Novel insights into alcoholic liver disease: Iron overload, iron sensing and hemolysis

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ABSTRACT

The liver is the major target organ of continued alcohol consumption at risk and resulting alcoholic liver disease (ALD) is the most common liver disease worldwide. The underlying molecular mechanisms are still poorly understood despite decades of scientific effort limiting our abilities to identify those individuals who are at risk to develop the disease, to develop appropriate screening strategies and, in addition, to develop targeted therapeutic approaches. ALD is predestined for the newly evolving translational medicine, as conventional clinical and health care structures seem to be constrained to fully appreciate this disease. This concept paper aims at summarizing the 15 years translational experience at the Center of Alcohol Research in Heidelberg, namely based on the long-term prospective and detailed characterization of heavy drinkers with mortality data. In addition, novel experimental findings will be presented. A special focus will be the long-known hepatic iron accumulation, the somewhat overlooked role of the hematopoietic system and novel insights into iron sensing and the role of hepcidin. Our preliminary work indicates that enhanced red blood cell (RBC) turnover is critical for survival in ALD patients. RBC turnover is not primarily due to vitamin deficiency but rather to ethanol toxicity directly targeted to erythrocytes but also to the bone marrow stem cell compartment. These novel insights also help to explain long-known aspects of ALD such as mean corpuscular volume of erythrocytes (MCV) and elevated aspartate transaminase (GOT/AST) levels. This work also aims at identifying future projects, naming unresolved observations, and presenting novel hypothetical concepts still requiring future validation.

Key words: alcoholic liver disease, iron overload; hemolysis, erythrophagocytosis, hepcidin, cd163, red blood cell

INTRODUCTION

Alcoholic liver disease (ALD) is the most frequent cause of severe liver disease in Europe. Based on the WHO database, more than 40% of the liver deaths are attributed to alcohol.[1] The number of performed liver transplantations for patients with ALD-related cirrhosis has increased over the past two decades, both in Europe and in the USA.[2-3] Despite the high burden of ALD, it is regrettable that most patients with ALD are diagnosed at the decompensation stage. Moreover, a large proportion of patients with newly diagnosed cirrhosis had recent consultations in primary care or emergency units,[4] without any intervention. As shown in Figure 1, ALD includes a wide spectrum of lesions ranging from steatosis to steatohepatitis, progressive liver fibrosis, cirrhosis and its complications.[5] Although steatosis is almost constant in heavy drinkers, it is estimated that only 10%–20% will eventually develop cirrhosis.[4] Since the presence of advanced fibrosis or cirrhosis in compensated patients is the main predictor of long-term survival,[6] it is of clinical importance to diagnose those patients with advanced fibrosis before decompensated stage, in order to promote abstinence and improve survival. Figure 1 also demonstrates another key feature of ALD, the hepatic iron accumulation over years, which is an independent factor for disease progression and long-term survival.
PATHOGENESIS OF ALD

Although many individuals consuming more than 60 g of alcohol per day (e.g., ½ a bottle of wine or more than 1 liter of beer) develop steatosis, only a minority of the patients with steatosis progress to alcoholic steatohepatitis (ASH) and 10%–20% eventually develop cirrhosis.[5] Genetic and non-genetic factors modify both the individual susceptibility and the clinical course of ALD.[7] The mechanisms of ALD are not completely understood. Most studies have been performed in rodents with chronic alcohol intake (e.g., Tsukamoto-French model or Lieber-DeCarli diet). However, these models basically induce moderate liver disease and severe fibrosis or liver damage do not develop. Few studies have been performed so far in livers from patients with ALD. These translational studies are needed to develop novel targeted therapies for these patients.[7]

HEPATIC ALCOHOL METABOLISM

The liver is the major alcohol-metabolizing organ in humans. Ethanol is metabolized in hepatocytes that represent approx 70% of the total liver mass. Hepatocytes oxidize ethanol to acetate mainly by three pathways[8] (Figure 2). Generally, hepatocytes express the highest levels of the major ethanol-oxidizing enzymes: (1) alcohol dehydrogenase (ADH) which is located in the cytosol. (2) cytochrome P450 2E1 (CYP2E1), which resides in the smooth endoplasmic reticulum (ER) of hepatocytes and is strongly induced in drinkers. (3) hepatic peroxisomal catalase (CAT), which decomposes hydrogen peroxide (H₂O₂) representing a relatively minor pathway of ethanol metabolism. However, it presents a larger ethanol-oxidizing function in the brain.[9]

OXIDATIVE STRESS IN ALD

Oxidative stress triggered by alcohol is another major contributor to the pathogenesis of the ALD.[10,11] Oxidative stress is defined as an imbalance in pro-oxidant/antioxidant reactive species (pro-oxidant side occupies the leading status) that lead to damage of cellular macromolecules. Reactive oxygen species (ROS) typically refer to superoxide, hydrogen peroxide, and the hydroxyl radical (Table 1). ROS have been implicated in promoting inflammation, apoptosis, necrosis, and carcinogenic DNA damage in ALD.[12-14] Alcohol-mediated ROS formation

Table 1: ROS/RNS and antioxidative defense systems

| ROS/RNS          | Enzymatic defenses             | Non-enzymatic defenses       |
|------------------|--------------------------------|------------------------------|
| Superoxide anion (O₂⁻) | SODs (against O₂⁻)            | Vitamin E/C/A                |
| Hydrogen peroxide (H₂O₂) | GSH (against H₂O₂)           | Ubiqinone                    |
| Hydroxyl radical (·OH)   | Glutathione peroxidases (GPx 1-8) | Uric acid                   |
| Peroxyl radical (ROO⁻)  | Catalase (CAT)               | Bilirubin, etc.              |
| Nitrix oxide (NO)       | Peroxiredoxins               |                             |
| Nitrogen dioxide (·NO₂⁻) | Heme oxygenases (HO)         |                             |
| Peroxynitrite (ONOO⁻)   | Thioredoxin (Trx) system     |                             |
|                    | Ferritin                       |                             |
|                    | Ceruloplasmin                 |                             |

SOD: superoxide dismutase; GSH: glutathione; ROS: Reactive oxygen species; RNS: reactive nitrogen species.
is triggered by various pathways including cytochrome P450 2E1 (CYP2E1), alcohol-induced inflammation, in vivo several enzymatic and non-enzymatic mechanisms (Figure 3). CYP2E1 is an effective ROS generator and potential mechanisms of ROS generation have been intensively discussed. Chronic alcohol consumption leads to structural and functional abnormalities in hepatic mitochondria, which have been also implicated in ROS overproduction. Excessive levels of reducing equivalents (e.g. NADH), produced by alcohol and ADH or CYP2E1 may enter the mitochondrial respiratory chain facilitating superoxide anion formation. Superoxide-removing superoxide dismutase (SOD) is affected by alcohol exposure. ROS-producing enzymes include also NADPH oxidases (NOXs) in Kupffer cells and neutrophils that can further promote the secretion of inflammatory cytokines, for instance, interleukin-1β (IL-1β) and recent studies have highlighted the role of e.g. NOX4 in ALD. Thus, inhibition of NOX4 ameliorated liver injury in rodent ALD models. Notably, conditions such as hypoxia typically seen during ethanol-metabolism can induce NOX4-mediated H$_2$O$_2$ generation in hepatocytes. Finally, Gram negative bacteria-driven lipopolysaccharide (LPS) plays a key role in ROS generation in ALD patients. LPS binds to Toll-like receptor 4 (TLR4) expressed on a wide variety of cells in the liver triggering hepatic inflammation and liver damage. Notably, Park et al. demonstrated that NOX4 interacts with the TLR4 resulting in ROS generation in monocytes under LPS participation.

**ALD PATHOGENESIS ACCORDING TO DISEASE STAGES**

There are four main pathogenic factors that drive the development of alcoholic fatty liver: (1) Increased generation of NADH caused by alcohol oxidation, favoring fatty acid and triglyceride synthesis and inhibiting mitochondrial β-oxidation of fatty acids. (2) Enhanced hepatic influx of free fatty acids from adipose tissue and of chylomicrons from the intestinal mucosa. (3) Ethanol-mediated inhibition of adenosine monophosphate activated kinase (AMPK) activity resulting in increased lipogenesis and decreased lipolysis by inhibiting peroxisome proliferating-activated receptor α (PPARα) and stimulating sterol regulatory element binding protein 1c (SREBP1c). (4) Damage to...
mitochondria and microtubules by acetaldehyde, resulting in a reduction of NADH oxidation and the accumulation on VLDL, respectively.\(^{38}\)

Regarding the development of inflammation (ASH), alcoholic fatty livers can develop parenchymal inflammation (mainly by polymorphonuclear cells) and hepatocellular damage, a prerequisite for progressing to fibrosis and cirrhosis. In cases of severe alcoholic hepatitis (AH), it is associated with a poor prognosis. Various factors may contribute to the development of ASH and AH including direct lipo-toxicity or acetaldehyde-induced toxicity.

1. Acetaldehyde binds to proteins and to DNA resulting in functional alterations and protein adducts, which activate the immune system by forming autoantigens. It also induces mitochondria damage and impairs glutathione function, leading to oxidative stress and apoptosis.\(^{34}\)

2. ROS generation as mentioned above and the resulting lipid peroxidation with DNA adduct formation.\(^{94}\) Main sources of ROS include CYP2E1-dependent MEOS, mitochondrial electron transport system of the respiratory chain, NADH-dependent cytochrome reductase and xanthine oxidase.\(^{97,98}\) Moreover, chronic alcohol intake markedly up-regulates CYP2E1, which metabolizes ethanol to acetaldehyde and parallels the generation of ROS and hydroxyl-ethyl radicals.\(^{99}\)

3. Pro-inflammatory cytokines. Alcohol metabolites and ROS stimulate signaling pathways such as NF-\(\kappa\)B, STAT-JAK, and JNK in hepatic resident cells, leading to the local synthesis of inflammatory mediators such as TNF\(\alpha\) and CXC chemokines (e.g. IL-8), as well as osteopontin.\(^{40}\) Alcohol abuse also results in changes in the colonic microbiota and increased intestinal permeability, leading to elevated serum levels of lipopolysaccharides that induce inflammatory actions in Kupffer cells via CD14/TLR4.\(^{42}\) The resulting inflammatory milieu in the alcoholic liver leads to polymorphonuclear(PMN) infiltration, ROS formation and hepatocellular damage.

4. Impaired ubiquitin-proteasome pathway leading to hepatocellular injury and hepatic inclusions of aggregated cytokeratins (i.e. Mallory-Denk Bodies).\(^{43}\)

**FIBROSIS PROGRESSION IN ALD**

Fibrosis progression in ALD is primarily observed in patients with ASH.\(^{44}\) In ALD, the fibrotic tissue is typically located in pericentral and perisinusoidal areas. In advanced stages, collagen bands are evident and bridging fibrosis develops. This condition precedes the development of regeneration nodules and liver cirrhosis. The cellular and molecular mechanisms of fibrosis in ALD are not completely understood.\(^{45}\) Alcohol metabolites such as acetaldehyde can directly activate hepatic stellate cells, the main collagen-producing cells in the injured liver. Hepatic stellate cells can also be activated paracrinally by damaged hepatocytes, activated Kupffer cells and infiltrating PMN cells. These cells...
release fibrogenic mediators such as growth factors (TGFβ1, PDGF), cytokines (leptin, angiotensin II, IL-8 and TNFα), soluble mediators (nitric oxide) and ROS. Importantly, ROS stimulate pro-fibrogenic intracellular signaling pathways in hepatic stellate cells including ERK, PI3k/AKT and JNK. They also up-regulate TIMP-1 and decrease the actions of metalloproteinases, thereby promoting collagen accumulation. Cells other than hepatic stellate cells can also synthesize collagen in ALD. They include portal fibroblasts and bone-marrow derived cells. Whether other novel mechanisms such as epithelia-to-mesenchymal transition of hepatocytes also play a role in liver fibrosis is under investigation.

