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Mesenchymal iron deposition is associated with adverse long-term outcome in non-alcoholic fatty liver disease

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Abstract

Background & Aims: Approximately one-third of patients with non-alcoholic fatty liver disease (NAFLD) show signs of mild-to-moderate iron overload. The impact of histological iron deposition on the clinical course of patients with NAFLD has not been established.

Methods & Results: For this retrospective study, 299 consecutive patients with biopsy-proven NAFLD and a mean follow-up of 8.4 (±4.1; range: 0.3-18.0) years were allocated to one of four groups according to presence of hepatic iron in the reticuloendothelial system (RES) and/or hepatocytes (HC): 156 subjects (52%) showed no stainable iron (NONE), 58 (19%) exclusively reticuloendothelial (xRES), 19 (6%) exclusively hepatocellular (xHC) and 66 (22%) showed a mixed (HC/RES) pattern of iron deposition. A long-term analysis for overall survival, hepatic, cardiovascular or extrahepatic-malignant events was conducted. Based on multivariate Cox proportional hazards models any reticuloendothelial iron was associated with fatal and non-fatal hepatic events. Specifically, xRES showed a cause-specific hazard ratio (csHR) of 2.4 (95%-CI, 1.0-5.8; P = .048) for hepatic as well as cardiovascular fatal and non-fatal events combined (csHR 3.2; 95%-CI, 1.2-8.2; P = .015). Furthermore, the mixed HC/RES iron pattern showed a higher rate of combined hepatic fatal and non-fatal events (csHR 3.6; 95%-CI, 1.4-9.5; P = .010), while xHC iron deposition was not associated with any defined events.

Conclusions: The presence of reticuloendothelial-accentuated hepatic iron distribution patterns is associated with detrimental long-term outcomes reflected in a higher rate of both liver-related and cardiovascular fatal and non-fatal events.

Abbreviations: AUC, area under the curve; BMI, body mass index; CI, confidence interval; csHR, cause-specific hazard ratio; FIB-4, Fibrosis-4 score; FU, follow-up; HC, hepatocytes; HC/RES, iron in hepatocytes and reticuloendothelial system; HCC, hepatocellular carcinoma; HR, hazard ratio; LSM, liver stiffness measurement; LTX, liver transplantation; MetS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NONE, no stainable iron in initial biopsy; RES, reticuloendothelial system; ROC, receiver-operating characteristic; SAF, Steatosis, Activity and Fibrosis score; SD, standard deviation; SF, serum ferritin; US, ultrasound; xHC, iron exclusively in hepatocytes; xRES, iron exclusively in reticuloendothelial system.

KEYWORDS

biopsy, cardiovascular, end-stage liver disease, iron overload, NASH, non-alcoholic fatty liver disease

1 | INTRODUCTION

With a prevalence of approximately 30%, non-alcoholic fatty liver disease (NAFLD) has become the most common liver disorder and developed into a major public health concern in Western societies.[1,2] Although NAFLD is regarded as a liver disease, morbidity and mortality are essentially determined by cardiovascular events and an increased incidence of various cancers.[3,4] Approximately one-third of patients with NAFLD show signs of mostly mild-to-moderate iron overload.[5] While iron plays an essential role in multiple physiologic processes, such as erythropoiesis or the synthesis of deoxyribonucleic acid, iron overload is detrimental via the formation of reactive oxygen species leading to cellular dysfunction and organ damage.[6,7] Excess iron is mainly stored in the liver and elevated serum ferritin (SF) concentrations have been associated with the stage of liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC) and a higher mortality rate in hereditary hemochromatosis and the general population.[8-10] While SF is well established as an indicator of body iron stores, its relationship with iron homeostasis in NAFLD and its role in disease progression has remained controversial, mostly as a result of the concurrent impact of adipose tissue and hepatic inflammation on iron homeostasis.[11,12] Although SF concentrations reflect higher liver iron stores in NAFLD, the pattern of iron distribution in the liver may vary. Iron deposition in the liver can be histologically distinguished as parenchymal, ie hepatocellular (HC) iron deposition; mesenchymal iron overload, ie iron in Kupffer cells and/or portal macrophages of the reticuloendothelial system (RES); or show a mixed pattern, defined by both parenchymal and mesenchymal deposition.[8,13] While parenchymal iron overload is a hallmark in early stages of iron overload and associated with oxidative stress, the presence of mesenchymal iron was further associated with fibrosis and increased apoptosis.[13-15] It is further noteworthy that different iron deposition patterns likely reflect differences in mechanisms of iron retention in cells as RES iron is typical for inflammatory changes while HC iron is typically observed in genetic or transfusion-related iron overload.[16] Among stainable hepatic iron, the proportions of the various patterns range between 22% and 36% for parenchymal, 21% and 31% for mesenchymal and 43% and 50% for mixed iron distribution.[14,15,17] With regard to the clinical relevance of iron in NAFLD, most studies have examined the link of intrahepatic iron deposition and/or SF concentrations in NAFLD or non-alcoholic steatohepatitis (NASH) with histological characteristics and have obtained conflicting findings.[8,18] Similarly, phlebotomies have been linked to improved clinical findings for various endpoints such as T2DM, insulin resistance or hypertension, while no clear benefit on NAFLD histological severity has been reported.[19-21]

Importantly, data on the relevance of liver iron findings regarding the natural history of NAFLD subjects have not been reported.

Lay summary

While iron plays an essential role in physiologic processes, iron overload is detrimental via the formation of reactive oxygen. In non-alcoholic fatty liver, excess iron is mainly stored in the liver and can be histologically distinguished as parenchymal, mesenchymal or a combination of both. Analysing the long-term outcome of subjects with non-alcoholic fatty liver and different patterns of iron deposition, this study indicates that the mesenchymal pattern of iron deposition is particularly linked to adverse outcome with increased overall mortality and risk of liver-related or cardiovascular events.

Furthermore, it remains elusive to date whether iron deposition patterns may differentially impact on the clinical course of the disease. Therefore, the aim of this study was to investigate the clinical course of NAFLD patients stratified according to the histological distribution of hepatic iron.

