Serum Iron Markers in Relation to Hepatocellular Carcinoma Risk Among Patients with Non-Alcoholic Fatty Liver Disease

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Abstract

Background: In recent years, non-alcoholic fatty liver disease (NAFLD) has been recognized as a new risk factor for HCC. Iron is an essential element for humans and hepatocytes are its main storage location. High absorption of iron from intestine due to iron-rich dietary pattern or hemochromatosis may result in hepatic iron overload. Epidemiologic data on the associations between different serum iron markers and risk of HCC are rare. In this study, we examined the association between four iron measurements in pre-diagnosed serum with the risk of HCC development in NAFLD patients. **Methods**: We identified 47,970 NAFLD patients in the electronic health records (EHRs) of the University of Pittsburgh Medical Center (UPMC) Healthcare System from 1/1/2004 through 12/31/2018. 19,925 of the population had at least one measurement of serum iron, transferrin saturation, total iron binding capacity and serum ferritin. A total of 192 NAFLD patients were diagnosed with HCC at least 30 days after measurement of serum iron markers with an average 4.15 years of follow-up. Cox proportional hazard regression model adjusted for age, sex, race, body mass index, history of diabetes and tobacco smoking were used to calculate hazard ratios (HRs) and the 95% confidence intervals (CIs) for HCC incidence associated with elevated levels of the four iron markers were obtained. **Results**: Serum iron and transferrin saturation were significantly elevated in NAFLD patients who developed HCC than NAFLD patients who remained free of HCC during the study period. HR of HCC for elevated serum iron >175 mcg/dl was 2.44 (95% CI 1.06 – 5.58) compared to its normal range at 75–175

mcg/dl. Similarly, HR for HCC associated with high transferrin saturation level (>35%) was 2.18 (95% CI 1.27 – 3.74) compare to its normal range at 25-35%. We did not find statistically significant associations for TIBC and serum ferritin with the risk of HCC. **Conclusions**: Elevated serum iron and transferrin saturation levels are significantly associated with increased risk of developing HCC in NAFLD patients. These findings could offer an important part in public health as serum iron level could be monitored clinically among NAFLD patients as early HCC prevention and detection.

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PREFACE

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1.0 INTRODUCTION

1.1 LIVER

The liver is the biggest solid internal organ in the human body. It is also one of the most important organs for various biological activities: a regulator of the blood system, a hub of the immune cells, a center of processing xenobiotic chemicals, and etc[1]. The liver also produces bile, which is stored in the gallbladder, and responsible for breaking down fat. Given its critical biological functions for human body, liver damage, especially in long-term condition, may lead serious health consequences.

Chronic infections with virus, heavy use of alcohol, and exposure to toxic compounds from the food, water, and drugs could lead to chronic liver damage, leading to liver cirrhosis and liver failure. Common syndromes and signs of liver failure include gastrointestinal bleeding, hepatic encephalopathy, and jaundice[2].

1.2 LIVER CANCER

Another long-term health outcome of chronic liver disease is liver cancer. Primary liver cancer includes hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, angiosarcoma and hemangiosarcoma, and hepatoblastoma. Liver cancer is the 3rd most common cause of cancer death globally[3]. In 2020, 42,810 new patients were projected to be diagnosed with primary liver cancer and 30,160 deaths from liver cancer[4]. At the same time, the incidence

rate and death rate of liver cancer could be up to 3-fold higher in men than women[5]. This increasing trend of liver cancer mortality rate is predicted to continue in the future and specifically moving towards younger generations[6,7].

1.3 HEPATOCELLULAR CARCINOMA (HCC)

Hepatocellular carcinoma (HCC) is the most common subtype of liver cancer[8]. Around 75% of primary liver cancer cases are HCC[9]. Hepatocytes are the major cell type of the liver organ. Several interruptions of molecular signaling pathways can be frequently found among HCC patients: Wnt, P53, chromatin remodeling, and abnormal cell cycle[10], which may explain why HCC is the most common subtype of liver cancer. From 1999 to 2016, the annual death of HCC in the US has doubled to 11,073. Across all race groups, only Asians and pacific islanders are the only ones that had reported improvements on their mortality rate (decreased by 2.7%)[11]. Males have 2-4 times higher incidence of HCC than females[12]. This is likely due to the risk factors of HCC commonly appear more frequently among males than females[13].

Similar to other cancers, HCC also results from abnormality of the cellular apoptosis[14], which emerges from different risk factors. Biologically, major risk factors of HCC including chronic viral infection of hepatitis C and hepatitis B, alcoholic drinking, aflatoxin, hemochromatosis, and nonalcoholic fatty liver disease[15-17]. This will be covered in more detail in section 2.0.

2.0 RISK FACTORS OF HEPATOCELLULAR CARCINOMA

2.1 NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

In recent years, non-alcoholic fatty liver disease (NAFLD) has become an emerging risk factor for HCC[18]. NAFLD is considered as the one of the most common chronic liver disease in developed nations[19]. It is estimated that 30 – 40% men and 15-20% women have NAFLD globally[20]. Among US adults, the prevalence rate of NAFLD is estimated to be 25%[21-23].

NAFLD is a wild spectrum of liver conditions from nonalcoholic fatty liver to nonalcoholic steatohepatitis (NASH), which could further progress to compensated and decompensated cirrhosis. Past studies have shown health conditions such as obesity and diabetes are highly associated with the development of NAFLD[24]. Around 30-40% of NAFLD patients will develop NASH[25], and up to 4% of NAFLD patients will develop cirrhosis[26-28]. The majority of HCC develops from a cirrhotic liver.

2.2 IRON AND HEPATOCELLULAR CARCINOMA

Iron is widely found in human cells. More than half of body iron is hemoglobin in matured red blood cells[29]. For humans, the major source of body iron is from diet. Around 10% of dietary iron will be absorbed by body[30]. Iron is not secreted through urine but naturally through the loss of blood[30]. The remainder of iron is mainly stored in the liver cells as ferritin[31,32]. Nevertheless, iron overload is a crucial reason for hepatotoxicity[33]. Excessive iron molecules is discharged into

the cytoplasm of liver cells and cause cellular damage[32]. As demonstrated, excessive iron may induce several biological pathways in cellular level: cellular iron compartmentalization and inflammation response[33,34]. Some common laboratory methodologies that evaluate iron levels include serum iron, TIBC, and serum ferritin. Transferrin saturation, which is another indicator of iron level, is calculated by serum iron level / TIBC[35]. Iron overload is quantified when the total amount of the iron surpasses 5 grams in the body[36]. In the US, 1 in 200 Caucasians have iron overload[37].