**UNEXPLAINED LABORATORY OBSERVATIONS IN PATIENTS WITH ALD**

Drinkers show typical laboratory findings. Some of them are textbook knowledge, some of them are less known although characteristic for ALD. For instance, glutamyltransferase (GGT) is frequently elevated (induced) in drinkers but the exact role of this bile-duct expressed enzyme and why it is upregulated is not known. In addition, it can be described as almost mandatory that aspartate transaminase (glutamic-oxaloacetic transaminase [GOT] / aspartate aminotransferase [AST]) is higher as alanine transaminase (glutamic-pyruvic transaminase [GPT] / alanine aminotransferase [ALT]) in patients with ALD (so called De Ritis Ratio GOT/GPT). Although it is generally assumed that both enzymes are liver derived, the reason for the elevated De Ritis Ratio is not really known and it is not plausible that mitochondrial GOT should be more leak in the presence of a toxin such as ethanol. An elevated mean corpuscular volume of erythrocytes (MCV) is also a quite frequent laboratory finding in patients with ALD and even part of formerly published diagnostic scores. Surprisingly, an assumed deficiency of folic acid or vitamin B12 is normally not confirmed in these patients. As we and others have shown repeatedly, a significant fraction of patients with ALD has elevated ferritin levels, in 15% even higher than 1000 ng/mL. Less obvious are the notoriety if only slightly reduced levels of hemoglobin (Hb). One major complicating factor with regard to the laboratory is that all these markers depend on the disease stage. For instance, transaminase levels will continuously decrease and even normalize once cirrhosis has developed. Of note, at this stage, GOT will be typically higher as GPT independent of the etiology. Another typical finding in ALD, namely during icteric episodes of ASH, AH or in cirrhotics, is elevated bilirubin. This is mostly conjugated bilirubin, not indirect, unconjugated bilirubin and indicates that bilirubin conjugation is normally not impaired in patients with ALD. In fact, icterus in ALD typically resembles the laboratory profile of mechanic cholestasis despite the absence of congested bile ducts e.g. in the ultrasound analysis. This observation still requires further confirmative studies but could be indeed explained by mechanic factors such as ballooning leading to compression of bile canaliculi and small bile ducts causing an impaired bile flow.

**HEPATIC IRON OVERLOAD IN ALD**

Hepatic iron accumulation and chronic alcohol consumption have been associated for a long time (Figures 1 and 4). In ALD patients, iron has been identified as independent risk factor both for survival and hepatocellular carcinoma (HCC) development comparable to patients with hereditary iron overload. These observations have driven for a long time the interest in molecular iron homeostasis, since iron is

![Figure 4: Hepatic iron accumulation in ALD. (A) Staining with Hematoxylin Eosin and iron marker Prussian blue indicating iron accumulation in hepatocytes and macrophages in a typical patient with ALD. (B) Statistical analysis in the Heidelberg ALD cohort indicates that almost 50% have pathological iron overload both in hepatocytes and macrophages (RES). ALD: alcoholic liver disease; RES: reticuloendothelial system.](image-url)
not only highly toxic but also essential for life (Figure 5A). The carcinogenic potential of tissue iron accumulation is highly attributed to Fenton-like reactions which occur in the presence of ferrous iron and \( \text{H}_2\text{O}_2 \) yielding to highly reactive hydroxyl radicals (Figure 5B).\(^{[52]}\) Several enzymes are able to generate \( \text{H}_2\text{O}_2 \) predominantly during the inflammation typically observed in ALD, however CYP2E1 and its induction by ethanol is regarded as key player in alcohol-mediated ROS production (Table 1).\(^{[53]}\) Thus, chronic alcohol consumption results in an up to 20 fold increased CYP2E1 expression.\(^{[53]}\) Furthermore, ethanol-induced oxidative stress is also the result of an impaired antioxidant defense mechanisms as well as impaired synthesis of mitochondria-encoded constituents of the respiratory chain particularly cytochrome b and the production of ROS by leakage from complex I and III of the respiratory chain.\(^{[37]}\) In addition, activated phagocytes also contribute to ROS.\(^{[37]}\) In addition, excessive iron accumulates in lysosomes that originates from auto-phagocytosed ferritin and hemosiderin. It often leads to fragile membranes via lipid peroxidation and subsequent lysosomal dysfunction with the loss of free iron into the cytoplasm.\(^{[54]}\) The iron-associated toxicity is due to mutagenic effects of free radicals resulting in DNA damage and structural alteration of lipids and proteins. An example is the cleavage of lipid hydroperoxides, resulting in aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which can react with the amino group of lysine and histidine residues\(^{[55]}\) and DNA bases\(^{[56]}\) forming extremely mutagenic adducts. Indeed, these adducts can specifically target the p53 tumor suppressor gene conferring apoptotic resistance to the cells.\(^{[57]}\) Therefore, 4-HNE has been used as an indicator of radical-mediated cellular damaged and oxidized hepatocytes and it correlates well with hepatic iron levels.\(^{[58]}\) Recent \textit{in vitro} studies indicate that mitophagy, the selective clearance of damaged mitochondria by autophagy in the liver, may also play a role in mediating hepatocyte apoptosis.\(^{[59]}\) Acute and chronic ethanol treatment in various animal models results in enhanced mitophagy which is associated with mitochondrial translocation of cytosolic Parkin (an E3 ubiquitin ligase maintaining mitochondrial homeostasis) caused by oxidative mitochondrial DNA damage. Parkin also co-localizes with accumulated 8-oxo-2'-deoxyguanosine, a marker of oxidative mitochondrial DNA damage in ethanol exposed hepatocytes which may be a stimulus for DNA repair and the prevention of carcinogenesis.\(^{[60]}\) For these reasons and because mitochondria are the main target organelles for alcohol toxicity, modulation of Parkin translocation may be a new therapeutic option in patients with ALD. Taken together, the actual role of iron (particular non-protein Fe complexes) and how alcohol favors iron accumulation are still under intensive investigation, but clinical data clearly show that iron overload and oxidative stress in ALD is tightly linked with the progression to hepatocellular damage, liver-related death and carcinogenesis.

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\*Figure 5: Schematic overview of iron toxicity. (A) Iron plays a bivalent role in cell physiology. It is both vital for many cell functions but also (B) highly toxic and carcinogenic through the generation of hydroxyl radicals (Fenton chemistry).*
IRON HOMEOSTASIS AND RECYCLING: IMPORTANT ROLE OF LIVER AND BLOOD

In the last decades, an enormous progress has been made to better comprehend the molecular mechanisms of iron regulation and homeostasis at the systemic and the cellular level.\(^\text{[61-62]}\) In humans, most of the body's iron (4 g) is found in the oxygen-carrying Hb of erythrocytes (Figure 6). The remaining iron is stored in hepatocytes (1 g) and in reticuloendothelial macrophages in the form of ferritin. Thus, the liver represents an important reservoir of excess iron while macrophages phagocytose the senescent red blood cells (RBCs) (erythrophagocytosis) ultimately recycling the iron from Hb to transferrin for novel synthesis pathways.\(^\text{[63]}\) Dietary uptake (1–2 mg/d) of iron is carried out by duodenal enterocytes and its efficient control is critical for the homeostasis, since iron is passively lost from regular sloughing of the mucosa and skin or during bleeding (Figure 6).\(^\text{[61]}\) About 90% of iron, however, is recycled from senescent erythrocytes that typically have a mean survival of 120 d.\(^\text{[63]}\) In other words, and considering that an average individual has 5 L total blood (4 g iron) approx 40 mL blood are recycled every day which equals ca. 30 mg iron. Likewise, 30 mg of iron are required daily for erythropoiesis in the bone marrow, which is mainly covered from sequestered RBCs via the reticuloendothelial system. These cells metabolize heme via HO1 and release iron into the serum, where it is distributed to tissues via serum transferrin (Figure 6B). Often overlooked, the liver plays also an important role in heme and iron recycling. While erythrocytes are first sequestered in the spleen, liver macrophages are also able

Figure 6: (A) Iron homeostasis and utilization in the body. Dietary iron is absorbed in the duodenum and binds to transferrin. Iron is then delivered to the bone marrow for erythropoiesis the major utilization pathway. Senescent RBCs are phagocytosed by macrophages (erythrophagocytosis) ultimately recycling the iron from Hb to transferrin for novel synthesis pathways. Excess iron is stored in ferritin in the liver. Regulation of iron metabolism by hepcidin and factors which influence hepcidin expression is also shown. Black circles indicate potential sites of alcohol interference. (B) Heme degradation by HO1. Notably, the heme binding respiratory chain-blocker CO is produced and toxic iron is release. In addition, bilirubin is ultimately conjugated in the liver and excreted through the biliary system. Bilirubin and bile acids can both cause eryptosis and hemolysis at higher concentrations. FPN: ferroportin; HFE: hemochromatosis protein; BMP: bone morphogenetic protein; LPS: lipopolysaccharide; RBC: red blood cell.
**Figure 7:** Present concepts of signaling pathways on hepcidin: erythropoiesis, iron, infection. These conventional concepts are not able e.g. why hepcidin is strongly suppressed in patients with severe hemolytic disease and why hepcidin is stimulated by iron *in vivo* but suppressed *in vitro*. BMP: bone morphogenetic protein; IL6: interleukin 6; STAT3: signal transducer and activator of transcription 3.

**Figure 8:** Important factors affecting hepcidin in patients with ALD. ALD: alcoholic liver disease.

Erythrocytes are either directly phagocytosed by macrophages or released toxic heme is bound to either haptoglobin (Hp) or hemopexin (Hpx) and internalized through the receptors CD163 or CD91. During erythropagocytosis, heme oxygenase (HO) catalyzes the enzymatic degradation of heme and produces equimolar amounts of carbon monoxide (CO), biliverdin and iron. In a coupled reaction, biliverdin is converted into bilirubin (BR) via biliverdin reductase (Figure 6B). Of the two genetically distinct HO isoforms, HO-2 is constitutively expressed and mainly found in brain and testis, whereas the inducible HO isozyme HO1 is expressed at low levels in most cells and tissues. HO1 is markedly up-regulated by its substrate heme and a variety of oxidative stress stimuli and HO1 induction has been considered a general adaptive response to protect against the toxicity of oxidative stress. HO1 deficient mice develop a chronic inflammatory disorder and are highly vulnerable to an experimental sepsis induced by the classical proinflammatory mediator endotoxin. Finally, Figure...
6A also highlights assumed and potential sides of impaired iron regulation by alcohol.\cite{123}

**SYSTEMIC IRON CONTROL BY HEPcidIN**

Systemically, iron is mainly controlled by the hormone hepcidin (Figures 6 and 8). This 25 amino-acid peptide is primarily expressed in hepatocytes although low expression levels have been also reported in macrophages\cite{80,81} representing about 10% of total serum hepcidin.\cite{138} By binding to the unique iron exporter, ferroportin, hepcidin efficiently inhibits duodenal iron absorption, iron recycling from macrophages and iron mobilization from hepatic stores. Hepcidin blocks the iron efflux into the plasma by binding ferroportin and consequently inducing the phosphorylation, internalization and lysosomal degradation of the complex by the proteasome.\cite{82} Deletion of hepcidin in mice or hepcidin deficiency in humans results in severe hepatic iron overload, increased serum iron levels and loss of iron in macrophage stores, caused by hyperabsorption of iron.\cite{83} In contrast, transgenic overexpression of hepcidin causes decreased serum iron leading to anemia by blocking the iron absorption in enterocytes and the release of iron from the hepatic stores.\cite{84-88} The conventional, present understanding of major regulatory pathways of hepcidin is summarized in Figure 7.

