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Dissertation

Non-biological liver support – improved

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Because falling's not the problem,
when I'm falling I'm at peace

It's only when I hit the ground
it causes all the grief

Florence Welch

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Abbrevations

ABiC	albumin binding capacity
ADVOS	advanced organ support system
ALEX	albumin exchange
AoCLF /ACLF	acute on chronic lifer failure
C-Doca	chenodeoxycholic acid
CLIF-C	chronic liver failure consortium
ELISA	enzyme-linked immunosorbent assay
ESR	electron paramagnetic resonance spectroscopy
FAS	first apoptosis signal receptor
FXR	farnesoid-X-receptor
HGF	hepatocyte growth factor
HMA	non-oxidized mercapt-albumin
HNA1	reversibly oxidized non-mercapt-albumin
HNA2	irreversibly oxidized non-mercapt-albumin
HSA	human serum albumin
HVPET	high volume plasma exchange therapy
Ig	immunoglobulin
IL-6	interleukin 6
IMA	ischemia modified albumin
IMAR	IMA/albumin
INR	internation normalized ratio
MARS	molecular adsorbent recirculating system
MELD	model for end stage liver disease
MW	molecular weight
OPAL	open albumin dialysis
SEPET	selective plasma exchange therapy
SIRS	systemic inflammatory response syndrom
SPAD	single pass albumin dialysis
TGF-beta1	transforming growth factor beta 1
TNF-alpha	tumor necrosis factor alpha
TNFR1	tumor necrosis factor receptor 1

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1. Introduction

1.1 Liver failure

Over the last 25 years the understanding of liver failure has evolved from a term loosely describing the impairment of liver functions in acute or chronic liver disease towards a more complex concept with sub entities. Local and systemic reactions orchestrate liver failure, lead to extra-hepatic organ involvement and manifest in clinically challenging inter-disciplinary problems with high mortality [1].

Whereas acute liver failure is characterized by a rapid deterioration of liver functions without pre-existing hepatic damages, acute-on-chronic liver failure precipitates from or adds insult to a previously compensated chronic liver disease like cirrhosis. Both can rapidly evolve multiple end-organ failures, are associated with higher risk for infections due to bacterial translocation, risk of coagulopathy due to synthesis derangements combined with increased consumption of clotting factors as well as an increase in portal pressure leading to vasoactive substances flooding the splanchnic and circulatory system. Portal hypertension and microcirculatory changes induce hemodynamic instability on a local and systemic level and are associated with the development of varices and hepatorenal syndrome [2]. Disease severity is aggravated by increasing extra-hepatic organ damages and in term lead to high short term mortality [3].

Liver transplantation has been shown to improve survival in these patients, however the field suffers from organ shortage as well as high costs that can range from roughly 60.000€ to 133.000€ with 5 year follow-up after transplantation [4]. Non-biological extracorporeal liver support therapies have emerged and add to the therapeutic arsenal by providing detoxification support [5]. This allows for a better organ allocation as patients can be identified that have chances of natural recovery, recovery with liver support, bridge to transplant using liver support or need for an organ transplant.

1.2 Toxins

Blood is the central compartment connecting all tissues and organ microcirculations. This allows for specific organs covering functions for the whole organism. In a disease like liver failure this results in extrahepatic organ involvement. Blood is made up from cellular and humoral components in aqueous solution. Whereas the cellular components cover erythrocytes, thrombocytes, leukocytes and are within micrometer size, humoral components make up the plasma and are within nm size. Extracorporeal blood purification treatments should be optimized to have no detrimental effects on cellular components while targeting the plasma fraction containing the dissolved toxins – either water soluble or protein bound. Anderson et al. showed that most constituting plasma proteins are smaller than 100 kDa [6].

Decrease of hepatic functions goes hand in hand with increasing metabolite concentrations to toxic levels. Water soluble and protein bound toxins have been identified that can serve as markers for liver failure and extrahepatic organ involvement [7].

Water soluble marker toxins

Liver failure is associated with a hampered urea synthesis. *Urea* is physiologically used to fix and remove nitrogen, originating from dietary uptake as well as protein turnover, from the circulation via renal excretion. Urea is able to generate cyanate and ammonia by a spontaneous equilibrium reaction. Free cyanate has been shown to interact with lysine residues of proteins leading to a carbamylation of the protein (often referred to as citrullination) [8]. Carbamylated proteins in term have been found to be predictors of mortality in end-stage renal disease (ESRD) concerning the development of cardiovascular problems by increasing plague formation [9]. Carbamylation of albumin is often seen on lysine 549 and Berg et. al were able to show that levels are increased in end stage renal disease and are associated with increased mortality [10].

Decreasing urea synthesis in term leads to an increase in *ammonia*, which is able to freely cross the blood-brain-barrier. Cerebral astrocytes are able to immobilize it by synthesis of glutamine. Excess intra-cellular glutamine levels lead to an increase in osmotic draft resulting in astrocyte swelling as well as mitochondrial damages accompanied with an increase in reactive oxygen species [11]. Additionally an increase of γ-aminobutyric acid activity is observed. This is aggravated by an elevated production of endogenous benzodiazepines and promotes an overall lowered cerebral energy delivery. Clinically this is presented by an increase in intracranial pressure and the development and progression of hepatic encephalopathy [12], [13].

Creatinine derives from physiological muscle metabolism and is excreted mainly by the kidneys via glomerular filtration. Creatinine is not considered to be a toxin, however due to its easy assessment in blood and urine it is widely used to assess remaining renal function and the development of hepatorenal syndrome [14].

Aromatic amino acids

Amino acids are physiologically used to build up proteins and, if energy deficiencies occur, can be used for gluconeogenesis. However, as no storage mechanism exists akin to fatty acids, amino acid catabolism is important in order to maintain low blood levels of ammonia [15]. Hepatic damages have been associated with decreased amino acid metabolism leading to an increase and fluctuations of serum amino acids. Briefly, branched chain amino acids decrease while aromatic amino acids increase and can be assessed as fischer's ratio (ratio of branched chain amino acids to aromatic amino acids) [16]. Elevated aromatic amino acids have been linked to disease progression and the development of hepatic encephalopathy – most likely due to a synthesis of false neurotransmitters resulting from increased cerebral uptake [17].