2 | MATERIALS AND METHODS

2.1 | Study cohort

The study cohort was analysed retrospectively and consisted of all consecutive subjects who underwent liver biopsy at the First Department of Medicine, Paracelsus Medical University Salzburg, Austria and the Department of Internal Medicine, Oberndorf Hospital, Austria between 1997 and 2017. A total of 466 subjects had received the final diagnosis of NAFLD (as reported in Denkmayr et al[22]), of whom the clinical course could be determined in 343 subjects, while 123 subjects were either lost to follow-up (FU) or had missing clinical data on the natural course after liver biopsy. Besides having a larger proportion of subjects without any stainable iron (72% compared to 52%, adjusted *P*-value = .022), the 123 patients with unknown clinical course did not differ from the finally analysed subjects in baseline characteristics (details in Table S4). Further 44 subjects diagnosed with a defined endpoint prior to the biopsy were excluded from the analysis. Finally, 299 subjects (215 male, 84 female) aged between 17 and 80 years were analysed (Figure 1). Subjects were referred to our liver outpatient clinic and underwent diagnostic liver biopsy because of (a) persistently elevated liver enzymes and/or (b) clinical suspicion of advanced liver disease and/or (c) evidence of unexplained fatty liver disease and/or unexplained

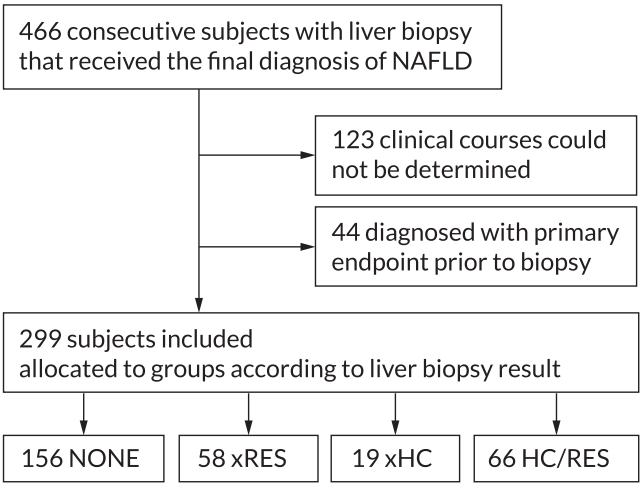

FIGURE 1 Flow diagram of study subject selection. Consecutive subjects who underwent liver biopsy between 1997 and 2017; NONE: subjects with no stainable iron residues, xRES exclusive reticuloendothelial system iron deposition, xHC exclusive hepatocellular hepatic iron deposition and HC/RES mixed pattern of iron deposition

hyperferritinaemia after the biochemical exclusion of infectious, autoimmune and hereditary causes of liver disease.

In case of unequivocal biochemical or clinical signs of cirrhosis, no biopsy was performed. These were (a) laboratory evidence of advanced liver disease with hyperbilirubinaemia, and/or thrombocytopenia, and/or prolonged prothrombin time together with imaging evidence of cirrhosis. No FibroScan® (Echosens, Paris, France) was available at baseline investigations in most subjects (until 2016); (b) history of past or recent hepatic decompensation (ascites, variceal bleeding and hepatic encephalopathy). Cirrhosis was defined clinically as (a) liver stiffness measurement (LSM) >20.0 kPa and/or (b) ultrasound examination showing irregular liver surface in conjunction with splenomegaly or oesophageal varices. The LSM cut-off was chosen for practicability and to avoid false-positive diagnoses of cirrhosis with high accuracy in the clinical evaluation.[23,24] Liver biopsy is generally not performed in our centres in these clinical constellations and such subjects are not included in our cohort.

2.2 | Clinical and laboratory assessment

At the time of the biopsy all subjects underwent a standard clinical examination and the medical history was obtained. Venous blood was drawn within a month of the liver biopsy for laboratory diagnostics including serum liver tests, viral hepatitis B and C markers, auto-antibodies, serum iron parameters, copper, ceruloplasmin, inflammation markers, lipid panel and fasting glucose. None showed signs of cardiac or renal insufficiency, infectious diseases or systemic autoimmune disorders. The aetiology of liver disease was judged to be non-alcoholic if other aetiologies were excluded and the alcohol consumption was reliably stated below clinically relevant amounts (<30 g/d in males, <20 g/d in females). The presence of metabolic syndrome (MetS) was determined by ATP III criteria.[25] For non-invasive fibrosis assessment, the Fibrosis-4 (FIB-4) score[26] was calculated from baseline data. To screen for associated hereditary hemochromatosis, genetic testing for C282Y and H63D mutations of the HFE gene was performed in all patients as described previously.[27] Subjects with homozygosity for the C282Y or compound heterozygosity (C282Y/H63D) had been excluded.

At the FU visit, a detailed clinical examination and medical history was obtained for determination of clinical endpoints as specified below. Particularly, besides laboratory measures, abdominal ultrasound (US) and liver stiffness measurement (FibroScan®) were performed for assessment of the stage of liver disease. Subjects were referred to computed tomography or MRI if HCC could not be reliably ruled out on US, and to gastroscopy for assessment of gastroesophageal varices if evidence of advanced liver disease was found on clinical, laboratory or imaging results at FU visit. Medical history was further specifically obtained with regard to present and meantime alcohol drinking and smoking habits.

Further hepatic evaluation for exploratory analysis was performed to detect development of advanced liver disease if the definition of fatal or non-fatal endpoint was not met and this included any diagnosis of compensated cirrhosis, defined as unequivocal imaging results on ultrasound, computed tomography or liver stiffness measurement by FibroScan® together with hyperbilirubinaemia and/or thrombocytopenia and/or prolonged prothrombin time, and the occurrence of oesophageal varices but without bleeding episodes on gastroscopy.