**TRANSCRIPTIONAL REGULATION OF HEPcIDIN**

The levels of circulating hepcidin are mostly controlled at the transcriptional level. Hepcidin promoter activity can be induced by iron signals, including serum iron concentrations and liver stores, or by inflammatory signals and suppressed during increased erythropoietic activity. In general, the transcriptional control of hepcidin by iron occurs via the bone morphogenic protein-SMAD (BMP/SMAD) pathway. High circulating concentrations of transferrin-bound iron (Tf-Fe) are the extracellular signal for transcriptional induction of hepcidin.\cite{89} Tf-Fe modulates the interaction between the transferrin receptors (TFR) 1 and 2 and hemochromatosis protein (HFE) by inhibiting the binding of HFE to TFR1. Consequently, HFE stabilizes activin receptor-like kinase 3 (ALK3), which activates BMP/SMAD signaling cascade.\cite{90} Increased Tf-Fe concentrations can also promote the association between HFE and TFR2 that can further form a membrane complex with the BMP the co-receptor HJV, promoting hepcidin transcription via BMP/SMAD pathway.\cite{91} Besides the iron, inflammatory signals as TGF-β, activin B and BMPs are also inducers of BMP/SMAD signaling while matripase-2 and furin act as suppressors by cleavage of the cell surface hemojuvelin (HJV) protein.\cite{92-93}

Increased erythropoiesis, caused by exposure to high altitude, anemia or other physiological conditions, is so far described as the major inhibitory stimuli of hepcidin synthesis. Increased erythropoietin (EPO) release by the kidney is the major feature of erythropoiesis, being the proposed critical factor for erythropoiesis-mediated downregulation of hepcidin.\cite{94} Despite many efforts over the last decade, there are still many open questions on how EPO suppresses hepcidin. Years ago, several studies have associated the suppression of hepcidin with two erythroid regulators (GDF15 and TWSG1), which are normally increased during erythropoiesis. However, the direct link between these proteins and hepcidin regulation is still missing.\cite{95} A more recent study has identified another erythroid regulator as part of the hepcidin-EPO axis, called erythropherrone (ERFE).\cite{95-98}

The induction of hepcidin during inflammation or infection constitutes an important evolutionary conserved mechanism known as “anemia of chronic disease”. This mechanism describes a host defense response against invading extracellular pathogens, in which IL-6 plays a key role as a major upstream regulator via STAT3 pathway leading to hepcidin induction.\cite{99} Recently, H$_2$O$_2$ has been suggested as additional important inflammatory cofactor and second messenger capable of upregulating hepcidin by activation of the STAT3 signaling cascade.\cite{100} In particular, hepcidin can be strongly induced by exposing hepatoma cells to sustained H$_2$O$_2$ concentrations similar to that released by inflammatory cells.\cite{101} Subsequent studies have confirmed the role of STAT3 in the H$_2$O$_2$-mediated hepcidin induction.\cite{99,102} Other studies reported contrary findings and demonstrated a suppression of hepcidin in alcohol-fed mice by ROS.\cite{103} However, no mechanistic details were provided and we had earlier shown that the concentration of peroxide is crucial for hepcidin transcription.\cite{104} While low levels induce hepcidin, toxic levels drastically block hepcidin most likely through unspecific inhibition of the transcription machinery.

In the context of ALD, H$_2$O$_2$ has been shown to have a complex, concentration-dependent and bivalent action on hepcidin. Since ethanol metabolism strongly affects hepatic oxygen homeostasis, liver hypoxia is thought to have an important impact on hepcidin regulation in vivo and in vitro.\cite{105}

**CELLULAR REGULATION OF IRON**

Erythrocyte cells as well as all other cell types depend on the delivery of iron via the iron carrier serum transferrin (Tf), a glycoprotein with two affinity sites for ferric iron. Diferric Tf binds with high affinity to cell surface transferrin receptor 1 (TFR1) and with lower affinity to transferrin receptor 2
hyd%0A%0A(TE%2F2).%0A%0ADespite their homology, TF%2F2 does not significantly contribute to iron import and it is regarded as a sensor of iron saturation. Genetic deletion of TF%2F1 in mice demonstrates its endocytotic role and ability to import iron into several cell types.104 In non-erythroid cells, iron is safely stored in ferritin complexes or can be incorporated into Hb of erythrocytes, being later reused for various synthesis pathways.106 Ferric iron stored in ferritin complexes (non-toxic form) must be subsequently released for biological use via lysosomal degradation of ferritin.106 This mechanism of autophagy dominates during iron deficiency and is mediated by the nuclear receptor coactivator 4 (NCOA4).107 It has been described that NCOA4 interacts with ferritin heavy chain targeting ferritin for degradation. In iron overloaded cells, NCOA4 expression decreases leading to suppression of ferritin autophagy.108 A recent study described the retention of iron within ferritin in NCOA4 KO mice, which led to iron-deficiency anemia, highlighting the important role ferritin autophagy mechanism on cellular and systemic iron homeostasis.109

At the cellular level, central regulators of iron homeostasis are controlled post-transcriptionally by iron responsive proteins (IRP1 and IRP2) which are able to bind to the iron responsive elements (IREs) of the RNA encoding for various iron-related proteins. While binding to the IRE located in the 5’ untranslated region of the mRNA results in a translational inhibition, the bind of IRPs to the 3’ untranslated region stabilizes and protects the transcripts from degradation. In particular, IRPs can cause upregulation of TF%2F1 or suppress the translation of mRNA encoding other proteins involved in iron metabolism, such as ferritins or ferroportin.110 During cellular iron deficiency, IRPs are in the active form (apo-IRP) and this results in TF%2F1 induction stimulating the acquisition of iron from plasma TF. In contrast, to counteract iron overload, IRPs become inactive (holo-IRP) for IRE binding leading to degradation of TF%2F1 mRNA and translation of ferritin mRNA.111

The interactions IRE-IRP allow an autonomous independent control of iron homeostasis for individual cells, however, this network can be also overwritten by additional mechanisms. Namely, TF%2F1 expression is also regulated at the transcriptional and translational levels.112,113 Interestingly, IRP1 is regulated in a complex manner by various ROS linking iron homeostasis to oxygen metabolism.114-116

REGULATION OF HEPcidIN BY HYPOxia

The hypoxic response is mainly controlled by hypoxia inducible factors (HIFs), which comprise an oxyge

(105)dependent α subunit (HIFα) and the constitutively expressed β subunit (HIFβ). Under adequate oxygen levels proly

Since the last two decades, the role of hypoxia and HIFs on hepcidin regulation has been extensively investigated and the first reports in this field have shown the in vitro and in vivo inhibition of hepcidin at the mRNA level under hypoxic conditions.118,119 In order to elucidate the molecular mechanisms involved in hypoxia-mediated hepcidin regulation, several authors have used HIF and VHL KO mouse models to dissect the role of HIFs in controlling the hepcidin promoter activity. A genetic study with iron deficient and VHL KO mice in conjunction with transcriptional assays have suggested that HIF1 stabilization downregulates hepcidin, however, HIF1 alone was insufficient to explain hepcidin suppression under hypoxia. The same study suggested the direct suppression of hepcidin via a putative hypoxia response element located in the hepcidin promoter120 but no further evidence could be provided until now. Moreover, subsequent reports suggested that HIF does not directly suppress the transcription of hepcidin.121,122 Most recently, in vivo studies have shown that HIF-mediated suppression of hepcidin occurs indirectly through EPO-induced erythropoiesis, highlighting the contribution of HIF2 on hepcidin regulation.123,124 Up to now, human studies have been published investigating the circulating hepcidin levels during the exposure to hypobaric hypoxia in conditions simulating high altitude.125,126 The role of a new factor, the platelet derived growth factor BB (PDGF-BB), was recently described in humans and mice. The circulating concentration of PDGF-BB highly correlates with hepcidin but not with other parameters. In addition, PDGF-BB could suppress hepcidin mRNA in hepatoma cells and primary hepatocytes.127

While these reports clarified the regulation of hepcidin by systemic hypoxia, in which renal EPO release induces erythropoiesis depleting the serum iron, the in vivo response to specific liver hypoxia is still lacking. The control of hepcidin by hepatospecific hypoxia has been extensively described in vitro, resulting in multiple contradictory findings.28 In general, it has been suggested that hypoxia induces the expression of hepcidin suppressors. Silvestri and colleagues identified HIF1α as down regulator of hepcidin transcription, through induction of furin.128 Another study has identified increased expression of matriptase-2 in conditions of severe hypoxia. The authors emphasized the role of HIF1α and HIF2α for the hypoxia-mediated suppression of hepcidin, while subsequent experiments performed in primary mouse hepatocytes discarded HIF2α as hepcidin regulator.124,129 At the same time, an in vivo study reported the ethanol-mediated
hepcidin suppression by stabilization of HIF in the liver. The direct role of HIF on hepcidin regulation was again suggested after experiments performed in VHL KO mice. Contrary to these findings, the upregulation of hepcidin under hypoxia was also reported in vitro in hepatoma cell lines. Moreover, downregulation of hepcidin could be obtained by co-culturing hepatoma cells with macrophages, however, the co-culture ratio used in this study did not reflect physiological conditions. In summary, conflicting and partly confusing data have been represented so far with regard to hepcidin regulation by hypoxia. We believe that these contradictions are mainly due to limitations in the interpretation of in vivo models of hypoxia due to many adaptive responses at various regulatory levels and to methodological limitations.

HEPCIDIN DYSREGULATION IN PATIENTS WITH ALD

Various conditions may affect hepcidin in patients with ALD depending on the disease stage (Figure 8). As shown in a confirming experiment (Figure 9A) from our center, acute exposure to ethanol (24 h) rapidly suppresses hepcidin in mice which is in contrast to human data (see below). It has been shown that ethanol downregulates hepcidin promoter activity and the DNA binding activity of C/EBP alpha (C/EBP alpha) but not C/EBP beta in mice leading to downregulation of hepcidin gene transcription thereby increasing duodenal iron transport. Recently it has been shown that alcohol exerted different effects on TGF-β-mediated SMAD2 activation and BMP-mediated SMAD1 and SMAD5 activation. Other data suggest the simultaneous inhibition of BMP-mediated SMAD activation and stimulation of TGF-β-mediated SMAD activation by alcohol in the involvement of hepcidin suppression by alcohol in vivo.

As shown in Figure 9B, our ongoing large trial of prospectively enrolled heavy drinkers does not confirm the data on acute ethanol exposure in rodents. In fact, we observe a clear upregulation of serum hepcidin levels in heavy drinkers as compared to healthy non-drinking controls. This is also the case, if drinkers and non-drinkers were matched for age. More information on these ALD cohorts is also provided in Table 4. Of note, elevated cytokines cannot solely explain upregulation of hepcidin. In conclusion, the observed short-term inhibition of hepcidin mRNA levels by acute and high alcohol exposure cannot be recapitulated at the serum level in heavy drinking humans. We assume and this will be discussed in more detail below that hemolysis contributes to hepcidin upregulation in drinkers.