Protein bound marker toxins

Bilirubin is a small sized toxic metabolite of heme catabolism and has low water solubility (solubility threshold in plasma is 0.004 mg/dL) [18]**.** Its transport in plasma is realized by high-affinity binding to albumin – in term increasing the size of the now toxin-protein complex. The liver features a highly permeable fenestrated endothelium – thereby enabling the protein-complex to reach hepatocytes. Transporter mediated uptake allows bilirubin to enter hepatocytes which results in the removal of bilirubin from albumin and subsequent metabolisation to conjugated glucoronic acid derivates. Conjugation leads to an increase in water solubility to the extent that they can be removed from hepatocytes via bile excretion and subsequent defecation. However, in liver failure excretion of bile as well as bilirubin metabolism are impaired and lead to accumulation of bilirubin in plasma and tissue. This is aggravated by low albumin production and displacement of bilirubin from albumin due to binding site competition of various endo- and exogenous compounds [19], [20]**.** Furthermore, conjugated bilirubin can spontaneously form covalently bound delta-bilirubin by non-enzymatical esterification to albumin. Delta-bilirubin itself cannot be excreted via bile or urine and can therefore remain elevated in plasma for as long as albumins serum half-life of approximately 15-17 days. This can be observed for example during cholestatic liver diseases, where decreased elimination of conjugated bilirubin leads to an increase of delta-bilirubin that remains elevated even after bile flow is restored [21].

Bile-acids are synthesis derivates of cholesterol that have detergent-like properties. Cholic acid as well as chenodeoxycholic acid (C-Doca) are primary bile-acids that are synthesized within the liver and secreted into the small intestine where contact with the localized flora leads to conversion to secondary bile-acids like deoxycholic acid or lithocholic acid. Notably, the enterohepatic circulation leads to readsorption and subsequent return of bile-acids to the liver. One of their main functions is to emulsify and help to absorb lipophilic dietary fats or vitamins. Physiologically low levels of bile acids are found in serum [22]. However, decreased hepatic secretion of bile acids leads to an increase of bile-acids within plasma. Their detergent-like properties lead to disturbances and damages to cell membranes triggering hepatocellular apoptosis. Furthermore, oxidative stress is increased by inducing mitochondrial dysfunction. Bile-acids also are discussed to play a role in the pathophysiology of the hepatorenal syndrome: owing their low solubility in water and specific microenvironment changes within the distal nephron – the formation of bile casts obstructing urinary flow has been observed [23] and is associated with damages to renal tubular cells [24]. Various bile acids are ligands with different affinities of the farnesoid-X-receptor (FXR) which is providing feedback on bile acid synthesis and excretion as well as homeostasis of glucose and amino acids [25].

1.3 Albumin

Albumin represents the most abundant plasma protein (35-55 g/l) in humans with about 40 % circulating intravascular and 60 % distributed within extravascular compartments. The absolute synthesis rate is roughly 150 mg/kg/day with a half-time of approximately 17 days – this translates to nearly 8.5 % of plasma albumin being synthesized daily and albumin making up 3 % of total body protein [26], [27].

Over the last decades several unique features of albumin have been identified:

- *antioxidant properties* are due to one reducable cystein residue (Cys34) on albumin [27],
- *buffering of blood pH* occurs owing to the high amino acid content of histidine in an albumin molecule (16 histidines; imidazole groups allow proton uptake) [28]
- being dissolved in aqueous compartments divided by capillary walls that prohibit diffusive passage of proteins (except e.g. liver – fenestrated endothelium) leads to the buildup and *maintenance of colloid oncotic pressure*
- *modulation of inflammation* is realized by a complex interplay of reversible ligand binding, antioxidant functions and the ability to immobilize endotoxins [29]
- prevents formation of protein aggregates thereby having a *chaperone-like function* [30]
- *attraction of positively charged molecules and ions* due to a negative surface net charge
- *transport functions* are realized by several binding sites for carboxyl-group containing molecules or heterocyclic compounds (Sudlow binding Sites I and II) or fatty acids [31]

Clinical analysis of albumin only includes control of plasma levels -no functional tests have made it into clinical routine, yet. Different efforts and technological approaches have been taken on quantification of albumins binding functions, post-translational modification as well as oxidative state.

Albumin binding capacity (ABiC)

Evaluation of albumin binding capacity was described by Klammt et. al and uses the binding site II specific fluorescent dye danzylsarcosine to assess remaining binding function. Notably, ABiC correlates with liver failure severity assessment score MELD which is made up from serum bilirubin, creatinine and international normalized ration (INR). Further, the authors were discussing the removal of bile-acids as a beneficiary factor to increase ABiC [32]**.**

Human non-mercapt-albumin 1/2 (HNA 1/2)

Albumins antioxidant properties are for the most part relying on the redox state of Cys34 which can be assessed using high performance liquid chromatography with an anion exchange column and specific buffers [33]**.** This leads to the identification of 3 different albumin fractions:

1. non-oxidized mercapt-albumin (HMA)

2. reversibly oxidized non-mercapt-albumin (HNA1)
3. irreversibly oxidized non-mercapt-albumin (HNA2)

Patient albumins HNA2 fraction are a better tool at predicting 30- and 90-day survival compared to the MELD-score in a group of cirrhosis and acute on chronic liver failure (AoCLF) patients [33]. As patients liver failure etiology was mainly of chronic alcoholic abuse (approx. 90 %), this gives weight to the idea of chronic liver failure being partly mediated by increased oxidative stress due to chronic inflammatory challenges and accumulating damages to albumin.

Ischemia modified Albumin (IMA)

During pathological conditions involving increased oxidative stress albumin can undergo conformational changes. The exact mechanism remains unknown to this date, however it has been suggested to be an N-terminal modification resulting in a decreased ability to bind cobalt or copper [34]. An indirect photometric cobalt binding assay is used to measure IMA: Briefly, patients' serum is incubated with cobalt chloride and dithiothretol is added. IMA is not able to bind cobalt while the addition of dithiothretol is used to bind to free cobalt and to determine the relation of non-modified albumin to IMA. IMA has been found to weakly correlate to the MELD score as well as serum albumin and -bilirubin levels in chronic liver disease patients however can be easily assayed and might be useful as a biomarker for disease progression [35]. Notably, when referenced to patients' serum albumin levels the relation between IMA/albumin (IMAR) displayed a strong correlation to the MELD score and was able to discriminate survivors from non-survivors in a small clinical study on ACLF from Jalan et. al [36].

Fatty acid binding and transport efficiency

Albumin has different binding sites for fatty acids which can be assessed using electron paramagnetic resonance spectroscopy (ESR): Briefly, different concentrations of a fatty acid spin probe (16-doxyl stearic acid) are incubated with albumin in different hydrophobic environments (ethanol) and are subjected to ESR [37]. Changes in mobility and localization of spin labels in term allows for characterization of structural changes to albumin while binding affinity is calculated from the amount of bound and unbound spin probe. As hydrophobic interactions are dominating the mechanism of compound release from albumin when interacting with albumin receptors or cell surface proteins, the change in hydrophobicity of surrounding environment is used to give insight on albumins transport efficiency. A study from Jalan et. al found albumins' transport efficiency to be significantly decreased in nonsurvivors compared to survivors in ACLF patients [36].