2.3 | Liver biopsy and histological examination

Liver biopsy specimens with at least 15-18 mm in length corresponding to a sufficient number of portal fields (>10) were used for histological analysis. All samples evaluated in this study were re-analysed in 2017 for current criteria for diagnosing, grading and staging of NAFLD as described below. Deparaffinized sections (4 µm) of each case were processed according to routine protocols and stained with haematoxylin, eosin and Masson's trichrome stain. All slides were evaluated and scored by two pathologists unaware of the clinical data first individually and in case of discordant results, slides were again analysed conjointly using a multiheaded microscope and a scoring decision was reached in consensus. Histological grading and staging of NAFLD components were performed as published by Kleiner et al[28] by application of numerical scores. Accordingly, scores were reported for steatosis (0-3), lobular inflammation (0-3), hepatocellular ballooning (0-2), Mallory-Denk bodies (0-2) and portal inflammation (0-1). Fibrosis stage was assessed on a 5-step scale including stages 0 (none), 1 (centrilobular or pericellular), 2 (centrilobular plus periportal), 3 (bridging) and 4 (cirrhosis). Ballooned hepatocytes were characterized by a marked increase in size (approximately two times the size of a normal hepatocyte), rounded cell shape and pale staining of the cytoplasm. The diagnostic algorithm for histological classification as NAFLD or NASH was followed as reported by

Bedossa et al[29] in the Steatosis, Activity and Fibrosis (SAF) score. Siderosis was determined semi-quantitatively upon histopathologic examination of Perl's-stained liver biopsy specimens: score 0, granules absent or barely discernible at a magnification of 400-fold (400×); 1, barely discernible at a magnification of 200 × but easily confirmed at 400×; 2, discrete granules resolved at 100 × magnification; 3, discrete granules resolved at a magnification of 25 × and 4, massive granules visible even upon 10 × magnification.[30]

2.4 | Group definitions

According to the histological presence and pattern of iron deposition in the RES and in hepatocytes, subjects were allocated to one of four groups: (a) NONE (n = 156) showed no stainable iron residues, (b) xRES (n = 58) had iron exclusively in the mesenchymal reticuloendothelial system, (C) in xHC (n = 19) exclusively iron in hepatocytes was found and (d) HC/RES (n = 66) showed iron depositions in both compartments (Figure 2). The study was approved by the local ethics committee (Ethikkommission des Landes Salzburg) and all patients gave written consent to be included in the study.

2.5 | Definition of endpoints

In order to evaluate the natural course of NAFLD subjects, endpoints for hepatic events, cardiovascular events and extrahepatic malignancies were defined as fatal and non-fatal for each category. Total events were defined as fatal and non-fatal events combined.

Hepatic endpoints thus included liver-related death, liver transplantation (LTX) and the combination thereof was defined as transplant-free survival. Non-fatal hepatic events were hepatic decompensation (defined as ascites, encephalopathy or variceal bleeding) and development of HCC. For exploratory analysis the above defined diagnosis of compensated cirrhosis at the time of FU was used.

Cardiovascular endpoints were defined as death from cardiovascular cause (myocardial infraction, stroke and peripheral artery disease), and the history of cerebrovascular stroke, myocardial infarction and acute heart failure and symptomatic peripheral artery disease between the time of liver biopsy and FU was counted as non-fatal cardiovascular events.

Any diagnosis of an extrahepatic malignancy was counted as fatal or non-fatal malignancy-related event.

2.6 | Statistical analysis

Subject characteristics are expressed as mean values and standard deviations (SD). Since all quantitative data were not normally distributed group, differences were analysed using Kruskal-Wallis test and Dunn's test for the according post hoc analysis. Categorical variables are reported as frequencies and compared with chi-squared test. P-values of group comparisons were adjusted for multiple testing using the Benjamini-Hochberg principle.[31] The overall and group-specific survival was conducted using Kaplan-Meier estimators and log-rank tests. The accuracy of SF to predict hepatic iron in biopsies is calculated as area under the curve (AUC) and 95%-CI of the respective receiver-operating characteristics (ROC) curve via 'pROC' package for R statistics.[32] The cumulative incidence rates of total endpoints were compared as competing risk events using Gray's test. To estimate group effects as well as RES and HC on mortality and total endpoints, univariate Cox proportional hazards models were calculated (Table S3). A multivariate model including RES and a second model with separate groups were calculated, respectively, adjusted for NASH and fibrosis. Variables with a p-value below 0.1 in the univariate analysis were added to a multivariate model using stepwise forward selection estimated by Akaike information criterion. Since only one event and no death occurred in xHC, it was combined with NONE for the models. Hazard ratios (HR) for overall survival and cause-specific HR (csHR) for total endpoints with 95% confidence intervals (95%-CI) were reported. Annualized incidence rates were calculated for all events and normalized to 100 subjects per year. A two-sided p-value below 0.05 was considered to indicate statistical significance. All statistical analyses were performed using R.[33]

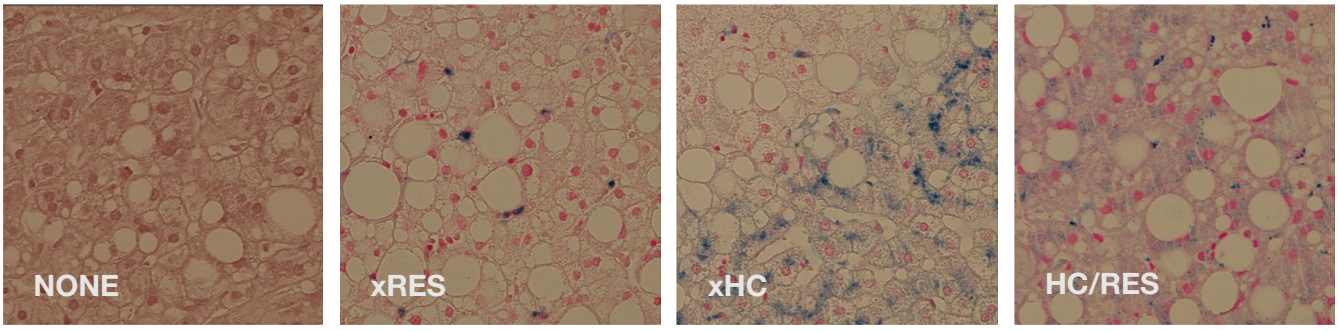

FIGURE 2 Histopathologic classification of baseline biopsy. Perl's-stained liver biopsy specimens. Subjects were allocated to one of four groups according to absence and presence of iron: NONE: no stainable iron residues, xRES: iron exclusively in the reticuloendothelial system, xHC: exclusively hepatocellular iron was found and HC/RES: iron depositions in both locations

