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Editorial Department of *World Journal of Gastroenterology*
77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com
<http://www.wjgnet.com>

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EDITING

Editorial Board of *World Journal of Gastroenterology*, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China
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E-mail: wjg@wjgnet.com

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How labile is gastric infection with *H pylori*?

M Hobsley, FI Tovey, J Holton

M Hobsley, FI Tovey, J Holton, Departments of Surgery and Medical Microbiology, Royal Free and University College Medical School, United Kingdom

Correspondence to: FI Tovey, Departments of Surgery and Medical Microbiology, Royal Free and University College Medical School, London W1W 7EJ, 5 Crossborough Hill Basingstoke RG 21 4AG, United Kingdom. frank.tovey@btinternet.com

Telephone: +44-1256-461521 Fax: +44-1256-461521

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Abstract

It is known that patients infected with *H pylori* can spontaneously become free from infection, and that the reverse change can occur. The time-scale of these conversions is expressed as percentages per year. Since they have been investigated in terms of serology, the changes are called sero-reversion and sero-conversion respectively. Using serological evidence to investigate these phenomena is open to the criticisms that positive serology can be present in the absence of all other evidence of infection, and that a time-lag of 6-12 mo or longer can occur between eradication of the infection and sero-reversion. Investigations using direct evidence of current infection are sparse. The few that exist suggest that some individuals can seroconvert or sero-revert within six to twelve weeks. If these findings are confirmed, it means that some patients have an ability that is variable in time to resist, or spontaneously recover from, *H pylori* infection. Evidence suggests that the deciding factor of susceptibility is the level of gastric secretion of acid.

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Key words: *H pylori*; Lability of infection; Serology; Conversion; Reversion

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EVIDENCE OF LABILITY OF *H PYLORI* INFECTION

Evidence from indirect tests: Spontaneous seroconversion and seroreversion

In papers concerned with human infections of the

gastroduodenum with *H pylori*, it is usually tacitly assumed that infection is stable, i.e., that a subject infected at any one moment will remain infected until the organism is eradicated with pharmacological agents. There is considerable evidence based on serological studies that *H pylori* infection can be more labile, with subjects undergoing spontaneous sero-reversion as well as sero-conversion. Reports from countries where the prevalence of *H pylori* infection is moderate (40%-60%) show that spontaneous cures may occur even more frequently than fresh infections, and more often in children and teenagers than in adults^[1-14]. The question is, do these figures adequately reflect the rates of the changes?

In children, of a total of 1134 children who were *H pylori* negative, 92 had converted to *H pylori*-positive in periods ranging from 9 to 14 years^[2-6]. The percentage conversion rates differed from 40 percent after 10 and 14 years to 5% or less after 2, 10 and 14 years. The same publications documented that of a total of 141 *H pylori*-positive patients 58 reverted to *H pylori* negative over the same periods. The sero-reversion rates in the five studies varied between 15% at 14 years and 80% at 10 years.

In these reports of children, there is no evidence that the length of follow-up is related to sero-conversion or sero-reversion rates. The lack of evidence of a link between the rates and the length of follow-up may be due to the (necessarily) small range of follow-up in an age group defined as children and teenagers. The salient feature of these results is that the sero-conversion rate overall was 92/1134 (8.1%), while the sero-reversion rate was 58/141 (41%). A small tendency for children to develop the infection as time passed was considerably outweighed by a five-fold tendency towards natural cure.

In adults, there is strong evidence that both sero-conversion and sero-reversion rates increase with the duration of follow-up. Eight publications^[7-14] yielded the following statistical results. Over a time-interval of 3-32 years, 94 (2.7%) of 3489 subjects sero-converted; regression analysis indicated that the number converting increased by 0.311 per cent per annum ($r^2 = 0.836$, $P = 0.0015$). The corresponding figures for sero-reversion were 109 (6.04%) of 1806 subjects; the regression values were an increased rate of reversion of 0.676 per cent per annum ($r^2 = 0.747$, $P = 0.0056$). In adults, therefore, conversion rates per annum were outweighed by a doubled rate of sero-reversion.

Comparisons between the two rates in adults and the two in children are strictly impossible because of the lack of correlation in children with length of follow-up. However, if one is prepared to accept that the yearly rates in children (in whom the average length of follow-up was

about 11 years) were, for sero-conversion $8.1/11 = 0.74\%$, for sero-reversion $41/11 = 3.73\%$, it is clear that infection status derived from antibody information in children is more labile in both directions than it is in adults.

The evidence from countries with a high prevalence of infection with *H pylori* is scanty. There are only three papers^[15-17] from Japan where, on the published evidence, the prevalence is variable (36%-87%), and only one^[15] of these papers gives data for children; and two from Brazil where the prevalence is very high (80%) - one for children^[18] and one for adults^[19]. Regression analysis to determine whether length of follow-up is related to the conversion rates is inappropriate. However, it is clear that in Japan sero-conversion rates are only slightly lower than sero-reversion rates, 5/86 (5.8%) versus 2/22 (8.1%) in children and 66/1038 (6.4%) versus 149/2103 (7.1%) in adults, whereas in Brazil the rates of sero-conversion are high 5/78 (6.41%) in less than 2 years in children, 5/46 (10.87%) in 3 years in adults, while in children there was a zero reversion rate and in adults only 1 of 173 *H pylori*-positive subjects reverted.

There seems little doubt that the sero-conversion rate rises with the overall prevalence of the infection in the population, that where the prevalence is moderate the tendency to spontaneous cure overtakes the rate of new infections, but where the prevalence is high there is practically no spontaneous cure. These conclusions depend on the assumptions that sero-positivity means the presence of infection, sero-negativity means its absence.

The time periods of the quoted studies range from 20 mo to 32 years. It is tacitly assumed by the authors that sero-reversion and sero-conversion rates represent the averages of a slow, single rate in each direction. However, it is also conceivable that during these times changes in infection status might have occurred several times in both directions.

These reports seem to assume that serological evidence of the presence or absence of antibodies to *H pylori* indicates the presence or absence of the infection. The fact is that the presence of antibodies indicates exposure to the infecting organism in the past, but does not indicate current infection. Indeed, there are reports of positive serology in the absence of other positive tests for infection^[20-23]. Moreover there is a known time lag of 6-12 mo^[24-27] or even longer between eradication of infection and reversion of serology to normal^[20-23].

Evidence from direct tests: Histology and urea breath test

Only a few reports base their opinions on direct methods such as the urea breath test (UBT) or histology. There are two reports of children showing changes either way within 3 mo^[28,29] and one reporting such changes within 6 mo in both children and adults^[30] using the urea breath test. There are two reports based on histology in adult patients, one showing 5/39 patients becoming *H pylori* negative over a ten year period^[31], and another reporting 9% of patients becoming positive and 9% becoming negative over a 6 years period^[32]. However, there is some direct evidence that infection can be even more labile than the above evidence suggests, There is one significant report

in a Master of Surgery Thesis^[33] involving adults. Some aspects of this study have been reported^[34]. Two hundred and eight patients undergoing endoscopy for dyspepsia were categorized as *H pylori*-positive or -negative, using the biopsy-rapid urease, culture and polymerase chain reaction tests. The patients received no anti-*H pylori* treatment. The first hundred of these patients to volunteer (14 duodenal ulcer, 5 gastric ulcer, 16 oesophagitis, 46 non-ulcer dyspepsia (NUD) and 19 Others) were examined between 6 and 12 wk later and re-categorized as positive or negative, using a non-invasive ¹³C-urea breath test. Of 42 patients positive for *H pylori* at endoscopy, 8 (19%) had become negative at the later breath test; and of 58 patients negative at endoscopy, 15 (26%) had become positive at the later breath test. The results suggest that *H pylori*-status in the adult can alter in both directions within a few weeks. The PCR test was done at the time of the endoscopy but, at the time of the follow-up, because it was not clinically justifiable to repeat endoscopy, the UBT was used.

It may be criticised that the results from two different tests may not be comparable. There is considerable evidence, however, that PCR and UBT vie with each other as the gold standard for *H pylori*-status, and therefore are highly unlikely to give divergent results^[24-26,35-40]. Indeed, it has been shown that PCR results can be used to determine the optimal cut-off point for the breath test results^[41], and that both tests can be used to determine not only the presence of, but also the weight of infection with, the organism^[42]. The evidence from the later breath tests can, therefore, be relied on as at least as satisfactory as that from the PCR tests at the time of endoscopy. It follows that in this study during a period of 6 to 12 wk there was a 20%-25% change of *H pylori*-status in both directions.

The possible effect of gastric pH on *H pylori* infection

One possible explanation is that the ability of *H pylori* to colonize the stomach (and gastric-type epithelium in the duodenum) is dependent on the local luminal pH. Extremes of pH in either direction kill the organism^[43,44]. The patients with peptic ulcer (whether gastric or duodenal), with reflux oesophagitis, and some of those with other lesions would have received acid-suppression agents during the period between the two examinations, and this fact might explain why patients negative at endoscopy later became positive. There is evidence that acid-suppression promotes gastritis associated with *H pylori* infection^[45]. For movements in status in the opposite direction, in patients given a clean bill of health (NUD) or those in the group with diagnoses that did not seem related to gastric hyperacidity, the later withdrawal of acid-suppressing agents given prior to the endoscopy that excluded an ulcer might be the cause of the reversion from positive to negative. It is interesting to recall that, when Marshall^[46] in 1985 swallowed a culture of *H pylori*, he took 600 mg of cimetidine 3 h before to reduce the acidity. Thereafter, stomach acidity would have returned to normal and, whilst stomach biopsies taken one week later were positive for *H pylori*, those taken at two weeks had become negative.

CONCLUSION

The above findings show that the *H pylori*-status of adults can alter in both directions in a matter of a few weeks and that the infection is much more labile than previously realised. The known time lag of 6-12 mo^[22-25] between eradication of infection and reversion of serology to normal compared with 6 wk for UBT^[22], and the unknown time lag between the inception of infection and seroconversion, are features that cast some doubt on whether serology could have demonstrated this lability.

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Should nonalcoholic fatty liver disease be regarded as a hepatic illness only?

Giovanni Tarantino

Giovanni Tarantino, Section of Hepatology in Internal Medicine, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Italy

Correspondence to: Giovanni Tarantino, MD, Section of Hepatology in Internal Medicine, Department of Clinical and Experimental Medicine, Federico II University Medical School of Naples, Italy. tarantin@unina.it

Telephone: +39-81-7462024 Fax: +39-81-5466152

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Abstract

The highly increasing prevalence of obesity and type 2 diabetes mellitus in the general population makes nonalcoholic fatty liver disease the most common diagnosis in every-day practices. Lifestyle changes (mainly exercise withdrawal and weight gain) have probably heightened the prevalence of nonalcoholic fatty liver disease. Mortality in patients with Nonalcoholic Fatty Liver Disease is significantly higher when compared with that of the same age-gender general population. Hepatologists claim to bear a new burden, being Nonalcoholic Fatty Liver Disease strongly linked to systemic diseases.

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Key words: Nonalcoholic fatty liver disease; Metabolic syndrome; Diabetes mellitus; Obesity; Polycystic ovary syndrome; Obstructive sleep apnea; Cardiovascular disease

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SCENARIO

Nonalcoholic fatty liver disease (NAFLD) has recently emerged as the most common cause of abnormal laboratory liver tests (LLTs) seen in patients presented to practicing hepatologists, with the overall prevalence of NAFLD in the developed world estimated to be between 20% and 30%. Changes in lifestyle have resulted in a dramatic increase in the prevalence of NAFLD. Apoptosis and insulin resistance (IR) play an important

role in the disease development and progression. Our current knowledge of the natural history of NAFLD can be summarized as follows: elevated body mass index (BMI) plays a key role; simple steatosis NAFL (Fatty liver, FL) does not generally progress to Non-Alcoholic SteatoHepatitis (NASH); patients with NASH progress, in relatively many cases, to cirrhosis; older age and advanced fibrosis are risk factors for hepatocellular carcinoma (HCC) in NASH; up to a third of patients develop liver-related morbidity or mortality. It is extremely important that physicians diagnose NASH accurately and perform appropriate treatments, because it represents an illness mirroring a systemic process, and an adjunctive cardiovascular disease (CVD) risk^[1]. A great deal of research highlight the need for surrogate serum markers for diagnosing NASH. Among them, serum cytokeratin 18 has captured certain interest^[2].

COULD NAFLD BE CONSIDERED A FURTHER EXPRESSION OF METABOLIC SYNDROME?

NAFLD and NASH are conditions gaining increasing recognition, obesity being (mainly of high grade) one of the more important risk factors. But, do other aspects of metabolic syndrome (MS) play a role? To answer this question, a prospective study was conducted in 127 consecutive obese patients (62% female, mean age 40 ± 11 years, mean BMI 42 ± 6 kg/m²) undergoing gastric bypass over a 20-month period. The report highlighted that arterial hypertension was present in 52 patients (41%) and type 2 diabetes mellitus (DM) in 18 (14%). However, NAFLD was confirmed in 80 patients (63%). Of them, 47 (37%) had FL, and 33 (26%) had NASH. Cirrhosis was found in 2 patients corresponding to 1.6% of the total population. For multivariate analysis, elevated HOMA independently predicted only NASH, (OR 4.18, 95% Confidence Interval, CI, 1.39-12.49). That NAFLD was frequently found, it is easy to deduce that the NAFLD presence coupled with obesity (visceral) and hypertension could be used as criteria to label the patients with MS^[3].

ARE PATIENTS' AGE OR LONGEVITY OF ILLNESS A CRITERION TO INFER NASH PRESENCE?

To characterize the spectrum of NAFLD in morbidly

obese adolescents, a cross-sectional study correlated in 41 adolescent subjects' (mean age, 16 years, 61% female, 83% non-Hispanic white, mean BMI 59 kg/m², undergoing bariatric surgery) liver histology with clinical features and compared data with those of the adults. The Authors wondered if NAFLD would be less severe as a result of younger age and shorter duration of obesity and which grade of portal inflammation and fibrosis would be present. Eighty-three percent had NAFLD: 24% FL, 7% isolated fibrosis with steatosis, 32% non-specific inflammation and steatosis, and 20% NASH. Twenty-nine percent had fibrosis; none had cirrhosis. Abnormal ALT was more prevalent in NASH. Mean fasting glucose was higher in NASH, but the percentage of MS was not different. The researchers concluded that NAFLD was very prevalent in morbidly obese adolescents, but severe NASH was uncommon. In contrast to morbidly obese adults, lobular inflammation, significant ballooning, and perisinusoidal fibrosis were rare, whereas portal inflammation and portal fibrosis were more prevalent, even in those who did not meet criteria for NASH. Presence of MS in morbidly obese adolescents did not distinguish NASH from FL. Conclusively; the answer is... yes it is^[4].

Could being overweight (not necessarily obese) be considered a risk factor for the NAFLD progression?

The realization that a proportion of patients with NAFLD can progress through NASH and fibrosis to full-blown cirrhosis and HCC has recently focused the attention of the liver-disease scientific community on this condition, previously considered to be benign. Although several studies have been performed on risk factors (presented in a single or combined way) and natural course of NASH, it seems that NASH is inclined to be more than a disease confined to classic boundaries. The objective of a recent study was to assess the clinical features and risk factors for NASH patients in an Iranian population. Fifty three patients (21 female, mean age 37.8 ± 11.3 years) with histologically confirmed NASH entered the study. Twenty-six patients (55.3%) were overweight, 15 (31.9%) obese, 40 (75.5%) dyslipidemic, and three patients (5.7%) were diabetic. Liver histology showed mild steatosis in 35.7%, moderate steatosis in 53.6%, and severe forms in 10.7%. In 80.2% of patients, portal inflammation was present, and 9.4% had cirrhosis. The amount of increase in LLTs bore no relationship with fibrosis, portal inflammation, and degree of steatosis. The interesting point of this research dwells with the overweight, not the obese, as having a risk factor for NASH. Obviously, a careful history was taken regarding alcohol intake^[5].

And what is the role of visceral fat?

NAFLD is increasing rapidly in the population of the Asia-Pacific region, representing a good model of study. The aim of an up-to-date research was to define the anthropometric, metabolic and histological characteristics of patients with NAFLD in these countries. Seventy-five patients with persistently raised LLTs and/or FL detected on ultrasonography (US) with exclusion of other liver disorders were prospectively enrolled (39 men, mean

age 47.0 ± 12.2 years). Fifty eight patients (77.3%) had visceral obesity, 29 (38.7%) were diabetic and 15 (20.0%) had impaired glucose tolerance (IGT). Insulin resistance was diagnosed in 62 out of 64 (96.9%) patients. FL, NASH and cirrhosis were diagnosed in three (4.3%), 59 (84.3%) and eight (11.4%) of 70 patients, respectively. The complete histological spectrum of NAFLD was seen in these patients. The majority of them were characterized by IR, central obesity and had either type 2 DM or IGT^[6].

Is isolated portal fibrosis the link between FL and NASH?

NASH is a progressive form of NAFLD that can lead to hepatic fibrosis and cirrhosis. Until now, portal fibrosis in the absence of NASH, called isolated portal fibrosis (IPF), has received less attention and has not been classified as a spectrum of NAFLD, but, when found in patients with HCV-related chronic hepatitis it is considered to be a key feature of viral infection. Liver biopsies from 195 morbidly obese subjects who underwent bariatric surgery after excluding all other causes of liver disease were analyzed. The prevalence of fatty liver (FL) only, IPF, and NASH was 30.3%, 33.3%, and 36.4%, respectively. Again, hyperglycemia was the only metabolic parameter associated with NASH (OR, 5.4; 95% CI, 2.4-12) and IPF (OR, 2.8; 95% CI, 1.2-6.5). MS was identified in 78.5% of subjects, and a significant trend for the clustering of MS criteria was observed across the spectrum of FL, IPF, and NASH^[7].

Is the exact prevalence of NASH in the severely obese known?

NAFLD has been consistently associated with every grade of obesity and IR. As previously stated, NASH is a histological entity within NAFLD that can progress to cirrhosis. The prevalence of NASH in morbidly obese patients is a crucial point of study, because they represent a well-defined population. It is unclear whether differences in insulin sensitivity exist among subjects with NASH and FL. To evaluate the prevalence and correlates of NASH and consequently liver fibrosis in this distinctive cohort of ninety-seven subjects, a recent study was employed. Thirty-six percent of subjects had NASH and 25% had fibrosis. No cirrhosis was diagnosed on histology. Markers of IR and MS, but not BMI were associated with the presence of NASH. Forty-six percent of patients suffering from NASH had normal transaminases. Subjects with NASH had more severe IR when compared to those with FL. In conclusion, NAFLD is associated with MS rather than excess adipose tissue in the severely obese^[8].

Are laboratory liver tests worth using to screen NAFLD?

A fascinating study was conducted to determine whether the current liver screening program for NAFLD has sufficient evidence to justify its continued implementation. The LLTs program to detect NAFLD was performed on 411 Japanese workers utilizing serum ALT, AST, and gammaglutamyl transpeptidase (GTP). Subjects with viral and alcohol hepatitis were excluded from the evaluation. The diagnosis of NAFLD was based on US findings. The program was evaluated by efficacy and effectiveness in comparison with the BMI. Effectiveness, based on

the efficacy determinations, was assessed by means of the positive predictive value (PPV) test performance, the disease characteristics, and the program cost. The diagnostic performances of ALT and BMI were far from excellent. The areas under the curves of the two indices were 0.69 and 0.63, respectively. The PPV ranged from 15 to 28% where the prevalence of fatty liver was 12.3%. The cost of the program was estimated at \$4 U.S. dollars per person based on the medical reimbursement fee rate. The efficacy of the liver screening program was found to be insufficient and, therefore, revealed that BMI monitoring may provide a more suitable and inexpensive alternative. The authors challenge the effectiveness of the LLTs, considering the high price of the program^[9].

What is the risk of cancer in patients with NAFLD?

The relation between NAFLD and cancer risk is poorly understood. Using the population-based National Registry of Patients, some Authors examined the incidence of cancer in 7326 patients discharged with a diagnosis of NAFLD from a Danish hospital during 1977-1993. Overall, 523 cancers were diagnosed during 47 594 person-years of follow-up, yielding a 1.7-fold increased risk (95% CI, 1.6-1.9) compared with the Danish general population. The risk of primary liver cancer was markedly elevated in patients with alcoholic liver disease (ALD) as well as NAFLD with a standardized incidence ratio of 9.5 (95% CI, 5.7-14.8) and 4.4 (95% CI, 1.2-11.4), respectively. Patients with ALD also had substantially increased risks of several types of cancer associated with alcohol and tobacco use (cancers of the lung, pharynx, larynx, esophagus, and stomach) and a moderately increased risk for cancers of the colon and breast. Among patients with NAFLD, an increased risk of some alcohol- and tobacco-related cancers was seen, and there was also an increased risk of colon and pancreas cancer^[10].

Then, what is the natural history of NAFLD in the community?

Authors sought to determine survival and liver-related morbidity among community-based NAFLD patients. Four hundred twenty patients diagnosed with NAFLD in Olmsted County, Minnesota, between 1980 and 2000 were identified using the resources of the Rochester Epidemiology Project. Overall survival was compared with the general population of the same age and sex. Overall, 53 of 420 (12.6%) patients died. Survival was lower than the expected survival for the general population ($P = 0.03$). Higher mortality was associated with age (hazard ratio per decade 2.2; 95% CI 1.7-2.7) and impaired fasting glucose (hazard ratio 2.6; 95% CI 1.3-5.2). Liver disease was the third leading cause of death (as compared with the thirteenth leading cause of death in the general population), occurring in 7 (1.7%) subjects. Twenty-one (5%) patients were diagnosed as being affected by cirrhosis, and 13 (3.1%) developed serious complications^[11].

And what about NAFLD in atopic/non-atopic children?

NAFLD in non-obese Japanese children was observed in 3.2% of non-atopic children and in 17.6% of patients

with atopic dermatitis or suffering from bronchial asthma, allergic rhinitis, in 2000. NAFLD was studied by abdominal US scans. The prevalence of NAFLD was increasing annually, and it reached 12.5% in non-atopic children, 13.1% in patients with bronchial asthma, 13.7% in patients with allergic rhinitis, or 33.9% in patients with atopic dermatitis, in 2003. Since NAFLD in childhood may be a risk factor for lifestyle-related diseases in future, care should be taken to prevent it^[12].

It would have been interesting to know how many of those subjects were on steroid therapy.

Could polyunsaturated fatty acids be used to improve an immunological disease and an apparently distant illness of liver, i.e., NAFLD?

The higher incidence of inflammatory diseases in Western countries might be related, in part, to a high consumption of saturated fatty acids and n-6 polyunsaturated fatty acids (PUFA) and an insufficient intake of n-3 fatty acids. In an intriguing study, Balb/C mice were fed for 3 wk either n-6 or n-3 PUFA-fortified diets. After inducing a contact or an atopic dermatitis, immunological parameters were analyzed by the authors to evaluate the anti-inflammatory potential of these n-3 PUFA. Accordingly, n-3 PUFA lessened innate and specific immune responses through inhibition of TH1 and TH2 responses, increase of immunomodulatory cytokines such as IL-10, and regulation of gene expression. Furthermore, reduction in edema, leukocyte infiltration, and enhancement of antioxidant defenses in the inflamed ears of mice from both models proved n-3 PUFA efficacy. Authors' data suggest that dietary fish oil-derived n-3 fatty acids could be useful in inflammatory disorders^[13].

On the other hand, is obesity a chronic low-grade inflammatory process or not? Because eicosapentaenoic acid, docosahexaenoic acid, and gamma-linolenic acid have anti-inflammatory, as well as lipid-modifying properties, the effects of supplement mixtures of these PUFA should have received much more attention by researchers in the field of NAFLD treatment.

What have two peculiar syndromes to do with NAFLD?

NAFLD and polycystic ovary syndrome (PCOS) are both associated with IR. Thus, women with PCOS may have an increased prevalence of NAFLD, including NASH. To determine the prevalence and characteristics of NASH and abnormal ALT in women with PCOS were retrospectively studied 200 women with PCOS, diagnosed with irregular menses and hyperandrogenism. Fifteen percent (29 out of 200) had AST and/or elevated ALT. Women with aminotransferase elevations had lower high-density lipoprotein (HDL), $P = 0.006$, higher triglycerides ($P = 0.024$), and higher fasting insulin ($P = 0.036$) compared with women with normal aminotransferases. Six women had NASH with fibrosis. The authors concluded that abnormal aminotransferase activity is common in women with PCOS and suggest a reflection of whether to screen PCOS women for liver disease at an earlier age than is currently recommended for the general population^[14].

Recently, obstructive sleep apnea (OSA) has been proposed as an independent risk factor for IR. The

objectives of this study were to document the prevalence of SOSA in patients with NAFLD and to determine whether prevalence rates for SOSA differ in NAFL versus NASH patients. The prevalence of OSA was similar in both biochemically ($P = 0.66$) and histologically ($P = 0.11$) defined NAFL and NASH patients. Other risk factors for NAFLD such as BMI, cholesterol and triglyceride levels, and prevalence of diabetes were also similar in the two groups. Approximately one-half of NAFLD patients, whether NAFL or NASH, have OSA^[15].

Could abnormalities in the estradiol to testosterone ratio (increased androgens and decreased estrogens) lead to insulin resistance and NASH?

Recently, the fourth case has been presented of an adult man (29 years old) affected by aromatase deficiency resulting from a novel homozygous inactivating mutation of the CYP19A1 (P450-aromatase) gene. The patient showed also a complex dysmetabolic syndrome characterized by IR, type 2 DM, acanthosis nigricans, NASH, and signs of precocious atherogenesis. The analysis of the effects induced by the successive treatment with high doses of testosterone, alendronate, and estradiol allows further insight into the roles of androgens and estrogens in several metabolic functions. High doses of testosterone treatment resulted in a worsening of IR and type 2 DM. Estrogen treatment resulted in an improvement of acanthosis nigricans, IR, and NASH, coupled with a better glycemic control and the disappearance of two carotid plaques. Data from this case provided new insights into the role of estrogens in glucose, lipid, and liver metabolism in men^[16].

CONCLUSION

For many years, research concerning acute and chronic liver diseases have been mostly confined to few areas of Medicine, i.e., virology and immunology. Vice versa, for the first time in decades, the liver comes back to have a pivotal role in Health Sciences, embracing endocrine, cardiovascular and oncological diseases, so the answer to the initial question, "Should nonalcoholic fatty liver disease be regarded as a hepatic illness only?," is... It should not!

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Nathan Subramaniam, PhD, Series Editor

HFE gene in primary and secondary hepatic iron overload

Giada Sebastiani, Ann P Walker

Giada Sebastiani, Venetian Institute of Molecular Medicine (VIMM), Padova and Digestive Diseases, Hepatology and Clinical Nutrition Department, Umberto I Hospital, Venice, Italy
Ann P Walker, Department of Medicine, University College London, London, United Kingdom

Correspondence to: Dr. Giada Sebastiani, Venetian Institute of Molecular Medicine (VIMM), Padova and Digestive Diseases, Hepatology and Clinical Nutrition Department, Umberto I Hospital, Venice, Italy. giagioseba@iol.it

Telephone: +39-49-8212293 Fax: +39-49-8211826

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Abstract

Distinct from hereditary haemochromatosis, hepatic iron overload is a common finding in several chronic liver diseases. Many studies have investigated the prevalence, distribution and possible contributory role of excess hepatic iron in non-haemochromatotic chronic liver diseases. Indeed, some authors have proposed iron removal in liver diseases other than hereditary haemochromatosis. However, the pathogenesis of secondary iron overload remains unclear. The High Fe (*HFE*) gene has been implicated, but the reported data are controversial. In this article, we summarise current concepts regarding the cellular role of the *HFE* protein in iron homeostasis. We review the current status of the literature regarding the prevalence, hepatic distribution and possible therapeutic implications of iron overload in chronic hepatitis C, hepatitis B, alcoholic and non-alcoholic fatty liver diseases and porphyria cutanea tarda. We discuss the evidence regarding the role of *HFE* gene mutations in these liver diseases. Finally, we summarize the common and specific features of iron overload in liver diseases other than haemochromatosis.

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Key words: Hereditary haemochromatosis; Chronic liver diseases; Chronic hepatitis C; Hepatic iron overload; *HFE* gene

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INTRODUCTION

Iron is an essential element for living cells because it is

a cofactor for enzymes of the mitochondrial respiratory chain and it co-ordinates the binding of oxygen by myoglobin and haemoglobin. However, excess iron is toxic, causing increased oxidative stress; the production of reactive oxygen species is thought to be responsible for the observed oxidation of lipids, proteins and nucleic acids. Thus, iron overload can cause serious damage to organs including the liver, heart, joints and endocrine glands.

Hereditary haemochromatosis (HH) is the paradigm of heavy iron overload which can eventually lead to multiple organ failure. HH is an inherited disease of iron metabolism^[1]. Absorption of dietary iron is inappropriately high in relation to body iron stores. This leads to increased deposition of iron, predominantly in parenchymal cells of the liver, heart, joints, pancreas and other endocrine organs. Undiagnosed and untreated, iron deposition can cause hepatocellular injury, activation of hepatic stellate cells (HSCs) and increased production of collagen and other components of the extracellular matrix^[2]. The liver is the major site for storage of excess iron, which probably explains the increased risk of hepatocellular carcinoma (HCC) in HH^[3].

In non-haemochromatotic liver diseases, hepatic iron may worsen liver injury or hepatic fibrosis. This has been investigated in common chronic liver diseases (CLDs) such as chronic hepatitis C (CHC) and alcoholic and non-alcoholic fatty liver diseases. In these CLDs, the progression towards end-stage liver disease is often unpredictable; many cofactors have been proposed to explain this variability. Iron overload has been proposed as such a cofactor, but its exact role remains unclear^[4]. Iron has also been implicated in the progression of hepatitis B virus (HBV) infection^[5], but this has not been widely studied. Other conditions that may cause CLD and that have been associated with iron overload include porphyria cutanea tarda (PCT) and insulin resistance-associated hepatic iron overload (IR-HIO) syndrome^[6,7]. The IR-HIO syndrome encompasses iron overload with hyperferritinaemia and normal transferrin saturation, type 2 diabetes mellitus and non-alcoholic steatohepatitis (NASH). In these forms of secondary (acquired) iron overload, however, the hepatic iron concentration is generally lower than that seen in HH. Although major advances have been made in understanding the pathogenesis of primary iron overload in HH, the mechanism(s) whereby pre-existing CLD may lead to iron overload remain unclear. In HH, there is a strong relationship with missense mutations in the *HFE* (High Fe) gene^[8]. In non-haemochromatotic diseases, several studies have investigated the possible role of *HFE* mutations in the pathogenesis of hepatic

iron overload, with somewhat discordant results. This review aims to describe the prevalence and role of *HFE* mutations in secondary iron overload. It will discuss the effects of moderate iron excess on the natural history and its possible relevance to therapeutic approaches in CLDs other than HH.

NOMENCLATURE OF IRON OVERLOAD

Hereditary, or primary, haemochromatosis is an inherited iron storage disease. Secondary iron overload is acquired as a result of another disease. The nomenclature and classification of iron overload states is shown in Table 1. Primary haemochromatosis may be due to mutations in the *HFE* gene, or to mutations in genes other than *HFE*. The main causes of acquired iron overload are haemolytic anaemias and CLDs. Untreated HH may develop severe iron overload, whereas in secondary iron overload due to CLDs, minimal to modest iron overload is usually seen^[2].

GENETICS OF PRIMARY IRON OVERLOAD

There are two common mutations of the *HFE* gene. The first results in a change of cysteine at position 282 to tyrosine (C282Y); the second results in a change of histidine at position 63 to aspartate (H63D). Numerous studies have shown that homozygosity for C282Y is associated with typical phenotypic HH in Caucasians. C282Y homozygosity ranged from 64% of Italian haemochromatosis cases up to 100% of cases in Australia^[9-11]. A recent study of almost 100 000 North American primary care patients analysed the distribution of *HFE* mutations in a racially diverse group^[12]. It confirmed that homozygosity for C282Y is most common in whites, consistent with the hypothesis that this mutation originated in a Caucasian “founder” individual. Individuals who are compound heterozygous for C282Y and H63D may also have iron overload in the range diagnostic for haemochromatosis, although the penetrance of this genotype is lower than for C282Y homozygosity^[13-15]. The H63D mutation is variably distributed worldwide. It is more prevalent than the C282Y mutation: approximately one in five of the European population are estimated to be H63D heterozygotes^[9,16]. The C282Y/H63D compound heterozygous and H63D homozygous genotypes have mostly been associated with only biochemical evidence of mild iron overload. The clinical penetrance of these genotypes is low although there have been reports of varied phenotypic presentation^[15,17].

A third point mutation (S65C) of the *HFE* gene was also identified. It was originally considered to be a neutral polymorphism, not associated with increased transferrin saturation^[18]. However, other evidence implicated the mutation in mild iron overload^[19,20]. Other, rare mutations have been described. Two missense mutations in exon 2 of the *HFE* gene (I105T and G93R) were detected in haemochromatosis patients with atypical *HFE* genotypes, such as heterozygosity for C282Y, H63D or S65C^[21]. A splice-site *HFE* mutation (IVS3+1G/T) that prevented normal mRNA splicing was identified in a patient with classical HH who was heterozygous for the C282Y muta-

Table 1 Nomenclature for iron overload states

Primary iron overload-hereditary haemochromatosis (HH)
<i>HFE</i> -associated HH
1 C282Y homozygosity
2 C282Y/H63D compound heterozygosity
3 Other mutations
Non <i>HFE</i> -associated HH
1 Juvenile haemochromatosis
2 Tfr2-related haemochromatosis
3 Autosomal dominant haemochromatosis
Secondary iron overload-acquired
Iron-loading anaemias
1 Thalassaemia major
2 Sideroblastic anaemia
3 Chronic haemolytic anaemias
Chronic liver diseases
1 Hepatitis C
2 Alcoholic liver disease
3 Non-alcoholic steatohepatitis
4 Porphyria cutanea tarda
5 IR-HIO
6 Post-portacaval shunting
7 Transfusional and parenteral iron overload
8 Dietary iron overload
Miscellaneous
1 Iron overload in sub-Saharan Africa
2 Neonatal iron overload
3 Acaeruloplasminaemia
4 Congenital atransferrinaemia

tion. This highlighted the possibility that other rare *HFE* mutations could explain the classical HH phenotype, particularly in C282Y heterozygotes with iron overload^[22]. In this review, we will consider only the two most frequent *HFE* mutations, C282Y and H63D. The other mutations are rare, thus they are unlikely to play a major role in CLDs other than HH.

HH has been described as the most common monogenic disorder in Celtic populations. Certainly, homozygosity for the C282Y mutation occurs in about 1 in 300 people of Northern European origin, with an estimated carrier (heterozygote) frequency of 1 in 10^[8]. However, the C282Y homozygous genotype has incomplete penetrance: not all patients with this genotype show an iron overload-related phenotype. The penetrance of HH is influenced by a variety of factors. Increasing age increases the penetrance, as the body has no means of active iron excretion, so iron accumulation progresses with time. Male gender also increases the penetrance, as women may be partially protected against iron overload by iron losses incurred in childbirth and menstruation. Dietary iron content may also influence penetrance. There has been considerable debate regarding the penetrance of HH. The “biochemical penetrance” of the C282Y homozygous state is generally agreed to be high, particularly in older males, but disease-related morbidity is less frequent. A study of almost a third (3011) of the residents of Busselton, Australia showed that four (all males) of the 16 C282Y homozygotes detected had fibrosis and/or cirrhosis. If attributable to iron overload, this equates to a disease-related morbidity of 25%^[23]. In contrast, recent large studies have reported lower penetrance. Beutler and colleagues studied over

40 000 individuals attending a health appraisal clinic, where expression of haemochromatosis was investigated by questionnaire. The results for C282Y homozygotes were compared with the control group who were wild type for C282Y and H63D. The clinical penetrance of ill health or shortened lifespan in C282Y homozygotes was reported to be less than 1%^[24]. Various factors, such as the exclusion of most patients who had a previous diagnosis of haemochromatosis from the assessment of ill health, would have tended to decrease this calculated clinical penetrance of C282Y homozygosity^[25]. Despite this, a similar, low value was obtained in a survey of about 1 000 000 individuals in two health authority regions in South Wales. It was concluded that a diagnosis of haemochromatosis had been made for only 1.2% of adult C282Y homozygotes. When only male homozygotes older than 45 years were considered, the diagnosis rate rose to 2.8%^[26]. Thus, the “severity” of the definition of penetrance (disease related morbidity or clinical expression as ill health/shortened life span) is an important factor influencing the observed value. It seems that the clinical penetrance of C282Y homozygosity is low.

HFE PROTEIN AND CELLULAR IRON HOMEOSTASIS

The predicted HFE protein has sequence homology to the major histocompatibility complex (MHC) class I molecules, a family of transmembrane glycoproteins of the immune system, which present peptide antigen to T cells^[8]. Like the class I proteins, HFE is made up of three extracellular loops (α_1 , α_2 , α_3)^[27], a transmembrane domain and a short cytoplasmic C-terminal region. Two disulphide bridges stabilize the tertiary structure of the HFE protein. One of these gives rise to the α_3 loop, that is crucial for interaction with β_2 -microglobulin and for translocation to the cell surface^[28,29]. The C282Y mutation prevents formation of this disulphide bond, thereby inhibiting correct cell surface expression of HFE^[8]. The H63D mutation causes a histidine to be replaced by aspartate in the α_1 domain^[8]. H63D is generally considered to have a milder effect on body iron stores than C282Y.

The role of HFE in cellular iron homeostasis remains only partially understood. Co-immunoprecipitation experiments showed that the HFE- β_2 -microglobulin complex interacted with transferrin receptor 1 (TfR1) in human placental membrane preparations^[30] and in human embryonic kidney cells over-expressing wild type HFE^[31]. Immunohistochemical studies of human duodenum showed that TfR1 staining overlapped that of HFE in the crypt cell enterocytes, consistent with the co-trafficking of these proteins^[32] (Figure 1). These studies provided a link between HFE and the transferrin-mediated endocytosis pathway of iron uptake into cells. Under conditions of iron excess, circulating iron-saturated transferrin is understood to bind the TfR1-HFE- β_2 -microglobulin complex on the cell surface, which is internalised within an endosome. At the acidic endosome pH, iron dissociates from transferrin and enters an intracellular chelatable iron pool. The transferrin-TfR1 complex recycles to the cell

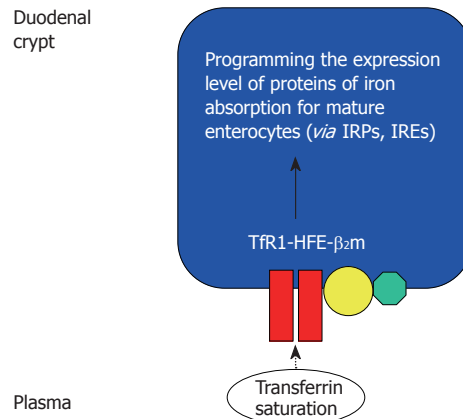


Figure 1 The duodenal “crypt cell hypothesis” of HFE function^[36]. HFE at the basolateral membrane of duodenal crypt cells co-localizes with β_2 -microglobulin and TfR1. The saturation of circulating transferrin, which reflects body iron stores, was proposed to be “sensed” by the TfR1-HFE- β_2 -microglobulin complex, through transferrin-mediated endocytosis. Wild type HFE was proposed to facilitate transferrin-mediated iron uptake. In haemochromatosis, deficiency of functional HFE would therefore decrease the iron pool within the crypt cell, despite increased body iron stores. This would increase the activity of iron responsive proteins (IRPs), leading to increased expression of genes involved in iron absorption, such as DMT1 and ferroportin. This could contribute to the iron overload seen in HH^[27,34,35]. However, recent evidence suggests that HFE may play more important roles in influencing iron metabolism in the liver.

surface, where transferrin dissociates at the pH of blood, around pH 7.4^[27,33].

However, investigations of the HFE-TfR1 interaction and its potential effect on iron metabolism in transfected cell lines gave conflicting results. Several studies indicated that overexpression of HFE may ultimately reduce iron uptake into cells *via* transferrin-mediated endocytosis^[34]. Biosensor- and radioactivity-based assays indicated that HFE competes with transferrin for binding to TfR1^[35]. Conversely, in stably transfected Chinese hamster ovary cells, HFE increased the rate of TfR1-mediated iron uptake and cellular iron concentrations, but only when co-transfected with β_2 -microglobulin^[36]. Also, in primary macrophages from haemochromatosis patients, transfection of the wild type HFE gene increased uptake of ⁵⁵Fe-transferrin and accumulation of ⁵⁵Fe in the ferritin iron pool^[37]. These latter studies are consistent with the observation that in haemochromatosis, duodenal enterocytes are paradoxically iron-deficient^[38].

Several hypotheses have been advanced to model the possible roles of HFE in the regulation of iron metabolism. It was originally proposed that the TfR1-HFE- β_2 -microglobulin complex on the basolateral surface of the duodenal crypt cell may sense the transferrin saturation (Figure 1). Where wild type HFE was present, the transferrin saturation was proposed to determine the crypt intracellular iron concentration, thereby setting the activity of iron responsive proteins 1 and 2 and ultimately the expression levels of key proteins of iron absorption in the mature enterocyte^[36]. The pathway of iron absorption in the mature enterocyte is shown in Figure 2. Duodenal cytochrome b ferric reductase (Dcytb) is expressed on the luminal surface of the enterocyte and reduces dietary ionic iron from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state.

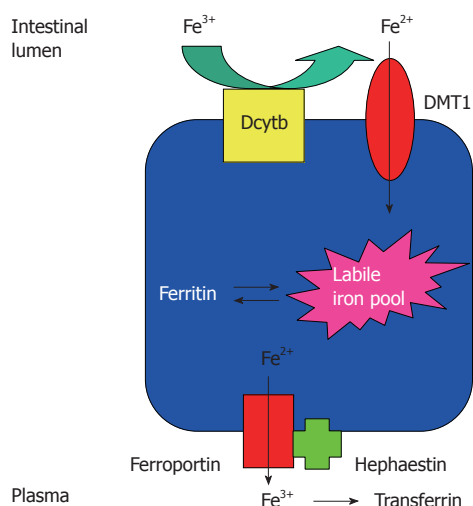


Figure 2 The pathway of iron absorption in the mature duodenal enterocyte. Dietary ferric iron is reduced to the ferrous state by ferric reductase(s), for example duodenal cytochrome b (Dcytb), which is expressed on the luminal surface of the enterocytes. Ferrous iron is taken up via DMT1 into the labile iron pool. Iron may be stored within the cell as ferritin or transferred across the basolateral membrane to the plasma by ferroportin. The exported iron is oxidized to the ferric state by hephaestin; ferric iron is then avidly bound by circulating transferrin^[39].

The ferrous iron is then taken up by the apical transporter, divalent metal transporter 1 (DMT1). Iron may be stored within the cell as ferritin or exported across the basolateral membrane by ferroportin. The exported iron is re-oxidized to the ferric state by hephaestin. Upon exiting the villus enterocyte, ferric iron can be avidly bound by circulating transferrin^[39].

The discovery of the peptide hormone hepcidin^[40-42] revived an alternative hypothesis, that the liver may be central to the regulation of iron metabolism^[43-46]. Hepatocytes act as a storage reservoir for iron, taking up dietary iron from the portal circulation and, in iron deficiency, releasing iron into the hepatic circulation. The liver is also the site of high expression of hepcidin, *TfR2* and haemojuvelin, three genes which when mutated result in HH.

Hepcidin is produced in the liver in response to dietary iron loading^[42]. Mutation of the hepcidin gene results in juvenile haemochromatosis, characterised by early onset and a rapid rate of iron loading^[47]. As in hepcidin-related haemochromatosis, inappropriately low hepcidin levels in relation to body iron stores are also seen in haemochromatosis resulting from mutation of the *HFE*, *TfR2* and haemojuvelin genes, suggesting that hepcidin may be the common pathogenic mechanism in haemochromatosis^[43,48]. Therefore, *HFE*, *TfR2* and haemojuvelin proteins may all be involved in the pathway sensing iron overload that leads to hepcidin synthesis. Experiments using cultured cells indicated that hepcidin may act by interaction with ferroportin, causing its degradation and reducing cellular iron export^[49]. The overall effect would be to “trap” iron within enterocytes, hepatocytes and macrophages. However, other studies, using parenteral injection of hepcidin into mice and analysis of iron absorption in tied off lengths of duodenum, found that hepcidin inhibited the uptake

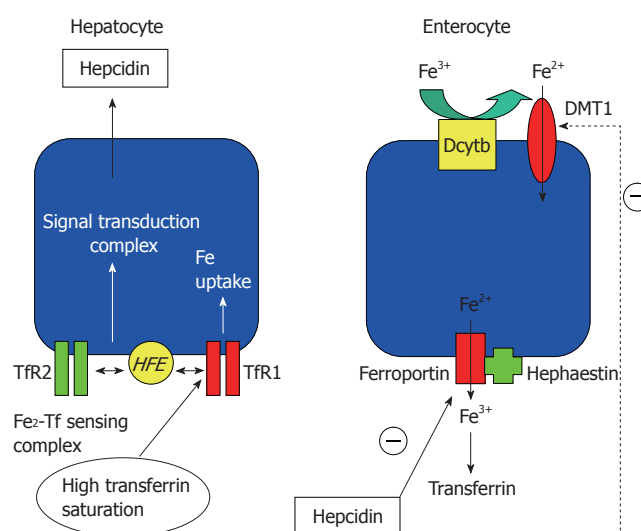


Figure 3 Current concepts regarding hepatic regulation of iron metabolism. Left panel, hepatocyte. At normal transferrin saturation, TfR1 may sequester HFE^[19]. At increased transferrin saturation, diferric transferrin competes with HFE for binding to TfR1^[55]. Freed HFE is proposed to bind TfR2; the complex conveys transferrin saturation status via a cytoplasmic signal transduction complex, leading to synthesis and secretion of hepcidin. In HH, mutations in the genes encoding HFE, TfR2, haemojuvelin or hepcidin may all disrupt this sensing system, leading to deficient hepcidin production and iron overload^[53]. Right panel, enterocyte. Circulating hepcidin may reduce iron absorption by interacting with ferroportin, causing its internalization and degradation^[49] and/or by reducing DMT1 expression^[50].

step of iron absorption. This did not require HFE. In this study, hepcidin did not influence the proportion of iron transferred into the circulation^[50]. Therefore it is not yet clear whether hepcidin acts at ferroportin in the enterocyte basolateral membrane, at DMT1 in the apical membrane, or both (Figure 3).