Genetic iron overload is hemochromatosis. From the national Human Genome Research Institute (NHGRI), around 1 million people in the US have hereditary hemochromatosis[38]. As of today, there are four different genes related to iron metabolism (*HFE*, *HAMP*, *HJV*, and *TFR2*)[30]. *HFE*-related hemochromatosis is the most common type of hemochromatosis[39]. In the US, 80 – 90% of Caucasians who are diagnosed with hemochromatosis had the *HFE* mutation[40,41]. The *HFE* is located on chromosome 6[42]. Mutations of *HFE* can occur in various patterns. The most common mutation of *HFE* hemochromatosis is C282Y[37]. The *HFE* gene encodes HFE protein, which serves as a regulator of hepcidin. Hepcidin, produced by the liver, is the main iron regulator. Hepcidin inhibits the absorption of iron in the small intestine and the function of ferroportin, which are the transporters in both iron release and circulation. With a mutated *HFE* gene, HFE protein would be malfunctional, which results in a decreased level of hepcidin, resulting in increased iron absorption from the small intestine, leading to excessive iron storage in the liver. On the other hand, excessive iron intake due to diet or blood transfusion could also lead to iron overload without hemochromatosis[43]. If not bound to transferrin, the excessive free iron within the cytoplasm may cause increased generation of hydroxyl radicals, which causes oxygen-related oxidative damage[44].

2.3 OTHER COMMON RISK FACTORS

2.3.1 Hepatitis B and hepatitis C virus

Chronic hepatitis virus (HBV, HCV) – related cirrhosis roughly account for half of the HCC cases in the US[45]. Geographic locations are the main determinant of HBV and HCV infection. It is estimated by the World Health Organization (WHO) that 257 million people lived with chronic HBV and 71 million lived with chronic HCV worldwide in 2015[46]. Underdeveloped nations or developing nations generally have higher prevalence rates of HBV and HCV infection[47]. The highest estimated prevalence rate of chronic HBV infection from 1965 to 2013 was reported in the Africa and Western Pacific as 8.8% and 5.3% respectively[48]. Highest estimated prevalent rate of HCV in 2015 was in Mongolia where approximate 8.5% of the population had chronic HCV infection[46].

One of the mechanisms of HBV associated HCC is the genome integration from virus genes into the liver cellular genes[49]. HBV DNA can be found in hepatocytes among 80% - 90% of HCC patients[50]. This genome progression could be found before the diagnosis of HCC[51]. Inflammation response induced by HBV virus could stimulate the progression of liver fibrosis and cirrhosis, and furthermore, HCC[52]. This immune response is mediated by tumor necrosis factor-alpha (TNF-alpha) and interleukin (IL) 1-beta [53]. Similar to HBV infection, chronic HCV infection could be reflected by the level of cytokines including IL-10 and IL-15, which could be associated with the progression to HCC[54].

2.3.2 Alcohol

Under the guidelines from the US Center for Disease and Control and Prevention (CDC), drinking alcohol increases the risk of many cancers including HCC[55]. It also promotes the reoccurrence of HCC[56]. Overall, 15% - 30% of HCC cases result from alcohol abuse[57]. Alcohol abuse (> 50 – 70g/day for several years[16]) could accelerate the speed of progression from cirrhosis to HCC by changing the liver's functional capacity[58]. Alcohol is processed in liver with generations of toxic acetaldehyde, acetate, fatty acids and cholesterol[59]. These compounds may prevent regular DNA damage repair procedures and accumulate somatic mutations in hepatocytes[60-62]. Cytokines and immune signals such as IL-17, IL-6, TNF-alpha are likely to mediate the progression of HCC prompted by alcohol abuse[63,64].

2.3.3 Dietary Aflatoxin B1 (AFB1)

Aflatoxin B1 secreted from fungi is another risk factor of HCC[65]. Produced by *Aspergillus* fungi and grown in common foods (corn, rice, and maize) [66], AFB1 is still a big challenge today in the global food supply chain[67]. Percentage of global population at risk of HCC because of AFB1 is estimated to be as low as 4.6% and as high as 28.2%[68]. Different pathways of carcinogenesis related to AFB1 were found by many studies. One crucial pathway is the epoxidation of AFB1 by CP450 enzyme, which influence P53 suppressor gene in cellular cycle[69]. Despite the role of AFB1 alone, there is also proposed interactions between AFB1 and HBV infection[66]. This interaction is achieved by the damage of DNA bonded by toxic protein while HBV virus could integrate into hepatocyte DNA easier[70].

2.3.4 Tobacco smoking

Tobacco smoking is well-known for its relationship with lung cancer. But it also increases the risk of many different types of cancers including HCC[71]. In the Liver Cancer Pooling Project, over 1,500,000 subjects were investigated, and the risk of HCC showed an increasing trend as the smoking history of one individual is longer[72]. Multiple studies in different populations also confirmed that the cessation of smoking reduced the risk of HCC[72-75]. Besides, a combined smoking and alcohol drinking lifestyle is likely to increase the risk of HCC even more than alcohol itself [76]. However, there has not been any strong evidence that tobacco smoking is associated with the mortality of HCC[77].

2.3.5 Type II Diabetes (T2D)

On average, T2D patients may have 2 times higher risk on developing HCC than non-diabetic people[78-81]. In the US, this risk is especially found among long-term T2D patients in both sexes[82]. Biologically, people with diabetes have higher level of several inflammatory cytokines such as IL-6, which is also linked with the development of HCC[83]. Insulin resistance makes substantial contributions to the progression of T2D and NAFLD [84-86].

2.3.6 Obesity

It is apparent that obesity is a major health crisis in many developed nations. Many studies conclude obesity is associated with various health conditions: hypertension, T2D, stroke, and in our case, the HCC. A common way to quantify this is to use body mass index (BMI), whose normal

range is generally between 18.5 – 25 kg/m^2. In 2003, 900,000 American adults were followed in a prospective study and the risk of dying from liver cancer among people with obesity (BMI > 25 kg/m^2) was almost 5 fold higher in men compared to people with normal range of BMI[87]. Obesity is also widely found among liver disease patients such as cirrhosis, such that it may increase the probability of the progression from liver disease to HCC[88]. A meta-analysis including around 7 million participants in 2007 concluded there was 17% higher risk of developing HCC among overweight individuals, and it was 89% higher among obese individuals[89].