3 | RESULTS

3.1 | Baseline characteristics

A total of 299 subjects (28% female) with a mean age of 49.5 years (SD ± 13.4; range: 17-80) and a mean body mass index (BMI) of 28.7 kg/m^2 (±3.8; range: 17.7-41.7) were included in this study. Among these, 156 subjects (52%) showed no hepatic iron (NONE), 58 (19%) exclusively reticuloendothelial (xRES), 19 (6%) exclusively in hepatocytes (xHC) and 66 (22%) reticuloendothelial as well as in hepatocytes (HC/RES) and were grouped accordingly. In group comparison, subjects with HC/RES showed a greater proportion of males compared to NONE and the mean age was significantly higher. At baseline, no differences were found for BMI and the presence of MetS between all groups. The results are summarized in Table 1, all details including post hoc analyses can be found in Table S1.

Serum iron, SF and transferrin saturation were significantly higher in all iron overload groups compared to NONE. Transferrin was significantly lower in iron overload groups except for xHC. While HC/RES had the highest average SF concentration, it did not reach significance compared to xRES or xHC. We aimed to identify the SF concentration which would predict histological iron deposition in our cohort. Therefore, ROC analysis was performed for male and female subjects separately. In male subjects, SF at an optimal threshold of 324 µg/L indicated stainable iron with an AUC 85.4% (95%-CI: 80.2%-90.7%), with a sensitivity of 85.3% and a specificity of 75.0%. For female subjects, a SF concentration of 387 µg/L represented the optimal threshold and indicated hepatic iron deposition with an AUC of 90.0% (95%-CI: 83.0%-97.0%) and a sensitivity of 85.2% and a specificity of 88.7%. Non-invasive estimation of the severity of liver disease using FIB4 score indicated the highest degrees of fibrosis in subjects with HC/RES with a significant difference to NONE.

3.2 | Histological characteristics

Differences of steatosis, ballooning or inflammation between groups reached no significance. Presence of any fibrosis was significantly higher in subjects with HC/RES compared to NONE; mostly attributable to a higher number of subjects with fibrosis stages 1, 2 and 3, but not the number of subjects with cirrhosis of stage 4 which was highest in xRES. A significantly higher portion of NASH subjects was observed in xRES with 22% (P = .041). The results are presented in Table 2 (for detailed post hoc results see Table S2).

3.3 | Mortality and transplantation-free survival

The overall mean follow-up period was 8.4 years (SD ± 4.2; range: 0.3-18.0 years) and during this period 36 deaths occurred. The major cause of death was liver related (n = 15; 42%), followed by extrahepatic malignancy (n = 7; 19%), cardiovascular (n = 7; 19%) and others (n = 7; 19%), specifically 2 accidents, 2 infectious causes, and 3 caused by chronic lower respiratory diseases (see Table 3). After a follow-up period of 10 years (120 months), the overall transplantation-free survival for all subjects was estimated at 88% (95%-CI,

TABLE 1 Baseline characteristics of the study population

Component	NONE	xRES	xHC	HC/RES	Adjusted *P*-value
Male, n (%)	101 (65%)	41 (71%)	15 (79%)	58 (88%)	.017
Age, years	47.0 ± 14.4	51.3 ± 11.2	46.4 ± 12.4	54.8 ± 11.2	.003
BMI, kg/m^2	28.7 ± 4.0	28.4 ± 4.1	30.1 ± 3.8	28.4 ± 3.1	ns
Systolic BP, mmHg	126.0 ± 18.0	125.8 ± 17.4	147.8 ± 19.2	130.8 ± 20.4	.041
Diastolic BP, mmHg	79.3 ± 11.6	79.4 ± 10.6	85.6 ± 12.4	78.7 ± 12.0	ns
Metabolic syndrome, n (%)	56 (36%)	28 (48%)	7 (37%)	28 (43%)	ns
Ferritin, µg/L	266.4 ± 232.4	768.2 ± 594.5	710.9 ± 378.6	891.5 ± 479.9	<.001
Serum iron, µg/dL	110.2 ± 39.2	141.1 ± 43.8	134.8 ± 35.5	129.5 ± 44.0	<.001
Transferrin, mg/dL	277.0 ± 36.6	254.5 ± 55.1	258.7 ± 50.4	242.1 ± 44.8	<.001
Transferrin saturation, %	30.3 ± 22.1	40.0 ± 15.2	38.2 ± 9.8	39.6 ± 14.5	<.001
AST, U/L	47.6 ± 28.5	50.9 ± 40.9	33.9 ± 23.5	44.7 ± 27.7	ns
ALT, U/L	76.7 ± 50.7	79.7 ± 60.1	57.1 ± 59.6	57.8 ± 38.1	.011
Total bilirubin, mg/dL	0.8 ± 0.4	1.1 ± 1.0	0.8 ± 0.6	1.0 ± 0.5	.001
Platelets, G/L	221.4 ± 62.5	209.5 ± 59.4	200.6 ± 51.7	196.5 ± 56.3	.025
Prothrombin time, %	99.6 ± 12.5	96.7 ± 11.6	96.0 ± 8.6	91.6 ± 13.7	.004
FIB4-Score	1.46 ± 1.59	1.56 ± 0.86	1.18 ± 0.64	2.07 ± 1.86	.011

Note: Data are means ± standard deviations or frequencies (%); *P*-values assessed by Kruskal-Wallis test for continuous data and by chi-squared test for categorical data; *P*-values are adjusted for multiple testing using the Benjamini-Hochberg principle.

Abbreviations: ALT/AST, alanine/aspartate transaminase; BMI, body mass index; BP, blood pressure; FIB4, Fibrosis 4; ns, not significant.