Because hepcidin is synthesised in hepatocytes, they have been proposed as the site of an iron sensing mechanism. TfR2 is expressed mainly in hepatocytes, haematopoietic cells and crypt cells of the duodenum, which are all also sites of HFE expression^[51,52]. Recent expression studies of intact HFE and TfR2 proteins in cultured cells showed interaction between these two proteins^[53]. It was proposed that when transferrin saturation is in the normal range, TfR1 may sequester HFE^[54]. At increased serum transferrin saturation, diferric transferrin has been shown to compete with HFE for binding to TfR1^[55]. Freed HFE is proposed to act as an iron sensor, binding TfR2 and conveying transferrin saturation status via TfR2 and a cytoplasmic signal transduction complex, leading to the synthesis of hepcidin (Figure 3). Haemojuvelin may form part of the signal transduction system. In HFE-, TfR2- or haemojuvelin-related haemochromatosis, mutations may disrupt this sensing system, leading to the observed deficiency of hepcidin; iron loading would therefore result^[53]. Thus, latest ideas propose that HFE-TfR2 interaction in hepatocytes may sense transferrin saturation, as an index of body iron status, determining production of hepcidin, which governs iron absorption^[53].

IRON AND *HFE* MUTATIONS IN CHRONIC HEPATITIS C

CHC remains a major health problem with around 200 million individuals affected worldwide^[56]. The natural course of CHC is characterised by progressive fibrosis in the inflamed liver with cirrhosis and haemodynamic changes which may be followed by end-stage complications^[57]. The progression of fibrosis in CHC is highly variable. Several factors may favour progression, including alcohol, young age at the time of infection and male gender. The role of iron in the pathogenesis of CHC has been debated. An association between iron and viral hepatitis was first described by Blumberg and colleagues^[5]. Following those observations, several studies noted elevated serum iron indices in CHC^[58-60]. Iron has been proposed as a cofactor that may both promote the progression of liver disease and reduce the response to antiviral therapy^[56,61,62]. Mechanisms proposed include production of reactive oxygen species, increased fibrogenesis through activation of HSCs and impairment of the host immune response^[63]. Several studies of CHC reported hepatic iron deposits in HSCs, which may contribute to liver damage^[64,65]. Many studies have also tried to investigate the pathogenesis of iron overload in CHC. Necroinflammation due to ongoing viral infection is considered the most important cause of iron overload. Additionally, the viral infection *per se* or the associated activation of cytokines have been proposed to modify iron metabolism in liver cells^[60,66]. A recent study showed that TfR1 expression was increased in CHC hepatic tissue irrespective of the degree of hepatic iron overload. This might, therefore, contribute to the accumulation of hepatic iron in CHC^[67]. Viral genotype may be another factor. In a large study of 242 patients, one of us analyzed the relationship between hepatitis C virus (HCV) genotypes and liver iron deposits in CHC^[68]. We found a higher prevalence of hepatic iron deposits in HCV-3 infected cases, concluding that hepatic iron deposition is HCV-genotype dependent. Indeed, the distribution of hepatic iron was mostly parenchymal in HCV-3 cases, while in non-HCV-3 patients, hepatic iron deposits were more frequently detected in the reticuloendothelial cells. These findings support the hypothesis that the expression of HCV-3 proteins in infected hepatocytes might cause specific metabolic changes resulting in enhanced oxidative stress. The non-HCV-3 cases had more severe fibrosis. Therefore their lesser hepatic iron deposits might reflect a more advanced stage of liver inflammation. A further relationship between viral genotype and iron was found in another study: Izumi and colleagues reported higher hepatic iron concentrations in HCV-1b compared with HCV-2 infected cases^[69].

A role of proteins that are involved in iron homeostasis has also been hypothesized. In a detailed histopathological study, Corengia and colleagues investigated *HFE* genotypes and hepatic iron score in hepatitis C^[70]. They found a significant relationship between *HFE* genotypes and iron deposition in hepatocytes. This suggested that some iron accumulation in CHC derives from increased iron absorption due to mutated *HFE* protein. Hecpudin has also been implicated. A study by Aoki and colleagues

evaluated the possible role of hepcidin in determining iron overload in hepatitis C^[71]. In patients with HCV, they demonstrated that hepatic hepcidin mRNA correlated with hepatic iron concentration (HIC). This suggested that iron stores regulate hepcidin expression normally; iron loading in CHC is not due to inappropriate hepcidin expression.

Serum iron indices are frequently abnormal in patients with CHC. Elevation of the serum ferritin concentration has been reported in 20%-60% of patients with chronic hepatitis C^[59,68,72,73]; stainable hepatic iron deposits were detected in 3%-38%. Furthermore, many cases of CHC, including those with elevated serum ferritin, elevated transferrin saturation, or both, showed no significant increase in HIC^[59,61,68,73-74]. In most of these studies, hepatic iron overload was generally mild to moderate and often not sufficient to be hepatotoxic *per se*^[61,75,76]. Elevated serum ferritin concentration in CHC may be explained by several associated conditions, in the absence of iron overload. Serum ferritin could be elevated as an acute phase protein because of the cytotoxic necroinflammation that is common in hepatitis C. Moreover, hepatic steatosis is frequent in patients with hepatitis C^[77,78] and has been associated with raised levels of serum ferritin^[79,80]. A recent study suggested that increased serum ferritin in CHC may be mainly due to diabetes mellitus, which is commonly associated with CHC^[81]. In contrast, other observations from one of us suggested that elevated serum ferritin in CHC may be mostly multifactorial^[82].

The distribution of iron has been examined in several studies, with some discrepancies. Some studies reported iron deposits mostly in hepatocytes^[70,83,84], others in reticuloendothelial cells^[85-87] and others reported a mixed distribution^[68,88,89]. This is a notable difference with HH where, until iron loading is severe, hepatic iron deposition is almost exclusively parenchymal.

The role of *HFE* mutations as a risk factor for iron overload in CHC has been studied in different populations, with somewhat discordant results (Table 2). Piperno and colleagues studied 110 Italian patients with chronic B or C viral hepatitis; they found that all male heterozygotes for the C282Y mutation had iron overload. The H63D mutation was significantly more frequent in patients with marked hepatic siderosis than in those with mild or no siderosis and in controls^[73]. A study of 137 CHC patients from the North of England reported that patients who carried the C282Y mutation had higher serum iron indices and more frequently had stainable hepatic iron, together with more advanced fibrosis or cirrhosis, than patients without the C282Y mutation^[90]. Similar results were obtained in other studies which correlated the presence of *HFE* mutations with increased serum iron indices, hepatic iron deposits and severe hepatic fibrosis^[70,91-94]. In an Austrian study of 184 patients with CHC versus 487 controls, Kazemi-Shirazi and colleagues found that serum iron indices were increased in patients carrying *HFE* mutations. In contrast with other studies, however, there was no evidence for more hepatic siderosis or advanced fibrosis in patients with *HFE* mutations^[95]. Other studies reported no relationship of *HFE* mutations with hepatic iron deposits and severe hepatic fibrosis^[83,89]. In a Scottish study, Thorburn and colleagues prospectively investigated

Table 2 Studies of the relationship between chronic hepatitis C, iron and *HFE* mutations

Reference	Cases <i>n</i>	Ethnicity	<i>HFE</i> relationship with serum iron indices	<i>HFE</i> relationship with HI	<i>HFE</i> relationship with fibrosis
[70]	206	Italian	Yes	Yes	Yes
[83]	120	Mostly Swiss and Italian	No	No	No
[88]	242	Mostly Caucasian	No	No	No
[89]	164	Mostly Caucasian	No	No	No
[90]	137	Caucasian	Yes	Yes	Yes
[91]	135	Brazilian	Yes	Yes	Yes
[92]	119	Mostly Caucasian, non-Hispanic	Yes	Yes	Yes
[93]	316	Mostly White	Yes	Yes	Yes
[94]	401	Mostly German	Yes	Yes	Yes
[95]	184	White, non-Hispanic	Yes	No	No
[96]	273	NA	Yes	Yes (only H63D)	No
[97]	1051	Mostly White, non-Hispanic	Yes	Yes	No

NA: not available; HI: hepatic iron (histological and/or biochemical evidence).

164 consecutive patients with HCV infection. They did not find a role for *HFE* mutations in the accumulation of iron or the progression of liver disease^[89]. Overall, only a few studies have suggested an increased prevalence of *HFE* mutations in CHC patients, with respect to the general population^[90,92,95]; this observation was not confirmed in other studies^[73,91,94].

A role for the H63D mutation in the iron overload of CHC was proposed in a few of the studies. Lebray and colleagues found that the histological hepatic iron score was higher in patients who were homozygous or heterozygous for H63D; this was surprisingly associated with an increased response rate to antiviral therapy^[96]. Another study reported increased hepatic iron deposits in male patients carrying the H63D mutation^[97]. Thus, the role of the H63D mutation is unclear and, as observed in HH, is minor with respect to the C282Y mutation.

Several studies suggested that higher levels of serum ferritin or HIC were associated with a diminished likelihood of response to antiviral therapy^[84,85,97]. Data from a recent Italian multicenter study indicated that iron removal by phlebotomy improved the rate of response to interferon^[98]. The influence of *HFE* mutations on the outcome of antiviral treatment has been investigated in a few studies. Chapman and colleagues suggested that a single mutation in the *HFE* gene had no impact on the outcome of interferon treatment; this was confirmed by subsequent reports^[99-101]. However, a study of 242 patients found that presence of the C282Y mutation was positively correlated with sustained response in a multivariate analysis^[88]. The reports by Lebray *et al* and Distant *et al* suggest that *HFE* may be part of a pattern of host genes which together influence response to antiviral therapy^[88,96]. Indeed, clearance of HCV is believed to be associated with different HLA alleles; the C282Y mutation of the

HFE gene is part of an extended founder haplotype which includes the HLA region on the short arm of chromosome 6^[101]. The positive effect of the C282Y mutation may reflect linkage disequilibrium between the *HFE* mutated allele and alleles at other loci implicated in the virological response, close to the *HFE* gene on chromosome 6. Several MHC class I and II loci have been shown to be associated with a sustained virological response to interferon treatment^[102-105]. Similarly, an American study investigated the role of iron overload and *HFE* mutations in the response to antiviral therapy in over 1000 patients with advanced CHC^[97]. The authors found that *HFE* mutations correlated with histological hepatic iron score in CHC. Subjects harbouring *HFE* mutations, particularly H63D, had significantly higher likelihood of both on-treatment virological responses (at 24 and 48 wk) and sustained virological responses (24 wk after the end of lead-in therapy) to re-treatment with pegylated interferon alpha-2α plus ribavirin. Again, both the *HFE* mutation and/or associated genetic variants were considered as possible causes of the improved response to therapy.

In conclusion, elevation of serum iron indices and hepatic iron deposits are a common feature in CHC. Hepatic iron overload is generally mild to moderate and it rarely reaches the severity seen in HH. In contrast to HH, the intrahepatic iron accumulation is generally mixed, with both parenchymal and reticuloendothelial distribution. The exact mechanism of hepatic iron accumulation in hepatitis C is still not clear. The pathogenesis is likely to be multifactorial and viral and host factors have been evaluated. The viral factors suggested by several studies include necroinflammation due to viral infection, direct influence on iron homeostasis mediated by cytokines, or HCV genotypes. Among the host factors, a role for *HFE* mutations has been proposed and extensively evaluated. On the basis of current knowledge, we conclude that *HFE* mutations may have a role in the elevation of serum iron indices and hepatic iron deposition observed in CHC, but they do not fully explain the observed abnormalities of iron homeostasis. Iron may also contribute to the rate of response to antiviral therapy.

IRON AND *HFE* MUTATIONS IN CHRONIC HEPATITIS B

Chronic hepatitis B (CHB) remains a serious global health concern. Approximately 350 million people are chronically infected, and 500 000 to 1.2 million deaths per year are attributed to HBV-associated complications^[106]. Among patients with active viral replication, cirrhosis will develop in 15%-20% within five years^[107]. For patients with cirrhosis, acute exacerbation can occur and the disease may progress to end stage complications^[107]. The histopathological pathway of progressive liver disease is characterised by fibrosis leading to increasing distortion of the hepatic architecture, that is the hallmark of evolution to cirrhosis. Liver fibrosis is the result of chronic injury and plays a direct role in the pathogenesis of hepatocellular dysfunction and portal hypertension. The progression of liver fibrosis is due to many viral and host

factors. In CHC it has been proposed that iron may be a cofactor, but data for patients with CHB are more scarce. An association between iron and hepatitis B was first described by Blumberg and colleagues^[5]. They found that serum iron indices were higher in patients who developed chronic hepatitis than in those who eliminated the virus. The same team also observed that haemodialysed patients with higher serum iron indices were less likely to achieve spontaneous recovery after acute hepatitis B^[108]. Other authors, assessing the presence of hepatic iron in Kupffer cells of patients with CHB, deduced that it is derived from hepatocytes destroyed by the virus^[109,110]. Zhou and colleagues studied 40 patients with hepatocellular carcinoma, 80% of whom were hepatitis B surface antigen (HBsAg) positive. They found a significant correlation between the presence of hepatitis B core antigen (HBcAg) and iron in hepatic tissue, suggesting that iron may accumulate predominantly in the hepatocytes in which HBV replication takes place^[111]. More recently, Martinelli and colleagues evaluated the prevalence of serum iron biochemical abnormalities and iron deposits in the liver of CHB patients^[112]. They found elevated transferrin saturation in 27.1% and liver iron deposits in 48.7% of cases. Patients with liver iron deposits presented with higher scores of necroinflammatory activity and fibrosis. The authors found no relationship between *HFE* mutations and elevation of serum iron indices or liver iron.

The effect of iron on the outcome of interferon alpha therapy in patients with CHB has been investigated by very few studies. In chronic viral hepatitis, Van Thiel and colleagues reported that low hepatic iron content may predict response to interferon therapy^[61]. More recently, a Polish group investigated iron metabolism and prognostic factors in interferon therapy in children with CHB. They showed that seroconversion for hepatitis Be antigen (HBeAg) was more frequently observed in children with lower iron and ferritin values^[113].

In conclusion, only a few studies have evaluated the prevalence and the physiopathological significance of iron overload in CHB. It has been suggested that iron deposits may occur mostly in hepatocytes. It seems that patients with higher levels of serum iron indices are less likely to achieve spontaneous recovery after acute hepatitis B. No role for the *HFE* gene mutations in iron overload has been detected. Iron could influence the response to antiviral therapy, but there is not sufficient evidence to permit a definitive conclusion.

IRON AND *HFE* MUTATIONS IN ALCOHOLIC LIVER DISEASE

Alcoholic liver disease (ALD) is one of the leading causes of end-stage CLD. It is well established that only a minority of heavy drinkers, estimated at between 10 and 30%, will ever develop advanced ALD; the risk increases with cumulative alcohol intake^[114,115]. Hence, in addition to alcohol, other factors are deduced to act synergistically to enhance its hepatotoxic effects. Patients with ALD commonly have elevation of transferrin saturation and serum ferritin concentration; significant hepatic iron

Table 3 Studies of the relationship between alcoholic liver disease, iron and *HFE* mutations

Reference	Cases <i>n</i>	Ethnicity	<i>HFE</i> relationship with serum iron indices	<i>HFE</i> relationship with HI	<i>HFE</i> relationship with ALD
[120]	257	Caucasian	NA	No	No
[121]	254	Caucasian	No	Yes	No
[129]	179	White	Yes (C282Y)	NA	No
		Hispanic			
[130]	61	White, non-Hispanic	NA	NA	NA

NA: not available; HI: hepatic iron; ALD: alcoholic liver disease (histological and/or biochemical evidence).

deposition is not infrequent^[116-118]. However, most patients with ALD have normal or slightly elevated HIC, with a mixed parenchymal and reticuloendothelial pattern of distribution^[119,120]. There is growing evidence that a mild degree of iron overload is sufficient to enhance alcohol-induced liver injury. The paradigm of synergy between iron and alcohol is HH. Patients with HH and significant alcohol consumption have a higher incidence of cirrhosis and hepatocellular carcinoma than those without heavy alcohol consumption or a history of alcohol abuse^[121,122]. In ALD, stainable hepatic iron was positively correlated with fibrosis in a multivariate analysis of risk factors in 268 French alcoholic patients^[123].

There are several potential causes for hepatic iron overload in alcoholic liver disease, including increased ingestion of iron, increased intestinal iron absorption, up-regulation of hepatic TfR1, secondary anaemia due to haemolysis, hypersplenism, ineffective erythropoiesis, hypoxaemia due to intrapulmonary shunts and portosystemic shunts^[4,124,125]. Increased iron absorption could arise through three main mechanisms: an increase in the reduction of luminal iron to the ferrous state; up-regulation of DMT1 in duodenal enterocytes; upregulation of ferroportin in duodenal enterocytes. The latter mechanism may be influenced by the down-regulation by ethanol of the hepatic production of hepcidin^[126].

Several studies suggested a genetic component to disease susceptibility. Significant associations have been reported between ALD risk and polymorphisms of the genes encoding cytochrome P450 and tumour necrosis factor (TNF) α ^[126,127]. Few studies assessed the possible role of *HFE* mutations as genetic cofactors in the development of ALD (Table 3). Lauret and colleagues found a significant association between carriage of the C282Y mutation and elevation of serum iron indices, but another study did not replicate this finding^[120,128]. One study found a relationship between *HFE* mutations and hepatic iron deposits^[120] but this observation was not confirmed by others^[119]. Moreover, none of these studies found that *HFE* mutations influenced the severity of ALD^[119,120,128,129].

In conclusion, patients with ALD tend to show mild increases in hepatic and serum measures of iron status. Iron is thought to play a role in worsening the course of ALD, although the mechanisms responsible are not resolved. To date, evidence from the literature does not

suggest a role for *HFE* mutations in determining iron overload or in influencing the course of ALD.

IRON AND *HFE* MUTATIONS IN NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as the most prevalent liver disease, at least in the West. In the adult population of the USA, 31% of men and 16% of women were found to have NAFLD^[130]. The disease has a spectrum ranging from fatty liver alone to steatohepatitis, and progressive steatofibrosis. Although fatty liver alone is considered non progressive, up to 20% of patients with non-alcoholic steatohepatitis (NASH) may develop cirrhosis, liver failure and HCC^[131]. Many cases of cryptogenic cirrhosis may be end-stage forms of NASH^[132]. The pathogenesis of NAFLD and the reasons why some patients with fatty liver develop NASH and have progressive liver disease are not entirely understood. A “two-hit” hypothesis has been proposed, involving the accumulation of fat in the liver (“first hit”), together with a “second hit” that gives rise to increased oxidative stress. Hepatic steatosis has been recognised as the first of two hits in the pathogenesis of NASH, since the presence of oxidisable fat within the liver is enough to trigger lipid peroxidation^[133]. However, many patients with fatty liver do not progress to steatohepatitis. Potential second hits for the development of NASH include all mechanisms contributing to the development of inflammation and fibrosis. Increased expression of ethanol-inducible cytochrome P450 2E1 (CYP2E1) and an increase in the intrahepatic concentration of free fatty acids could result in oxidative stress via peroxisomal oxidation^[134]. TNF α has also been implicated, since administration of anti-TNF α antibody ameliorated liver damage in animal models^[135].

There is controversial evidence that hepatic iron may play a role in the pathogenesis of NASH. It has been proposed that iron, in relatively low concentrations, could synergize with lipid overload and induction of CYP2E1 to increase oxidative stress in hepatocytes^[136]. Elevation of serum iron indices has been found in several studies^[126,127]. In most cases hepatic siderosis was mild and HIC was only rarely elevated^[137-140]. The distribution of hepatic iron was mixed parenchymal and sinusoidal^[79]. Strong support for an important role of iron in NAFLD and NASH was recently provided by studies on iron-depletion therapy, which improved both serum aminotransferases and insulin sensitivity^[141,142].

The role of iron has been re-evaluated since the discovery of the strict pathogenetic link between NASH and insulin resistance^[143]. Iron has been proposed to contribute to the development or exacerbation of insulin resistance, which is the most important risk factor for development of NAFLD and NASH^[79,80]. Mendler and colleagues reported a new syndrome characterized by mild to moderate hepatic iron overload and features of insulin resistance; the new syndrome was named insulin resistance-hepatic iron overload (IR-HIO). In their study, liver steatosis and NASH were present in 25% and 27% of IR-HIO cases, respectively^[7,144]. A subsequent study confirmed the high prevalence of NAFLD (59.7%) in patients with

Table 4 Studies of the relationship between NAFLD-NASH, iron and *HFE* mutations

Reference	Cases <i>n</i>	Ethnicity	<i>HFE</i> relationship with serum iron indices	<i>HFE</i> relationship with HI	<i>HFE</i> relationship with fibrosis
[139]	51	Australian	Yes with transferrin saturation, no with ferritin	Yes	Yes
[142]	31	Italian	No	Yes	No
[146]	263	Italian	Yes (C282Y)	No	No
[151]	57	Caucasian	Yes	Yes	Yes
[152]	32	Mostly Caucasian	No	No	No
[153]	38	Asian	No	No	No
[154]	31	Asian Indian	No	No	No
[155]	93	Mostly Caucasian	No	Yes	No

NA: not available; HI: hepatic iron (histological and/or biochemical evidence).

insulin resistance-associated iron overload^[145]. Moreover, as in NASH, phlebotomy allows for normalization of body iron stores in IR-HIO^[146]. Indeed, IR-HIO shares some features with NASH, such as hyperferritinemia with mostly normal transferrin saturation. However, not all patients with IR-HIO have hepatic steatosis. It is likely that NASH and IR-HIO share some pathogenetic mechanisms. It has been proposed that IR-HIO may represent one end of the spectrum of NASH in which HIC is increased. Another point of view is that IR-HIO may represent a coincidental convergence of hepatic iron overload with a very common liver disorder in the general population^[128].

Genetic factors have also been proposed to play a role in the pathogenesis of NASH. Investigations of the possible contribution of *HFE* mutations in the pathogenesis of NASH have given sometimes discordant results (Table 4). George and colleagues reported increased intrahepatic iron (Perls' grade > 1) in 41% of cases; 23% of patients had HIC above the upper limit of normal^[138]. The increased HIC was attributed to the higher prevalence of homozygotes and heterozygotes for C282Y in these Australian patients with NASH versus control subjects (31% *vs* 13%, respectively). No significant difference in the prevalence of the H63D mutation was reported. A subsequent study by Bonkovsky and colleagues reported a significantly increased prevalence of H63D heterozygosity in patients versus controls (44.4% *vs* 26.4%)^[147]. Heterozygosity for C282Y was not statistically different between the two groups. Fargion and coworkers also reported an increased prevalence of *HFE* mutations in patients with respect to controls (65% *vs* 26%). Patients carrying *HFE* mutations did not present with increased serum iron indices, but showed increased HIC^[79]. In contrast to these results, other investigators have failed to observe significant associations between hepatic iron accumulation, *HFE* mutations and the severity of liver disease in patients with NASH^[139,140,80,148-151]. Angulo and colleagues found that HIC was normal in patients with NASH and abnormal serum iron indices^[139]. Another study by Chitturi and colleagues did not find any correlation

between HIC and any features of NASH, including the severity of fibrosis^[151]. The authors did not find any increase in stainable hepatic iron in the majority (90%) of liver biopsies from patients with NASH. Although they, like others, found a higher prevalence of C282Y heterozygosity in patients with NASH, no relationship with fibrotic severity was identified. Other studies did not find either significant abnormalities of serum and liver iron or an increased frequency of *HFE* mutations^[80,148,149].

Explanations for discrepant results include ascertainment bias, varied power of the studies and possible ethnic differences in the study populations. It should be underlined that elevation of serum iron indices in NASH, especially serum ferritin, may be due to insulin resistance, steatosis and inflammation, rather than to iron overload. Indeed, the discrepancies could be partially attributed to the differences between databases: the patients could be selected among subjects with hepatic iron overload or among population routinely attended a gastroenterology outpatient clinic. The association between NASH and *HFE* mutations has been described mainly in Australian and North American Caucasians^[138,147]. This hypothesis is supported by a recent study by Chitturi and colleagues that found an increased frequency of C282Y heterozygosity in NASH only in Anglo-Celtic patients^[151]. However, studies showing a positive correlation may have come from tertiary centres with an interest in iron storage disorders.

An increased prevalence of C282Y and H63D mutations has also been reported in patients with IR-HIO, but this finding was not connected with increased iron burden^[7]. A recent study of ours described four cases of H63D homozygosity associated with hyperferritinemia, macrovesicular steatosis, mild parenchymal and sinusoidal hepatic siderosis, with a granular pattern that could be related to NASH. Furthermore, three of the cases had one or more metabolic disorders which are part of the insulin resistance syndrome. The study could be consistent with proposals of a possible biological effect of the H63D mutation in IR-HIO and/or fatty liver^[152].

In conclusion, NAFLD has a heterogeneous spectrum of disease. It can progress to NASH and to end-stage liver disease, but the exact mechanism of fibrosis progression is not completely clear. A “two hits” hypothesis has been proposed and iron has been evaluated as a potential “second hit” that can cause progression of simple fatty liver to NASH. The available data are discordant about prevalence and effect of iron overload in NAFLD and NASH. Patients with coexisting NASH and hepatic iron stores undoubtedly exist, but the discordant data of the literature suggest that iron overload in NASH may be an epiphenomenon rather than have a main causative role. Insulin resistance syndrome has emerged as a key player in NAFLD and in the development of NASH. Iron may exacerbate insulin resistance; although iron overload is fairly common in NAFLD, its amount is rarely clinically significant when considered in isolation. A possible role of *HFE* mutations in NASH has been described in two studies from countries with predominantly Caucasian populations. However, this was not confirmed in subsequent studies of more heterogeneous populations. *HFE* mutations may be part of a genetic pattern

Table 5 Studies of the relationship between porphyria cutanea tarda, iron and *HFE* mutations

Reference	Cases n	Ethnicity	HFE relationship with serum iron indices	HFE relationship with HI	HFE relationship with fibrosis
[163]	41	Caucasian	NA	NA	NA
[164]	108	Australian	Yes	Yes	No
[165]	70	North American	NA	NA	NA
[167]	23	Brazilian	Yes	No	Yes
[168]	36	Southern France	No	NA	NA
[170]	190	German	No	No	No
[172]	68	Italian	No	NA	NA
[174]	62	German	Yes	NA	NA

NA: not available; HI: hepatic iron (histological and/or biochemical evidence).

contributing to the progression of NASH in populations of Celtic origin.

IRON AND *HFE* MUTATIONS IN PORPHYRIA CUTANEA TARDA

Porphyria cutanea tarda (PCT) is the most common of the human porphyrias. It is caused by deficient activity of hepatic uroporphyrinogen decarboxylase. Most cases of PCT are acquired; the major risk factors are CHC, alcohol abuse, iron overload and oestrogen use. The familial form of the disease is observed in 20%-25% of patients^[4]. Both sporadic and familial PCT are iron-dependent disorders. The association of PCT with iron overload has been recognized for decades. Independent of the cause of liver disease, the majority of patients with sporadic PCT have biochemical evidence of iron overload, liver siderosis and increased body iron stores. However, hepatic siderosis is generally mild or moderate, reaching the lower end of the haemochromatosis range in less than 10% of cases^[153-154]. The causes of iron overload in patients with PCT appear to be heterogeneous. Indeed, altered iron status may be secondary to cofactors such as alcohol and chronic infection with HCV, that are frequently associated with sporadic PCT^[4]. Clinical and experimental data suggest that an iron-dependent process reversibly inactivates uroporphyrinogen decarboxylase^[155]. Indeed, as initially observed by Lundvall, venesection therapy may induce remission of cutaneous lesions and an improvement of liver function tests, whereas replenishment of iron stores leads to relapse^[156,157]. Phlebotomy may also be beneficial in patients without biochemical or histological evidence of iron overload^[157]. Altered iron homeostasis, even in the absence of systemic iron overload, could reduce uroporphyrinogen decarboxylase activity^[158].

Several studies have investigated the possible association between PCT and mutations of the *HFE* gene (Table 5). Investigators from UK reported that 44% of patients with PCT *vs* 11% of the control group carried the C282Y mutation, whereas no significant difference was found for the H63D mutation^[159]. Similar results were

reported by Stuart and colleagues in Australian patients and by Bonkovsky and colleagues in patients from North America^[160,161]. A detailed study comprising 108 US patients with PCT reported a C282Y frequency of 30% compared to 6% in controls^[162]. HIC, transferrin saturation and serum ferritin were highest in PCT patients who were homozygous for the C282Y mutation. The authors also reported a high frequency of comorbidity due to factors such as alcohol and hepatitis C. They concluded that homozygosity for the C282Y mutation and HCV infection, especially with heavy alcohol consumption, are the strongest risk factors for PCT. Martinelli and colleagues reported an association between C282Y and PCT with respect to controls (17.4% *vs* 4%) in a population of Southern European ancestry^[143]. Similar results were obtained in two studies of French patients^[164,165]. In a large study of 190 sporadic PCT cases from Germany the C282Y and H63D mutations were found in 39% and 45%, respectively^[166]. C282Y was significantly more frequent in patients than controls. Serum iron, transferrin saturation, ferritin concentration, HIC and liver enzymes did not differ significantly between patients with or without *HFE* mutations. Investigators from South Africa determined the frequency of *HFE* mutations in a racially-mixed group of patients with PCT in Cape Town^[167]. They found that both the C282Y and H63D mutations were highly prevalent in South Africans of European origin. In cases of mixed or Asian origin, the H63D mutation was common but the C282Y mutation was very rare. Neither mutation was found in any African subject. They concluded that both mutations were associated with PCT, but the association was dependent on the ethnic origin of the patient. Interestingly, one study from Italy reported a strong association of PCT with the H63D mutation, which was present in half of the patients. The presence of the H63D mutation was not related to the iron status of patients. However, a subtle abnormality of iron metabolism induced by this mutation could escape detection by the standard parameters of iron status^[168]. Some major factors may account for some differences between these studies. Firstly, the C282Y mutation is more frequent in cases of Celtic ancestry, although the prevalence of this mutation appears to be lower in Southern European countries^[9,11]. Secondly, the distribution of factors predisposing for PCT also shows relevant geographical differences. In Italy and other Mediterranean countries, hepatitis C is present in 70%-90% of PCT patients while it is rare in Northern European countries, where alcohol is the prevalent aetiological agent for CLD associated with PCT^[169]. Stolzel and colleagues investigated the relationship between *HFE* gene mutations and response to chloroquine in PCT patients^[170]. Chloroquine therapy was accompanied by clinical remission and reduced urinary porphyrin excretion in 39% of patients without *HFE* mutations versus 56% of *HFE* heterozygous patients. Interestingly, all patients homozygous for the C282Y mutation had high serum iron, transferrin saturation and serum ferritin concentration, and failed to respond to chloroquine treatment.

In conclusion, PCT is frequently associated with elevated serum iron indices and sometimes with hepatic iron overload. The pathogenesis of altered iron

homeostasis in PCT is not completely understood, although iron is thought to reduce the activity of uroporphyrinogen decarboxylase. Venesection therapy may be beneficial in PCT patients. Several cofactors have been proposed to play a role in the impairment of iron homeostasis observed in PCT, such as alcohol and CHC. A role of *HFE* mutations has also been reported. The available data support a role for the C282Y mutation in many cases of PCT, especially the C282Y homozygous genotype. This is particularly true in Celtic ancestry cases, thus confirming that the importance of *HFE* mutations as modifiers of disease varies according to the ethnic group. Most studies did not detect a relationship between *HFE* mutations and serum and hepatic iron overload. This suggests that *HFE* mutations, particularly C282Y, may contribute to the pathogenesis of PCT either through immunological mechanisms that could be iron-independent, or via subtle changes in hepatic iron metabolism that may act in concert with other co-factors to inhibit the activity of uroporphyrinogen decarboxylase.

IRON AND *HFE* MUTATIONS IN HEPATOCELLULAR CARCINOMA OCCURRING IN CLDS OTHER THAN HH

HCC is a common cause of death in patients with compensated cirrhosis^[171]. European studies have reported HCC as the cause of liver-related deaths in 54%-70% of cases of compensated cirrhosis of varied aetiology and in 50% of cases with cirrhosis due to HCV^[172]. The annual incidence of HCC in patients with liver cirrhosis has been estimated at 3%-5%^[173,174]. Thus, it is important to identify patients at high risk of HCC, to increase the rate of early detection. Several risk factors for the development of HCC have been identified in Western patients with cirrhosis, including male sex, age, persistently raised serum α -fetoprotein levels, severity of cirrhosis and genetic background^[173]. A possible carcinogenic role for iron has been suggested by *in vitro* studies. Iron may promote cellular oxidative stress through the production of reactive oxygen species. These have the potential to cause lipid peroxidation as well as damage to other cellular components, including proteins and nucleic acids^[151]. *In vitro* studies showed that iron can reduce the levels of two systems that normally protect against reactive oxygen species, vitamin E and superoxide dismutases^[175-176]. Furthermore, *in vivo* studies in mammals showed that tumor growth is enhanced by iron supplementation and inhibited by iron deficiency^[175,177]. Finally, neoplastic cells highly express TfR1 and can synthesize their own transferrin^[177].

Apart from the experimental evidence, the role of iron in the step by step process that leads to HCC in CLDs other than HH is controversial. A report of 133 cases by Boige and colleagues did not find any difference in the grade of hepatic iron staining between cirrhotic patients with and without HCC^[178]. This finding was also in keeping with a previous study where no significant relationship was observed between the hepatic iron score and the occurrence of HCC in alcoholic and HCV-related

cirrhosis^[179]. A role of *HFE* mutations as part of a genetic pattern promoting carcinogenesis has been evaluated in some studies, with somewhat discordant results. In a German study, C282Y heterozygosity was significantly more common in 137 HCC cases with no history of HH versus 107 cirrhotic patients without HCC and 126 healthy controls. C282Y heterozygote HCC patients had significantly increased hepatic iron score in both HCC and non-tumorous tissue^[180]. Other studies reported that the prevalence of C282Y heterozygosity was increased above control levels in patients with HCC and alcoholic and virus-related liver disease^[129,182]. An excess of the C282Y mutation, mostly in the heterozygous genotype, has also been reported in patients with HCC developed in non cirrhotic liver. In that study, Blanc and colleagues showed that mild iron overload is frequent (54%) and that in patients with HCC in non cirrhotic liver and iron overload, C282Y mutations are frequent (36.8% of cases) and significantly increased compared to HCC in non cirrhotic liver without iron overload^[183]. In contrast with the above results, some studies did not find a relationship between HCC and mutations in the *HFE* gene. Cauza and colleagues evaluated the prevalence of *HFE* mutations in patients with HCC developed on cirrhosis of viral and alcoholic aetiology. The authors found that, except for C282Y homozygotes, *HFE* mutations did not increase the risk of HCC in patients with cirrhosis^[184]. A prospective study of 133 consecutive cirrhotic patients without HH did not find an increased prevalence of *HFE* mutations in cirrhotic patients who developed HCC compared to cases without HCC^[178]. Similarly, a study from Italy found that in patients with HCC in the absence of HH, the frequency of *HFE* mutations was not increased, compared to the controls. The authors concluded that mutations of the *HFE* gene do not play a significant role in the pathogenesis of HCC^[185].

In conclusion, iron has a clear pathogenetic role in the development of HCC in HH. The carcinogenic role of iron is deduced to be mediated through production of reactive oxygen species leading to lipid peroxidation and damage of proteins, nucleic acids and other cellular components in hepatocytes. However, similar results have not been obtained for other CLDs. The available studies about the role of *HFE* mutations in HCC in liver diseases other than HH indicate that C282Y heterozygosity may play a role in liver iron deposition and could contribute to hepatocarcinogenesis, possibly by playing a part in the immunogenetic pattern of the patient or through subtle changes in iron metabolism acting together with other cofactors.

HIGHLIGHTS

Apart from HH, a number of CLDs cause hepatic iron overload. These include hepatitis C and B, alcoholic and nonalcoholic steatohepatitis and PCT. Secondary iron overload due to CLD presents with some clinical, histological and genetic differences with respect to HH. Since the occurrence of HH is clearly related to the presence of specific genetic patterns, the role of mutations

Table 6 Secondary iron overload: common features and differences

Common features	
1	Mixed distribution of hepatic iron (parenchymal and reticuloendothelial)
2	Mild to moderate iron overload in most cases, rarely severe iron overload
3	Phlebotomy possibly improves the course of disease and response to therapy
Differences	
1	C282Y: accepted role in PCT, possible cofactor in CHC and NAFLD (especially in populations of Northern European descent)
2	H63D: unclear role in NAFLD and PCT
3	No role for <i>HFE</i> mutations in ALD or CHB

PCT: porphyria cutanea tarda; CHC: chronic hepatitis C; NAFLD: non-alcoholic fatty liver disease; ALD: alcoholic liver disease; CHB: chronic hepatitis B; CLDs: chronic liver diseases.

of the gene most commonly responsible for HH has been investigated in liver diseases other than HH. Liver diseases that cause secondary iron overload share some common features, but they also show differences (Table 6). The evidence in the literature regarding secondary iron overload in comparison to HH are consistent in showing that: (1) in HH iron overload is widespread in many organs while in secondary iron overload due to CLDs, iron is confined to the liver; (2) in HH the histological distribution of iron is mostly parenchymal while in secondary iron overload due to CLDs, iron shows mostly a mixed distribution, with both reticuloendothelial and parenchymal localization; (3) point mutations in the *HFE* gene are the most common genetic factor underlying HH; their prevalence varies according to geographic area and ethnic group. In secondary iron overload, data are controversial. When present, the penetrance of *HFE* mutations may be more influenced by cofactors, since the aetiology of CLDs is multifactorial; (4) in HH, genetics is the “primum movens” of the disease while in secondary haemochromatosis it has been proposed as a cofactor that may increase the severity of disease expression. Evidence from the literature suggests that the C282Y mutation may play a role in NASH, PCT and possibly CHC in cases of Celtic ancestry. The importance of *HFE* mutations as modifiers of disease, therefore, varies between different ethnic groups. H63D has been reported by few authors as having a role in some cases of mild iron overload in NAFLD and PCT, although any effect is generally minor, in agreement with the findings in HH. *HFE* mutations do not seem to determine iron overload or to influence the course of ALD and CHB. Iron may contribute to the rate of response to antiviral therapy in CHC and to chloroquine in PCT and a positive influence of *HFE* mutations has been suggested in CHC.

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TOPIC HIGHLIGHT

Nathan Subramaniam, PhD, Series Editor

Non-HFE haemochromatosis

Daniel F Wallace, V Nathan Subramaniam

Daniel F Wallace, V Nathan Subramaniam, Membrane Transport Laboratory, The Queensland Institute of Medical Research, Brisbane, Queensland, Australia
Correspondence to: Dr. V Nathan Subramaniam, Membrane Transport Laboratory, The Queensland Institute of Medical Research, 300 Herston Road, Herston, Brisbane, QLD 4006 Australia. nathan.subramaniam@qimr.edu.au
Telephone: + 617-3362-0179 Fax: + 617-3362-0191
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Abstract

Non-HFE hereditary haemochromatosis (HH) refers to a genetically heterogeneous group of iron overload disorders that are unlinked to mutations in the HFE gene. The four main types of non-HFE HH are caused by mutations in the *hemojuvelin*, *hepcidin*, *transferrin receptor 2* and *ferroportin* genes. Juvenile haemochromatosis is an autosomal recessive disorder and can be caused by mutations in either *hemojuvelin* or *hepcidin*. An adult onset form of HH similar to HFE-HH is caused by homozygosity for mutations in *transferrin receptor 2*. The autosomal dominant iron overload disorder ferroportin disease is caused by mutations in the iron exporter ferroportin. The clinical characteristics and molecular basis of the various types of non-HFE haemochromatosis are reviewed. The study of these disorders and the molecules involved has been invaluable in improving our understanding of the mechanisms involved in the regulation of iron metabolism.

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Key words: Haemochromatosis; Iron overload; Non-HFE; Juvenile haemochromatosis; Hemojuvelin; Heparin; Transferrin receptor 2; Ferroportin

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INTRODUCTION

After the identification of the *HFE* gene in 1996^[1] it became apparent that not all cases of haemochromatosis are caused by mutations in *HFE*. HFE-associated HH (HFE-HH) or type 1 HH is the most common form,

especially in populations of Northern European origin, where the C282Y mutation has a high allele frequency^[2]. Haemochromatosis that is unrelated to mutations in the *HFE* gene are collectively referred to as non-HFE haemochromatosis. Non-HFE haemochromatosis occurs in populations world wide and makes up a larger proportion of HH cases in areas where the C282Y mutation is less common, such as Southern Europe^[3] and Asia^[4]. Non-HFE HH can be further differentiated according to the gene mutated. There are four main types of non-HFE HH. The molecules mutated in all forms of HH are related in pathways involved in the regulation of iron homeostasis. Heparin the central regulator of iron homeostasis and hemojuvelin are mutated in juvenile or type 2 HH^[5,6]. Transferrin Receptor 2 is mutated in type 3 HH^[7] and the iron exporter ferroportin is mutated in the autosomal dominant type 4 HH or ferroportin disease^[8,9]. The genetic, clinical and laboratory features of the various types of HH are outlined in Table 1. This review will describe in detail the four main types of non-HFE HH and review the current literature in this area.

JUVENILE HAEMOCHROMATOSIS (TYPE 2)

An early onset form of juvenile haemochromatosis (JH), distinct from the typical HFE-HH has been recognised for some time^[10]. As with HFE-HH, JH or type 2 HH is an autosomal recessive disorder, and is characterised by elevated serum iron indices and iron deposition in parenchymal cells. JH usually presents before the age of 30 years and has a more rapid and severe course than HFE-HH. Unlike HFE-HH both sexes are affected equally^[10]. Cardiomyopathy and hypogonadism are more prominent features of JH, hypogonadism being the most common symptom at presentation^[11]. The rapid accumulation of iron in patients with JH can often be fatal, death usually resulting from heart failure^[11].

When the *HFE* gene was identified in 1996, it became apparent that JH was indeed a disorder genetically distinct from the typical HFE-HH. Linkage to the *HFE* gene region on chromosome 6 was ruled out, in a study utilising microsatellite markers in five Italian JH families^[12]. This was followed by a genome wide search that identified linkage to the chromosome 1q21 region in nine JH families. Subsequently a subset of families who did not have linkage to 1q21 were found to have mutations in the *hepcidin* (*HAMP*) gene on chromosome 19^[5]. This subset of JH has been termed type 2B HH and is described in more detail later. The chromosome 1 form of JH has been termed type 2A HH.