3.0 GAPS IN KNOWLEDGE

Research on the association of iron overload and HCC has become clearer. A large number of studies have examined the association between iron overload and HCC. Clearly, genetic hemochromatosis iron overload has proven to be related to chronic liver disease and HCC[90-94]. Nevertheless, epidemiological data on iron overload without hemochromatosis is sparse. While non-hemochromatosis iron overload could be a potential pathway for NAFLD patients develop HCC, the available studies only showed some associations between iron overload and HCC without proper exclusions of major underlying causes such as HBV/HCV[95,96].

4.0 PUBLIC HEALTH SIGNIFICANCE

As the number of NAFLD patients is increasing in the world, liver cancer prognosis becomes crucial in reducing the mortality rate of liver cancer or HCC. Regardless of the existing information about the relationships between alcoholic drinking, HBV / HCV infections and HCC, finding potential pathways of HCC among NAFLD is critical in clinical settings. If identified, iron could be served as a tool in HCC's early detection and prevention. Furthermore, since iron-related laboratory measurements are easy to access for the majority of communities with a relatively low cost, having iron as part of the clinical setting is not hard to achieve, thus providing a potential novel public health approach to early detection and prevention of HCC

5.0 OBJECTIVE

In this study, we are able to access the electronic health records of patients' clinical visits from the largest health system network in the Commonwealth of Pennsylvania. We were able to use real-time iron-related measurements with a large enough cohort. With proper exclusions of potential underlying causes of HCC and hemochromatosis, we aim to exam four different iron-related measurements (serum iron, transferrin saturation, total iron binding capacity, serum ferritin) through a 12-year period of time for each NAFLD patients and their status of HCC. We hypothesized that a high serum iron level, which is an indicator of iron overload, is associated with an increased risk of developing HCC among the NAFLD population in the US.

6.0 MATERIAL AND METHODS

6.1 STUDY DESIGN AND STUDY POPULATION

We used a retrospective cohort study design in this project. Study subjects were retrieved from electric health records (EHRs) of the University of Pittsburgh Medical Center (UPMC) Healthcare System from 1/1/2004 to 12/31/2018. UPMC is a major local medical service provider with 40 hospitals and 700 doctors' offices and outpatient sites that serves more than 3 million patients annually throughout western Pennsylvania (PA), USA. Patient's demographics, weight, height, tobacco smoking, alcohol intake, vitals, and disease diagnosis were recorded upon their clinical visits. Briefly, 47,970 NAFLD patients with a clinical diagnosis of NAFLD between 40-90 years old without alcoholic use disorder, autoimmune hepatitis, biliary cirrhosis, chronic viral disease, hemochromatosis, and Wilson's disease were identified from 1/1/2004 to 12/31/2018. Detailed steps are described in section 5.2 exclusion criteria and Figure 1.

For our study, subjects were selected if they have any of the four iron measurements (serum iron, transferrin saturation, TIBC, and serum ferritin). Given the complexity of the original dataset, intensive data cleanings of primary exposure (serum iron, transferrin saturation, TIBC, serum ferritin) and outcome (HCC) were performed discussed in section 5.3.

All diseases are classified by the International Classification of Diseases (ICD) codes. The ICD system was developed for physicians and healthcare providers to classify diagnoses, symptoms, and medical records. The specific names for each ICD code used in this study are provided in Appendix A.

6.2 EXCLUSION CRITERIA

6.2.1 Defining Exposure

The primary exposure variables of interest as identifiers of iron levels were serum iron, transferrin saturation, TIBC, and serum ferritin. Patients who did not have any one of the four iron markers in their HER were excluded from the NAFLD cohort (N = 27,749 excluded, Figure 1).

To define the NAFLD population, we included patients who were diagnosed with the followings by International classification of disease (ICD) 9 and 10 code: (1) NASH (ICD9 = 571.40, 571.41, 571.49; ICD10 = K75.81, K75.89; (2) NAFL (ICD9 = 571.8; ICD10 = K76.0); (3) Cirrhosis (ICD9 = 571.5, 571.9, 572.2, 572.8; ICD10 = K72.1, K72.9, K74.0, K74.2, K74.61, K74.69); (4) liver transplant (ICD9 = V42.7; ICD10 = 94.4).

6.2.2 Defining the Primary Outcome

The primary outcome of interest was diagnosis of primary HCC. We used three ICD codes to define HCC: C22.0 (liver cell carcinoma) and C22.8 (malignant neoplasm of liver, primary) for ICD 10; 155.0 (liver cell carcinoma) for ICD 9.

6.2.3 Defining the Time at Risk

Time at risk was differentiated based on populations. For the NAFLD HCC population, it was calculated from the earliest four iron measurements to the HCC diagnosis date. For the

NAFLD non-HCCs, it was calculated from the four iron measurements to death date or last contact date with UPMC.

6.2.4 Excluding Bleeding due to Decompensate Cirrhosis within 6 Months to Iron Measurements

Bleeding due to decompensated cirrhosis (Gastrointestinal hemorrhage, and esophageal varices with bleeding) patients had severe liver conditions, which were very likely to impact hepatic iron level. We further excluded bleeding events due to decompensated cirrhosis within 6 months to the iron measurement (N = 61 excluded, Figure 1). This exclusion was also based on the related ICD codes (ICD9: 456.0, 456.20; ICD10: I85.01, I85.11, K92.2).

6.2.5 Excluding Underlying Causes of HCC

The study cohort was expected to be free of health conditions that were underlying causes of HCC. Individuals with the following disease / disorder were excluded by their ICD codes: (1) alcoholic use disorder (ICD9: 291.0, 291.8, 291.9, 303.00, 303.9–303.93, 305.0-305.03, 571.0–571.3; ICD10: F10.10–F10.90, K70.0–K70.9); (2) biliary cirrhosis (ICD9: 571.6; ICD10: K74.3–K74.6); (3) autoimmune hepatitis (ICD9: 573.2; K75.4); (4) hemochromatosis (ICD9: 275.0; ICD10: E83.11x); (5) Wilson's disease (ICD9: 275.1; ICD10: E83.01); (6) Chronic viral disease (ICD9: 070.2, 070.3, 070.4, 070.5, 070.6, 070.9; ICD10: B18.00 – B18.99, B19.00 – B19.99).

