Article type : Original

Low Serum Hepcidin is Associated with Reduced Short-term Survival in Adults with Acute Liver Failure

Short title: Hepcidin and Acute Liver Failure

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/hep.30486

Keywords: Acute Liver Failure, Iron, Hepcidin, Survival

Electronic word count: 4463

Number of figures and tables: 4 Figures + 3 Suppl. Figures; 4 Tables + 6 Suppl. Tables

Conflict of interest statement: The authors declare that they do not have any conflict of interest to disclose.

Financial support statement: This work was supported by the German Research Foundation grant STR 1095/4-1, IZKF research group funding, Else Kröner Exzellenzstipendium (to P.S.) and SFB/TRR57 (to P.S. and C.T.)

Authors contributions:

Study concept and design: WL, RF, PS, IS, VD, CM

Acquisition of data: IS, JA, PS

Analysis and interpretation of data: mice: IS, PS; human data: IS, JA, PS, CM

Drafting of the manuscript: IS, PS, JA

Critical revision of the manuscript for important intellectual content: all authors.

Statistical analysis: JA, CM, IS, PS

Obtained funding and study supervision: WL, RF, PS, CT, VD, CM

Abstract:

Background and Aims: The liver has an important role in iron homeostasis through the synthesis of the serum transporter transferrin and the iron hormone hepcidin. The aim of this study was to analyze parameters of iron metabolism in a multicenter cohort of adult patients with acute liver failure (ALF) and in an acetaminophen (APAP)-induced ALF mouse model. **Method:** A representative subset of 121 ALF adults (including 66 APAP-related patients) had baseline serum samples tested for ferritin, transferrin, iron, and hepcidin. Outcomes at 3 weeks after enrollment were categorized as spontaneous survivor (SS) vs. death/transplantation (NSS). Mice were assessed prior to (controls), four and 18 hours after injection of 300 mg/kg APAP. **Results:** ALF patients as well as APAP-treated mice displayed increased ferritin, diminished serum hepcidin and hepcidin/ferritin ratio. SS had lower iron (29.1 vs. 34.5 µmol/l; $p<0.05$) and transferrin saturation (60.9 vs. 79.1%; $p<0.01$), but higher hepcidin levels (8.2 vs. 2.7 ng/ml; $p<0.001$) and hepcidin/ferritin ratio (0.0047 vs. 0.0009; $p<0.001$) than NSS. In a multivariate analysis, a log transformed hepcidin-containing model displayed similar prognostic power as the established ALFSG index (C-statistic 0.87 vs. 0.85) and was better than MELD score (C-statistic 0.76). In mice, hepcidin levels inversely correlated with the surrogate of liver injury. **Conclusion:** Our findings demonstrate that several serum iron parameters significantly associate with 3-week outcomes in adults with

ALF. Among them, hepcidin decreases early during experimental APAP-induced ALF, and is an independent predictor and might be a useful component of future prognostic scores.

Introduction

Acute liver failure (ALF) is an uncommon condition characterized by a rapid loss of liver function in individuals without a pre-existing liver disease [1]. In the US, nearly 50% of ALF cases are due to acetaminophen (APAP) overdose, while idiosyncratic drug-induced liver injury and indeterminate ALF are the most common causes of non-APAP related ALF and associated with a high rate of short-term mortality [1]. Although liver transplantation is an effective therapy in selected patients, the decision for/against transplantation is challenging because of the fast pace of disease development, lack of donor organs and a resulting need for life-long immunosuppressive therapy [2, 3, 4]. To facilitate this decision, multiple prognostic markers/scores have been developed. The Model of End-Stage Liver Disease (MELD) and King´s College Criteria (KCC) are both commonly used prognostic models [3, 4, 5]. In addition, the US Acute Liver Failure Study Group (ALFSG) index is a useful prognostic tool [6] that includes the clinical/demographic parameters of ALF etiology, the need for vasopressor use, grade of encephalopathy and liver-related lab values of bilirubin and INR [3, 7]. Given that the currently available scores have only a limited ability to detect a potentially lethal ALF, there is a constant search for novel predictors [8, 9, 10].

Iron is an essential, but potentially toxic element that causes oxidative stress and promotes the development of bacterial infections [11, 12, 13]. Iron is physiologically sequestered as transferrin in the serum and ferritin in the parenchymal cells [14]. Hepatocytes constitute the major parenchymal iron storage pool and contain large amounts of ferritin. A small fraction of ferritin is released into the serum. In the absence of liver injury, serum ferritin is used as a surrogate for the parenchymal iron load [13, 14, 15]. The ratio of serum iron and transferrin is termed transferrin saturation (TSAT) indicating the amount of serum iron load [14]. Iron metabolism is regulated by hepcidin, a hormone produced primarily in the hepatocytes that blocks the uptake of iron in the intestine and release of iron from macrophages [13, 15]. Hepcidin is a short-living hormone (serum half-life of several minutes) [16] and is subjected to a complex regulation with hypoxia, anemia and iron deficiency being the major suppressors while inflammation and iron overload are the major inducers [17].

Multiple studies demonstrated that parameters of iron metabolism represent useful predictors of liver disease outcome. For example, decreased transferrin and elevated TSAT associated with an adverse outcome in patients with liver cirrhosis, ALF and acute-on-chronic liver failure [11, 18, 19, 20]. In contrast, increased ferritin indicated a life-threatening disease in some, but not all analyses [11, 18, 19, 21]. In studies that measured hepcidin levels,

decreased serum hepcidin associated with a poor survival in patients with alcoholic liver cirrhosis [22]. In acute-on-chronic liver failure, the association between low hepcidin and increased mortality was seen in one, but not the other report [18, 19]. Given these data, we aimed to analyze the changes in iron metabolism occurring in ALF and to delineate their prognostic usefulness. For this purpose, we assessed a mouse model of acetaminophen related ALF and also investigated iron parameters in a multi-center US ALF cohort that were prospectively followed for 3-week outcomes.

Materials and Methods

Animal experiments

10 to 12 week old wild-type C57BL/6N mice were kept under standardized conditions (12 h day/night cycle, 20-24 ℃, humidity 50 %). In the APAP-induced acute liver injury model, mice were fasted for 12 hours prior to an intraperitoneal injection of 300 mg APAP / kg body weight (Sigma-Aldrich, St. Louis, MO, USA) and received free access to food and water thereafter (23). The animals were sacrificed four or 18 h after APAP administration by cervical dislocation after an inhalational anesthesia with isoflurane (AbbVie AG, Ludwigshafen, Germany). Control mice of the same age, sex and genetic background received no treatment. Blood was collected through a cardiac puncture and serum was obtained via centrifugation for 15 min at 2000 g. Levels of serum aminotransferases were measured in the Clinical Chemistry Department of Aachen University Hospital. Levels of hepcidin (LSBio LS-F5905, LifeSpan Biosciences, Seattle, USA), ferritin (ab157713, Abcam, Cambridge, United Kingdom) and transferrin (ab157724, Abcam) were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits as recommended by the suppliers. RNA isolation and complementary DNA (cDNA) preparation were performed with the RNeasy mini kit (Qiagen, Hilden, Germany) and Superscript II reverse transcriptase (Invitrogen, Darmstadt, Germany), respectively. Quantitative PCR was carried out with a 7500 fast Real Time PCR Sequence Detection System (Applied Biosystems, Foster City, USA). The specific primers are listed in suppl. table 1. The relative expression levels of mRNA were determined with the help of ddct method and the ribosomal RNA gene L7 was employed as internal control.