Glycated albumin

A non-enzymatic reversible addition of glucose and glucose derivates to proteins' amino acids lysine and arginine has been observed in serum. Elevated glucose levels and an environment

displaying increased oxidative stress can lead to the irreversible formation of advanced glycation endproducts that induce protein aggregation and loss of function. Albumin has a long plasma half-life (approx. 17 days) and a high content of lysine (59 residues) and arginine (24 residues) and is therefore a promising candidate when looking at reversible and irreversible glycation. Interestingly, it has been shown that addition of glucose derivates to albumin leads to biophysical and biological property changes resulting in increased or decreased drug binding kinetics. This is partly explained by structural modifications resulting from a net reduction of alpha helical protein structure or covalent binding of glucose to the Sudlow binding site I [38]. Currently, 8 different methods are being described in the literature to assess the degree and position of albumin glycation, however strongly vary in methodology and still need to be referenced in clinical studies for their potential use as biomarkers in liver failure entities.

The described methods portray the importance of functional albumin during liver failure even though its role within the underlying disease pathology is not fully understood. However, clinical relevance is given by their correlation to survival or disease progression in specific subgroups of liver failure. A systematic approach correlating available methods among each other and with disease progression is still needed.

1.4 Immune system

The liver is a first-hit organ for many nutrients and toxins coming from the gastrointestinal tract due to its connection to the portal vein. This ensures for nutrient uptake and conversion as well as detoxification of toxins and pathogens. Chronic alcoholic abuse or viral etiology often seen in AoCLF leads to hepatic cell death and replacement with connective tissue clinically assessed as liver fibrosis or the more severe form of liver cirrhosis. On a local level this remodeling of tissue is being performed by stellate cells. Furthermore, disruptions of the gastrointestinal barrier occur – thereby enhancing bacterial translocation and inflammatory stress [39], [40]. This is accompanied by hepatic necrosis and apoptosis and leads to an increase in cytokines produced by hepatic immune cells – kupffer cells. Cytokines are small-to-middle molecular sized proteins that serve as messengers between tissues and the immune system. A variety of pro- and anti-inflammatory cytokines with pleiotropic functions have been identified and coordinate the complex interplay of cellular reactions to injurious stimuli and tissue homeostasis.

Rozga et. al [41] classified cytokines and immune-competent mediators according to their size and biological activity in liver failure: whereas smaller sized pro-inflammatory cytokines like Interleukin 6 (IL-6, 22-27 kDa), Tumor necrosis factor alpha (TNF-alpha, trimer 51 kDa), Transforming growth factor beta 1 (TGF-beta1, 44 kda) and endotoxin components (lipid A, 10-20 kDa [42]) or anaphylatoxins (C3a, 10 kDa) contribute to disease severity by being negative

regulators of hepatocyte proliferation, larger molecular sized hepatocyte growth factor (HGF, 190 kDa), immunoglobulins (IgG, 150 kDa; IgA 320 kDa; IgM, 900 kDa) and components of the complement system (30 – 200 kDa) contribute to physiological function and hepatocyte recovery. This is aggravated by decreased elimination and increased synthesis of cytokines observed in chronic liver failure [43].

TNF-alpha induces hepatocellular apoptosis by binding to a death receptor (Tumor necrosis factor receptor 1; TNFR1) [44] and is expressed by liver kupffer cells as well as infiltrating mononuclear cells in hepatic failure. This is boosted by an increase in hepatic TNFR1 expression as well as a simultaneous induction of first apoptosis signal receptor (FAS) genes, priming hepatocytes for additional apoptosis inducing pathways [45].

IL-6 is a cytokine known for its multifaceted role within the acute phase response and is a downstream mediator of TNF-alpha. Effects include the increased production of acute phase response proteins within the liver (e.g. C-reactive protein) as well as the recruitment of leukocytes. While these mechanisms promote liver tissue regeneration and homeostasis [46], the cytokines also sieve into the systemic circulation and lead to the development of hepatic encephalopathy [47] as well as problems associated with hyperdynamic circulation [48]. Notably, TNF-alpha and IL-6 induce liver regeneration by priming hepatocytes for growth factor stimulation showing that immunomodulation coordinates liver regeneration and is impaired in liver disease [49].

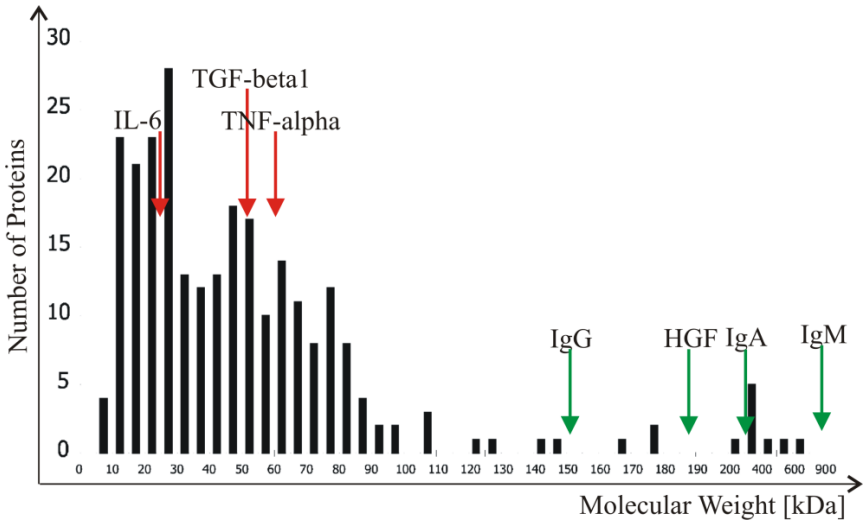

Figure 1 Overview of constituting plasma proteins modified after Andersen et. al [6] to include negative (red) and positive (green) markers of liver failure recovery according to Rozga et. al [41]

Larger molecular sized endotoxins (100-1000 kDa) are being released as breakdown products of gram-negative bacteria originating from increased bacterial gut-liver translocation that further contribute to inflammation and can lead to a cytokine storm. Jalan et. al gave rise to the idea of

looking at the neutrophil function during liver failure concerning their oxidative burst capacities and phagocytic activity: Oxidative burst measurements of isolated neutrophils form patients and ex vivo challenging of neutrophils with patient plasma were used to elucidate the role of endotoxins as hypothesised key players in the development of neutrophil abnormal functions in patients with alcoholic hepatitis. A higher risk of infection seen in alcoholic AoCLF might be explained by constant endotoxin challenge from ongoing bacterial gut-liver translocation resulting in decreased neutrophil function [50]. Identifying patients developing "immune paralysis" akin to the model of compensatory anti-inflammatory syndrome during sepsis might be a useful tool for treatment options improving survival [51]. Last but not least, the presence of clinically assessed systemic inflammatory syndrome (SIRS) was shown to correlate with increased mortality in patients with cirrhosis and renal failure [52].