Furthermore, subjects who tested positive for HBV or HCV by laboratory tests were also excluded. Because the HBV and HCV diagnosis and lab tests were located in different data files when we requested it, it is likely to miss someone who does not record as HBV or HCV diagnosis

while being tested positive for the virtual infection. This step was to ensure we excluded HBV or HCV infections more precisely.

6.2.6 Exclude Extreme Values

We also examined the potential outliers of the four iron measurements. All four iron-related measurements' values which were 5 standard deviations (SD) from the mean were removed (N=8 excluded, Figure 1). The appearance of these extreme values was likely due to human error when being entered into the system by medical practitioners. For example, it is very unlikely to observe someone with a serum iron level over 5000 mcg/dl. And someone cannot have a negative value in their serum iron. We believe values within 5 SD from the mean would be reliable to use for our dataset since this step only excluded a small proportion of the study population.

6.2.7 Exclude Short Follow-up time for HCC Cases

Individuals who had iron measured less than 30 days before their HCC diagnosis were removed. A feature of the EMR database was that patients could be diagnosed with HCC when measured iron level on the same day. Since the goal of the study was to test whether iron could serve as a predicting risk factor for HCC, we removed follow-up time within 30 days since they were too close the diagnosis date (N=227 excluded, Figure 1).

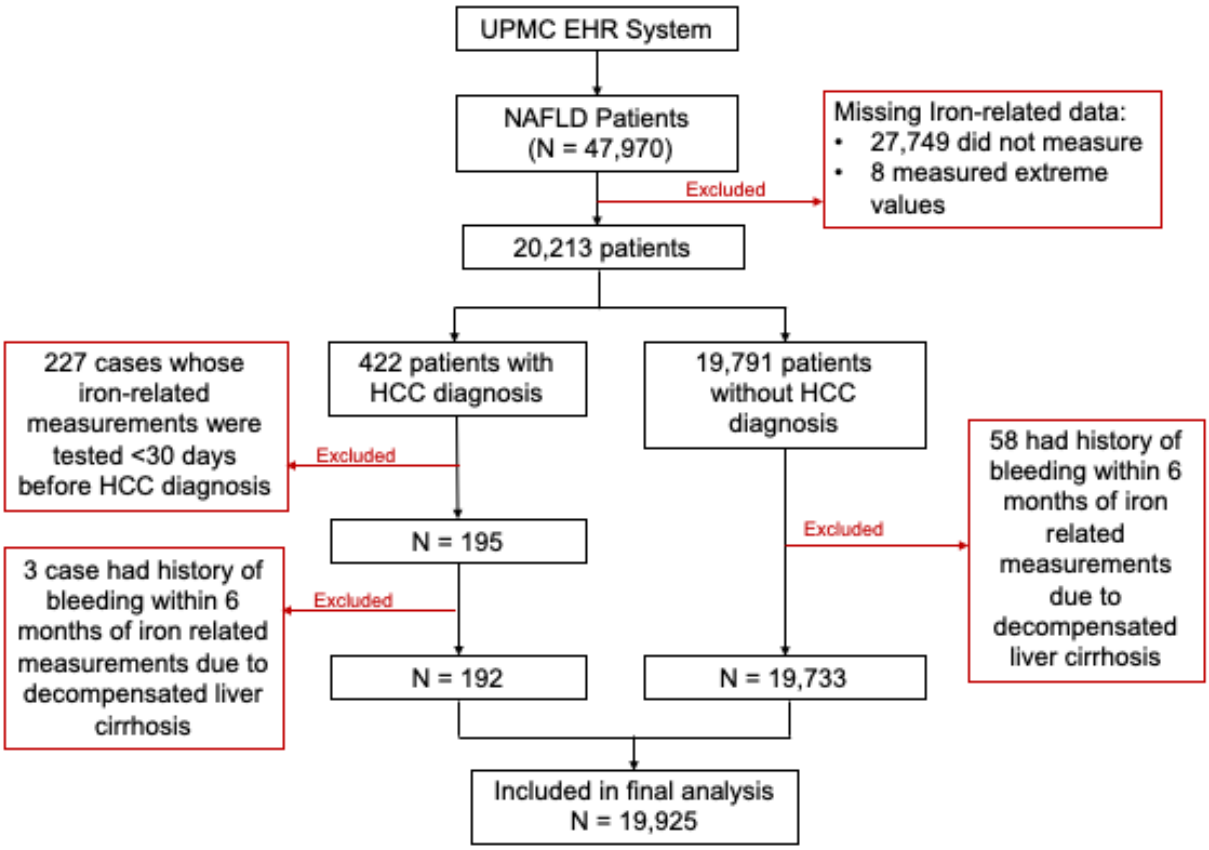

Figure 1: Diagram of Electronic Health Records from UPMC

6.3 STATISTICAL METHODS

Chi-square test and pooled two-sample *t* test was used to compare the differences in the distributions of categorical variables and means of continuous variables between NAFLD patients with HCC and those without HCC, respectively.

Because the four iron measurements were not normally distributed, we used the non-parametric Wilcox test to examine the median differences between NAFLD patients with HCC and those without HCC. Spearman correlations among the four iron measurements were obtained based on all available iron measurements tested on the same day among subjects without HCC.

Serum ferritin level was log transformed in the correlation calculation to minimize the skewness of serum ferritin level.

The Cox proportional hazard regression method was used to assess the association between levels of iron measurements and risk of developing HCC. Concentrations of a given iron measurement were grouped into low, normal, and high groups according to the most recent recommendations for clinical use: the normal range of serum iron was 75–175 mcg/dl[97,98], transferrin saturation was 25% -35%[99,100], total iron-binding capacity was 240–450 mcg/dl[100,101], and serum ferritin was 30–300 ng/ml for male and 10 – 200 ng/ml for female[102,103]. Concentrations below the lower limit of normal range were classified into the low group whereas those above the upper limit of normal range into the high group. The NAFLD cohort was also grouped into quartiles levels according to their concentrations of iron measurements due to the variability of iron measurements' normal ranges. The multivariate Cox regression models included following covariates for adjustments: age (years), sex, BMI (kg/m^2), race, type II diabetes, and smoking status.

All statistical analyses and data cleanings were carried out in SAS software version 9.4 (SAS Institute, Cary, NC). All P values reported are two-sided. The $P < 0.05$ was considered to be statistically significant.