Livers were removed, cut and placed in 10% buffered formaldehyde overnight. After that, they were dehydrated, embedded in paraffin and cut into 3 µm thick sections. To study the overall tissue architecture, the specimens were stained with hematoxylin and eosin (H&E). Images were obtained with a Leica light microscope (Leica, Solms, Germany) equipped with a digital camera and Leica Application Suite software V4.1 (Leica Microsystems, Heerbrugg, Switzerland). The animal experiments were approved by the responsible Institutional Animal Care Committee.

Patients

The analyzed cohort was randomly selected from a pool of 2244 adult patients that were prospectively identified and recruited at 31 tertiary US centers participating in the ALFSG [1]. All subjects met the ALF criteria, i.e. presence of coagulopathy (international normalized ratio ≥ 1.5) and hepatic encephalopathy occurring within 26 weeks of the first symptoms in individuals without a pre-existing liver disease [1]. The selection of patient samples was carried out by the ALFSG staff not involved in the analysis of obtained results. The institutional review boards (IRB) of all participating centers approved the research and the clinical investigation has been conducted according to the principles expressed in the 1975 Declaration of Helsinki. As the patients enrolled had by definition an altered mental status, written informed consent was obtained from the legal next of kin. Monitoring and therapeutic interventions were implemented by each center according to institutional standards of care. Demographic, clinical, laboratory, radiologic, and 21 day transplant free outcomes data were recorded prospectively.

Laboratory Parameters

The measurements of ferritin (electrochemiluminescence; Elecsys ferritin, cat. nr. 04491785 190), transferrin (turbidimetry; Tina-quant Transferrin ver.2) and serum iron (photometry; Iron Gen.2) were performed by the Clinical Chemistry Department of Aachen University Hospital using the Cobas 8000 system (Roche Diagnostics, Mannheim, Germany). The reference ranges were: serum iron 5.8-35 µmol/l; transferrin 200-360 mg/dl; TSAT 25-45%; ferritin 13-150 ng/ml (males), 30-400 ng/ml (females). For the determination of serum hepcidin concentrations, a commercially available ELISA-kit was used (EIA-5782; DRG Instruments, Marburg, Germany) [19, 22, 24]. Measurements of ferritin, transferrin, iron and hepcidin provided valid results in 120 (99 %), 121 (100 %), 121 (100 %) and 113 (93 %) patients, respectively. All analyzed samples were obtained from the first two visits after the inclusion of the patient into the study (i.e. day one or two).

Statistical Analysis

Continuous variables were presented as the mean ± standard deviation and compared using the student's t-test, while continuous non-parametric variables were displayed as median ± interquartile range and compared with the Wilcoxon rank-sum test. Categorical variables were presented as number (percentage) and comparison for proportion was done using the Chi-squared test or Fisher's exact test. Spearman's correlation coefficient was used to analyze correlations between variables.

The study of associations with transplant-free survival at 21 days post study enrollment was done using a univariate logistic regression model and all the clinically relevant covariates with $p < 0.2$ were chosen for a stepwise multi-variable logistic regression model. All continuous variables were assessed and the appropriate transformations were done as necessary. All continuous variables were also analyzed for co-linearity. The final multivariable model was assessed using the area under the receiver operating curve (AUROC) or concordance c-statistic - a measure of discriminatory ability. The performance of the final model was compared with two other SS prediction models based on outcome indices namely MELD and ALFSG Prognostic Index using AUROC.

Statistical significance was defined as a two-sided p-value < 0.05. Statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA) and RStudio: Integrated Development Environment for R, version 1.1.383 (Rstudio Inc., Boston, MA, USA)

Results:

APAP-induced Acute Liver Injury in Mice Results in Altered Serum Iron Parameters

The impact of experimental ALF on parameters of iron metabolism was studied in mice subjected to APAP overdose. 18 hours after APAP administration, we observed a marked increase in serum AST and ALT levels (Suppl. Table 2) and the appearance of characteristic centrilobular necrosis in H&E-staining (Fig. 1A). Moreover, elevated levels of acute phase response and inflammatory marker genes were noted (Suppl. Fig. 1). While APAP treatment for 18 hours did not result in obvious changes in transferrin (Fig. 1C), it led to significantly elevated serum iron (38.5 ± 26.1 vs. 28 ± 6.8 µmol/l; $p<0.01$) (Fig. 1B), ferritin (18830 ± 33987 vs. 871 ± 384 g/l; $p<0.001$; Fig. 1D) and diminished hepcidin levels (25.5 ± 15.3 vs. 53.3 ± 24.4 ng/ml; $p<0.01$; Fig. 1E). Interestingly, hepcidin levels in APAP treated mice negatively correlated with ALT ($r=-0.72$; $p<0.01$) and AST ($r=-0.62$, $p<0.05$). On the other hand, a significant positive correlation with ALT and AST was seen for ferritin (ALT: $r=0.91$; AST: $r=0.84$; $p<0.0001$ for both; Suppl. Table 3). These results suggest that serum ferritin reflects the hepatocellular injury while serum hepcidin may mirror the resulting decrease in the synthetic capacity of the liver. In line with this hypothesis, the hepatic mRNA levels of ferritin, transferrin and hepcidin did not differ significantly between control animals and mice exposed with APAP for 18 hours thereby demonstrating that the observed serum alterations are not due to transcriptional changes (Suppl. Fig. 2).

To test whether the observed changes in serum iron, ferritin and hepcidin levels occur early after APAP-induced injury, we analyzed mice exposed with APAP for four hours. Even at this early time point, the animals displayed a marked elevation in serum AST, ALT and ferritin

levels as well as decreased serum hepcidin values (Suppl. Table 4; Suppl. Fig. 3). On the other hand, serum iron levels did not differ significantly between the untreated and APAP-exposed animals (Suppl. Fig. 3).

Characteristics of the ALF patient cohort

To determine the role of iron parameters in human ALF, we analyzed serum samples at the time of enrollment in 121 ALF patients randomly selected from the prospective multi-center US ALFSG registry (Fig. 2). Our subcohort was representative of the entire registry (Suppl. Table 5) and consisted of 66 APAP and 55 non-APAP cases (Fig. 2). In the non-APAP group, autoimmune hepatitis, idiosyncratic drug-induced liver injury and hepatitis B were the most common etiologies (Suppl. Fig. 4). At day 21 after enrollment, 59 subjects (48.8 %) were classified as spontaneous survivors (SS), whereas 30 subjects (24.8 %) deceased and 32 subjects (26.4 %) received a liver transplant. The latter two categories were analyzed together and labeled as non-SS (NSS). In line with previous data [1], spontaneous survival tended to be more frequent in APAP- vs. non-APAP-induced ALF (57.6 vs 38.2 %; p=0.05; Fig. 2). Moreover, individuals with APAP etiology were younger, had higher transaminases and higher grades of hepatic encephalopathy, both when admission and peak levels were considered. In contrast, non-APAP ALF cases had higher bilirubin and alkaline phosphatase values and less frequently needed ventilator therapy (Table 1).