1.5 Liver support techniques

Liver support techniques can be divided into *biological and non-biological systems*.

Non-biological liver support systems are designed to provide mainly detoxification support and use filtration and adsorption techniques to remove water-soluble and protein-bound toxins. Biological liver support systems are designed to further provide hepatic synthesis support by including a therapy specific liver cell compartment into the extracorporeal circuit. Cell lines used so far have been primary porcine hepatocytes as well as human immortalized liver cancer cell lines. The cells are supposed to metabolize toxins and procure specific synthesis products enhancing regeneration [53]. This work focuses on non-biological liver support techniques.

Single Pass albumin dialysis (SPAD) is the simplest way of performing albumin dialysis and can be set up using nephrology equipment. In principle, blood is being perfused against an albumin enriched dialysate over a semi-permeable membrane. The "clean" dialysate albumin is used as a molecular adsorbent that enhances removal of patients protein-bound toxins and will be subsequently fed to waste. Membranes can be selected according to clinical experience [54].

Using *MARS* (Molecular Adsorbent Recirculating System) the molecular adsorbent albumin is being recycled within an albumin recirculation loop separated from the patient via the MARSflux membrane (cut-off 60 kDa): after transfer of toxins from patient to dialysate albumin, the dialysate solution is passed over a charcoal and ion-exchange adsorber in order to remove protein-bound toxins. Water soluble toxins are being removed using a secondary small pore sized low-flux hemofilter within the recirculation circuit.

Open albumin dialysis (OPAL) was developed to improve toxin removal over the primary hemofilter into an albumin circuit by employing a transfer-optimized membrane (Opal Premium: EMIC², Fresenius) and an optimized charcoal adsorbent (Hepalbin12, Albutec) for removal of

albumin bound molecules. Removal of water soluble toxins is achieved by incorporation of a high-flux membrane into the albumin loop.

The Prometheus system uses two hemofilters in order to remove albumin bound toxins and water soluble toxins separately. The albumin filter (AlbuFlow, cut-off 250 kDa) allows for efficient sieving of patients´ albumin into a secondary circuit, where two adsorbents (neutral resin, anion exchange resin) remove toxins from albumin. The cleaned albumin is subsequently reinfused. Removal of water soluble toxins is achieved by introducing a small pore sized low-flux hemofilter into the patient circuit according to standard dialysis care. Notably, using two extracorporeal filters for removal of toxins leads to an increased extracorporeal blood volume in already hemodynamically unstable patients.

MARS and Prometheus trials lead to speculations on albumins oxidized fractions as a therapeutic target in liver failure treatments [55], [56]. Albumin can undergo reversible and irreversible oxidation processes in liver failure as portrayed by HNA1 and HNA 2 fractions. The *DIALIVE* system is designed to remove modified albumin using an albumin sieving hemofilter. Replacement of albumin is realized using an infusion of clean, high binding capacity albumin. Further an endotoxin and cytokine adsorption column is used to cater to inflammatory stimuli [57].

Another approach on non-biological liver support is the complete removal of plasma loaded with toxins and potentially unknown factors from patients using plasmapheresis techniques omitting adsorbents. High cut off membranes (500-2000 kDa cut-off) are used for plasma filtration followed by subsequent infusion of fresh frozen plasma from healthy donors. This requires high volumes of fresh frozen plasma as 3 days of therapy will amount to 24-36 liters. *High volume plasma exchange therapy* (HVPET) has been studied by Larsen et. al and has shown to improve transplant free survival as well as provide immunomodulatory effects in patients with fulminant hepatic failure [58].

The *selective plasma exchange therapy* (SEPET) had a similar approach, however limiting the plasma fraction to be smaller than 100 kDa - limiting the loss of albumin, complement factors, immunoglobulins, clotting factors and the hepatocyte growth factor. Fluid losses are replaced by electrolyte solution, human albumin solution and, if needed, fresh frozen plasma [59]. A novel SEPET membrane was developed based on extensive research on humural and cellular mechanisms on liver recovery. Notably, the SEPET approach allowed the usage of standard dialysis machines, which can in term shorten the time to initiation of treatment. Although animal studies looked promising and showed improvement of survival, no clinical data exists up to date [41]. Recently, the authors have introduced another novel membrane (ALEX – Albumin exchange filter) with a molecular cut-off around 150 kDa in order to replace functionally impaired albumin using albumin apheresis [60].

A novel therapeutic approach is under investigation using the *advanced organ support system* (ADVOS), where an albumin recirculation loop is used akin to the MARS system, however albumin is regenerated by carefully monitored pH changes that result in toxin displacement. Water soluble and displaced toxins are being removed via hemofiltration techniques using small pore sized membranes [61].

1.6 Membranes and Sorbents

As most toxins are soluble in plasma, it is the therapeutic target of extracorporeal liver support systems. Separation of plasma from blood is performed using hollow fiber membrane filtration. Those membranes have to minimize damages to cellular components while allowing for efficient plasma component sieving. A variety of parameters can be addressed to fine tune membranes according to their usage. These include membrane architecture, membrane material, surface modifications and the distribution and molecular weight cut-off of pores [62]. Progressing biomaterial research and technological improvements on membrane spinning for example lead to novel membranes with sharper molecular weight cut-off profiles mimicking the glomerular filtration barrier.

The removal of water soluble small sized toxins can be achieved using hemodialysis techniques where blood or plasma is pumped against clean aqueous dialysate over a semi-permeable small pore sized membrane. This allows for diffusion mediated removal of e.g. ammonia, urea and creatinine while limiting the effects on physiologically important plasma proteins. In order to maintain electrolyte balance the dialysate solution is made-up to meet patients' needs.

In order to remove protein-bound toxins the affinity of the accepting material for the specific toxin needs to be higher. Therefore sorbents have been applied to the field of liver support. They represent are a class of materials that is able to collect and incorporate target molecules by sorption processes according to their physical properties. Sorbent characteristics can be tuned to have higher binding affinity towards target molecules compared to proteins, which lead to the development of different blood- or plasma-perfusion devices.

Water-insoluble toxins are being physiologically transported by binding to plasma proteins like albumin. High binding affinity combined with the high abundance of albumin in plasma ensures effective metabolite transport. Physiologically albumin can be classified as a transport protein rather than a sorbent protein as binding to albumin is reversible and not used to immobilize toxins. However, when used within extracorporeal liver support systems for the removal of patients albumin bound toxins, it can be classified as a molecular adsorbent. The separation of "donor" and "acceptor" albumin allows for regeneration of acceptor albumin using different technological approaches.