TABLE 2 Histological characteristics of the study population

Component	NONE (n = 156)	xRES (n = 58)	xHC (n = 19)	HC/RES (n = 66)	*P*-value
Steatosis, %	30.96 ± 20.60	35.86 ± 19.22	28.95 ± 19.83	29.39 ± 18.64	ns
Grade 1 (5%-33%)	93 (60%)	28 (48%)	13 (68%)	40 (61%)	ns
Grade 2 (33%-66%)	52 (33%)	26 (45%)	5 (26%)	23 (35%)	ns
Grade 3 (>66%)	11 (7%)	4 (7%)	1 (5%)	3 (5%)	ns
Inflammation[a]	23 (15%)	13 (22%)	4 (21%)	17 (26%)	ns
Ballooning[a]	22 (14%)	15 (26%)	3 (16%)	13 (20%)	ns
Fibrosis Stage	0.40 ± 0.81	0.69 ± 1.11	0.47 ± 0.70	0.73 ± 1.00	.035
Stage 0	114 (73%)	35 (60%)	12 (63%)	36 (55%)	.042
Stage 1	29 (19%)	15 (26%)	5 (26%)	19 (29%)	ns
Stage 2	8 (5%)	2 (3%)	2 (11%)	5 (8%)	ns
Stage 3	2 (1%)	3 (5%)	0 (0%)	5 (8%)	ns
Stage 4	3 (2%)	3 (5%)	0 (0%)	1 (2%)	ns
NASH[b]	14 (9%)	13 (22%)	2 (11%)	10 (15%)	.041
Siderosis Stage[c]	0.03 ± 0.16	1.10 ± 0.44	1.21 ± 0.56	1.33 ± 0.49	<.001

Note: Data are means ± standard deviations or frequencies (%); *P*-values assessed by Kruskal-Wallis test for continuous data and by chi-squared test for categorical data.

NASH, non-alcoholic steatohepatitis; ns, not significant; SAF, Steatosis, Activity and Fibrosis.

[a]Histological score above 0 described in Kleiner et al.[28]

[b]The Incidence of NASH was determined as described by Bedossa et al.[29]

[c]Siderosis was determined semi-quantitatively upon histopathologic examination of Perl's-stained liver biopsy specimens.[30]

83%-94%). The survival of subjects without stainable iron (NONE) of 91% (95%-CI, 85%-98%) was not significantly different from subjects with any stainable iron of 85% (95%-CI, 78%-92%). Among subgroups, subjects with xRES showed an estimated survival of 82% (95%-CI, 71%-94%) and subjects with HC/RES 82% (95%-CI, 72%-94%) at 10 years, which corresponds to an annualized mortality rate of 2.3 and 2.2 per 100 person-years, respectively, compared to 0.9 in NONE (see Table 3). Among xHC subjects no death was observed. A higher mortality for subjects with any RES (ie xRES and HC/RES combined) compared to subjects without RES (ie NONE and xHC combined) was found based on a Kaplan-Meier estimator (log-rank $P = .006$, see Figure S1). In multivariate Cox regression models, the presence of RES was associated with a HR of 1.4 (95%-CI, 0.6-3.0) for overall mortality and further a HR of 1.7 (95%-CI, 0.7-4.3) and 1.2 (95%-CI, 0.5-2.9) for xRES and HC/RES, respectively, (see Table 4) without reaching significance. Both models were adjusted for age and fibrosis on overall mortality.

3.4 | Total events

The total number of events combining fatal and non-fatal events was estimated for liver, cardiovascular and malignant diseases. After a follow-up period of 10 years, the cumulative incidence for all events was 16% for NONE and 34% for any stainable iron; 34% for xRES and 30% for HC/RES respectively (see Figure 3). For xHC no event occurred during the 10-year follow-up period.

3.5 | Total hepatic events

The cumulative incidence for hepatic events after 10 years FU was 4% (95%-CI, 0%-7%) for NONE and 12% (95%-CI, 6%-18%) for any stainable iron, showing statistical significance (Fine and Gray: $P = .036$). The subgroup analysis showed a cumulative incidence of 12% (95%-CI, 3%-21%) for xRES and 16% (95%-CI, 5%-26%) for HC/RES, which was significantly different (Fine and Gray: $P = .019$). Comparing all subjects with RES (14%) to subjects without RES (3%, Fine and Gray: $P = .003$) likewise showed significantly higher rates. Based on multivariate Cox proportional hazards models, any RES was associated with a csHR of 2.8 (95%-CI, 1.2-6.6; $P = .015$) for hepatic events (Table 4). Specifically, xRES with a csHR of 2.4 (95%-CI, 1.0-5.8; $P = .048$) and 3.6 (95%-CI, 1.4-9.5; $P = .010$) for HC/RES. The adjustment for fibrosis stages showed a high csHR for the occurrence of hepatic events for advanced fibrosis. A higher BMI was associated with fewer hepatic events while the adjustment for NASH did not reach significance (see Table 4).

We also performed a separate multivariate regression analysis for advanced fibrosis (F3 and F4 combined) as a single variable. Further adjusted for NASH and the forward-selected BMI the influence of any RES could be confirmed with a csHR of 2.3 (95%-CI, 1.0-5.2; $P = .042$). In the second model, xRES and HC/RES showed a csHR of 2.1 (95%-CI, 0.8-5.3) and 2.5 (95%-CI, 0.9-6.6), respectively, with HC/RES trending without reaching significance. Both models are led by advanced fibrosis and its dominant association with hepatic events (find detailed results in Table S5).