Next, we investigated factors associated with spontaneous survival at day 21. As reported previously [1], females were more likely to display spontaneous survival (84.7 % in SS vs 64.5 % in NSS; $p<0.05$) and SS had lower bilirubin, INR and MELD-scores, both when admission and peak values were considered (Table 2). In contrast, NSS had lower platelet counts, higher grades of hepatic encephalopathy and more frequently needed vasopressor therapy and ventilator support (Table 2).

Iron Parameters are Altered in ALF and Differ Between Spontaneous Survivors and Non-spontaneous Survivors

In ALF patients, serum iron levels were slightly elevated or at the upper limit of normal. ALF individuals also displayed somewhat diminished transferrin values and strongly increased TSAT and ferritin levels (Table 1 and methods section). While the reference range for hepcidin remains to be defined, the detected values tended to be lower than the ones obtained previously in healthy population controls [24]. With regard to ALF etiology, subjects

with APAP-induced ALF had higher transferrin (198.0±81.0 vs. 165.0 ± 58.5 mg/dl; p<0.01), hepcidin (6.8 ± 13.6 vs. 3.3 ± 6.7 ng/ml; p<0.01) and ferritin levels (4179.0 ± 19481.7 vs. 1935.0 ± 4421.8 ng/ml; p<0.05) than non-APAP cases (Table 1).

Compared to NSS, SS displayed significantly lower TSAT levels (60.9 ± 47.2 vs. 79.1 ± 21.1 %; p<0.01) and lower levels of serum iron (29.1 ± 22.3 vs. 34.5 ± 20.6 µmol/l; p<0.05), but had higher hepcidin values (8.2 ± 14.2 vs. 2.7 ± 5.9 ng/ml; p<0.001) and hepcidin/ferritin ratios (0.0047 ± 0.0219 vs. 0.0009 ± 0.0029; p<0.0001; Table 2). In this sample, ferritin levels were similar among SS and NSS groups (2925 ± 13716 vs. 2755 ± 10018 ng/ml; p=0.058), and no differences in the pattern of association by gender were observed. Although the average serum iron and hepcidin levels differed somewhat between APAP and non-APAP cases (Table 1), the above-described differences between SS and NSS with regard to serum iron, TSAT, hepcidin and hepcidin/ferritin ratios were concordant in both subgroups (Fig. 3).

Among the iron parameters, hepcidin/ferritin ratio correlated negatively with iron (r=-0.47; p<0.001) and TSAT (r=-0.63; p<0.001), while TSAT and ferritin displayed a positive correlation (r=0.59; p<0.001). Hepcidin/ferritin ratio also exhibited a weak negative correlation with parameters of a liver injury/dysfunction such as AST (r=-0.37; p<0.001) MELD (r=-0.32; p<0.001) and INR (r=-0.51; p<0.001) and a similar association was noted between hepcidin and bilirubin (r=-0.30; p<0.001). Along the same lines, the ALFSG prognostic index positively correlated with both hepcidin (r=0.51, p<0.001) and hepcidin/ferritin ratio (r=0.33, p<0.001; for details see Suppl. Table 6).

Hepcidin Constitutes an Independent Predictor of 21-Day Transplant-free Survival

To evaluate the prognostic significance of iron parameters for 21-day SS, we performed univariate logistic regression. It revealed a significant predictive value for iron (p<0.05), TSAT (p<0.01), logarithmic transformed (hepcidin+1) (p<0.001) and square-root transformed hepcidin/ferritin ratio (p<0.001) (Table 3). We also conducted multivariate analysis including all potentially relevant markers. Log (INR) (p<0.05), coma grade 3/4 (p<0.001), platelet count (p<0.001), APAP etiology (p<0.01) and log (hepcidin+1) (p<0.05) were found as the only parameters that independently associated with 21-day SS (Table 3). To determine the potential usefulness of log (hepcidin+1) in the clinical routine, we investigated whether its combination with other established markers improves the currently used outcome indices, i.e. the MELD and the ALFSG model [6]. Forward-selection procedures identified a combination of platelet count, log (INR), APAP etiology, coma grade (3/4) and log (hepcidin+1) as the most parsimonious and best predictive score (Table 4). Compared with both MELD and ALFSG models, it achieved the highest sensitivity (81 %), specificity (79.6%) and the performance of this model was good as defined by the area under the curve (AUC 0.87; 95

% CI 0.80 - 0.93) (Table 4, Fig. 4). In contrast, MELD and ALFSG model reached an AUC (95% CI) of 0.76 (0.67 – 0.85) and 0.85 (0.78 – 0.92), respectively (Table 4, Fig. 4).

Discussion

Our study demonstrated that ALF patients display markedly altered iron parameters. However, the observed changes clearly differed from the alterations seen in other liver disorders. In line with a previous report, we saw a massive increase in serum ferritin that surpassed the levels detected in other diseases such as compensated liver cirrhosis, acute-on-chronic liver failure or sepsis [11, 19, 18, 21, 24]. The rise in ferritin was recapitulated in our experimental ALF model and is not surprising since ferritin is present in large amounts in hepatocytes and is released into serum during liver injury [25]. Accordingly, we observed a strong correlation between serum ferritin and the serum liver enzyme levels. Consequently, the high ferritin levels mirror the acute, massive liver damage characteristic for ALF.

Our study and the work from Anastasiou et al. [11] reported very similar levels of serum iron and transferrin. Serum iron exceeded the values seen in decompensated liver cirrhosis, acute-on-chronic liver failure and severe alcoholic hepatitis [19, and unpublished data]. Moreover, the experimental model demonstrated that the increased serum iron levels constitute a late event during APAP toxicity. These high levels likely reflect the large cell turnover and the diminished hepcidin production [12]. Moreover, the transferrin levels observed in both human studies and the experimental ALF model were above the values detected in individuals with sepsis or decompensated liver cirrhosis [19, 24]. These fairly preserved transferrin levels are likely due to the rather long serum half-life of transferrin (8-10 days) [26] and the lack of inflammation that suppresses transferrin production [24, 27].

Because of the high serum iron levels, ALF subjects exhibited TSAT values that were above the ones seen in decompensated cirrhosis or acute on chronic liver failure [18, 19, 22]. These data indicate that ALF leads to a breakdown of the serum iron metabolism. This is not surprising since serum iron represents a very small and dynamic fraction of the total iron body stores that is altered in multiple human disorders [18, 19, 24]. In particular, the serum iron overload is likely facilitated by decreased iron consumption that is common in critically ill patients [28] as well as by an increased release of iron from damaged cells and red blood cell transfusions.

