högt blodsocker påverkar blodkärl och således hela det cirkulära systemet och leder följaktningen till kardiovaskulära problem. Ateroskleros (åderförkalkning) är ofta slutresultatet och är en del av den kardiovaskulära påverkan då detta medför förträngningar och stelare blodkärl.

Detta är ett stort globalt problem, då uppskattningsvis 70 miljoner personer lider av nedsatt njurfunktion och 2 till 3 miljoner av dessa har ett så pass långt framskridet sjukdomsförlopp att de behandlas med någon form av dialys. Att tillägga är 250 miljoner personer 2010 diagnostiserade med diabetes och denna population beräknas öka till 300 miljoner år 2025 och 75 % av dessa diabetespatienter uppskattas dö av kardiovaskulära följder.

Delarbete I

I denna avhandlings första del, undersökte vi GDP-innehåll i kommersiellt tillgängliga värmesteriliserade glukosinnehållande infusionsvätskor, i jämförelse med vätskor med samma innehåll men utan GDP. Dödliga koncentrationer (LC50) av olika GDPer på vita blodkroppar utforskades och dess effekter på inflammatoriska markörer studerades. Dessutom analyserades blodprover från post-operativa patienter som fått GDP-innehållande lösningar och jämfördes mot en kontrollgrupp. GDPer och ”advanced glycation end products” (AGE), som är nästa steg i reaktionen då GDPer reagerat vidare med protein mättes över tid.

Resultaten var tydliga då vi hittade GDPer i olika koncentrationer i samtliga av de vätskor vi undersökte, dessutom var koncentrationerna i flera fall jämförbara med de värdena som var dödliga för vita blodkroppar. De kommersiella lösningarna inducerade dessutom mer celldöd och gav en ökning av inflammatoriska markörer, dessutom kunde vi hitta GDPer och AGE i blod och urin från patienter upp till 9 timmar efter att infusionen avslutats.

Delarbete II

I nästa del av avhandlingen gick vi djupare in på några av de signalvägar som identifierats och kopplas till markörer som är tänkbara för hyperglycemi-inducerad skada på blodkärlens innersta cellager, de så kallade endotelcellerna. Endotelceller har viktiga funktioner i kroppens immunförsvar, samt reglerar vad som får komma igenom

blodkärlen och in i närliggande vävnad, de kan påverkar blodflöde och således också blodtrycket.

I detta delarbete tillsatte vi också citrat till cellerna för att se om vi kunde minska inducerad skada. Citrat är en komponent i citronsyracykeln som hjälper våra celler att producera energi i form av adenosine tri-fosfat (ATP), citrat används som en antikoagulant och har kända antiinflammatoriska och antioxidativa egenskaper, ofta genom att binda kalcium.

I detta arbete visade vi att tillsatts av glukos eller GDPer inducerar celldöd i endotelceller, både via apoptos, som är en kontrollerad form av celldöd, och nekros som är okontrollerad och leder till skada även i omkringliggande vävnad. Vi såg också att genomsläppligheten av vita blodkroppar ökade över endotellagret efter denna behandling, samt en uppreglering av adhesionsmolekyler och inflammatoriska cytokiner som hjälper de vita blodkropparna att lokalisera och att ta sig igenom endotellagret.

Proteinkinas C (PKC), är en grupp av enzymer som aktiveras av och vid aktivering leder till mer oxidativ stress, inflammation, förändringar i kärlbildning och celldöd. En specifik form av PKC, PKC-β har blivit identifierad som den som aktiveras av för höga glukos koncentrationer och detta kunde vi bekräfta i vår studie. Tillsats av citrat visade sig sänka samtliga markörer och proteinuttryck och kan därför ha terapeutiska möjligheter värda vidare utforskning, som till exempel arbeta vidare med konceptet att ge dialyspatienter citrat via dialysvätskan.

Delarbete III

Spårämnen är essentiella grundämnen som behövs i mycket små koncentrationer i vårt biologiska system för att allt ska fungera korrekt. En del spårämnen fungerar som antioxidanter, ofta som delar av eller genom aktivering av viktiga enzym. Somliga har dessutom förmågan att när de finns i överskott inducerat oxidativ stress genom kemiska reaktioner. Vid dialys avlägsnas många små molekyler inklusive vissa spårämnen, samtidigt finns det litteratur som visar på att spårämnen istället ansamlas hos dialyspatienter. Detsamma gäller diabetespatienter, som precis som dialyspatienter redan är extra utsatta för inflammation och oxidativ stress.

I det sista delarbetet, valde vi en klinisk inriktning med syfte att mäta spårämnesnivåer i plasma och blod, före och efter dialys, samt i den använda dialysvätskan. Dessutom mätte vi markörer för inflammation, oxidativ stress och AGE bildning. Detta jämfördes med mätningar från en grupp friska frivilliga. Vi undersökte även specifikt en undergrupp av dialyspatienter med diabetes och jämförde med dem utan diabetes. Syftet var att se om det finns några samband mellan specifika spårämnen, AGE bildning, inflammation och oxidativ stress.

Resultaten av denna delstudie var att dialyspatienter lider av signifikant brist på spårämne selen och rubidium jämfört med friska. En signifikant koppling mellan lågt

plasmaselen och höga nivåer av markörer för oxidativ stress i dialyspatienter med diabetes kunde också observeras. Andra spårämnen som kan leda till ökad oxidativ stress, så som krom och koppar, var förhöjda hos dialyspatienter jämfört med friska.

Sammanfattningsvis visar denna doktorsavhandling komplexiteten av glukos och dess metaboliter om de överstiger normala koncentrationer och förhållanden, särskilt i utsatta populationer så som, dialys- och diabetespatienter. Det orsakar mycket skada att utsätta dessa patienter för exponering av för höga glukoskoncentration och således, då också GDPer. Vilket lämnar oss till den återstående frågan, vad kan vi göra för att minska denna skada? Är möjliga antioxidanter, såsom citrat svaret, eller kanske spårämnes- komplettering eller borttagning?

Sammanfattningsvis är en terapeutisk aspekt nödvändigt och genom den se möjligheterna att använda citrat och ta kontroll över spårämnes nivåer. Ytterligare arbete med att förbättra dialysvätskor kan vara ett sätt att inte bara tillföra ämnen utan tillföra dem där de kan ha en omedelbar effekt, det vill säga på blodcellerna och endotelceller.

INTRODUCTION

Approximately 70 million people worldwide suffer from chronic kidney disease (CKD) and 2 to 3 million of these are treated for end stage renal disease (ESRD) and the numbers are expected to increase with at least 7% annually [24]. Patients with CKD suffer from increased oxidative stress and inflammation due to uraemia and dialysis treatment. A diabetic patient does not have a normal glucose metabolism, either due to failure of the insulin production by the β-cells in the pancreas (type 1) or due to cells non-responsiveness to insulin, so called insulin resistance (type 2) – or a combination of both [25]. Diabetic nephropathy is one of the primary sources of renal failure, and dialysis patients with diabetes are particularly sensitive to oxidative stress and inflammation as they are also exposed to hyperglycaemia and other diabetic complications leading to increased risk of endothelial dysfunction, atherosclerosis and cardiovascular diseases (CVD) [23, 25, 26]. The number of people worldwide with diabetes was estimated to over 250 million in 2010, and is expecting to increase to 300 million by 2025, thus, representing more than 6% of the world's adult population [26, 27]. In addition, at least 75% of this diabetic population is expected to die due to CVD complications [28, 29].

Oxidative stress

Oxidative stress is a consequence of the imbalance between reactive oxygen species and antioxidants in a biological system. A reactive species is an unstable molecule with one or several unpaired electrons, making them extremely potent to react with other molecules in order to gain stability. Oxygen (O_2), reacts to yield superoxide ion ($\cdot O_2^-$) and further reversible to hydrogen peroxide (H_2O_2). The Fenton reaction describes the next reaction where H_2O_2 forms the hydroxide ion OH^- and

hydroxyl radical ·OH with the help of trace elements, that are able to donate or accept free electrons, figure 1 [30, 31]. Hyperglycaemia has been shown to elevate oxidation of proteins, lipids and DNA [30 27, 31-35].