Charcoals have been used in non-biological liver support systems as they display high affinity towards organic compounds which include bilirubin and bile-acids. Furthermore, pore texture (macro-, meso-, micropores), surface modifications and mineral matter content can be tuned to increase accessible surface area (=activated charcoal) and increase or decrease affinities towards specific organic compounds. Clinically, activated charcoals have been used for extracorporeal blood purification or e.g. ingested as an emergency treatment for acute poisoning [63].

Ion exchange resins can be used to capture anions or cations depending on their functionalization: Whereas the incorporation of acidic groups leads to an increase in attraction of positively charged molecules, basic groups allow for removal of negatively charged molecules. Sulfonated polystyrene beads (cation exchange resin) for example have been clinically used to remove potassium in hyperkalemia. In liver failure treatments employing plasma separation techniques anion exchange resins have been used to remove a variety of negatively charged small molecules and albumin bound molecules [64].

Another sorbent approach based on the molecular size of target molecules is represented by *size exclusion filtration* or molecular sieving. When plasma is passed over a selectively pore sized matrix the traversal speed of molecules is influenced by their ability to permeate into the matrix. This has been used for example as first separation layer using the Cytosorbents technology [65] where middle molecular sized proteins are able to enter the Cytosorb-beads and can be captured on the chosen material (polystyrene co-polymer optimized to adsorb hydrophobic compounds) , whereas larger blood components pass by.

A different approach on toxin removal from albumin has been studied using the ADVOS system: displacement of toxins from albumin is achieved by *changing the pH* of albumin's surrounding medium to acidic or basic conditions. This leads to conformational changes which results in lower binding affinity and the release of bound molecules. Unbound molecules are subsequently removed by hemofiltration using small pore sized filters [66].

An overview of membranes and adsorbents is given in the following table:

Table 1 Overview of currently available artificial liver support therapies with denoted membranes, cut-off values as well as employed sorbent modalities

Therapy	**Membrane** (Cut-off)	**Adsorbent**
SPAD	According to clinical experience	Acceptor Albumin
MARS	MARSflux (<60 kDa)	Acceptor Albumin, regenerated by AC250 *(activated granular charcoal)* IE250 *(anion exchange resin)* DIAflux (hemodialysis; ~10kDa)
OPAL	Cordiax FX1000 (~60 kDa) or EMiC² (~60 kDa)	Acceptor Albumin, regenerated by Hepalbin12 *(activated powdered charcoal)*
DIALIVE	Septex (>100 kDa)	

	Oxiris		PolyethyleneImine *(treated fiber surface for endotoxin removal)*
Prometheus	Albuflow AF 01 Dialyser F 60S	(<250 kDa) (~10 kDa)	Prometh 01 *(neutral synthetic resin)* Prometh 02 *(anion exchange resin)*
HVPET	Plasmafilter	(500-2000 kDa)	/
SEPET	SEPET ALEX	(~100 kDa) (~150 kDa)	/
ADVOS	FX 80	(<60 kDa)	Acceptor albumin, regenerated by pH mediated toxin displacement coupled with hemofiltration

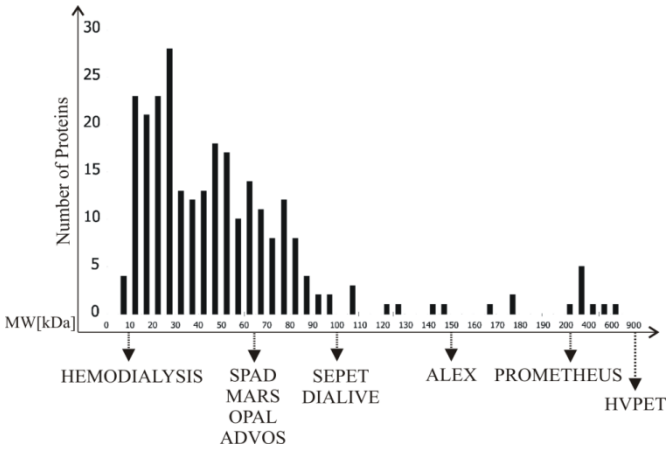

Figure 2 Proposed therapeutical plasma target fraction of non-biological liver support therapies modified after Andersen et. al [6] to include cut-off values of used hemofilters

1.7 Aim

The aim of this thesis is to summarize our research efforts taken on improving albumin dialysis.

We compared novel clinically available membranes and sorbents concerning marker molecule removal (water soluble, protein bound, cytokines) and potential to increase albumin binding function to the existing MARS technology using an established in-vitro two-compartment model developed for the comparison of liver support techniques.

2. Methods /Results

2.1 2-Compartment Model

During liver failure a range of toxins is accumulating within patients' blood as well as extra-vascular tissues. These toxins can be in their free form or protein-bound to e.g. albumin. Therefore liver failure presents itself as a highly dynamic disease. Extra-corporeal treatments need not only to remove toxins from the central compartment but also be able to counterbalance the rate of endogenous redistribution over time. Therefore, we chose a dynamic two-compartment model initially developed by Stiffel et al. [67]. Whereas compartment one is accessible for an extracorporeal treatment and features 1 liter of human plasma spiked with water-soluble and protein-bound toxins, compartment two is the continuously added toxin cocktail mimicking

rebound phenomena. This allows for the comparison of moving parts such as membranes and absorbents as well as technological approaches (e.g. flow rates) or even complete systems.

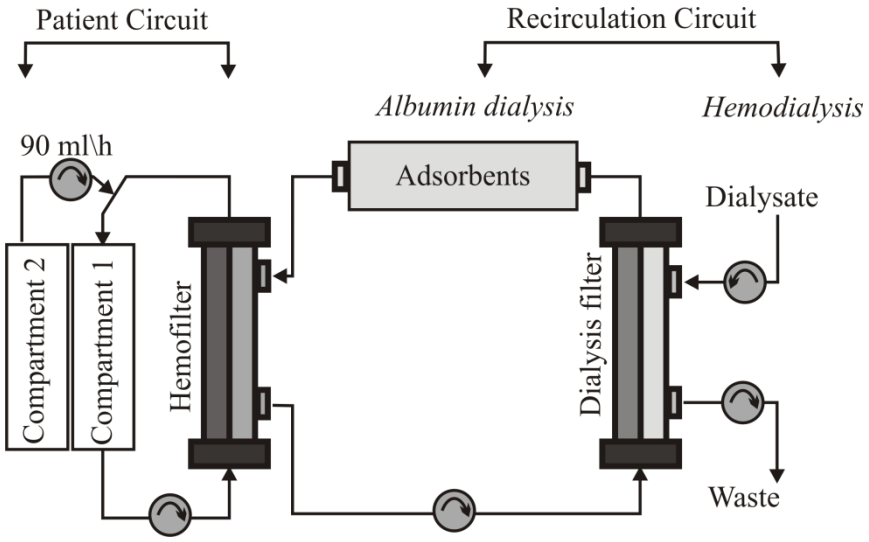

Figure 3 Schematic representation of the two-compartment model with denoted circuits, compartments, filters and adsorbents

2.2 Efforts on marker molecule removal

Dominik et al. "Reduction of elevated cytokine levels in acute/acute-on-chronic liver failure using super-large pore albumin dialysis treatment: an in vitro study" [68]

Using this two-compartment model we investigated the influence of novel medium-to-large pore sized hemofilters Synclear0.2 (Membrana), EMiC² (Fresenius) and Septex (Gambro) against the MARSflux hemofilter on marker molecule removal.