Even more interestingly, TSAT levels were significantly elevated in NSS individuals compared to survivors. While a causal impact of elevated TSAT on ALF development remains to be proven, several mechanisms might play a role. First, increased TSAT leads to generation of the highly reactive non-transferrin bound iron that is known to cause oxidative

stress and endothelial damage [29, 30]. Moreover, higher iron availability might be exploited by microorganisms and thereby predispose to development of microbial infections [12]. While microbial infection and oxidative stress are of great importance in ALF [31, 32], they play also a crucial role in multiple other disorders. In that respect, high TSAT has been shown to constitute a negative outcome predictor in critically ill patients, individuals with decompensated liver cirrhosis or acute lymphocytic leukemia [18, 19, 24, 33].

Compared to healthy subjects [24], ALF individuals displayed decreased hepcidin levels. This is not surprising since diminished hepcidin was reported in patients with impaired liver function [34, 35]. In line, our experimental ALF model revealed a strong negative correlation between serum hepcidin levels and the markers of liver injury and similar, albeit less pronounced correlation was observed in ALF patients. In addition to that, hypoxia, the serum iron overload and oxidative stress are further factors commonly seen in ALF patients that are known to suppress hepcidin production [12, 34]. While hepcidin is a well-known acute phase reactant, the above-described suppressive factors were obviously more potent than the potential inducers.

Hepcidin levels were particularly low in NSS and hepcidin constituted an independent predictor of ALF-related survival. These data are reminiscent of the situation in compensated cirrhosis, where low hepcidin levels also associated with a poor survival [22]. In contrast, hepcidin did not predict survival in cohorts with significant amount of microbial infections including critically ill patients and individuals with acute-on-chronic liver injury [19, 24]. Collectively, these observations suggest that in absence of inflammation or in a situation where other factors outweigh the existing inflammation, hepcidin may serve as a marker of liver function, however, also provides added benefits, as demonstrated in the multivariate analysis. Notably, in comparison to the established liver function parameters such as albumin or INR, hepcidin has a much shorter half-life [36] and in the hepcidin changes in the experimental ALF model occurred at an early time point after APAP exposure. Thus hepcidin might better reflect the dynamic changes occurring in ALF. In contrast, transferrin might be the better predictor in disorders associated with a more pronounced inflammation [18, 19, 24].

As a possibility to enhance the prognostic usefulness of hepcidin, we analyzed hepcidin/ferritin ratio. While serum hepcidin and ferritin significantly correlate in healthy subjects [37], this relationship is altered in advanced liver disease [34] and even more in ALF, that displays low hepcidin, but strongly elevated ferritin as a surrogate of the hepatocellular injury [our study; 11, 25]. While hepcidin/ferritin ratio greatly differed between ALF survivors and NSS, multivariate analysis revealed that it may not provide a better predictive value than hepcidin alone.

In conclusion, our study detected unique alterations in iron parameters in ALF individuals and demonstrated that they may serve as useful predictors of disease outcome. While they seem to primary serve as surrogates for the extent of liver injury and decreased liver function, they may also play a causal role in ALF development. Further studies are needed to explore this intriguing possibility as well as to address the usefulness of iron parameters in different ALF etiologies.

FIGURE LEGENDS:

Figure 1. Acetaminophen (APAP)-induced liver injury leads to altered parameters of iron metabolism. (A) Hematoxylin and Eosin (H&E) staining reveals the liver architecture in non-treated mice (control; a)) and animals exposed with APAP for 18 hours (b)). Scale bar = 100 µm. (b). Serum iron (B), transferrin (C), log (ferritin) (D) and hepcidin levels (E) are displayed as means ± SD. N highlights the amount of analyzed mice. ** $p<0.01$; *** $p<0.001$

Figure 2. Overview of the analyzed patients with acute liver failure (ALF). APAP: acetaminophen-induced ALF.

Figure 3. Parameters of iron metabolism in patients with acute liver failure (ALF) grouped by etiology and survival status. Log (ferritin) (A), iron (B), log (hepcidin+1) (C), hepcidin/ferritin ratio (D), transferrin levels (E) as well as transferrin saturation (TSAT) (F) were determined in the highlighted subgroups of ALF patients. Boxplots display median with first and third quartile, while whiskers indicate smallest and largest non-outlier observations. Outliers are depicted by empty circles. APAP/non-APAP refers to the ALF etiology (acetaminophen-related or not). Non-spontaneous survivals (NSS) are individuals, who in contrast to spontaneous survivals (SS) either deceased or required a liver transplantation within 3 weeks of enrollment. * $p<0.05$; *** $p<0.001$

Figure 4. Diagnostic accuracy of selected models to predict 21-days spontaneous survival in patients with acute liver failure. Receiver operating characteristics (ROC) curves are shown for the Model Of End-Stage Liver Disease (MELD), ALFSG (consisting of ALF etiology, need for vasopressor therapy, INR, bilirubin and coma grade ≥ 3) and iron model (consisting of log (hepcidin+1), platelet count, log (INR), ALF etiology and coma grade ≥ 3). Areas under the curves (AUC) are indicated as C-statistic.

Supplementary Figure 1. Exposure with acetaminophen (APAP) induces the expression of inflammatory and acute phase response genes. Serum amyloid A1-percursor protein (A), CD68 (B), as well as CXCL1 (C) mRNA levels were quantified by RT-qPCR in non-treated animals (control) and animals exposed with acetaminophen (APAP) for 18 hours. L7 ribosomal mRNA was used as an internal reference. Results are displayed as means ± SD. N highlights the amount of analyzed mice. * $p<0.05$; *** $p<0.001$.

Supplementary Figure 2. Acetaminophen (APAP)-induced liver injury does not alter the hepatic expression of iron-related genes. Ferritin light chain 1 (FTL1), transferrin and hepcidin antimicrobial peptide 1 (HAMP1) mRNA levels were quantified by RT-qPCR in untreated animals (control) and mice exposed with APAP for 18 hours. L7 ribosomal mRNA was used as an internal reference. Results are displayed as means ± SD. N highlights the amount of analyzed mice.

Supplementary Figure 3. Alterations in parameters of iron metabolism occur early during acetaminophen (APAP) induced liver-injury. Serum iron (A), log (ferritin) (B) and hepcidin levels (C) were determined in non-treated mice (control) and animals exposed with APAP for 4 hours and are displayed as means ± SD. N highlights the amount of analyzed mice. ** $p<0.01$

Supplementary Figure 4. Etiologies of acute liver failure (ALF) in the analyzed patient cohort. DILI: drug-induced liver injury (induced by drugs other than acetaminophen). APAP: acetaminophen-induced ALF. Auto Hepatitis: Autoimmune Hepatitis. Hep B: Hepatitis B.