Table 1 Genetic, clinical and laboratory features of the various types of Hereditary Haemochromatosis

HH Type	Gene	Inheritance	Clinical features	Laboratory findings	Liver pathology	Functional consequences of mutations
1	<i>HFE</i>	Autosomal recessive	May include: fatigue, lethargy, arthropathy, skin pigmentation, liver damage, diabetes mellitus, endocrine dysfunction, cardiomyopathy, hypogonadotropic hypogonadism	↑ serum ferritin, ↑ transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	Impaired hepcidin regulation by iron, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
2A	<i>Hemojuvelin (HJV)</i>	Autosomal recessive	As for HFE. Earlier onset (< 30 yr). Cardiomyopathy and hypogonadism more prevalent.	↑ serum ferritin, ↑ transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	Loss of hepcidin regulation, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
2B	<i>Hepcidin (HAMP)</i>	Autosomal recessive	As for HFE. Earlier onset (< 30 yr). Cardiomyopathy and hypogonadism more prevalent.	↑ serum ferritin, ↑ transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	No/inactive hepcidin, leading to maximal iron absorption and release of iron from reticuloendothelial cells
3	<i>Transferrin Receptor 2 (TfR2)</i>	Autosomal recessive	As for HFE.	↑ serum ferritin, ↑ transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	Impaired hepcidin regulation by iron, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
4	<i>Ferroportin (Fpn), SLC40A1, IREG1, MTP1</i>	Autosomal dominant	Typical presentation: as for HFE, except generally milder. May have mild anaemia and lower tolerance to venesection. Atypical: as for HFE	↑ ↑ serum ferritin, normal transferrin saturation	Predominant Kupffer cell iron loading, fibrosis	Reduced ferroportin iron transport ability, leading to accumulation of iron in reticuloendothelial cells
				↑ serum ferritin, ↑ transferrin saturation	Predominant hepatocyte iron loading, fibrosis, cirrhosis	Loss of ferroportin regulation by hepcidin, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells

HEMOJUVELIN-ASSOCIATED HAEMOCHROMATOSIS (TYPE 2A)

The gene responsible for the chromosome 1 form of JH was identified in 2004^[6]. Fine mapping of the JH locus was performed in 12 JH families of Greek, Canadian and French origin. Sequencing of genes in this region revealed a novel gene that was mutated in all affected individuals^[6]. The gene originally named *HFE2* encodes hemojuvelin (HJV), a protein with homology to the repulsive guidance molecule (RGM) family of proteins. Six mutations were initially identified in the affected individuals either in the homozygous or compound heterozygous state. One mutation G320V was present in nine of the 12 families^[6]. Since the identification of *HJV*, numerous mutations have been identified in JH families worldwide^[6,13-24]. An Italian study identified 16 more mutations among 34 JH patients from various European backgrounds^[13]. Figure 1 illustrates the structure of hemojuvelin and position of disease-causing mutations. Most mutations are private and were detected in single families. A few have been detected in more than one population. One mutation in particular (G320V) is significantly more frequent and has been reported in JH patients in many populations^[6,13,16-20,24].

Patients with HJV-HH were shown to have low levels of urinary hepcidin^[6]. This suggested that HJV may be involved in regulating hepcidin expression in response to iron. The generation of mouse knockouts confirmed that HJV was critical for the regulation of iron homeostasis and the induction of hepcidin^[25,26]. Recent studies have suggested that HJV regulates hepcidin expression through signalling pathways involving bone morphogenic proteins (BMPs)^[27].

HEPCIDIN-ASSOCIATED HAEMOCHROMATOSIS (TYPE 2B)

While most cases of juvenile haemochromatosis have been linked to a locus on the long arm of chromosome 1 (1q21) and mutations in *HJV*, a small number of families have been described with a JH-like disorder unlinked to 1q21^[28]. This subset of patients was found to have mutations in the *hepcidin (HAMP)* gene. Two consanguineous families of Italian and Greek origin were originally reported with linkage to a region on chromosome 19, encompassing the region containing the *hepcidin* gene^[5]. Homozygosity for mutations in the *hepcidin* coding sequence was detected in both families. One family harboured a one base pair deletion (93delG), causing a frameshift and an abnormal extended protein (T31fsX180). The other family carried a single base pair substitution (166C>T) causing the replacement of an arginine with a stop codon (R56X), in the predicted cleavage site for prohormone convertases. Both of these mutations severely affect the protein sequence and would result in the absence of any mature hepcidin peptide^[5].

Hepcidin mutations remain a rare cause of JH. However, since the first report, other cases and mutations have been described^[5,29-37]. Mutations described in the *hepcidin* gene are shown in Figure 2. These include mutations affecting two of the eight highly conserved cysteine residues (C70R and C78T), important for the complex disulphide bonded structure of mature hepcidin^[33-35]. A mutation in the 5'UTR of the *hepcidin* mRNA (-25G>A) has been described in two Portuguese families^[36,37]. This mutation creates a new initiation codon upstream from the original

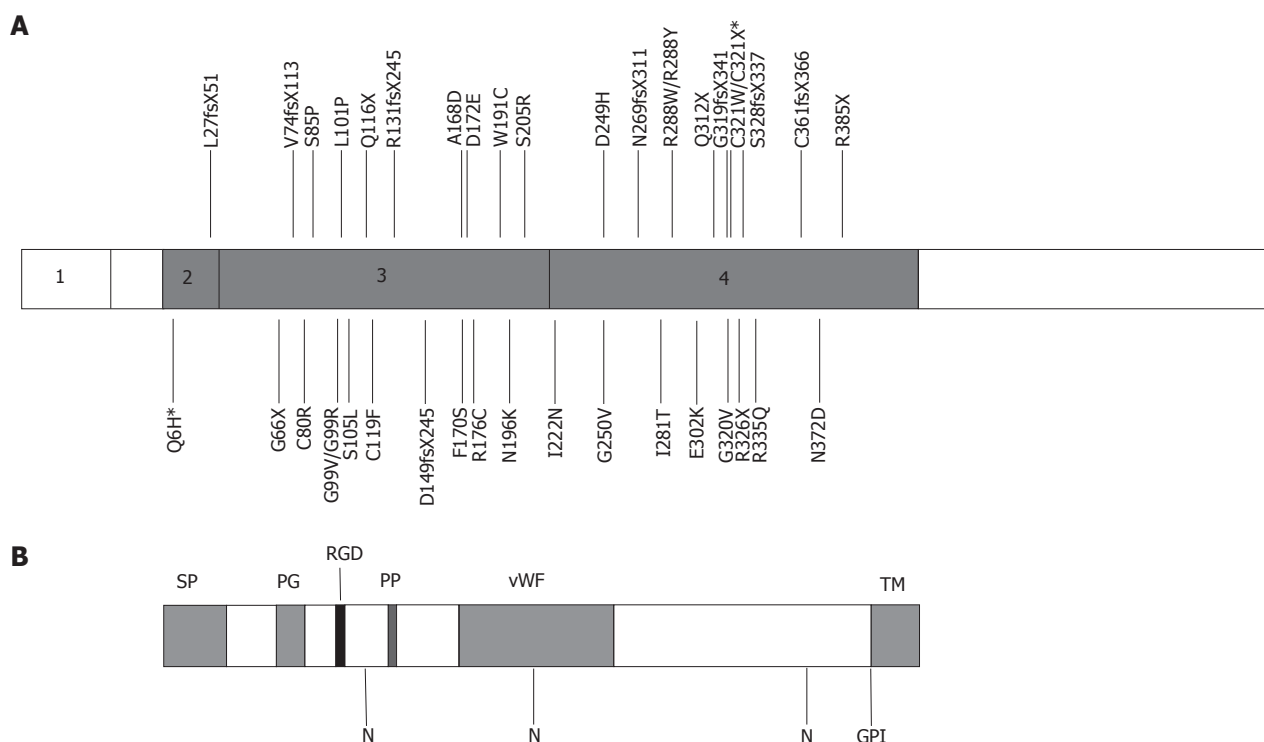


Figure 1 Structure of human hemojuvelin and positions of mutations. **A:** The exon structure of human *hemojuvelin* is shown with positions of known mutations marked^[16,13-24,30,39]. *Q6H was found associated with C321X; **B:** Predicted structure of the full length HJV protein, showing the positions of structural domains and motifs. SP, signal peptide; PG, poly-glycine sequence; RGD, RGD motif; PP, poly-proline sequence; vWF, partial von Willebrand factor type D domain; N, potential N-linked glycosylation sites; GPI, GPI-attachment site; TM, transmembrane domain, cleaved after GPI attachment.

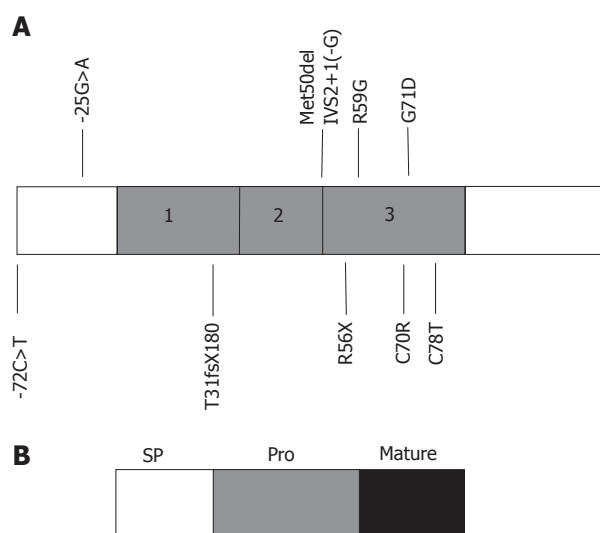


Figure 2 Structure of human *hepcidin* and positions of mutations. **A:** The exon structure of human *hepcidin* is shown with positions of known mutations marked^[5,29-37]. **B:** Predicted structure of the hepcidin peptide. SP, signal peptide; Pro, pro-region; Mature, 25 amino acid mature hepcidin peptide.

ATG. Measurement of urinary hepcidin in a patient homozygous for this mutation, suggests that steady state transcription of *hepcidin* from the original ATG codon does take place. But there is loss of upregulation of *hepcidin* transcription in response to iron^[37]. In another study it was shown *in vitro* that the out of frame upstream initiation codon was functional and prevented normal transcription from the original ATG^[38].

DIGENIC INHERITANCE AND MODIFIERS OF HFE

The remaining mutations in *hepcidin* have been detected in the heterozygous state in patients carrying *HFE* mutations. Merryweather-Clarke *et al*^[29,30] described patients with haemochromatosis who carried mutations in both *HFE* and *hepcidin*. One patient carried a four base pair deletion in *HAMP* (Met50del IVS2+1(-G)) and had a JH-like phenotype. Another family carried the G71D mutation in combination with either heterozygous or homozygous C282Y, and adult-onset iron overload. This was the first description of iron overload due to digenic inheritance of mutations in two separate genes. Two studies have detected *hepcidin* mutations in large cohorts of patients with HFE-HH. Jacolot *et al* detected *HAMP* mutations in five individuals from a cohort of 392 C282Y homozygotes and found that these were among the more iron-loaded. In addition, four of 31 subjects with iron overload, but at least one chromosome lacking an *HFE* mutation also carried a *HAMP* mutation. This supports the concept that digenic inheritance of *HFE* and *HAMP* mutations can lead to iron overload. Biasiotto *et al*^[30] also screened for *hepcidin* mutations in iron overload patients carrying the C282Y allele and detected sequence variations in some. They concluded that a novel substitution in the *hepcidin* promoter (-72C>T) may aggravate iron loading in patients with *HFE* mutations.

HJV mutations have also been detected in patients with HFE-HH. Two studies suggested that heterozygosity for *HJV* mutations may aggravate the phenotype in HFE-

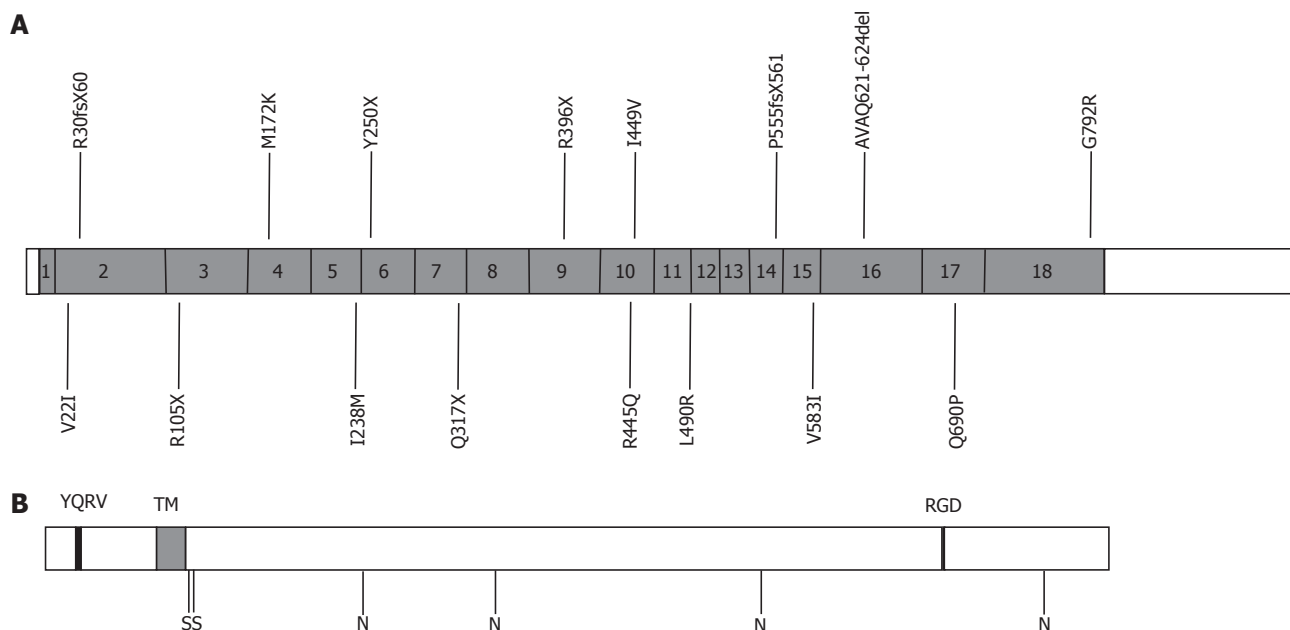


Figure 3 Structure of human transferrin receptor 2 (TfR2) and positions of mutations. **A:** The exon structure of human *TfR2* is shown with positions of known mutations marked^[7,30,40-52]. The frameshift mutations R30fsX60 and P555fsX561 are also known as E60X and V561X respectively. G792R may be associated with R396X; **B:** Predicted structure of TfR2 protein. YQRV, endocytosis signal; TM, transmembrane domain; RGD, RGD motif; S, predicted interchain disulphide bonds; N, potential N-linked glycosylation sites.

HH^[30,39]. Le Gac *et al.*^[39] reported nine of 310 C282Y homozygous patients with additional *HJV* mutations. Eight of the nine patients appeared to have a more severe phenotype, suggesting that heterozygosity for mutations in *HJV* were having a modifying effect. A similar effect of *HJV* mutations was reported by Biasotto *et al.*^[30], the N196K mutation being associated with abnormally high iron indices in a C282Y/H63D compound heterozygote. The effect of *HJV* mutations on phenotypic expression of HFE-HH is small, and has not been detected in all studies. Lee *et al.*^[24] did not detect any *HJV* mutations in a group of 49 C282Y homozygotes. Wallace *et al.*^[7] reported a G320V heterozygous relative of a JH patient, who was also a C282Y/H63D compound heterozygote, but with normal iron indices.

TRANSFERRIN RECEPTOR 2-ASSOCIATED HAEMOCHROMATOSIS (TYPE 3)

Transferrin Receptor 2 (TfR2)-HH was first described in 2000^[7]. This was the first HH syndrome to be attributed to non-HFE mutations. Mutations in *TfR2* were first detected in six members of two Sicilian families. The defect was linked to a region on the long arm of chromosome 7 (7q22), and affected individuals were found to be homozygous for a nonsense mutation (Y250X) in *TfR2*^[7]. Affected individuals had iron overload with a similar phenotype to HFE-HH. TfR2-HH is a rare condition; however, several mutations have been reported worldwide associated with haemochromatosis^[7,30,40-52]. Mutations reported in *TfR2* are illustrated in Figure 3.

TfR2 is a homologue of the classical transferrin

receptor (TfR1)^[53], the molecule responsible for the uptake of transferrin-bound iron into cells. Unlike the ubiquitous expression of TfR1, TfR2 expression and activity is restricted almost exclusively to the liver^[54]. Rather than being involved in the uptake of transferrin bound iron, it appears that the main function of TfR2 is as a sensor of iron levels and regulator of hepcidin. In both patients and animal models with TfR2-HH hepcidin levels are low in relation to iron stores^[55-57].

The clinical features of TfR2-HH resemble that found in HFE-HH. Onset is usually in adulthood and is associated with increased serum iron indices and iron accumulation in parenchymal cells. Clinical features reported in patients with TfR2-HH include abnormal liver function, liver fibrosis, cirrhosis, arthritis, diabetes, hypogonadism, cardiomyopathy and skin pigmentation^[41,44]. All of these features are typical of HFE-HH. A direct comparison of phenotype between HFE-HH and TfR2-HH is difficult, due to the low prevalence of *TfR2* mutations and small number of reported cases. It appears, however, that TfR2-HH may have a more severe phenotype. Early onset of disease has been reported in a number of cases. Two adolescent siblings homozygous for the R105X mutation were reported with elevated transferrin saturation^[51]. However, the serum ferritin in both cases was normal and liver biopsy was not performed. Two unrelated cases, presenting at ages 3 and 16 years were reported, homozygous for the Y250X mutation^[40]. Both had raised serum iron, transferrin saturation and hepatic iron. The 16-year-old, who presented with fatigue, also had raised serum ferritin. Other cases suggestive of an earlier onset and more severe phenotype than HFE-HH have also been reported^[42, 44].

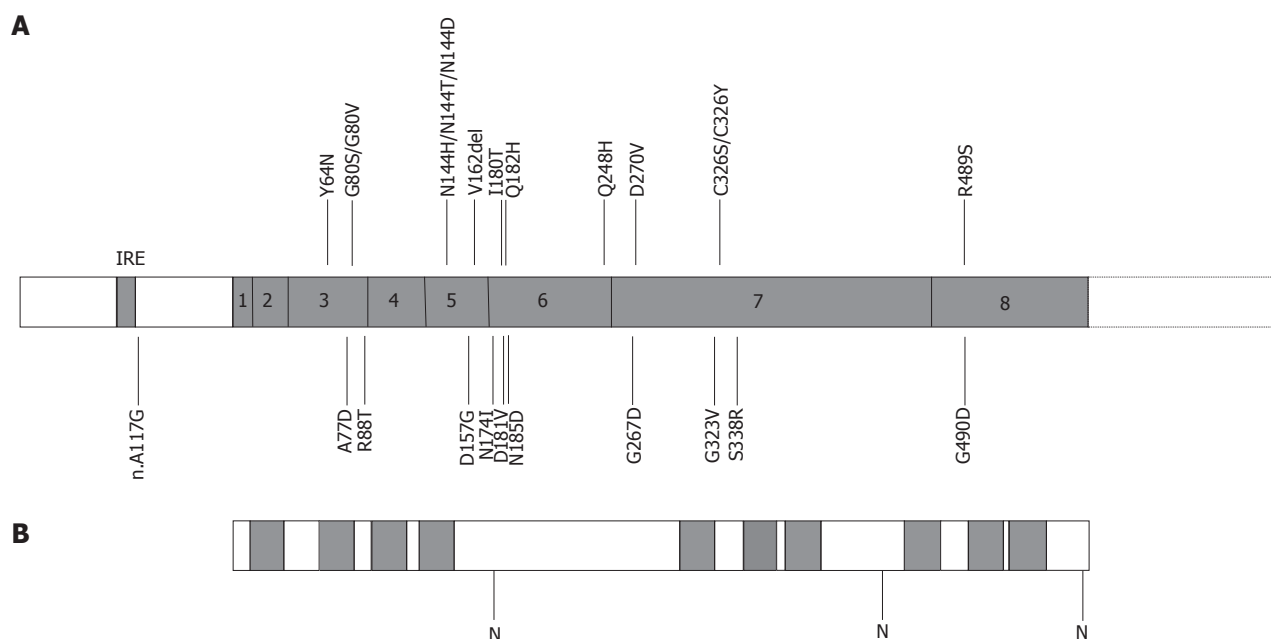


Figure 4 Structure of human *ferroportin* and positions of mutations. **A:** The exon structure of human *ferroportin* is shown with positions of known mutations marked^[8,9,60-85]. IRE, iron response element; **B:** Predicted structure of *ferroportin* protein. Predicted transmembrane domains are shaded; N, potential N-linked glycosylation sites.

Pietrangelo *et al*^[52] recently reported an unusual family with both adult and juvenile onset haemochromatosis. Two siblings presented in their early twenties with features typical of JH. These included hypogonadotropic hypogonadism, cardiomyopathy and cirrhosis. Both were found to be homozygous for a mutation Q317X in *TfR2*. In addition compound heterozygosity for *HFE*-C282Y/H63D was also present. A brother who had a less severe adult onset phenotype was homozygous for the Q317X *TfR2* mutation, but had wild type *HFE* sequence. This is the first and only report of juvenile haemochromatosis due to mutations in two genes normally associated with adult-onset haemochromatosis^[52]. This observation suggests that defects in either *HFE* or *TfR2* can be compensated to some extent by the other. Homozygosity for mutations in either one can lead to appreciable iron overload with onset normally in adulthood. The combination of mutations in both genes has an additive effect on iron loading, leading to an earlier onset and JH-like phenotype. Both *HFE* and *TfR2* are thought to regulate hepcidin expression in the liver through as yet unidentified signalling pathways. It is possible that *HFE* and *TfR2* work through either parallel or converging signalling pathways resulting in the induction of hepcidin. This would explain why the loss of one can be compensated to some extent by the other. The loss of both, however, would lead to complete loss of regulation of hepcidin by iron, as occurs in JH. Further studies will be needed to clarify the relationship between *HFE* and *TfR2*, and the signalling pathways they are involved in.

FERROPORTIN DISEASE

Ferroportin disease differs from other genetic iron overload disorders in that it is inherited in an autosomal dominant pattern. An autosomal dominant form of

haemochromatosis was first reported in 1990, in a Melanesian pedigree from the Solomon Islands^[58]. In a large 96 member pedigree, 31 of 81 members tested were affected in at least three generations. All affected individuals had raised transferrin saturation and serum ferritin levels. Liver biopsies in 19 individuals showed a pattern of iron staining consistent with *HFE*-HH, with iron present in hepatocytes and Kupffer cells. Some degree of fibrosis or cirrhosis was present in the majority of cases. This study was performed before the identification of the *HFE* gene; hence, analysis of *HFE* mutations could not be performed. However, linkage to the *HFE* locus on chromosome 6 was excluded, by HLA typing of affected and non-affected family members^[58]. Another large pedigree with multiple affected individuals, but without pathogenic mutations in the *HFE* gene was described in 1999^[59]. This Italian family consisted of 53 living members, with 15 affected across three generations. Linkage to the *HFE* region on chromosome 6 was excluded by typing of microsatellite markers.

Mutations in *ferroportin*, associated with autosomal dominant haemochromatosis were first described in 2001. An asparagine to histidine mutation (N144H) was identified in a large multi-generation family from the Netherlands^[9]. At the same time an alanine to aspartate mutation (A77D) was reported in the large Italian pedigree described previously^[8]. Since these first reports, many more *ferroportin* mutations have been described in association with autosomal dominant haemochromatosis^[60-85]. Figure 4 shows the mutations reported in the literature to date. *Ferroportin* mutations have been reported in populations throughout the world. Most of the reported *ferroportin* mutations are private and restricted to single families. Some mutations, however, are more common and have been reported in diverse populations. The most prevalent is the deletion of one of a group of three valine residues

(V162del). This mutation has been reported in families from Australia, the UK, Italy, Greece, Sri Lanka and Austria^[61-66]. It has been proposed that this mutation has occurred independently, several times, due to slippage mispairing in a repeat sequence. Other mutations reported in multiple populations include A77D (Italy and Australia)^[8,60] and Q248H^[75-79]. It is unclear whether Q248H is a mutation or polymorphism. It has been reported at high frequencies in African and African-American populations, in both controls and individuals with iron overload, where it can occur in the heterozygous or homozygous state. There is suggestive evidence that it may contribute to slightly higher serum ferritin levels^[76-79], but this effect is very small compared to other *ferroportin* mutants. There are three mutations affecting asparagine 144, suggesting that this residue is important for the functioning of ferroportin. In the original Dutch family reported by Njajou *et al*^[9], it was mutated to a histidine (N144H)^[9], in an Australian family to an aspartate (N144D)^[71] and in a Solomon Island patient to a threonine (N144T)^[67]. Whether this mutation is responsible for the autosomal dominant Solomon Island iron overload syndrome reported by Eason *et al*^[58], remains to be determined.

The phenotypic features of most cases of ferroportin disease differ significantly from that of HFE-HH. The typical features are an early elevation in serum ferritin, with normal transferrin saturation, and iron accumulation preferentially in the Kupffer cells of the liver. With increasing age, iron stores increase, and iron is seen in hepatocytes as well as Kupffer cells, and the transferrin saturation can be elevated. In these cases liver damage is minimal, with fibrosis occurring in some individuals. Venesection therapy is not always tolerated, with anaemia developing, especially in early cases when the transferrin saturation is low. It is now apparent that some cases of ferroportin disease differ from this typical pattern. A second atypical phenotype has been proposed, with features that more closely resemble HFE-HH. Atypical features include an early rise in transferrin saturation, and iron accumulation preferentially in hepatocytes, with some Kupffer cell iron apparent in some cases. Venesection therapy in these cases is usually tolerated well, but liver damage would appear to be more prevalent, with two reports of cirrhosis^[71,80].

The heterogeneity of ferroportin disease has led to the suggestion that mutational differences account for the phenotypic variation observed in patients. In general each mutation can be classified as leading to either the typical or atypical ferroportin disease phenotype. It has been proposed that particular mutations affect the function of ferroportin in different ways. The *ferroportin* gene, also referred to as *IREG1*, *MTP1*, *SLC11A3* and *SLC40A1*, encodes a multiple transmembrane domain iron transporter, highly expressed in duodenum, liver and reticuloendothelial cells. It is responsible for iron transport across the basolateral surface of enterocytes into the blood and recycling of iron in the reticuloendothelial system. Mutations such as A77D and V162del lead to the typical phenotype of reticuloendothelial iron storage, with relatively low transferrin saturation. A non-functional

ferroportin molecule would be predicted to lead to this phenotype. Heterozygosity for a non-functional mutant would be predicted to lead to haploinsufficiency for ferroportin, with only half the amount of functional ferroportin present on the surface of cells at any one time. In the reticuloendothelial system, where the vast majority of daily iron turnover occurs, this would be predicted to cause a blockage in the release of iron back into the circulation. Hence, iron would accumulate in the reticuloendothelial macrophages, and serum iron concentrations would be relatively low. These low levels of circulating iron would in turn lead to an increase in iron absorption in the duodenum, possibly involving sensors in the liver, such as HFE and Tfr2 and signalling *via* the hepcidin pathway. The turnover of iron in the reticuloendothelial system far outweighs that in the duodenum. Hence, the ferroportin required to transport iron across the basolateral surface of enterocytes would probably be sufficient to transport more iron into the body, even if a non-functional mutant was present. Over a long period of time body iron stores would increase to a point where the capacity of the reticuloendothelial cells to store iron would be reached, and iron would accumulate in parenchymal cells such as hepatocytes. This is seen in advanced typical ferroportin disease and is usually accompanied by an increase in the transferrin saturation.

It was recently reported that ferroportin expression on the cell surface can be regulated by hepcidin^[86]. It was shown that hepcidin could bind to ferroportin on the cell surface and induce its internalisation and degradation. In this way hepcidin could rapidly reduce iron absorption in the intestine and release of iron from the reticuloendothelial system, resulting in a reduction in serum iron. It has been proposed that the mutations which cause the atypical ferroportin disease phenotype affect the ability of hepcidin to internalise ferroportin. Failure to internalise would lead to a permanently “switched on” ferroportin molecule. This would lead to increased iron absorption in the duodenum and release from reticuloendothelial cells, resulting in high serum iron levels and storage of iron in parenchymal cells. Having a permanently “switched on” ferroportin molecule would effectively be the same as having hepcidin deficiency, as they both result in the same end point. This explains why the atypical form of ferroportin disease phenotypically resembles other forms of haemochromatosis, which all result from hepcidin deficiency.

CONCLUSION

There are four main genes implicated in non-HFE haemochromatosis. Mutations in these genes occur in populations world wide and account for the majority of HH cases not linked to HFE. The study of these disorders has led to a greater understanding of how the body regulates iron homeostasis. All the genes implicated in the different forms of haemochromatosis are involved in the regulation and maintenance of iron homeostasis. Hepcidin is at the centre of the iron regulatory pathway. Its expression in the liver can be regulated by the activities of HFE, Tfr2 and HJV. Hepcidin itself can regulate the activity of the iron

exporter ferroportin. Mutations in any one of these genes can disrupt the regulation of iron homeostasis and lead to iron overload. Further study of these molecules, their relationships to each other, and signalling pathways they are involved in will further illuminate our understanding of iron metabolism and its regulation.

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Iron overload and cofactors with special reference to alcohol, hepatitis C virus infection and steatosis/insulin resistance

Yutaka Kohgo, Katsuya Ikuta, Takaaki Ohtake, Yoshihiro Torimoto, Junji Kato

Yutaka Kohgo, Katsuya Ikuta, Takaaki Ohtake, Yoshihiro Torimoto, Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Midorigaoka-higashi 2-1, Asahikawa 078-8510, Japan
Junji Kato, Fourth Department of Internal Medicine, Sapporo Medical University, South-1, West-14, Chuoku, Sapporo 060-0030, Japan

Correspondence to: Yutaka Kohgo, MD, PhD, Professor of Medicine, Chairman, Division of Gastroenterology and Hematology/Oncology, Department of Medicine, Asahikawa Medical College, Midorigaoka-higashi 2-1, Asahikawa 078-8510, Japan. yk1950@asahikawa-med.ac.jp

Telephone: +81-166-682462 Fax: +81-166-682469

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Abstract

There are several cofactors which affect body iron metabolism and accelerate iron overload. Alcohol and hepatic viral infections are the most typical examples for clarifying the role of cofactors in iron overload. In these conditions, iron is deposited in hepatocytes and Kupffer cells and reactive oxygen species (ROS) produced through Fenton reaction have key role to facilitate cellular uptake of transferrin-bound iron. Furthermore, hepcidin, antimicrobial peptide produced mainly in the liver is also responsible for intestinal iron absorption and reticuloendothelial iron release. In patients with ceruloplasmin deficiency, anemia and secondary iron overload in liver and neurodegeneration are reported. Furthermore, there is accumulating evidence that fatty acid accumulation without alcohol and obesity itself modifies iron overload states. Ineffective erythropoiesis is also an important factor to accelerate iron overload, which is associated with diseases such as thalassemia and myelodysplastic syndrome. When this condition persists, the dietary iron absorption is increased due to the increment of bone marrow erythropoiesis and tissue iron overload will thereafter occurs. In porphyria cutanea tarda, iron is secondarily accumulated in the liver.

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Key words: Iron overload; Cofactors; Alcohol; Chronic hepatic C; Non-alcoholic steatohepatitis; Insulin resistance; Hepatocellular carcinoma

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INTRODUCTION

In hereditary hemochromatosis, patients having HFE trait are more susceptible to iron overload when cofactors such as alcohol, hepatitis viruses, and abnormal porphyrin metabolism are present. Even in the absence of hereditary hemochromatosis, there are several conditions associated with secondary iron overload in which iron deposition is rather mild^[1]. For example, in alcoholics and patients with chronic hepatitis C, intrahepatic iron is increased and liver injury is accelerated, followed by development of fibrosis, cirrhosis and hepatocellular carcinoma (HCC). In addition, abnormal copper metabolism and several causes for iron-loaded anemia are also important cofactors which influence the background iron overload. Furthermore, there is accumulating evidences that fatty acid accumulation without alcohol and obesity itself modifies insulin resistance through iron^[2] and fibrogenesis of the liver^[3]. In this review, the role of cofactors on iron overload will be discussed in three categories such as alcohol, hepatitis C virus infection and steatosis with obesity, the most common cofactors in liver iron overload.

COFACTORS AFFECTING BODY IRON METABOLISM AND IRON OVERLOAD

There are several factors which affect body iron metabolism and accelerates iron overload. Table 1 lists cofactors and disease conditions which are known to accelerate hepatic iron accumulation independent from responsible genes for hereditary hemochromatosis. Alcoholic and hepatic viral infections are the most typical examples for clarifying the role of cofactors in iron overload. In addition, abnormal copper metabolism and several causes for iron-loaded anemia such as thalassemia and myelodysplastic syndrome are also important factors which influence the background iron overload. When this condition persists, the dietary iron absorption is increased due to the increment of bone marrow erythropoiesis^[4] and tissue iron overload will occur thereafter. These patients are usually anemic in spite of increased body iron stores (iron-

Table 1 Cofactors of iron overload

- | | |
|---|--|
| 1 | Alcohol (Alcoholic liver disease) |
| 2 | Infection (Hepatitis C virus infection, <i>etc</i>) |
| 3 | Obesity and insulin resistance (Nonalcoholic steatohepatitis) |
| 4 | Copper (Ceruloplasmin deficiency) |
| 5 | Porphyria (Porphyria) |
| 6 | Ineffective erythropoiesis (Thalassemia, myelodysplastic syndrome) |
| 7 | Others |

loaded anemia), and require frequent blood transfusions, which further exaggerate secondary iron overload, in which conditions of **new oral iron chelators are effective**^[5]. In patients with ceruloplasmin deficiency, anemia and secondary iron overload in liver and neurodegeneration are reported^[6]. Furthermore, there are accumulating evidences that fatty acid accumulation without alcohol and obesity itself modifies iron overload states. Ineffective erythropoiesis is also an important factor to accelerate iron overload. This condition is associated with diseases such as thalassemia, aplastic anemia, and myelodysplastic syndrome. In porphyria cutanea tarda, iron is secondarily accumulated in the liver and phlebotomy and oral iron chelators are effective as well as in hemochromatosis.

ALCOHOL

Alcohol is one of the most important cofactors to modify or enhance iron accumulation in the liver. Excess intake of alcohol induces alcoholic liver diseases (ALD) such as fatty liver, fibrosis, hepatitis, and cirrhosis, in which iron overload is frequently associated^[7]. By **Perls' iron stain**, excess iron accumulation was found in hepatic tissues with ALD, but not in any normal hepatic tissues^[8]. In ALD, iron is deposited in both hepatocytes and reticuloendothelial (Kupffer) cells. In advanced cases of ALD, which is also called as "alcoholic siderosis", the reticuloendothelial iron deposition is dominant. In earlier stages of ALD such as fatty liver and fibrosis, iron deposition is mild and is preferentially present in hepatocytes **rather than in** Kupffer cells, which finding is more frequently observed in Japanese patients who have mild clinical phenotype comparing with those in US^[9].

The **reactive oxygen species (ROS) produced** play an important role in the development of ALD^[10]. The expression of 4-hydroxy-2-nonenal (HNE)-protein adducts, which is a lipid peroxidative product is **increased** in oxidized hepatocytes^[11]. Chronic alcohol ingestion in experimental animals is associated with oxidative stress as reflected by increased hepatic levels of lipid peroxidation products such as malondialdehyde and HNE, both of which have been implicated in hepatic fibrogenesis in the intragastric ethanol infusion model^[12]. Furthermore, lipid peroxidation products induce gene expression of procollagen α -1 (I) and increase collagen production by several folds in cultured hepatic stellate cell^[13]. In human ALD, there is a positive correlation between iron deposition and histological intensity of HNE-protein adduct^[14]. As shown in Figure 1, the **distribution of** HNE-protein adducts and iron granules appeared to be

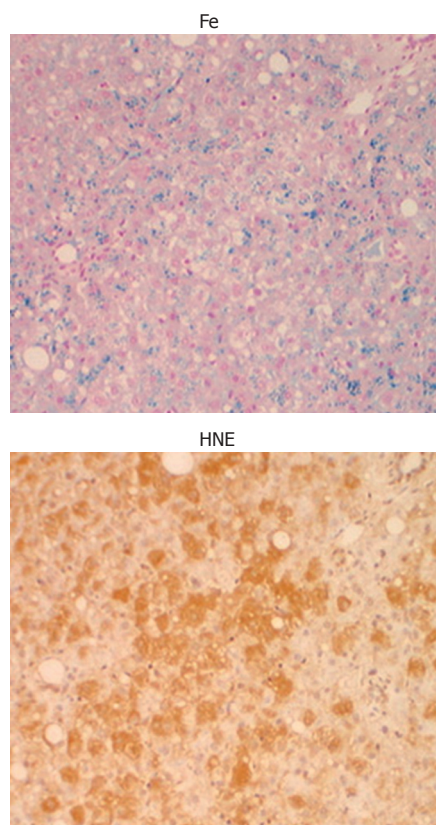


Figure 1 Iron staining and immunohistochemical staining of 4-hydroxy-2-nonenal-modified protein (HNE-protein) adducts in human alcoholic liver disease. The localization of HNE-protein adducts and iron in hepatocytes appeared to be identical (from ref. 14 with some modifications).

identical, suggesting that iron may be associated with the production of HNE-protein adduct. As hepatic iron is **visualized by Perls' reaction as an insoluble protein-bound iron** such as hemosiderin, this form of iron may be inactive for the production of ROS. But, the free iron responsible for Fenton reaction should be present close to the protein-bound iron, and may be involved in the production of HNE-protein adducts. There are two pathways to generate ROS through ethanol metabolism. Oxidation of ethanol by alcohol dehydrogenase to form acetaldehyde, which is subsequently oxidized to acetate and ultimately carbon dioxide and water. During the oxidation process of acetaldehyde involving aldehyde oxidase and xanthine oxidase, superoxide (O_2^-) is produced^[15]. In addition, cytochrome P450 is involved in the metabolism of ethanol, in which ROS are also generated in microsomes^[16]. Among ROS, hydroxy radical (OH) is most potent, which is produced via Fenton reaction in the presence of free iron and the resulted OH can easily cause **cell damage by oxidizing lipid, proteins, and nucleic acids**. In an intragastric infusion mouse model of ALD, supplementation of carbonyl iron advanced peri-venular fibrosis to bridging fibrosis and cirrhosis^[17]. Oxidative stress arising from hepatocytes and macrophage activates hepatic stellate cells by increasing the production of cytokines such as transforming growth factor- β (TGF β), directly or indirectly. The **dietary iron supplementation** was associated with increased NF- κ B activation^[18], and the up

regulation of NF- κ B responsive proinflammatory genes such as IL-1 β , TNF α , and MIP-1^[19].

In advanced cases of ALD, iron is accumulated more prominently in Kupffer cells than in hepatocytes, mainly due to repeated endotoxemia and hyper-cytokinemias of TNF α and IL-1 β ^[20]. These cytokines induced hepatic uptake of transferrin iron *in vitro*^[21] and *in vivo*^[22]. In mild cases of ALD, iron is preferentially stained in hepatocytes, rather than in Kupffer cells, suggesting that hepatocyte is the main site of early iron storage in the liver. However, it is not clear why iron is accumulated in liver parenchymal cells of alcoholics in such conditions. Two possibilities can be drawn: one is the increased uptake of iron in hepatocytes, and another is the increased iron absorption through hepcidin, which is a newly found antimicrobial peptide, and is a negative regulator of iron absorption and reticuloendothelial iron releases^[23]. Hepatocytes have several pathways for iron uptake. Iron in serum is usually bound to transferrin and iron-bound transferrin is taken up via transferrin receptor (TfR) with high affinity or via other unknown mechanism with greater capacity, but low affinity independent of high affinity receptor^[24]. There are two molecules of transferrin receptor: transferrin receptor 1 (TfR1) and transferrin receptor 2 (TfR2). TfR1 has a high affinity to serum transferrin and considered to be functional, while the function of TfR2 is not clear yet, even though the TfR2 gene is responsible for genetic hemochromatosis^[25]. In normal hepatocytes, TfR2 is constitutively expressed. But, TfR1 is down-regulated, suggesting that TfR1 does not contribute to the steady state hepatic iron uptake. Recently, Wallace *et al*^[26] reported that homozygous TfR2 knockout mice had no TfR2 associated with typical iron overload, and there was no upregulation of hepcidin mRNA, suggesting that TfR2 is required to iron regulated expression and is involved in a pathway to HFE and hemojuvelin. In addition, DMT1 may be involved when serum iron concentration exceeds transferrin iron binding capacity^[27]. It is noteworthy that TfR1 is regulated by cellular iron levels or oxidative stresses post-transcriptionally and it is possible that ethanol may augment TfR1 expression by producing oxidative stresses. According to immunohistochemical investigation, TfR1 expression was increased in hepatocytes in 80% of hepatic tissues with ALD, but was not detected in any normal hepatic tissues^[28]. It is noteworthy that the mean duration of abstinence of patients who demonstrated positive TfR1 expression in hepatocytes was significantly shorter than that of patients who demonstrated negative TfR1 expression.

Ethanol exposure in the presence of iron to the primary cultured-hepatocytes demonstrated an increase of TfR expression, and this augmentation was suppressed by the inhibitor of alcohol dehydrogenase, 4-methoxy pyrazole, but enhanced by a inhibitor of acetaldehyde dehydrogenase, cyanamide, suggesting that ethanol metabolite acetaldehyde itself is involved for the induction of TfR1 by ethanol^[29]. By functional uptake assay using ⁵⁹Fe-transferrin, the additional ethanol exposure increased transferrin-iron uptake into hepatocytes, while non-transferrin-bound iron (NTBI) uptake^[30] was not increased. It has been reported that TfR1 expression was

Table 2 Speculated effects of iron on HCV

1	Immunological modification (Immunological escape of HCV)
	Decrease of Th1 activity
	Impaired function of macrophage and Kupffer cells
	Decrease of innate immunity (Natural resistance macrophage protein 2)
2	Increase of liver toxicity by iron-mediated radical formation
	Reactive oxygen production through fenton reaction
	Induction of apoptosis
	Acceleration of fibrinogenesis
	DNA damage and carcinogenesis
3	Effect on cell signalling
	Decrease of interferon responsiveness by NF κ B activation
4	HCV proliferation
	Activation of translation initiation factor 3 (eIF3)
	Suppression of HCV RNA polymerase (NS5B) activity

up-regulated both transcriptionally^[31] and posttranscriptionally^[32]. This regulation is induced either by iron deficiency state or oxidative stress such as H₂O₂ and nitric oxide via iron regulatory protein, IRP^[33]. In addition to the direct cell toxicity, acetaldehyde produces free radicals^[34] and free radicals modify IRP activity^[35,36].

Body iron homeostasis is strictly regulated by a balance between the processes such as dietary iron absorption in enterocytes, iron transport by transferrin in circulation, iron utilization and storage in bone marrow and liver. The increase of intestinal iron absorption was one of the mechanisms of the hepatic iron deposition in alcoholics^[37]. In patients with hereditary hemochromatosis, serum pro-hepcidin is lower than that of normal controls, suggesting that iron absorption is increased in spite of high iron storage^[38]. It is speculated that down-regulation of hepcidin might be one of important factors for pathogenesis of iron overload in ALD^[39]. Serum pro-hepcidin concentration in ALD was significantly lower than that in healthy subjects, and pro-hepcidin/ferritin ratios in ALD were lower than healthy subjects^[40]. In the ethanol-loaded mouse model which has a mild steatotic change, the hepcidin mRNA and protein expression were significantly lower than that of control. In addition, alcohol-loading might disrupt the sensing signal of inflammatory cytokines, and then down-regulate hepcidin expression, following the increased iron absorption from small intestine. Recently, the mechanism of hepcidin downregulation by alcohol has been elucidated: a decreased hepcidin expression in mouse liver is accompanied with an increase of DMT1 and ferroportin1, and a decrease of hepcidin promoter activity and DNA-binding activity of CCAAT/enhancer-binding, protein α (C/EBP α)^[41].

HEPATITIS C VIRUS INFECTION

Hepatitis C virus infection is one of the most common disorders in liver diseases involving chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Table 2 summarizes the effect of iron on hepatitis C virus infection. In the Third National Health and Nutrition Examination Survey, HCV infection is significantly associated with higher serum levels of ferritin and iron in the US population^[42]. The mean serum levels of

ferritin and iron were significantly higher among subjects with HCV infection than among subjects without liver disease^[43]. In addition, serum ferritin levels were directly and significantly correlated with serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ -glutamyl transpeptidase, whereas platelet counts were inversely correlated with serum ferritin. It is also found that lipid peroxidative products such as malondialdehyde are increased in hepatic tissues with CH-C^[44]. In 1994, an initial report was published that phlebotomy was effective in improving the serum ALT level in patients with CH-C^[45] and a national prospective study confirmed the results^[46]. Since then, it was reported that hepatic iron accumulation in CH-C predict a response to interferon (IFN) therapy^[47], and phlebotomy before and during IFN therapy improved virological and histological response to short-term IFN therapy evaluated at the end-of-treatment^[48]. This observation is reasonable considering the finding that oxidative stress impairs interferon alpha signal by blocking JAK-STAT pathway^[49]. The standard therapy for hepatitis C is now a combined therapy of interferon- α and ribavirin, in which patients with viral response to treatment seemed to develop higher soluble transferrin receptor levels^[50] with decline in serum iron and ferritin than non-responders, revealing intracellular reduction of iron store depending on the result of treatment including hemolytic reaction by ribavirin^[51]. This is an interesting observation that decrease of iron status may be an additional effect of the combination therapy with interferon and ribavirin. Moreover, HFE mutations are also associated with increased sustained virologic responses by antiviral long-term treatment, while it is well known that HFE mutations are associated with increased iron loading^[52]. However, some reports suggest that iron depletion was unable to trigger interferon response, so that there are conflicting data. It should be further investigated whether hepatic iron content modify the response to interferon^[53,54].