PRELIMINARY LESSONS FROM THE ONGOING PROSPECTIVE SURVIVAL COHORT OF HEAVY DRINKERS IN HEIDELBERG: HEMOGLOBIN AS AN IMPORTANT PROGNOSTIC MARKER

Mortality data are essential to identify important confounders of disease progression. So far, no prospective long-term data on survival are available for patients with ALD. In 2007, the Heidelberg Center for Alcohol Research started to prospectively enroll heavy drinkers. Most patients are presenting for alcohol detoxification allowing to also collect data after approx one week of alcohol withdrawal.
The study is still ongoing and covers now almost 15 years. In an interim analysis, information of survival status was obtained in 602 of 943 screened patients that had presented from 2007–2017 with a mean daily consumption of alcohol of 178 g. Mean observation time was 3.7 years and mean duration of heavy drinking was 14.0 years. During the observation time, 99 patients had passed away. The cause of death could be clarified in 39%. In 38%, death was liver related. As shown in Table 2 and of most interest within the context of this concept paper, Hb was among the three major factors that were associated with long-term mortality next to liver stiffness and alkaline phosphatase (AP). Notably, low Hb levels are associated with increased long-term mortality.

To get more insights into potential causes of the anemia, we had a closer look at our database with more than 1000 ALD patients and we finally divided them into four groups according to the two major iron compartments, Hb and serum ferritin. We are aware that especially ferritin can be also elevated by other causes and does not necessarily reflect intracellular iron content. However, even ferritin elevation during acute inflammation indicates filled iron storage due to hepcidin-mediated blockage of iron export by ferroportin. In total, four groups were obtained when dividing the ALD patients according to low and high Hb and ferritin (Table 3). Using this strategy, the four groups had an identical size of approx 250 patients. Mean levels of Hb and ferritin differed markedly between the groups and were either ca. 200 or 1200 ng/mL for ferritin and 15 or 12.2 mg/dL for Hb. Moreover, no differences were observed between the low and high Hb groups with regard to ferritin levels and the selection sequence did not affect the outcome. In other words, same data were obtained whether patients were first sorted according to Hb and then ferritin or vice versa. As shown in Table 3, group 3 (high ferritin, low Hb) had the worst outcome and this group is characterized by an impaired liver function (higher liver stiffness, more fibrosis, reduced liver function, elevated bilirubin). Group 3 also showed more liver apoptosis (elevated levels of M30 and M65). The marked elevation of the erythropagocytosis marker CD163 both in groups 1 and 3 (high ferritin levels) clearly indicates that enhanced erythrophagocytosis is responsible for the ferritin elevation. In contrast to group 1 (normal Hb, elevated ferritin), prognostically unfavorable group 3 (low Hb, high ferritin) has a significantly higher reticulocyte count, MCV and LDH. Thus, these typical laboratory findings are highly suggestive for enhanced hemolysis or eryptosis. We also tested whether B₁₂ or folic acid deficiency could account for the elevated MCV in group 3 but did not find any significant differences (not shown). Based on the CD163 levels, the erythrocyte turnover seems to be similar in groups 1 and 3 so that an enhanced turnover could also not account for e.g. “a relative B₁₂ or folic acid deficiency”.

The major difference between group 1 and 3 is the ability of group 1 patients to reuse iron for novel Hb synthesis.

| Items                  | Univariate | Multivariate (stepwise) |
|------------------------|------------|-------------------------|
|                        | P-value    | HR 95% CI                | P-value    | HR 95% CI                |
|                        |            | Lower | Upper | Lower | Upper |
| Liver stiffness        | <0.001     | 1.026 | 1.02  | 1.033 | 1.005 | 1.021 |
| AP                     | <0.001     | 1.006 | 1.004 | 1.007 | <0.001 | 1.003 | 1.002 | 1.005 |
| Bilirubin total        | <0.001     | 1.119 | 1.085 | 1.153 |                |       |
| Hemoglobin             | <0.001     | 0.757 | 0.701 | 0.818 | 0.001 | 0.858 | 0.785 | 0.938 |
| Age                    | <0.001     | 1.043 | 1.025 | 1.061 | 0.002 | 1.028 | 1.010 | 1.046 |
| INR                    | <0.001     | 1.775 | 1.362 | 2.314 |                |       |
| Platelets              | <0.001     | 0.995 | 0.993 | 0.998 | 0.047 | 0.998 | 0.995 | 1.000 |
| GGT                    | <0.001     | 1.000 | 1.000 | 1.001 |                |       |
| AST                    | 0.024      | 1.002 | 1.000 | 1.003 |                |       |
| Urea                   | 0.040      | 1.009 | 1.000 | 1.017 |                |       |
| CAP                    | 0.335      | 1.002 | 0.998 | 1.007 |                |       |
| Sex (0: female, 1: male)| 0.337     | 1.233 | 0.804 | 1.888 |                |       |
| ALT                    | 0.777      | 1.000 | 0.997 | 1.002 |                |       |
| Lipase                 | 0.789      | 1.000 | 0.996 | 1.003 |                |       |
| Leukocytes             | 0.996      | 1.000 | 0.955 | 1.047 |                |       |
| BMI                    | 0.999      | 1.000 | 0.958 | 1.044 |                |       |
| Creatinine             | 0.999      | 1.001 | 0.472 | 2.122 |                |       |

AP: alkaline phosphatase; INR: international normalized ratio; GGT: glutamyltransferase; AST: aspartate aminotransferase; CAP: controlled attenuation parameter; ALT: alanine aminotransferase; BMI: body mass index; HR: hazard ratio; ALD: alcoholic liver disease. Parameters are marked in bold if they are significantly associated with mortality in multivariate analysis.
Group 3 also has higher EPO levels further indicating anemia is not due to the insufficient production of this important hormone but, in contrast, trying to compensate for the low Hb levels. We next performed a correlation analysis with the systemic iron master-switch hepcidin as hepcidin was both elevated in groups 1 and 3 with high ferritin levels. The clear evidence of masked hemolysis could also at least contribute to hepcidin suppression. In addition, hepcidin is elevated in group 3, but not as high as group 1 despite similar ferritin elevations and even more pronounced iron overload. Hepcidin is seemingly paradoxically and profoundly suppressed in hemolytic diseases such as thalassemia [134] or chronic liver diseases [52] in the presence of iron excess e.g. released from an increased red blood cell turnover. [52,135] Finally, it is mechanistically interesting that transferrin is significantly suppressed in group 3. Low levels of transferrin have been described as prognostically unfavorable in ALD patients. In summary, lessons from our ongoing prospective survival study indicate that anemia is an important factor of mortality and this anemia is associated with enhanced erythrocyte turnover due to elevated hemolysis (masked hemolysis) and/or eryptosis.

| Parameter                                | Group 1        | Group 2        | Group 3        | Group 4        |
|------------------------------------------|----------------|----------------|----------------|----------------|
| Hemoglobin (g/dL)                        | 15.40 ± 0.99   | 15.31 ± 1.14   | 12.04 ± 1.64   | 12.16 ± 1.75   |
| Ferritin (ng/mL)                         |                |                |                |                |
| LIC (mg/g dry weight)                    |                |                |                | 5.41 ± 17.44   |
| Transferrin (g/L)                        | 2.33 ± 0.55    | 3.54 ± 14.29   | 1.76 ± 0.63    | 2.44 ± 0.60    |
| Transferrin saturation (%)               | 51.2 ± 24.1    | 34.8 ± 18.1    | 55.4 ± 28.7    | 32.9 ± 22.2    |
| Hepcidin (ng/mL)                         | 21.40 ± 13.36  | 12.40 ± 10.49  | 17.49 ± 13.86  | 9.63 ± 8.25    |
| Fibrosis stage                           | 1.40 ± 1.37    | 1.11 ± 1.32    | 2.29 ± 1.62    | 1.76 ± 1.79    |
| Liver stiffness (kPa)                    | 14.99 ± 18.06  | 9.28 ± 12.12   | 31.2 ± 28.51   | 22.27 ± 26.26  |
| AST (U/L)                                | 140.7 ± 135.4  | 55.7 ± 50.1    | 141.8 ± 122.1  | 70.5 ± 67.8    |
| ALT (U/L)                                | 104.7 ± 115.7  | 46.6 ± 44.0    | 74.7 ± 59.7    | 46.0 ± 73.9    |
| M30 (U/L)                                | 725.0 ± 883.5  | 264.8 ± 245.5  | 899.2 ± 1033.4 | 543.9 ± 1134.2 |
| M65 (U/L)                                | 1262.2 ± 1409.4| 480.0 ± 484.9  | 1628.1 ± 1661.3| 1096.4 ± 3509.6|
| Bilirubin total (mg/dL)                  | 1.17 ± 1.73    | 0.60 ± 0.46    | 3.66 ± 5.96    | 1.65 ± 3.51    |
| Quick (%)                                | 102.0 ± 17.9   | 108.0 ± 16.4   | 87.4 ± 27.7    | 92.4 ± 24.9    |
| MCV (fL)                                 | 94.2 ± 10.4    | 90.6 ± 7.5     | 98.5 ± 11.0    | 92.0 ± 8.2     |
| LDH (U/L)                                | 244.1 ± 75.7   | 207.0 ± 63.0   | 299.5 ± 195.1  | 219.4 ± 75.2   |
| CD163 (ng/mL)                            | 1643.8 ± 712.0 | 1095.2 ± 772.2 | 2231.6 ± 1246.9| 1782.4 ± 1301.2|
| CD91 (µg/mL)                             | 7.03 ± 2.82    | 5.34 ± 4.33    | 5.61 ± 4.52    | 4.55 ± 3.82    |
| ERFE (ng/mL)                             | 0.59 ± 0.55    | 0.27 ± 0.36    | 1.3 ± 3.57     | 0.33 ± 0.35    |
| B2MG (ng/mL)                             | 9.39 ± 3.72    | 8.34 ± 3.31    | 12.40 ± 7.10   | 8.54 ± 2.97    |
| Hemopexin (mg/mL)                        | 0.45 ± 0.27    | 0.52 ± 0.41    | 0.37 ± 0.33    | 0.33 ± 0.26    |
| Haptoglobin (mg/dL)                      | 1.50 ± 0.79    | 1.62 ± 0.72    | 1.38 ± 1.09    | 1.31 ± 0.76    |
| BMP6 (ng/mL)                             | 0.25 ± 0.73    | 0.18 ± 0.33    | 0.28 ± 0.75    | 0.08 ± 0.03    |
| CRP (mg/L)                               | 5.41 ± 17.44   | 2.80 ± 5.14    | 17.12 ± 29.30  | 8.40 ± 15.02   |
| IL-1b (pg/mL)                            | 137.59 ± 297.51| 29.94 ± 29.8   | 31.75 ± 44.33  | 20.95 ± 1.33   |
| IL-6 (pg/mL)                             | 140.54 ± 293.99| 25.11 ± 21.35  | 59.72 ± 119.62 | 23.18 ± 14.94  |

**Table 3:** Groups in ALD patients according to hemoglobin (<14 g/dL or >14 g/dL) and ferritin (<400 ng/mL or >400 ng/mL)