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Table 1 – Comparison of demographics, clinical characteristics and iron results by ALF etiology

Variable	N	Non-APAP (N=55)	APAP (N=66)	P-Value
Age (Years)^	121	46.0±19.0	36.0±14.2	<0.001
Sex (% Female)	121	35(63.6)	55(83.3)	0.024
Race (% Caucasian)*	121	35(63.6)	59(89.4)	0.002
Race (% African American)*	121	16(29.1)	6(9.1)	
Race (% Other)*	121	4(7.3)	1(1.5)	
Ethnicity (% Not Hispanic or Latino)*	121	48(87.3)	64(97.0)	0.07
21 day outcome				
Spontaneous Survival (% Yes)	121	21(38.2)	38(57.6)	0.05
Admission Labs				
ALT (IU/l)^	119	639.0±1167.0	3549.5±4229.0	<0.001
AST (IU/l)^	120	533.5±1259.5	4717.0±6897.0	<0.001
Alkaline Phosphate (IU/l)^	120	172.0±103.8	120.5±56.0	<0.001
Bilirubin (mg/dl)^	120	18.0±12.3	4.4±3.6	<0.001
Creatinine (mg/dl)^	121	1.0±1.8	1.6±1.9	0.008
Creatinine^2 (mg/dl)**	121	1.1±2.9	2.1±3.0	0.02
Hemoglobin (g/dl)^	119	11.5±3.2	10.6±2.9	0.13
INR^	121	2.5±1.4	3.3±2.7	0.005
MELD^	120	30.2±13.7	33.7±8.0	0.47
Platelet Count^	119	134.0±117.5	122.5±120.8	0.38
Toxin Screen (% Yes)	95	13(33.3)	36(64.3)	0.006
Venous Ammonia^	76	79.5±73.2	103.0±66.2	0.09
Leucocyte Count (x10^9/l)^	119	8.9±5.9	9.5±9.3	0.21
Peak Labs				
ALT Peak (IU/l)^	121	697.0±1147.5	4542.5±4192.8	<0.001
AST Peak (IU/l)^	121	566.0±1285.0	5995.0±7403.0	<0.001
Bilirubin Peak (mg/dl)^	121	21.1±9.9	7.5±5.9	<0.001
Creatinine Peak (mg/dl)^	121	1.7±1.9	2.5±2.6	0.015
INR Peak^	121	3.0±2.3	3.9±2.6	0.11
MELD Peak^	121	35.4±13.5	35.2±9.9	0.61
Platelet Count Nadir ^	121	99.0±93.0	70.5±62.5	0.012
Venous Ammonia Peak^	81	106.0±84.5	105.0±71.2	0.61

Table 1 – Comparison of demographics, clinical characteristics and iron results by ALF etiology

Variable	N	Non-APAP (N=55)	APAP (N=66)	P-Value
Admission Vitals				
Diastolic Blood Pressure (mm/Hg)^	121	68.0±21.0	67.0±24.5	0.96
Systolic Blood Pressure (mm/Hg)^	121	124.0±18.5	120.0±26.5	0.28
Weight (kg)^	117	81.5±29.5	73.0±26.0	0.06
BMI^	105	28.4±7.0	25.2±7.9	0.06
Clinical Parameters at Admission				
HE Grade^	121	2.0±2.0	3.0±2.0	<0.001
Coma Grade (% 3/4)	121	16(29.1)	42(63.6)	< 0.001
Pressors (% Yes)	121	6(10.9)	13(19.7)	0.28
RRT (% Yes)	121	10(18.2)	14(21.2)	0.85
Ventilator (% Yes)	121	15(27.3)	44(66.7)	< 0.001
Transfusion (Prior to Specimen Day)				
Pre admission FFP (% Yes)	118	20(38.5)	29(43.9)	0.68
Pre admission to Specimen Day FFP (% Yes)	121	31(56.4)	48(72.7)	0.09
Admission to Specimen Day RBC (% Yes)	121	16(29.1)	16(24.2)	0.69
Admission to Specimen Day RVIIA (% Yes)*	121	0(0.0)	3(4.5)	0.25
Admission to Specimen Day Platelets (% Yes)	121	9(16.4)	13(19.7)	0.81
Admission to Specimen Day Vitamin K (% Yes)	121	4(7.3)	10(15.2)	0.29
Admission to Specimen Day Blood Product (%Yes)	121	33(60)	48(72.7)	0.2
RRT (Prior to Specimen Day)				
Admission to Specimen Day RRT (% Yes)	121	11(20.0)	16(24.2)	0.74
Clinical Parameters during Hospitalization				
Peak Coma Grade during Hospitalization^	121	2.0±2.0	4.0±2.0	0.009
Pressors during Hospitalization (% Yes)	121	14(25.5)	16(24.2)	1

Table 1 – Comparison of Demographics, Clinical Characteristics and Iron Results by APAP Etiology

RRT during Hospitalization (% Yes)	121	13(23.6)	21(31.8)	0.43
Ventilator during Hospitalization (% Yes)	121	22(40.0)	52(78.8)	< 0.001
Diabetes				
Diabetes (% Yes)	121	13(23.6)	10(15.2)	0.34
Transplant				
Ever listed for Transplant (% Yes)	121	23(41.8)	25(37.9)	0.8
LTx within 21 days (% Yes)	121	17(30.9)	15(22.7)	0.42
Iron Indices				
Ferritin (ng/ml)^	120	1935±4422	4179±19482	0.018
Iron (µmol/l)^	121	30.6±17.4	34.5±25.9	0.08
Transferrin (mg/dl)^	121	165.0±58.5	198.0±81.0	0.008
Hepcidin (ng/ml)^	113	3.3±6.7	6.8±13.6	0.008
Transferrin Saturation (%)^	121	71.9±40.7	71.4±32.3	0.83
Hepcidin/Ferritin ratio ^	112	0.0018±0.0063	0.0016±0.0079	0.80

Data expressed as median (IQR) for continuous variables and n (%) for categorical variables.

^: Presented as Median (IQR) with Wilcoxon Rank Sum p-value.

*: The p-value is calculated by the Fisher's exact test.

**: If on Renal Replacement Therapy at admission then Creatinine set to 4.0.

Abbreviations: ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; INR: International Normalized Ratio; MELD: Model of End Stage Liver Disease; RRT: Renal Replacement Therapy; FFP: Fresh Frozen Plasma; RBC: Red Blood Cells; rVIIa: Recombinant Factor VII; LTx: Liver Transplantation

Table 2 - Comparison of demographics and clinical characteristics of the patient cohort by NSS / SS-status