From these observations, iron and related molecules seem to be key factors in the hepatocytes to influence the disease condition of CH-C, and also development of cirrhosis and maybe hepatocellular carcinoma. Clinical data on phlebotomy on CH-C generally indicates that phlebotomy does not influence the viral load in vivo. On the other hand, in vitro study on HCV replication is controversial: iron promotes HCV translation by up-regulating expression of the translation initiation factor eIF3 by reporter assay^[55], whereas iron suppresses HCV replication by inactivating the RNA polymerase NS5B^[56].

As previously described, hepatocytes have two iron uptake systems, transferrin-mediated and nontransferrin-bound iron-mediated pathway. Transferrin and TfR1 are molecules involved in the classical pathway of cellular iron uptake, but are faintly expressed in normal hepatocytes, and is down-regulated in iron-loaded hepatic tissues with hemochromatosis. Concerning the post-transcriptional regulation of TfR1, two mechanisms are postulated through the activity change of IRP which is already mentioned. In CH-C, TfR1 expression was up-regulated and DMT1 expression was down-regulated in the condition of hepatic excess iron accumulation, suggesting that regulation of DMT1 expression is iron-

dependent, but that of TfR1 expression is iron-independent in CH-C^[57]. In patients with CH-C, serum values of inflammatory cytokines such as IL-1 β , IL-6, and TNF α have been reported to be high in comparison with those in normal controls. In addition, TfR1 was up-regulated by IL-1 β , IL-6, and TNF α in HepG2. Administration of IL-6 augments hepatic uptake of transferrin-bound iron (⁵⁹Fe), and this is mainly mediated through hepatocytes, but not through Kupffer cells. These results suggest that the up-regulation of TfR1 expression in CH-C might be caused by increase of inflammatory cytokines that proceeded from HCV infection, although there is a possibility that the components of HCV themselves may induce TfR1 expression directly or indirectly.

Like wise, the up-regulated TfR1 might act as a key molecule for hepatic excess iron accumulation in CH-C; however, there are several candidate molecules which cause this condition. For instance, each mutant of HFE, TfR2, hepcidin, hemojuvelin and ferroportin1 (also known as Ireg1 or MTP1) with substitution of amino acid causes the similar phenotype of hemochromatosis. That is, these facts indicate that at least 5 molecules are involved in the familiar hemochromatosis^[58]. In hepatocytes, TfR2 predominantly expresses in the normal condition^[59] and the disruption of TfR2 gene caused the hepatic iron overload, a phenotype of hemochromatosis, suggesting that TfR2 should also have important role in hepatic iron metabolism^[60]. This receptor might act as a sensor of iron status because hepatic TfR2 protein level was increased in iron loaded rats and was decreased in iron deficient rats. Recently, Takeo *et al*^[61] reported that in CH-C TfR2 protein expression is increased parallel with ferroportin1, although the meaning of this TfR2 elevation is still to be elucidated^[62].

In addition, there was a significant correlation of hepcidin mRNA expression in the liver with hepatic iron concentration and serum ferritin, but did not correlate with ALT, AST, HAI, or viral load. In inflammatory conditions, hepcidin is regulated transcriptionally by IL-6^[63] and IL-1 β ^[64] independent of liver iron content. It is noteworthy that, in contrast to other inflammatory states, hepcidin mRNA expression in the liver was independent of markers of inflammation in hepatitis C, suggesting that iron stores in patients with hepatitis C regulate hepcidin expression, and that iron loading in chronic hepatitis C is not due to inappropriate hepcidin expression^[65]. However, there is still a controversial result concerning the hepcidin metabolism in chronic hepatitis C that serum pro-hepcidin is down-regulated^[66]. The role of hepcidin in chronic hepatitis C seems to need further consideration.

The role of iron on the hepatocellular carcinoma (HCC) development in patients with chronic hepatitis C is another major concern. In primary hemochromatosis, iron could be involved in the development of HCC in associated with cirrhosis, suggesting a strong link between heavy iron overload and HCC development. In cases of chronic hepatitis C, it is also known that HCC are developed 20 to 30 years after the infection of hepatitis C virus through the progression of the disease from chronic hepatitis and cirrhosis. In Long-Evans Cinnamon (LEC) rat, an animal model of human Wilson

disease which spontaneously developed hepatitis and liver fibrosis, HCC is frequently developed after the rats have recovered from initial fulminant hepatitis and subsequent liver fibrosis. This is considered to relate to progressive iron accumulation in the animal^[67], and iron depletion prevents their development of hepatic cancer^[68]. Even though the iron deposition in chronic hepatitis C is mild compared with that in hemochromatosis, iron may be an independent factor on the risk of HCC. It is reported that liver fibrosis is a favorable environment of proliferation of cancer cells by releasing transforming growth factor β , and there is a strong link between liver fibrosis and liver iron deposition. In clinical trials of phlebotomy, the hepatic content of 8-OH deoxyguanosine is decreased and fibrotic score is improved. An important issue in hepatocarcinogenesis in chronic hepatitis C is the closely related sustained production of ROS during inflammation and fibrosis. Moriya *et al*^[69] reported that HCC developed in HCV core transgenic mice after the age of 16 mo, and showed high hepatic lipid peroxidation levels in old (more than 16 mo) core transgenic mice, than in control. However, the association of HCV transgenic mice, and HCC development disappeared with advanced passaging of animals, suggesting that HCC development in HCV transgenic mice cannot be simply explained by HCV infection, but requires additional cofactors. A recent study by Furutani *et al*^[70] clearly showed that hepatic iron overload induces HCC in transgenic mice expressing HCV polyprotein. Transgenic animal carrying full length polyprotein-coding region (core to NS5B, nts 342-9378) by using pAlb promoter/enhancer was fed with excess iron diet. After 6 mo feeding, the transgenic mice showed marked steatosis and increased 8 hydroxy-2'-deoxyguanosine content in association with the hepatic iron accumulation. Twelve months after feeding, 45% of transgenic mice developed hepatic tumors including HCC. It is noteworthy that the steatosis does not accompany with inflammation but a remarkable ultrastructural alteration of mitochondria associated with decreased degradation activity of fatty acids.

STEATOSIS AND INSULIN RESISTANCE

Nonalcoholic steatohepatitis (NASH) is a clinical entity characterized by the development of histopathological changes in the liver that are nearly identical to those induced by excessive alcohol intake, but in the absence of alcohol abuse; the presence of macrovesicular steatosis and mixes inflammatory infiltrate associate with varying amounts of Mallory's hyaline, glycogenated nuclei, and focal hepatocyte ballooning degeneration. Clinical features of NASH include obesity, hyperlipidemia, diabetes mellitus, and hypertension. In US population, approximately 25% is obese, and at least 20% of the obese individuals have hepatic steatosis. Thus, non-alcoholic liver disease (NAFLD) is the most common cause of liver dysfunction, and it is believed that NASH becomes a cause of cryptogenic cirrhosis and hepatocellular carcinoma (HCC). In patients with homozygote of HFE-related hemochromatosis, obesity and steatosis affect liver disease progression, and will be cofactors for iron overload. There

is one study of Australia that showed that the prevalence of abnormal genotype of HFE in NASH is 31% compared to a normal prevalence of 13% in the general population, suggest that excess iron might be important. A study on North American subjects showed similar results that the prevalence of the HFE gene mutation associated with hereditary hemochromatosis are increasing in patients with NASH^[71]. In the study dealing Japanese NASH patients, who had no HFE gene mutations, a significant staining of liver iron and increased level of thioredoxin, a marker of oxidative stress in addition to the increase of serum ferritin, was observed.

As diabetes and obesity were background conditions of NAFLD, and is thought to be a initial triggering factor, insulin resistance is now considered the fundamental operative mechanism. Insulin resistance is probably the "first step" in NASH, and a close correlation between insulin resistance and iron is speculated. Even though it is not still clear whether secondary iron accumulation increases insulin resistance, or vice versa, oxidative stress may be the elusive "second" hit of possibly multiple steps in the progression of steatosis to fibrosing steatohepatitis^[72]. This may be due to the activation of stellate cells^[73].

Because hepatic iron promotes oxidative stress, it seems that iron is a contributory cofactor in NASH. This proposal is strengthened by an association with hepatic fibrosis with NASH^[74] and was confirmed by measuring serum markers of oxidative stress^[75-77]. Excess hepatic iron also occur in insulin resistance-associated iron overload (IRHIO), characterized by hyperferritinemia with normal to mild increases in transferrin saturation. There is an interesting clinical study that venesections and restricted diet are effective in patients with IRHIO^[78]. As in IRHIO, restriction of dietary calories, fat and iron improved NAFLD in addition the decrease of levels of serum aminotransferases and ferritin^[79]. It seems that the simultaneous disorder of iron and glucose and/or lipid metabolism, in most cases associated with insulin resistance, is responsible for persistent hyperferritinemia and identifies patients at risk for NASH^[80]. However, it is still unclear why iron is deposited in IRHIO and NAFLD. There is an interesting report by Bekri *et al*^[81] that there is an increase of hepcidin in adipose tissue of the severely obese but of liver, suggesting that severe obesity itself cause hypoferrremia due to the overproduction of hepcidin in the adipocytes. This finding may explain the hypoferrremia in severe obese patients, but does not show the mechanism of hepatic iron deposition in IRHIO and NASH. Further studies are needed to clarify this issue, including an increase of transferrin iron influx into hepatocytes in NAFLD.

In patients with NASH, increased transferrin saturation correlated positively with the severity of fibrosis in univariate analysis, although it became insignificant when age, obesity, diabetes, and AST/ALT ratio were controlled. A recent study showed improvement in insulin sensitivity with the use of venesection in 11 patients with NASH. Biweekly phlebotomy until serum ferritin concentration became lower than or equal to 30 ng/mL reduced mean serum ALT activity without a significant change of

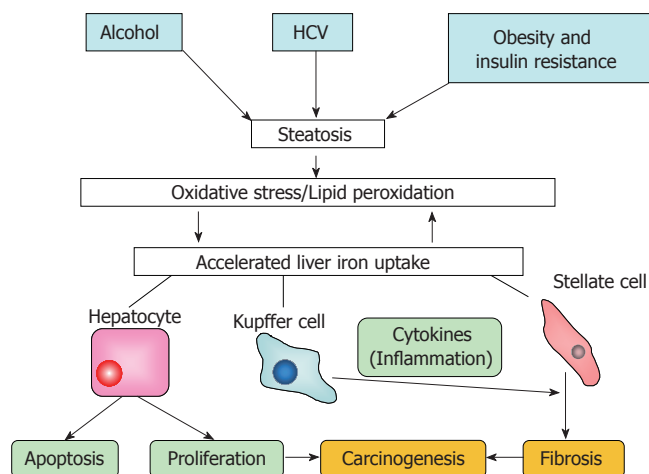


Figure 2 Postulated schema of liver damage occurred by alcohol, HCV infection, obesity and insulin resistant. A common pathway through steatosis/oxidative stress may be responsible for the development of liver fibrosis and carcinogenesis by iron.

body weight, suggesting that iron reduction therapy by phlebotomy will be one of the promising therapies for NASH^[82], although this approach cannot be implemented without extensive review.

The natural history of NASH is still unclear, but some patients follow advanced liver fibrosis progressing to cirrhosis and sometimes HCC^[83]. It is also known that diabetes increases the risk of hepatocellular carcinoma in US^[84]. Further studies are needed to clarify this issue, especially the relation between hepatocarcinogenesis from mild iron accumulation in NASH.

As shown in Figure 2, a common pathway through steatosis/oxidative stress may be present for the development of liver fibrosis and carcinogenesis by iron.

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Iron overload and immunity

Graça Porto, Maria De Sousa

Graça Porto, Maria De Sousa, ICBAS, Abel Salazar Institute for the Biomedical Sciences, Porto, IBMC, Institute of Molecular and Cellular Biology, Porto, Portugal

Graça Porto, HGSA, Santo António General Hospital, Porto 823 4150, Portugal

Supported by Portuguese Foundation for Science and Technology and Calouste Gulbenkian Foundation

Correspondence to: Graça Porto, Institute of Molecular and Cellular Biology, Rua do Campo Alegre, Porto 8234150, Portugal. gporto@ibmc.up.pt

Telephone: +351-22-6074956

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Abstract

Progress in the characterization of genes involved in the control of iron homeostasis in humans and in mice has improved the definition of iron overload and of the cells affected by it. The cell involved in iron overload with the greatest effect on immunity is the macrophage. Intriguing evidence has emerged, however, in the last 12 years indicating that parenchymal iron overload is linked to genes classically associated with the immune system. This review offers an update of the genes and proteins relevant to iron metabolism expressed in cells of the innate immune system, and addresses the question of how this system is affected in clinical situations of iron overload. The relationship between iron and the major cells of adaptive immunity, the T lymphocytes, will also be reviewed. Most studies addressing this last question in humans were performed in the clinical model of Hereditary Hemochromatosis. Data will also be reviewed demonstrating how the disruption of molecules essentially involved in adaptive immune responses result in the spontaneous development of iron overload and how they act as modifiers of iron overload.

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Key words: Iron; Iron overload; Innate immunity; Adaptive immunity

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THE TWO IMMUNITIES

The last decade has seen a growing understanding of the

numerous functions of the immune system beyond the two classical attributes of adaptive immunity: antigen specificity and memory. The key effector cell of adaptive immunity is the lymphocyte, a cell no longer known from its two principal origins in mammals, the thymus (T) and bone marrow (B), but with several subtypes characterized by different function and cytokine production profiles^[1]. “Behind” adaptive immunity lays a complex world of cells and molecules involved in less antigen-specific tasks collectively dedicated to the function of innate immunity. Two competing models have sought to explain innate immunity. One where evolutionary conserved features of pathogens are recognized by pattern recognizing receptors^[2] known generally as toll-like-receptors (TLRs). A second model, known as the danger model, is based on the assumption that products from damaged or stressed cells provide a danger signal to the host, thus evoking an innate immunity response^[3]. The effector cells of innate immunity are mostly myeloid cells and lymphocytes whose interaction with target cells does not depend on the recognition of the Major Histocompatibility Complex (MHC). A third cell, the dendritic cell (DC), was first described by Steinman and Cohn in 1973^[4]. DCs have been described most elegantly as “the nexus for translating signals from innate recognition into cells guiding adaptive immune function”^[5].

One hallmark of immune system cells, whether involved in innate or adaptive immunity, is their capacity to circulate and migrate from the blood compartment into lymphoid and non-lymphoid tissues (innate immunity cells) from non-lymphoid into lymphoid tissues (DCs) and from the blood into the lymph through the peripheral lymphoid organs (lymphocytes). The other is their role in protection from pathogen infections. Since early descriptions of immune cell functions in iron overload^[6] knowledge of the reciprocal interactions between immune cell responses, intracellular iron load and response to micro-environmental changes in iron levels has increased considerably. A decisive contribution to this understanding was the clarification of the genes involved in iron homeostasis. Reviews of the functions of such genes and their link to the reticuloendothelial system and immunity have been published recently^[7,8]. The present review will focus on the evidence that the cells of the immune system are equipped to modulate iron homeostasis through the expression of several iron related genes and proteins, and how do innate and adaptive immune cells respond in conditions of iron overload. In addition, the reader will be reminded of the evidence indicating that defective immune system models, particularly experimental models,

Table 1 Relevant genes/proteins involved in iron homeostasis and iron overload, and their related immunological functions

Genes/Proteins	Main role in iron homeostasis	Related immunological functions
Lactoferrin	Iron binding, transport and storage Iron chelator	Bactericidal and antiviral ^[13,14] . Immunoregulatory effects on Th1/Th2 cell activities ^[15]
Transferrin	Iron transporter	Present in monocyte/macrophages and T lymphocytes ^[16] Required for early T-cell differentiation ^[17]
Transferrin receptor 1	Cellular iron uptake	Iron uptake by activated lymphocytes and required for DNA synthesis and cell division of T lymphocytes ^[18]
Ferritin	Iron storage	Synthesised by macrophages and T lymphocytes ^[19,20]
Nramp1 (<i>SCLA11</i>)	Iron transfer in late phagolysosome	Resistance to infection with intracellular pathogens ^[21,22]
Nramp2/DMT-1	Iron uptake and transfer in late phagolysosome	Resistance to infection with intracellular pathogens ^[21,22]
Ferroportin 1 (IREG1)	Cellular iron exporter	TLR4 mediated downmodulation in infection ^[23]
Lipocalin	Unknown	Limits bacterial growth by sequestering the siderophore ^[24]
Regulators and modifiers of iron overload		
Hepcidin	Key regulator of iron homeostasis ^[25] . Gene disruption results in hemochromatosis ^[26] . Mutations associated with severe Juvenile Hemochromatosis (JH) ^[27]	Liver derived antimicrobial peptide ^[28] . TLR4 mediated expression in myeloid cells in response to bacterial pathogens ^[23]
Hemojuvelin	Regulation of hepcidin expression ^[29,30] . JH associated gene ^[31]	Unknown
IL-6 and IL-1	Involved in iron deprivation during infection and inflammation, through hepcidin induction and ferroportin down-modulation ^[32,33]	Derived from macrophages during infection and inflammation ^[32,33]
Heme-oxygenase 1	Required for mammalian iron reutilization. Targeted <i>Hmox1</i> mutations induce spontaneous iron overload ^[34]	High CD4:CD8 ratios with activated CD4+ cells in <i>Hmox1</i> deficient mice ^[34]
HFE	Hereditary Hemochromatosis associated gene ^[35] . HFE gene knockout produces mouse model of hereditary hemochromatosis ^[36]	Non-classical MHC-class I molecule ^[37]
β2-Microglobulin	β2m deficiency induces spontaneous iron overload and modifies the phenotype of HFE deficient mice ^[38,39]	Critical to the folding of MHC-class I molecules and selection of MHC class I molecule-associated peptides ^[40]
MHC-class I	MHC-class I deficient mice develop hepatic iron overload ^[41]	Critical molecule for viral peptide presentation ^[42]

are associated with the spontaneous development of iron overload^[9]. The mechanisms underlying what could be called the “reverse” side of this same coin, i.e. immunity and iron overload are less well understood. However, taking into account that practically all cells of the immune system express iron related genes their contribution to systemic iron homeostasis should no longer be ignored.

IRON AND INNATE IMMUNITY

As a critical element of cellular activity, iron plays a pivotal role in the fight for survival between mammalian hosts and their pathogens, each displaying a wide range of mechanisms for controlling iron acquisition and utilization. Micro-organisms have developed a large number of strategies to acquire iron from the environment and to transport the element to sites of incorporation into biologically important molecules^[10,11]. On the other side, the host has developed the capacity to modulate cellular iron metabolism, not only for its optimal utilization as a catalyst for the generation of reactive oxygen species acting as strong antimicrobial molecules, but also in order to make iron less available for the micro-organisms^[11,12]. A number of genes and proteins primary involved in iron homeostasis, namely in iron binding, transport and storage are now recognised to display related immunological functions. These are summarized in Table 1^[13-24]. In addition, it is becoming clear that the cells of the innate immune system, as part of a non-specific defense against

infection, are equipped to express genes and proteins that can modulate iron homeostasis both at the cellular and the systemic levels (see also Table 1)^[25-42]. One central player in this modulation is hepcidin, first described as a liver derived antimicrobial peptide^[28], and now well recognised as a key regulator of iron homeostasis and the anaemia of inflammation, at the interface of innate immunity and iron metabolism^[43]. Liver-derived hepcidin is strongly induced during infection and inflammation, causing intracellular iron sequestration and decreased plasma iron levels, a process mediated by the inflammatory cytokine cascade, namely macrophage derived IL-6 and IL-1^[32,33]. The mechanism underlying intracellular iron sequestration is mediated by the hepcidin-induced internalization and degradation of ferroportin, the only known iron exporter^[44]. The reduction in extracellular iron concentrations is believed to limit iron availability to invading microorganisms, thus contributing to host defense. Recently, hepcidin has been shown to be endogenously expressed by innate immune cells, i.e. macrophages and neutrophils, capable of migrating from the blood to a site of infection, constituting a newly recognised component of the local innate immune response to bacterial pathogens^[23]. Myeloid endogenous hepcidin mRNA expression in response to bacterial pathogens was shown to be dependent on the specific activation by the toll-like receptor 4 (TLR-4), the key pattern recognition receptor for LPS^[45]. This activation also produces down-regulation of the iron exporter

ferroportin. Endogenous myeloid hepcidin production is not stimulated by iron, pointing to different pathways of hepcidin activation in response to infection or iron overload^[23]. The mechanisms underlying the differential stimulatory effects of infection and iron overload on hepcidin are still not understood. Interestingly, it was recently shown that hepcidin levels in fish also respond both to iron overload and infection, demonstrating the evolutionary conservation of hepcidin's dual function^[46].

In addition to the demonstrated role of neutrophils and macrophages on hepcidin gene activation and ferroportin down-modulation, other iron genes and proteins involved in iron transport from the human phagosome into the cytosol also play critical roles in TLR-4 mediated innate immunity. The natural resistance-associated macrophage protein 1 (Nramp1) is expressed in circulating phagocytes, and is recruited from the lysosomal compartment to the phagosome membrane where it functions as an efficient antimicrobial through a mechanism of iron deprivation^[22]. Nramp1 mutations in mice are shown to cause susceptibility to infection with various intracellular pathogens including *Salmonella*, *Mycobacterium* and *Leishmania*^[21]. Mutations in the human homologue of the gene, NRAMP1, were also shown to strongly affect the susceptibility to tuberculosis^[47]. Nramp2, also known as the divalent metal transporter 1 (DMT1) is another important iron transporter in mammals^[48,49] and, as Nramp1, it is also induced by infection with intracellular pathogens, namely *Mycobacterium*^[50]. In addition, it is suggested to play an important role in recycling iron from RBC-containing phagosomes to the cytoplasm^[51]. The amount of available iron in the phagosome is also decreased by the entry in macrophages of neutrophil derived lactoferrin (Lf), another well known potent iron chelator found recently to have also a bridging role between innate and adaptive immunity. Both *in vitro* and *in vivo* studies showed that Lf is able to stimulate the proliferation and differentiation of T lymphocytes from their immature precursors into the Th1 or the Th2 phenotypes thus having an immunoregulatory effect on Th1/Th2 activities^[15].

Finally, lipocalin 2 has been recently described as a pivotal component of the innate immune system and the acute phase response^[52]. Upon infection the toll-like receptors on immune cells stimulate the transcription, translation and secretion of lipocalin 2 which then limits bacterial growth by sequestering the iron-loaded siderophore^[24].

INNATE IMMUNITY AND IRON OVERLOAD

If one or all of the above described iron related immune response genes either fail or the cells are overwhelmed by continuous iron overloading such as seen in transfusional iron overload, one consequence is the development of infection. Infections including rare microorganisms^[53] are among the major complications in patients with thalassemia, a group of common genetic disorders of hemoglobin synthesis clinically characterized by severe anemia and blood transfusion-dependent iron overload. Besides the well-known risks of blood borne viral infections associated with multiple transfusions, the

increased susceptibility of these patients to infection is known to be associated with a wide spectrum of immune abnormalities which are, at least in part, due to the effect of iron overload, including defective chemotaxis and phagocytosis by neutrophils and macrophages and decreased natural killer cell activity^[54].

In contrast with the findings in transfusional iron loading, patients with severe iron overload due to Hereditary Hemochromatosis (HH) do not show evidence of increased susceptibility to infections or iron loading of macrophages. HH is a common genetic disorder of iron overload, the majority of HH patients being homozygous for the C282Y mutation in *HFE*, a gene encoding a protein of the Major Histocompatibility Complex class I (MHC class I). The C282Y mutation disrupts the correct folding of the $\alpha 3$ domain of the protein, interfering with its interaction with $\beta 2$ -microglobulin ($\beta 2m$) and consequently abolishing the cell surface expression of the molecule^[35]. The surface expression of *HFE* was shown to have a prominent role in the regulation of iron export from macrophages^[55,56].

Monocyte/macrophage abnormalities in HH

Anomalies in monocyte/macrophage cells have been consistently described in HH patients, including low TNF- α production by peripheral blood macrophages upon stimulation with lipopolysaccharide^[57] and a significant increase in iron regulatory protein (IRP) activity in monocytes^[58], an anomaly that was corrected after phlebotomy treatment. Interestingly, subjects with a tissue iron burden similar to HH patients, but due to secondary iron overload have an IRP activity significantly decreased suggesting that the increased IRP activity in iron overload is specific to the *HFE* related hereditary form of hemochromatosis^[58]. In addition, a study that investigated the release of erythrocyte-derived iron from purified human monocytes obtained from controls and HH patients showed that although HH monocytes phagocytosed less than half the number of erythrocytes taken up by control monocytes, they released twice as much iron in the form of LMW-Fe complex than controls^[59]. More recently, increased iron content was described in macrophages from HH patients transfected with wt *HFE* when compared with HH macrophages transfected with an empty vector^[56]. These observations are consistent with previously reported observations in macrophage cell lines derived from C282Y mutated HH patients, where *HFE* was shown to lose its ability to inhibit iron release leading to a relative macrophage iron deficiency^[55]. Interestingly, *Mycobacterium tuberculosis* (*M.tb*) residing within phagosomes of macrophages from HH patients exhibit a profound defect in their ability to acquire iron from exogenous transferrin and lactoferrin relative to *M.tb*-infected macrophages from normal controls^[60]. Moreover, macrophages from HH patients failed to induce Nef-mediated iron and ferritin accumulation upon HIV-1 infection in contrast to macrophages expressing wild-type *HFE*^[61], thus suggesting that *HFE* mutated macrophages may be better equipped to protect from HIV-1 infection, compatible with the description of a long survival in a patient with AIDS

and hereditary hemochromatosis^[62]. One must therefore wonder whether the failure of macrophages from C282Y *HFE* HH patients to hold on to the iron is the expression of a putative selective advantage protecting from infection brought by the appearance and the establishment of such a mutation.

Iron and the dendritic cells

Very little work has addressed the interaction between iron loading and dendritic cell (DC) function. There is, however, recent evidence indicating that, upon endotoxin induced maturation, DCs increase significantly the expression of Tfr1 and down regulate expression of the export molecule ferroportin^[63], an observation compatible with an earlier finding of Kramer *et al*^[64] who reported that DCs generated under iron deprivation conditions were phenotypically undifferentiated and could not stimulate T cells.

IRON OVERLOAD AND ADAPTIVE IMMUNITY

The postulate that the immunological system could have a role in monitoring tissue iron toxicity, as part of its surveillance function, was first advanced in 1978, based on studies on lymphocyte traffic and positioning^[65-67]. It was implicit in that postulate that the lymphomyeloid system, and its circulating components participate in the recognition and binding of metals as a protective device against metal toxicity, and the preferential use of indispensable metals, such as iron, by bacteria or transformed cells. While a vast number of studies have clarified the reciprocal interactions between myeloid innate immunity cells and iron metabolism, fewer studies addressed this question in lymphocyte populations. Lymphocyte activation and expansion depend on the expression of transferrin receptors, required for DNA synthesis and cell division^[18] and both activated and non-activated T lymphocytes synthesize ferritin^[19,20]. Lymphocytes could, therefore, act as a “mobile” and easily “mobilizable” iron-storage compartment protecting from iron-mediated toxicity^[65]. This hypothesis motivated the study of lymphocyte function in iron overload^[68]. The influence of iron on the expansion of different T-cell subsets was demonstrated both *in vitro*^[69] and *in vivo*, namely in patients with thalassemia^[70]. Results in this clinical model, however, are difficult to interpret due to the inseparable effects of blood transfusion, splenectomy, iron chelation therapy and infection. Imbalances of the relative proportions of CD4+ and CD8+ T lymphocytes, with abnormally high CD4/CD8 ratios were later reported in HH patients^[71,72], a clinical model where iron overload is not complicated by the effects of transfusion, splenectomy or desferrioxamine. Curiously, in one experimental model of iron overload seen in mice generated with targeted *Hmox1* mutations, high CD4:CD8 ratios have been seen in the splenic cell populations of older mice aged 50 wk^[34]. These mice also exhibited numerous activated CD4+ cells. Hepatic inflammatory cell infiltrates were seen in the mice, a finding similar to that reported by Rodrigues *et al*^[73] in

aging *Hfe* deficient mice.

T lymphocyte abnormalities in HH

Abnormalities in the relative proportions of the two major T lymphocyte subpopulations have been consistently described in HH patients. Reimão and co-workers first described that patients with abnormally high CD4/CD8 ratios displayed a faster re-entry of iron into the serum transferrin pool after intensive phlebotomy treatment than patients without those abnormalities^[71]. It was shown later that the amount of iron mobilized by phlebotomy correlated significantly with the number of CD8+ T cells, but not with CD4+ T cells^[72,74]. An independent study examining patients homozygous for the C282Y mutation showed that the low percentages of CD8+ T cells seen in the peripheral blood of HH patients were associated with low numbers of the same cells in the liver and with higher levels of hepatic tissue iron^[75]. HH patients had been shown earlier to have reduced percentages of CD8+ CD28+ T cells in peripheral blood^[76]. No anomalies of CD28 expression were found in the CD4+ subset. The apparent failure of the CD8+ CD28+ T cell population to expand coincided with an expansion of CD8+ CD28- T cells in peripheral blood of HLA-A3+ but not HLA-A3-HH patients^[76]. Although the described abnormalities in lymphocyte populations were systematically found in the sub-population of CD8+ T lymphocytes, the association with total body iron stores is also reflected in the total lymphocyte counts. Low total lymphocyte counts were found associated significantly with a higher degree of iron overload in *HFE*-linked HH, but not in African iron overload^[77]. More recently, Barton *et al*^[78] also described a significant inverse relationship of total blood lymphocyte counts and severity of iron overload in hemochromatosis probands with *HFE* C282Y homozygosity. Fabio *et al*^[79] confirmed that the presence of low numbers of total lymphocyte counts and CD8+CD28+ T cells in C282Y homozygous patients was inversely related to the transferrin saturation. In addition, they found low numbers of CD4+ T and NK cells, and a major increase in IL-4 and IL-10 production in the CD3+ CD8+ T cell subset^[79]. A study of the V α / β T cell receptor (TcR) repertoire in a population of C282Y homozygous HH patients showed that the frequency of V α / β TcR expansions within the CD8+ pool in the group of HH patients was significantly higher in those with iron overload related pathology (9/16) than in patients (1/16) without pathology^[80]. In the same study it was found that control subjects heterozygous for the C282Y mutation had an absence of expansions of the V β 5.2 and V β 12 chains in the CD8+ pool, suggesting that *HFE* could have an effect in the shaping of T cell receptor repertoire.

Functional abnormalities in CD8+ T lymphocytes were also described in HH patients. The level of autophosphorylation of the CD8-associated p56lck as well as its phosphotransferase activity, as determined by phosphorylation of an exogenous substrate, was significantly reduced by two- to three-fold in HH patients relative to a control population of healthy donors^[81]. By contrast, the level of CD4-p56lck activity did not show an

overall decrease relative to controls. The decreased CD8-p56lck activity seen in patients was not corrected by iron depletion. A significantly higher percentage of HLA-DR+, but not CD45RO+ cells was also found within the peripheral CD8+ T cell subset in HH patients relative to controls^[76]. Moreover, functional studies showed that CD8+ cytotoxic T lymphocytes (CTL) from HH patients exhibited a diminished cytotoxic activity when compared with CD8+ CTL from healthy controls^[76].

The finding of a significant association of abnormally low CD8+ T lymphocytes with a more severe clinical expression of hemochromatosis in HH patients^[72,82] raises the obvious question: are if these anomalies the follow or precede the development of iron overload. The fact that those abnormalities are remarkably stable in each individual patient, that they are not corrected by phlebotomy treatment, and that they are observed in asymptomatic patients at young ages, favors the hypothesis that they are intrinsic to the genetic defect and not a consequence of the progressive iron overload. More recently it was shown that the numbers of CD8+ T lymphocytes are genetically determined, in association with other genetic determinants at the MHC class I region close to HLA and HFE^[83,84]. It is, therefore, conceivable that the inherited abnormalities in CD8+ T lymphocytes in HH are modifiers of the clinical expression of the disease as proposed by Cruz *et al.*^[82] or genetically located close to a yet unidentified modifier gene of iron metabolism.

THE REVERSE TOPIC: ADAPTIVE IMMUNITY AND IRON OVERLOAD

Genetically manipulated animal models have, therefore, become wonderful and decisive tools to address the questions of the effect on iron overload of a specific gene or protein at the systemic level. Several animal models of spontaneous iron overload were described that illustrate the influence of proteins of the adaptive immunological system on iron homeostasis, all pointing to the putative importance of the MHC class I region.

The $\beta 2m$ -microglobulin deficient ($\beta 2m^{-/-}$) mice constitute the first described model of spontaneous iron overload^[38,85,86]. These mice develop a hepatic iron overload with a distribution similar to that seen in HH liver pathology, i.e., mainly in the liver parenchyma with no evidence of iron loading in the Kupffer cells^[38,86]. These mice present severe decreased cell surface expression of the MHC-class I molecules and consequently almost no CD8+ T lymphocytes^[87,88]. Intestinal uptake of ferric iron and the subsequent transfer into the plasma is inappropriately increased in $\beta 2m^{-/-}$ mice^[86]. Upon treatment with hematopoietic cells derived from normal mice fetal liver iron overload is attenuated and shifted from the parenchymal to the Kupffer cells^[86,89]. However, TfSat and intestinal iron absorption remain high, suggesting that the primary defect of iron overload is not corrected. With the discovery of the HFE gene and the demonstration that the C282Y mutated form failed to bind to $\beta 2m$ ^[35] it was assumed that the earlier findings in $\beta 2m^{-/-}$ were due to an impaired HFE function. However, several subsequent studies showed that this was not sufficient to explain the

pathology of $\beta 2m$ deficient mice. In contrast to $Hfe^{-/-}$, the $\beta 2m^{-/-}$ mice display increased expression of the duodenal iron transporters DMT1 and ferroportin1, implicating a broader role of $\beta 2m$ in mammalian iron overload^[90]. More recently, Rodrigues *et al.*^[73] described results of a comparative study of these two models in older mice. The results confirmed that the $\beta 2m^{-/-}$ old mice present a more severe hepatic iron overload than the $Hfe^{-/-}$ counterparts. $\beta 2m^{-/-}$ old mice which also showed liver steatosis, probably as a reflection of the higher hepatic iron content causing lipid peroxidation. Earlier Levy *et al.*^[91] had reported the finding that in mice lacking both the Hfe and the $\beta 2m$ molecules, liver iron deposition is observed in greater levels than in mice lacking Hfe alone.

The $\beta 2m^{-/-}$ - $Rag1^{-/-}$ double knock out mice lack mature T and B lymphocytes as well as MHC-class I and Hfe expression. $Rag1$ is required for normal T and B lymphocyte development. $\beta 2m$ is required for correct folding and cell surface expression of MHC-class I like proteins. These mice present a more severe body iron overload than each of the single knock out models^[39]. Besides liver iron deposition in the parenchymal cells, the $\beta 2m^{-/-}$ - $Rag1^{-/-}$ also showed iron deposition in pancreas and heart. Older mice under an iron-enriched diet develop heart fibrosis, which could be prevented by treatment with normal fetal liver hematopoietic cells. To determine whether the effect of the $\beta 2m$ deficiency in the $\beta 2m^{-/-}$ - $Rag1^{-/-}$ double knock out mouse was only due to lack of Hfe expression, double knock out mice for Hfe and $Rag1$ were generated^[92]. $Hfe^{-/-}$ - $Rag1^{-/-}$ double knock out mice showed increased liver iron overload compared to each of the single knock out, or the $\beta 2m^{-/-}$ - $Rag1^{-/-}$. The distribution of the iron loading in $Hfe^{-/-}$ - $Rag1^{-/-}$ mice did not recapitulate the iron loading of the $\beta 2m^{-/-}$ - $Rag1^{-/-}$, since they did not present heart or pancreas iron loading.

The δ TCR^{-/-} mice lack the $\gamma\delta$ intraepithelial lymphocytes. Following the administration of an iron supplemented diet, these mice showed an increased liver iron accumulation in relation to control mice^[93]. In addition, δ TCR^{-/-} mice had a marked reduction of tumor necrosis factor alpha (TNF- α) production by intraepithelial lymphocytes when compared with controls suggesting a role for this cytokine in intestinal iron regulation.

Finally, mice deficient in the MHC class I molecules H2K^b and D^b have a strong reduction in CD8+ T-lymphocyte numbers^[94]. These mice present a spontaneous increase of iron content in the liver preferentially in hepatocytes with occasionally Kupffer cells iron staining^[41]. The liver iron content in this model was shown to correlate directly with the number of residual CD8+ T lymphocytes (Cardoso E and M de Sousa, unpublished observations).

The above described double knockout mouse models provide a good illustration of the modifier effect of the components of the adaptive immune system, namely a MHC class I dependent effect, on the iron overload phenotype, a conclusion also reached separately by Muckenthaler *et al.*^[90] in a study of differential gene expression in $\beta 2m$ deficient mice. Altogether the results described in animal models of hemochromatosis may help to explain why immunological anomalies

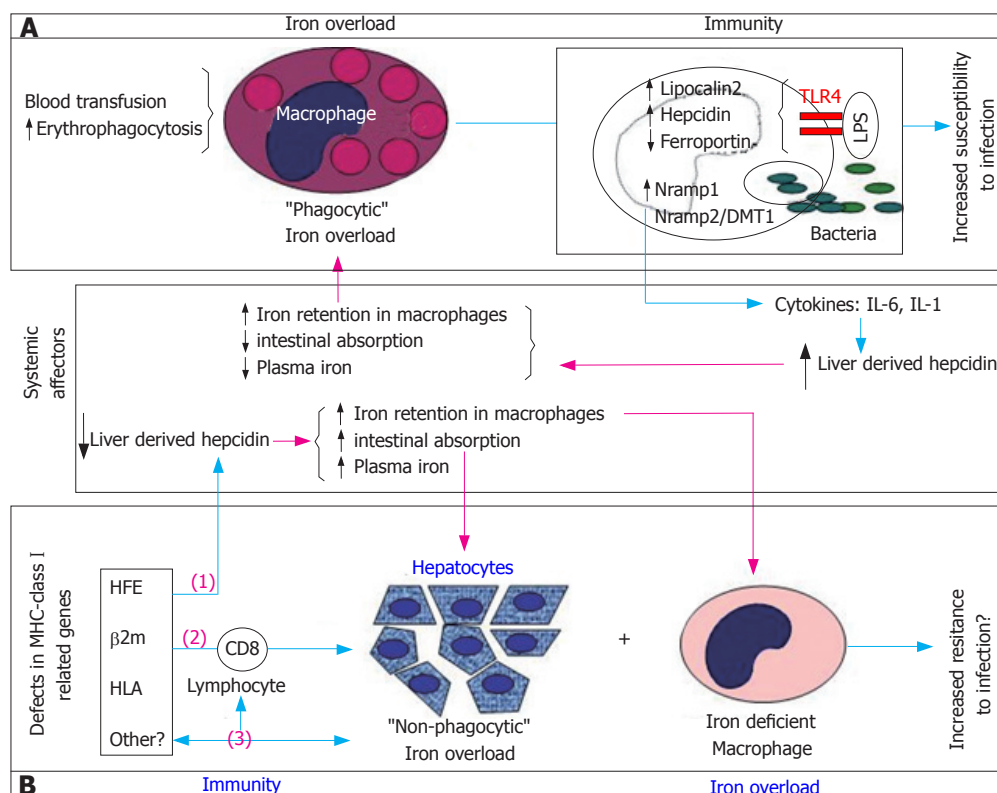


Figure 1 A model illustration of the two topics addressed in this review: how innate immunity is affected and affects "phagocytic" iron overload (Box A) and the evidence that defective immune system models, namely natural and experimental defects in MHC-class I related genes (*HFE*, β 2-microglobulin and *HLA*) are associated with the spontaneous development of "non-phagocytic" or parenchymal iron overload (Box B). **A:** Macrophages "overwhelmed" by continuous iron overloading as seen in conditions of chronic transfusion and increased erythrophagocytosis are compromised in their function of surveillance of bacterial growth resulting in increased host susceptibility to infection. As part of the natural response to bacterial infection macrophages modulate the expression of a number of genes involved in the regulation of cellular iron metabolism. By activating the cytokine cascade they induce up-regulation of liver hepcidin synthesis with further increase in macrophage iron retention and perpetuation of this cycle; **B:** A "non-phagocytic" or parenchymal iron overload is observed in hepatocytes of several defective immune system models associated with a relative iron deficiency in macrophages, recapitulating the findings observed in the human model of Hereditary Hemochromatosis. The mechanisms underlying the spontaneous development of iron overload in these models are not yet understood. Three possible (not mutually exclusive) mechanisms are represented in this figure: (1) the fact that both *Hfe* and β 2m knockout mouse models lack the liver hepcidin response to iron overload^[80,97,98] may support the notion of a liver-derived, systemically induced iron overload. The involvement of liver hepcidin in the development of iron overload in mice deficient in the MHC-class I molecules *H2K^b* and *D^b* (*HLA*) was never tested; (2) the iron overload seen in the β 2m^{-/-} *Rag1*^{-/-} and *Hfe*^{-/-} *Rag1*^{-/-} mouse models support the involvement of lymphocytes themselves as modifiers of the severe parenchymal iron overload phenotype seen in those mice^[39]; (3) finally, the recent demonstration of a strong link between the MHC-class I region and the genetic transmission of low CD8⁺ T lymphocyte numbers in humans and the association of total lymphocyte numbers with a more severe iron overload phenotype in patients with Hereditary Hemochromatosis^[78,82,83] opens a new possibility that another, still unidentified MHC-class I associated gene(s) may simultaneously regulate CD8⁺ T lymphocyte numbers and influence the development of parenchymal iron overload^[84].

modify the severity of iron overload in Hereditary Hemochromatosis^[82,95].

CLOSING REMARKS

The growing knowledge and availability of genetic techniques dissecting the fine components of the host response to the challenge of infection have strengthened the opportunity of revising the topic "iron and immunity". When the main concern for this topic resided in the effects of iron deficiency on the immune response, Weinberg pioneered the opposite concern for iron overload and infection^[12]. Today it is evident that in response to infection numerous innate immunity components display metal chelating properties, including synthesis and release of lactoferrin, lipocalin and hepcidin, as discussed above. The cells involved in that response are neutrophils and macrophages. But, during evolution the macrophage, particularly the splenic macrophage, assumed the key

physiological role of recognizing senescent red blood cells and recycling the iron in hemoglobin^[96]. Exhaustion of that pool *in vivo* leads to "phagocytic" iron overload, such as seen in transfusional iron overload, with the expected consequences in the development of infection. If on the other hand the macrophage fails, it is to be expected that "parenchymal" iron overload will develop. This is the case in *HFE*-Hereditary Hemochromatosis. Therefore, iron overload must not be seen as one entity, but two separate entities with different relationships to immunity (Figure 1). Evidence was provided in this review that innate immunity is affected and affects "phagocytic" iron overload. On the reverse side, defective immune system models, namely natural and experimental defects in MHC-class I related genes (*HFE*, β 2-microglobulin and *HLA*) are associated with the spontaneous development of "non-phagocytic" or parenchymal iron overload.

The large new question looming in the horizon of this topic with which we wish to close this brief review is: does

the MHC class I region harbor, in addition to *HFE*, other gene or genes that besides regulating lymphocyte numbers also influence the development of “non-phagocytic” or parenchymal iron overload? The search for the answer to this question will probably guide many research interests for years to come.

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TOPIC HIGHLIGHT

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Molecular mechanisms involved in intestinal iron absorption

Paul Sharp, Surjit Kaila Srai

Paul Sharp, Nutritional Sciences Division, King's College London, London SE1 9NH, United Kingdom

Surjit Kaila Srai, Department of Biochemistry & Molecular Biology, University College London, London NW3 2PF, United Kingdom

Correspondence to: Dr. Paul Sharp, Department of Nutrition & Dietetics, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH,

United Kingdom. paul.a.sharp@kcl.ac.uk

Telephone: +44 -20-78484481

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Abstract

Iron is an essential trace metal in the human diet due to its obligate role in a number of metabolic processes. In the diet, iron is present in a number of different forms, generally described as haem (from haemoglobin and myoglobin in animal tissue) and non-haem iron (including ferric oxides and salts, ferritin and lactoferrin). This review describes the molecular mechanisms that co-ordinate the absorption of iron from the diet and its release into the circulation. While many components of the iron transport pathway have been elucidated, a number of key issues still remain to be resolved. Future work in this area will provide a clearer picture regarding the transcellular flux of iron and its regulation by dietary and humoral factors.

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Key words: Haem; Non-haem iron; DMT1; IREG1; Dcytb; Hephaestin

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INTRODUCTION

Iron is an essential trace metal for all organisms. In humans it plays numerous biochemical roles, including oxygen binding in haemoglobin and as an important catalytic centre in many enzymes, for example the

cytochromes. In normal healthy adults, some 0.5-2 mg of iron is lost each day due to blood loss and the constant exfoliation of iron-containing epithelial cells that line the gastrointestinal and urinary tracts, skin and hair. Therefore, the same amount of iron from dietary sources is required each day to replace the lost iron and maintain body iron homeostasis. Even though iron is an essential metal in human metabolism, it is highly toxic to cells and tissues if present in elevated levels. Perversely, humans do not possess the necessary machinery to rid the body of excess iron and, therefore, the absorptive process must be tightly regulated within defined physiological limits to avoid pathologies associated with both iron deficiency and overload.