Increasing the pore size of membranes did not reflect better removal of water-soluble small sized toxins ammonia, urea or creatinine. However, removal of tightly albumin-bound indirect bilirubin was remarkably increased. No difference was seen for bile-acids. Notably, all membranes, except the MARSflux, allowed for sieving of cytokines into the recirculation circuit. Large pore sized membranes Synclear0.2 and Septex achieved near complete removal of IL-6 and TNF-alpha from the patient circuit. Septex membrane results also indicated a net-protein loss over time compared to MARS.

Pfensig et al. "A New Application for Albumin Dialysis in Extracorporeal Organ Support: Characterization of a Putative Interaction Between Human Albumin and Proinflammatory Cytokines IL-6 and TNFα" [69]

Analysis of cytokine spiked albumin samples by enzyme-linked immunosorbent assay (ELISA) indicated an interaction of albumin concentration on cytokine recovery: measured cytokines were diminished with increasing albumin concentrations (5 % to 20 %). To further investigate, samples

were subjected to size exclusion chromatography, fractionated according to retention time and ELISAs were performed for albumin, IL-6 and TNF-alpha. Cytokines IL-6 (28 kDa) and TNF-alpha (17 kDa monomer/ 51 kDa homotrimer) were found in albumin fractions (65 kDA) further adding evidence towards a putative interaction.

To see whether this interaction might be of clinical relevance, we compared 6 hour albumin dialysis with and without albumin in dialysate using the two-compartment model. Notably, as the MARSflux membrane did not allow for cytokine sieving in previous experiments, the middle pore sized membrane EMiC² was chosen. The addition of albumin to dialysate significantly facilitated the removal of IL-6 (5.77 % vs 19.32 %; p<0,001). This effect was not observed for TNF-alpha (47 % vs 50 %, p=0.842).

2.3 Targeting albumin binding function

Dominik et al. "Targeting Albumin Binding Function as a Therapy Goal in Liver Failure: Development of a Novel Adsorbent for Albumin Dialysis" [70]

Clinical data suggested that an improvement of albumin binding function correlates with survival in liver failure. Therefore we developed and followed an evolutionary test schedule for the design of novel adsorbents focusing on improving albumin binding capacity as well as increasing absorption capacity for marker toxins. MARS adsorbents diaMARS AC 250 and diaMARS IE 250 were used as reference.

Commercially available albumin solutions contain stabilizers that lower albumin binding capacity. Therefore batch tests were designed to compare different charcoal formulations concerning ABiC improvement and stabilizer reduction. Powdered activated charcoal "Hepalbin" outperformed granulated charcoals (diaMARS AC 250 is made up from Norit ROX 0.8 granulated charcoal).

The powdered "Hepalbin" charcoal was then introduced into a cellulose framework and resulted in the development of the Hepalbin adsorbent. Adsorbent capacity for marker toxins bile-acids, direct and indirect bilirubin were evaluated using single pass perfusion tests. Comparison with diaMARS AC250 revealed an at least 15-fold increase for marker toxin removal using Hepalbin.

In order to compare the novel adsorbent at the model limits concerning albumin binding capacity as well as toxin load on adsorbents, we performed a modified two-compartment model test: removing the primary hemofilter as a transfer barrier we used the albumin based toxin solution of compartment 1 without the addition of human plasma directly within the recirculation circuit while compartment 2 solution was continuously added. Tests were performed using an upscaled Hepalbin adsorbent (Hepalbin12) against both MARS adsorbents diaMARSAC250 and diaMARS IE250 in series. Empty sorbents were used as control.

Post 6 hour data revealed that only Hepalbin12 adsorbents were able to significantly increase ABiC. Marker toxins bile acids, direct and indirect bilirubin were nearly completely removed (<5 %) using Hepalbin12 when compared to both MARS adsorbents.

3. Discussion

Large clinical studies using extracorporeal liver support therapies MARS and Prometheus failed to improve overall survival [55], [56], however yielded an immense data pool on liver failure. As limitations of study success were discussed, novel insights in pathophysiological processes and involved toxins were gathered and allowed new therapies to be developed. The biggest study on albumin dialysis was conducted using MARS during the RELIEF trial and was not able to show an improvement in survival undergoing MARS treatment in AoCLF patients, even though renal functions and grade of hepatic encephalopathy scores improved. Suggested drawbacks of this study were concerning (i) the therapeutic schedule (number of therapy sessions, length of session), (ii) MARS adsorbent saturation over a single treatment session, (iii) whether albumin function could be increased and (iv) whether patient stratification was sufficient [55]. Further clinical studies revealed (v) MARS to have no influence on patient plasma cytokine levels over the course of seven days treatment [49], [71].

3.1 Increasing therapeutic dose

During liver failure effects of auto-intoxication can be observed: As the capacity to remove water soluble toxins via glomerular filtration and the possibility to remove albumin-bound toxins via bile excretion are diminishing - plasma concentrations rise to toxic levels. This in term promotes further hepatocellular damage and deterioration of secondary organ functions even if the precipitating event has been taken care of. Non-biological liver support systems based on providing detoxification support are supposed to break through this vicious circle of auto-intoxication and thereby open up a window for regeneration. The RELIEF trial hinted at improving the therapeutic dose and adsorbent capacity for improving patient survival. Therefore we used the MARS membrane and adsorbents as reference and tested the effects of novel membranes and a novel charcoal adsorbent in-vitro.

Concerning membrane selection for albumin dialysis all tested membranes allowed for higher removal of direct and indirect bilirubin fractions. Notably, the MARS membrane did not allow for any transfer of cytokines IL-6 and TNF-alpha in-vitro. On one hand the performed experiments hinted at the MARSflux membrane not being optimized for cytokine transfer, on the other hand the therapeutic dose of MARS administered might not have been able to counterbalance the underlying inflammatory disease pathology in-vivo as seen by Sen and Illonen [49], [71].

Nonetheless, cytokines increase disease severity by deteriorating circulatory functions or interfering with hepatocellular regeneration. The work of Rozga et. al [41] on designing an optimized membrane for liver failure treatments by increasing the removal of proteins <100 kDa underlines the importance of novel larger pore sized membranes being tested.