Antioxidants

Antioxidants are the natural way to manage fluctuations of reactive oxidants in our systems, working both enzymatically and non-enzymatically. Some antioxidants work as primary scavengers of ROS by transformation of free oxygen radicals to hydrogen peroxide and then further to water and some act secondary by binding metal ions or proteins involved in the formation of ROS [31]. Deficiencies in the natural oxidative defence mechanism, such as decreased glutathione levels, have been observed in dialysis and diabetes patients, making them extra vulnerable [14].

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is a group of enzymes could be considered the first line of defence since it contributes in the first step of eliminating ROS by transforming $\cdot O_2^-$ to H_2O_2, figure 1. Copper, zinc and manganese are trace elements, and as a parts of superoxide dismutases, they are important antioxidants. Zn-Cu SOD is located in the cytosol, while Mn SOD is located in the mitochondrial matrix [31, 36]. SOD has been shown to improve hyperglycaemia-induced harm on endothelial cells [27].

Glutathione and catalase

GSH is the reduced form of glutathione and work as a non-enzymatic radical scavenger by being oxidized to GSSG. It is also a co-substrate for glutathione peroxidase. Glutathione peroxidise belongs to the second line of defence mechanism; here selenium is located at the active site, reducing oxidative stress by transformation of hydrogen peroxide to water. Catalase also belongs to the second line of defence mechanisms, and works faster than glutathione peroxidas. The exact mechanism of catalse is not fully understood, but a two step reaction using iron in there active site is proposed. Catalase mostly exists in the peroxisomes in the liver, while glutathione can be found in the mitochondria and in the cytosol in all organs, figure 1 [31].

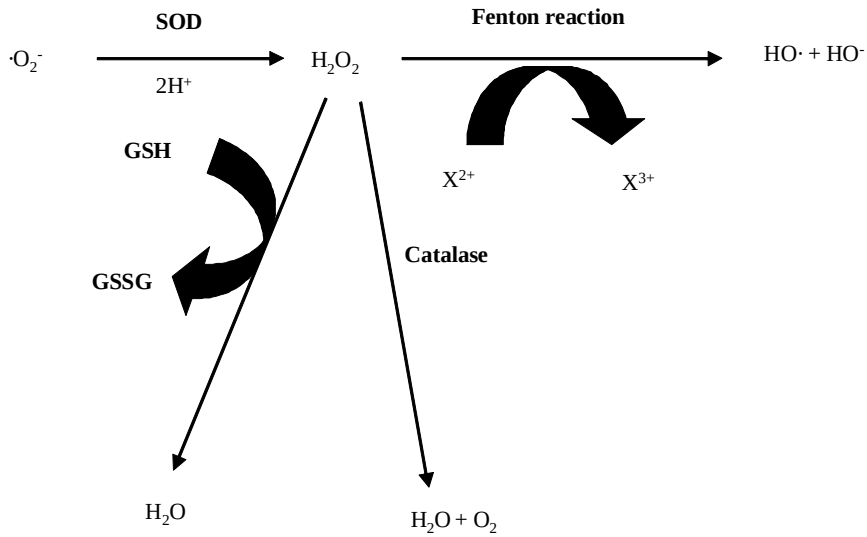

Figure 1 ROS formation by the Fenton reaction and elimination by SOD, glutathinone and catalase SOD enzymatically faciltates the formation of $\cdot O_2^-$ to H_2O_2, where glutathione is oxidased from GSH to GSSG and catalase is used to eliminate H_2O_2 to yield H_2O and O_2. In addition, H_2O_2, can react further by the help of trace elements or Fe^{2+} forming the reactive oxygen species HO^- and $HO\cdot$.

Inflammation

Inflammation is the body's attempt of self-protection with the aim to remove harmful stimuli, including damaged cells, irritants or pathogens, and begin the healing process.

The immune system is divided into two different parts, the innate immunity and the adapted immunity. An acute inflammation is the first playground for the key players from the innate immunity that are always present and activated up on stimuli, such as the endothelial cells and the phagocytic leukocytes. If the inflammatory condition is not reduced, the adapted immunity takes over, involving other important mechanisms and activation of lymphocytes [37].

Acute inflammation

Acute inflammation is a rapid response, starting with the production of pro-inflammatory cytokines, such as tumour necrosis factor (TNF-α) and interleukin 1 (IL-1), secreted to amplify the inflammatory response. Acute inflammation leads to recruitment of leukocytes, to complement activation and to mast cell secretion of histamine, NO and prostaglandins. This causes vasodilatation and increases the blood flow. Upon inflammation, endothelial cells and leukocytes express selectins and integrins. The selectins bind weakly to their ligands on the opposite cells, resulting in leukocyte "rolling" as a result of these weak bindings. To firm the binding, endothelial cells increase the expression of adhesion molecules, including I-CAM, V-CAM and integrins, which bind to activated integrins on the leukocytes. The adhered leukocytes start to migrate through the endothelial cell layer towards a chemokine gradient, such as CXCL8, figure 2 [37, 38].

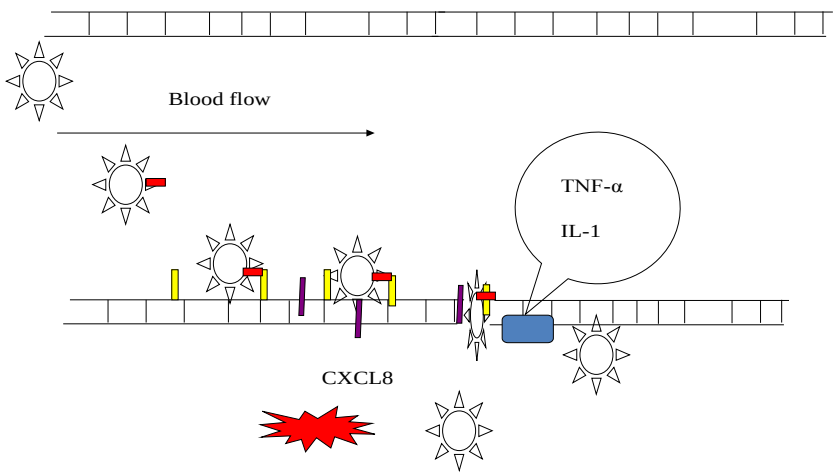

Figure 2 Neutrophil recruitment and transmigration
Pro-inflammatory cytokines IL-1 and TNF-α are secreted into the blood stream as an answer to inflammatory stimuli, resulting in increased blood flow and attraction of leukocytes. Adhesion molecules help the neutrophils to attach to the endothelial cell layer and start to roll along the cell surface and finally transmigrate into the tissue, where chemo-attractants guide them to the site of infection.

Chronic inflammation

It is sometimes hard to determine the origin of a chronic inflammation, because many conditions are either the result of, or the cause of a chronic inflammation. In addition, over time can the acute inflammation passes to chronic. This response involves the lymphocytes, T and B cells, of specific immunity. If these cells fail to eliminate the subject that causes the inflammation, or if there is an autoantibody response or if there is a constant low density of irritant present, the inflammation passes to a long-term chronic state, which can last for several weeks and even years [39]. Chronic inflammation can eventually cause severe diseases, such as cancer, rheumatoid arthritis and atherosclerosis. In these patients the concentrations of measurable inflammatory markers are constantly low to moderate, compared with an acute inflammatory response where the concentrations peak at a limited time [39, 40].

Inflammatory markers

There are many substances activated or secreted upon inflammatory stimuli and it is of high importance to be able to evaluate these parameters in order to understand the condition of the patient. There is constantly an ongoing debate of new markers to use; especially when it comes to evaluating the patients' condition. Here is a short summary of some of the mostly important markers that are mentioned in the literature and relevant for this work.

Cytokines and chemokines

Cytokines are small proteins involved in cell signalling and can increase due to inflammatory stimuli. First we have the pro-inflammatory cytokines, such as IL-1 secreted by macrophages in the tissue or the endothelial cells to recruit leukocytes and up-regulates adhesion molecules. Furthermore, TNF-α is probably the most important pro-inflammatory cytokine, able to start a variety of events. TNF-α is secreted by macrophages or neutrophils due to antigen identification. Its presence increases the up-regulation of adhesion molecules on endothelial cells, and this cytokine is involved in complement activation, increased CRP secretion from the liver and can also affect the hypothalamus to induce fever [39].