Dietary iron is found in two basic forms, either as haem-found in meat and meat products-or non-haem iron-present in cereals, vegetables, pulses, beans, fruits *etc* in a number of forms ranging from simple iron oxides and salts to more complex organic chelates. Non-haem iron predominates in all diets comprising some 90%-95% of total daily iron intake. The absorption of both haem and non-haem iron takes place almost exclusively in the duodenum and the bioavailability of iron from these sources is influenced by a number of variables, e.g. the iron content of foods, the type of iron present, i.e. haem or non-haem, and other dietary constituents. Importantly, absorption is also regulated in line with metabolic demands that reflect the amount of iron stored in the body, and the requirements for red blood cell production. These humoral mechanisms are further reviewed in this volume^[1].

Despite accounting for only 5%-10% of dietary iron in western countries, haem is the most bioavailable source of iron amounting to is some 20%-30%^[2]. In contrast, the bioavailability of non-haem iron is low-only 1%-10% of the dietary load is absorbed-and is profoundly influenced by other dietary components that can enhance or inhibit non-haem iron bioavailability. The most potent enhancer is ascorbic acid (vitamin C), which acts by reducing ferric iron to the more soluble and absorbable ferrous form. Phytates found in cereal products and polyphenolic compounds found in all plant products are the most potent dietary inhibitors of non-haem iron absorption. However, it is important to note that on an equimolar basis the pro-absorptive action of dietary ascorbic acid can counteract the inhibitory effect of phytates and polyphenols^[3].

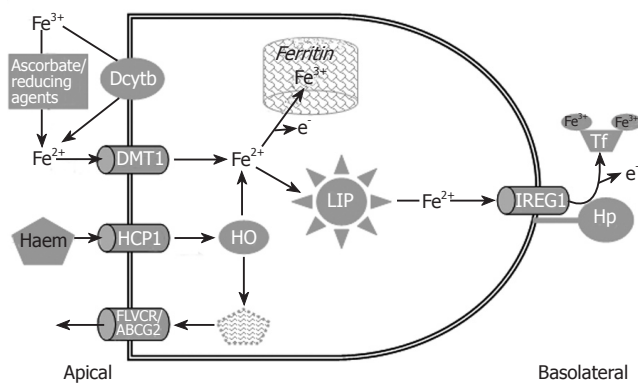


Figure 1 The cellular mechanisms involved in intestinal iron absorption. Dietary non-haem iron (mostly ferric) is reduced by the actions of the ferric reductase Dcytb and reducing agents in the diet to yield Fe^{2+} , which subsequently enters the enterocytes via DMT1. Haem is absorbed via HCP1, broken down by haem oxygenase 1 (HO) to liberate Fe^{2+} (this joins a common pool with iron from the non-haem pathway) and bilirubin (which might be removed from the cell by the efflux proteins FLVCR and ABCG2). If body iron stores are high, iron may be diverted into ferritin and lost when the cell is shed at the villus tip. Alternatively, iron passes into the labile iron pool (LIP) and is subsequently processed for efflux via IREG1 (as Fe^{2+}). The exiting iron is re-oxidised to Fe^{3+} through hephaestin (Hp) to enable loading onto transferrin (Tf).

MECHANISMS INVOLVED IN INTESTINAL IRON TRANSPORT

In recent years our understanding of the mechanisms involved in dietary iron absorption by duodenal enterocytes has increased dramatically. Both haem and non-haem iron are taken up in this proximal region of the small intestine, though their transport across the apical membrane of the enterocytes occurs through totally independent pathways (Figure 1).

Ferrous iron

The majority of dietary non-haem iron enters the gastrointestinal tract in the ferric form. However, Fe^{3+} is thought to be essentially non-bioavailable (see below) and, therefore, it must first be converted to ferrous iron prior to absorption. There are numerous dietary components capable of reducing Fe^{3+} to Fe^{2+} , including ascorbic acid^[4], and amino acids such as cysteine^[5] and histidine^[6]. It is believed that the action of these dietary reducing agents takes place in the acidic environment of the gastric lumen. Indeed the essential requirement for an acid environment in iron absorption is exemplified by the fact that achlorhydria is commonly associated with iron deficiency anaemia^[7,8]. However, ferric iron reaching the duodenal enterocytes may still be reduced by the cells endogenous reducing activity. A number of studies have demonstrated that the brush-border surface of duodenal enterocytes and cultured intestinal cells possess ferric reductase enzymic activity^[9-11]. Using a subtractive cloning strategy designed to identify intestinal genes involved in iron absorption, Dcytb (for duodenal cytochrome b), a homologue of cytochrome b_{561} , was identified as the enzyme responsible for this process^[12]. Like cytochrome b_{561} , Dcytb is a haem-containing protein with putative binding sites for ascorbate and semi-dehydroascorbate. The protein is expressed on

the brush border membrane of duodenal enterocytes, the major site for the absorption of dietary iron. Moreover, antibodies raised against Dcytb block the endogenous ferric reductase activity of the duodenal brush border membrane, providing further evidence for a functional role in dietary iron processing^[12].

Intriguingly, a recent study has reported that the targeted disruption of the *Cybrd1* gene in mice (which encodes Dcytb) does not lead to an iron deficient phenotype^[13], implying that Dcytb is not necessary for intestinal iron absorption in mice. However, an important caveat to this study is the fact that mice are able to synthesize significant quantities of ascorbic acid-unlike humans who are reliant on dietary vitamin C to satisfy their requirements-and may, therefore, have less need for a duodenal surface ferric reductase.

Following reduction either by Dcytb or dietary reducing agents, the resulting Fe^{2+} becomes a substrate for the divalent metal transporter, DMT1-also known as the divalent cation transporter, DCT1^[14], and natural resistance associated macrophage protein, Nramp2^[15]. The relatively low pH of the proximal duodenum together with the acid microclimate present at the brush border membrane^[16,17] stabilises iron in the ferrous form and provides a rich source of protons that are essential for driving iron uptake across the apical membrane of the intestinal epithelium^[14,18].

The role of this transporter in intestinal iron homeostasis has been highlighted by a number of studies. Our work^[18] and that of others^[19] have shown that antibodies to DMT1 can significantly inhibit iron absorption. Furthermore, targeted disruption of DMT1 in mice has revealed the essential role of this transporter in both intestinal iron absorption and in erythroid precursor development^[20]. In addition to these biochemical and molecular manipulations, two rodent models, the *mk/mk* mouse^[15] and the Belgrade (*b*) rat^[21], which possess a spontaneous mutation (G185R) in the DMT1 gene, exhibit defective intestinal iron uptake and microcytic anaemia. More recently a number of mutations in human DMT1 have also been identified^[22-25] which in turn lead to the development of microcytic anaemia.

The molecular identity of the functional DMT1 isoform in intestinal epithelial cells has been the subject of recent debate. At least four isoforms exist through alternate splicing in exon 16^[26] and the presence of two transcription start sites-named exon 1A and 1B^[27]. Exon 16 splicing gives rise to two variants which differ in their terminal 19-25 amino acids and their 3' untranslated sequence (UTR). Interestingly one of these variants contains an iron responsive element in its 3' UTR^[26]. The significance of this is discussed later in this review. All four isoforms can be detected at varying levels in intestinal epithelial cells^[27], but the exon 1A/IRE-containing variant has been suggested to be the major functional isoform in absorptive enterocytes.

Ferric iron

We stated earlier that Fe^{3+} is thought to be non-bioavailable. However, some workers have proposed that Fe^{3+} might be absorbed by intestinal enterocytes *via* a

mechanism that is distinct from DMT1 (reviewed in^[28]). In this model, ferric iron, which is insoluble at physiological pH, is released from the food matrix in the acidic environment of the stomach, and is chelated by mucins on the duodenal brush border surface, which maintain the iron in the ferric state. Fe^{3+} enters the enterocyte across the apical membrane via interaction with β_3 -integrin and mobilferrin (a calreticulin homologue). In the cytosol, this complex combines with flavin monooxygenase and β_2 -microglobulin to form a larger conglomerate (approximately 520 kDa) known as paraferitin, which has ferric reductase activity resulting in the conversion of the absorbed Fe^{3+} to Fe^{2+} . Recent evidence suggests that the paraferitin complex may also contain DMT1^[29], which may permit the delivery of ferrous iron to intracellular organelles.

Ferritin

In animal and plant tissues, the major iron storage protein is ferritin. Most nutrition texts focus only on haem and non-haem iron, and generally ignore the possibility that ferritin may be an important nutritional source of iron. While the issue of ferritin bioavailability is still controversial, a number of studies have shown that both plant and animal ferritin sources are absorbed in humans^[30-32] with a bioavailability equivalent to that of ferrous sulphate. The ferritin iron uptake mechanism is yet to be determined. One possibility is that ferritin is broken down by protease activity in the upper gastrointestinal tract and the released iron is absorbed *via* the Dcytb/DMT1 route. However, studies have shown that ferritin is largely resistant to high temperature, low pH and protein denaturing agents^[33,34]. Therefore, it is possible that ferritin may be absorbed intact and broken down intracellularly (in the lysosomes) to liberate its iron load. In support of this latter possibility, one study has reported that iron and ferritin protein are both taken up by the intestinal Caco-2 cell line^[35]. Such a mechanism would require the presence of a ferritin receptor on the apical membrane of intestinal enterocytes. While the presence of ferritin receptors has been postulated on liver^[36] and placental^[37] plasma membranes, none has yet been identified in intestinal tissue. Taken together this evidence suggests that the molecular identity of at least one important intestinal iron transport gene may remain to be discovered.

Lactoferrin

In breast-fed infants, a major proportion of iron is bound to the human milk protein lactoferrin, an iron-binding protein capable of binding two ferric ions^[38]. Specific receptors for lactoferrin have been identified on the brush border surface of foetal enterocytes^[39] and subsequent studies have shown that these receptors mediate the uptake of lactoferrin-bound iron in intestinal epithelial cell cultures^[40]. Interestingly, a recent study looking at ontogenic changes in the expression of iron transport proteins in mouse small intestine, has suggested that the lactoferrin receptor may be the principal iron transport pathway in early life^[41]. Intriguingly, a recent human volunteer study has indicated that the nutritional importance of lactoferrin

may not be limited to infants since it is also a bioavailable source of iron (with equivalent bioavailability to ferrous sulphate) in young adult females^[42].

Haem

Non-vegetarian diets provide an additional and important source of iron in the form of haem (largely from haemoglobin and myoglobin). While haem comprises only approximately 10% of dietary iron intake, because it is more bioavailable than non-haem iron, it may contribute as much as half of the total iron absorbed from western meat-rich diets^[43]. Despite the wealth of information available on the uptake of non-haem iron, the mechanisms involved in haem iron absorption are only just beginning to emerge. Early work on intestinal absorption suggested that haem binds to the duodenal brush border membrane and is absorbed as an intact molecule^[43,44]. In support of this proposed mechanism, workers have reported the existence of haem binding proteins on pig enterocytes^[45,46] and intestinal Caco-2 cells^[47]. More recently, a number of candidate haem binding proteins have been identified in the intestinal epithelial cells including the ATP-binding cassette protein, ABCG2^[48], the feline leukaemia virus C receptor protein, FLVCR^[49] and the haem carrier protein, HCP1^[50]. Of these candidate haem transporters, ABCG2 and FLVCR mediate haem efflux and only HCP1 acts as a haem import protein. The high duodenal expression of HCP1 suggests that it may be the protein involved in haem uptake from the diet. However, the precise role of HCP1 in iron metabolism remains to be fully elucidated. This issue has been complicated by recent data indicating that HCP1 may also function as a proton-coupled folate transporter, independent from its haem transporting properties^[51].

Following absorption, haem is detectable in membrane-bound vesicles within the cytoplasm^[52,53]. Within these vesicles, it is thought that the iron contained within the protoporphyrin ring is excised by the action of haem oxygenase 1^[54] yielding ferrous iron which enters a common intracellular pool along with the iron absorbed *via* the non-haem transport pathways. Digestion appears to be complete within the enterocytes since a number of studies have failed to detect intact haem efflux across the basolateral membrane^[44,47]. One intriguing possibility is that the efflux proteins ABCG2 and FLVCR, also expressed in the duodenum, may act to remove bilirubin formed as a by-product of haem degradation from the enterocytes.

Intracellular storage and translocation of iron

At this stage, the absorbed iron has two fates depending on the body's requirements. If the body stores are replete, and there is no increased erythropoietic drive, a significant amount of newly absorbed iron will be stored in the enterocytes as ferritin. Because duodenal enterocytes turnover very rapidly (their lifespan is approximately 3-4 d) and the majority of enterocytes contributing to absorption lie in the mid/upper villus region, this intracellular ferritin iron is quickly lost into the intestinal lumen as the ageing cells are sloughed off at the villus tip. Interestingly, in human subjects there is a positive correlation between

dietary iron bioavailability and faecal ferritin content which supports the above mechanism^[55,56]. Indeed it is likely that this is a very important mechanism for controlling the release of iron into the circulation.

The mechanism by which iron is translocated from the apical pole of the enterocytes so that it becomes available for export across the basolateral membrane is poorly understood. A body of evidence has emerged from studies in Caco-2 cells for a vesicular transport pathway that co-ordinates the transcellular movement of iron. Central to this mechanism is apo-transferrin (apo-Tf) which when added at the basolateral surface of the Caco-2 cell monolayer stimulates transepithelial iron flux in a dose-dependent manner^[57-59]. Interestingly, in Caco-2 cells apo-Tf and Fe-Tf, once taken up from the basolateral medium, appear to be directed into distinct endosomal vesicular fractions^[59,60]. The apo-Tf containing endosomes are routed towards the apical pole of the cell where they co-localise with vesicles containing DMT1^[61]. It is proposed that the iron entering the cell along with DMT1 is transferred to apo-Tf within these endocytic vesicles, and is subsequently exocytosed into the basolateral medium as Fe-Tf. Using a combination of biochemical inhibitors to disrupt this vesicular network, it is estimated that this pathway may count for as much as 50% of the transepithelial iron flux in Caco-2 cells^[62,63].

While on the face of it, the above studies provide compelling evidence for a co-ordinated mechanism for the transcellular routing of iron, a number of caveats must be taken into consideration. (1) This model requires the expression of both DMT1 on the apical surface and transferrin receptors (TfR) on the basolateral membrane of the same enterocytes. While this requirement holds for Caco-2 cells^[64], the evidence from rat and mouse intestine suggests that TfR are predominantly expressed in the proliferating crypt and lower villus enterocytes^[65-69] while dietary iron uptake through apical membrane DMT1 occurs in the upper villus enterocytes^[70-73]. (2) Recent studies have shown that intestinal-specific inactivation of IREG1 (the basolateral iron transporter) results in anaemia confirming the essential role of this pathway in iron homeostasis^[74]. (3) Caco-2 cells, while an excellent model of the intestinal epithelium, exhibit some non-enterocyte properties including the ability to synthesize and secrete transferrin^[75-76]. Taken together, all of these studies highlight the need for further investigation into the transcellular iron transport mechanisms and their role in maintaining body iron homeostasis.

Iron export

Efflux of iron across the basolateral surface of enterocytes is achieved through the co-ordinated action of a transport protein IREG1^[77]-also known as ferroportin^[78] and MTP1^[79]-and a ferrioxidase, hephaestin^[80]. Studies in which IREG1 was expressed in *Xenopus laevis* oocytes indicate that this is a unidirectional efflux transporter of ferrous iron^[77,78]. Interestingly, this efflux function is up-regulated in the presence of ceruloplasmin, a copper binding ferrioxidase, plus transferrin to bind the newly liberated iron^[77]. This suggests that while ferrous iron is released through IREG1 it must be oxidised to ferric iron to

facilitate its loading onto transferrin for onward transport in the circulation. Interestingly studies with the yeast ceruloplasmin homologue, Fet3p, have highlighted the requirement for ferrioxidase activity in iron accumulation by transferrin^[81,82].

As stated above, the use of knockout mice has elegantly demonstrated the essential role of IREG1 in basolateral iron efflux^[74]. In addition, a second genetic mutant mouse model-the sex-linked anaemia (sla) mouse-has highlighted the critical requirement for oxidation of iron leaving the enterocytes for normal iron homeostasis. The sla mouse develops a moderate to severe microcytic hypochromic anaemia^[83]. It has been shown subsequently that these mice exhibit normal uptake of iron into enterocytes^[84], but the subsequent release of iron into the circulation is diminished^[85]. As a result, iron accumulates in enterocytes, and is lost when these cells are sloughed at the villus tip^[86]. While the *in vitro* studies described earlier^[77] used the ferrioxidase activity of ceruloplasmin to drive iron efflux, in the intestine the oxidation of iron is achieved by a ceruloplasmin homologue, hephaestin, which is also a multicopper ferrioxidase^[87]. In the sla mouse, the hephaestin gene is defective leading to a truncated form of the protein^[80], which is mislocalised within the enterocytes^[88] and has reduced ferrioxidase activity^[87].

REGULATION OF INTESTINAL IRON TRANSPORT

The regulation of intestinal iron absorption is complex and relies on mechanisms which sense dietary iron content as well as iron storage levels in the body and erythropoietic iron requirements (Figure 2). The iron regulatory hormone hepcidin is likely to be an important intermediate in signalling the storage and erythroid requirements and this aspect of iron homeostasis will be dealt with in an accompanying review^[1].

Basal transporter expression

In the healthy physiological state intestinal transporter expression will reflect body iron status exemplified by the circulating levels of iron bound to transferrin. Cells in the duodenal crypts of Lieberkühn express both Hfe^[89,90], the protein mutated in more than 80% of haemochromatosis patients^[91], and TfR on their basolateral surface. It is believed that Hfe binds to TfR regulating the rate at which transferrin-bound iron can enter the cell^[92,93]. One suggestion is that the cellular iron concentration established as a result of this interaction ultimately determines the basal level of expression of the proteins involved in iron absorption in the mature absorptive cells in the upper third of the villus. Importantly, in a modification to this hypothesis, we propose that in response to humoral signals, such as hepcidin, iron transport^[94] and transporter expression^[95] in mature epithelial cells, can be modified rapidly without the need to re-programme the crypt cell sensing mechanism.

The role of dietary iron

Rapid regulation of intestinal transporter expression in

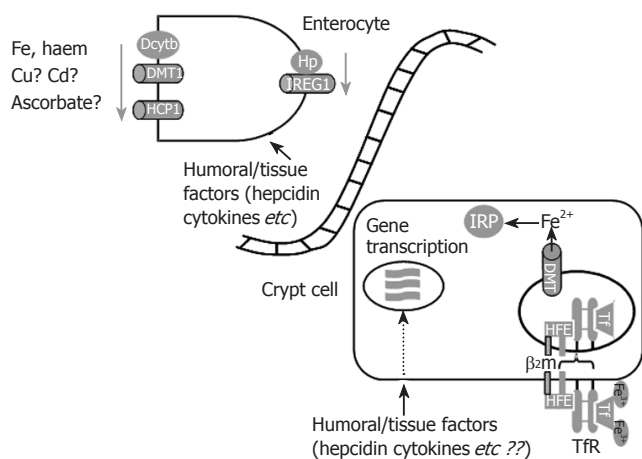


Figure 2 The regulation of intestinal iron transport. The duodenal crypt-villus axis represents a differentiation pathway that can be influenced by the dietary and humoral signals that ultimately regulate iron absorption. Immature proliferating cells in the crypt take up transferrin from the circulation via transferrin receptors (TfR). This process is governed by interactions between Hfe, β_2 microglobulin and TfR. The crypt cells are thus sensitive to the circulating levels and the iron-saturation of Tf. Iron regulatory proteins (IRP) recognise the cellular iron content and regulate iron responsive mRNAs posttranscriptionally. In addition, transcription control of iron metabolism may be exerted by a number of humoral and tissue-derived factors, such as hepcidin or cytokines interacting with their individual specific receptors. Together, these processes set the basal level of iron transporter protein expression in enterocytes as they mature and travel up the villus. Importantly, this basal level of transporter expression can be modified rapidly in mature enterocytes in response to changes in the levels of dietary factors (e.g. the iron content of the diet, other metals etc) and humoral and local tissue mediators (especially hepcidin and pro-inflammatory cytokines). This combination of basal programming in the crypts and fine tuning in villus enterocytes permits tight control of intestinal iron transport.

response to dietary factors is probably sensed by the villus enterocytes. More than half a century ago the “mucosal block” hypothesis was formulated following studies which demonstrated that a large oral dose of iron could reduce the absorption of a smaller dose administered several hours later^[96,97]. It was argued that due to the short time interval between doses, the initial dose must be having a direct effect on the mature enterocytes rather than the crypt cells. Whether such a phenomenon occurs with meaningful dietary iron levels is not clear but this may be a considerable problem with supplemental iron levels^[98]. Using the Caco-2 cell model, we have addressed the issue of whether non-haem iron can regulate iron transporter expression within a timescale and at concentrations that are consistent with digestion and absorption of a meal. We found that DMT1 (the IRE-containing isoforms) protein expression on the apical surface of these cells is decreased by iron concentrations as low as 20 $\mu\text{mol/L}$ ^[64]. The decrease in DMT1 transporter expression was rapid, occurring within 1-4 h following the exposure to iron^[99]. Further analysis revealed that DMT1 protein was internalised and targeted towards a late endosomal/lysosomal compartment. Interestingly, these iron-dependent effects were restricted to the apical uptake pathway-IREG1 protein expression was unaltered and were fully reversible (DMT1 protein levels returned to their original basal levels within 4-8 h) following the removal of iron^[99]. Our findings in this pertinent cell

culture model are consistent with those observed in rats following oral gavage with an iron bolus^[100-102] suggesting that the redistribution of DMT1 between different cellular compartments may be important physiologically for optimising iron absorption from a meal so that it matches better the body's metabolic requirements.

Iron regulatory proteins and iron responsive elements

In addition to trafficking of iron transport proteins, a number of intestinal iron metabolism genes can be regulated post-transcriptionally through interactions between cytosolic iron regulatory proteins (IRP) which bind to iron responsive elements (IRE), stem loop structures in either the 3' or 5' untranslated region (UTR) of several mRNA species, under conditions of cellular iron deficiency. TfR mRNA contains five IREs in its 3' UTR, and is stabilised following IRP binding as this blocks a target site for endonuclease activity^[103-106]. Interestingly, two isoforms of DMT1 contain a single IRE in the 3'UTR^[26]. While the DMT1 IRE can bind IRP *in vitro*^[107,108], its role in regulating DMT1 expression remains to be fully determined.

In contrast to the reported role of the 3'IRE sequences in conferring mRNA stability, the expression of mRNAs possessing 5'IREs, such as ferritin, are poorly translated with cellular iron low. But, expression is increased by high iron levels in duodenal enterocytes^[109]. This is because IRP/IRE binding blocks the association of the eukaryotic initiation factor complex to the 43S ribosomal unit^[110]. Interestingly, IREG1 mRNA contains a single IRE in the 5'UTR^[77,79]. However, the role of IRP/IRE interactions in the regulation of IREG1 transporter expression is even more controversial than its role in regulating DMT1 expression. Evidence suggests that the response to changes in iron status is tissue-specific-IREG1 expression in the liver^[79] and lung^[111] and in macrophages^[112] is up-regulated by high iron whereas in the intestine expression is elevated by iron deficiency^[77]. This may indicate that transcriptional, translational and post-translational processing of IREG1 varies between cell types^[113]. Clearly, the mechanisms involved in iron-dependent regulation of IREG1 in the intestine require further attention.

Local tissue factors

While the majority of this review has focussed on the transport pathways in the enterocytes it is important to bear in mind that the intestinal cell population is a highly heterogeneous affair. It is likely, therefore, that cross-talk between the epithelial cells and other cell types, such as macrophages, neutrophils, intraepithelial lymphocytes etc will be important in the overall regulation of intestinal iron transport. One intriguing hypothesis in this regard is the possible physiological role of the pro-inflammatory cytokine TNF α in regulating intestinal iron transport. TNF α is not only synthesized by peripheral blood monocytes and macrophages in response to inflammatory stimuli, but is also released by intraepithelial lymphocytes (IEL) that reside within the intestine in response to high iron intakes^[114]. These findings led to the formation of a hypothesis (named the piggyback-sensor model^[115])

which suggested that interaction between Hfe in the developing enterocytes with specific epitopes on the IELs was essential for regulating local TNF α production. Once released, TNF α leads to iron deposition within intestinal enterocytes *via* a TNF receptor 2-dependent mechanism^[116]. Further studies by our group^[117] and others^[118,119] have shown that TNF α has a direct influence on intestinal iron transporter expression and iron transport. These studies have opened the way for further investigations into the role of cell to cell cross-talk and the role of local tissue factors in regulating intestinal iron transport.

SUMMARY AND FUTURE DIRECTIONS

Clearly, our understanding of the molecular components of the intestinal iron transport pathway has increased exponentially in the last decade. However, there are still a number of important questions that remain unanswered: (1) How is iron shunted across the enterocytes from the apical pole to the basolateral membrane? There is some evidence for the presence of a tubulovesicular pathway. But, this work has largely been carried out in cell lines, and needs to be explored further in “normal” intestinal tissue. A role for calreticulin, a proposed component of the paraferitin pathway, remains a possibility though is still unproven. (2) What are the relative contributions of ferritin (and possibly lactoferrin) to iron nutrition? Could these iron sources be exploited for new supplemental therapies to treat iron deficiency? (3) Is there a role for cross-talk between enterocytes and other intestinal cell types in the local regulation on intestinal iron transport? If so, what are the cellular mechanisms involved? Are local tissue factors (such as TNF α) relevant physiologically in the control of iron absorption? (4) Hcpidin - how does it regulate intestinal iron transport? Is it an important physiological regulatory or is its main role in iron-related pathologies such as iron deficiency anaemia, haemochromatosis and anaemia of chronic disease?

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Liver iron transport

Ross M Graham, Anita CG Chua, Carly E Herbison, John K Olynyk, Debbie Trinder

Ross M Graham, Anita CG Chua, Carly E Herbison, John K Olynyk, Debbie Trinder, School of Medicine and Pharmacology, Fremantle Hospital, University of Western Australia, Fremantle, Western Australia, Australia

John K Olynyk, Debbie Trinder, Western Australian Institute for Medical Research, Nedlands, Western Australia, Australia

Supported by The National Health and Medical Research Council of Australia

Correspondence to: Dr. Debbie Trinder, School of Medicine and Pharmacology, Fremantle Hospital, University of Western Australia, PO Box 480, Fremantle, 6959, Western Australia, Australia. dtrinder@cyllene.uwa.edu.au

Telephone: +618-9431-3640 Fax: +618-9431-2977

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Abstract

The liver plays a central role in iron metabolism. It is the major storage site for iron and also expresses a complex range of molecules which are involved in iron transport and regulation of iron homeostasis. An increasing number of genes associated with hepatic iron transport or regulation have been identified. These include transferrin receptors (TFR1 and 2), a ferrireductase (STEAP3), the transporters divalent metal transporter-1 (DMT1) and ferroportin (FPN) as well as the haemochromatosis protein, HFE and haemojuvelin (HJV), which are signalling molecules. Many of these genes also participate in iron regulatory pathways which focus on the hepatic peptide hepcidin. However, we are still only beginning to understand the complex interactions between liver iron transport and iron homeostasis. This review outlines our current knowledge of molecules of iron metabolism and their roles in iron transport and regulation of iron homeostasis.

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Key words: Liver; Iron homeostasis; Iron uptake; Iron release; Iron transporters; Hereditary haemochromatosis

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INTRODUCTION

Iron is an essential trace element for almost all forms of

life. However, under physiological conditions, the free form of iron is practically insoluble and potentially toxic. Thus, iron is always found bound to specific ligands in such a way as to render it both soluble and non-toxic. The toxicity of iron stems from its ability to redox cycle. The release of an electron from ferrous iron, if uncontrolled, may result in the formation of highly reactive oxygen species capable of oxidising lipids, proteins and DNA^[1] causing damage to the structures and processes in which they are involved. However, many catalytic and other biological processes rely on the redox properties of iron; hence, iron must be available in a form which allows it to donate and accept electrons without causing non-specific damage.

In mammals, iron is transported around the plasma bound mainly to the glycoprotein transferrin, although other forms are also present in small amounts. In normal human plasma, transferrin has a concentration of between 25 and 50 $\mu\text{mol/L}$, and is usually about one-third saturated with iron. The remaining, unoccupied, binding sites on transferrin provide a large buffering capacity in case of an acute increase in plasma iron levels, an important consideration given the toxicity of free iron. Following uptake by the tissues, iron is transferred into a cytosolic pool (the "transit pool") from where it is distributed to ferritin for storage or to iron-requiring moieties, such as haem or iron-sulphur clusters. The majority of hepatocellular iron is contained in ferritin (80%) with 2%-3% present as haem; the remainder is either bound to transferrin or present in the transit pool^[2].

The liver plays a central role in iron metabolism. It is responsible for approximately 8% of plasma iron turnover in humans^[3], most of which is mediated by hepatocytes^[4,5] and it has long been known that it is the major site for storage of iron. Histologically, iron is distributed around the periportal regions of the liver with a decreasing gradient towards the centrilobular regions. In iron overload disorders, this gradient becomes more pronounced^[6], involving mainly hepatocytes with the resident liver macrophages, Kupffer cells, leading to a much lesser extent^[7].

More recently, it has been shown that the liver expresses a complex range of molecules which regulate iron homeostasis. The liver and, more specifically, hepatocytes, also express the vast majority of genes that have been associated with hereditary iron disorders. Our understanding of these disorders as well as the normal function of the liver in iron homeostasis is, as yet, incomplete. This review focuses on iron metabolism in hepatocytes, with a specific section on the role of Kupffer

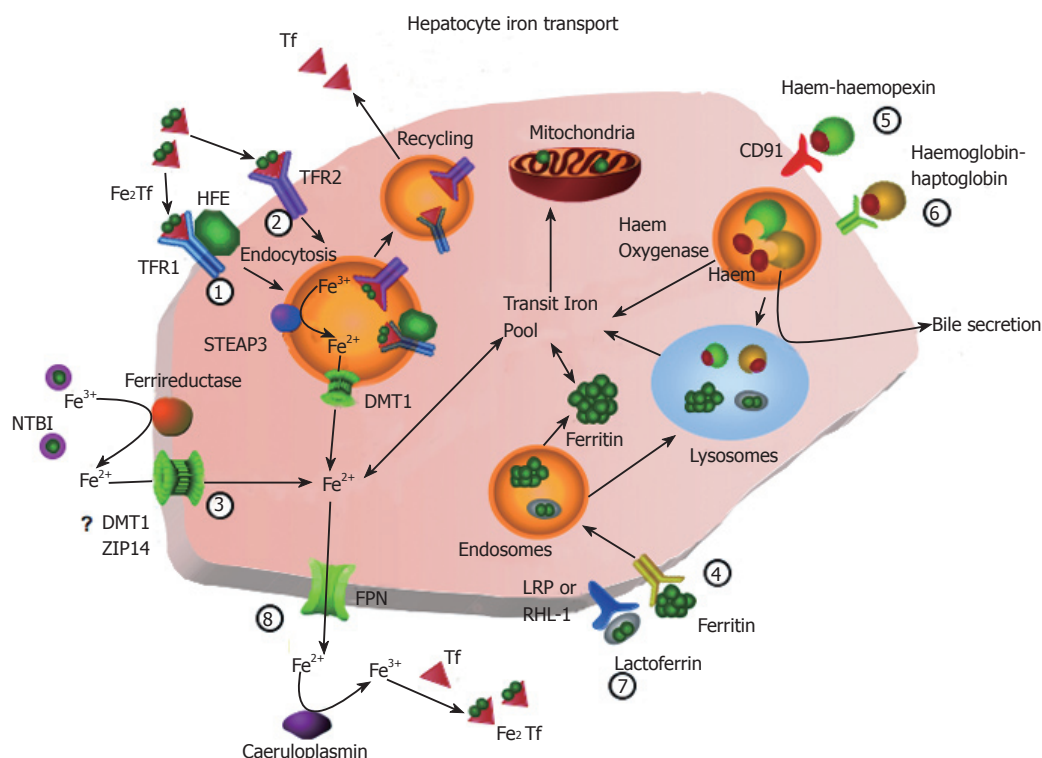


Figure 1 Hepatocyte iron transport. (1) TFR1-mediated uptake of diferric transferrin. Diferric transferrin binds to its specific receptor and is endocytosed. The endosome is acidified and Fe^{3+} is reduced by STEAP3. The iron is released and transported out of the endosome via DMT1 and apotransferrin is exocytosed. (2) TFR2-mediated uptake of transferrin. This mechanism is similar to the TFR1-specific mechanism except that transferrin binds to TFR2. (3) Uptake of NTBI. Iron is reduced and is transported into the cell via a carrier-mediated process. (4) Uptake of ferritin. Ferritin binds to its specific receptor and is endocytosed. The endosome is directed to lysosomes and the iron is transferred to the transit pool or endogenous ferritin. (5) Uptake of haem-haemopexin. The haem-haemopexin complex binds to its specific receptor CD91 and is endocytosed. Haem is removed and is degraded by haem oxygenase. (6) Uptake of haemoglobin-haptoglobin. The haemoglobin-haptoglobin complex binds to a specific receptor. Following endocytosis, the complex may be directed to the canalicular membrane for release into the bile or to the lysosomes for degradation. (7) Uptake of lactoferrin. Lactoferrin binds to LRP or RHL-1 and is endocytosed and targeted to the lysosomes for degradation. (8) Iron release. Iron is released by FPN and oxidised by caeruloplasmin and binds to apotransferrin. TFR1, transferrin receptor 1; TFR2, transferrin receptor 2; STEAP3, six-transmembrane epithelial antigen of the prostate 3; DMT1, divalent metal transporter 1; NTBI, non-transferrin bound iron; ZIP14, zinc-regulated transporter and iron-regulated transporter-like protein 14; LRP, low-density lipoprotein receptor-related protein; FPN, ferroportin.

cells, and on the molecules known to be involved in iron metabolism in the liver and their role in our current understanding of liver iron transport.

TRANSFERRIN RECEPTOR 1 (TFR1)-MEDIATED UPTAKE OF TRANSFERRIN

TFR1-mediated uptake of diferric transferrin is, perhaps, the best described process of iron uptake (Figure 1, pathway 1). Briefly, transferrin binds to TFR1 and is endocytosed^[2,5]. At the pH of the extracellular fluid, diferric transferrin is bound preferentially, the affinity of the receptor being higher for diferric transferrin than for either monoferric- or apo- transferrin^[7-10]. Following formation of the endosome, it is acidified which results in a decrease in the affinity of transferrin for iron and subsequent detachment of the metal^[11,12]. The affinity of the receptor for the (now) apotransferrin is increased by the acidic environment^[7] and apotransferrin remains bound to the receptor as the endosome returns to and fuses with the plasma membrane. At the higher extracellular pH, the affinity of the receptor for apotransferrin decreases^[7] and apotransferrin is released back into the circulation^[2]. The entire process takes between 3.8 and 15 min^[13-15].

TFR1

TFR1 expression is regulated by iron primarily by a post-transcriptional mechanism. The transcript contains five iron-responsive elements (IRE) in its 3' untranslated region (UTR) along with a number of instability elements that facilitate breakdown of the message^[16-19]. Under low-iron conditions, iron regulatory proteins (IRP) bind to the IREs, placing an inhibition on the instability elements^[18,19], increasing the half-life of the mRNA and, hence, increasing translation. When iron is abundant, IRPs do not bind IREs, resulting in a decrease of the stability of the transferrin receptor message. Two isoforms of IRP have been identified. The first (IRP1) is an iron-free form of cytosolic aconitase^[20-22]. The second (IRP2) does not exhibit any aconitase activity^[23] and appears to be the physiologically active IRP since it can respond to iron under conditions of low oxygen tension^[24], a situation which occurs in the liver *in vivo*.

TFR1 is also regulated by other mechanisms. The gene contains an hypoxia response element in its promoter region which mediates up-regulation of transcription in the presence of hypoxia-inducible factor 1^[25-27]. Transcription is also up-regulated by cytokines, such as interleukin-2, mitogens and growth factors^[28-30]. Furthermore, *TFR1*

expression is increased in proliferating cells and reduced in quiescent cells^[31-33], consistent with cellular demand for iron during periods of growth.

HFE

A mutation in HFE was the first to be shown to be causative for the iron overload disorder, haemochromatosis^[34]. HFE is a major histocompatibility complex-like protein and was originally designated HLA-H. The gene is widely expressed, with highest expression in the liver and small intestine. HFE requires the protein β 2-microglobulin for its correct localisation to the cell surface^[35].

Despite this knowledge, the normal function of the protein has been difficult to elucidate. The crystal structure of the transferrin-TFR1 complex^[36] indicates that the C-lobe of transferrin interacts with the helical domain of one of the TFR1 monomers. In contrast, the N-lobe of transferrin appears to interact partially with the helical domain, partially with the protease-like domain and, unusually, with the stalk connecting the extracellular region of TFR1 to its transmembrane region. HFE also interacts with the helical domain of TFR1^[37,38], competing with transferrin for its binding site^[39,40]. The resulting inhibition causes a reduction in transferrin-bound iron uptake in a variety of cell types^[41-44], suggesting that HFE is involved in the regulation of iron uptake by TFR1, possibly by limiting the amount of iron released from transferrin^[44]. HFE cycles with TFR1^[44], but its effect on cycling is controversial, with different groups reporting no effect^[44], a reduction in endocytosis^[45] or a reduction in exocytosis^[43].

The physiological consequences of the HFE-TFR1 interaction are difficult to ascertain given that the affinity of TFR1 for HFE is one to two orders of magnitude lower than for diferric transferrin^[46], implying that, at normal transferrin concentrations, almost no HFE would be associated with the receptor. However, these measurements were conducted on isolated proteins, so it is possible that, *in vivo*, the local environment of these proteins changes their interactions leading to a shift in the balance of competition between HFE and transferrin.

STEAP3

Following endocytosis and vesicle acidification, iron is reduced to its ferrous form prior to being transferred across the endosomal membrane. It was suggested some years ago that the transferrin receptor appeared to facilitate detachment of iron from transferrin in the endosome^[47]. More recently, it was shown that, at endosomal pH, the reduction potential of ferric iron co-ordinated by transferrin is increased when diferric transferrin is complexed to TFR1^[48], confirming the earlier observation and suggesting that reduction of iron occurs prior to release from transferrin. However, purely chemical reduction is unlikely to result in the highly efficient process of iron uptake seen in biological systems.

Despite evidence of endosomal ferrireductase activity^[49], it wasn't until recently that a candidate ferrireductase was identified^[50]. The gene *STEAP3* ("six-

transmembrane epithelial antigen of the prostate 3") is one of four genes wholly or partially deleted in the *nm1054* iron deficiency anaemia mouse. Under normal conditions, it is highly expressed in the liver, and its product is a protein which co-localises in endosomes with TFR1 and DMT1 (divalent metal transporter 1; see below). It is predicted to be a haemoprotein containing an N-terminal flavin-NADH binding domain. Most importantly, ferrireductase activity and iron uptake were lower in reticulocytes obtained from *nm1054* and *steap3* knockout mice and overexpression in HEK293T cells resulted in increased ferrireductase activity. A follow-up paper from the same group^[51] showed that the remaining three members of the Steap family (STEAP1, 2 and 4) were also ferri- and cupric- reductases. The four genes are ubiquitously expressed; however, different members are expressed more highly in some tissues than others. Foetal liver expresses all four transcripts, but adult liver expresses predominantly STEAP3 with a small amount of STEAP1^[50,51]. Like STEAP3, the other Steap proteins co-localise, at least partially, in an endosomal compartment with transferrin and TFR1.

Divalent metal transporter 1 (DMT1)

The released ferrous iron is transported from the interior of the endosome to the cytosol by DMT1 (also known as "natural resistance-associated macrophage protein 2", NRAMP2, "divalent cation transporter 1", DCT1, or "solute carrier family 11 member 2", SLC11A2). This protein is a transmembrane glycoprotein with 12 predicted transmembrane helices^[52,53] although there is no structure currently available to confirm this.

There are four known isoforms of DMT1, resulting from splice variation at the mRNA level. Alternative first exons (1 A or 1 B) give rise to the first level of variation^[54]. Secondly, each of the 5' splice variants may contain one of two 3' splice variations^[55]. The first of these contains an IRE in its 3'UTR. The second results in replacement of the final 18 codons of the open reading frame with a different sequence of 25 codons and a different 3'UTR, which, importantly, does not contain an IRE^[55]. The predominant form in the liver is the exon 1B + IRE form, although a small amount of the 1B-IRE form may also be present^[54].

Studies comparing the variants of DMT1 have indicated that the +IRE isoform is localised predominantly to the plasma membrane, exhibits slower internalisation kinetics than the -IRE isoform, and is targeted to lysosomes. In contrast, the C-terminal region of the -IRE isoform contains peptide signals which are required for efficient endocytosis and subsequent targeting to recycling endosomes^[56,57]. Thus, it is possible that the +IRE isoform is predominantly involved in iron transport across the plasma membrane whereas the -IRE isoform is involved in endosomal transport.

Evidence that DMT1 is the endosomal transporter is supported by the finding that DMT1 co-localises with TFR1^[58-60] and cycles through the endosomal compartment, appearing in acidic endosomes^[61]. DMT1 transports iron optimally at pH 5.5^[62], consistent with its presence in acidic endosomes and suggesting the energy for iron transport may be provided by a proton gradient. However, considerable transport also occurs at pH 7.4,

and a model for metal transport by DMT1 has been proposed which is consistent with symport of Fe^{2+} and H^+ from acidic endosomes and uniport of Fe^{2+} from a neutral environment^[63].

DMT1 appears to be regulated by iron levels with protein expression increased in iron loaded liver, lower in control liver, and not detected in iron deficient livers^[64]. Similar results have been obtained with the HepG2 hepatoma cell line^[65]. These findings are inconsistent with an IRE located in the 3'UTR of the transcript, which would be expected to result in a decrease in mRNA stability in iron loading with a concomitant decrease in protein expression. However, regulation of DMT1 is complex, and it is possible that the 5'UTR of the transcript or the N-terminal domain of the protein may modify the regulatory effects of the IRE in a tissue-specific manner^[54]. Additionally, regulation based around the stability of the protein cannot be ruled out.

LOW AFFINITY TRANSFERRIN UPTAKE

A second transferrin-mediated route of iron uptake (Figure 1, pathway 2) has been recognised in hepatocytes for many years^[14,66,67] and is probably responsible for the bulk of iron uptake by hepatocytes since, at the concentrations of transferrin present in the plasma, TFR1 would be saturated^[15,66,68]. The mechanism of uptake is similar to that of the TFR1-mediated pathway^[5,14,69]. After binding to the low affinity binding site, transferrin is endocytosed and iron is removed following acidification of the vesicle. Iron is sequestered away from the vesicle and apotransferrin is exocytosed^[69].

Transferrin receptor 2 (TFR2)

In 1999, Kawabata and colleagues^[70] reported the cloning of transferrin receptor 2 (TFR2), a type II transmembrane protein which shared significant sequence similarity to TFR1. It is currently the best candidate gene to code for the low-affinity binding site, with which it shares many similarities. TFR2 binds diferric transferrin specifically in a pH-dependent manner with an affinity 25-30 times lower than TFR1^[38,71]. In the liver, it is expressed predominantly in hepatocytes^[70,72,73] and mediates cellular transferrin and iron uptake^[70,73].

Regulation of TFR2 is different from regulation of TFR1. TFR2 mRNA does not contain any iron-responsive elements and cellular iron levels do not appear to change TFR2 mRNA or protein expression. Dietary and pathological iron loading do not result in decreased hepatic expression of TFR2 mRNA and neither does iron deficiency result in increased hepatic expression^[74]. Instead, TFR2 appears to be regulated at the protein level by cell cycle, with proliferating cells expressing approximately twice as many receptors as stationary cells^[75] and by the presence of diferric transferrin. Diferric transferrin causes an upregulation of receptor number and a redistribution of the protein to the cell surface in liver and hepatoma cells^[72,76]. The upregulation is caused by an increase in the half-life of the receptor conferred by its binding diferric transferrin. Removal of diferric transferrin results in a return to baseline expression^[77]. Consistent with these

findings, TFR2 protein levels were decreased with iron deficiency, and increased with iron loading in genetic models of iron overload, such as haemochromatosis, but not in the atransferrinaemic mouse which has impaired transferrin synthesis^[76]. Evidence suggests that TFR2 is a sensor of transferrin saturation and controls iron metabolism by regulating hepcidin expression^[78,79]. However, it is still not known whether changes in levels of TFR2 expression are correlated with changes in transferrin-bound iron uptake by the liver.