7.0 RESULTS

7.1 CHARACTERISTICS OF STUDY POPULATION

Table 1 shows the comparisons of selected characteristics between NAFLD HCC and NAFLD non-HCC. A total of 192 patients were diagnosed with HCC in our cohort. The mean ± SD age at diagnosis for NAFLD HCC and NAFLD non-HCC were 65.5 ± 10.7 and 59.9 ± 12.0, respectively, ($P<0.001$). The mean time of follow-up was 4.15 years. There was no difference in BMI between the NAFLD HCC (32.8 ± 7.4) and NAFLD non-HCC (33.7 ± 7.4), $P=0.105$). Those NAFLDs with HCCs were predominately Caucasian (95.3%), ever smokers (45.8%), type II diabetics (72.9%), hypertensive (84.9%), and had dyslipidemia (57.8%). For the NAFLD non-HCCs, the cohort tended to be female (62.2%), Caucasian (90.6%), never smokers (48.5%), type II diabetics (50.8%), and had dyslipidemia (70.4%), and hypertension (77.7%). Between the NAFLD HCC and NAFLD non-HCCs, statistical significances were found in age ($P<0.001$), sex (<0.001), race ($P = 0.026$), smoking status (<0.017), type II diabetes (<0.001), dyslipidemia ($P<0.001$), and hypertension ($P=0.017$) (Table 1).

Table 1: Distribution of demographics for hepatocellular carcinoma patients and free of hepatocellular carcinoma among the non-alcoholic fatty liver disease cohort from UPMC health electric health record: 2004 – 2018

Characteristics	NAFLD with HCC N (%) or Mean ± SD	NAFLD without HCC N (%) or Mean ± SD	P
Age (years), mean ± SD	65.5 ± 10.7	59.9 ± 12.00	<0.001*
BMI* (Kg/m^2), mean ± SD	32.8 ± 7.4	33.7 ± 7.4	0.105*
Sex			
Women	96 (50.0)	12,274 (62.2)	<0.001**
Men	96 (50.0)	7,459 (37.8)	
Race			
White	183 (95.3)	17,879 (90.6)	0.026**
Non-white	9 (4.7)	1,854 (9.4)	
Smoking Status			
Never Smoker	77 (40.1)	9,568 (48.5)	
Ever Smokers	88 (45.8)	8,348 (42.3)	0.017
Missing	27 (14.1)	1,817 (9.2)	
Type 2 diabetes			
No	52 (27.1)	9,699 (49.2)	< 0.001**
Yes	140 (72.9)	10,034 (50.8)	
Dyslipidemia			
No	81 (42.2)	5,834 (29.6)	< 0.001**
Yes	111 (57.8)	13,899 (70.4)	
Hypertension			
No	29 (15.1)	4,401 (22.3)	
Yes	163 (84.9)	15,332 (77.7)	0.017

*Two sample T-test
**Chi-square test

7.2 IRON MEASUREMENT LEVELS AND RISK OF NAFLD HCC

Table 2 shows the median and interquartile values of the four iron-related measurements for NAFLD HCCs and NAFLD non-HCCs. The median of serum iron levels among NAFLD (median = 76 mcg/dl) HCCs were higher than NAFLD non-HCC (69 mcg/dl). We observed the

same levels of transferrin saturation for both groups. TIBC and serum ferritin levels showed lower levels among NAFLD with HCCs. However, none of the comparisons were statistically significant (P > 0.05).

Table 2: Median values of iron-related laboratory measurements for hepatocellular carcinoma and free of hepatocellular carcinoma among the non-alcoholic fatty liver disease cohort from UPMC health electric record: 2004 – 2018

	NAFLD HCC		NAFLD without HCC		
Iron Measurements	N	Median (25%, 75%)	N	Median (25%, 75%)	P*
Serum Iron (mcg/dl)	151	76 (45.0, 102.0)	16,091	69.0 (44.0, 95.0)	0.059
Transferrin Saturation, %	141	20.0 (14.0, 32.0)	14,162	20.0 (13.0, 28.0)	0.224
TIBC** (mcg/dl)	173	345.0 (283.0, 420.0)	16,785	349.0 (297.0, 401.0)	0.612
Serum Ferritin (ng/ml)	178	80.5 (31.0, 221.0)	16,596	98.0 (38.0, 222.0)	0.451
*Wilcoxon Rank Sum Test **Total iron binding capacity					

Table 3 presents nonparametric Spearman's rank correlations among the four iron-related measurements. Same day tested serum iron and transferrin saturation were highly correlated among the NAFLD population ($\rho = 0.89$). Conversely, same day tested TIBC had negative correlations with log serum ferritin ($\rho = -0.57$). The other correlation pairs showed weaker, non-significant correlations with each other among the NAFLD population.

Table 3: Spearman's correlation table between serum iron, transferrin saturation, total iron binding capacity, and serum ferritin among the non-alcoholic fatty liver disease patients in UPMC health electric record: 2004 – 2018

	Serum Iron (P)	Transferrin Saturation (P)	TIBC** (P)	Log Serum Ferritin (P)
Serum Iron	1	0.89 (<0.001)	0.08 (<0.001)	0.24 (<0.001)
Transferrin Saturation	-	1	-0.31 (<0.001)	0.45 (<0.001)
TIBC**	-	-	1	-0.57 (<0.001)
Serum Ferritin	-	-	-	1
*The correlations were obtained only if individuals have two iron-related measurements tested on the same day **TIBC: total iron binding capacity				

Table 4 shows the associations between iron measurement levels and risk of HCC among NAFLD patients. After adjusting age, BMI, sex, type II diabetes, and tobacco smoking, the risk of HCC among NAFLD patients who had serum iron level above normal (> 175 mcg/dl) was 144% higher than those were within normal range (P = 0.036). The risk of HCC among NAFLD among those had serum iron level below the normal were 33% lower than those with normal serum iron level (P_{trend} = 0.001). There was a statistically significant 29% increase risk of HCC with doubling iron concentration (p for tread = 0.012). Similarly, a higher level of transferrin saturation (>35%) was also associated with a statistically significant >2-fold increased risk of HCC (HR = 2.18, 95% CI = 1.27 – 3.74). We did not find any significant associations for TIBC or serum ferritin with HCC risk among the NAFLD population.