IL-6 is an example of a cytokine, which often is secreted later into the inflammatory cascade and not categorised as pro-inflammatory. A chemokine is a cytokine that works as a chemo-attractant, and help attracting leukocytes to the site of inflammation; CXCL8 is one example [39].

Cell adhesion molecules

ICAM-1 and VCAM-1 are both expressed on endothelial cells as a response to stimuli or pro-inflammatory cytokines such as IL-1 and TNF-α. They bind to leukocytes and help them transmigrate trough the endothelial cell layer. ICAM-1 can also facilitate virus entry by a similar mechanism to that of the leukocytes [39, 40].

Integrins and selectins are two groups of receptors that facilitates cell to cell or cell to extracellular matrix interactions [39, 40]. Selectins are expressed on endothelial cell surface upon inflammatory stimuli. During transmigration of leukocytes adhesion molecules both on leukocytes and on the endothelial surface bind and help the leukocytes to roll and finally migrate through the endothelial cell layer [39, 40].

Acute inflammatory markers

C reactive protein (CRP) and pentraxin-3 (PTX-3) both belong to the family of pentraxins and are upregulated and secreted due to different acute immunological responses. CRP is a well-used parameter for bacterial infections and PTX-3 has been suggested as a relevant marker for dialysis-induced inflammation [41, 42].

The kidney and renal failure

The kidney is a vital organ needed for regulation of body fluids, ion homeostasis and excretion of waste products. The kidneys are also necessary for production and secretion of the following hormones: erythropoietin (EPO, stimulating erythrocyte production), rennin (regulating part of blood pressure) and active vitamin D (needed for calcium absorption in the intestine). Renal failure occurs when the kidney is not working properly, resulting in a lack of urine production and thereby fluid overload and accumulation of uraemic toxins. Renal failure is observed by measuring proteins in urine, urea in the blood and the glomerular filtration rate (GFR), which is a measure of blood-rate filtration in the kidney. Blood clearance of creatinine is often used to calculate GFR [43, 44].

Renal failure and dialysis treatment

CKD is affecting 7% of the worldwide adult population, that is older than 30 years old. CKD is divided in to five stages, depending on kidney function, and in the last stage, (also called ESRD) treatment is needed [24]. In addition renal failure can be either chronic, due to underlying diseases such as diabetes, or acute due to intoxication or external injury. Regardless of the cause, when the kidney´s function is lower than 5%, renal failure can only be treated with kidney transplantation or different dialysis modalities, such as intermittent hemodialysis (HD), including continuous renal

replacement therapy (CRRT), performed on patients with acute injury and peritoneal dialysis (PD). During intermittent HD- and CRRT treatments the excess fluid and uraemic toxins are removed extra-corporally. In contrast to PD where the patients' peritoneum is used as a filter, and excess fluid and waste products are removed via diffusion and osmotic pressure across the peritoneum [43].

Kidney damage and associated biomarkers

As for inflammation, it is important to be able to measure the biological function of the kidneys, to determine the grade of functionality. Presented here are some of the biomarkers related to kidney performance.

Neutrophil gelatinase-associated lipocalin (NGAL) binds to iron whereby it reduces bacterial growth. Upon acute kidney damage, NGAL is secreted in the urine and blood, making it an important biomarker. NGAL´s normal function is in the innate immunity, where it is expressed by several cell types, but mainly by the neutrophils [45].

Creatinine is, unlike NGAL (which is a marker for kidney damage), a marker for kidney function. It is a waste product of muscle metabolism and it is not reabsorbed by the kidney. This property makes creatinine blood concentration a suitable marker for kidney function, where the clearance of creatinine often is calculated (GFR) [24, 43, 45] .

Basic principles for chronic- and acute-dialysis

The therapeutic effect of dialysis treatment is removal of waste products and excess fluid from the patients. This is carried out by pumping the patient's blood in one direction and dialysis fluid in the opposite direction, separating the different flows by a semi-permeable membrane, figure 3.

The flow rate is decided by the machine settings and a pressure gradient, and so called convection helps removing some of the excess fluid and waste products from the blood. In addition, the concentration gradient of waste products and other molecules are also removed from the blood side by diffusion. The principle of diffusion and the pore size of the semi-permeable dialysis membrane also make it possible to retain or add essential molecules to the blood side by adding them to the dialysis fluid, making the composition of a dialysis fluid very important. The compositions of dialysis fluids may vary, but in general they all contain essential electrolytes, such as sodium (Na^+), calcium (Ca^{2+}), potassium (K^+), magnesium (Mg^{2+}), low concentrations of glucose and a buffer [43].

Dialysis-induced oxidative stress and inflammation

30% to 60% of the European and Northern American dialysis patients have increased inflammatory markers as a result of the uraemic condition before dialysis (pre-dialysis). The dialysis treatment per se contributes to further increase of inflammation and oxidative stress [41, 46].

A dialysis treatment is more or less biocompatible, depending of filter, flow and dialysis fluids used. However, even in the best cases, the blood is still re-circulated outside the body, which activates the complement system, coagulation and leukocytes mechanically by the dialysis filter, through exposure to air or by microbiologic exposure [14].

Uraemic conditions contribute to vascular dysfunction by the up-regulation of inflammatory markers and ROS, which are linked to increased arthrosclerosis, arterial stiffness, NO regulation and calcifications. Uraemic toxins such as para-cresol sulphate and indoxyl sulphate are associated with increased mortality and activated leukocytes, as well as induced ROS production [47].

A uraemic patient treated with dialysis is subsequently exposed to further risks by the treatment itself. After a dialysis treatment, over-stimulated leukocytes increase intracellular ROS production, endothelial cells up-regulate the expression of adhesion molecules, and elevated levels of inflammatory markers CRP and PTX-3 are found in the blood. In addition, dialysis patients show increased levels of circulating markers for advanced lipid oxidation products, i.e. MDA and oxidised LDL [14, 36, 41, 42, 47-50].

Oxidation of proteins is also a problem in hemodialysis patients and advanced oxidative protein products (AOPP) could be determined in plasma and correlated with pentosidine and phagocytosis. Both uraemic dialysis patients and untreated uraemic patients show elevated levels of AOPP that correlates with AGE concentrations in plasma, inflammatory markers and apoptosis [47]. Another risk factor is dose-dependent AGE-modification of β2-microglubulin, resulting in ß2-m amyloidosis and reduced phagocytosis [51]. Furthermore, altered lipid metabolism is one of the major risk factors in the development of atherosclerosis and several abnormalities such as increased serum levels of triglycerides are noticed in hemodialysis patients [51].

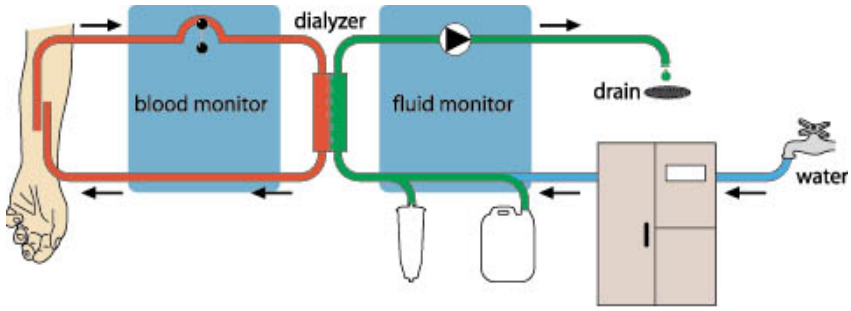

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Figure 3 Scematic picture of hemodialysis
During hemodialysis the blood is recirculated extra-corporal across a semi-permeable membrane and excess fluid and waste products are removed. The dialysis fluid is on the opposite site of the membrane, helping to remove waste products and replace the small- to medium sized molecules in the blood by flow rate, pressure and diffusion over the membrane. A concentration gradient between the dialysis fluid and the blood makes it possible to selectivly keep, remove or give molecules to the dialysis patient.