Variable	N	NSS (N=62)	SS (N=59)	P-Value
Age (Years)^	121	40.5±16.8	38.0±21.0	0.84
Sex (% Female)	121	40(64.5)	50(84.7)	0.019
Race (% Caucasian)*	121	48(77.4)	46(78.0)	0.83
Race (% African American)*	121	12(19.4)	10(16.9)	
Race (% Other)*	121	2(3.2)	3(5.1)	
Ethnicity (% Not Hispanic or Latino)*	121	58(93.5)	54(91.5)	0.74
Admission Labs				
ALT (IU/l)^	119	1629.5±2846.2	2333.0±4233.0	0.21
AST (IU/l)^	120	1524.0±4350.0	2043.0±6041.5	0.45
Alkaline Phosphate (IU/l)^	120	136.0±83.0	143.0±96.0	0.99
Bilirubin (mg/dl)^	120	10.8±14.6	4.9±10.3	<0.001
Creatinine (mg/dl)^	121	1.4±1.8	1.2±1.7	0.27
Hemoglobin (g/dl)^	119	11.2±2.9	10.7±2.7	0.56
INR^	121	3.1±2.6	2.3±2.2	0.007
MELD-Score^	120	36.2±11.1	28.6±8.9	<0.001
Platelet Count^	119	92.0±102.0	159.5±119.8	<0.001
Venous Ammonia (µmol/l)^	76	101.0±80.5	90.0±48.2	0.49
Leucocyte Count (x10 ^{9}/l)^	119	9.6±7.6	8.8±7.3	0.47
Peak Labs				
ALT Peak (IU/l)^	121	1629.5±3725.2	2471.0±4108.5	0.2
AST Peak (IU/l)^	121	1603.5±5036.0	2319.0±6074.5	0.38
Bilirubin Peak (mg/dl)^	121	16.8±15.1	8.5±10.5	<0.001
Creatinine Peak (mg/dl)^	121	2.3±2.0	1.6±2.5	0.15
INR Peak^	121	3.9±3.1	2.8±2.4	<0.001
MELD-Score Peak^	121	38.8±8.2	30.3±8.5	<0.001
Platelet Count Nadir ^	121	63.5±53.2	106.0±85.5	0.002
Venous Ammonia Peak (µmol/l)^	81	128.0±90.0	91.5±70.8	0.07
Etiology				
APAP (% Yes)	121	28(45.2)	38(64.4)	0.05
Admission Vitals				
Diastolic Blood Pressure (mmHg)^	121	67.0±24.8	69.0±22.0	0.70
Systolic Blood Pressure (mmHg)^	121	120.0±24.2	124.0±21.0	0.83

Table 2 - Comparison of demographics and clinical characteristics of the patient cohort by NSS / SS-status

Weight (kg)^	117	76.9±29.0	77.0±25.5	0.82
Body Mass Index^	105	25.9±8.1	27.3±8.9	0.55
Clinical Parameters at Admission				
Hepatic Encephalopathy Grade^	121	3.0±2.0	2.0±2.0	0.002
Coma Grade (% 3/4)	121	38(61.3)	20(33.9)	0.005
Vasopressor Use (% Yes)	121	16(25.8)	3(5.1)	0.004
RRT (% Yes)	121	17(27.4)	7(11.9)	0.06
Ventilation (% Yes)	121	37(59.7)	22(37.3)	0.023
Variable	**N**	**NSS (N=62)**	**SS (N=59)**	**P-Value**
Transfusion (Prior to Specimen Day)				
Pre admission FFP (% Yes)	118	32(54.2)	17(28.8)	0.009
Pre admission to Specimen Day FFP (% Yes)	121	47(75.8)	32(54.2)	0.021
Admission to Specimen Day RBC (% Yes)	121	25(40.3)	7(11.9)	< 0.001
Admission to Specimen Day rVIIa (% Yes)*	121	3(4.8)	0(0.0)	0.24
Admission to Specimen Day Platelets (% Yes)	121	17(27.4)	5(8.5)	0.01
Admission to Specimen Day Vitamin K (% Yes)	121	6(9.7)	8(13.6)	0.70
RRT (Prior to Specimen Day)				
Admission to Specimen Day RRT (% Yes)	121	20(32.3)	7(11.9)	0.013
Clinical Parameters during Hospitalization				
Coma Grade during Hospitalization^	121	4.0±1.0	2.0±3.0	<0.001
Vasopressor Use during Hospitalization (% Yes)	121	25(40.3)	5(8.5)	< 0.001
RRT during Hospitalization (% Yes)	121	22(35.5)	12(20.3)	0.1
Ventilation during Hospitalization (% Yes)	121	48(77.4)	26(44.1)	< 0.001
Transplant				
Ever listed for Transplant (% Yes)	121	39(62.9)	9(15.3)	< 0.001
Iron Indices				
Ferritin (ng/ml)	120	2755±10018	2925±13716	0.58
Iron (µmol/l)	121	34.5±20.6	29.1±22.3	0.016
Transferrin (mg/dl)	121	172.5±80.2	186.0±79.5	0.34
Hepcidin (ng/ml)	113	2.7±5.9	8.2±14.2	<0.001
Transferrin Saturation (%)	121	79.1±21.1	60.9±47.2	0.001

Table 2 - Comparison of demographics and clinical characteristics of the patient cohort by NSS / SS-status

Hepcidin/Ferritin ratio	112	0.0009±0.0029	0.0047±0.0219	<0.001

Data expressed as median (IQR) for continuous variables and n (%) for categorical variables.

^: Presented as Median (IQR) with Wilcoxon Rank Sum p-value.

*: The p-value is calculated by the Fisher's exact test.

Abbreviations: SS = Spontaneous Survival; NSS: Non-Spontaneous Survival; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; INR: International Normalized Ratio; MELD: Model of End Stage Liver Disease; APAP: Acetaminophen; RRT: Renal Replacement Therapy; FFP: Fresh Frozen Plasma; rbc: Red Blood Cells; rVIIa: Recombinant Factor VII.

Table 3 – Univariate and multi variable logistic regression analysis for 21-day spontaneous survival

	Univariate (N=121)		Multivariate (N=121)	
VARIABLE	**ESTIMATE**	**P-VALUE**	**ESTIMATE**	**P-VALUE**
Bilirubin (mg/dl)	0.07015	0.002		
Creatinine^2 (mg/dl)*	0.20672	0.07		
Log (INR)	1.00832	0.009	1.4888	0.012
MELD	0.13839	<0.001		
Platelet Count	-0.00797	0.002	-0.0111	<0.001
Height (cm)	0.03276	0.09		
Iron (µmol/l)	0.02723	0.013		
Transferrin Saturation (%)	0.01979	0.004		
Log (Hepcidin+1) (ng/ml)	-0.83198	<0.001	-0.7257	0.01
Square root (Hepcidin/Ferritin)	-14.8873	<0.001		
APAP (Yes)	-0.39361	0.035	-2.2672	0.002
Sex (Female)	-0.55848	0.013		
Vasopressors (Yes)	0.93522	0.005		
RRT (Yes)	0.51594	0.037		
Ventilation (Yes)	0.45595	0.015		
Coma Grade (3/4)	0.56364	0.003	2.4221	<0.001
Blood product (Yes)	0.49684	0.013		
Prior To Specimen Day RRT (Yes)	0.63170	0.009		

Probability of death or transplant within 21 days is modeled.

*: If on Renal Replacement Therapy at admission then Creatinine set to 4.0.