**ALD:** alcoholic liver disease; **IL:** interleukin; **LIC:** liver iron concentration; **AST:** aspartate transaminase; **ALT:** alanine transaminase; **MCV:** mean corpuscular volume; **LDH:** lactate dehydrogenase; **ERFE:** erythroferrone; **BMP6:** bone morphogenetic protein 6; **CRP:** C reactive protein; **Hb:** hemoglobin; **Fer:** ferritin.
Table 4: Spearman correlations of serum hepcidin and MCV with different serum parameters in a large cohort of ALD patients

| Parameter                          | Hepcidin (ng/mL) | MCV (fL) |
|------------------------------------|------------------|----------|
| Ferritin (ng/mL)                   | 0.398***         | 0.344*** |
| Iron macrophages (score 0-4)       | 0.413***         | 0.223*   |
| Iron hepatocytes (score 0-4)       | 0.361***         | 0.046    |
| Erythrocytes (i/pL)                | 0.206***         | -0.636***|
| Hematocrit (%)                     | 0.199**          | -0.287***|
| ALT (U/L)                          | 0.192**          | -0.001   |
| Hemoglobin (g/dL)                  | 0.192**          | -0.315***|
| MCV (fL)                           | -0.164**         | 1.000    |
| LDH (U/L)                          | 0.175*           | 0.249*** |
| Bilirubin total (mg/dL)            | 0.150*           | 0.276*** |
| Fibrosis stage (cut-off adapted)   | -0.133*          | 0.385*** |
| Kleiner score (0-4)                | -0.002*          | 0.342**  |
| Transferrin saturation (%)         | 0.143            | 0.205**  |
| Liver stiffness (kPa)              | -0.101           | 0.380*** |
| Alcohol consumption (g/day)        | -0.082           | -0.106   |
| GGT (U/L)                          | 0.076            | 0.363*** |
| GDF15 (U/L)                        | 0.112            | 0.323*** |
| Serum iron (µg/dL)                 | 0.073            | -0.022   |
| AST (U/L)                          | 0.054            | 0.243*** |
| Quick (%)                          | 0.046            | -0.279***|
| M65 (U/L)                          | 0.044            | 0.416*** |
| Platelets (/nL)                    | -0.040           | -0.176** |
| Haptoglobin (mg/dL)                | 0.035            | 0.121    |
| CRP (mg/L)                         | 0.014            | 0.070    |
| CAP (dB/m)                         | -0.006           | 0.076    |
| Leukocytes (/nL)                   | -0.003           | -0.082   |
| Transferrin (g/L)                  | -0.003           | -0.381***|
| M30 (U/L)                          | -0.002           | 0.399*** |

*P < 0.05, **P < 0.01, ***P < 0.001.

MCV: mean corpuscular volume of erythrocytes; ALD: alcoholic liver disease; ALT: alanine aminotransferase; MCV: mean corpuscular volume of erythrocytes; GGT: glutamyltransferase; AST: aspartate aminotransferase; CAP: controlled attenuation parameter; LDH: lactate dehydrogenase; CRP: C reactive protein; GDF15: growth differentiation factor 15.

Table 5: Mechanisms for RBC recycling either by erythrophagocytosis or after direct hemolysis.

| Mechanisms                                                      | Examples                                      |
|----------------------------------------------------------------|-----------------------------------------------|
| 1. Direct hemolysis and elimination by RES*                     |                                               |
| Hypo-osmosis                                                    |                                               |
| Membrane-destabilization by detergents                          | Bile acids, drugs                             |
| Direct toxicity                                                 | Bilirubin                                     |
| Mechanic shear stress                                           |                                               |
| Heme-recycling through haptoglobin or hemopexin                 | CD163 or CD91 mediated heme uptake            |
| 2. Induction of RBC suicidal death (eryptosis)                  |                                               |
| Phenylhydrazine                                                |                                               |
| Continuous exposure to H₂O₂                                     | glucose oxidase                               |
| Bilirubin                                                      |                                               |
| 3. Erythrophagocytosis of intact RBCs                          |                                               |
| Oxidative modification of RBCs                                  | Treatment with copper sulfate                 |
| Continuous exposure to H₂O₂, e.g. through glucose oxidase      |                                               |

*It is partly not yet known whether some pathways are overlapping. RBC: red blood cell; RES: reticuloendothelial system.
WHAT FACTORS CONTRIBUTE TO HEMOLYSIS DURING ETHANOL-METABOLISM?

If we consider Hb as the major iron storage compartment in humans (80%) (Figure 6A), all forms of enhanced erythrocyte turnover whether eryptosis, masked or direct hemolysis become highly relevant for iron release and hepatic overload (Table 5 and 6). This is further underlined by the fact that the liver macrophages are an essential part of erythropoiesis next to the spleen and bilirubin as major heme degradation product is exported primarily through the liver. Under the microscope, the erythrocytes are observed as mouth-like or stoma-like shape, with a shortened lifespan. In former studies more than 25% of alcoholics were found to have an increased number of peripheral stomatocytes. Spur-cells are considered the most severe form of hemolysis related to alcohol consumption and they are characterized by spikelike protrusions on the cell surface. These spur cells with an irreversible rigid membrane will be prematurely removed by spleen macrophages. Spur-cell associated hemolysis is observed in ca. 3% of chronic heavy drinkers ultimately leading to progressive fatal anemia.

As shown in Table 5, RBCs can be directly hemolyzed e.g. by hyposmotic or mechanic shear stress conditions. Free heme or Hb released from the hemolyzed RBCs is recognized by hemopexin or haptoglobin in the serum, forming heme-hemopexin complex or hemoglobin-haptoglobin complex. The complexes are then recognized by CD91 or CD163 on the membrane of macrophages and catabolized by HO1 in macrophages.

The finding that αa. 50% of all drinkers show signs of enhanced erythropagocytosis or hemolysis leads to the direct question what molecular mechanisms are involved in ethanol-mediated hemolysis (see also Table 3). Generally, humans have sophisticated, efficient and partly redundant mechanisms to sequester and eliminate senescent or fragile RBCs, and to remove release heme as heme is highly toxic to all cells including the vascular endothelial bed. Senescent erythrocytes are normally recognized by Fc-receptor. Under alcoholic circumstance, some injured erythrocytes may also start an apoptosis-like suicidal cell death, called eryptosis. It remains an open question whether formerly discovered spur-cells are related to what is now called eryptosis. This mechanism enables the elimination of defective cells without destroying the cell membrane integrity and release of cytosolic material to the exterior and can thus be compared to apoptosis in epithelial cells. The phospholipid membrane of eryptotic cells is characterized by the translocation of phosphatidyserine (PS) from the inner leaflet to the exterior regulated by flippases. Eryptotic erythrocytes may further display alterations in their membrane elasticity and are recognized by PS receptors on macrophages including direct receptors such as Tim-1, Tim-4, Stabilin-2 and CD300 and indirect receptors such as TAM receptors and αvβ3/β5 integrins. These erythrocytes are then

Table 6: The effects of ethanol and its metabolites on erythropoiesis and RBCs

| Molecules       | Targets                      | Effects                                      | Ref.                        |
|-----------------|------------------------------|----------------------------------------------|-----------------------------|
| Ethanol         | Intestine                    | Folate deficiency                            | Medici et al. 2013          |
| Ethanol         | Erythroid precursors         | Vacuolization and cell death                 | Roselle et al. 1986         |
| Ethanol         | Erythroblasts                | Iron deposit and ring sideroblasts, sideroblastic anemia | Yeung et al. 1988           |
| Ethanol         | Erythrocytes                 | Elevated MCV, macrocytic anemia              | Pierce et al. 1976          |
| Acetaldehyde    | Hemoglobin                   | Forming adducts with hemoglobin A            | Lindenbaum et al. 1980      |
| Acetaldehyde    | DNA                          | Blocking DNA translation                     | Maruyama et al. 2001        |
| ROS             | Erythrocytes                 | Disorder of erythrocyte cytoskeleton, stomatocytes and spur-cells | Stevens et al. 1998         |
| ROS             | Erythroblasts                | Disturbing the enucleation                   | Yu et al. 2010              |
| Fatty acid ethyl ester | Erythrocytes | Incorporating into the membrane and causing hemolysis | Morse et al. 1990            |

RBC: red blood cell; MCV: mean corpuscular volume of erythrocytes; ROS: Reactive oxygen species.
Figure 10: Experimental erythrophagocytosis and hepcidin expression in THP-1 macrophages. (A) Erythrophagocytosis of human erythrocytes (oxidized by copper sulfate) by PMA-differentiated THP-1 cells. (B) HO1 continuously increases with increasing numbers of oxidized RBCs (shown as % hematocrit = htc). No HO1 induction is seen with non-oxidized control RBCs. (C) In contrast, hepcidin mRNA shows a so-called "bivalent response" to increasing concentrations of oxidized RBCs (heme). Low levels of heme increase hepcidin while higher levels cause hepcidin suppression. ERFE cannot explain these *in vitro* observations. RBC: red blood cell; Htc: hematocrit; ERFE: erythroferrone; HO1: hemeoxygenase 1.

Figure 11: Direct exposure of human RBCs with EtOH and AA for 24 h. Note, that hemolysis or modification only occurs at quite high levels of ethanol and acetaldehyde. CO: normal control (no addition of ethanol); EtOH: ethanol; AA: acetaldehyde.
Figure 12: Eryptosis and in vitro erythrophagocytosis in the presence of calf bile acids and bilirubin. (A) Induction of an eryptosis phenotype by bile acids or for three hours. (B) Experimental set up to assess erythrophagocytosis. THP-1 cells were differentiated over 24 hours by PMA to attaching macrophages and exposed to 1% Htc bile acid or bilirubin treated RBCs for another 24 h. Cells were then collected for mRNA quantitation by RT-PCR. (C) HO1 mRNA induction (erythrophagocytosis) in the presence of identical concentrations of bile acid- and bilirubin-treated RBCs. The experiment underlines that cholestasis can initiate a vicious cycle of hemolysis and heme-degradation-mediated hemolysis. This vicious cycle is deteriorating in endstage ALD and may also contribute to AH. Htc: hematocrit; RBC: red blood cell; ALD: alcoholic liver disease; HO1: hemeoxygenase 1; AH: alcoholic hepatitis; Htc: hematocrit.

Figure 13: RBCs in ALD patients are more fragile than RBCs in normal controls. RBCs from both groups were treated with the hemolytic agent phenylhydrazine for various time points and hemolysis was measured by absorption spectroscopy in the supernatant. After 60 min, the hemolysis rate of ALD patients was significantly higher than in healthy controls. It indicates that RBCs seem to be generally more fragile and prone to turn over in drinkers. ALD: alcoholic liver disease; RBC: red blood cell; CO: control patients (healthy volunteers without alcohol consumption).
An efficient way to mark erythrocytes for erythropagocytosis is oxidative modification of the membrane (Table 5). In the laboratory, copper sulfate is regularly used in a Fenton-like manner. Figure 10A shows erythropagocytosis of human erythrocytes by differentiated THP-1 macrophages with and without copper sulfate treatment. The efficient erythropagocytosis can be further documented by mRNA expression of the major heme degradation enzyme HO1 (Figure 10B). Of note, hepcidin is increased at low RBC numbers (till 0.05% hematocrit) but suppressed at higher concentrations (bivalent response of hepcidin towards iron and heme).