Given the similarities between TFR1 and TFR2, it was thought that, like TFR1, TFR2 may bind HFE. Co-localisation studies suggested an interaction in the duodenum. TFR2 was shown to co-localise with wild-type HFE in an early endosomal compartment whereas, in the presence of HFE^{C282Y}, the mutation predominantly associated with haemochromatosis type 1, TFR2 was distributed mainly to basolateral membrane^[80]. Despite this, initial *in vitro* binding studies indicated that there was no detectable interaction between soluble HFE and the soluble TFR2 ectodomain^[38]. More recently, an interaction has been demonstrated between the full-length, membrane-anchored HFE and TFR2 proteins^[81] suggesting that HFE may, indeed, be involved in TFR2-mediated iron uptake and TFR2-dependent regulation of hepcidin.

It has also been shown that TFR2, unlike TFR1, is present in lipid rafts and binding of diferric transferrin to TFR2 can activate the ERK1/2 and p38-MAPK signalling pathways^[82]. However, any connection of this to the hepcidin signalling pathway has yet to be demonstrated.

NON-TRANSFERRIN-BOUND IRON UPTAKE

The liver is one of the major sites of accumulation of iron delivered as low molecular weight chelates (Figure 1, pathway 3)^[83,84]. The pathophysiological relevance of such a process is apparent in diseases of iron overload such as hereditary haemochromatosis^[85]. The form of this low molecular weight plasma pool is likely to comprise several species; however, citrate appears to be the major component in both normal^[86,87] and haemochromatotic^[88] sera. In experimental situations, hepatocytes and their derivatives have been shown to take up iron from a variety of chelators^[89-105]. Iron taken up from these chelators has been shown to be distributed to haem and ferritin in hepatocytes^[90,101,103].

NTBI uptake by hepatocytes is linear for at least the first 15 to 60 min of incubation^[91,94,101,104]. It is also concentration dependent, with both ferrous and ferric iron, delivered as a variety of low molecular weight chelates, showing saturation kinetics^[95,97,106-108], indicating that this process is carrier-mediated. Uptake of iron as ferric citrate has been shown to be most efficient in normal rat hepatocytes at neutral pH^[92].

DMT1 as a major transporter of NTBI

NTBI uptake is increased in cells in which DMT1 mRNA and protein expression are upregulated^[109,110]. Furthermore, NTBI uptake appears to share at least one common pathway with TBI uptake since diferric transferrin has

consistently been shown to competitively inhibit uptake of NTBI^[93,102,111]. These observations, together with findings that DMT1 is active at neutral pH^[62], are consistent with DMT1 being a major transporter of NTBI in hepatocytes.

The specificity of NTBI uptake has been investigated by a number of groups and it has been generally observed that Cd, Co, Cu, Mn and Zn decrease iron uptake by normal and transformed hepatocytes^[97,104,108,109,112,113]. These observations match the range of divalent metals transported by DMT1^[53,114], adding further credence to the suggestion that DMT1 is a transporter of NTBI. Indeed, Mn and Cd appear to be transported by DMT1 with higher affinity than Fe^[114]. Although this observation is probably not relevant under normal physiological conditions, where the concentration of Fe is considerably higher than either Mn or Cd, it may become important in pathological conditions such as heavy metal poisoning in which competition for the transporter may result in a reduction in iron uptake. The alternative N and C termini conferred by the splice variants do not appear to affect the metal transport abilities^[62].

Certain inconsistencies in the data showing that some metals cause inhibition in some cell types, but not others have led to the suggestion of a family of transporters for iron and other transition metals^[101,111,115]. Several candidate transporters have been identified including calcium channels and specific transporters of other metals such as the zinc transporter, ZIP14 (zinc-regulated transporter and iron-regulated transporter-like protein 14).

ZIP14

ZIP14 (SLC39A14) is a transmembrane protein with eight predicted transmembrane helices^[112,113]. It is highly expressed in the liver, and is localised to the plasma membrane^[112]. There are two splice variants of the transcript; however, the biological functions of these two forms are yet to be determined. Originally shown to transport zinc, ZIP14 has also been shown to transport non-transferrin bound iron^[112,113,116]. But, it is not currently known whether ZIP14 is involved in hepatic iron loading. Importantly, ZIP14 has also been shown to be upregulated by interleukin-6^[112], which also upregulates hepcidin during inflammation^[117].

Calcium channels

The role of calcium channels in uptake of NTBI by the liver remains unclear. It has been suggested that L-type calcium channels are responsible for a significant component of ferrous iron uptake by cardiomyocytes, particularly under iron loaded conditions^[118,119]. However, information about any role for calcium channels in liver iron uptake is scant. Available evidence indicates that the transcripts coding for calcium channel subunits are expressed at low levels in the liver^[120], suggesting their participation in iron uptake by the liver is likely to be minor. However, levels of mRNA do not take into account any post-transcriptional modifications or functional regulation such as gating. Hence, the contribution of calcium channels to iron uptake by the liver requires further investigation.

It has also been suggested that calcium itself plays a functional role in NTBI uptake; however, this, too, needs

further clarification. Some studies have reported stimulation of NTBI uptake in cell types including hepatocytes^[99,101,104,121], whilst other studies have reported inhibition^[122] or no effect^[92]. It is possible that this spectrum of observations is due to variable chelation of calcium by the variety of chelators used to solubilise the iron^[123].

TRANSPORT OF OTHER IRON COMPLEXES

A number other forms of iron are recognised as being cleared from the circulation by the liver; however, these are likely to be mechanisms of clearance for their respective ligands rather than for uptake of iron, *per se*.

Specifically, these are ferritin, lactoferrin, the haem-haemopexin complex and the haemoglobin-haptoglobin complex. Circulating ferritin contains very small amounts of iron^[124-126] and, as such, it is not a major source of iron in the normal human. Nevertheless, the liver clears ferritin by a method involving binding to a specific ferritin receptor^[127-129] followed by endocytosis (Figure 1, pathway 4). There are several possible fates for endocytosed ferritin including catabolism of the protein in lysosomes^[130-132], excretion in the bile or inclusion in the endogenous ferritin pool^[133]. Any iron released is distributed to the mitochondria and endogenous ferritin^[130,132].

The uptake of the haem-haemopexin complex is mediated by its specific receptor, CD91 (Figure 1, pathway 5)^[134]. Following endocytosis, haem is degraded by haem oxygenase. Like transferrin, haemopexin was thought to be recycled back to the circulation^[135-137]; however, this fate has recently been questioned with evidence suggesting that it is substantially degraded in lysosomes^[134].

The haemoglobin-haptoglobin complex also binds to a high-affinity specific receptor and is endocytosed (Figure 1, pathway 6)^[138]. However, from this point, two possibilities exist for the fate of the complex. Both haemoglobin and haptoglobin may be directed to lysosomes for degradation^[139] or transported to the canalicular membrane of hepatocytes where haemoglobin is released into the bile and the receptor is recycled to the sinusoidal membrane^[140]. Clearance of the haemopexin and haptoglobin complexes by the liver is of importance in haemolytic states, especially those associated with intravascular haemolysis.

Lactoferrin is an iron-binding protein similar to transferrin which is present mainly in milk. Two lactoferrin binding sites have been reported on hepatocytes, although neither is specific for lactoferrin. The first is low-density lipoprotein receptor-related protein (LRP)^[141] and the second is the major (RHL-1) subunit of the asialoglycoprotein receptor^[142]. Lactoferrin appears to be cleared *via* receptor-mediated endocytosis regardless of its binding site (Figure 1, pathway 7)^[141,142]. Most of the internalised lactoferrin is directed to lysosomes for degradation^[143].

IRON RELEASE

Ferroportin (FPN)

The transporter, ferroportin (FPN; “solute carrier family 40 member 1”, SLC40A1; IREG1 or “metal transporter protein-1”, MTP1) was reported independently by three groups in 2000^[144-147] and appears to be the sole mediator

of iron release from hepatocytes (Figure 1, pathway 8)^[148]. Although it has not been shown directly, FPN appears to transport ferrous iron. Evidence comes from the apparent requirement of transport for ferroxidase activity. Caeruloplasmin knockout mice exhibit impaired hepatocellular and reticuloendothelial iron efflux which can be rescued by injection of caeruloplasmin^[149]. Similarly, mice with mutations in the membrane-bound ferroxidase, hephaestin, also exhibit impaired iron efflux^[150]. Further, iron efflux was stimulated in *Xenopus* oocytes over-expressing FPN in the presence of caeruloplasmin^[147]. There have been no reports to date indicating whether FPN-mediated iron transport is linked to transport of any other ion or whether there is any energy requirement for the process.

The structure and membrane topology of FPN is currently unclear with various models predicting between nine and twelve transmembrane helices^[151-153]. However, both the N- and C-termini appear to be located intracellularly^[153,154], which precludes an odd number of transmembrane segments. Similarly, the quaternary structure of FPN has been the subject of debate. Initial reports suggested that FPN was oligomeric^[155,156], but later reports cast doubt on this, suggesting a monomer^[152,157]. Recently, a comprehensive study by de Domenico *et al*^[154], demonstrated that FPN was most likely a dimer.

The quaternary structure of FPN may have important implications for regulation of iron homeostasis. Under the oligomeric model, the dominant negative phenotype of FPN-associated haemochromatosis (type 4) can be interpreted as interaction between wild-type and mutant forms of the protein interfering with its normal function^[155,156]. The alternative interpretation, haploinsufficiency, is less likely given that mice heterozygotic for a FPN knockout demonstrated a very mild phenotype and homozygotic knockout mice died *in utero*^[148]. Also, the majority of reports of human FPN-associated haemochromatosis with demonstrable iron loading involve heterozygotic point mutations which are at least partially functional^[156,158,159].

Like ferritin, FPN mRNA contains a functional IRE in its 5'-UTR^[144,147,160] indicating that translation should be augmented when iron is abundant. This has been shown to be true in HepG2 and Kupffer cells but not in the duodenum^[144,160,161] suggesting that regulation of FPN is cell-specific and one or more other regulatory mechanisms may be involved.

OTHER MOLECULES INVOLVED IN IRON HOMEOSTASIS

Hepcidin

Hepcidin is a 25 residue peptide containing four internal disulphide bonds which is produced in hepatocytes under conditions of iron sufficiency^[162-164]. It is created as a pre-pro-peptide which undergoes post-translational cleavage^[163], and its expression is regulated by inflammation and hypoxia as well as iron levels^[164,165]. It appears to be the focal point of an iron-regulatory pathway involving HFE, TFR2 and HJV, since disruption of these genes in haemochromatosis results in decreased hepcidin expression^[166-168]. Its expression is enhanced by cytokines such as interleukin-6 (IL-6)^[117].

In 2004, it was shown that FPN was a receptor for hepcidin^[169]. In HEK293 cells, the peptide was shown to bind to FPN and induce its internalisation in a dose-dependent manner. The complex was targeted to lysosomes for degradation^[169]. This is consistent with results that show increased FPN in the duodenum under conditions of iron deficiency, when hepcidin levels would be low^[144], and offers a mechanism for hepcidin-mediated anaemia of inflammation^[117,170] in which FPN levels are decreased resulting in a reduction of iron efflux to the plasma. The N-terminus of hepcidin is necessary for binding and internalisation of FPN, and the disulphide bonds appear to be necessary for its stability in the plasma^[171]. It is unclear whether hepcidin acts *in vivo* as an autocrine hormone, signalling to FPN in hepatocytes or as a paracrine hormone, signalling to FPN in Kupffer cells.

Haemojuvelin (HJV)

HJV is a protein known to play a very important role in hepatic iron homeostasis although its exact function and whether it plays a role in iron transport have yet to be ascertained. It is expressed in adult skeletal muscle, and foetal and adult liver in the periportal hepatocytes^[168,172]. Both soluble and membrane anchored forms have been demonstrated in Hep3B cells^[173]. Identified as the protein mutated in many cases of juvenile haemochromatosis^[168], human HJV shares 48% sequence identity with repulsive guidance molecules which are important in retinal development^[174]. Absence of functional HJV results in increased plasma transferrin saturation and ferritin in humans^[175,176] and studies in HJV knockout mice demonstrate decreased hepatic hepcidin expression and increased liver iron loading^[172,177]. The major function of HJV appears to be regulation of hepcidin levels, and it has been shown that HJV can bind bone morphogenic protein-2 (BMP-2), a member of the TGF- β superfamily of cytokines and activate hepcidin transcription *via* SMAD-4^[178,179]. This pathway is independent of HFE, TFR2 and IL-6^[180].

KUPFFER CELLS

Kupffer cells are the resident macrophages of the liver. Their main function in iron metabolism appears to be as a clearing house for iron from phagocytosed red blood cells^[181]. Haem breakdown is catalysed by haem oxygenase, and the products are ultimately excreted in the bile^[182]. Iron can be stored in Kupffer cells as ferritin. But, much of it is released back into the circulation^[183]. Consistent with this, Kupffer cells have been shown to strongly express both FPN transcript and protein^[184,185]; indeed, FPN is more highly expressed in Kupffer cells than hepatocytes^[144,184,186].

No functional studies on the role of FPN in iron release by Kupffer cells have been carried out. However, a number of studies have been undertaken using bone marrow-derived macrophages or macrophage cell lines. Following erythrophagocytosis or experimentally induced iron loading, the expression of many genes involved in iron metabolism, including FPN and haem oxygenase 1, are upregulated^[185,187]. In these cells, FPN is localised to intracellular vesicles, redistributing to the cell surface

following erythrophagocytosis^[188]. The upregulation of FPN results in an increase in iron release and its down-regulation results in a decrease in iron release^[189], consistent with involvement in iron recycling by Kupffer cells.

That FPN has been observed localised to intracellular vesicles in the absence of hepcidin suggests that FPN may play a role in intracellular redistribution of iron within Kupffer cells^[190] as well as in iron export. Addition of hepcidin resulted in rapid disappearance of FPN from the cell membrane, and subsequent degradation of the protein^[188], suggesting that in the absence of hepcidin, FPN may be able to cycle to intracellular compartments as necessary.

Kupffer cells also express TFR1^[191] indicating that they can obtain iron from transferrin if necessary. Interestingly, Kupffer cells also express high levels of HFE^[192]; however, they appear to be spared the level of iron loading associated with hepatocytes in HFE-associated haemochromatosis^[7]. This may indicate a difference in regulation of HFE in macrophages compared to hepatocytes or simply that iron loading of Kupffer cells is partially negated by the high level of iron exported from these cells^[183]. A recent report has suggested that GAPDH functions as a transferrin receptor in macrophages^[193]. However, the affinity of the interaction was extremely low and its importance is yet to be determined.

LIVER IRON TRANSPORT IN DISORDERS OF IRON METABOLISM

In the absence of any relevant genetic defects, an increase in plasma iron would result in an increase in transferrin saturation followed by a rise in the concentration of NTBI. Gene expression in the liver would change to sequester the iron in a non-toxic form, and signal to the duodenum to reduce iron absorption. In primary iron overload disorders, such as hereditary haemochromatosis, mutations in HFE, HJV or TFR2 result in a decrease in hepcidin production, and subsequent misregulation of iron absorption by the duodenum (as, of course, does a lack of functional hepcidin)^[194]. The mechanistic consequences of this for the liver are difficult, if not impossible, to dissect out from the resulting iron overload. This leads to the paradox of TFR2, an iron transporter which is sub-functional in type 3 haemochromatosis, resulting in hepatic iron overload^[71,195] rather than hepatic iron deficiency. This apparent paradox is probably the most telling demonstration of the liver's repertoire of iron transport and regulatory mechanisms.

Secondary iron overload is often a consequence of blood transfusions required for the treatment of certain types of anaemia such as β -thalassaemia or sideroblastic anaemia. The source of the excess iron is haem from transfused erythrocytes which are broken down in the normal way with the haem being catabolised, *inter alia*, by Kupffer cells. As with primary iron overload disorders, gene expression in secondary iron overload will change to reflect the cellular iron loading and the increase in plasma transferrin saturation and NTBI concentration despite the underlying anaemia.

Iron deficiency may be caused by a number of factors

including genetic disorders, pregnancy, an increased requirement for iron during growth, or simply by lack of dietary iron intake. The initial stage of iron deficiency corresponds to mobilisation of storage iron from the liver with decreases in hepatocyte ferritin, and an increase in iron uptake proteins such as TFR1^[196]. Plasma NTBI is generally considered to be non-existent or at very low levels in iron deficiency^[85].

CONCLUSION

Iron transport by the liver is, of necessity, tightly regulated because of the liver's myriad of transport pathways, and its role in iron homeostasis. The explosion of information in the past ten years describing many of the genes involved in liver iron transport has not only provided insight into the mechanisms involved, but also confirmed the complexities evident from the literature from previous decades. Nevertheless, much work remains to be done in piecing together this information in order to fully understand how the pathways of iron transport, the distribution of iron in the liver and the regulatory pathways interact and how they contribute to iron homeostasis.

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Liver-gut axis in the regulation of iron homeostasis

Deepak Darshan, Gregory J Anderson

Deepak Darshan, Gregory J Anderson, Iron Metabolism Laboratory, The Queensland Institute of Medical Research, Brisbane, Australia

Correspondence to: Gregory J Anderson, PhD, Iron Metabolism Laboratory, Queensland Institute of Medical Research, PO Royal Brisbane Hospital, Brisbane Queensland 4029, Australia. greg.anderson@qimr.edu.au

Telephone: +61-7-3362-0187 Fax: +61-7-3362-0191

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Abstract

The human body requires about 1-2 mg of iron per day for its normal functioning, and dietary iron is the only source for this essential metal. Since humans do not possess a mechanism for the active excretion of iron, the amount of iron in the body is determined by the amount absorbed across the proximal small intestine and, consequently, intestinal iron absorption is a highly regulated process. In recent years, the liver has emerged as a central regulator of both iron absorption and iron release from other tissues. It achieves this by secreting a peptide hormone called hepcidin that acts on the small intestinal epithelium and other cells to limit iron delivery to the plasma. Hepcidin itself is regulated in response to various systemic stimuli including variations in body iron stores, the rate of erythropoiesis, inflammation and hypoxia, the same stimuli that have been known for many years to modulate iron absorption. This review will summarize recent findings on the role played by the liver and hepcidin in the regulation of body iron absorption.

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Key words: Iron; Homeostasis; Intestinal iron absorption; Liver; Hepcidin

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INTRODUCTION

Intestinal epithelial cells or enterocytes take up the predominating ferric iron from the diet through the combined action of an iron reductase (duodenal

cytochrome B or DcytB is a strong candidate) and a ferrous iron transporter known as divalent metal-ion transporter (DMT1) on the brush border membrane^[1,2]. Heme iron, on the other hand, appears to be absorbed through a separate system, and a recently identified apical membrane protein, HCP1, has emerged as a candidate heme transporter^[3]. Irrespective of the form in which iron crosses the brush border, enterocytes export iron into the circulation by the combined action of an iron reductase, hephaestin, and a basolateral membrane iron transporter, ferroportin (FPN). The newly absorbed iron is then bound to circulating transferrin which distributes it around the body to sites of utilization and storage.

The amount of iron transported across the enterocytes is ultimately influenced by body iron requirements. Thus, for example, when body iron levels are low or when erythropoietic demand is increased, iron absorption is elevated. The factors that alter iron absorption exert their effects by influencing the duodenal expression of the major iron transport molecules, particularly DMT1, Dcytb1 and ferroportin^[1,2,4]. Early kinetic studies suggested that it was the efflux of iron out of the enterocytes and into the circulation that was rate limiting for absorption^[5], and more recent molecular studies have provided support for this concept^[6,7]. In particular, this work suggested that basolateral iron transfer by ferroportin was most likely the primary regulatory step. But, how signals from distant sites modulate iron release from enterocytes has until recently remained poorly understood. The missing link in this regulatory pathway has now emerged as the liver-derived peptide hormone hepcidin, and thus we know that the liver plays a central role in the regulation of body iron homeostasis. Hepcidin regulates plasma iron levels by controlling the cell surface expression of ferroportin, and this in turn limits the efflux of iron from enterocytes, macrophages and a number of other cell types.

HEPCIDIN

Hepcidin was first discovered as an antimicrobial peptide in human blood ultrafiltrate^[8] and urine samples^[9]. The gene encoding hepcidin (*HAMP*) is very strongly expressed in the liver. But, weak expression has also been detected in heart, spinal cord, stomach, intestine and lungs^[8-10]. The first evidence linking hepcidin to iron metabolism came almost simultaneously from two groups. One group, using suppressive subtractive hybridization, demonstrated that the levels of the hepcidin transcript were greatly increased in the livers of iron loaded mice^[10]. The other group

<i>Homo sapiens</i>	D	T	H	F	P	I	C	I	F	C	C	G	C	C	H	R	S	K	C	G	M	C	C	K	T
<i>Canis familiaris</i>	D	T	H	F	P	I	C	I	F	C	C	G	C	C	K	T	P	K	C	G	L	C	C	K	T
<i>Sus scrofa</i>	D	T	H	F	P	I	C	I	F	C	C	G	C	C	R	K	A	I	C	G	M	C	C	K	T
<i>Bos taurus</i>	D	T	H	F	P	I	C	I	F	C	C	G	C	C	R	K	G	T	C	G	M	C	C	R	T
<i>Mus musculus</i>	D	T	N	F	P	I	C	I	F	C	C	K	C	C	N	N	S	Q	C	G	I	C	C	K	T
<i>Rattus norvegicus</i>	D	T	N	F	P	I	C	L	F	C	C	K	C	C	K	N	S	S	C	G	L	C	C	I	T
<i>Danio rerio</i>	Q	S	H	L	S	L	C	R	F	C	C	K	C	C	R	N	K	G	C	G	Y	C	C	K	F
<i>Salmo salar</i>	Q	I	H	L	S	L	C	G	L	C	C	N	C	C	H	N	I	G	C	G	F	C	C	K	F

Figure 1 Sequence alignment of hepcidin from various species.

inadvertently engineered a mouse strain with negligible hepcidin expression (considered in more detail below), and found that these animals accumulated high levels of body iron^[11]. *HAMP* was mapped to human chromosome 19, and it encodes an 84 amino acid pre-pro-peptide^[9,10]. Pre-pro-hepcidin is ultimately processed into several smaller peptides that consist of the 20, 22 or 25 C-terminal amino acids^[9]. The 25 amino acid peptide has eight cysteine residues forming four intramolecular disulfide bonds, and is the biologically active form of hepcidin^[9,12]. The eight cysteine residues are highly conserved among species from zebra fish to humans (Figure 1). Two-dimensional nuclear magnetic resonance (NMR) spectroscopy showed that hepcidin forms a distorted hairpin-like structure, and NMR diffusion studies demonstrated that the 25 residue peptide forms aggregates involving the first five N-terminal residues^[13]. The 20 and 22 amino acid forms, that lack the N-terminal residues, do not form aggregates and have much reduced iron regulatory capacity^[12,13]. Removal of individual disulfide bonds did not reduce the hepcidin function significantly *in vitro*. However, serial deletions of the N-terminal residues progressively reduced hepcidin activity^[12].

The first animal model describing the relationship between hepcidin and iron homeostasis came serendipitously from the knockout of an adjacent gene encoding upstream stimulatory factor 2 (USF2)^[11]. The USF2 knockout mice of this strain (Paris USF2) had very low levels of hepcidin transcript in the liver and developed multi-organ iron overload (but with relative sparing the spleen) and high transferrin saturation, a phenotype consistent with the human iron loading disease hereditary hemochromatosis^[11]. These results provided the first indication that hepcidin was a negative regulator of iron uptake from the intestine and of iron release from macrophages. Another USF2 knockout mouse strain (Houston USF2)^[14] had normal hepcidin levels, and showed no aberrations in iron metabolism^[15], indicating that the Paris USF2 knockout phenotype was due to hepcidin deficiency. Since these original studies, a specific hepcidin knockout mouse has been generated, and it also shows an iron loading phenotype^[16]. In contrast, mice over expressing hepcidin display decreased body iron levels and a microcytic hypochromic anaemia typical of severe iron deficiency^[15]. The majority of these mice die within a few hours after birth suggesting that hepcidin also inhibits placental iron transport^[15]. A similar situation has been described in humans, and patients with hepatic adenomas producing abnormally high levels of hepcidin suffer from a severe iron refractory anaemia that only resolved after resection of the tumour^[17]. As might be expected from these results, injection of synthetic hepcidin peptide into mice leads to inhibition of intestinal iron absorption and consequently hypoferrremia^[18].

Taken together, these data provide strong evidence that hepcidin is the central regulator of body iron levels.

In humans, mutations in the *HAMP* gene result in a severe form of iron loading disease that presents at early age, and is aptly named juvenile hemochromatosis (JH). *HAMP*-associated JH is inherited in an autosomal recessive manner, and two mutations have been described (93delG and C166T) that are associated with iron loading when present in the homozygous state^[13,19]. While heterozygosity for these mutations alone does not lead to iron loading, compound heterozygosity between two other *HAMP* mutations (Met50del IVS2+1(-G) and G71D) and C282Y, the most common mutation in patients with HFE-associated iron loading, has been reported to result in hemochromatosis^[20]. Thus *HAMP* mutations may act as modifiers of the HFE-associated hemochromatosis phenotype.

Clearly hepcidin plays a major role in the regulation of intestinal iron absorption. But, how does it exert its effects? Soon after the link between hepcidin and iron was recognised, a close inverse correlation between *HAMP* expression and iron absorption and the expression of duodenal iron transporter transcripts was described^[21]. It was suspected that hepcidin interacted with a receptor on the basolateral surface of the enterocytes, thereby activating one or more signal transduction pathways that ultimately led to changes in the expression of the iron transport genes. The truth turned out to be elegantly simple. Hepcidin acts by directly binding to the sole basolateral iron export molecule, ferroportin, and causing its internalisation and subsequent degradation^[22]. Thus ferroportin is the hepcidin “receptor”. This loss of ferroportin on the cell surface reduces iron export from the cells leading to intracellular iron accumulation. As ferroportin is responsible for iron export from both enterocytes and macrophages, loss of this protein will result in reduced supply of iron to the plasma and, hence, will cause hypoferrremia and, ultimately, anaemia. Consistent with this mechanism is the observation that mice lacking hepcidin show decreased iron in the spleen, an organ rich in macrophages, in the face of increased hepatic iron^[15]. Similarly, J774 mouse macrophages treated with hepcidin peptide showed decreased levels of ferroportin and reduced the efflux of iron^[23]. Hepcidin likely acts on iron export from other cell types, such as hepatocytes, in a similar fashion, and this can explain its key role in regulating iron traffic into and around the body.

Since hepcidin interacts with FPN, it might be expected that mutations in FPN that alter this interaction could essentially mimic hepcidin deficiency. This has been found to be the case. Two classes of FPN mutations have been identified in human subjects and both lead to iron loading.

However, there are subtle differences in the phenotypes, with one being consistent with reduced iron transport by the protein and, the other consistent with impaired interaction with hepcidin^[24].

SYSTEMIC FACTORS THAT REGULATE HEPcidIN

Consistent with its role as a central regulator of body iron metabolism, hepcidin levels are modulated by the same factors that alter iron homeostasis. Changes in body iron stores, the rate of erythropoiesis, inflammation and hypoxia all influence iron absorption and iron release from macrophages and these are the major systemic factors that regulate *HAMP* mRNA levels in the liver.

Hepcidin levels are increased in response to oral and parenteral iron loading and decreased under iron deficient conditions^[10]. This inverse relationship is seen with chronic changes in body iron status. But, it can also occur quite quickly, and *HAMP* mRNA levels in the liver can decrease within days of transferring rats from a control to iron deficient diet^[21]. The regulation of hepcidin by body iron levels acts as a feedback mechanism to allow sufficient iron to enter the plasma when demand is high, but to limit iron intake/release in times of iron sufficiency. How hepcidin responds to changes in body iron levels is incompletely understood. Since hepcidin expression is largely restricted to the liver, it is highly likely that the hepatocyte is the site of action of the regulatory stimulus. But, whether hepatocyte iron levels per se play a primary role or whether an external signal is involved is unclear. This will be considered in more detail in the following section.

Interestingly, *in vitro* loading of hepatocyte cell lines and primary hepatocytes with iron decreases *HAMP* mRNA expression, the opposite effect to that seen *in vivo*^[25]. Why this is the case has proved difficult to resolve. One possibility is that the iron supplied to the cultured cells is of a different form to that presented to the liver *in vivo*. This may be the case, but the *in vitro* reduction in hepcidin expression is seen when both iron salts and transferrin-bound iron is presented to the cells. A second possibility is that during the isolation and culture procedure liver-derived cells lose some factor that is critical for their normal physiological response to iron. Since the same effect is seen on freshly isolated primary hepatocytes this appears unlikely. But, it remains possible. Potentially the most satisfying explanation for the observations is that the response of the liver to iron *in vivo* requires the interaction of two or more types of cells, and that this interaction is lost after the cells are isolated. The liver macrophages or Kupffer cells are strong candidates for cells that might interact with hepatocytes to regulate iron homeostasis. But, several studies have now shown that when animals are depleted of macrophages (including Kupffer cells) their livers respond normally by increasing hepcidin expression in response to iron^[26,27]. Thus it appears that macrophages are not required for hepcidin regulation in the liver in response to iron *in vivo*. A final possibility is that reduced expression of hepcidin is the normal physiological response of hepcidin to iron, and that the *in vivo* situation is complicated by a range of interacting stimuli that

influence expression of the *HAMP* gene. Further work is required to resolve this issue.

The largest single sink for iron in the body is haemoglobin in the red blood cells, and consequently iron demand is closely linked to the rate of erythropoiesis. Thus when erythropoiesis is stimulated, following phenylhydrazine-induced hemolysis for example, hepcidin expression is suppressed to allow increased iron flow into the plasma and consequently to the developing red cells^[15,28]. This hepcidin response is observed only in the presence of erythropoiesis as suppression of erythropoiesis by irradiation or by post-transfusion polycythemia leads to increased hepcidin levels^[29,30]. The regulation of hepcidin mRNA levels by erythropoiesis is independent of direct erythropoietin effects^[30], and is likely to reflect several stimuli. The iron requirement of the erythroid marrow is certainly a major factor. But, hypoxia too is also likely to be important as reduced haemoglobin may reduce oxygen delivery to the tissues. The response of hepcidin to hypoxia is considered in more detail below. In a recent study by Ganz and colleagues, it was concluded that in addition to iron requirements and hypoxia, there is an erythropoiesis-specific factor that affects hepcidin expression^[30]. However, this factor has yet to be characterized.

Another situation where body iron homeostasis is perturbed is during inflammation or infection. Under these circumstances, iron absorption declines and iron is sequestered in macrophages, with the consequence that the plasma iron level is decreased (hypoferrremia). With chronic inflammation or infection, anemia may result, and this condition is often called the anemia of chronic disease^[31]. Consistent with the reduction in plasma iron is the demonstration that inflammatory stimuli positively regulate hepcidin levels^[32-35]. Increased hepcidin means decreased iron entry into the plasma. That hepcidin is responsible for the hypoferrremia accompanying inflammation has been shown by studies with *Hamp* null mice. These animals mount a standard inflammatory response to a stimulus such as bacterial lipopolysaccharide (LPS), but the expected hypoferrremia does not occur^[32].

One of the major mediators of the inflammatory response is the cytokine IL-6. IL-6 infusion in humans or administration to experimental animals leads to an increase in hepcidin production and decrease in serum iron levels within a few hours^[36]. A time course analysis in human subjects injected with LPS revealed a strong temporal correlation between increases in serum IL-6 and urinary hepcidin, and the decrease in serum iron^[34]. Similarly, IL-6, other pro-inflammatory cytokines like IL-1 α and IL-1 β and LPS stimulate hepcidin in primary hepatocytes and hepatoma cell lines^[36,37].

Many inflammatory processes have a systemic component, and these are able to influence hepcidin expression in hepatocytes. However, there is increasing evidence that hepcidin may also be relevant in the local, extra-hepatic setting. For example, in an *in vivo* murine granulomatous pouch model of infection the host animals responded to bacterial infection by upregulating hepcidin at the local level, presumably to limit availability of iron to the pathogens in the immediate vicinity^[35]. The

cells responsible for the local production of hepcidin are unknown, but may be infiltrating macrophages and neutrophils. Indeed hepcidin expression has been detected in myeloid cells in response to systemic infection^[38]. Hepcidin production also has been demonstrated in adipose tissue. But, again the responsible cell type is not known^[39].

At the whole body level, hypoxia is usually associated with a reduced amount of circulating hemoglobin, and the body's response to this deficiency is to stimulate erythropoiesis. This in turn requires an increased iron supply. It thus comes as no surprise that hepcidin levels drop 2-4 d after animals are placed in a hypoxic chamber^[32], and that luminal iron uptake is increased in the small intestine under the same conditions^[40]. Some of the *in vivo* effects of hypoxia cannot be attributed to the direct repression of hepcidin expression by low oxygen as increased erythropoiesis may reduce hepcidin levels by other mechanisms^[30]. But, the demonstration that hypoxia down regulates hepcidin mRNA levels in human hepatoma cell lines^[32] suggests that the *HAMP* gene itself may be regulated by hypoxia. In addition, hypoxia could also trigger a stress response in cells and animals, and *HAMP* is a known stress response gene^[33].

The factors that regulate hepcidin levels described above vary in their relative strength, and in certain situations, where more than one stimulus is present, one may predominate. A good example of this is in β -thalassemia. In both mice and humans with this disorder, hepcidin levels are initially low despite increased levels of storage iron, and thus the erythropoietic stimulus is predominating^[41-43]. However, as the disease advances the effect of the increasing iron stores become relatively stronger and hepcidin levels increase. A similar situation is found in hypotransferrinaemic mice^[17] and in iron loaded animals treated with PHZ to induce anaemia and erythropoiesis^[32] where the erythropoietic stimulus predominates.

THE MOLECULAR BASIS OF HEPCIDIN REGULATION

While the major physiological factors that alter hepcidin expression have been identified, how these stimuli signal the liver to alter hepcidin expression, and how the changes in expression are brought about is complex and only partially understood. Important clues in dissecting these regulatory pathways have come from the analysis of human iron loading disorders and their equivalent murine models. Patients with mutations in the *HFE*, transferrin receptor 2 (*TfR2*), hemojuvelin (*HJV*) or *HAMP* genes all show a histological pattern of iron deposition that is similar and consistent with elevated iron absorption. Furthermore, *HFE*, *TfR2* and *HAMP* all show their highest expression in the liver and *HJV* shows strong expression in this organ. These similarities suggested that the proteins these genes encode may form part of the same regulatory pathway. This has now proved to be the case.

Classical, adult onset hereditary hemochromatosis (HH) results from mutations in the *HFE* gene, and both human

patients and mouse strains with a disrupted *HFE* gene show periportal iron deposition with relative sparing of the Kupffer cells^[44,45]. *HFE* is expressed on the cell surface as a complex with β_2 -microglobulin (β_2m) and mouse models of β_2m deficiency show a similar iron loading phenotype^[46-48]. Since hepcidin expression is increased with iron loading, it was expected that disruption of the *HFE* gene would lead to enhanced hepcidin levels. When this was investigated, however, the opposite was seen. Human patients with *HFE*-associated hemochromatosis showed significantly lower levels of liver hepcidin expression than control subjects^[49]. In *Hfe* knockout mice, hepcidin levels were similar to those of wild-type animals. But, the level remained low even when the knockout animals were fed a high iron diet^[50]. Taken together, these observations show that *HFE* is an upstream regulator of hepcidin, and not a downstream target as was previously believed^[49]. Proof of this was provided by Nicolas and colleagues who showed that mice over expressing hepcidin, but null at the *Hfe* locus, did not accumulate excess iron^[51]. Despite low hepcidin levels in patients with HH, hepcidin levels still increase as the body iron load increases indicating that hepcidin regulation is not completely disrupted when *HFE* is mutated^[49,52]. This is consistent with the milder phenotype of patients with *HFE*-associated HH compared to those with mutations in the *HAMP* gene^[24].

If *HFE* acts as an upstream regulator of hepcidin, how does it respond to changes in body iron demand? The answer to this question is not clear. But, the level of circulating diferric transferrin has emerged as a potential regulator^[53,54]. *HFE* is able to bind to TfR1, the major cell-surface diferric transferrin binding protein^[55,56], and *HFE* and transferrin are able to compete for TfR1 binding. Such competition could modulate the amount of *HFE* that is not bound to TfR1 that in turn could transduce a signal to alter hepcidin expression. It is also possible that *HFE* interferes with the cellular uptake of transferrin-bound iron, and that this in turn affects *HAMP* expression^[57]. Support for diferric transferrin as a signal to alter *HAMP* expression has come from the demonstration that another transferrin binding protein, TfR2, also is involved in hepcidin regulation.

Transferrin receptor 2 encodes a protein that shares 45% identity with TfR1^[58]. However, in contrast to its widely expressed homolog, TfR2 expression is restricted to the liver, spleen, brain and heart, with highest expression in the liver^[58]. Mutations in TfR2 lead to iron overload in human patients^[59] with a clinical picture similar to classical *HFE*-associated hereditary hemochromatosis^[60]. The same phenotype is seen in TfR2 knockout and mutant mice^[61,62]. As in *HFE*-associated iron loading, hepcidin expression is decreased in the liver of TfR2 mutant mice^[62,63], and patients with TfR2-related hemochromatosis also show decreased urinary hepcidin levels^[64]. The severity of TfR2-related hemochromatosis is much less than that associated with mutations in *HAMP* or *HJV*, but is similar to *HFE*-associated hemochromatosis.

Like *HFE*, TfR2 appears to be an upstream regulator of hepcidin. But, how it exerts its effects is unknown. It has been reported that, unlike TfR1, TfR2 does not bind to *HFE*, and shows 25 times less affinity for transferrin compared to TfR1^[65] making it unlikely that TfR2 makes a

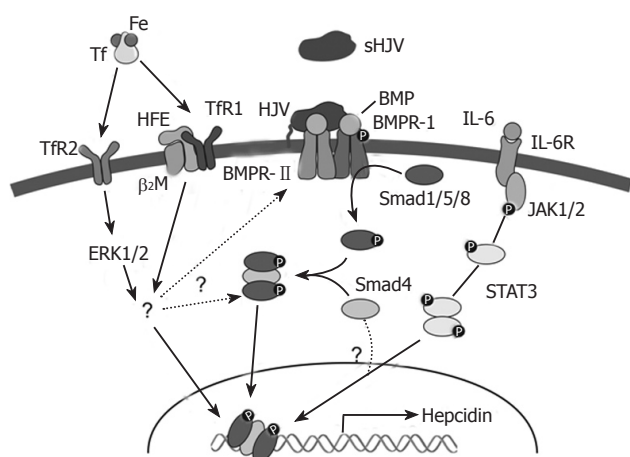


Figure 2 Signalling pathways for *HAMP* regulation.

major contribution to cellular iron uptake. Indeed, in *Tfr2* knockout mice, the liver accumulates iron very efficiently indicating that *Tfr2* is not essential for hepatic iron uptake. In contrast, a recent study has shown that when *Tfr2* and *HFE* are over expressed together in the same cells they can be co-immunoprecipitated^[66]. Whether this interaction is physiologically relevant or represents an overexpression artefact remains to be determined. Interestingly, two independent studies have demonstrated that *Tfr2* protein levels are upregulated by diferric transferrin, but do not respond to apotransferrin or non-transferrin bound iron^[67,68]. This effect of diferric transferrin is not observed at the transcript level, and appears to be post-translational^[68]. Recently, it has been reported that the interaction between diferric transferrin and *Tfr2* activates ERK1/ERK2 and p38 MAP kinase, but only when *Tfr2* is present on the lipid rafts of the exosomes^[69]. However, the precise mechanisms and signalling pathway of *Tfr2*-hepcidin axis are not yet understood and remain to be further explored.

The third important protein known to be involved in the regulation of hepcidin is hemojuvelin (HJV, RGMc). HJV, is a member of the repulsive guidance molecule (RGM) family, and shares some common features with RGMa and RGMb, including a C-terminal glycosylphosphatidylinositol-linked membrane anchor (GPI-anchor), N-terminal signal sequence, proteolytic cleavage site and partial von Willebrand factor type D domain^[70,71]. Mutations in the *HJV* gene cause severe iron overload, and lead to juvenile hemochromatosis due to greatly decreased hepcidin levels in human patients^[72] and mice^[73,74]. Moreover, knocking down HJV with siRNA in Hep3B cells leads to a decrease in hepcidin levels^[74]. Interestingly, HJV expression is strongest in heart and skeletal muscle, but also shows moderate expression in the liver^[72], where *HAMP* is most strongly expressed. Treating primary hepatocytes with soluble HJV led to a decrease in hepcidin levels suggesting that a binding competition exists between soluble and cell-associated hemojuvelin^[75]. In addition, increasing iron concentrations led to a decrease in soluble HJV (sHJV) in cells over expressing HJV^[75], indicating that iron could regulate HJV at the post-transcriptional level.

HJV acts as a co-receptor for the bone morphogenetic proteins (BMPs), in a similar fashion to other molecules of the Rgm family, and HJV mutants have impaired BMP signalling^[76]. BMPs represent a subfamily of transforming growth factor-beta (TGF- β) ligands that signal by binding to and bringing together type I and type II BMP receptors on the cell surface, and then propagating the signal through phosphorylation of the Smad proteins^[77] (Figure 2). BMPs phosphorylate receptor-regulated Smads 1, 5 and 8 that in turn form heteromers with the co-mediator Smad 4. The activated complex then translocates to the nucleus and, in combination with other factors, regulates target genes such as *HAMP*^[77]. Cells transfected with the co-receptor HJV or treated with the ligand BMP-2 showed increased levels of hepcidin, and BMP-2 induction was enhanced in the presence of HJV^[76]. Other BMPs, BMP-4 and BMP-9, have been shown to have a similar effect on hepcidin expression independent of *HFE* and *Tfr2* status^[78]. This suggests that HJV acts *via* a *HFE*/*Tfr2* independent pathway to alter *HAMP* levels. Further insight into this aspect of hepcidin regulation comes from the studies of the liver-specific Smad 4 knockout mouse. These animals showed markedly decreased levels of hepcidin, increased duodenal transporters and iron overload^[79]. Overexpression of Smad 4 led to transcriptional activation of *HAMP* due to epigenetic modification of histone H3 protein^[79]. Smad 4-deficient hepatocytes showed no increase in hepcidin levels upon treatment with BMP, iron, TGF- β or IL-6^[79]. This response to BMP is expected as Smad 4 acts downstream in the signalling pathway^[79]. However, the result with IL-6 is interesting as it indicates that the IL-6 pathway and the BMP-Smad pathway converge at some point. Overall, these data indicate that the BMP/SMAD pathway plays an important role in the regulation of *HAMP* gene expression. Whether this is the major pathway operating or other pathways are dominant remains to be determined, as the relationship between *HFE*, *Tfr2* and HJV (Figure 2).

Pro-inflammatory molecules like LPS and FCA positively regulate hepcidin expression by inducing the expression of cytokines such as IL-6. This induction of hepcidin by pro-inflammatory cytokines appears to be independent of *HFE*, β_2m and *Tfr2*^[80,81], although one study provided evidence that these proteins may play some role^[82]. The differences in these studies could be due to the difference in the timing and dosage of the treatments. IL-6 alters *HAMP* transcription through the classical JAK-STAT pathway^[83,84]. This pathway ultimately leads to activation of STAT3 that binds to an element in the proximal 100 bases of the *HAMP* promoter. The interesting observation that the IL-6 dependent stimulation of the *HAMP* gene is abrogated in Smad 4 knockout mice indicates that Smad 4 is involved in the signalling process as well. This has yet to be investigated in detail, as have the mechanisms by which other pro-inflammatory cytokines stimulate *HAMP* expression.