Abbreviations: INR: International Normalized Ratio; MELD: Model of End Stage Liver Disease; APAP: Acetaminophen RRT: Renal Replacement Therapy

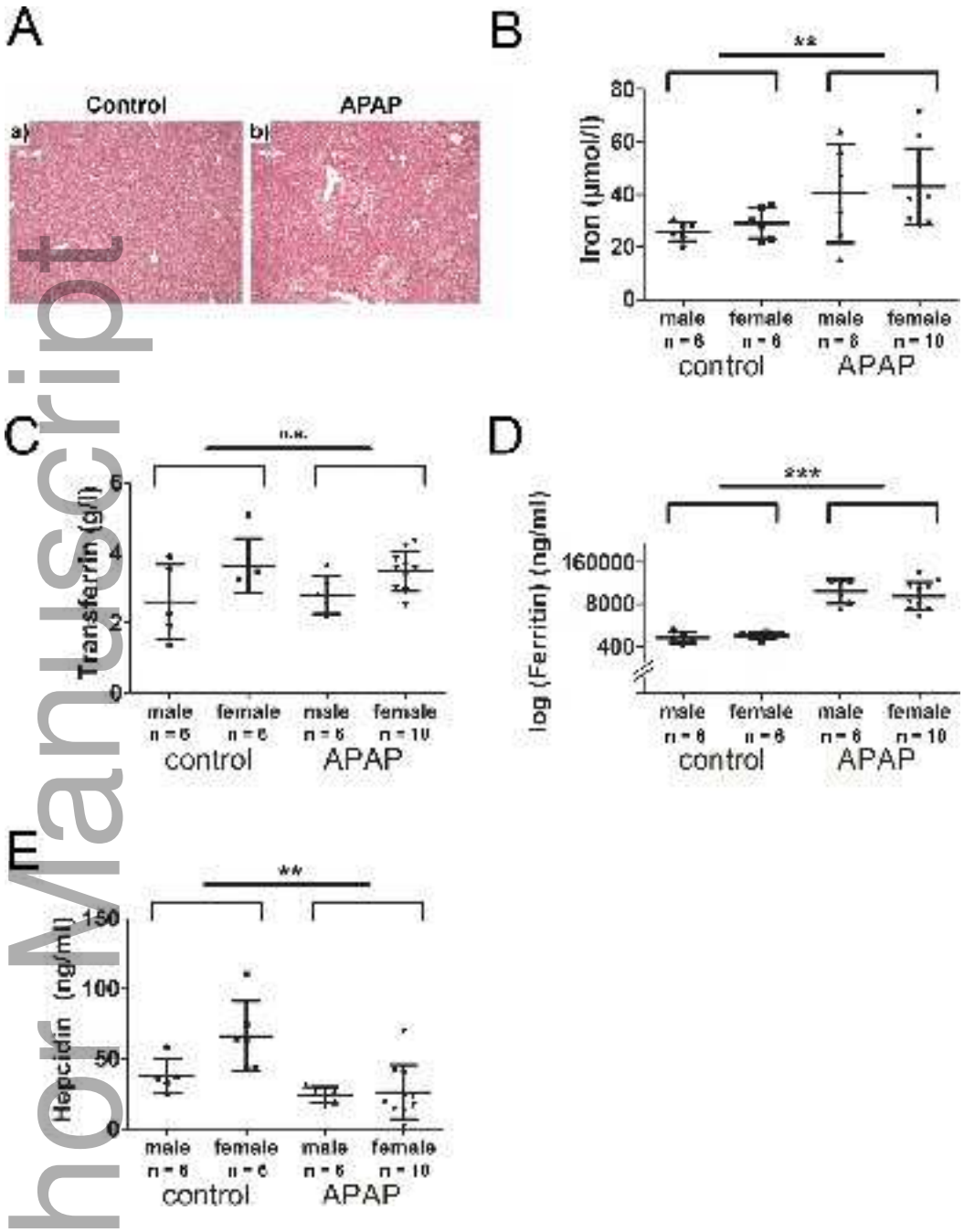
Table 4 - Iron, MELD and ALFSG prognosis model results

	MELD Model		ALFSG Model		Iron Model	
	OR (95% CI)	**P-Value**	**OR (95% CI)**	**P-Value**	**OR (95% CI)**	**P-Value**
MELD	1.15 (1.1 -1.22)	<0.001	---	---	---	---
Favorable Etiology (Yes)*	---	---	0.4 (0.08 - 1.91)	0.25	---	---
APAP Etiology (Yes)	---	---	---	---	0.10 (0.02 - 0.45)	0.002
Log (INR)	---	---	6.96 (2.24 - 21.59)	<0.001	4.43 (1.38 - 14.23)	0.012
Log (Bilirubin)	---	---	3.69 (1.6 - 8.75)	0.003	---	---
Platelet Count	---	---	---	---	0.99 (0.98 - 1)	<0.001
Log (Hepcidin+1)	---	---	---	---	0.48 (0.28 - 0.84)	0.01
Coma Grade (3/4)	---	---	7.61 (2.13 - 27.21)	0.002	11.27 (2.91 - 42.8)	<0.001
Vasopressors (Yes)	---	---	3.02 (0.64 - 14.3)	0.16	---	---
Sensitivity (%)	65.6		73.8		81.0	
Specificity (%)	69.5		69.5		79.6	
c (95% CI)	0.76 (0.67 - 0.85)		0.85 (0.78 - 0.92)		0.87 (0.81 - 0.94)	

The Sensitivity and Specificity is based on a cut-point of 0.50.

*Favorable Etiologies – Acetaminophen-induced ALF, Hepatitis A, Pregnancy related ALF, Ischemic Liver Injury

Abbreviations: ALFSG: Acute Liver Failure Study Group; APAP: Acetaminophen; c: C-statistic; CI: Confidence Interval; INR: International Normalized Ratio; MELD: Model of End Stage Liver Disease; OR: Odds Ratio



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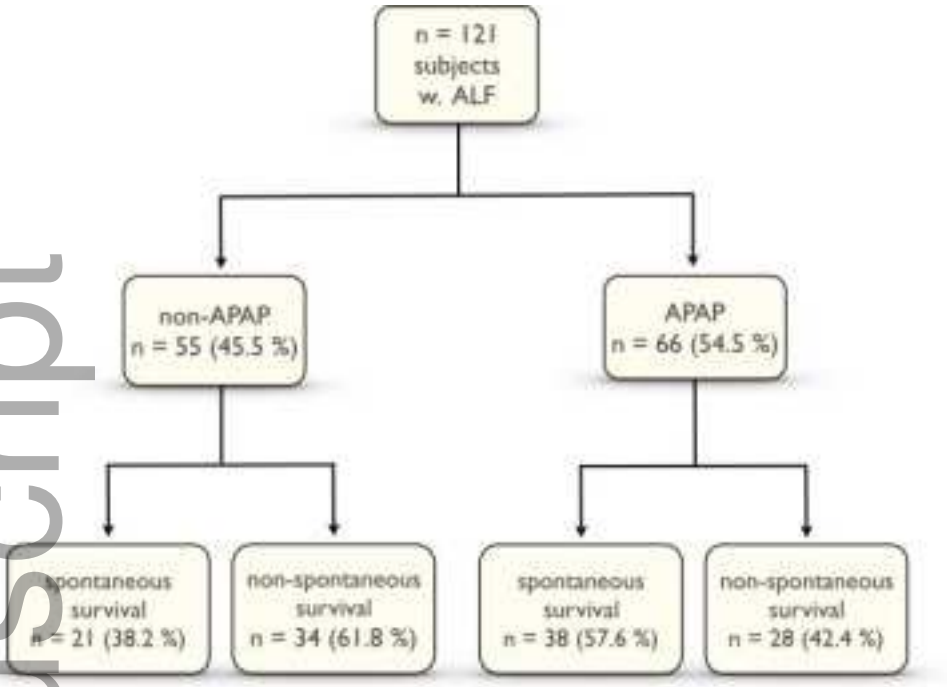