Table 4: The association between serum iron measurements laboratory measurements and hepatocellular carcinoma risk among the non-alcoholic fatty liver disease patients in UPMC health electric record: 2004 – 2018

	N. of NAFLD HCC	Total Person-Years	Adjusted HR* (95% CI)	P
Serum Iron (mcg/dl)				
Low	71	37293.1	0.67 (0.48, 0.93)	0.016
Normal	74	28896.4	1.00 (Reference)	
High	6	881.8	2.44 (1.06, 5.62)	0.036
P for trend				0.001
Continuous (Log2)			1.29 (1.06,1.58)	0.012
Transferrin Saturation (%)				
Low	88	40289.6	1.06 (0.68, 1.65)	0.797
Normal	26	13277.2	1.00 (Reference)	
High	27	5631.6	2.18 (1.27, 3.74)	0.005
P for trend				0.009
Continuous (Log2)			1.19 (0.98, 1.45)	0.073
Total Iron Binding Capacity (mcg/dl)				
Low	17	6257.1	0.82 (0.49, 1.37)	0.438
Normal	134	62645.1	1.00 (Reference)	
High	22	8770.6	1.27 (0.81, 2.00)	0.299
P for trend				0.173
Continuous (Log2)			1.28 (0.87, 1.89)	0.208
Serum Ferritin (ng/ml)				
Low	22	7217.5	1.28 (0.81, 2.02)	0.293

Normal	118	53341.5	1.00 (Reference)	
High	38	15520.5	0.96 (0.66, 1.38)	0.817
P for trend				0.354
Continuous (Log2)			0.96 (0.89, 1.04)	0.368

Adjusted by age, sex, race, body mass index, type II diabetes, smoking status

In a sensitivity analysis, we used the same approach but excluded all decompensated cirrhosis in the eligible population (data not shown). Decompensated cirrhosis is in the progression to the HCC stage. By excluding all decompensated cirrhosis, we lose a large proportion of NAFLD HCC cases (N = 82), where clinical reference ranges could not be applied any longer.

8.0 DISCUSSION

We investigated the association between four different iron measurements and HCC risk with a retrospective design of 19,925 NAFLD patients. As hypothesized, NAFLD patients who had evaluated serum iron and transferrin saturation had a statistically significant 144% and 118% higher risk of HCC than NAFLD patients who had normal level of serum iron and transferrin saturation, respectively. These positive associations between serum iron, transferrin saturation and HCC were in a level-dependent manner. While we do find strong associations among HCC, serum iron test, and transferrin saturation test, the associations were not found for TIBC and serum ferritin.

To date, the majority of studies related to iron overload and HCC mainly focus on hemochromatosis iron overload. A few studies in Africa reported a higher risk of HCC development and dietary iron overload among Africans in different regions[104-106]. However, among the three studies, one study did not have sufficient indications on the role of hemochromatosis in dietary overload[104]. The other two studies did not adjust for the effects of HBV and HCV infections[105,106]. In addition, one study in south-western Taiwan also found a correlation between excessive iron intake in drinking water and HCC[107]. Similarly, the study also did not take into account justifications on underlying causes of HCC such as HBV and alcoholic drinking. Growing evidence have suggested iron overload could be linked with underlying causes such as HCV infections, excessive alcoholic drinking[90,95,108-111]. However, the picture of iron overload and HCC remains unclear if without sufficient exclusions of major underlying causes of HCC. As Pietrangelo A. indicated in the article *Iron in NASH, chronic liver disease and HCC: how much iron is too much?*[112], we proposed this retrospective study in order to

clarify the pathway of iron overload controlling major risk factors of HCC among the NAFLD population.

Our study did not find any associations between serum ferritin, TIBC, and HCC. Currently, both TIBC and ferritin are used clinically as indicators of iron levels. A low TIBC value or a high serum ferritin value are indicators of iron overload[113,114]. This unexpected result is likely due to the NAFLD itself. TIBC value may be low among liver conditions such as cirrhosis[114]. Similar, raised serum ferritin levels could result from liver disease or metabolic syndromes[115]. Since our study population is among the NAFLD population, the NAFLD itself could show lower TIBC values or higher serum ferritin levels without iron overload. Thus, the TIBC or serum ferritin may not be reliable tests for HCC risks among the NAFLD population. In addition, our study reported an even number of males and females among the NAFLD HCCs. Nevertheless, there are more incidences of HCC among males than females in most countries[116].

Several strengths of this study are worth noting. This is a retrospective cohort study using iron-related laboratory tests in a large population size with NAFLD patients with proper exclusions on underlying causes of HCC and genetic iron overload hemochromatosis. Since iron measurements were included 30 days before the HCC diagnosis, potential impact caused by HCC treatment and HCC progression on iron levels was minimized. In addition, all the underlying causes of HCC and primary outcomes were precisely recorded as ICD codes, and therefore, increased the precision of defining outcome and exclusion criteria.

However, the study also has some limitations. Firstly, random errors of the four iron measurements which were possibly due to data entry could not be avoided. Although we used a conservative approach to rule out some potential errors, there was still a likelihood random errors existed. These random errors could shift the results depending on their values and grouping.

Secondly, the sample size for the high serum iron level relatively small. The small sample size gives us limited options to do further stratifications. Thus, subgroup analysis based on patients' characteristics could not be done. Thirdly, the conclusion may not be applied to US minority groups. Because over 90% of our study population was reported as Caucasian, the conclusion may not be generalizable among other ethnic groups. Fourthly, potential confounding effects of alcohol drinking is still a possibility. Although we have excluded alcohol abuse and alcohol disorders from the entire NAFLD population based on ICD code, alcoholic consumption generally cannot be fully recorded in clinical settings. Fifthly, decisions on whether to test iron or not is likely to be a confounder. Generally, patients who report to experience fatigue or muscle weakness are recommended for iron tests. In our study population, there were 27,749 individuals that were not tested with any of the four iron-related measurements. However, we did not have information on why the 20,213 patients were tested with iron. There might be some common clinical differences between the two groups that could not be identified, which may serve as confounders. Sixthly, the study is based on single measurement of iron-related tests. Body iron level could change throughout the day and week. A single measurement may not fully predict the trajectory of iron level changes in the populations. Lastly, even though multiple covariables were controlled, unmeasured confounding factors could still exist.

Overall, the study showed that serum iron and transferrin saturation are significantly associated with the risk of NAFLD HCC among NAFLD patients. Our findings provide stronger evidence on iron overload on the risk of HCC among the NAFLD population without hemochromatosis and other major risk factors of HCC. This is the first study to comprehensively investigate the role of clinical iron-related measurements on the risk of HCC among the NAFLD population. The study provides an insightful view of iron as a risk factor of HCC among the

growing NAFLD population globally. Specifically, serum iron test or transferrin saturation test could be potentially used as a tool to identify NAFLD patients who may have higher risks progressed to HCC. For future studies, more evidence is needed for serum iron and transferrin saturation measurements as HCC risk factors in large cohort studies with a more diverse population. In addition, whether elevated serum ferritin and TIBC levels could be associated with a higher risk of HCC among the NAFLD population need to be further examined.