Although it is widely considered that the *HAMP* gene is regulated predominantly at the level of transcription, relatively few promoter analyses have been carried out. As noted above, STAT3 is known to bind to the promoter, and several other transcription factors have been

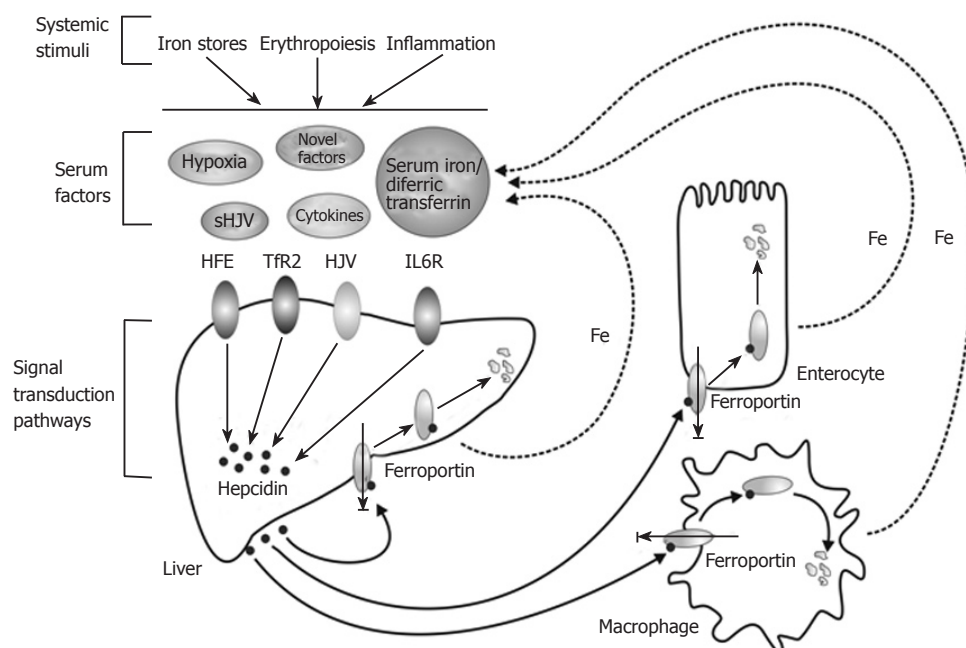


Figure 3 A model for the liver-dependent regulation of iron homeostasis.

identified. C/EBP α , a liver-enriched transcription factor, has been shown to bind the *HAMP* promoter 230-250 bp upstream of the transcription start site^[85], and may drive basal transcriptional activity of the gene. Supporting such a role, C/EBP α knockout mice showed decreased hepcidin levels and increased iron staining in their livers^[85]. In another study, the role of the basic helix-loop-helix leucine zipper (bHLH-ZIP) family of transcriptional regulators, and notably USF2, in *HAMP* transcription was studied in order to investigate the reasons behind the lack of hepcidin expression in the USF2 knock out mouse. It was demonstrated using site-directed mutagenesis, chromatin immunoprecipitation assays and mobility shift assays that USF1/USF2 and c-Myc/Max bind to E-boxes in the hepcidin promoter, and regulate its transcription^[86]. Because some genes with E-boxes show rhythmicity, it has been proposed that hepcidin might also be under pulsatile or rhythmic transcriptional control. However, the signals and cellular pathways that lead to the activation of these proteins in the context of hepcidin regulation remain to be resolved.

A MODEL FOR THE LIVER-DEPENDENT REGULATION OF IRON HOMEOSTASIS

The discussion above has highlighted various factors that regulate hepcidin, and the current knowledge about the molecular mechanisms behind their effects. We previously proposed a model to explain the regulation of hepcidin in a physiological context^[53], and present an updated version of the model here (Figure 3). In this model, signals that alter body iron homeostasis (iron stores, erythropoiesis, inflammation, hypoxia) act on the hepatocytes in the liver to modulate *HAMP* gene expression. Some of these stimuli may act directly on the liver cells (e.g. hypoxia), while others may act indirectly e.g. iron stores may act through changing the levels of diferric transferrin in the circulation. How the signals alter *HAMP* expression at the

molecular level is incompletely understood. HFE, TfR2 and HJV are clearly involved in the process and, in the case of the latter; signalling through the BMP/SMAD pathway is a likely mode of action. Pro-inflammatory cytokines such as IL-6 act through the JAK/STAT pathway to regulate *HAMP* transcription. Hepcidin secreted by the liver acts on mature enterocytes in the proximal small intestine to reduce iron export into the circulation. Thus, high hepcidin means reduced iron absorption and vice versa. Hepcidin also acts on macrophages, hepatocytes, and likely other cells in the body to regulate their release of iron, so it plays a universal role in iron homeostasis.

Hepcidin levels show a positive correlation with transferrin saturation that is independent of the liver iron content^[21] indicating that the levels of iron bound to transferrin may be a major signal that regulates the expression of hepcidin in the liver. Diferric transferrin is essential for iron delivery to the tissues making it suitable as an indicator of plasma iron status. Transferrin saturation thus decreases with iron deficiency and increases with iron loading. Diferric transferrin competes with HFE for binding to TfR1, and their binding sites overlap. But, it has a higher affinity for its principal receptor than HFE^[87], and thus out-competes HFE for binding to TfR1. This leaves HFE “free” on the cell surface to initiate a signal to stimulate hepcidin expression. Similarly, diferric transferrin binds to TfR2, although with lower affinity, and sends a signal via the proposed ERK1/2 and MAPK pathways^[69]. The signal transduction pathways driven by HFE and TfR2 that lead to the regulation of hepcidin expression are not yet clear. HFE and TfR2 may form a stable complex with each other as has been proposed^[66] or may interact with HJV/BMPRs to propagate the signalling *via* the Smad1, 5, 8/Smad4 pathway. But, the precise details have yet to be elucidated.

In iron deficiency, TfR1 and transferrin levels increase, and transferrin saturation drops. The balance shifts towards monoferric transferrin that has lower binding affinity for TfR1 compared to diferric transferrin. This

results in increased binding of HFE to TfR1, decreased signalling, and consequently lower hepcidin production. Similarly, as the plasma iron level falls, TfR1 binds residual diferric transferrin more efficiently than TfR2. This down regulates the TfR2-ERK signalling pathway, and thus hepcidin synthesis. When body iron levels are high the opposite pattern is seen, and hepcidin expression is increased. Stimulated erythropoiesis is another major stimulus for iron absorption, and at least one mechanism by which it might exert its effects on reducing hepcidin expression is *via* reduced transferrin saturation.

CONCLUSION

Recent developments in understanding the molecular mechanisms of iron homeostasis have greatly enhanced our knowledge of iron absorption in the gut. The most important advance in this area has been the recognition that the liver-derived peptide hepcidin responds to variations in body iron demand, and acts on the proximal small intestine to regulate iron efflux into the plasma. This shows that the liver plays a central role in the regulation of body iron homeostasis. Various systemic stimuli including iron stores, the rate of erythropoiesis, and oxygen levels regulate hepcidin expression, and consequently iron absorption. But, how systemic signals are received by the liver, and how these signals are transduced into changes in *HAMP* gene expression are incompletely understood. Strong evidence now suggests that signalling through the BMP/Smad pathway plays a major role in regulating hepcidin. But, how universal this pathway is has yet to be resolved.

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TOPIC HIGHLIGHT

Nathan Subramaniam, PhD, Series Editor

Role of iron in hepatic fibrosis: One piece in the puzzle

Marie A Philippe, Richard G Ruddell, Grant A Ramm

Marie A Philippe, Richard G Ruddell, Grant A Ramm, Hepatic Fibrosis Group, The Queensland Institute of Medical Research, PO Royal Brisbane and Women's Hospital, Brisbane 4029, Australia
Supported by NHMRC Program Grant 339400

Co-first-authors: Richard G Ruddell

Correspondence to: Grant A Ramm, PhD, Associate Professor, Hepatic Fibrosis Group, The Queensland Institute of Medical Research, PO Royal Brisbane and Women's Hospital, Brisbane 4029, Australia. grant.ramm@qimr.edu.au

Telephone: +61-7-33620177 Fax: +61-7-33620108

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Abstract

Iron is an essential element involved in various biological pathways. When present in excess within the cell, iron can be toxic due to its ability to catalyse the formation of damaging radicals, which promote cellular injury and cell death. Within the liver, iron related oxidative stress can lead to fibrosis and ultimately to cirrhosis. Here we review the role of excessive iron in the pathologies associated with various chronic diseases of the liver. We also describe the molecular mechanism by which iron contributes to the development of hepatic fibrosis.

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Key words: Iron; Fibrosis; Oxidative stress; Hepatic stellate cell; Haemochromatosis; Hepatitis C; Non-alcoholic fatty liver disease; Alcoholic liver disease

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INTRODUCTION

Iron, created by stellar nucleosynthesis, is the most abundant element on Earth in terms of mass, making up 35% of total planetary mass. Iron, in its ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms, is critical to all life forms from the simplest filamentous algae through to the most complex multicellular organisms. Iron is an essential element mainly present within the cells in association with haemoprotein (haemoglobin, myoglobin) or within an iron-sulphur cluster of various metalloproteins (e.g., aconitase and

Rieske proteins of the respiratory chain)^[1]. Iron is involved in the redox-driven processes of oxygen transport, electron transport and various enzymatic reactions such as DNA synthesis, transcriptional regulation, catalysis as well as nitric oxide (NO) and oxygen sensing^[1]. Mammals do not have any major physiological pathway for iron excretion. Therefore, iron uptake and storage is closely regulated so as to avoid deficiency, and excess. In humans, iron deficiency is manifested as anaemia and with increasing severity can eventually result in cardiac failure. Iron overload, especially at sites of storage, enhances oxidative stress ultimately leading to lipid, nucleic acid and protein peroxidation. Within the liver, which is the major site of iron storage, enhanced oxidative stress can lead to fibrosis, cirrhosis, hepatocellular carcinoma (HCC) and death.

In this review, we outline the hepatic disease states where iron is an important factor in disease progression. We also discuss the role of iron in promoting liver fibrosis and those cells and mechanisms most important in the underlying wound healing/fibrotic processes.

HEPATIC FIBROSIS

Hepatic fibrosis is promoted by various pathogenic, mechanical and toxic insults to the liver, and is part of a physiological wound healing response. If the injurious stimuli are chronic, the degree of fibrosis worsens, leading to cirrhosis, and eventually to hepatic failure and death. The interactions between various resident hepatic cell populations and immune cells that lead to the establishment of fibrosis are complex, and not yet fully understood. However, some profibrogenic pathways and clinical outcomes are common to several disease states, and these will be briefly outlined below.

In the normal liver, a balance is struck between extracellular matrix (ECM) deposition and degradation, a process that is tightly regulated by matrix metalloproteinases (MMP) and their specific inhibitors (TIMPs)^[2]. Fibrosis is associated with major quantitative and qualitative changes in the ECM. These changes are mainly due to an increase in the expression of TIMP-1^[3] and an increase in the expression of various ECM components, which include fibrillary collagens I and III, collagen IV, fibronectin, elastin and laminin^[4].

The hepatic stellate cell (HSC) resides within the space of Disse, and is responsible for the majority of ECM deposition in the normal and fibrotic liver^[5,6]. In the normal liver the primary role of the HSC is the regulation of

vitamin A homeostasis and storage^[6]. In times of chronic injury, the HSC transdifferentiates into a myofibroblastic cell exhibiting contractile, proliferative, inflammatory and fibrogenic properties. Transdifferentiation occurs in response to soluble fibrogenic and proliferative factors released mainly by Kupffer cells (KC), (PDGF, TGF β 1) or by damaged hepatocytes (IGF-1, TNF α , EGF)^[2]. Once transdifferentiated, the HSC expresses a number of myogenic markers (including α smooth muscle actin (α SMA), c-myc and myocyte enhancer factor-2) that allows them to be readily identified by immunohistochemical techniques^[4]. In addition, HSC also express a number of neuroendocrine proteins (e.g., glial fibrillary acidic protein or GFAP, synaptophysin and nestin^[4]) and receptors for various neurotransmitters^[7]. Once activated, HSC also release TGF β 1 and PDGF-BB, thereby ensuring a self-sustained phenotypic change^[8,9]. Fibrosis was once thought to be irreversible; however, a growing body of evidence now suggests that once the underlying source of liver injury is treated, fibrosis, and even cirrhosis, to some extent, are reversible. Fibrosis resolution is known to be accompanied by HSC apoptosis, and to some degree of reversion of HSC to their original phenotype^[10].

IRON AND DISEASES OF THE LIVER

Iron is proposed to play a role in promoting hepatic fibrosis in a number of different chronic liver disease states. The evidence supporting or contradicting those propositions is outlined in the following section.

Haemochromatosis

Haemochromatosis is a term used to describe excessive iron loading of the liver leading ultimately to cirrhosis and frequently to HCC^[11]. The knowledge regarding the underlying causes of haemochromatosis is gradually expanding, and the term has now been subdivided according to the specific genetic mutation involved. Iron loading of the liver is a life long process in patients with haemochromatosis, occurring without any apparent overt inflammation or elevation of serum liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The symptoms associated with hereditary haemochromatosis (HH) include; hepatomegaly, malaise, insulin resistance and arthritis, to name but a few. When hepatic iron reaches the threshold concentration of 60 μ mol/g dry weight, HSC begin to exhibit the early stages of cellular activation (namely α SMA expression), a key event in the initiation of hepatic fibrosis^[12]. Evidence also suggests that there is a hepatic iron concentration threshold (about 250 μ mol/g dry weight) beyond which cirrhosis can develop in patients with HH^[13,14]. Initial iron loading of the liver occurs in hepatocytes located in Rapoport zone 1, progressively extending to hepatocytes in zones 2 and 3. In the later stages of the disease, KC also accumulate iron and it is the co-loading of both hepatocytes and KC that is, believed to allow fibrosis to become established^[15]. Patients with well established hepatic iron loading are also at a significantly greater risk of developing HCC^[11]. Phlebotomy is widely used to reduce iron burden

in patients with haemochromatosis and, is also an effective way to reverse hepatic fibrosis^[14,16], although regression of cirrhosis remains controversial^[14,16].

Transition of the iron-loaded liver from non-fibrotic to fibrotic, and then onwards to cirrhotic is not always a clear-cut process, and there is growing evidence to suggest that other factors, such as excessive alcohol consumption^[17], viral hepatitis^[18] and steatosis^[19] among others, have an important role in that transition. In fact, iron overload has been shown to be important in the pathology of other liver disease such as non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD) and chronic hepatitis C.

Non-alcoholic fatty liver disease

Increase in caloric intake and an evermore sedentary lifestyle have led to a surge in the prevalence of people being overweight or obese. Current estimates suggest that as much as 60% of the over 18 age group in Australia will be overweight or obese by the year 2010^[20]. Obesity impacts the liver in the form of NAFLD, which is now recognised as the most common form of liver disease. NAFLD begins with steatosis (fat accumulation within the liver) and can remain in this state without any apparent associated pathology. In its more advanced form NAFLD is often referred to as non-alcoholic steatohepatitis (NASH). It is believed that in order for steatosis to advance to NASH a secondary liver insult is also required often termed the "second hit"^[21]. Iron is among several co-factors known to increase hepatic oxidative stress, and constitutes a "second hit" agent that is postulated to contribute to the progression of steatosis to NASH.

Several studies have highlighted mutations (C282Y and H63D) in the HFE gene that positively correlate with the presence of NAFLD/NASH^[19,22-24]. These studies suggest that while combined NASH/NAFLD and HFE mutation have a negative effect on disease severity as highlighted by serum ALT concentrations and fibrosis grade, hepatic iron concentration is not always elevated. Interestingly Mendler and colleagues also noted that patients with an elevated iron burden almost always displayed insulin resistance irrespective of liver damage^[24]. Insulin resistance is known to play a central role in the accumulation of triglycerides within the hepatocytes, and in the initiation of the inflammatory cascade ultimately leading to cirrhosis^[25]. The correlation between elevated hepatic iron and disease severity in patients with NAFLD/NASH is not always consistent. Several studies have failed to find any evidence of increased hepatic iron levels in patients with NAFLD/NASH, with histological severity being linked to the presence of ballooning hepatocyte degeneration, Mallory hyaline, older age, obesity and the presence of diabetes mellitus^[26-28]. The role of iron in the pathologies associated with NAFLD is, however, further supported by several studies that demonstrate the beneficial effects of iron depletion on serum ALT concentrations and insulin response in patients with NAFLD^[29]. How and why steatosis and iron combined lead to exacerbated fibrosis is possibly linked to the oxidative stress that both can exert.

Alcoholic liver disease

ALD is caused by high risk alcohol consumption over a number of years. Of those engaging in high risk alcohol consumption, only approximately 30% go on to develop liver cirrhosis^[21]. This would suggest that other factors influence disease severity and progression in patients with ALD. Iron is one such factor known to affect the pathogenesis of ALD. In pre-cirrhotic ALD, approximately 29% of patients demonstrate an elevated hepatic iron concentration^[30]. As the disease progresses to cirrhosis, as many as 57% of patients have iron overload ($> 25 \mu\text{mol/g}$)^[31]. It is also apparent that iron is an independent risk factor for the development of fibrosis^[32] in ALD, and higher iron concentrations also correlate with reduced survival^[33]. Alcohol has been postulated to enhance iron uptake by a number of different mechanisms, which have recently been reviewed by Brittenham^[34]. In addition, recent work by Bridle and colleagues suggest that ethanol may perturb IL-6-mediated hepcidin expression resulting in enhanced absorption of iron and hemosiderosis^[35]. This is important when one considers that mammals lack an effective mechanism by which excess iron can be eliminated from the body^[34].

Hepatitis C

Viral hepatitis encompasses a range of different entities from hepatitis A through E, which are caused by distinct viruses. Of these, hepatitis B and C are perhaps the most significant in terms on patient morbidity and mortality. Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family. Estimates suggest that between 3% and 6% of the world's population are infected with HBV, with up to one third having been previously exposed^[36]. Hepatitis C virus (HCV) is a member of the *Flaviviridae* family and is thought to infect somewhere between 150 and 200 million people worldwide. Both types of viral hepatitis contribute to the development of cirrhosis and HCC. As is common with many forms of liver disease, the severity and rate at which fibrosis progresses are influenced by a great number of external factors and in viral hepatitis these include male gender, alcohol consumption, iron status and age at infection^[37].

The association between elevated serum iron and hepatitis B was first described by Blumberg *et al* in 1981^[38]. Some years later, a link was also found between serum iron, ferritin, transferrin saturation levels and HCV infection^[39,40]. However, these results did not correlate with an increased hepatic iron concentration. Where hepatic iron was found to be elevated in association with HCV infection (in 5% of cases), it was seldom to levels deemed hepatotoxic^[39]. In HCV-infected patients, iron is deposited in hepatocytes, sinusoidal cells, and portal mesenchymal cells, with the degree of portal mesenchymal iron deposition correlating with both hepatic inflammation and fibrosis^[41]. Martinelli and colleagues demonstrated a significant correlation between liver iron scores and the number of GFAP and αSMA -positive HSC in patients with HCV. These αSMA -positive or "activated HSC" were located primarily in Rappaport zones 1 and 3^[42]. Similar findings were also reported by Rigamonti and colleagues,

who also described a correlation between hepatic iron concentration and HSC activation. They suggested that, in patients with HCV infection, iron was important in both HSC activation and fibrosis progression^[43]. Besides being directly correlated with inflammation and fibrosis, hepatic iron has also been linked to a lack of response to IFN α in chronic hepatitis C patients^[44,45]. One mechanism by which this may occur was suggested by Di Bona and colleagues, who found that oxidative stress prevented IFN α induced phosphorylation of STAT-1 & 2 and subsequent upregulation of the antiviral proteins MxA and interferon regulatory factor 9, thereby impairing the antiviral action of IFN α ^[46].

The link between iron and fibrosis in chronic hepatitis C patients has, however, been challenged by Guyader and colleagues who looked at other confounding factors also known to influence both iron overload and fibrosis^[47]. In their studies, liver iron was elevated in only 17% of patients, and correlated with age, male sex, and alcohol intake. They found no association between liver iron and fibrosis after adjusting for confounding variables, and suggested that iron should be considered more as a surrogate marker for disease severity rather than as a fibrogenic factor^[47]. Iron is still further implicated in chronic hepatitis C progression by a number of groups investigating the effects of iron depletion on HCV-associated fibrosis. Kaito and colleagues demonstrated that phlebotomy treatment of HCV-infected patients alone was enough to reduce markers of liver damage (AST and ALT values), lipid peroxidation and oxidative stress^[48]. In addition, patients treated by maintenance phlebotomy (keeping patients in a state of near iron deficiency with a serum ferritin level of 10 ng/mL) showed less inflammation and suppressed fibrosis progression^[49]. Phlebotomy has also been used to increase the therapeutic effect of IFN α treatment of chronic hepatitis C by a number of groups^[50,51]. How and why HCV should interfere with iron homeostasis is open to conjecture. However, there is some evidence to suggest that HCV infection may influence the expression of the iron homeostasis peptide, hepcidin. Nagashima and colleagues suggested that a failure in the regulation of serum prohepcidin levels (mediated by HCV) leads to elevated serum ferritin concentrations, and subsequently to progression of liver injury by iron overload in chronic hepatitis C patients^[52]. Conversely, Aoki and colleagues found hepcidin mRNA expression in the liver did not correlate with AST, ALT levels, or viral load and no differences in hepcidin mRNA were found based on viral genotype or the presence of fibrosis^[53].

MOLECULAR MECHANISMS OF IRON-INDUCED FIBROSIS

This section outlines the mechanisms by which iron is able to cause liver damage and the subsequent pathways activated that lead to hepatic fibrosis.

Role of iron in oxidative stress

In hepatocytes and KC, iron catalyses the production

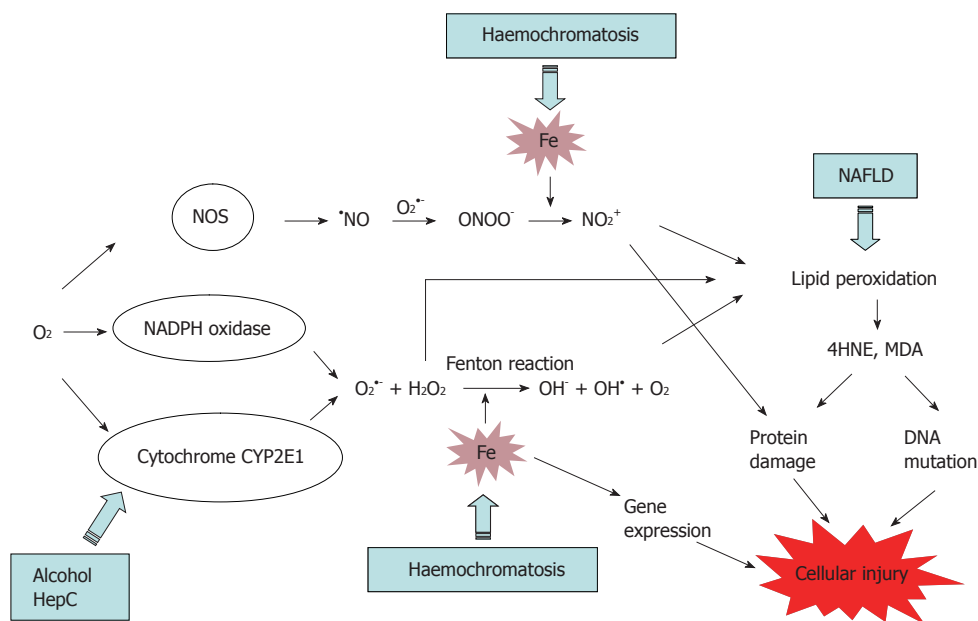


Figure 1 The involvement of iron in oxidative stress and its cytotoxic consequences. Iron catalyses the production of the reactive molecules OH^\bullet (via the Fenton reaction) and NO_2^+ , which promote lipid peroxidation and protein damage leading to cellular injury. NOS, nitric oxide synthase; NO , nitric oxide; ONOO^\bullet , peroxynitrite; $\text{O}_2^{\bullet-}$, superoxide radical; OH^\bullet , hydroxyl radical; H_2O_2 , hydrogen peroxide; NO_2^+ , nitronium anion; Fe, iron; NAFLD, non-alcoholic fatty liver disease.

of hydroxyl radical (OH^\bullet) from reactive oxygen species (ROS), superoxide ($\text{O}_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) *via* a chemical reaction known as the Fenton reaction (Figure 1). ROS are the by-products of aerobic respiration reactions by cytochrome P450 (CYP) 2E1, and are also produced by the membrane-bound NADPH oxidase complex^[54]. In addition, Fe catalyses the formation of NO_2^+ from peroxynitrite (ONOO^\bullet). ONOO^\bullet is formed when the cellular level of $\text{O}_2^{\bullet-}$ is elevated as this later reacts with nitric oxide (NO), produced by the constitutive and inducible nitric oxide synthase (NOS)^[55,56] (Figure 1). Interestingly, nitric oxide has also a protective effect against oxidative stress, as it can inhibit lipid peroxidation and the generation of OH^\bullet by reacting with Fe^{3+} ^[56,57]. NO_2^+ and OH^\bullet induce oxidative deterioration of biomolecules (lipid, protein and DNA), leading to tissue injury and cell death. In addition, iron can modulate gene expression in the cells leading to an alteration of cell function^[58,59].

Alteration of essential biomolecules: NO_2^+ and OH^\bullet catalyse lipid peroxidation which is the process whereby electrons are transferred from the lipids in cell membranes to the free radical, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It affects polyunsaturated fatty acids, as they contain multiple double bonds, between which lie methylene $-\text{CH}_2-$ groups that are especially susceptible to peroxidation. Lipid decomposition leads to the generation of thiobarbituric acid (TBA)-reactants and breakdown by-products 4-hydroxynonenal (4HNE) and malondialdehyde (MDA), which are used as marker of lipid peroxidation.

Lipid peroxidation affects the plasma membrane of the cell, but also increases the membrane fragility of a number of different cell organelles, such as lysosomes, which store excess iron, mitochondria and endoplasmic reticulum, leading to impaired cell function^[2]. Lipid peroxidation of the mitochondrial membrane can lead to an increase in their permeability resulting in loss of the electrochemical gradient and release of the proapoptotic cytochrome C.

This phenomenon is called mitochondria permeability transition. Damaged mitochondria generate yet more ROS that further enhance cell damage and activate proapoptotic signals^[60].

Another consequence of lipid peroxidation is the damage of DNA and proteins, as lipid peroxidation products such as 4HNE and MDA can react with DNA bases^[61] and the ϵ - NH_2 group of lysine and histidine residues^[62]. The presence of acetaldehyde, resulting from ethanol oxidation, increases the binding of MDA and its own binding to proteins in a synergistic manner, generating new hybrid adducts called MDA-acetaldehyde adducts^[63]. These adducts may play a role in the development and progression of liver fibrosis as they have been shown to stimulate the secretion of several cytokines and chemokines by liver endothelial cells (TNF α , MCP-1, MIP-2, fibronectin) and HSC (MCP-1, MIP-2, uPA)^[64-66].

Gene expression modulation: Iron or iron-induced oxidative stress have also been found to activate cell signaling cascades triggering apoptosis and necrosis pathway *via* NF- κ B and AP-1 pathways respectively^[60]. NF- κ B promotes the synthesis and release of cytotoxic, proinflammatory and fibrogenic factors such as TNF α , IL-6 and MIP-1 that alter KC and hepatocyte function, and trigger HSC activation^[58,59]. In HSC, AP-1 transcription factors are involved in the regulation of procollagen (I)^[56]. In addition, AP-1 and NF- κ B-dependent gene products modulate hepatocyte death induced by oxidative stress^[67].

Regulation of intracellular levels of ROS: In the normal liver, hepatocytes are able to remove or neutralize oxidative molecules *via* enzymatic and non-enzymatic antioxidant processes, thereby maintaining a safe cellular level of ROS. For example glutathione (GSH) is a tripeptide that neutralizes free radicals and ROS directly *via* a chemical reaction or *via* enzymatic reactions involving glutathione-reductase or glutathione-peroxidase^[68]. Antioxidant agents such as vitamin A, C and E are also able to impair

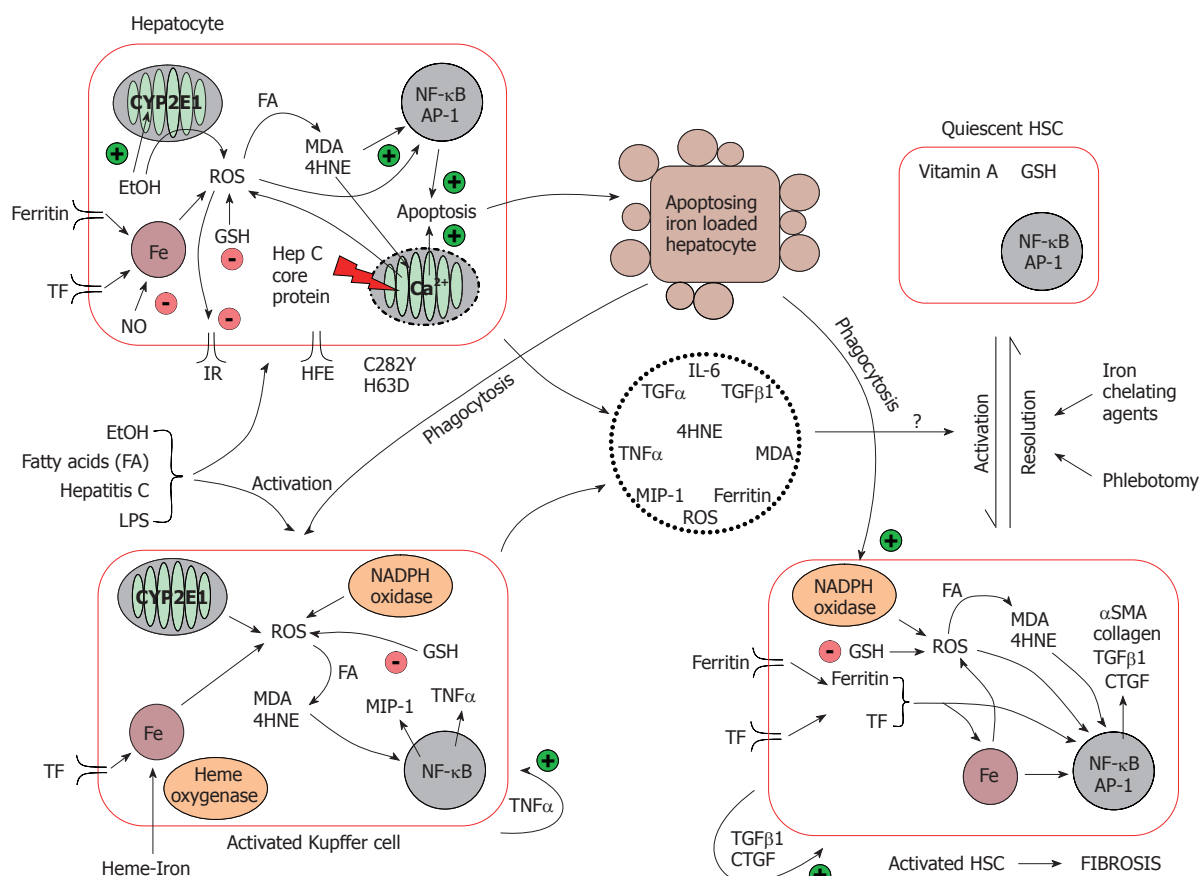


Figure 2 Mechanisms of iron-induced liver injury and fibrosis. Iron catalyses the formation of several reactive oxygen species in hepatocytes. Under normal circumstances, hepatocytes are able to effectively cope with oxidant stress. When the liver is subjected to a secondary insult that enhances hepatic oxidant stress, hepatic fibrosis begins to develop. Increased oxidative stress and other pathological modes of action of HCV, ethanol and steatosis, lead to mitochondrial dysfunction and hepatocyte apoptosis. Kupffer cell activation is achieved by phagocytosis of apoptosing hepatocytes in conjunction with the direct effects of iron, HCV infection, ethanol and steatosis on Kupffer cells. The concomitant hepatocyte damage/apoptosis and Kupffer cell activation is able to drive and maintain hepatic stellate cell activation leading to fibrosis and ultimately cirrhosis if left unchecked. Fe, iron; TF, transferrin; ROS, reactive oxygen species; FA, fatty acids; IR, insulin receptor; GSH, glutathione; TNF α , tumour necrosis factor α ; NO, nitric oxide; IL-6, interleukin 6; TGF β 1, transforming growth factor β 1; CTGF, connective tissue growth factor; α SMA, α smooth muscle actin; MDA, malondialdehyde; 4HNE, 4-hydroxynonenal; MIP-1, macrophage inflammatory protein-1; TGF α , transforming growth factor α ; NF- κ B, nuclear factor κ B; AP-1, activating protein-1.

lipid peroxidation by breaking the chain reaction. Other antioxidants can act as inhibitors of CYP2E1, such as diallylsulfide^[56]. However, when cellular defences are overwhelmed, there is an accumulation of ROS, which trigger cellular damage and apoptosis and, when this occurs in the liver, it can lead to fibrosis.

Iron driven oxidative stress promotes the development of hepatic fibrosis: Iron from the diet is absorbed into the enterocyte, and is either stored bound to ferritin or exported out into the plasma coupled to transferrin. In the liver, iron is taken up mainly by hepatocytes, and secondarily by KC. Hepatic iron uptake is mediated via HFE and the transferrin receptor, and iron is stored within the storage protein, ferritin^[69]. Macrophages phagocytose senescent blood cells, and recycle the iron back to plasma. In iron-loading disorders, although hepatocytes remain the dominant site of iron deposition, KC can also store iron^[70]. As mentioned earlier, the interplay between hepatocytes, KC and HSC and other extracellular proteins in the liver is important in the progression of hepatic fibrosis, and can be influenced by iron. Iron, as a key player in the oxidative reaction, may contribute directly and indirectly to HSC activation although the precise pathways remain

unclear (Figure 2).

Indirect activation of HSC: Hepatocytes and KC are the main cells where iron-induced oxidative damage occurred as described earlier, i.e. lipid peroxidation and activation of proapoptotic/necrotic signalling. It is proposed that HSC activation occurs as a result of soluble factors (TGF- α / β 1, TNF α , MIP-1, IL-6) produced by injured hepatocytes or KC following oxidative stress^[71-73]. Indeed, the gene expression of two markers of HSC activation, collagen type I and α SMA, is increased in studies where HSC are incubated with conditioned medium from iron loaded hepatocytes^[72,74]. Collagen upregulation was also observed when HSC were co-cultured with hepatocytes expressing CYP2E1, which is the principal cytochrome involved in the oxidation of ethanol when the concentration of ethanol is elevated^[74]. This effect was even more pronounced in the presence of iron, and was prevented by several antioxidants suggesting a oxidation mediated upregulation of these profibrogenic genes^[74].

KC have been shown to play a role in HSC activation. When KC and HSC are co-cultured, HSC proliferation is increased along with HSC expression of α SMA and collagen type I, when compared to the culture of HSC

alone^[73]. Friedman and Arthur demonstrated that KC conditioned medium activated HSC and stimulated HSC proliferation^[75]. After exposure to several stimuli such as LPS, ethanol, fatty acid, HCV infection or the phagocytosis of injured hepatocytes, KC become activated and produced ROS *via* PKC dependant activation of NADPH oxidase and *via* the CYP1E2^[55,59,76,77]. Deugnier and colleagues observed KC containing phagocytosed hepatocellular debris in hemochromatosis liver tissue^[78]. Tsukamoto and colleagues demonstrated that haeme-derived iron primed hepatic macrophages for NF- κ B activation, and enhanced the expression of the pro-inflammatory genes TNF α and MIP-1^[79]. TNF α is known to play a key role in promoting KC activation^[80], and also prevents the HSC from undergoing apoptosis^[81] thereby promoting fibrosis.

In addition to the production of profibrogenic and proinflammatory cytokines that activate HSC, KC release iron-loaded tissue ferritin that may interact with the ferritin receptor on activated HSC^[82]. Furthermore, hepatocyte and KC-generated ROS can be released from the cell, and enhance the perpetuation of HSC activation^[83]. In contrast, the role of the toxic by-products of lipid peroxidation such as MDA and 4HNE has been studied by Olynyk and others who showed that these compounds did not directly activate HSC^[84,85].

Direct activation of HSC: Activated HSC express a specific receptor for H-ferritin^[86] which appears to regulate the expression of α SMA^[82]. Ruddell and colleagues have preliminary evidence which suggest that ferritin upregulates genes involved in HSC activation via a PKC ζ dependent pathway^[87]. In addition, activated HSC are also known to express a transferrin receptor which can enhance the expression of α SMA and collagen type I within these cells^[88]. The role of free iron in the induction of HSC activation has been studied by Gardi and colleagues who demonstrated that iron can stimulate type I collagen gene expression but this was not mediated by lipid peroxidation^[89]. This is supported by other studies which rule out the involvement of intracellular lipid peroxidation in the activation of HSC^[84,85,90].

However, in other studies, intracellular oxidative stress has been shown to induce collagen expression in HSC, and that HSC lipid peroxidation can be triggered by hepatocytes^[91,92]. A recent study by Zhan and colleagues supports the role of lipid peroxidation in HSC in the upregulation of procollagen expression^[93]. They showed that HSC are able to phagocytose apoptotic bodies (i.e. injured hepatocytes), which triggers the activation of the stellate cells NADPH oxidase and its production of superoxide within and outside the cell. They also showed that the upregulation of procollagen I following the phagocytosis of apoptotic bodies was NADPH oxidase-dependent.

In conclusion, iron may have a direct effect on the activation of HSC or *via* the modulation of intracellular oxidative stress. However, this may be a minor role in the activation of the HSC when you consider that hepatocytes are the main cells that take up iron, and that quiescent HSC do not express the transferrin or ferritin

receptors^[86,88]. The inherent ability of iron to catalyse the production ROS in hepatocytes and KC appears likely the main mechanism by which it is able to influence hepatic disease progression. Iron, in conjunction with other factors, may overwhelm the liver in terms of its innate ability to cope with oxidative stress thereby causing disease.

Iron and other oxidation-related molecules

As described in Figure 1, iron is a key player in oxidative reactions that produced toxic ROS. However, the cellular level of other molecules, such as H₂O₂ and unsaturated lipids, also influence the degree of oxidation that occurs in the cells and thereby the degree of fibrosis. Indeed, the role of iron along with alcohol, steatosis and HCV in exacerbating liver injury has been the subject of fairly intensive study. The effects of feeding rats a diet supplemented with iron and alcohol was found to enhance hepatic localisation of MDA and 4HNE as well as increase serum levels of ALT and AST to two-fold that of rats fed ethanol alone^[94]. Both liver tissues and HSC isolated from rats fed ethanol and iron were also found to express elevated levels of procollagen α_1 (I) and TGF β 1 mRNA compared to those fed ethanol alone^[94]. Kato and colleagues also suggested TGF α derived from alcohol exposed hepatocytes may also contribute to hepatic fibrosis in ALD^[95]. Ethanol is known to upregulate the expression of CYP2E1^[74] which would exacerbate the potential oxidative stress exerted by iron in hepatocytes due to the catalysis of more ROS. In the same manner, accumulation of fatty acid within the cells will increase oxidative damage due to the enhanced peroxidation of lipid.

HCV alone has been shown to increase ROS generation and also lipid peroxidation in hepatocytes^[96]. It does this *via* the core protein, which is able to alter mitochondrial Ca²⁺ uptake, and also it induces endoplasmic reticulum (ER) stress and enhances ER to mitochondria Ca²⁺ transfer^[97]. Iron overload is also able to induce mitochondrial dysfunction (as visualised by ultrastructural alterations), and in mice over expressing the HCV core polyprotein, iron increases the risk of those mice going on to develop HCC^[98].

CONCLUSION

Iron plays an integral part in the progression of hepatic fibrosis, and it does this via its ability to catalyse the formation of highly reactive and damaging ROS. The damage done by ROS includes lipid peroxidation, protein and DNA modification leading ultimately to apoptosis and necrosis. However, the literature also indicates that co-factors such as steatosis, ethanol and HCV infection contribute to iron-induced hepatic injury. Antioxidant therapies have been tested in clinical trials with limited success with regards to the prevention and reversal of liver fibrosis. Iron depletion therapies have also been used with varying degrees of success to treat all four diseases of the liver outlined in this review. Continued investigation into the molecular mechanisms of iron toxicity, and how it leads to hepatic fibrosis may give a better understanding of the role of iron as a cofactor in the

progression of liver disease leading ultimately to novel anti-fibrotic therapies.

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Pathology of hepatic iron overload

Yves Deugnier, Bruno Turlin

Yves Deugnier, Liver Unit and CIC INSERM 0203, Pontchaillou University Hospital, Rennes 35033, France
Bruno Turlin, Department of Pathology and INSERM U 522, Pontchaillou University Hospital, Rennes 35033, France
Correspondence to: Yves Deugnier, Professor of Hepatology, Liver Unit and CIC INSERM 0203, Pontchaillou University Hospital, Rennes 35033, France. yves.deugnier@univ-rennes1.fr
Telephone: +33-2-99284297 Fax: +33-2-99284112
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Abstract

Although progress in imaging and genetics allow for a noninvasive diagnosis of most cases of genetic iron overload, liver pathology remains often useful (1) to assess prognosis by grading fibrosis and seeking for associated lesions and (2) to guide the etiological diagnosis, especially when no molecular marker is available. Then, the type of liver siderosis (parenchymal, mesenchymal or mixed) and its distribution throughout the lobule and the liver are useful means for suggesting its etiology: HLA-linked hemochromatosis gene (HFE) hemochromatosis or other rare genetic hemochromatosis, nonhemochromatotic genetic iron overload (ferroportin disease, aceruloplasminemia), or iron overload secondary to excessive iron supply, inflammatory syndrome, noncirrhotic chronic liver diseases including dysmetabolic iron overload syndrome, cirrhosis, and blood disorders.

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Key words: Iron; Liver; Biopsy; Hemochromatosis; Ferroportin; HLA-linked hemochromatosis gene; Hfe; Metabolic syndrome

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INTRODUCTION

Progress in molecular genetics and in liver imaging have allowed for the noninvasive diagnosis of most cases of inherited disorders of iron metabolism. However, liver pathology remains often useful to assess associated lesions—especially fibrosis—in patients with HLA linked hemochromatosis gene (HFE) and, mainly, nonHFE hemochromato-

sis, and to guide the etiological diagnosis of iron overload in the absence of a molecular marker. Moreover, because iron is more and more considered as a putative (co) factor of morbidity in patients with chronic liver diseases of various causes, iron excess must be recognized, indicated, qualified, quantified and interpreted even when it seems to be contingent upon a well established hepatic disorder.

ASSERTION OF IRON OVERLOAD

In the normal liver, iron is present at a concentration lower than 20 $\mu\text{mol/g}$ of dry weight^[1]. But, it is not histologically visible. Iron deposits are usually difficult to identify on usual stains unless they are abundant. Therefore, every liver biopsy should be routinely stained using not only hematoxylin-eosin-safron and connective stains, but also iron stain. Because it is easier and more reproducible than the Tirmann-Schmeltzer's method, Perl's staining is the most widely used, despite, it identifies Fe^{2+} only^[2].

DESCRIPTION OF IRON OVERLOAD^[2,3]

Distribution

The cellular distribution of iron deposits within hepatocytes, sinusoidal and portal macrophages, sinusoidal and portal endothelial cells, and biliary cells must be precisely described according to lobular areas in order to differentiate the following types of liver siderosis.

Parenchymal iron overload: It is related to intestinal iron hyperabsorption. Then, because it comes to the liver through the portal vein, iron deposits within hepatocytes as fine granules at the biliary pole of cells, and is distributed throughout the lobule according to a decreasing gradient from periportal to centrolobular areas. Mesenchymal iron deposits may be found at a latter stage when the amount of hepatocytic iron is high and responsible for sideronecrosis.

Mesenchymal iron overload: It corresponds to iron deposition within Kupffer cells and/or portal macrophages. Iron loaded cells are either isolated or grouped together without any lobular systematization. When associated, hepatocytic iron deposits are rough, sparse and usually located within cells close to iron loaded macrophages.

Mixed iron overload: It presents with the histological characteristics of the previous two types and corresponds usually to complex conditions or to massive iron loading.

Table 1 Histological grading of iron storage^[2]

Grade	Ease of observation and magnification required
0	Granules absent or barely discernible at × 400
1	Granules barely discernible at × 250 and easily confirmed at × 250
2	Discrete granules resolved at × 100
3	Discrete granules resolved at × 25
4	Masses visible at × 10, or naked eye

Table 2 Histological grades of iron storage from Deugnier Y and Turlin B^[3-12]

Hepatocytic iron	0, 3, 6, 9 or 12	HIS
	According granules size	0-36
	In each Rappaport area	
Sinusoidal iron	0, 1, 2, 3 or 4	SIS
	According granules size	0-12
	In each Rappaport area	
Portal iron	0, 1, 2, 3 or 4	PIS
	According to % of iron overloaded macrophages, biliary cells, and vascular walls	0-12
Total iron score		0-60

HIS: hepatocytic iron score; SIS: sinusoidal iron score; PIS: portal iron score.

It is also important to precise whether iron distribution throughout the liver is homogeneous or not, i.e. whether lobules (or, in case of cirrhosis, nodules) are equally iron loaded or not^[4,5].

Quantification

Biochemical hepatic iron concentration: Irrespective of the method used (colorimetry or atomic absorption), the biochemical determination of hepatic iron concentration (HIC) is considered as the reference method for quantifying iron in the liver^[1,6]. In normal subjects, HIC ranges from 10 to 36 μmol/g of dry weight. Iron excess is considered as mild up to 150, moderate between 150 and 300, and important above 300. Cases with HIC greater than 1000 are exceptionnal. Results obtained from fresh tissue and from deparaffinized blocks are equivalent^[7]. Therefore, determination of HIC on deparaffinized tissue should be the rule because it allows for histological control. This is especially relevant when iron distribution is heterogeneous as in the cirrhotic liver^[4,5].

Histological semiquantitative assessment: Several scoring systems have been proposed. The Scheuer's scoring system, either in its original presentation^[8] or modified according to Rowe *et al*^[9] or to Searle *et al*^[2] is widely used because it is simple (Table 1). However, it was not satisfactorily validated. The system proposed by the authors (Table 2) was well validated in both hemochromatotic^[10,11] and nonhemochromatotic iron overload disorders^[12]. But, it remains mainly used for research purposes due to its relative complexity.