Trace element and dialysis

Trace elements are essential micronutrients needed in very small quantities in our biological systems for everything to work properly. Some serve as antioxidants, often as part of, or activators of, important enzymes, in addition some of them also have the ability to induce oxidative stress by chemical reactions, such as the Fenton reaction when present in excess. Dialysis patients have elevated risk of both trace element deficiency and accumulation due to the dialysis treatment and renal failure. In addition, complications affecting trace element levels are anaemia, residual renal function and malnutrition, as a result of poor appetite, dietary restrictions, drug intake and dysgeusia [18, 52, 53]

Excess fluid, low-weight to medium-weight molecules and uraemic toxins are removed during dialysis, but some low-weight to medium-weight molecules, such as glucose, sodium, calcium and potassium, are replaced by being included in the dialysis fluid. However, small substances that are not present in the dialysis fluid are often removed from the patients during treatment. Trace element disturbances might occur

in dialysis patients, and theses disturbances can lead to either chronic or acute intoxications. The main source of trace element contamination, when treating dialysis patients, is poor quality of the water used for preparing the dialysis fluids. In addition, patient accumulation can further be due to patients' disability to utilize the substances. Recommendations on daily trace element supplementation for dialysis patients exist, but there is a lack of scientific evidence for most of them [18, 19, 54-59].

Glucose and hyperglycaemia

Normally glucose is metabolised by glycolysis in order to generate cellular energy in form of adenosine triphosphate (ATP) and NADPH. Its metabolism is also important for lipid and amino acid synthesis. In the medical field, glucose is an important component in several fluids used to provide nutrition, or as an osmotic agent. Critically ill patients and diabetic patients often have a condition called hyperglycaemia, often due to impaired glucose metabolism.

The definition of hyperglycaemia is plasma glucose concentrations exceeding 11.1 mmol/l, and chronic elevated plasma concentrations above 7 mmol/l can cause organ damage [25]. Diabetic patients (that are not under strict metabolic- and insulin control) may suffer from chronic hyperglycaemia. Patients with renal failure with or without diagnosed diabetes are at a greater risk for hyperglycaemia, since the glycemic control is more difficult to mange in this patient group. Most orally taken diabetic drugs are removed during dialysis treatment and diabetic hemodialysis patients have altered insulin secretion and decreased insulin clearance, compared with diabetic patients without renal failure [60]. There is a correlation between long-term hyperglycaemia and mortality of dialysis patients [61].

Glucose degradation products

When excess glucose is metabolised or when glucose-containing fluids are transported or stored, glucose degradation products (GDP) are formed. Furthermore, temperature, pH and storage time are critical factors that can increase the formation of GDPs [62]. GDPs are a group of molecules, where some are more reactive and cytotoxic than others. 3-DG and 5-HMF are examples of less reactive ones that can be found in glucose-containing fluids and in plasma of hyperglycaemic patients. Formaldehyde and 3,4-DGE are examples of GDPs that has been proven to be highly reactive and cytotoxic, and to induce inflammatory response and apoptosis *in vitro*. An equilibrium has been proposed, suggesting that the concentrations of the less reactive GDPs correlates with the concentrations of more reactive and cytotoxic GDPs, for which no reliable analytically methods exists, due to their ability of instantly reacting [1-4, 7, 62, 63].

The Maillard reaction- AGE formation and RAGE activation

When excess glucose or reactive GDPs enter the circulation they instantly react with proteins and glycated proteins, called advanced glycation end products (AGE) are subsequently irreversible formed, by the so called "Maillard reaction". Formation of AGE by the Maillard reaction was first observed in 1912 by a French physician and chemist called Louis-Camille Maillard, while trying to reproduce biological protein synthesis. The reaction described is non-enzymatic and illustrates a reaction between amino groups and glucose [8, 64, 65].

RAGE activation

AGEs are involved in oxidative stress, inflammation and apoptosis by binding to their receptors for advanced glycation end products (RAGE). RAGE is a member of the immunoglobulin super-family and exist both as soluble and as a cell surface receptor, expressed by several cell types including endothelial cells and leukocytes [5].

RAGE was originally named after it's exclusive capability to bind AGEs, but later studies have shown several other ligands are able to bind to RAGE with high affinity. Furthermore, enhanced levels of RAGE correlate with inflammatory markers in patients with diabetes [66-68] and are involved in the immune defence by facilitating cytokine secretion, leukocyte recruitment and up-regulation of adhesion molecules [65]. RAGE signalling activates pathways responsible for acute- and chronic inflammation by the production of the pro-inflammatory cytokines TNFα, IL-6 and IL-1. AGEs can also directly cause glycation of intracellular proteins and lipids. AGE and its intermediates can undergo fast auto-oxidation, generate ROS and contribute to advanced atherosclerosis in the future. There are at least 20 different identified AGEs, such as carboxymethyl lycine (CML) and pentosidine, where the prior have been shown to increase RAGE signalling. AGEs are accumulating in patients with kidney failure due to increased production and impaired metabolism of glucose [5, 65, 69, 70].

Endothelial dysfunction and atherosclerosis

Endothelial dysfunction

We have approximately 10^{13}-10^{14} endothelial cells, with a total weight of almost 1 kg, forming the thin layer of cells lining our blood vessels in direct contact with the circulating blood [13, 27]. The endothelial cells have unique functions in the system such as regulating the influx of substances to and from circulation and adjacent tissue. They are also involved in the inflammatory process by regulating the transmigration of

leukocytes and regulating the blood pressure by secreting vasoconstrictors and vasodilators.

eNOS protects the endothelial cells by producing NO, involved in vasodilatation and prevention of leukocyte adhesion. Although decreased eNOS expression might result in endothelial dysfunction, over-expression is most likely a superior risk factor, since increased O_2^-, due to up-regulation of NADPH oxidase (NOX), might contribute to increased expression of eNOS. Together, NO· and O_2^- form peroxynitrite anion ($ONOO^-$), which can result in eNOS-induced endothelial dysfunction and increased ROS formation [71]. Furthermore, some studies suggest that endothelial cells have incapacity to regulate the influx of glucose making them a vulnerable target for hyperglycaemia- and GDP-induced damage [8, 72, 73].

Atherosclerosis

Atherosclerosis is an inflammatory condition that results in endothelial dysfunction through thickening of the vessel wall due to accumulation of oxidized fat, making the arteries inflexible and thick [30, 74, 75]. The definition for atherosclerosis is that it is a chronic inflammatory process characterized by plaque formation within the vessel wall of arteries with extensive necrosis and fibrosis of surrounding tissues [76]. It is suggested to be a down stream effect of endothelial dysfunction, and hyperglycaemia promotes atherosclerosis by several pathways. In addition, glycosylation of LDL is mostly investigated as a specific target, correlating with glucose concentration. Two parts of the LDL molecule can be glycosylated, resulting in oxidized LDL (ox-LDL) unrecognizable for the LDL receptor. This transformed LDL molecule binds to a nonspecific receptor on macrophages that promotes intracellular cholesterol accumulation [14, 74, 76, 77].

Basic mechanism for atherosclerosis

1. Adhesion of neutrophils and monocytes to the endothelial cell layer
2. Transmigration to the tissue
3. Maturation of monocytes to macrophages in the tissue
4. Elevated levels of ox-LDL, results in foam cell formation. A foam cell is a macrophage that engulfs due to endocytosis of lipids, such as LDL
5. Accumulation and necrosis of foam cells [75, 76]

Hyperglycaemia induces endothelial dysfunction and promotes atherosclerosis by several pathways

There are four main pathways proposed for glucose-induced damage, resulting in altered gene expressions, increased inflammation and oxidation of protein and lipids, and finally potential atherosclerosis

1. The polyol pathway results in increased ROS formation by decreased GSH activity, osmotic stress and increased DAG synthesis

Excess glucose enters the polyol pathway whereupon sorbitol is enzymatically formed by aldose reductase, using NADPH as a co-factor. In a normal environment, this enzymatic process is used to inactive alcohols by converting aldehydes generated by ROS and excess ROS are eliminated as GSSG is reduced to GSH, thereby consuming NADPH.

If the NADPH instead is used to convert glucose to sorbitol, is less GSH subsequently formed, figure 4A. Next mechanism proposed is that sorbitol diffuses slowly and might not be able to cross the cell membrane, resulting in osmotic stress, figure 4B. The NADH produced, when sorbitol is further metabolised to fructose generates more DAG and activates the PKC pathway, figures 4C and 5 [9, 10, 26, 29, 78-80].