APPENDIX A TABLE OF ICD CODES

Disease Name	ICD Code	Code Type	Descriptions
NAFL	571.8	ICD9CM	Other chronic nonalcoholic liver disease
	K76.0	ICD10CM	Fatty (change of) liver, not elsewhere classified
NASH	571.4	ICD9CM	Chronic hepatitis
	571.40	ICD9CM	Chronic hepatitis, unspecified
	571.41	ICD9CM	Chronic persistent hepatitis
	571.49	ICD9CM	Other chronic hepatitis
	K75.8	ICD10CM	Other specified inflammatory liver diseases
	K75.81	ICD10CM	Nonalcoholic steatohepatitis (NASH)
	K75.89	ICD10CM	Other specified inflammatory liver diseases
Liver Transplant	V42.7	ICD9CM	Liver replaced by transplant
	Z94.4	ICD10CM	Liver transplant status
Cirrhosis	571.5	ICD9CM	Cirrhosis of liver without mention of alcohol
	571.9	ICD9CM	Unspecified chronic liver disease without mention of alcohol
	K74.2	ICD10CM	Hepatic fibrosis with hepatic sclerosis
	K74.0	ICD10CM	Hepatic fibrosis
	K74.6	ICD10CM	Other and unspecified cirrhosis of liver
	K74.69	ICD10CM	Other cirrhosis of liver
HCC	155.0	ICD9CM	Malignant neoplasm of liver, primary
	C22.0	ICD10CM	Liver cell carcinoma
	C22.8	ICD10CM	Malignant neoplasm of liver, primary, unspecified as to type
Decompensated Cirrhosis	572.2	ICD9CM	Hepatic encephalopathy
	572.8	ICD9CM	Other sequelae of chronic liver disease
	K72.1	ICD10CM	Chronic hepatic failure
	K72.9	ICD10CM	Hepatic failure, unspecified
	348.3, 348.39	ICD9CM	Encephalo pathy
	789.51, 789.59	ICD9CM	Ascites
	G93.40, G93.49	ICD10CM	Encephalopathy

	R18.0, R18.8	ICD10CM	Ascites
Bleeding due to Decompensated Cirrhosis	456.0, 456.20	ICD9CM	Esophageal varices with bleeding
	578.0, 578.9	ICD9CM	Gastrointestinal hemorrhage
	I85.01, I85.11	ICD10CM	Esophageal varices with bleeding
	K92.2	ICD10CM	Gastrointestinal hemorrhage
Alcoholic Use Disorders	291.0	ICD9CM	Alcohol withdrawal delirium
	291.8	ICD9CM	Other specified alcohol-induced mental disorders
	291.9	ICD9CM	Unspecified alcohol-induced mental disorders
	303.00	ICD9CM	Acute alcoholic intoxication in alcoholism, unspecified
	303.9	ICD9CM	Other and unspecified alcohol dependence
	303.90	ICD9CM	Other and unspecified alcohol dependence, unspecified
	303.91	ICD9CM	Other and unspecified alcohol dependence, continuous
	303.92	ICD9CM	Other and unspecified alcohol dependence, episodic
	303.93	ICD9CM	Other and unspecified alcohol dependence, in remission
	305.0	ICD9CM	Alcohol abuse
	305.00	ICD9CM	Alcohol abuse, unspecified
	305.01	ICD9CM	Alcohol abuse, continuous
	305.02	ICD9CM	Alcohol abuse, episodic
	305.03	ICD9CM	Alcohol abuse, in remission
	571.0	ICD9CM	Alcoholic fatty liver
	571.1	ICD9CM	Acute alcoholic hepatitis
	571.2	ICD9CM	Alcoholic cirrhosis of liver
	571.3	ICD9CM	Alcoholic liver damage, unspecified
	F10.1	ICD10CM	Alcohol abuse
	F10.10	ICD10CM	Alcohol abuse, uncomplicated
	F10.11	ICD10CM	Alcohol abuse, in remission
	F10.12	ICD10CM	Alcohol abuse with intoxication
	F10.120	ICD10CM	Alcohol abuse with intoxication, uncomplicated
	F10.121	ICD10CM	Alcohol abuse with intoxication delirium
	F10.129	ICD10CM	Alcohol abuse with intoxication, unspecified
	F10.14	ICD10CM	Alcohol abuse with alcohol-induced mood disorder
	F10.15	ICD10CM	Alcohol abuse with alcohol-induced psychotic disorder

	F10.150	ICD10CM	Alcohol abuse with alcohol-induced psychotic disorder with delusions
	F10.151	ICD10CM	Alcohol abuse with alcohol-induced psychotic disorder with hallucinations
	F10.159	ICD10CM	Alcohol abuse with alcohol-induced psychotic disorder, unspecified
	F10.18	ICD10CM	Alcohol abuse with other alcohol-induced disorders
	F10.180	ICD10CM	Alcohol abuse with alcohol-induced anxiety disorder
	F10.181	ICD10CM	Alcohol abuse with alcohol-induced sexual dysfunction
	F10.182	ICD10CM	Alcohol abuse with alcohol-induced sleep disorder
	F10.188	ICD10CM	Alcohol abuse with other alcohol-induced disorder
	F10.19	ICD10CM	Alcohol abuse with unspecified alcohol-induced disorder
	F10.2	ICD10CM	Alcohol dependence
	F10.20	ICD10CM	Alcohol dependence, uncomplicated
	F10.21	ICD10CM	Alcohol dependence, in remission
	F10.22	ICD10CM	Alcohol dependence with intoxication
	F10.220	ICD10CM	Alcohol dependence with intoxication, uncomplicated
	F10.221	ICD10CM	Alcohol dependence with intoxication delirium
	F10.229	ICD10CM	Alcohol dependence with intoxication, unspecified
	F10.23	ICD10CM	Alcohol dependence with withdrawal
	F10.230	ICD10CM	Alcohol dependence with withdrawal, uncomplicated
	F10.231	ICD10CM	Alcohol dependence with withdrawal delirium
	F10.232	ICD10CM	Alcohol dependence with withdrawal with perceptual disturbance
	F10.239	ICD10CM	Alcohol dependence with withdrawal, unspecified
	F10.24	ICD10CM	Alcohol dependence with alcohol-induced mood disorder
	F10.25	ICD10CM	Alcohol dependence with alcohol-induced psychotic disorder