TABLE 3 Annualized incidence rates of fatal and non-fatal events

	NONE (n = 156)		xRES (n = 58)		xHC (n = 19)		HC/RES (n = 66)	
	n	Rate (95%-CI)	n	Rate (95%-CI)	n	Rate (95%-CI)	n	Rate (95%-CI)
Hepatic	15	1.3 (0.8-2.1)	10	2.1 (1.1-3.8)	0	—	18	2.9 (1.8-4.5)
Liver transplantation	1	0.1 (0.0-0.6)	1	0.2 (0.0-1.5)	0	—	1	0.2 (0.0-1.1)
Hepatic decompensation	6	0.5 (0.2-1.1)	7	1.5 (0.7-3.0)	0	—	8	1.3 (0.6-2.5)
Hepatocellular carcinoma	2	0.2 (0.0-0.7)	1	0.2 (0.0-1.5)	0	—	3	0.5 (0.2-1.5)
Variceal bleedings	2	0.2 (0.0-0.7)	1	0.2 (0.0-1.5)	0	—	1	0.2 (0.0-1.1)
Cardiovascular	7	0.6 (0.3-1.2)	9	1.9 (1.0-3.6)	1	0.5 (0.1-3.2)	8	1.3 (0.6-2.5)
Cerebral stroke/ TIA	2	0.2 (0.0-0.7)	5	1.0 (0.4-2.5)	0	—	3	0.5 (0.2-1.5)
Myocardial infarction	2	0.2 (0.0-0.7)	5	1.0 (0.4-2.5)	0	—	0	—
Acute heart failure	5	0.4 (0.2-1.0)	1	0.2 (0.0-1.5)	1	0.5 (0.1-3.2)	6	1.0 (0.4-2.1)
Extrahepatic Malignancy	10	0.8 (0.5-1.6)	8	1.7 (0.8-3.3)	1	0.5 (0.1-3.2)	11	1.7 (1.0-3.1)
Death	11	0.9 (0.5-1.7)	11	2.3 (1.3-4.1)	0	—	14	2.2 (1.3-3.7)
Hepatic death	6	0.5 (0.2-1.1)	5	1.0 (0.4-2.5)	0	—	4	0.6 (0.2-1.7)
Cardiovascular death	2	0.2 (0.0-0.7)	4	0.8 (0.3-2.2)	0	—	1	0.2 (0.0-1.1)
Extrahepatic malignancy	1	0.1 (0.0-0.6)	0	—	0	—	6	1.0 (0.4-2.1)
Other death	2	0.2 (0.0-0.7)	2	0.4 (0.1-1.7)	0	—	3	0.5 (0.2-1.5)

Note: The absolute number of incidences over the complete observed time is shown for iron deposition groups, respectively. The incidence rates are normalized to 100 subjects per year, with 95% confidence intervals.

Abbreviation: TIA, transient ischaemic attack.

In order to provide an additional assessment of hepatic morbidity and mortality, we calculated liver-related death and transplantation as a composite endpoint. This analysis of 16 events revealed a higher cumulative incidence in RES (10%; 95%-CI, 4%-17%) compared to non-RES subjects (5%; 95%-CI, 0%-9%). The subgroup analysis showed 6% (95%-CI, 0%-11%) in NONE, 12% (95%-CI, 2%-22%) in xRES and 9% (95%-CI, 0%-17%) in HC/RES. Although both comparisons did not reach significance, the analysis substantiated the association of RES iron with liver-related endpoint.

As an exploratory analysis we aimed to corroborate our findings by additionally including development of non-decompensated cirrhosis as hepatic events, this analysis confirmed higher cumulative incidence of hepatic endpoints in those with RES iron (xRES and HC/RES), however, RES iron was not significantly associated with outcomes following adjustment for age and fibrosis (see Supporting Data 1).

3.6 | Total cardiovascular events

The cumulative incidence for fatal and non-fatal cardiovascular events combined after 10 years follow-up was 5% (95%-CI, 1%-8%) for NONE and 5% (95%-CI, 2%-9%) for any stainable iron. The subgroup analysis showed cumulative incidences of 12% (95%-CI, 1%-23%) for xRES and 2% (95%-CI, 0%-4%) for HC/RES. The Cox proportional hazards models showed a csHR of 3.2 (95%-CI, 1.2-8.2; $P = .015$) for xRES in cardiovascular events adjusted for age, NASH and fibrosis stages 1 and 2, while there were no subjects with stages 3 and 4 with a cardiovascular event. The annualized event rate for cardiovascular events was 0.6 (95%-CI, 0.3-1.2) for NONE, 1.9 (95%-CI, 1.0-3.6) for xRES, 0.5 (95%-CI, 0.1-3.2) for xHC and 1.3 (95%-CI, 0.6-2.5) for HC/RES per 100 person-years.

3.7 | Total extrahepatic malignancies

We observed 30 extrahepatic malignancies in our cohort and these were as follows: 6 prostate, 5 colon, 4 kidney, 3 pancreas, 2 central nervous system, 2 lungs, 2 skin and 1 each in the thyroid, breast, urothelium, ovary, cervix and vagina. The cumulative incidence for all extrahepatic malignancies after 10 years of follow-up was 8% (95%-CI, 2%-14%) for NONE and 10% (95%-CI, 4%-16%) for any stainable iron. The subgroup analysis showed cumulative incidences of 10% (95%-CI, 2%-19%) for xRES and 13% (95%-CI, 3%-23%) for HC/RES. HC, RES or HC/RES was significant in univariate Cox proportional hazards models (see Table S2). The annualized event rate for extrahepatic malignancy endpoints was 0.8 (95%-CI, 0.5-1.6) for NONE, 1.7 (95%-CI, 0.8-3.3) for xRES, 0.5 (95%-CI, 0.1-3.2) for xHC and 1.7 (95%-CI, 1.0-3.1) for HC/RES subjects per 100 person-years (Table 3).