Besides marking the RBCs through oxidative modification, they can be also directly lysed either through hypotonic conditions, by detergents, other toxins or even mechanic stress as is seen in patients with artificial heart valves (Table 5). The leaked heme can be detected either directly in the supernatant (Figure 11) or by other assays such as the ultra-sensitive luminol-enhanced chemiluminescence.\textsuperscript{145,146} Since ethanol and acetaldehyde also possess lipophilic properties that could destabilize the lipid membrane, direct effects can also not be ruled out (Figure 11). We have reassessed the ability of ethanol or its major metabolite acetaldehyde to induce erythropagocytosis/hemolysis. As shown in Figure 11, both compounds are able to induce hemolysis but at very high concentrations. Acetaldehyde has been shown to react with the amino termini of Hb to form stable cyclic imidazolidinone derivatives.\textsuperscript{147} It has been recently shown that either the detergent or direct toxic effects of bile acids and conjugated bilirubin are able to induce eryptosis or erythropagocytosis.\textsuperscript{148,149} Confirmative preliminary experiments from our own lab show (Figure 12A-C) that both bile acids and bilirubin can induce a phenotype of eryptosis (Figure 12A) eventually triggering erythropagocytosis in differentiated THP-1 cells. We have also established an ex vivo assay to test the resistance of erythrocytes towards the hemolytic agent phenyl hydrazine. Figure 13 shows the hemolysis measured by direct photometric heme determination in the supernatant. After 60 and 90 min, blood from ALD patients clearly showed more hemolysis as healthy control persons. The test is attractive since it measures the overall fragility of RBCs towards a standard stress situation but does not detect the specific cause of hemolysis (membrane rupture, reduced antioxidative defense \textit{etc}). Taken together, various conditions in ALD patients may induce eryptosis, erythropagocytosis or direct hemolysis finally resulting in enhanced heme turnover in these patients.

**PRESENT CHALLENGES IN UNDERSTANDING IRON SENSING BY HEPCIDIN: IMPLICATIONS FOR ALD**

Although various upstream regulators of hepcidin have been discovered (such as C/EBP\textalpha, BMP6, SMAD 1/5/8, SMAD4, SMAD6, SMAD7, TNPRESS6, IL-6, CREBH, CHOP and TLR4), the overall regulation, namely the exact sensing of iron by hepcidin, is still far from being understood. Two major experimental and clinical observations are especially difficult to understand. Although hepcidin suppression has been recognized as major mechanisms of chronic iron accumulation in most hereditary iron overload diseases such as hemochromatosis,\textsuperscript{150} hepcidin is seemingly paradoxically and profoundly suppressed in hemolytic diseases such as thalassemia\textsuperscript{151} or chronic liver diseases\textsuperscript{152} in the presence of iron excess \textit{e.g.} released from an increased red blood cell turnover.\textsuperscript{153,154} Another major unresolved observation is that hepcidin responds differently to iron overload \textit{in vitro} and \textit{in vivo}.\textsuperscript{155} In contrast to the profound induction of hepcidin by iron under \textit{in vitro} conditions, hepcidin expression is typically inhibited in the presence of iron in cell culture models.\textsuperscript{152} The complexity of iron sensing by hepcidin could neither be resolved by further mechanistic insights from intercellular communication between hepatocytes and endothelial cells or macrophages\textsuperscript{150,153} or additional mechanistic insights of known or novel molecules such as Tfr1, ERFE or GDF15.\textsuperscript{156,157} An alternative explanation for the paradox response of hepatocellular hepcidin to direct iron exposure could be the fact that the actual location of “iron sensing” occurs in a different type of cells \textit{e.g.} in the endothelial bed which is then indirectly communicated to hepatocytes by intercellular signaling though the BMP6/SMAD pathway.

We believe that despite the complexity, iron sensing should be recapturable \textit{in vitro}. First, we need to fully understand all conditions that modulate hepcidin at the single cell level not only including protein factors such as transcription factors but also non-protein factors such as hypoxia, reactive oxygen species or iron itself. We then should better understand the crosstalk between neighboring cells that are relevant for iron sensing. Third, the crosstalk should be regarded within the context of the whole organisms. This will finally also include anatomic specificities \textit{e.g.} of the liver and spleen but also the liver pathology itself with the development of cirrhosis and all its \textit{e.g.} hemodynamic consequences that should be highly relevant for iron sensing. For instance, it is not discussed in the literature what are the consequences once portal hypertension has developed and humoral factors will not have direct access anymore to the liver. Moreover, although quite evident,
the loss of function of necrotic or apoptotic cells is rarely considered. For instance, the loss of hepatocytes during ethanol-mediated liver damage will ultimately cause a loss of total hepcidin secretion which can further lead to enhanced iron uptake.

In addition, some confusions arise from seemingly contradictory reports in the literature that are simply due to errors or artificial conditions. Thus, and as mentioned above, hypoxia and iron are interrelated in a complex manner at various organization levels. It matters whether an animal is exposed to hypoxia and shows all the adaptive responses or whether a single cell is studied which is by far better adaptable to hypoxia. This may explain why earlier studies postulated a hypoxia-mediated hepcidin suppression and even hypoxia-responsive elements within the hepcidin promoter (which could not be confirmed later), while detailed in vitro studies with different hypoxia systems demonstrated in fact a stimulatory effect of hypoxia on hepcidin. Moreover, oxidative stress was postulated to block hepcidin using artificial bolus experiments e.g. of H$_2$O$_2$ while later studies with physiology-mimicking conditions clearly demonstrated that H$_2$O$_2$ is a potent up regulator of hepcidin through the STAT3 pathway. Likewise, iron accumulation in ALD has been generally attributed to a direct hepcidin blocking effects of alcohol but this could only be demonstrated in short term animal models. As shown in Figure 9, however, a large prospective study of heavy drinkers showed highly elevated hepcidin levels in drinkers. As shown in Table 3, this is not due to drastic inflammation or cytokine storm. We rather believe that the major topic of this concept paper, hemolysis, contributes to hepcidin expression in ALD.

**NOVEL INSIGHTS INTO THE CROSSTALK OF MACROPHAGES, ENDOTHELIAL CELLS AND HEPATOCYTES**

We and other have recently studied in more detail the crosstalk of various cells such as macrophages or endothelial cells with hepatocytes. As mentioned above, BMP6 has been identified as one of the most potent upstream inducers of hepcidin. BMP6 knockout mice exhibited hepcidin deficiency and severe tissue iron overload, although BMP2 and BMP4 can also bind to HJV. Recently, a study demonstrated that liver sinusoidal endothelial cells (LSECs) are the predominant source of basal BMP6 secretion and participate in iron-regulated hepcidin expression. Although the role of both LSECs and endothelial cells (EC) in systemic iron regulation has been only marginally studied, a few studies on the blood-brain barrier and retina suggest that they may be involved in iron sensing and transport. Moreover, ECs are highly sensitive to iron changes and, finally, they secrete the autocrine protein BMP-binding endothelial cell precursor-derived regulator (BMPER), able to antagonize BMPs activity and regulate hepcidin. Finally, a recent study provided the first insights into molecular iron sensing by ECs. Accordingly, Nrf2 is activated by iron-induced, mitochondria-derived pro-oxidants and drives BMP6 expression in liver sinusoidal endothelial cells.

In a more detailed in vitro study, we could recently show that iron directly blocks hepatocellular hepcidin signaling through the SMAD pathway independent of STAT3. All iron III forms including ferric ammonium citrate (FAC) efficiently blocked hepcidin mRNA expression at non-toxic dosages in Huh7 cells or primary hepatocytes in a time and dose-dependent manner which could be efficiently blunted by iron chelators (Figure 14A). FAC also inhibited BMP6, hypoxia, IL-1β, IL-6-mediated and LPS-mediated hepatic hepcidin induction in a co-culture model with macrophages. Moreover, FAC reduced SMAD6 mRNA and p-SMAD1/5/8 protein expression at basal or upon stimulation by BMP6 or under hypoxia. However, FAC had no significant effect on p-STAT3 protein expression at basal or upon stimulation by various stimuli. In another detailed still unpublished study, we focused on the crosstalk between ECs and HCs. HCs (Huh7 cells) were direct co-cultured with ECs (HUVECs and SK hep). EC-conditioned media (CM) were also employed to culture Huh7 cells and primary mouse hepatocytes. Both direct co-culture with ECs or EC-CM significantly increased HC-hepcidin. The upstream SMAD pathway of hepcidin, including pSMAD1/5/8, SMAD1 or Id1, were induced by EC-CM. Efficient blockage of this EC-mediated hepcidin upregulation by an ALK2/3 inhibitor or BMP6 siRNA identified BMP6 as a major hepcidin regulator in this co-culture system, which highly fits the hepcidin regulation by iron in vivo. In addition, EC-derived BMP6 and hepcidin responded highly sensitive to levels of ferric iron but also heme as low as 500 nmol/L. We have been also able in preliminary experiments to demonstrate a bivalent response of an EC-HC co-culture system in response to heme (Figure 14B). At low levels, both direct HC-mediated hepcidin response but also BMP6 secretion from ECs are upregulated. At higher levels, HC-derived hepcidin is partly blocked. At higher levels (<10 µmol/L heme), iron toxicity is observed in ECs and no further upregulation of hepcidin can be observed. We therefore propose that even in the absence of other systemic regulators such as ERFE, the bivalent response of EC-HCs to iron can explain the behavior during mild and severe hemolysis. Figure 15 shows the response of hepcidin in mice treated either mildly or more severely with the hemolytic agent phenyldiazine (PHZ). As seen in Figure 15, mild hemolysis causes hepcidin upregulation while, in contrast, the severe form causes profound inhibition of hepcidin. Figure 16 shows...
Figure 14: The in vitro bivalent response of hepatocytes hepcidin to iron levels. A. Hepcidin expression in Huh7 cells was inhibited by all ferric iron forms (FAC 50 μmol/L, FeCl₃ 50 μmol/L, FC 50 μmol/L, Ferrlecit 50 μmol/L and Hemin 50 μmol/L). B. Hepcidin expression in Huh7 cells co-cultured with BMP6-secreting endothelial cells (HUVEC)-derived conditioned medium was upregulated by low hemin (0.1 μmol/L) but inhibited by higher hemin concentrations; FAC: ferric ammonium citrate.

Figure 15: Hepcidin expression in mice liver with mild or severe hemolysis. One or two injections the hemolytic agent PHZ (60 mg/kg body weight) during two consecutive days (the last injection is t = 0) were applied to mice as models of mild or severe hemolysis. Hepcidin transcription levels were measured at 4, 8, 12, 24, 48 h after the injection. In the mild hemolysis model, hepcidin was upregulated after 4 h and recovered at 48 h. In contrast, in the severe hemolysis model, hepcidin was inhibited at 48 h as is seen in patients with severe hemolysis; PHZ: phenylhydrazine.