ProteinLoss - Constant infusion of compartment 2 solution within the used model mimics rebound phenomena. As the toxins can only be dissolved bound to albumin in a physiological environment, this leads to an increase in albumin concentration over the experiments time. Post 6 hour albumin concentrations were compared and used to indicate a possible loss of important plasma factors within and below the molecular size of albumin under therapy in-vivo. As this was a test of clinically available hemofilters the influence of surface area or other membrane associated parameters was not investigated. Changes in albumin concentration in this case are a representation of increased transfer over the membrane as well as protein adsorption on the used membrane. An increase in concentration was observed for Synclear0.2 and EMiC² - the Septex membrane however displayed a 5 % loss compared to MARS. The EMiC² membrane performed as a good compromise in our experiments and allowed for increased toxin and cytokine transfer while showing no signs of protein-loss.

Concerning adsorbent selection for albumin dialysis treatments we tested the upscaled version of the Hepalbin charcoal adsorbent (Hepalbin12) against both MARS adsorbents diaMARS AC250 and diaMARS IE250 for removal of albumin bound toxins. The two-compartment model was modified to neglect membrane passage and plasma protein effects by direct infusion of albumin-bound and water-soluble toxins into the recirculation circuit while continuously adding compartment 2 solution. This allowed for the highest possible toxin concentrations within the models limit to reach sorbents.

The novel Hepalbin12 adsorbent displayed significant higher removal of all employed marker toxins and shows no saturation effects. Notably, no ion-exchange adsorbent was needed. Owing its modular concept the incorporation of other sorbent materials within the cellulose framework of Hepalbin is possible and leaves potential for future investigations.

Summing up, when introducing these novel membranes and high capacity adsorbents to albumin dialysis treatments it is expected that the therapeutic dose administered can be increased. Further, as no saturation of novel adsorbents within model limits was observed single therapy session efficacy should also improve.

3.2 Improving albumin function parameters

Due to its high osmotic effect albumin is mainly used as a plasma expander in diseases or injuries associated with hypovolemia [72]. The production of commercially available albumin solutions is

based on fractionation of albumin from pooled human plasma. A critical step in the production of infusion products is the inactivation and removal of microbial contaminants and viruses. Virus inactivation is realized by heat inactivation. During this process the addition of high amounts of stabilizers (5:1) octanoate and n-acetyl-tryptophanate (5:1) protects albumin from heat degradation. These stabilizers are discussed to be vasodilators and contribute to hepatic encephalopathy [73]. Mitkov et. al were able to show that decreasing octanoate uptake in patients leads to an increased hepatic synthesis of urea which in term decrease ammonia concentrations [74]. Therefore it is important to remove stabilizers from albumin products when used for patients with decreased hepatic function or increased susceptibility to circulatory failure. Last but not least, the addition of stabilizers leads to a reduction in albumin binding capacity when using commercially available albumin products [75].

In our experiments we used the albumin binding function as assessed by ABiC as target parameter in order to develop a novel adsorbent for albumin dialysis treatments. In-vitro tests showed no increase in ABiC using MARS adsorbents while the introduction of the micro-charcoal Hepalbin12 adsorbent did. This could be attributed to an optimized micro-structure and incorporated charcoal formulation resulting in a more efficient toxin transfer from albumin to sorbent and an increased capacity for said toxins. Effects of tested sorbents on albumin function parameters like HNA2 levels or IMAR are of interest and need future investigation.

3.3 State of the art

Gerth et. al did a retrospective analysis on the RELIEF trial [76] using a novel clinical scoring system: the CLIF-C (chronic liver failure consortium) organ failure score for ACLF as developed by the CANONIC study group [77]. Briefly, a 3-point organ failure score for liver (bilirubin levels), kidneys (creatinine levels and need for renal replacement therapy), brain (hepatic encephalopathy assessed via West-Haven grade), coagulation status (international normalized ratio; INR), circulatory function (mean arterial pressure and need for vasopressor support) and respiratory function (partial pressure of arterial oxygen) is used to stratify patients and was shown to be a better predictor of survival then the existing MELD scoring system. The retrospective analysis revealed patients to have higher 14-day survival by extracorporeal therapy using MARS when presenting ACLF grades >1. This highlights the fact, that patient allocation for liver support trials included a variety of underlying disease etiologies and might not have been sufficient enough in finding the right target population.

Clinical studies using non-biological liver support therapies or plasma-exchange therapies during ALF and ACLF revealed that liver failure mortality can be stratified according to biomarkers and clinical scores:

- toxin levels and coagulation status as in MELD score,
- organ failure sub-scoring as in CLIF-C ACLF score,
- albumin binding capacity as in ABIC,
- irreversibly oxidized albumin fractions as in HNA 1/2,
- ischemia modified albumin to albumin ratio as in IMAR,
- fatty acid binding capacity and transport efficiency as in ESR measurements,
- occurrence of SIRS or neutrophil dysfunction as immune system markers

This clearly underlines the complex nature of liver failure and puts emphasis on the fact that - apart from mere toxins being responsible for hepatic and extra-hepatic challenges - poor albumin function and deranged systemic immune reactions are contributing to mortality and need to be addressed. Whether albumin dysfunctions are of causal nature, symptomatic or accelerator of liver disease pathology needs be elucidated further.

Looking at the technological approaches taken on extracorporeal liver support therapies a hemofilter is most often used as first separation layer between therapeutic target and patient. Concerning therapeutic targets albumin dialysis was intended to remove albumin-bound and water soluble toxins while minimizing effects of plasma fractions larger than albumins itself. The RELIEF trial was able to demonstrate reduction of albumin-bound bilirubin as well as water-soluble creatinine, however hinted at increasing dose for increasing survival further and revealed no effects on plasma cytokine levels in patients [55]. This leads to the idea of immunomodulatory effects being sparse to observe when using small to medium pore sized membranes.

In contrast to increasing predominantly detoxification support using albumin dialysis, the concept of HVPET and SEPET was that not all disease mediators are within this range, but rather a complex composition of toxins, released macromolecules from hepatic cell death as well as mediators of the immune system [41], [58]. This mixture of chemically diverse compounds cannot be specifically and completely adsorbed using available sorbent technologies and on top contains hepatoprotective proteins (e.g. HGF) that should be retained. Therefore, applying plasmapheresis techniques using super large pore sized membranes seamed feasible when substitution with fresh frozen plasma is used to counterbalance the removal of albumin and other physiologically important factors if needed. Though SEPET was cleared for a phase 2/3 clinical study by the FDA, lack of funding lead to no further data being gathered [78]. Future studies using the novel ALEX filter for albumin exchange will be interesting, however no data exists by now. A clinical study using HVPET observed positive effects on survival and hepatic regeneration in ALF by a

discussed efficient modulation of local and systemic immune challenges deriving from hepatocellular damage [58]. Just like the RELIEF trial one limitation of this study was determining the optimal dosing and timing of intervention. Further the absence of sorbents leads to toxin removal being solely mediated by the amount of exchanged plasma. This limits the amount of treated volume of blood when compared to recirculating albumin dialysis treatments. A continuous treatment might be necessary to counterbalance rebound phenomena.