4 | DISCUSSION

In this study we aimed to assess the relationship between patterns of hepatic iron distribution and the long-term natural outcome in

TABLE 4 Multivariate Cox proportional hazards models

	Mortality or transplantation		Total Hepatic Events		Total cardiovascular events	
	HR (95%-CI)	*P*-value	csHR (95%-CI)	*P*-value	csHR (95%-CI)	*P*-value
Model 1						
Age (y)	1.1 (1.0-1.1)	<.001	–	–	1.1 (1.0-1.1)	.039
NASH	1.9 (0.8-4.9)	.158	1.8 (0.7-4.9)	.240	1.1 (0.4-3.1)	.820
BMI (kg/m^2)	–	–	0.8 (0.7-0.9)	.005	–	–
Groups:						
RES	1.4 (0.6-3.0)	.425	2.8 (1.2-6.6)	.015	1.6 (0.7-4.1)	.290
Fibrosis:						
F0	*Ref.*		*Ref.*		*Ref.*	
F1	1.6 (0.6-4.1)	.317	10.7 (1.3-86.9)	.026	0.7 (0.2-2.1)	.480
F2	2.3 (0.7-7.7)	.183	23.4 (2.2-245.0)	.009	2.2 (0.8-6.2)	.130
F3	6.3 (1.6-24.9)	.008	117.1 (10.1-1360)	<.001	–	–
F4	5.9 (1.4-25.1)	.016	757.4 (56.0-10200)	<.001	–	–
Model 2						
Age (y)	1.1 (1.0-1.1)	<.001	–	–	1.1 (1.0-1.1)	.031
NASH	1.9 (0.8-4.7)	.175	1.8 (0.6-4.8)	.270	1.1 (0.4-2.8)	.880
BMI (kg/m^2)	–	–	0.8 (0.7-1.0)	.009	–	–
Groups:						
NONE & xHC	*Ref.*		*Ref.*		*Ref.*	
xRES	1.7 (0.7-4.3)	.256	2.4 (1.0-5.8)	.048	3.2 (1.2-8.2)	.015
HC/RES	1.2 (0.5-2.9)	.695	3.6 (1.4-9.5)	.010	0.8 (0.2-2.8)	.740
Fibrosis:						
F0	*Ref.*		*Ref.*		*Ref.*	
F1	1.7 (0.7-4.4)	.266	10.4 (1.3-83.7)	.028	0.8 (0.2-2.4)	.640
F2	2.3 (0.7-7.7)	.181	22.9 (2.1-244.0)	.010	2.4 (0.9-6.4)	.078
F3	6.7 (1.7-26.3)	.007	108.3 (8.8-1340)	<.001	–	–
F4	6.5 (1.5-27.4)	.011	830.6 (58.3-11800)	<.001	–	–

Note: Analysis of mortality/liver transplant with multivariate Cox proportional hazard models; Total hepatic, cardiovascular events as well as all extrahepatic malignancies are analysed as competing risks, 95% confidence intervals are shown; Extrahepatic malignancies were not included in the models as no differences were observed between groups. Models are adjusted for NASH and fibrosis as semi-quantitative covariate; variables with a p-value below 0.1 in univariate analysis are added using forward selection; In Model 1 the impact of RES (ie xRES and HC/RES combined) is evaluated, in Model 2 these groups are evaluated separately; Since only one event and no death occurred in xHC it was combined with NONE for the models.

Abbreviations: BMI, body mass index; csHR, cause-specific hazard ratio; HR, hazard ratio; NASH, non-alcoholic steatohepatitis.

299 adults diagnosed with NAFLD from 1997 to 2017 in Austria. The key finding of our analysis was that particularly the mesenchymal pattern of iron deposition was associated with worse hepatic and cardiovascular outcome measures.

The potential role of iron in NAFLD has been extensively studied for over two decades since hyperferritinaemia has been established as a frequent clinical finding in NAFLD subjects and NAFLD is now the most common underlying aetiology in the work-up of hyperferritinaemia. The potentially detrimental effect of iron on the disease course is commonly attributed to its ability to augment oxidative stress and thereby promote cell damage.[6,7] This principle is well known from iron excess in hereditary causes or transfusion-related iron overload.[6] However, the contribution of excess iron in the context of metabolic syndrome spectrum disorders such as NAFLD is by far less clear. First, the extent of iron overload is significantly lower compared to hemochromatosis, even for similar degrees of ferritin concentrations. Second, SF concentrations reflect both, mild iron overload as well as elevation via inflammatory stimuli arising from adipose and liver tissue inflammation. And third, while hemochromatosis shows iron deposition quite uniformly in parenchymal cells, the pattern of iron deposition in NAFLD subjects varies from exclusively mesenchymal or parenchymal to mixed patterns of different magnitude. Hence, in a number of studies NAFLD biopsies were aimed to assess disease severity and iron with inconclusive and conflicting results.[8,14,15,17,18] Interventional studies, ie iron depletion via phlebotomies, are few and observational, while one well-designed

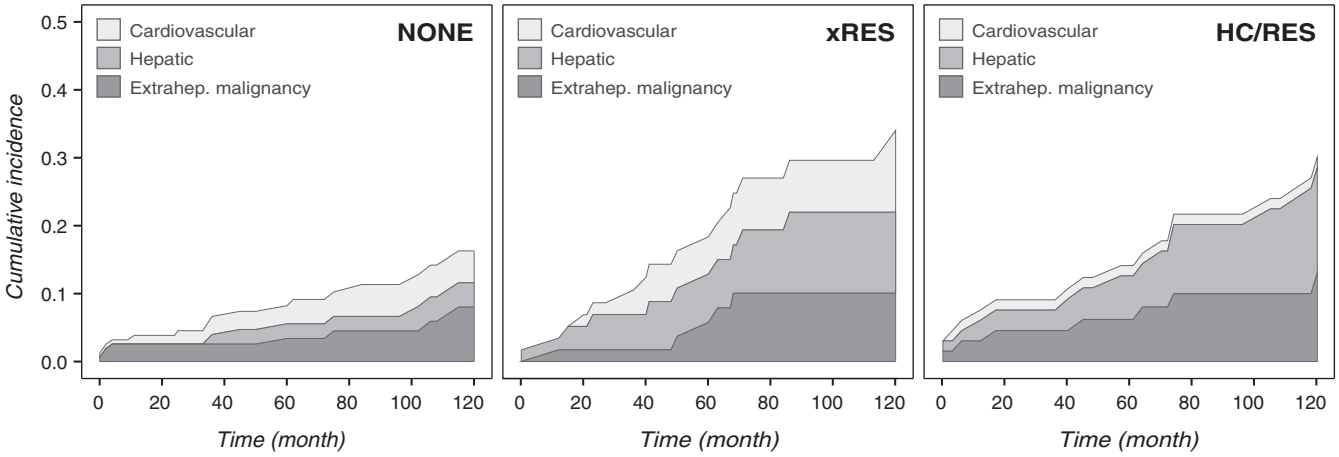

FIGURE 3 Cumulative incidence analysis of fatal and non-fatal events combined. Hepatic events, cardiovascular events and extrahepatic malignancies were analysed as competing risks and are presented for each group separately. (xHC not shown, as only two events occurred during follow-up.)

study[34] has included subjects without documented iron overload for phlebotomies over a relatively short period of time which also limits the conclusions that can be derived from the data. On this background, we aimed to determine the natural course of our entire cohort of NAFLD subjects who underwent liver biopsy and study the impact of iron and iron deposition patterns at baseline for the subsequent natural course. The study carries the inherent limitations of a retrospective study. However, the detailed analysis and high rate of follow-up have yielded a substantial and reliable data set to link iron deposition with the development of clinical endpoints.