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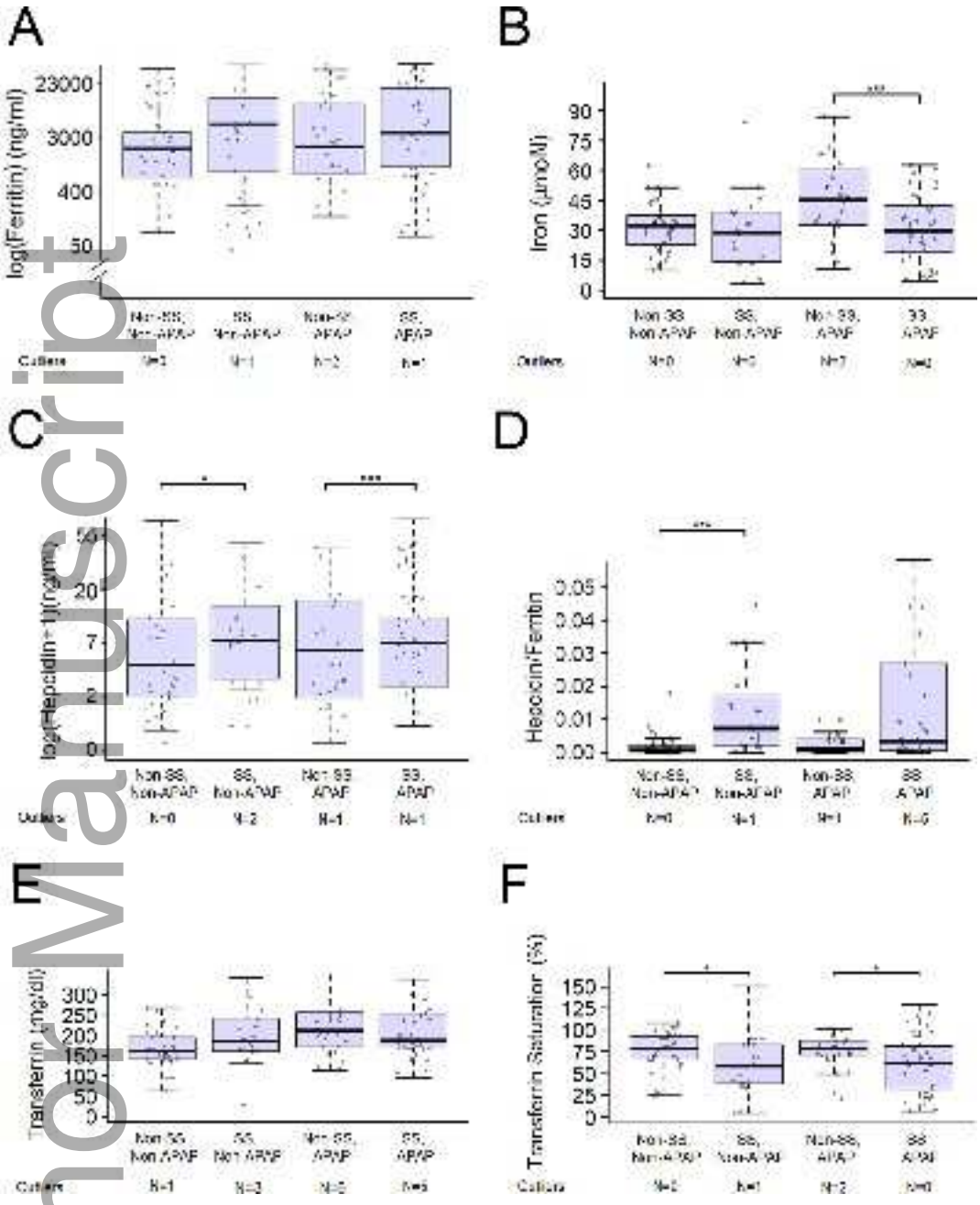

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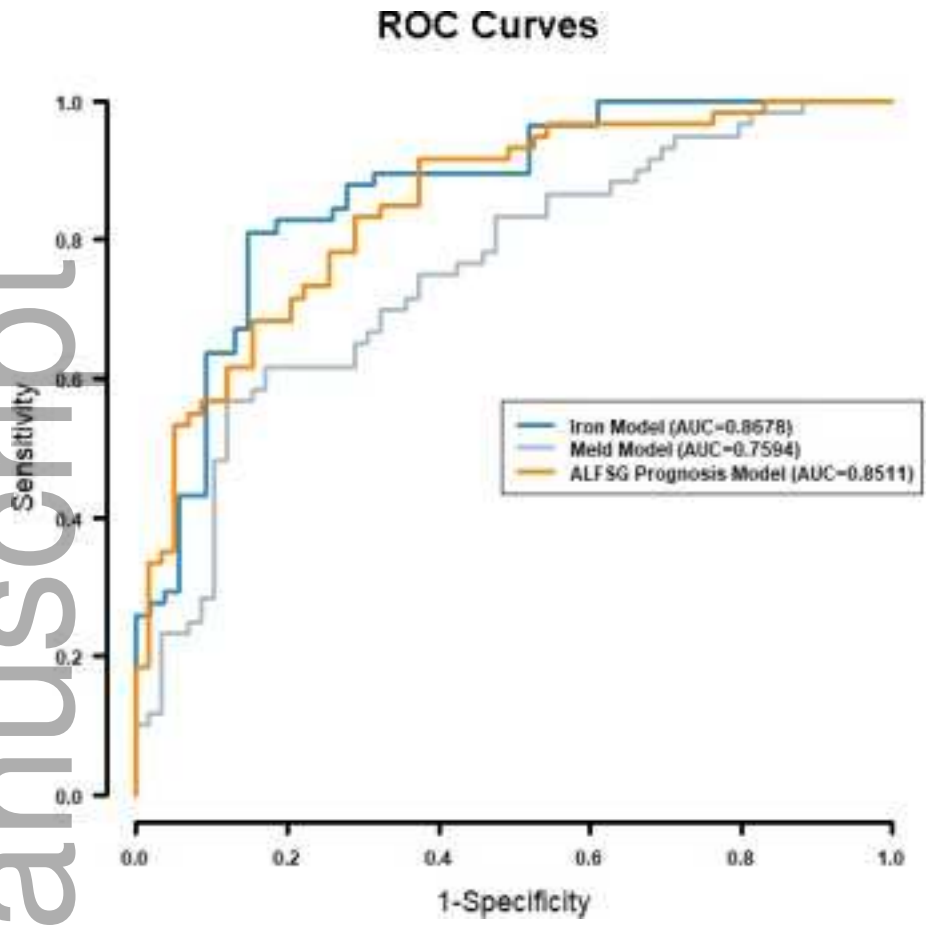

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