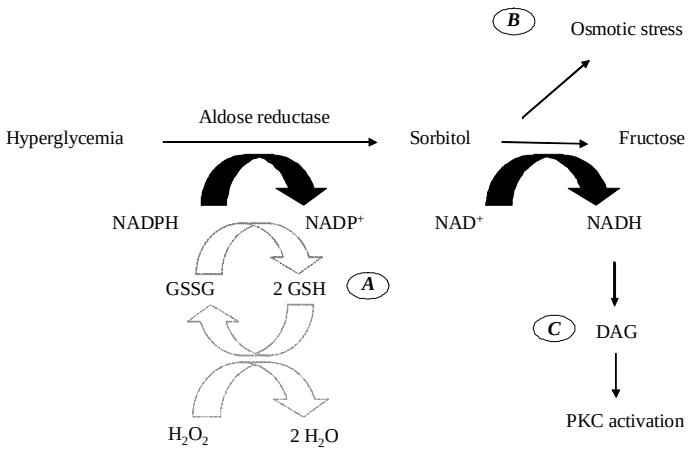

Figure 4 Three different aspects of the polyol pathways, inducing oxidative stress
A, less GSH is formed as a consequence of sorbitol formation. B, osmotic stress due to sorbitol formation and C, sorbitol metabolism alters DAG production, activating PKC.

2. Auto-oxidation of glucose gives rise to AGE and activates RAGE

Auto-oxidation of glucose can occur in a hyperglycaemic environment, forming GDPs and AGE precursors. Several intracellular proteins are modified by interacting with AGE, including proteins involved in endocytosis and growth factors. Furthermore, AGE interaction induces protein cross-linking and decreased blood vessel elasticity, which also interferes with matrix-cell interactions and several other binding proteins.

Many constellations of AGE-proteins also act as ligands to RAGE, triggering a cascade of pro-inflammatory and inflammatory events, including altered gene-expression of nuclear factor-κB (NF-κB) and altered regulation of adhesion molecules and cytokines. Some ligands induce VEGF expression and are by that proposed to be responsible for hyper-permeability of the vessel wall [9-11, 26, 30, 47, 81, 82].

3. Increased PKC activation impairs gene expression and induces vessel damage, apoptosis and inflammation

Hyperglycaemias increase diacylglycerol (DAG) synthesis by glucose metabolism, forming glycerol-3-phosphate and phosphatidic acid that incorporate and activate DAG.

Protein kinase C (PKC) is a family of multifunctional enzymes activated by three phosphorylation steps; (1) phophorylation of the regulatory domain, necessary for maturation of the protein, followed by (2) auto-phosphorylation, stabilizing the enzymes hydrophobic parts, in this step PKC is still in its inactive form, helped by increased cytosolic Ca^{2+} due to external stimuli. Ca^{2+} binds to PKC in one domain, increasing the affinity for DAG interactions on another domain. In the next step (3), PKC binds to DAG in the cell membrane, followed by phosphorylation, activating the kinase activity of the protein [22, 83-85].

PKC exists in more than 10 identified isoforms; however, PKC-α, PKC- δ and PKC-β is most abundant in vascular cells and the δ- and β-isoforms is by far most activated by an intracellular hyperglycaemic condition [22, 82]. PKC-α, β_1, β_2 and γ are activated by increased DAG or/and increased Ca^{2+}, while PKC δ, ε and θ are DAG but not Ca^{2+} dependent, and a third group of PKC, which is either DAG or Ca^{2+} dependent has also been identified. In addition, all three PKC groups can be activated by phospholipids [80, 83-87].

The activation of the DAG-PKC pathway impairs the regulation of vascular permeability and abnormal angiogenesis by increasing VEGF, decreasing eNOS and increasing ET-1, the later working as a vasoconstrictor that leads to impaired blood flow. These events aggravate even more in combination with vessel thickening due to increased TGF-β expression, increasing collagen and fibronectin production, figure 5. Furthermore, PKC increases the expression of several inflammatory mediators by NF-κB activation. PKC activation also leads to activation of several pro-apoptotic and apoptotic mediators, figure 5 [9, 10, 22, 28, 29, 86-90].

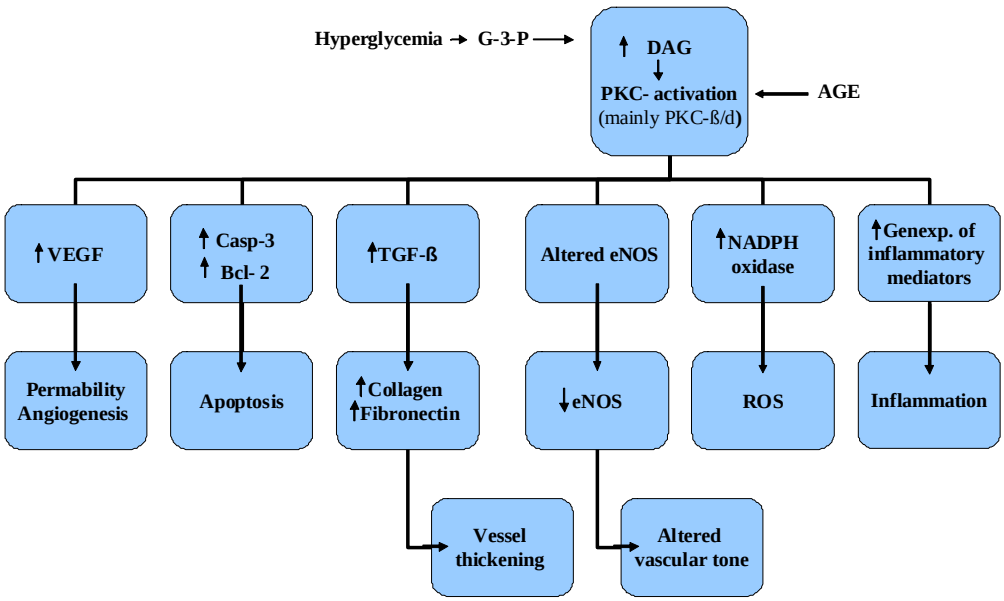

Figure 5 Forthcoming events of PKC activation
A schematic picture illustrating the effects PKC activation by increased DAG synthesis. The final outcome is increased angiogenesis and endothelial permeability, cell death in form of apoptosis, vessel thickening, altered vascular tone, increased ROS production and inflammation.

4. Increased flux through the hexosamine pathway

Excess intracellular glucose doubles the flux rate into the hexosamine pathway. During normal conditions this is a branch from the glycolysis where ~ 3% of the total glucose is utilized and metabolised to fructose-6-posphate and further to glucoseamine-6-phosphate, in order to produce uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is a co-enzyme required for the synthesis of glycoprotein, glycolipids and proteoglycans which are major components of the extracellular matrix.

Increased glucose and by that higher flux through the hexosamine pathway leads to modulated transcription factors and insulin resistance. The mechanism explaining insulin resistance is not yet fully understood, but among different theories impaired pancreatic β-cell function and induced apoptosis have been observed [9, 10, 26, 91-94].

Cell death by apoptosis or necrosis

Cell death, as a response of any kind of injury can occur through two main pathways. The first is the apoptotic pathway (controlled), where the cells shrink, defragment and are removed by phagocytosis by macrophages and the second is the necrotic pathway (uncontrolled) where the cells are swollen, burst and induce inflammation and damage to nearby tissue [95, 96].

Apoptosis

Apoptosis or programmed cell death is an energy-dependent form of cell death that can occur due to cellular injury or when the cell has fulfilled its purpose. The apoptotic process is mediated by a group of proteolytic enzymes called caspases [95, 96]. All cells with nuclei have inactive pro-caspases waiting for an activation signal to execute the cell. There are two major pathways leading to cellular apoptosis; the extrinsic pathway and the intrinsic pathway which both finally lead to the "execution pathway" thereby killing the cell [95-97].

The extrinsic- and intrinsic pathway

The extrinsic pathway requires extracellular stimuli for activation of death receptors on the cell surface, followed by formation of death-inducing signalling complex (DISC) and activation of caspase-8 before entering the execution pathway [95-97].

The events in the intrinsic pathway are mitochondria initiated and dependent on intracellular signalling. Stimuli, such as viral infections, toxins, cytokines and ROS cause changes in the mitochondrial trans-membrane potential, releasing cytochrome c and several pro-apoptotic proteins.