	F10.250	ICD10CM	Alcohol dependence with alcohol-induced psychotic disorder with delusions
	F10.251	ICD10CM	Alcohol dependence with alcohol-induced psychotic disorder with hallucinations
	F10.259	ICD10CM	Alcohol dependence with alcohol-induced psychotic disorder, unspecified
	F10.26	ICD10CM	Alcohol dependence with alcohol-induced persisting amnestic disorder
	F10.27	ICD10CM	Alcohol dependence with alcohol-induced persisting dementia
	F10.28	ICD10CM	Alcohol dependence with other alcohol-induced disorders
	F10.280	ICD10CM	Alcohol dependence with alcohol-induced anxiety disorder
	F10.281	ICD10CM	Alcohol dependence with alcohol-induced sexual dysfunction
	F10.282	ICD10CM	Alcohol dependence with alcohol-induced sleep disorder
	F10.288	ICD10CM	Alcohol dependence with other alcohol-induced disorder
	F10.29	ICD10CM	Alcohol dependence with unspecified alcohol-induced disorder
	F10.9	ICD10CM	Alcohol use, unspecified
	F10.92	ICD10CM	Alcohol use, unspecified with intoxication
	F10.920	ICD10CM	Alcohol use, unspecified with intoxication, uncomplicated
	F10.921	ICD10CM	Alcohol use, unspecified with intoxication delirium
	F10.929	ICD10CM	Alcohol use, unspecified with intoxication, unspecified
	F10.94	ICD10CM	Alcohol use, unspecified with alcohol-induced mood disorder
	F10.95	ICD10CM	Alcohol use, unspecified with alcohol-induced psychotic disorder
	F10.950	ICD10CM	Alcohol use, unspecified with alcohol-induced psychotic disorder with delusions
	F10.951	ICD10CM	Alcohol use, unspecified with alcohol-induced psychotic disorder with hallucinations

	F10.959	ICD10CM	Alcohol use, unspecified with alcohol-induced psychotic disorder, unspecified
	F10.96	ICD10CM	Alcohol use, unspecified with alcohol-induced persisting amnestic disorder
	F10.97	ICD10CM	Alcohol use, unspecified with alcohol-induced persisting dementia
	F10.98	ICD10CM	Alcohol use, unspecified with other alcohol-induced disorders
	F10.980	ICD10CM	Alcohol use, unspecified with alcohol-induced anxiety disorder
	F10.981	ICD10CM	Alcohol use, unspecified with alcohol-induced sexual dysfunction
	F10.982	ICD10CM	Alcohol use, unspecified with alcohol-induced sleep disorder
	F10.988	ICD10CM	Alcohol use, unspecified with other alcohol-induced disorder
	F10.99	ICD10CM	Alcohol use, unspecified with unspecified alcohol-induced disorder
	K70.0	ICD10CM	Alcoholic fatty liver
	K70.1	ICD10CM	Alcoholic hepatitis
	K70.10	ICD10CM	Alcoholic hepatitis without ascites
	K70.11	ICD10CM	Alcoholic hepatitis with ascites
	K70.2	ICD10CM	Alcoholic fibrosis and sclerosis of liver
	K70.3	ICD10CM	Alcoholic cirrhosis of liver
	K70.30	ICD10CM	Alcoholic cirrhosis of liver without ascites
	K70.31	ICD10CM	Alcoholic cirrhosis of liver with ascites
	K70.4	ICD10CM	Alcoholic hepatic failure
	K70.40	ICD10CM	Alcoholic hepatic failure without coma
	K70.41	ICD10CM	Alcoholic hepatic failure with coma
	K70.9	ICD10CM	Alcoholic liver disease, unspecified
Biliary cirrhosis	571.6	ICD9CM	Biliary cirrhosis
	K74.3	ICD10CM	Primary biliary cirrhosis
	K74.4	ICD10CM	Secondary biliary cirrhosis
	K74.5	ICD10CM	Biliary cirrhosis, unspecified
	K74.6	ICD10CM	Other and unspecified cirrhosis of liver
Autoimmune hepatitis	573.2	ICD9CM	Hepatitis in other infectious diseases classified elsewhere
	K75.4	ICD10CM	Autoimmune hepatitis

Hemochromatosis	275.0	ICD9CM	Disorders of iron metabolism
	E83.11	ICD10CM	Hemochromatosis
	E83.110	ICD10CM	Hereditary hemochromatosis
	E83.111	ICD10CM	Hemochromatosis due to repeated red blood cell transfusions
	E83.118	ICD10CM	Other hemochromatosis
	E83.119	ICD10CM	Hemochromatosis, unspecified
Wilson’s disease	275.1	ICD9CM	Disorders of copper metabolism
	E83.01	ICD10CM	Wilson's disease
Chronic viral hepatis	070.2	ICD9CM	Viral hepatitis B with hepatic coma
	070.3	ICD9CM	Viral hepatitis B without mention of hepatic coma
	070.4	ICD9CM	Other specified viral hepatitis with hepatic coma
	070.5	ICD9CM	Other specified viral hepatitis without mention of hepatic coma
	070.6	ICD9CM	Unspecified viral hepatitis with hepatic coma
	070.9	ICD9CM	Unspecified viral hepatitis without mention of hepatic coma
	B18	ICD10CM	Chronic viral hepatitis
	B18.0	ICD10CM	Chronic viral hepatitis B with delta-agent
	B18.1	ICD10CM	Chronic viral hepatitis B without delta-agent
	B18.2	ICD10CM	Chronic viral hepatitis C
	B18.8	ICD10CM	Other chronic viral hepatitis
	B18.9	ICD10CM	Chronic viral hepatitis, unspecified
	B19	ICD10CM	Unspecified viral hepatitis
	B19.0	ICD10CM	Unspecified viral hepatitis with hepatic coma
	B19.1	ICD10CM	Unspecified viral hepatitis B
	B19.10	ICD10CM	Unspecified viral hepatitis B without hepatic coma
	B19.11	ICD10CM	Unspecified viral hepatitis B with hepatic coma
	B19.2	ICD10CM	Unspecified viral hepatitis C
	B19.20	ICD10CM	Unspecified viral hepatitis C without hepatic coma
	B19.21	ICD10CM	Unspecified viral hepatitis C with hepatic coma
	B19.9	ICD10CM	Unspecified viral hepatitis without hepatic coma

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