Figure 16: Blood parameters in mild and severe hemolysis in mice. Two PHZ injections (with 60 mg/kg body weight) in two consecutive days (the last injection is t = 0) were applied to mice. Blood samples were collected at 24 and 48 h after the injection. Hemolytic mice developed anemia, elevation of GOT, GPT and LDH. Transferrin also increased while hepcidin showed a bivalent response. GOT: glutamic-oxaloacetic transaminase; GPT: glutamic-pyruvic transaminase; LDH: lactate dehydrogenase; PHZ: phenylhydrazine.
corresponding levels for hemoglobin, GOT, GPT, LDH, transferrin and hepcidin for the severe hemolysis models. As already shown for macrophages (Figure 10B) and cultured hepatoma cells (Figure 4), hepcidin is induced at low levels of heme exposure but suppressed at high levels. Also note that transferrin is strongly upregulated during hemolysis. Several studies by Tom Ganz’ team have demonstrated that repressed hepcidin actually causes continued and fatal hepatic iron overload in hemolytic diseases such as thalassemia.[96,169] Some other preliminary studies seem to be highly relevant within this context. Figure 17 shows the response of HO1 expression in macrophages (THP-1 cells) in the presence of increasing hemin concentrations. Starting from 0.5% hematocrit or hemin, no further HO1 expression is observed. Moreover, first signs of autophagy (and pre-toxicity) are observed at concentrations of 15 µmol/L. This indicates that the heme degradation is somehow self-limiting potentially because the release of carbon monoxide (CO) and iron from heme degradation (see also Figure 6B) are highly toxic. It is still too early to speculate whether CO itself plays an important role in this auto-limitation (regulatory loop). In another preliminary set of experiments, we could show that both hemopexin (Hpx) and haptoglobin (Hp) lower the HO1 expression in response to equal amount of hemin (Figure 18), which was coherent with recent data from other labs.[170] This suggests that both Hp and Hpx are mechanisms to safely internalize toxic heme into macrophages. The data also suggest that these save pathways prevent rapid heme degradation with subsequent release of toxic CO and iron. So far, it can only be speculated whether CO plays a critical feedback role e.g. by inhibiting the respiratory chain.

ALCOHOL-RELATED DAMAGE OF BONE MARROW AND ERYTHROPOIESIS

In human embryos, the early production of erythroid lineage cells occurs in the yolk sac.[171] The mesodermal cells migrate into the yolk sac to form blood islands containing red blood cells and endothelial cells.[172,173] Then primitive erythroblasts (PE) expressing embryonic globin emerge in yolk sac blood islands, along with platelet progenitor cells and macrophages.[174,176] Later, erythro-myeloid progenitor (EMP) emerges from yolk sac and migrates to the fetal liver, producing definite erythroblasts.[175,177] Then, hematopoietic stem cells (HSC) emerge and migrate to the fetal liver and eventually to the adult bone marrow producing definite erythroblasts.[178,179] Adult HSCs in the bone marrow give rise
to either a common myeloid progenitor (CMP) or common lymphoid progenitor (CLP). The CMP then differentiates into either a granulocyte monocyte progenitor (GMP) or megakaryocyte erythroid progenitor (MEP). The committed MEPs further differentiate into erythroid precursor cells with distinct morphologies, gradually reducing in cell and nuclear size, and finally to form enucleated reticulocytes.\textsuperscript{180}

Alcohol abuse appears to be toxic on bone marrow, interfering with erythropoiesis, granulopoiesis and thrombopoiesis.\textsuperscript{180} It directly damages erythroid precursors with vacuolization.\textsuperscript{182-184} The most commonly observed effects of alcohol on bone marrow are large vacuoles in early RBC precursor cells which usually emerge in the pronormoblasts within 7 days after heavy alcohol intake and disappear within 1–2 weeks after abstinence.\textsuperscript{182,192} Morphological examinations also reveal that bone marrow from alcoholics exhibits a significant vacuolization in erythroid and megakaryocytic lineages.\textsuperscript{184,193} Culturing normal marrow cells in nutrient medium with alcohol can induce cytoplasmic vacuolization as well, and the proportion of cells developing vacuoles appears to correlate with the concentration of alcohol.\textsuperscript{184}

ALCOHOL DIRECTLY IMPAIRS HEMATOPOIETIC PROGENITOR CELLS

Alcohol causes damage to hematopoietic cells both directly and indirectly.\textsuperscript{189,193,199} The direct toxic targets of excessive alcohol include bone marrow, blood cell precursors, mature erythrocytes, white blood cells, and platelets (Figure 19).\textsuperscript{139} The most commonly observed effects of alcohol on bone marrow are large vacuoles in early RBC precursor cells which usually emerge in the pronormoblasts within 7 days after heavy alcohol intake and disappear within 1–2 weeks after abstinence.\textsuperscript{182,192} Morphological examinations also reveal that bone marrow from alcoholics exhibits a significant vacuolization in erythroid and megakaryocytic lineages.\textsuperscript{184,193} Culturing normal marrow cells in nutrient medium with alcohol can induce cytoplasmic vacuolization as well, and the proportion of cells developing vacuoles appears to correlate with the concentration of alcohol.\textsuperscript{184}

Accumulation of vacuole can also indicate cell stress in hematopoietic precursor cells. Vacuolization reflects an adaptive response for cell survival,\textsuperscript{194} which has the potential to lead to different forms of cell death subsequently.\textsuperscript{194,195} In addition, alcohol induces oxidative stress, disrupts protein production in the ER, and inhibits ubiquitin-proteasome activity in cells.\textsuperscript{196,198} These negative effects of alcohol on cell functions may potentially contribute to the formation of vacuoles.

Figure 18: Presence of Hp or Hpx lowers HO1 expression in THP1 cells. Exposure of THP-1 macrophages to lysed RBCs or 30 μmol/L hemin causes induction of HO1 mRNA expression. HO1 mRNA is significantly suppressed in the presence of heme scavenging Hp and Hpx. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\). Hp: haptoglobin; Hpx: hemopexin; RBC: red blood cell; HO1: hemeoxygenase 1.
METABOLITES OF ETHANOL EXERT NEGATIVE EFFECTS ON BONE MARROW

Besides the direct influence of alcohol on bone marrow, the cellular metabolites such as acetaldehyde and reactive oxygen species (ROS) can also cause toxicity. Acetaldehyde covalently binds to proteins and nucleic acids while ROS causes damage to lipids, proteins and nucleic acids. Acetaldehyde adducts have been found both in blood and bone marrow of patients with ethanol-induced erythrocyte abnormalities. While ethanol facilitates both acidic and oxidative hemolysis, acetaldehyde only caused destabilization in the case of oxidative hemolysis. In RBCs, catalase is considered as the only enzyme which can metabolize ethanol to acetaldehyde. Fatty acid ethyl esters incorporated into membranes erythrocytes have been also shown to cause increased hemolysis.

In addition, acetaldehyde is a genotoxic compound that reacts with DNA to primarily form dysfunctional adducts which ultimately and efficiently block DNA synthesis. ROS are also produced during ethanol metabolism. Normally, ROS decreases in erythroblasts in later stages when the cells are preparing for enucleation. However, in alcoholics, ROS accumulation of terminal erythropoiesis will interfere the initiation of enucleation. In summary, alcohol consumption causes impairment of structure, signaling, metabolism, proliferation and differentiation of hematopoietic cells. HSCs and the upstream multipotent progenitors are more resistant to the negative effects exerted by ethanol and acetaldehyde in comparison to the myeloid and the downstream progenitor cells which is thought to be related to aldehyde dehydrogenase (ALDH) activity. Whether the Zieve syndrome, a rare but severe form of hemolysis during heavy alcohol consumption, is caused by bone alcohol-mediated marrow damage remains an open question.

MACROCYTIC ANEMIA AND SIDEROBLASTIC ANEMIA IN HEAVY DRINKERS

Excessive alcohol consumption is one of the most common causes of macrocytosis and non-megaloblastic macrocytic anemia and alcoholic detoxification can improve the elevated MCV. On the other side, it is also commonly observed that MCV remains elevated for months despite abstaining from alcohol.

Neutrophil hyper-segmentation is a useful sign of folate depletion in patients, whereas serum folate concentrations are not reliable. The Michigan Alcoholism Screening and γ-glutamyl transferase levels are highly sensitive tests for detecting alcoholics with macrocytosis,
who may also exhibit other typical symptoms such as gynecomastia, caput medusae, and jaundice. Significant folate depletion in alcoholism has been described as important reason for macrocytosis. The normal production and maturation of erythroid precursor cells require folic acid and other B vitamins. Although ethanol itself has been considered as the main reason of folate deficiency in alcoholics, there are conflicting results we and others have not seen a stringent association between an elevated MCV deficiency of folic acid and vitamin B\textsubscript{12} (see also text to Table 3).

Sideroblastic anemia is characterized by the emergence of ring sideroblast in bone marrow. In some patients with alcoholism, iron cannot be properly incorporated in hemoglobin. These pathological erythroblasts which have particular iron accumulation in perinuclear mitochondria are called ringed sideroblast and cannot further develop into functional erythrocytes. Acquired sideroblastic anemia is a common complication in heavy drinkers. Furthermore, alcoholic sideroblastic anemia is often associated with myelodysplastic syndrome. It has been shown that ring sideroblast usually disappear within one week of abstinence.

In first preliminary experiments with a mouse alcoholic mouse model from our laboratory in cooperation with Marina Scheller-Wendorff from Heidelberg, both short and long-term ethanol exposure led to a reduced number of erythrocytes (Ter 119\textsuperscript{+} population) in the bone marrow whereas increased cell numbers were observed in the spleen. Increased weight for spleen was observed in chronic mice possibly due to hemolysis and to compensate for reduced hematopoietic activity. Analysis of terminal erythroid differentiation revealed that ethanol increased both number and production rate of proerythroblast. In contrast, the number of late erythroblasts was drastically decreased. These preliminary data suggest that ethanol exposure may cause ineffective erythropoiesis due to a differentiation arrest of late erythroblasts. Further studies are needed to better understand the molecular mechanisms of ethanol-mediated damage of bone marrow stem cell function.

**SUMMARY IRON SENSING AND MECHANISMS OF ETHANOL-MEDIATED IRON OVERLOAD**

**Summary 1: Normal iron sensing and hepcidin expression**

Based on the literature and our preliminary data, we propose a slightly different scenario for iron sensing and hepcidin expression (Figure 20). Accordingly, and as shown in Figure 6A, about 0.8\% of all RBCs are recycled.
every day under normal, physiological conditions. The release of heme and subsequent heme degradation causes a permanent iron flow and basal ferritin expression. Under these low iron/heme concentrations, endothelial cell-derived BMP6 will enhance hepatocyte hepcidin expression through the SMAD/BMP pathway. Under normal conditions, total serum hepcidin will be derived at 90% from hepatocytes and 10% from macrophages. Especially hepatocyte-derived hepcidin shows a bivalent response (Figure 20 and Figure 14B). At low iron levels, hepcidin linearly depends on iron levels while it is suppressed at high iron levels. Bleeding will reduce the total amount of RBCs and consequently reduce the daily RBC recycling. This will naturally reduce the iron/heme-driven basal levels of hepcidin. Excess hemolysis will suppress hepcidin despite elevated BMP6 levels and cause further iron uptake such as seen in thalassemic patients. In addition, hepcidin will be further suppressed by either bone marrow secreted ERFE, by loss of hepatocytes during apoptosis or necrosis (liver damage) or during development of cirrhosis since most portal blood will bypass the liver and the function bowel-liver axis does not exist anymore. The right side of Figure 21 depicts the inflammation pathway through STAT3 that eventually causes iron accumulation in ferroportin-containing cells such as macrophages and hepatocytes limiting the availability of serum iron. It will consequently lead to microcytic anemia since iron will also become limiting for erythropoiesis. In contrast to present concepts, the continuous upregulation of hepcidin through RBC-recycling is critical for the understanding of iron sensing. The novel concept is able to harmonize the seeming discrepancies between in vitro and in vivo responses to iron. It still, however, needs to be resolved at the molecular level why hepatocytes show the bivalent response to increasing iron levels.

**Summary 2: Single cells versus multicellular hepcidin responses to iron**

Another important aspect of our novel concept is the autocrine versus systemic function of hepcidin. So far, the major role of hepcidin is seen in an efficient blockage of the iron exporter ferroportin that is only expressed in hepatocytes, macrophages and duodenal enterocytes. Although it has been already described that macrophage-secreted hepcidin can directly block macrophage-ferroportin and thus prevent iron export, the clear meaning of this mechanism is still missing. It has been also not widely conceived why hepatocytes also have ferroportin and most studies focus on duodenal and macrophage hepcidin. The above mentioned clinical observation that hepcidin is profoundly suppressed during severe hemolysis is also poorly understood and cannot be fully understood.