There are two families of pro-apoptotic proteins; Bcl-2 and BH3-only proteins. Members of the prior family can either promote or suppress apoptosis. Bax and Bim belong to the part of the Bcl-2 family of proteins that promote apoptosis [96]. Furthermore, the pro-aptotic proteins are located in the innermembrane of the mitochondria and are released to the cytosol upon apoptotic stimuli, where Bim-cleavage activates the death receptor [97, 98]. The BH3-only family members; Bid, Puma, Noxa and Bad, do all promote apoptosis by mitochondrial damage. Secondary activation of Bax or Bim leads to mitochondrial cytochrome C leakage and further caspase-9 and caspase-3 activation and finally entering of the execution pathway [95, 97-100].

The execution pathway

Finally, the execution pathway, where several other caspases and enzymes are activated, leads to cell degradation. The cell nucleus defragments and apoptotic bodies are formed, still containing functional organelles. The apoptotic bodies are then phagocytosed by macrophages [95-97, 101].

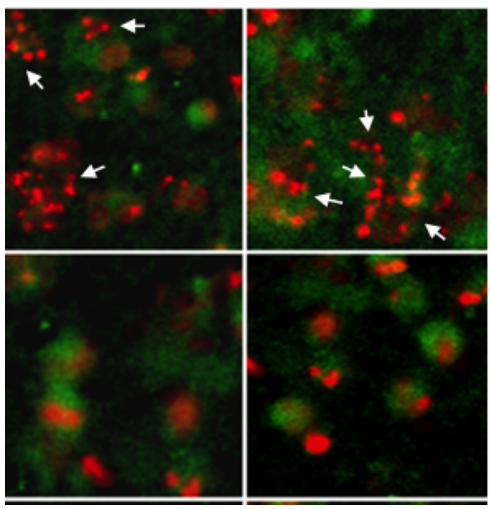

Figure 6 Apoptotic and intact endothelial cells
Apoptotic and non-apoptotic endothelial cells illustrated by confocal microscopy. Cellular membrane (green), stained for chemokine receptor 2 (CXCR2), and cell nucleus (red) stained with propidium iodine, illustrating apoptotic cells on the two upper pictures and intact cells on the lower.

Necrosis

The classical way of looking at necrosis is that it is an uncontrolled, non-energy dependent process that often leads to local inflammation due to a cellular burst. It is initiated by extracellular stimuli, such as toxins or infection. Here the cell membrane ruptures, the organelles are dysfunctional and necrotic blebs are formed. High levels of ROS and highly oxidized LDL often results in necrosis, while low to medium levels of these factors generally are observed in apoptosis. These events have been shown in the development of atherosclerosis [102]. Necrotic cells induce inflammation by releasing pro-inflammatory proteins, which bind to RAGE and increase the expression of vascular adhesion molecules [97, 102, 103].

Proposed mechanisms for glucose induced apoptosis

PKC activation due to a hyperglycaemic condition leads to apoptosis indirectly, by increased levels of inflammatory markers and ROS, and directly by altered levels of intracellular tumour suppressor p53 and p38 mitogen-activated protein kinase (MAPK) phosphorylation. Furthermore, elevated expression of PKC leads to a reduction of mitochondria membrane potential, cytochrome c release and activation of Bcl-2 proteins and further casapse-3 activation, figure 5 [29, 89, 90]. 3,4-DGE and high concentrations of glucose have been shown to promote caspase-3 activation and overall apoptosis in leukocytes [3, 4].

PRESENT STUDIES

The overall aim of this thesis was to investigate glucose and its degradation products. When wrongly handle they contribute to increased inflammation and oxidative stress, both on a cellular level by looking at leukocytes and endothelial cells and with a clinical approach, reinforced by the negative influences of uraemia and dialysis treatment.

In addition, citrate, by its suggested antioxidative properties, was added to glucose-harmed endothelial cells and inflammatory markers were evaluated. Further, correlations between trace elements, AGE formation, oxidative stress and inflammation in dialysis patients with and without diabetes was evaluated.

The results confirmed that hyperglycaemia and GDPs increase oxidative stress and inflammation both at a cellular level and in patients receiving GDP containing infusion fluids. Some of the induced cellular harm could further be reduced by citrate addition. We also observed an unbalance in trace element status in dialysis patients, compared with a healthy control group, and a significant correlation between low plasma selenium and high markers of oxidative stress in diabetic dialysis patients.

Paper I. Infusion fluids contain harmful glucose degradation products

Background

Toxic GDPs have previously been found in glucose containing PD-fluids, formed during sterilisation and storage [62]. When GDPs enter the circulation they react with proteins and AGE is formed. AGE can lead to increased inflammation and oxidative stress [1, 5, 7, 69, 70].

This was investigated in *Paper I*

- GDPs in commercially available glucose-containing infusion fluids by HPLC
- LC50 for the different GDPs on human neutrophils, fluorometrically by the MTT-assay
- Comparison of cell viability on cells exposed to GDP-containing infusion fluids with GDP-free containing infusion fluids, fluorometrically by the MTT-assay
- Comparison of post-operative patients receiving GDP-containing infusion fluids with a control group not given glucose containing fluids; looking at serum GDPs found and AGE formation pre- and post infusion, by HPLC
- Looked at the inflammatory response of neutrophils during bacterial infection by measuring cytokines IL-6 and CXCL8 as well as neutrophil oxyburst after exposure of GDPs, by ELISA and flow cytometry

Results

Patients that receive normal infusion fluids at the admission to intensive care unit (ICU) are also infused with reactive GDPs, in concentrations similar to neutrophil LC50 values for the most cytotoxic GDPs, i.e. 3,4-DGE and formaldehyde. GDPs remain in the circulation up to 9 h after infusion and there is a significant correlation between infused GDPs and AGE formation. GDPs and GDP containing infusion fluids also modulate the inflammatory response by suppressing neutrophil cytokine secretion and neutrophil microbial killing during infection.

Paper II. Citrate treatment reduces endothelial death and inflammation under hyperglycaemic conditions

Background

Hyperglycaemia and GDP-caused endothelial dysfunction plays a key role in the pathogenesis of diabetic complications and are linked to oxidative stress and inflammation [5, 69, 70]. Citrate is an intermediate of the citric acid cycle and possesses anticoagulant and antioxidant capacities. Although the clinical usage of citrate is gaining popularity, in-depth knowledge about its anti-inflammatory mechanisms was unknown prior to this study, *paper II* and there were no studies published on citrate treatment during hyperglycaemic conditions.

This was investigated in *Paper II*

Primary endothelial cells (HUVEC) during hyperglycaemia or after exposure of 3,4-DGE. Citrate or citrate-gluconate was added subsequently and following events were measured:

- Apoptotic and necrotic endothelial cells, by using flow cytometry
- Apoptotic endothelial cells were visualised by confocal microscopy
- PKC-β expression, by western blotting
- Neutrophil migration across endothelial cell layer by a transwell model
- Expression of adhesion molecules ICAM-1 and VCAM-1, quantitatively by flow cytometry
- Cytokine secretion (IL-6 and CXCL8), by ELISA

Results

A hyperglycaemic condition or addition of 3,4-DGE increased both the fraction of necrotic and apoptotic endothelial cells compared with controls. More cells are necrotic than apoptotic and the addition of citrate reduced both types of cell death. Furthermore, the adhesion molecule ICAM-1 and PKC-β were upregulated, as was the secretion of the cytokine IL-6, and increased fraction of migrated neutrophils was observed. Adding citrate showed beneficial results in all experiments mentioned above.

Paper III. The complexity of inflammation, oxidative stress and trace element status in non-diabetic and diabetic hemodialysis patients

Background

Hemodialysis patients are at elevated risk for oxidative stress and inflammation in combination with altered trace element levels. Deficiencies of some essential trace elements could potentially lead to impaired inflammatory defence and to oxidative stress, while elevated concentrations trace elements might result in increased generation of reactive oxygen species (ROS) [53, 54, 104, 105]. For diabetic HD patients the risks are further elevated due to the effects of the disease [106]. We investigated correlations between inflammation, oxidative stress and trace element concentrations in HD patients and, in particular, if there was any correlation between diabetic and non-diabetic HD patients.