4.1 | Baseline disease severity and serum ferritin differences between groups

There are some conclusions that appear relevant from our baseline data. We identified a similar SF cut-off in men (324 μg/L) and women (387 μg/L) to be predictive of stainable iron deposition in the biopsy specimen which was surprisingly low as it is only slightly above accepted upper-limits of normal and close to the limits known from hemochromatosis which were reported 300 μg/L in men and postmenopausal women and 200 μg/L in premenopausal women.[35] Hence, the inflammatory contribution to SF elevation appears moderate and SF cut-offs around 350-400 μg/L may be useful in the clinical care of NAFLD subjects to identify subjects with stainable iron.

With regard to NAFLD disease severity, we observed that xRES pattern was linked with the highest degree of steatosis and numerically also of NASH. This is in accordance with findings from Nelson et al[15] and Maliken et al[14] who found the presence of RES associated with NASH, even though the proportion of NASH subjects was higher in both studies with 68% and 76% respectively. However, an Italian study found that the mixed pattern was linked to NASH.[17] Thus, our findings are in support of the relevance of the mesenchymal pattern of iron deposition to be detected in a NAFLD patient.

4.2 | Link of iron with specific endpoints

Our key aim was to obtain data on the link of baseline liver biopsy findings with long-term clinical outcomes, as to our knowledge these data are not available. We observed that subjects with any iron deposition had a higher rate of hepatic events compared to those without iron in the baseline biopsy. With regard to the various subgroups of iron deposition patterns, we reported that patients with an xHC pattern had significantly better outcomes compared to those in whom iron was also or exclusively found in the mesenchymal compartment. Although this finding was hampered by the small number with xHC iron, it was consistent across different analyses and endpoints. We further observed that the mesenchymal pattern of iron deposition was linked to adverse clinical outcomes with regard to overall mortality, hepatic cause-specific mortality and morbidity. Specifically, xRES iron deposition was associated with a higher mortality and with total number of cardiovascular events while a mixed mesenchymal and hepatocellular pattern (HC/RES) was linked to the highest risk of hepatic events. Concerning the mortality our findings differ from previous conclusions by Hagström et al,[36] where no difference in survival was found in a group comparable to our RES group, but with a third of the group size. Importantly, our analysis using advanced fibrosis as the discriminator confirmed previous findings that in case of established progressed liver disease, the clinical outcome is almost exclusively determined by the development of hepatic events.[3,37] In this case, any influence of iron appears to be abolished.

Hence, in summary, our data suggest that iron detected in the mesenchymal compartment is relevant to future clinical outcomes in NAFLD subjects unless advanced fibrosis is present. Although our data require confirmation, ideally from prospective investigations, at this stage we hold that the following conclusions can be drawn: Determination of the pattern of iron deposition in the liver biopsy may indicate a subgroup of patients with higher risk to develop hepatic or vascular outcomes compared to those without iron or iron

only in the parenchymal compartment independent from NASH or early stages of fibrosis. As these causes of mortality and morbidity are the most common sequelae in NAFLD, such subjects may profit from a closer and more detailed cardiovascular and hepatic follow-up in order to detect complications at a treatable stage.

Our data do not allow conclusions on causality. Phlebotomies have not conclusively shown benefit in subjects with the metabolic syndrome and NAFLD/NASH and particularly mesenchymal iron retention points towards potential underlying inflammatory and thus systemic mechanisms which may be causative for the adverse clinical consequences. In addition, mesenchymal iron stores respond less well to phlebotomy therapy as evidenced from the treatment of patients with ferroportin disease which is equally characterized by a predominance of iron deposition in mesenchymal cells.[38] Mesenchymal iron may therefore equally well be a marker for disease risk rather than the causative factor. From a mechanistic point of view, the reproducible association of RES iron but not HC iron in our data set allows speculation on potential underlying causes. Iron retention in macrophages usually indicates a different mechanism compared to iron in hepatocytes, as macrophage iron is linked to inflammatory mechanisms and hepatocellular iron is rather representative of true iron overload. Our observations where this inflammatory pattern of iron deposition is linked to hepatic and vascular events suggest that RES iron is more likely an indicator of systemic and hepatic inflammatory mechanism than iron toxicity resulting from true iron overload and oxidative stress formation. Thus, we interpret our data as not primarily indicating a detrimental effect on the clinical outcome via oxygen radical effects typical of iron excess but more likely an effect of underlying systemic and hepatic inflammatory mechanisms which over time promote adverse outcomes.

In conclusion, our observational study provides evidence that the mesenchymal pattern of iron deposition in a comprehensive cohort of NAFLD subjects from a restricted Central European area may be of particular relevance to the further clinical development. Hence, liver biopsy with assessment of the iron deposition pattern may identify subjects at risk from vascular or hepatic events who may profit from detailed further investigations and closer monitoring.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Data analysis and interpretation, drafting and writing of manuscript: SKE; Patient recruitment and data acquisition: AF, GS, SZ, DN, MS, SR and BP; Data analysis: JK; Histopathological analysis: HH and KS; Patient recruitment and revision of manuscript for important intellectual content: CD and FS; Study concept and design, analysis and interpretation of data, outlining and revising the manuscript: EA

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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