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**Figure 21: Proposed novel hepcidin signaling and iron sensing integrating the important role of continuous hemolysis and the bivalent response of hepcidin.**

For comparison, see also the conventional concept of hepcidin signaling in Figure 7. In normal conditions, iron released from RBC recycling is sensed by endothelial cells and the signal is transduced to hepatocytes by BMP6, which further control TFR2-HFE-HJV complex to upregulate hepcidin. During blood loss, hemolysis-driven hepcidin is suppressed because hemolysis-driven hepcidin expression will be decreased. This effect is further supported by GDF15 and ERFE. However, under conditions of severe hemolysis, the excess iron has an additional inhibitory effect on hepatocyte hepcidin, next to anemia-mediated effects, ultimately causing further iron accumulation. RBC: red blood cell; BMP: bone morphogenic protein; IL6: interleukin 6; STAT3: signal transducer and activator of transcription 3; ERFE: erythroferrone.
explained by e.g. data on ERFE. One explanation for these observations is the fact that hepcidin may have at least two functions: First, it controls the iron flow and export of hepatocytes and macrophages in an autocrine manner and, therefore, can be considered a single cell control. An increase of iron or heme will buffer the iron within these cells and lead to transient storage in ferritin which passively follows the control by ferroportin. Second, hepcidin has a systemic role, especially the hepatocyte-derived hepcidin that represents ca. 90% of total serum hepcidin. This hepcidin will also block ferroportin far from its origin of secretion such as the duodenum and macrophages. The bivalent response of hepcidin towards iron could represent a decision-making conflict between the single cell and the systemic responsibilities. For instance, if hepcidin is drastically suppressed under conditions of excess iron, ferroportin is upregulated and will allow the release of iron from e.g. hepatocytes or macrophages. Under these conditions of acute danger, the management tolerates the fact that further iron is absorbed via duodenum. In conclusion, iron sensing and control should not be seen as an absolute iron level-driven process but as a collective self-organization depending on various demands whether of single cells or systemic origin.

Summary 3: Molecular mechanism of iron overload during ethanol consumption

Based on our preliminary clinical studies and recent in vitro studies on the crosstalk between hepcidin-secreting and other neighboring cells, enhanced RBC turnover seems to be a key mechanism responsible for the iron accumulation in ALD patients. According to our long-term prospective study on heavy drinkers, about 50% develop hepatic iron overload. In ca. 15%, ferritin levels higher than 1000 ng/mL are observed that rapidly normalize within 1–4 weeks after abstaining from alcohol. In our histological studies, iron is found both in macrophages and hepatocytes with huge variations among individual patients. The long-term mortality data clearly show that blood count (RBC number, Hb content or hematocrit) are among the first three top prognostic factors for long-term survival. A closer look at iron related parameters in this cohort of over 1000 patients indicates that indeed ca. 50% show an enhanced erythropagocytosis as evidenced by increased CD163 levels (Table 3). Studies about the potential causes of the enhanced RBC turnover are still too complex to interpret. Although major ethanol-metabolites such as acetaldehyde and ethanol are able to cause hemolysis, quite high concentrations are necessary that may only transiently be reached e.g. in the portal vein. However, many other causes of hemolysis are present in alcoholics and may be related to ROS or at later stages of ALD to toxic effects of accumulating bile acids, bilirubin, hypalbuminemia and hypoosmolality. The various mechanisms of enhanced oxidative stress in ALD may further contribute to heme-turnover since oxidative marking of RBCs is known to initiate their sequestration. Notably, all these factors will worsen during disease progression. First, cholestasis will develop in cirrhotic causing accumulation of bile acids and bilirubin, causing hyposmotic stress due to water retention. In addition, the protective effect of albumin will be weakened during the continuously developing hypalbuminemia.

We have also noted that this hemolysis seems to account for the typical laboratory finding in ALD with GOT levels prevailing over GPT levels since RBCs also contain significant amounts of GOT (see Figure 22A and B). It remains still to be elucidated how an excessive hemolysis also causes liver damage. However, the liver is essential for hemolytic pathways. For instance, the important heme degradation end product bilirubin needs to be conjugated by the liver and excreted through the bile. The fact that bilirubin or bile acids can further enhance hemolysis could represent a novel vicious cycle of hemolysis within ALD patients most likely contributing to the rare but severe and still poorly understood alcoholic hepatitis. It should be also noted that, in ALD, liver maintains its ability to conjugate bilirubin and typical laboratory findings in icteric ALD patients indicate a so-called mechanic cholestasis. It should be also mentioned that the term hemolysis is used in a very broad sense and also subsumes all forms of suicidal death such as erythropoietin or regular forms of RBC sequestration through erythropagocytosis. Direct hemolysis is certainly the most severe form of immediate RBC rupture. We have also learned from our large ALD cohort (Table 3) that ca. 25% are able to safely handle enhanced RBC turnover by promoting RBC re-synthesis ultimately storing iron in the largest and safest compartment, the RBC. Why only a smaller proportion of ALD patients are able to allow for RBC re-synthesis remains to be resolved since both levels of erythropoietin and ERFE are upregulated in these patients (Table 3). There are first indications that bone marrow toxicity, most likely at the stem cell compartment, contributes to this impaired RBC synthesis. It could also explain why MCV is elevated in ALD and considered a hallmark of chronic alcohol consumption for many decades. In this context, it is also quite elucidating that MCV levels remain elevated for many months despite complete alcohol withdrawal. Vitamin analysis by us and others also indicated that the seemingly first explanation, deficiency of either vitamin B12 or/and folic acid, are not the cause of MCV elevation in the majority of ALD patients. These observations also suggest that alcohol itself, so it may be the trigger for stem cell impairment,
Figure 22: Evidence that elevated GOT levels in ALD are, at least in part, due to enhanced RBC turnover. (A) Measurement of GOT, GPT, GGT and LDH in diluted lysed RBCs and serum. Note that at 0.5% hematocrit, RBC-associated GOT elevation can be observed (and LDH). (B) GOT measured in hemolyzed RBCs (in distilled water 1:10) according to hematocrit. Consequently, ethanol-mediated RBC-turnover will be most likely responsible for GOT elevation in ALD. In addition, the heme degradation product bilirubin needs to be conjugated and excreted by the liver. During jaundice in endstage ALD, accumulation of bilirubin and bile acids will further cause hemolysis (eryptosis) initiating a vicious cycle of hemolysis and bilirubin elevation (see also Figure 6A). So far, RBC-GOT cannot be discriminated from liver-GOT. ALD: alcoholic liver disease; GOT: glutamic-oxaloacetic transaminase; RBC: red blood cell; GPT: glutamic-pyruvic transaminase; GGT: glutamyltransferase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase.

is not directly involved. One potential hypothesis could be the concept that iron accumulation itself could cause stem cell compartment toxicity as iron overloaded erythroid precursor cells have been observed in drinkers for a long time.

IMPLICATIONS FOR FUTURE TREATMENT STRATEGIES OF ALD-ASSOCIATED IRON OVERLOAD

At present, no validated treatment options are readily available for ethanol-mediated iron overload. The idea of iron depletion by phlebotomy has been controversially discussed in patients with HCV that equally develop iron overload and phlebotomy is highly propagated e.g. in countries such as Japan. With regard to ALD, there has been one trial (Tirrox) initiated in France in 2012 (REF: ClinicalTrials.gov NCT01342705 Title: “Phlebotomy in risk of hepatocellular carcinoma in patients with compensated alcoholic cirrhosis”). This study, however, was stopped because of delayed patient recruitment. Based on the novel insights and concepts outlined within this concept paper, the following targeted molecular therapeutic approaches can be proposed:

(1) Molecular strategies to enhance hepcidin signaling or supplement hepcidin (e.g. mini-hepcidins) could help to prevent further iron uptake in patients with ALD.

(2) Targeted approaches to stabilize RBCs and lower RBC turnover should be very powerful strategies in preventing ALD-mediated iron overload. The documented efficacy of the antioxidant N-acetylcysteine (NAC) at least in one study points to one potential treatment option. As the mechanisms of RBC turnover are quite complex, various strategies should be explored that include supplementation with protective heme carriers (hemopexin, haptoglobin), iron chelators, or supplementation with RBC stabilizing vitamins such as folic acid or B12.

(3) It will be essential to better understand the bone marrow stem cell compartment and the mechanisms of toxicity during alcohol consumption. A better understanding of the underlying mechanisms could lead to stem cell refreshing therapies in order to
produce more and more stable RBCs.

(4) In addition, we need to better understand why a significant fraction of ALD patients with enhanced RBC turnover are able to re-use the released iron for erythropoiesis while ca. 25% are not able to do so. Novel insights could also lead to novel targeted therapies to re-use and store iron safely in newly synthesized hemoglobin.

**FUTURE STUDIES FOR A BETTER UNDERSTANDING OF ALCOHOL-RELATED IRON OVERLOAD**

As is discussed in this concept paper, disturbed iron homeostasis seems to be a major part of ethanol pathology in drinkers. As has been outlined above, clinical and experimental evidence suggests that an increased RBC turnover is critical for survival of chronic drinkers, in cirrhotics but also complications such as alcoholic hepatitis. The following future directions are listed that may help to better understand iron management and RBC turnover in ALD patients in order to improve diagnosis, risk assessment and therapy for these patients.

**RBC TURNOVER**

(1) Study RBS damage through ethanol-related mechanisms including suicidal death (eryptosis) and erythrophagocytosis.

(2) Study the role of heme-protective clearing pathways such as haptoglobin and hemopexin through CD163 and CD91.

(3) Study the genetics of antioxidative defense of RBCs.

(4) Study the role of hemolysis for liver damage.

(5) Study the mechanisms of macrophage-mediated heme degradation and bilirubin transition to hepatocytes and bile excretion.

(6) Study the role of nuclear receptors such as FXR in heme-degradation and ALD.

(7) Differentiate between RBC-GOT and liver-GOT.

**MECHANISMS OF IRON SENSING**

(1) Role of HFE in heme-mediated iron sensing.

(2) Role of endothelium in hepcidin/iron sensing.

(3) Complex interplay between oxidative stress, hypoxia and iron metabolism including ALD-induced oxidases such as NOX4.

**ALD THERAPY**

(1) How can we interpret present beneficial concepts in the light of enhanced RBC-turnover such as the effects of N-acetyl cysteine or steroids.

(2) If hemolysis is important for ALD progression, how can it be blocked?

(3) If there is a vicious hemolytic cycle in patients with ALD, how can it be interrupted? Could albumin dialysis improve severe ALD?

(4) How can the bone marrow stem cell compartment be stimulated to store iron more safely in RBCs?

(5) Develop tests to specifically measure GOT and GPT in RBCs and compare it with liver transaminases.

(6) Better define the role of AP and GGT in alcohol-toxicity which could be also linked to heme-toxicity.

(7) Study role of cholestasis on ALD progression.

(8) Better explain a rare form of alcohol-related hemolysis, the Zieve Syndrome.

**Source of Funding**

This study was funded by a grant of the DFG to SM (RA 2677/1-2). CC and SJW were supported by a fellowship from the China Scholarship Council (CSC).

**Conflict of Interest**

None declared.

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