This was investigated in *Paper III*

Hemodialysis patients with and without diabetes pre- and post-treatment compared with a healthy control group by measuring:

- Plasma AGE formation (Pentosidine), by HPLC
- Marker for acute inflammatory response (PTX-3) in plasma, by ELISA
- Plasma oxidative stress marker in form of oxidized DNA (8-OHdG), by ELISA
- Trace element concentrations of chromium, manganese, cobalt, copper, zinc, selenium, rubidium and molybdenum in plasma, whole blood and effluent, by coupled plasma-mass spectrometry (ICP-MC)

Results

All HD patients had significantly increased plasma levels of markers for AGE, inflammatory response and oxidative stress pre-dialysis, compared with controls. PTX-3 levels increased even further during dialysis, while the concentration of 8-OHdG decreased.

The highest pentosidine concentration was unexpectedly found in non-diabetic HD patients. All HD patients had elevated concentrations of chromium, manganese,

cobalt, copper and molybdenum compared with controls. In contrast, significantly lower concentrations of selenium and rubidium was found in HD patients compared with controls. Selenium concentrations were lower in the diabetic HD patients. Furthermore, there was a significant correlation in concentrations between decreased plasma selenium and increased 8-OHdG in the diabetic HD group.

DISCUSSION

This thesis confirms that too much or wrongly handled glucose contributes to increased inflammation and oxidative stress, both on a cellular level and with a clinical approach. In addition, trace element status is affected in dialysis patients and the addition of citrate, working as a proposed antioxidant, reveals positive results *in vitro*, figure 7.

From a future perspective, preventing hyperglycaemia and terminating the forthcoming cascades are steps towards a better outcome. Nevertheless, a therapeutic aspect is also necessary and here is where the focus needs to be, looking at the possibilities of using citrate and taking control over trace element reductions and supplementations. Further work improving dialysis fluids might be a way of controlling these substances and administrate them where they may have an immediate effect, i.e. on the blood cells and the endothelial cells.

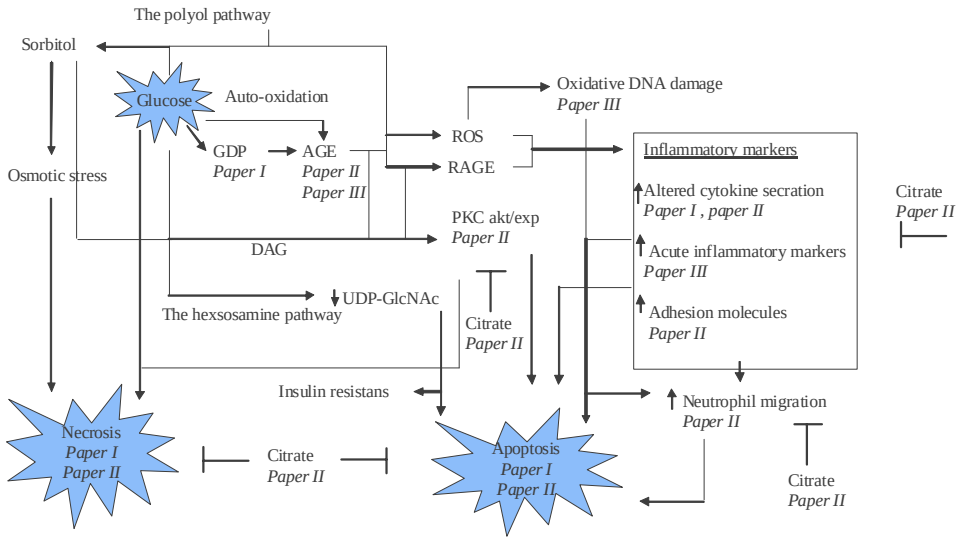

Figure 7 Pathways involved in hyperglycaemia induced harm, discussed in this thesis
Schematic picture illustrating the complexity of the different aspects of this thesis. With glucose, as a main player, inducing oxidative stress, inflammation and cell death by apoptosis and necrosis by auto-oxidation, the polyol pathway, the hexosamine pathway and PKC activation. The addition of citrate is also an important part of this study and the positive effects of citrate addition to glucose-damaged endothelial cells are illustrated here and discussed later.

Apoptotic or necrotic pathways; wrong focus?

In *paper I* neutrophil viability was evaluated by the MTT assay, measuring mitochondrial activity, and in *paper II* we used specific markers for endothelial apoptosis and necrosis. Although experiential settings differed due to optimization of the procedures and methods used, the results from both papers were consistent, i.e. illustrating increased cell death after exposure of GDPs and high concentrations of glucose.

However, the mechanisms behind GDP- and glucose-induced cell death are still unclear. We do know that a majority of exposed endothelial cells die due to necrosis and not apoptosis, strengthening the theory of overproduction of ROS, which results in

necrosis and not apoptosis [103]. Less oxidized LDL and proteins often follow the apoptotic pathway, but there is an ongoing debate on whether necrosis is an uncontrolled and non-energy dependent process [102, 103, 107, 108]. In addition Goldstein et al suggests that early mitochondrial dysfunction, such as ATP depletion, is a specific event related to a forthcoming necrotic death [103]. This theory could explain the reduced mitochondrial activity observed in the neutrophils during the MTT assay [103]. TNF-α produced by PKC activation [87] can also be an explanation of necrotic cell death, since TNF-α overproduction can result in a quick ROS burst that knock out the mitochondrial membrane-potential [109]. Passive release of pro-inflammatory high-mobility group protein B1 (HMGB1), from necrotic cells due to changed redox-potential, could possible be an explanation [5], though HMGB1 binds to RAGE and are thus able to induce forthcoming events [37].

Cells like cortical neurons have a switch, that switches from necrotic cell death to apoptotic when the glucose concentration is increased in the cell growth medium [110]. High glucose is also known to suppress the necrotic markers in favour of the expression of the apoptotic markers such as Bax and Bim [101, 110, 111]. The increased ATP production due to more available glucose could be a possible explanation, making the cells able to process with the apoptotic cascade. Catalan et al showed that 3,4-DGE, of the investigated GDPs, is responsible for accelerated caspase-3 mediated apoptosis in leukocytes, confirmed by results on apoptosis and overall cell death [3, 4]. In addition, most available literature on glucose and GDP-induced cell death does not compare the necrotic fraction with the apoptotic [4, 112].

Most studies focus on the apoptotic cell population, which in our study, *paper II*, were a rather small population compared with the necrotic ones. In addition it is known that macrophages undergoing necrosis contribute to advanced atherosclerosis, but this is not applicable to the observed endothelial necrosis in our experiments, since the macrophages form foam cells and engulf due to endocytosis of fat as previous described [102, 113, 114].

Antioxidants or PKC-blockers as a strategy to eliminate hyperglycaemia-induced oxidative stress and inflammation

Hyperglycaemia can increase ROS formation by several pathways as previously described. In addition, a hyperglycaemic condition can also reduce cellular capacity to cope with ROS and a recent study suggested that endothelial cells exposed to high glucose had a decreased H_2O_2-degradation compared cells treated with normal glucose [115]. The capacity of antioxidants has been studied in several *in vitro* experiments, showing that antioxidants can reduce intracellular ROS and increase cell survival. The

α-lipoic acid, coenzym-Q10 and quercetin metabolites decrease Bax, caspase-3 and caspase-9 expression in hyperglycaemic treated human endothelial cells [74, 116-118], and turine inhibit ROS-induced apoptosis but not necrosis [119].

Furthermore, supplementation of SOD and catalase modifications have been suggested to improve ROS elimination [120]. Vitamin C and vitamin E have been suggested as a supplement to CVD patients, diabetic patients and elderly, but the antioxidative properties have been hard to prove clinically [15-17]. Dietary supplementation of different antioxidants has been tested with varying results in hemodialysis patients [14]. Decreased lipid oxidation and improved anaemic status was shown after oral vitamin E and C supplementation. Vitamin E on the dialysis membrane and vitamin C in the dialysis fluid has also been used in attempts to reduce dialysis induced oxidative stress with conflicting results [14]. In addition vitamin E is shown to reduce PKC activity and inhibit LDL oxidation after hyperglycaemic activation [14, 27, 28].

In vitro, citrate protects cells from CaOx crystallization (kidney stone) induced injury by preventing lipid peroxidation through decreased ROS production [33, 51, 121-124].

Omi et al pointed out PKCδ as the most relevant apoptotic protein [90] and activation of this specific PKC isoform has been identified as an apoptotic inducer of vascular smooth muscle cells [89]. Quagliro et al suggested that hyperglycaemia leads to increased oxidative stress by activating a PKC-β and linked this activation to the caspase-3 and Bcl-2 mediated apoptosis in endothelial cells, since blocking PKC activity reduces parts of the apoptotic cell fraction [88].