The Prometheus therapy uses a similar approach: a plasmalike filtrate (<250 kDa) is being removed from the patient, however is being treated using a neutral and an anion exchange sorbent and is subsequently being returned to the patient. As unadsorbed molecules are being reinfused the therapeutic efficacy relies on efficient sorbent function. Whereas the Prometheus system has been shown to be clinically safe and decrease serum bilirubin levels no increase in survival in AoCLF patients has been observed [56]. Discussed drawbacks of the study were similar to the RELIEF trial and included delivered treatment dose, timing of intervention and unsufficient patient stratification according to liver failure etiology. Notably, 50 % of treated patients developed bacterial infections. Their presence and alcoholic and viral etiology were the strongest observed independent risk factors. This clearly demonstrates immunological challenges not being addressed by the Prometheus system. Employed granular neutral and anion exchange resin sorbents do not allow for efficient cytokine removal which has also been reported by others [79], [80]. Data on endotoxin removal as well as albumin function parameters are unavailable up to date.

DIALIVE is designed to exchange patients toxin loaded and functionally impaired albumin. Removal of disease modified albumin with lower binding transport characteristics or irreversible oxidation to HNA2 will be achieved using the Septex membrane. Treatment of commercially available albumin solutions by Hepalbin is used for the generation of high binding capacity albumin as albumin substitute. Currently no data on HNA1/2 levels after micro-charcoal treatment for infusion exists, but will be expected. In a pre-clinical study using a porcine acetaminophen acute liver failure model DIALIVE was able to reduce animals' HNA2 levels efficiently and reduce severity of endotoxaemia [57]**.** Notably, the described reference albumin for HNA1/2 analysis was a stabilizer containing commercial albumin solution by Behring. Analysis for anti-oxidant capacity revealed that only 65 % of albumin from the bottle are non-oxidized HMA and 5 % being irreversibly modified HNA2. As Hepalbin treatment increased the overall albumin binding capacity, it could be suggested that it will also lead to a decrease in functionally impaired albumin fraction from the bottle. However, as no correlation between functional albumin parameters has been published so far – this remains speculative. DIALIVE further includes an endotoxin adsorbing filter which is used to provide immunomodulatory effects based on neutrophil function being challenged by constant endotoxin exposure. Clinical data will provide

insight on whether disease severity can be lightened without means of detoxification support, as removal of water soluble and protein bound substances comes at the slow rate of albumin containing filtrate removal. This could potentially still require fresh frozen plasma units for substitution if derangements within plasma proteins lead to bleeding problems, as observed in their animal study.

As toxin removal using ADVOS is mediated by pH adjustments that could potentially lead to denaturation of albumin, it will be interesting to see data on functional parameters. On one hand it could lead to a thermodynamic stabilization of the protein when bound molecules are dissociating and pH is readjusted, on the other hand it could potentially lead to a change in secondary or tertiary structure that could render albumin unfunctional. In a first clinical study ADVOS allowed for efficient removal of water soluble creatinine and bilirubin and appeared to be a safe intervention [66].

At this point only larger controlled clinical trials will show which non-biological liver support therapy will improve survival. As the removal of water soluble marker toxins is an already established intervention, focus should be put on improving functional parameters of albumin liver disease. This is emphasized by their correlation to survival.

4. Conclusion

Clinical studies on liver failure using non-biological liver support techniques have lead to new insights in disease pathology as well as short-comings of current devices. The emerging of novel disease correlated biomarkers revolving around albumin function lead to new approaches on albumin dialysis. Apart from routinely assessed clinical blood parameters during liver failure, these biomarkers could be added to the spectrum of diagnostics either for patient stratification or efficacy evaluation of treatments.

Efforts taken on membrane technology lead to novel medium-to-high cut-off membranes being available for mass separation of therapeutic target molecules from patients blood. By using larger pore-sized and optimized membranes the removal of albumin-bound toxins can be increased and options for cytokine removal exist. Targeting the increase of albumin binding function and toxin adsorption capacity as discussed drawbacks of the MARS system a micro-charcoal adsorbent was developed that offers a valuable tool for the regeneration of albumin used as a molecular adsorbent in extracorporeal liver support systems or as an infusion product. Liver failure toxin adsorption capacity was remarkably increased compared to MARS adsorbents. Further, an interaction of albumin with the pro-inflammatory cytokine IL-6 was discovered that could attribute to immunomodulatory effects of albumin dialysis and needs further investigation.

A combination approach of efficient toxin removal while retaining hepatoprotective plasma proteins, allocation of functional, high-binding-capacity albumin and the option to cater to inflammatory disease pathology is proposed to help ameliorate survival even further - is however not on the market. The incorporation of relevant novel biomarkers concerning toxin transfer, albumin function, oxidative state or proposed therapeutic immunomodulatory effects should be considered in the future and might provide new insights in disease pathology as well as therapeutic targets.

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Appendix

Statutory declaration

I declare that I have developed and written the enclosed dissertation “Non-biological liver support – improved“completely by myself, and have not used sources or means without declaration in the text. Any thoughts from others or literal quotations are clearly marked. The dissertation was not used in the same or in a similar version to achieve an academic grading or is being published elsewhere.

Rostock,

Date

Signature

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Blood Purif. 2020;1-10.
DOI: 10.1159/000508810

Posters

Poster ISAD 2010 „Antithrombin III loss is a useful parameter for membrane selectivity in liver support devices“ *1st price*

Poster ISAD 2014 „Reduction of elevated cytokine levels in Acute Liver Failure/Acute on chronic Liver Failure using super-large pore albumin dialysis treatment: an *in vitro* study“ *1st price*

Poster ISAD 2014 „Albumin; a tool for the depletion of proinflammatory cytokines?“

Poster World Transplant Congress 2014 „Protein content assessment during treatment with the ELAD bioartificial liver support system“

Poster ISAD 2015 „Bilirubin Removal by Cytosorb is based on Albumin-Toxin Complex Adsorption and has no impact on Albumin Binding Capacity.“ *1st price*

Poster ISAD 2016 „Evolutionary Test schedule for the development of novel adsorbent materials that increase albumin binding capacity “*1st price*

Poster ISAD 2016 „Comparison of cytokine adsorption by different materials“

Poster ISAD 2018 „Analysis of Hepatic Cells in Bioartificial liver support using e-Cadherin as a marker for morphology and cellular activity“

Poster ISAD 2018 „Comparison of four different Human Serum Albumins solutions exhibits differences in the amount of co-purified proteins“

Poster ISAD 2018 „Albumin influences C3A hepatocytes responses to serum starvation“

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