Citrate is an intermediate in the citric acid cycle, generating ATP in the mitochondrial membrane of the cells. It works as an anticoagulant by chelating calcium ions in the clotting cascade and is primarily preferred over heparin due to heparins unspecific affinity of binding proteins, contributing to increased inflammation. In addition patients treated with heparin as an anticoagulant have also an increased bleeding risk, compared to the ones treated with citrate [125, 126]. We showed that citrate has anti-inflammatory properties in *paper II*, by decreased levels of inflammatory cytokines, transmigration, PKC-β expression and reduced cell death. Citrate could block PKC-β, and end the subsequent cascade; PKC-β needs Ca^{2+} in order to be activated, and the chelating effect of citrate to Ca^{2+} might be part of the inhibitory step.

The pathway from increased PKC expression to cell death by apoptosis has been analysed *in vitro* in several cell types, similar to what we showed in *paper II*, suggesting that citrate is beneficial for the cells during this condition, figure 7 [102, 113, 114, 127]. Especially since hyperglycaemia also up-regulates other, non-Ca^{2+} sensitive, isoforms of PKC [22], and further investigations of this would be of interest.

Another theory might be that citrate binds increased cytosolic calcium that is upregulated due to apoptotic stimuli, before the mitochondrial rupture [97] and by that prevent the forthcoming cascade of apoptosis. Terminating the inflammatory- and ROS-generating cascade prior to PKC activation by AGE inhibitors has been

investigated with several substances, including aspirin [69, 128], often working with chelating mechanisms similar to those of citrate.

ICAM-1 expression, due to increased levels of inflammatory mediators, such as pro-inflammatory cytokines, is well investigated and have a crucial roll in the regulation of vascular permeability and atherosclerosis [129-131]. Hyperglycaemia has been identified as an inducer of increased endothelial ICAM-1 expression, *paper II*. Anti-diabetic drugs have been observed to decrease the levels of ICAM-1 leading to elimination of the inflammatory events [132]. Since calcium chelating agents were not effective in their study, the authors proposed an alternative mechanism, an aldose reductase inhibitor, which prevents sorbitol production (the popyol pathway) and decreases ICAM-1 expression following this pathway; this might explain the other mechanisms of citrate capacity in our study [132].

Trace element deficiency or accumulation

Nevertheless, to add another key player to the puzzle, we also investigated the possible role of trace element accumulation or deficiency and the inflammatory and oxidative status of diabetic and non-diabetic HD patients, *paper III*. Diabetic patients on hemodialysis have an altered vascular tone when using HD fluids with higher glucose content compared with non-diabetic HD patients [133]. It is also well documented that both the uraemiccondition and diabetes contribute to increased AGE formation, oxidative stress and inflammation in hemodialysis patients [46, 104, 106]. Considering the double nature of trace elements, being able to induce ROS production by the Fenton reaction and being able to act as co-factors of important antioxidants, makes the present uncontrolled way of supplement additional trace elements to dialysis patients questionable [58, 134].

Our observed increased chromium and copper concentrations and decreased selenium, in combination with dialysis and diabetes could explain parts of the altered oxidative and inflammatory status in hemodialysis patients, *paper III*. The overall picture seems to be more complex, although copper, besides inducing ROS, is being a part of Zn-Cu SOD and chromium is necessary in glucose metabolism [135]. The correlation between high levels of the oxidative stress marker 8-OHdG and low plasma selenium levels in diabetic hemodialysis patients could be explained by decreased active glutathione peroxidase. Selenium deficiency in combination with diabetic complications can explain the decreases in active glutathione.

In addition, this event is not linked to pentosidine production, which surprisingly was higher in the non-diabetic hemodialysis group, *paper III*. According to Spavaro et al pentosidine does not bind to RAGE with high affinity, on the other hand, CML, measured in *paper I*, is able to bind to RAGE and by that start an inflammatory and

apoptotic cascade and therefore it would be of great interest to correlate this AGE instead of pentosidine with forthcoming events of RAGE activation [5, 70].

Malnutrition-inflammation complex syndrome (MICS) is a combination of protein-energy malnutrition and increased inflammation observed in hemodialysis patients due to inadequate nutrition intake, dialysis removal, uraemictoxins, volume overload, increased ROS production and decreased levels of antioxidants [46, 52]. Rubidium deficiency has been linked to protein-malnutrition and depression which might lead to anorexia. Zinc deficiency can result in altered taste, contributing to anorexia and decreased levels of Zn-Cu SOD, which is an important antioxidant [53, 57, 134, 136].

Supplementations or reductions of trace elements are like playing with a weight-bowl. The question is not whether we need them or not, but, rather, how do we find the right dosage and administration route? Administration of selected trace elements through dialysis fluids could also help providing this equilibrium, *paper III.*

Future perspective

As illustrated in figure 7, the multipart pathways involved in hyperglycaemia-induced cellular damage, together with dialysis and trace element abnormities, are still not yet fully explored. More focus is needed on the necrotic endothelial cells and to find whether this cascade is ATP dependent or not. Other possible therapeutic targets need to be mapped, even though PKC seems to play a central roll. In addition, targeting a protein responsible for the subsequent cellular events, such as PKC, might have the opposite effect, since we depend on cellular apoptotic signalling in order to prevent tumour formation. Here, blocking the apoptotic cascade, by blocking PKC activation in a tumour cell, is one example [85].

Targeting early hyperglycaemia, before the onset of diabetes, showed positive results on micro-vascular damage and nephropathy [26, 94]. The vascular trauma should also be taken into consideration, which might lead to irreversible premature aging of the endothelial cells that might include irreversible gene-expression due to exposure of hyperglycaemia – a so called permanent phenotypic change [27]. Madonna et al further suggests that targeting already formed ROS might be difficult to achieve, and that instead focus should be to prevent its formation [94]. This strategy is promising and we would like to continue our work on citrate, as citrate induce boosting of the Krebs cycle, generates ATP and chelates ROS inducers by calcium and metal ion binding. Our results suggest that citrate may have therapeutic potential by reducing hyperglycaemia-induced endothelial inflammation and by abolishing endothelial dysfunction.

But citrate metabolism needs further investigations, since addition of citrate generates unspecific Ca^{2+} binding and CO_2 as a metabolite that is of clinical relevance.

Nevertheless, the opposite function has also been proposed for citrate, suggesting a promotion of anaerobic glycolysis due to infectious stimulation, similar to tumour cells [137, 138]. ATP generation by the Krebs cycle is then turned off, in favour for glycolysis and the phosphate pathway, shutting down the mitochondrial metabolism, preventing apoptosis and promoting fatty-acid synthesis. O'Neill et al suggests that this metabolic switch makes citrate a precursor for oxidative stress, by being withdrawn from the Krebs cycle and transposed out of the mitochondria, generating oxaloacetat and further NO and ROS [137, 138]. Targeting the citrate carrier (CIC), which transport citrate from Krebs cycle has further been investigated as possible therapeutic target, decreasing ROS and NO production [139].

Despite these findings the future approaches of reducing hyperglycaemic-induced oxidative stress and inflammation, both in patients with or without renal failure, look positive, since there are several molecular pathways to target. Further, we have the therapeutic aspect – the market is indeed growing, due to a continually increased number of both diabetic- and renal failure-patients [24, 25].

Here, the administration-route might be the essential part for a proper therapy. The negative reactions often start in the blood when the glucose or GDPs present in excess meet the leukocytes and the endothelial cells. Nevertheless, during renal failure the blood is exposed to uraemic toxins and also to external interactions during dialysis treatment. So this is perhaps where we would like to put in our efforts, preventing and targeting these events on the spot.

One way of getting there is by improving the dialysis fluids. This is a perfect opportunity, controlling supplementation and removal over the membrane, by diffusion and convection when the blood meets the dialysis fluid. As a result of this thesis we know that citrate can decrease the inflammatory response created by hyperglycaemia, even though the exact mechanism is still to explore. We also posses a great amount of data regarding trace element status in dialysis patient with and without the diabetic aspect. Taken together, from a therapeutic point of view, this opens up a lot of possibilities of further exploring the synergetic effects of citrate addition and selectivity of trace element supplementation or removal.

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APENDIX: Paper I-III