ETIOLOGICAL DIAGNOSIS

Both the type of iron overload and associated hepatic

Table 3 Main causes of hepatic iron overload according to the histological type of siderosis and associated lesions

Parenchymal iron overload
With normal liver
Early genetic hemochromatosis
Nontransfused dysmyelopoiesis
Hereditary aceruloplasminemia
With cirrhosis
Iron overload secondary to cirrhosis
Mixed iron overload
With normal liver
Insulin resistance syndrome
Ferroportin disease
Transfused dysmyelopoiesis
Oral or parenteral iron supplementation
With steatosis or steatohepatitis
Insulin resistance syndrome
Chronic alcoholism
With chronic hepatitis
Hepatitis C or B
Wilson's disease
With intrahepatocytic inclusions
Porphyria cutanea tarda
With cirrhosis
Late genetic hemochromatosis
Mesenchymal iron overload
With normal liver
Inflammatory syndrome
Repeated transfusions

lesions may guide towards the right etiology (Table 3).

Genetic iron overload

Genetic hemochromatosis: Genetic hemochromatosis^[3] corresponds to 4 disorders transmitted as autosomal recessive traits, two with late onset (adult type: HFE hemochromatosis and iron overload related to mutation on the receptor transferrin 2 gene) and two with early onset (juvenile type related to mutations on the hemojuvelin or the hepcidin gene). From a histological point of view, these disorders are very similar because of a common pathophysiology consisting in an impairment of hepcidin production whose degree modulates the severity of iron burden^[13].

In early GH, iron remains located within hepatocytes, at the biliary pole of cells. It is distributed according to a decreasing gradient from periportal to centrilobular areas. This results in a typical parenchymal iron overload pattern.

With the passage of time, hepatocytic iron load increases, and then periportal sideronecrosis occurs. Sideronecrosis is responsible for macrophage activation, which leads to both development of fibrosis, and redistribution of iron towards nonparenchymal cells. In the absence of other cause of chronic liver disease, cirrhosis develops when hepatic iron concentration exceeds 400 μmol/g. GH related cirrhosis consists of large fibrous septa resembling biliary cirrhosis, while preserving the vascular architecture of the liver for a long time. This likely explains why portal hypertension and hepatic failure are rare features in GH patients. According to series, 25% to 50% of GH patients are still diagnosed at the cirrhotic stage.

Liver cancer is a frequent complication in GH^[10]. Most cases are hepatocellular carcinomas (HCC) developed in

a cirrhotic liver. However, some cases of HCC have been reported in GH patients with no cirrhosis, and frequency of cholangiocarcinoma reports is increasing^[14]. Two types of preneoplastic lesions have been reported and should be sought for at histological examination: (1) Iron-free-foci^[15] consist of sublobular nodular clusters of hepatocytes devoid of iron or with a low iron content within an otherwise iron-overloaded liver. Most often, they exhibit a proliferative pattern with either large or small cell dysplasia in 50% of cases. More than half the patients with IFF on their initial liver biopsy will develop. (2) Von Meyenburg complexes have also been reported as abnormally numerous in the surrounding liver of patients with GH complicated with cholangiocarcinoma^[14].

Nonhemochromatotic genetic iron overload

The ferroportin disease: The ferroportin disease^[16] is a dominant hereditary iron overload disorder characterized by phenotypic variability. In most cases, iron deposits are found within macrophages (mesenchymal type) with no significant fibrosis. This corresponds to the classical asymptomatic form with elevated hyperferritinemia contrasting with normal or mildly increased transferrin saturation. Rarely, iron is predominantly located within parenchymal cells, and the histological picture is similar to that of genetic hemochromatosis with, in some cases, either severe fibrosis or cirrhosis. Then, transferrin saturation is usually markedly elevated.

Hereditary aceruloplasminemia: Hereditary aceruloplasminemia^[17,18] is an exceptional disease transmitted as a recessive trait. Under the lens, iron is found predominantly in parenchymal cells. No case of liver cirrhosis has been described even in the most iron overloaded cases.

African iron overload : African iron overload^[19] is a rare disorder characterized by a mixed hepatic iron overload frequently complicated with cirrhosis. It is related to excessive iron intake, and likely underlain by nonHFE genetic factors.

Excessive iron supply

When brought parenterally: (i.e. through multiple transfusions), iron is initially localized within Kupffer cells and portal macrophages. With time, it is usually redistributed towards surrounding parenchymal cells, which results in a mixed and heterogenous pattern.

In case of excessive chronic iron intake: mixed hepatic iron overload may develop as reported in elite road cyclists^[20].

Inflammatory syndrome

It is a frequent cause of mesenchymal hepatic siderosis related to a defect of iron release from Kupffer cells due to increased production of hepcidin^[13]. Iron deposits are usually sparse and distributed throughout the lobule.

Noncirrhotic chronic liver diseases.

Dysmetabolic iron overload syndrome (DIOS): DIOS

is a frequent condition corresponding to the association of an unexplained hepatic iron overload with usually normal transferrin saturation and features of metabolic syndrome^[21,22]. The histological pattern of DIOS is mixed, both mesenchymal (throughout the entire lobule) and parenchymal (predominating in periportal area)^[23]. Iron excess is usually mild and averages 100 $\mu\text{mol/g}$. However, in 30% of cases, the hepatic iron concentration to age ratio exceeds 2, a threshold previously considered as highly suggestive of GH before the discovery of the HFE gene. Either steatosis or steatohepatitis is present in 50% of cases. Bridging fibrosis or cirrhosis is found in 12% of cases. Whether iron may be involved in the development of fibrosis in DIOS patients, and its removal may be beneficial remains debated.

Alcoholic liver disease: Mixed and mild hepatic siderosis is found in 5% to 20% of chronic alcoholics, even in the absence of cirrhosis. A direct effect of alcohol on hepcidin production could be involved^[24].

Chronic hepatitis: Hepatic iron deposition is found in 35% to 56% of patients with chronic hepatitis. This was especially demonstrated in patients with chronic hepatitis C. The histological pattern is usually mesenchymal with frequent iron deposits in endothelial cells. Iron excess has been shown to be correlated with necrotico-inflammatory changes, and to decrease after interferon therapy. Moreover, iron removal before or at the time of interferon therapy could result in histological improvement, even in nonresponders^[25].

Wilson disease: Mixed iron overload is frequently found in the liver of patients with Wilson disease. Its mechanism is likely multifactorial, and involves low serum ceruloplasmin levels, hemolysis, necrotico-inflammatory changes and cirrhosis.

Cirrhosis: Significant liver siderosis is found in 35 to 78% of patients with end-stage cirrhosis, irrespective of the cause of cirrhosis^[4,5]. Iron deposits are mainly located within hepatocytes, predominate in remaining periportal areas, and, in some cases, can mimic GH. However, liver siderosis secondary to cirrhosis is distributed very heterogeneously from a nodule to another and absent from fibrous septa, biliary cells and vascular walls. This usually allows for correct diagnosis, and points to the need, in case of cirrhosis, for interpreting hepatic iron concentration according to histological findings. It is likely that nontransferrin bound iron (NTBI) plays a key role in the development of iron overload in cirrhosis^[26]. Indeed, in severe cirrhosis, serum transferrin levels are low due to hepatic failure which results in increased saturation of transferrin and, then, in the appearance of NTBI, a special form of iron that is avidly taken up by hepatocytes.

Hepatocellular carcinoma: Parenchymal or mixed iron overload is frequently present in the nontumorous part of the liver of patients with hepatocellular carcinoma (HCC), whether they have cirrhosis or not^[27]. This supports the

(co)carcinogenic role of iron suggested by numerous experimental and epidemiological studies^[28].

Blood disorders

Porphyria cutanea tarda: Mixed, heterogeneous and mild hepatic siderosis is encountered in 60% to 70% of patients with porphyria cutanea tarda (PCT). Searching for intrahepatocytic inclusions when faced with mixed iron overload is suitable, because the clinical diagnosis of PCT may be missed.

Dyserythroipoiesis: In well compensated dyserythropoietic syndromes, intestinal iron absorption is increased secondary to impairment in iron incorporation into red cell precursors. With time, severe hepatic iron overload resembling GH may develop, even in the absence of blood transfusions. Once transfusions are required, iron deposits in both parenchymal and mesenchymal cells resulting in a mixed pattern.

PLACE OF LIVER BIOPSY IN THE MANAGEMENT OF IRON OVERLOAD DISORDERS

Recent progress in both imaging and genetics have resulted in reducing the role of liver biopsy in the diagnosis of hepatic iron overload.

Positive diagnosis: MRI allows for a specific and sensitive detection and reliable quantification of excessive hepatic iron content when comprised between twice the upper limit of normal and 300 $\mu\text{mol/g}$ dry weight^[29]. Then, liver biopsy is no longer necessary to ascertain iron overload.

Aetiological diagnosis: MRI also allows for classifying iron excess as parenchymal, mesenchymal or mixed according to organ iron deposition (liver and/or spleen and/or spine)^[30]. Then it provides guidance for the aetiological diagnosis process, especially with respect to the first choice of genotyping tests. This was recently well illustrated by Pietrangelo *et al*^[31] in patients with ferroportin mutations. Moreover, in these patients, MRI contributed to establish phenotypic/genotypic correlations, and to understand the pathophysiology of the disease by demonstrating, beside the classical mixed pattern of iron accumulation, a non-classical parenchymal pattern related to the N144H ferroportin mutation^[31].

Disease severity: Once the positive and aetiological diagnosis of hepatic iron overload has been made, it is mandatory to assess the degree of hepatic damage, that is to determine whether severe fibrosis has developed or not. This will remain the major goal of liver biopsy as long as noninvasive tests for fibrosis-including biochemical markers, and elastometry-are not validated in patients with iron overload syndromes.

Currently, indication of liver biopsy can be discussed according to the phenotypic presentation of iron excess.

In case of hemochromatotic phenotype (= increased transferrin saturation with parenchymal iron deposition),

performing liver biopsy depends on HFE genotyping:

In C282Y homozygotes, liver biopsy is no longer necessary for diagnosis, but remains suitable with respect to prognosis. Guyader *et al*^[32] demonstrated that, when the liver was not clinically enlarged AND serum ferritin level was lower than 1000 ng/mL AND serum AST level was normal, there was never significant liver fibrosis (i.e. grade 3 or 4 fibrosis according to the METAVIR scoring system). On the contrary, when one, two or all these conditions were not met, there was a significant risk of fibrosis calculated as $1/(1 + \exp[-(-6.7620 + 3.2934 \text{AST}_{(\text{iu/l})} + 0.0013 \text{ferritin}_{(\text{ng/ml})} + 2.5317 \text{hepatomegaly}_{(0:1})])$. Accuracy of Guyader's algorithm was further validated in Canadian patients^[32]. Since then, other equations of prediction of (non)fibrosis have been proposed^[33,34] based upon age and serum ferritin level or serum ferritin level, serum AST level and platelets count. But, either they were not further correctly validated or they were not as simple for clinical use as Guyader's algorithm. Then, currently there is a global consensus to perform liver biopsy for fibrosis evaluation in C282Y homozygotes with either increased liver size, serum ferritin level higher than 1000 ng/mL or abnormal serum AST levels except when the diagnosis of cirrhosis is clinically obvious or when the predictive equation gives a risk close to 100%. Recently, Powell *et al*^[35] showed that obesity-related steatosis may have a role as a cofactor in liver injury-especially fibrosis-in C282Y homozygotes. This has clinically important implications, but does not modify indications of liver biopsy in these patients.

In a C282Y-H63D compound heterozygote presenting with mild increase in transferrin saturation-usually comprised between 45% and 60% and in serum ferritin-usually < 500 ng/mL and with no biochemical abnormalities and clinical liver symptoms, it can be reasonably assumed that the HFE genotype is responsible for the abnormalities in iron metabolism^[36], and that the patient is free of risk of fibrosis. Then, liver biopsy is not necessary.

In all other cases, the diagnostic procedure must be conducted irrespective of HFE genotype. Indeed, C282Y and H63D heterozygosity as well as H63D homozygosity not only are frequent (up to 1/3 of subjects in European general populations), but do not result, in a given subject, in clinically relevant perturbances of iron metabolism even if large genotyping studies^[36-39] have shown that some of them induced slight but significant increase in serum ferritin and/or transferrin saturation. Then, liver biopsy remains suitable to search for an additional cause of either iron overload or chronic liver disease. The most frequent finding is heterogeneous parenchymal iron overload complicating alcoholic or viral liver cirrhosis^[4,5,40]. Much more rarely, liver biopsy discovers marked iron overload suggesting an associated mutation on another gene involved in iron metabolism. In that case, the precise description of iron deposition, and associated lesions may participate in defining the choice of diagnostic molecular tests: mesenchymal or mixed iron deposition with no significant fibrosis is suggestive of ferroportin disease (which sometimes presents with TS > 60%) while parenchymal iron overload suggests the diagnostic

of juvenile haemochromatosis in a young adult with usually severe fibrosis (mutation on the hemojuvelin or hepcidin gene) and that of transferrin receptor 2-related haemochromatosis in an adult with or without fibrosis.

In the absence of hemochromatotic phenotype (= low, normal or slightly elevated transferrin saturation), the question is whether increased serum ferritin levels are related to iron overload or not. MRI can replace liver biopsy to answer this question, and histological examination of the liver can be restricted to patients with significant iron deposition at MRI (i.e. hepatic iron concentration > 100 $\mu\text{mol/g}$ dry weight) and/or elevated serum transaminase levels and/or abnormal noninvasive predictive tests of fibrosis^[41]. In such a situation, the most frequent finding is mild and mixed iron overload with either metabolic or alcoholic steatohepatitis or chronic hepatitis C or porphyria cutanea tarda. Much more rarely, histological examination reveals marked iron overload with no significant fibrosis corresponding to ferroportin disease (mesenchymal type-normal or slightly increased transferrin saturation) or to hereditary aceruloplasminemia (parenchymal type-low transferrin saturation).

Due to the widespread use of genotyping, of MRI and of noninvasive predictive markers of hepatic fibrosis, liver biopsy is less and less performed for diagnostic and prognostic purposes in C282Y homozygous patients. Conversely, it remains often necessary in other patients in order to guide the etiological diagnosis of hepatic iron overload by describing and semi-quantifying iron excess and by assessing associated lesions.

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Inhibition of histone deacetylase for the treatment of biliary tract cancer: A new effective pharmacological approach

Thilo Bluethner, Manuel Niederhagen, Karel Caca, Frederik Serr, Helmut Witzigmann, Christian Moebius, Joachim Mossner, Marcus Wiedmann

Thilo Bluethner, Joachim Mossner, Marcus Wiedmann, Department of Internal Medicine II, University of Leipzig, Philipp-Rosenthal-Str. 27, Leipzig 04103, Germany
Frederik Serr, Helmut Witzigmann, Christian Moebius, Department of Surgery II, University of Leipzig, Liebigstrasse 20a, 04103 Leipzig, Germany
Manuel Niederhagen, Institute of Pathology, University of Leipzig, Liebigstr. 26, Leipzig 04103, Germany
Karel Caca, Department of Internal Medicine I, Klinikum Ludwigsburg, Posilipstr. 4, Ludwigsburg 71640, Germany
Supported by the Deutsche Krebshilfe, No. 10-2106-Wi I
Correspondence to: Dr Marcus Wiedmann, Department of Internal Medicine II, University of Leipzig, Philipp-Rosenthal-Str. 27, 04103 Leipzig, Germany. wiedm@medizin.uni-leipzig.de
Telephone: +49-341-9712230 Fax: +49-341-9712239
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significant *in vivo* activity and potentiated the efficacy of gemcitabine. Therefore, further clinical evaluation of this new drug for the treatment of biliary tract cancer is recommended.

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Key words: Histone deacetylase inhibitor; Biliary tract cancer; Cholangiocarcinoma; NVP-LAQ824; NVP-LBH589

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Abstract

AIM: To investigate *in vitro* and *in vivo* therapeutic effects of histone deacetylase inhibitors NVP-LAQ824 and NVP-LBH589 on biliary tract cancer.

METHODS: Cell growth inhibition by NVP-LAQ824 and NVP-LBH589 was studied *in vitro* in 7 human biliary tract cancer cell lines by MTT assay. In addition, the anti-tumoral effect of NVP-LBH589 was studied in a chimeric mouse model. Anti-tumoral drug mechanism was assessed by immunoblotting for acH4 and p21^{WAF-1/CIP-1}, PARP assay, cell cycle analysis, TUNEL assay, and immunohistochemistry for MIB-1.

RESULTS: *In vitro* treatment with both compounds significantly suppressed the growth of all cancer cell lines [mean IC₅₀ (3 d) 0.11 and 0.05 µmol/L, respectively], and was associated with hyperacetylation of nucleosomal histone H4, increased expression of p21^{WAF-1/CIP-1}, induction of apoptosis (PARP cleavage), and cell cycle arrest at G2/M checkpoint. After 28 d, NVP-LBH589 significantly reduced tumor mass by 66% (bile duct cancer) and 87% (gallbladder cancer) *in vivo* in comparison to placebo, and potentiated the efficacy of gemcitabine. Further analysis of the tumor specimens revealed increased apoptosis by TUNEL assay and reduced cell proliferation (MIB-1).

CONCLUSION: Our findings suggest that NVP-LBH589 and NVP-LAQ824 are active against human biliary tract cancer *in vitro*. In addition, NVP-LBH589 demonstrated

INTRODUCTION

Interactions between histones and DNA are regulated by the acetylation status of histones, which in eukaryotic cells, plays a pivotal role in chromatin remodeling and in the regulation of gene expression: hyperacetylation determines transcription activation while hypoacetylation transcription repression. The balance between two classes of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), can affect the acetylation status of histones. Altered HAT or HDAC activity has been identified in several cancers^[1]. HDACs have been found to be associated with aberrant transcription factors, and can mediate the function of oncogenic translocation products in specific forms of leukemia and lymphoma. They are divided into three classes: class I (HDAC 1-3, 8, 11) is generally nuclear; class II (HDAC 4-7, 9, 10) is generally tissue-dependent for the expression, and can shuttle between the cytoplasm and the nucleus; and class III requires nicotinamide adenine dinucleotide (NAD) as cofactor with substrate; they were discovered recently and are poorly characterized^[1]. To date, several structural unrelated classes of HDAC-inhibitors (HDACIs) demonstrating anti-tumor activity both *in vitro* and *in vivo* in animal models have been identified. These classes include carboxylic acids such as phenylbutyrate (PB), phenylacetate (PA), sodium butyrate (SB), AN-9 (Pivanex) and valproic acid; cyclic tetrapeptides such as trapoxin A; cyclic peptides such as depsipeptide or FK-228 and apicidine; benzamides such as MS27-275 and CI-994

(*N*-acetyldinaline); ketones such as trifluoromethyl ketone and α -ketomides; hydroxamic acids such as trichostatin A (TSA), suberoylanilide hydroxamic acid (vorinostat, SAHA), azelaic bis-hydroxamic acid (ABHA), scriptaid, oxamflatin, pyroxamide, *m*-carboxycinnamic acid bis-hydroxamide (CBHA), and the recently developed NVP-LAQ824, NVP-LBH589, and PXD101^[2]. Multiple phase I, II clinical trials are either completed or currently ongoing with several HDACIs, either as single agent or in combination with conventional chemotherapy, or biologicals. Clinical studies published so far have shown that HDACIs can be administered safely in humans and that treatment of some cancers with such agents seems to be beneficial^[3]. NVP-LAQ824 and NVP-LBH589 are a new chemical entity belonging to a structurally novel class of cinnamic hydroxamic acid compounds^[4-6] which are currently in phase I clinical evaluation in advanced refractory solid tumors and hematologic malignancies^[7-12]. However, little is known about their potential efficacy in biliary tract cancer, a rare tumor with a grim prognosis and up to now only limited treatment options. Therefore, the objectives of the current study were to investigate the efficacy of *in vitro* and *in vivo* treatment with the two novel pan-HDACIs NVP-LAQ824 and NVP-LBH589 and to evaluate the combination with gemcitabine.

MATERIALS AND METHODS

Materials

Seven biliary tract cancer cell lines - five extra-hepatic bile duct cancer cell lines (EGI-1, TFK-1, CC-SW-1, CC-LP-1, and SK-ChA-1)^[13-17] and two gallbladder cancer cell lines (Mz-ChA-1, Mz-ChA-2)^[16] were examined. All cell lines were cultured with appropriate media and incubated at 37°C in a humidified atmosphere containing 50-100 mL/L CO₂ in air, and the media were changed every three days. The HDACIs NVP-LAQ824 and NVP-LBH589 were provided by Novartis (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) (10 mmol/L stock). Hoechst dye, sodium butyrate and monoclonal (mc) β -actin antibody were purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), mc p21^{WAF-1/Cip-1}, and polyclonal (pc) cleaved-poly(ADP-ribose) polymerase (PARP) antibodies from Cell Signaling (Cell Signaling Technology, Beverly, USA), mc acH4 antibody from Upstate (Upstate Biotechnology, Lake Placid, USA), mc MIB-1 antibody from Dako (Glostrup, Denmark), and gemcitabine [diluted in 50 g/L dextrose in water (D5W) and 50 mL/L DMSO] and etoposide (dissolved in normal saline to 10 mmol/L stock) from our hospital pharmacy. Six to eight-week-old female athymic NMRI nude mice were supplied from Taconic (Taconic Europe, Ry, Denmark) and held under pathogen-free conditions. Humane care was administered, and study protocols complied with the Institutional Guidelines.

Inhibition of cell growth detected by MTT assay

Cytotoxic effects of both drugs were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldridge Chemie GmbH Munich, Germany) assay. About 1.5×10^3 cells were seeded in

triplicate in 96-well plates (100 μ L/well) and allowed to attach overnight. The medium was then replaced with medium (100 μ L) containing the designated drug or vehicle control (50 mL/L DMSO in D5W), followed by an incubation for 3 or 6 d. For the 6-d experiment, medium was changed after 3 d. Three hours before the end of the incubation period, 10 μ L of phosphate-buffered solution (PBS) containing 5 g/L MTT was added to each well. Following this, the medium was removed. The precipitate was then resuspended in 100 μ L of lysis buffer (DMSO, 100 g/L SDS). The absorbance was measured on a plate reader at 590 nm and a reference wavelength of 630 nm. Each experiment was performed in triplicate.

Immunoblotting

Cell culture monolayers were washed twice with ice-cold PBS, and lysed with RIPA-buffer containing Tris-HCl (50 mmol/L, pH 7.4), NP-40 (10 g/L), sodium-desoxycholate (2.5 g/L), NaCl (150 mmol/L), EDTA (1 mmol/L), sodium-orthovanadate (1 mmol/L), and one tablet of complete mini-EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany) (in 10 mL buffer). Histones for anti-acH4 immunoblotting were isolated by acid extraction [cells were lysed in ice-cold lysis buffer (HEPES 10 mmol/L; pH 7.9), MgCl₂ (1.5 mmol/L), KCl (10 mmol/L), DTT (0.5 mmol/L), PMSF (1.5 mmol/L), and additional protease inhibitor]. One molar HCl was added to a final concentration of 0.2 mol/L, followed by incubation on ice for 30 min, and centrifugation at 13000 *g* for 10 min. The supernatant was kept and dialysed against 200 mL of 0.2 mol/L acidic acid twice for 1 h and against 200 mL of H₂O overnight. Proteins were quantified by Bradford protein assay (Bio-Rad, Munich, Germany) and stored at -80°C, and 50 μ g of cell or tissue lysates were separated on SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution [50 g/L dry milk in 10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20 (TBS-T)], followed by incubation with the primary antibody at 4°C overnight (50 g/L BSA in TBS-T). The membranes were then washed in TBS-T, and incubated with horseradish peroxidase (HRPO)-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemoluminescence reaction (SuperSignal West Dura, Pierce, Rockford, USA).

Cell cycle analysis

Cells (2×10^5) were seeded in T-25 flasks, treated with various concentrations of NVP-LAQ824 or NVP-LBH589 or vehicle control (50 mL/L DMSO in D5W) for 72 h, washed with PBS, trypsinized, centrifuged, and fixed in 750 mL/L ice-cold ethanol/phosphate-buffered saline containing 10 g/L EDTA. DNA was labelled with 100 mL/L propidium iodide. Cells were sorted by FACScan analysis, and cell cycle profiles were determined using ModFitLT V2.0 software (Becton Dickinson, San Diego, USA). Each experiment was performed in triplicate.

Animal studies

Tumors were induced by injecting 5×10^6 Mz-ChA-2 or

EGI-1 cells in 200 μ L of PBS subcutaneously into the flank region of NMRI nude mice. Treatment was started when an average tumor volume of 150 mm³ was reached (usually after 2 wk). The verum groups intraperitoneally received either NVP-LBH589 (40 mg/kg, 5 \times weekly) or gemcitabine (5 mg/kg, 2 \times weekly) or a combination of both (NVP-LBH589 at 20 mg/kg, 5 \times weekly plus gemcitabine at 5 mg/kg, 2 \times weekly), whereas the control group received placebo (carrier solution 50 mL/L DMSO in D5W) only. Treatment was continued for 28 consecutive days, tumors were daily measured with a Vernier caliper and tumor volumes were calculated using the formula: tumor volume = $0.5 \times L \times W^2$, where L represents the length and W the width of the tumor. When treatment was finished, animals were sacrificed and tumors were excised and weighed.

TUNEL POD test

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (*In Situ* Cell Death Detection Kit, POD) was used to detect apoptosis in paraffin sections from mouse tumor tissue. TUNEL test was carried out following the manufacturer's instructions (Roche, Penzberg, Germany) as previously described^[18]. Apoptotic cells (red) were counted under a light microscope after fluorescence signal conversion using antibody with conjugated peroxidase and the substrate for peroxidase (DAB, Roche, Penzberg, Germany). The number of positive cells was counted by an experienced pathologist in a total of 8 high-power fields (HPFs) and expressed as mean percentage of total cells in these fields of the tumor. Necrotic tumor cells were excluded from cell count.

Immunohistochemical staining

For MIB-1 staining, we used paraffin sections following a protocol that has been described elsewhere^[19]. The number of positive cells was counted in a total of 4 HPFs and expressed as mean percentage of total cells in these fields of the tumor.

Statistical analysis

Statistical calculations were performed using SPSS version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean \pm SD or SEM. Inter-group comparisons were performed using Student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Inhibition of cellular growth by NVP-LAQ824 and NVP-LBH589

After 3 d of incubation, all tested cell lines were sensitive to NVP-LAQ824 [mean IC₅₀ (3 d) = 0.11 ± 0.06 μ mol/L] and even more to NVP-LBH589 [mean IC₅₀ (3 d) = 0.05 ± 0.05 μ mol/L]. There was no significant difference in response between the group of extra-hepatic bile duct cancer cell lines [mean IC_{50/LAQ824} (3 d) = 0.10 ± 0.07 μ mol/L and mean IC_{50/LBH589} (3 d) = 0.05 ± 0.06 μ mol/L] and the group of the two gallbladder cancer cell lines [mean IC_{50/LAQ824} (3 d) = 0.12 ± 0.03 μ mol/L and mean IC_{50/LBH589} (3 d) = 0.06 ± 0.05 μ mol/L].

Inhibition of cell growth was more pronounced if incubation time was extended to 6 d, with a mean IC₅₀ value of 0.05 ± 0.02 μ mol/L for NVP-LAQ824 and 0.02 ± 0.01 μ mol/L for NVP-LBH589. Once again, there was equal response between the group of extra-hepatic bile duct cancer cell lines [mean IC_{50/LAQ824} (6 d) = 0.05 ± 0.02 μ mol/L and mean IC_{50/LBH589} (6 d) = 0.01 ± 0.01 μ mol/L] and the group of the two gallbladder cancer cell lines [mean IC_{50/LAQ824} (6 d) = 0.06 ± 0.03 μ mol/L and mean IC_{50/LBH589} (6 d) = 0.02 ± 0.02 μ mol/L] (Figure 1 and Table 1). In addition, DMSO, the solvent of NVP-LAQ824 and NVP-LBH589, alone had no influence on cell growth (data not shown).

Mechanism of drug action

Treatment of cell lines EGI-1 and Mz-ChA-2 with 0.1 μ mol/L NVP-LAQ824 or 0.1 μ mol/L NVP-LBH589 for 24 h resulted in acetylation of histone H4 (Figure 2A). Protein extract from HELA cells that were treated with 5 mmol/L sodium butyrate served as positive control. The same treatment caused an induction of p21^{WAF-1/CIP-1} expression (Figure 2B). Cell lysate from HEK 293 cells served as positive control. A dose increase to 0.2 μ mol/L NVP-LAQ824 or 0.2 μ mol/L NVP-LBH589 corresponded with an increase of p21^{WAF-1/CIP-1} levels. Histone H4 acetylation was higher in treated Mz-ChA-2 than EGI-1 cells, whereas p21^{WAF-1/CIP-1} expression was higher in treated EGI-1 cells. Immunoblotting for cleaved-PARP was positive when cells were treated with 0.1 μ mol/L NVP-LAQ824 or 0.1 μ mol/L NVP-LBH589 for 24 h (Figure 2C, Lanes 2 and 5). PARP cleavage was more pronounced when the concentration of NVP-LAQ824 or NVP-LBH589 was increased to 0.2 μ mol/L (Figure 2C, Lanes 3 and 6). Lysate from untreated NIH-3T3 cells (Figure 2C, Lane 7) and from cells that were treated with 25 μ mol/L etoposide for 5 h [Figure 2C, Lane 8 (lane *)] served as negative control. Lysate from NIH-3T3 cells that were treated with 10 μ mol/L etoposide for 24 h [Figure 2C, Lane 9 (lane **)] served as positive control. Positive and negative controls were selected according to the recommendations of the manufacturers of antibodies. Staining with β -actin antibody confirmed equal protein loading in all immunoblots.

Treatment of cell lines EGI-1 and Mz-ChA-2 with 25 nmol/L NVP-LAQ824 or 25 nmol/L NVP-LBH589 for 72 h resulted in G2/M phase arrest. This arrest was more pronounced if the dose of NVP-LAQ824 or NVP-LBH589 was increased to 50 nmol/L. For both concentrations, the effect of NVP-LBH589 was stronger than the effect of NVP-LAQ824 (Table 2). Further increase of the NVP-LAQ824 and NVP-LBH589 concentration to 100 nmol/L and 200 nmol/L led to no further significant changes in G2/M phase arrest (data not shown). Sub-G1-peak percentage was not analyzed.

Inhibition of tumor cell growth by NVP-LBH589 \pm gemcitabine in nude mice

Tumors were induced in nude mice by subcutaneous injection of Mz-ChA-2 and EGI-1 cell lines. These cell lines were selected because they had shown the best growth capability in nude mice in our previous studies^[20,21].

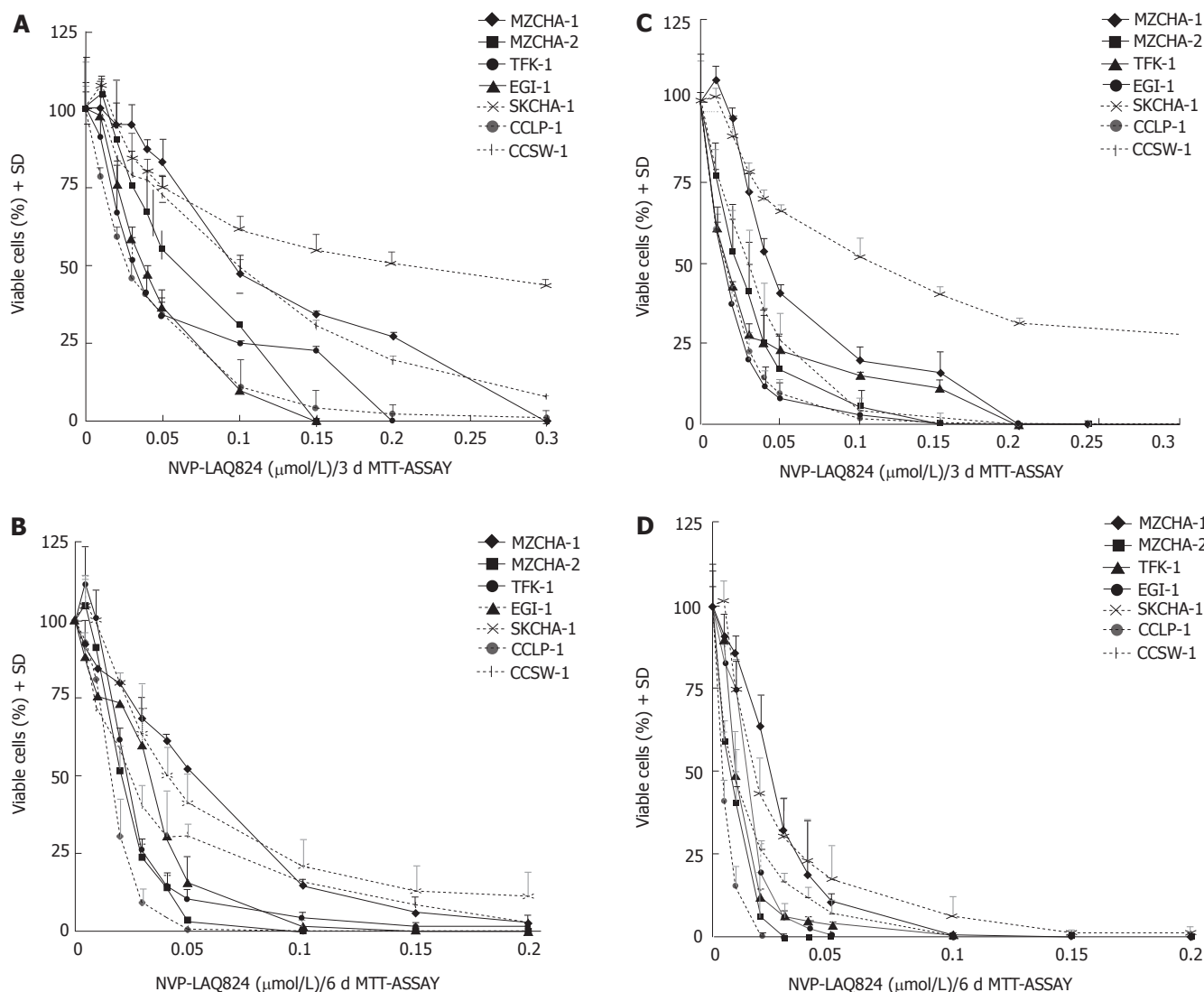


Figure 1 *In vitro* treatment of biliary tract cancer cell lines with NVP-LAQ824 and NVP-LBH589. **A:** 3-d incubation with NVP-LAQ824 ($n = 3$); **B:** 6-d incubation with NVP-LAQ824 ($n = 3$); **C:** 3-d incubation with NVP-LBH589 ($n = 3$); **D:** 6-d incubation with NVP-LBH589 ($n = 3$).

Table 1 Inhibition of cell growth by NVP-LAQ824 and NVP-LBH589

Cell line	IC ₅₀ (μmol/L)			
	NVP-LAQ824		NVP-LBH589	
	3 d	6 d	3 d	6 d
TFK-1	0.06	0.05	0.01	0.01
EGI-1	0.07	0.05	0.01	0.01
CC-LP-1	0.05	0.02	0.01	0.01
CC-SW-1	0.13	0.05	0.04	0.01
Sk-ChA-1	0.21	0.08	0.15	0.04
Mz-ChA-1	0.14	0.07	0.09	0.04
Mz-ChA-2	0.10	0.04	0.02	0.01

Since NVP-LBH589 seemed to have a higher *in vitro* activity than NVP-LAQ824 in these two cell lines (Table 1), only NVP-LBH589 was investigated *in vivo* in order to save costs. Treatment of mice consisted of intraperitoneal injections with NVP-LBH589, gemcitabine, NVP-LBH589 plus gemcitabine (COMBO) or placebo (50 mL/L DMSO in D5W). Starting at d 19, respectively at d 26, of the

experiment, EGI-1 cell tumors showed a significantly reduced volume in comparison to control after treatment with NVP-LBH589 or COMBO ($n = 7$ for each group; $P < 0.05$). The statistically significant difference was maintained until the end of the experiment. In contrast to that, treatment of mice with gemcitabine alone did not result in any significant reduction of tumor growth compared to control (Figure 3A). Treatment of Mz-ChA-2 tumors with NVP-LBH589, COMBO, or gemcitabine resulted in a significantly reduced volume in comparison to control at d 5, 5, and 8 ($P < 0.01$), respectively ($n = 7$ for each group; Figure 3B). The statistically significant difference was maintained until the end of the experiment. Regarding tumor latency (representing the time for the tumor to increase to 150% of the initial volume when treatment was initiated), the extrapolated data were as follows for EGI-1: placebo d 6, LBH d 26, COMBO d 12, and gemcitabine d 4. The data for Mz-ChA-2 were: placebo d 3, LBH d 14, COMBO "not reached", and gemcitabine d 7. At the end of the experiment at d 29, tumor mass in EGI-1 cells-bearing mice was significantly diminished as compared to placebo after

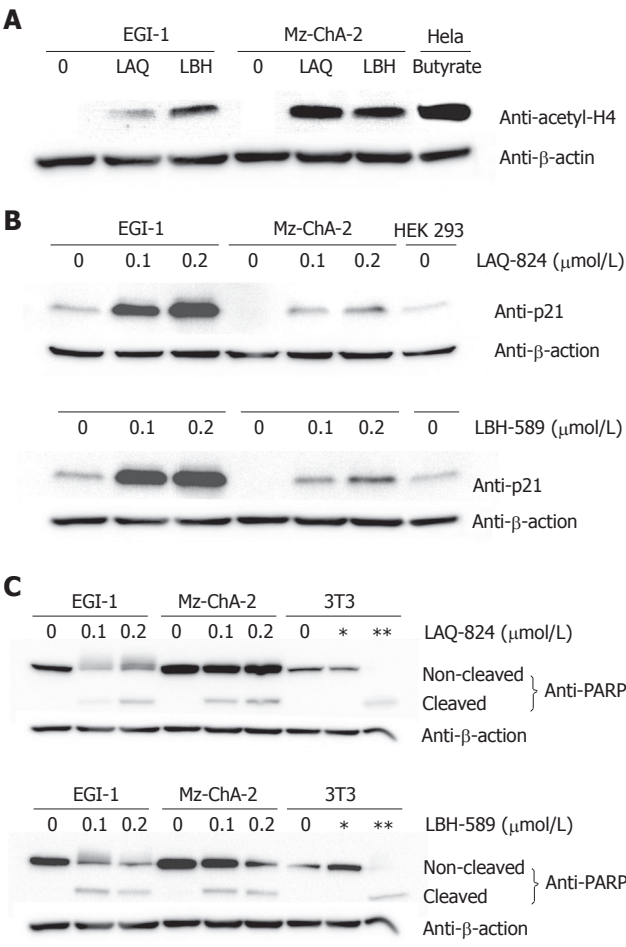


Figure 2 Mechanism of drug action after *in vitro* treatment with NVP-LAQ824 and NVP-LBH589 for 24 h. Lane *: NIH-3T3 cells treated with 25 $\mu\text{mol/L}$ etoposide for 5 h (negative control); lane **: NIH-3T3 cells treated with 10 $\mu\text{mol/L}$ etoposide for 24 h (positive control). **A**: acetylation of histone H4; **B**: p21^{WAF-1/CIP-1} expression; **C**: PARP cleavage.

Table 2 Cell cycle analysis (mean \pm SD, %)								
	MZ-CHA-2			EGI-1				
	G0/G1	S	G2/M	G0/G1	S	G2/M		
CTRL	38.3 \pm 3	44.7 \pm 3	17.0 \pm 3	58.7 \pm 5	29.2 \pm 2	12.1 \pm 1		
LAQ824 (25 nmol/L)	37.0 \pm 1	42.8 \pm 2	20.1 \pm 2	50.1 \pm 7	31.9 \pm 4	18.1 \pm 3		
LAQ824 (50 nmol/L)	36.9 \pm 2	38.7 \pm 5	24.4 \pm 4	41.2 \pm 2	34.8 \pm 2	24.1 \pm 4		
LBH589 (25 nmol/L)	35.5 \pm 1	27.6 \pm 3	36.9 \pm 2	38.8 \pm 5	33.8 \pm 3	27.4 \pm 4		
LBH589 (50 nmol/L)	38.8 \pm 2	9.0 \pm 3	52.3 \pm 2	46.0 \pm 1	15.1 \pm 2	38.9 \pm 1		

treatment with NVP-LBH589 (-66%) or COMBO (63%) ($P < 0.05$; Figure 3C). In contrast to that, treatment of mice with gemcitabine alone (-6%) did not result in any significant reduction of tumor mass as compared to control (Figure 3C). For Mz-ChA-2 cells-bearing mice, tumor mass was significantly diminished after treatment with either NVP-LBH589 (-87%), COMBO (96%), or gemcitabine (-74%), respectively ($P < 0.01$; Figure 3C). For both cell lines, EGI-1 and Mz-ChA-2, the combination of NVP-LBH589 and gemcitabine was more effective in tumor mass reduction in comparison to gemcitabine alone ($P < 0.05$ and $P < 0.01$, respectively). Moreover, for tumor

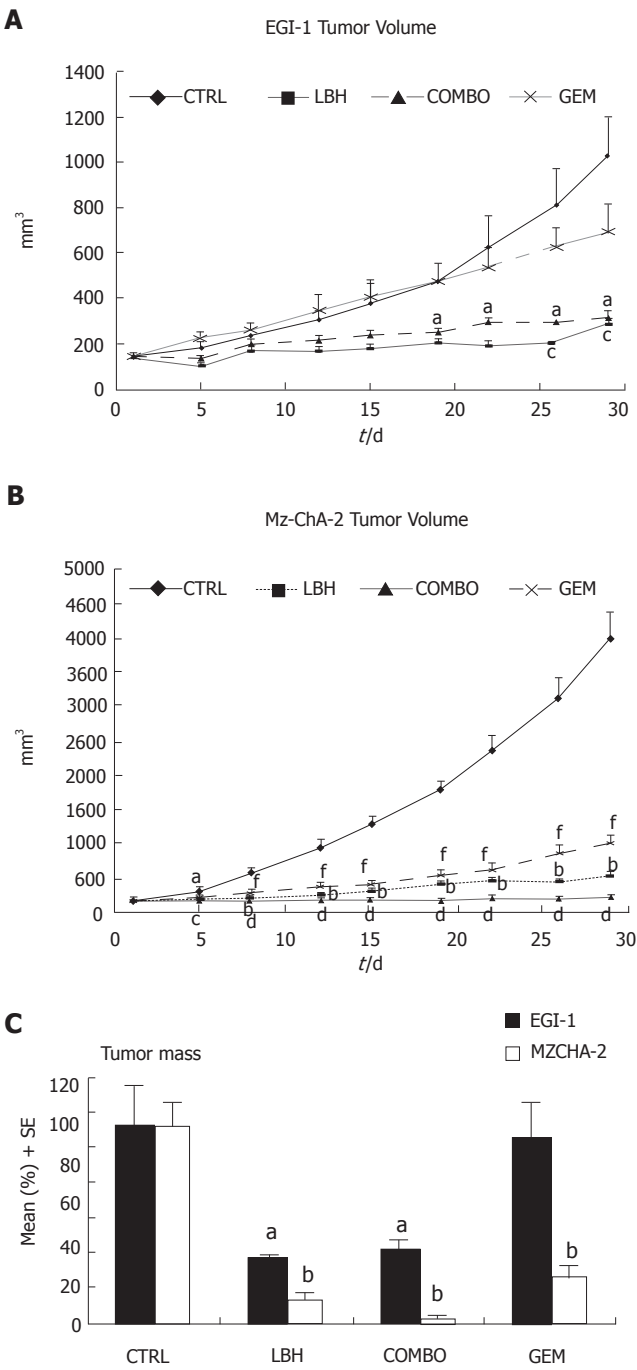


Figure 3 *In vivo* treatment with NVP-LBH589 \pm gemcitabine in chimeric mice. **A**: Effect on tumor volume of EGI-1 cells ($^aP < 0.05$, NVP-LBH589 vs control; $^cP < 0.05$, COMBO vs control); **B**: Effect on tumor volume of Mz-ChA-2 cells ($^aP < 0.05$, $^bP < 0.01$, NVP-LBH589 vs control; $^cP < 0.05$, $^dP < 0.01$, COMBO vs control; $^eP < 0.01$, gemcitabine vs control); **C**: Effect on tumor mass ($^aP < 0.05$, NVP-LBH589 or COMBO vs control; $^bP < 0.01$, NVP-LBH589 or COMBO or gemcitabine vs control).

cell line Mz-ChA-2, COMBO reduced tumor mass at a higher degree than NVP-LBH589 alone ($P < 0.05$), although NVP-LBH589 was administered at half dose in the COMBO group. Concerning side effects of the different drugs used in our experiments with EGI-1 and Mz-ChA-2 cells tumor-bearing mice, NVP-LBH589 alone (-17% and -19%, respectively) and COMBO (-7% and -10%, respectively) induced weight loss in the animals at d 29 of therapy as compared to the initial body weight, whereas

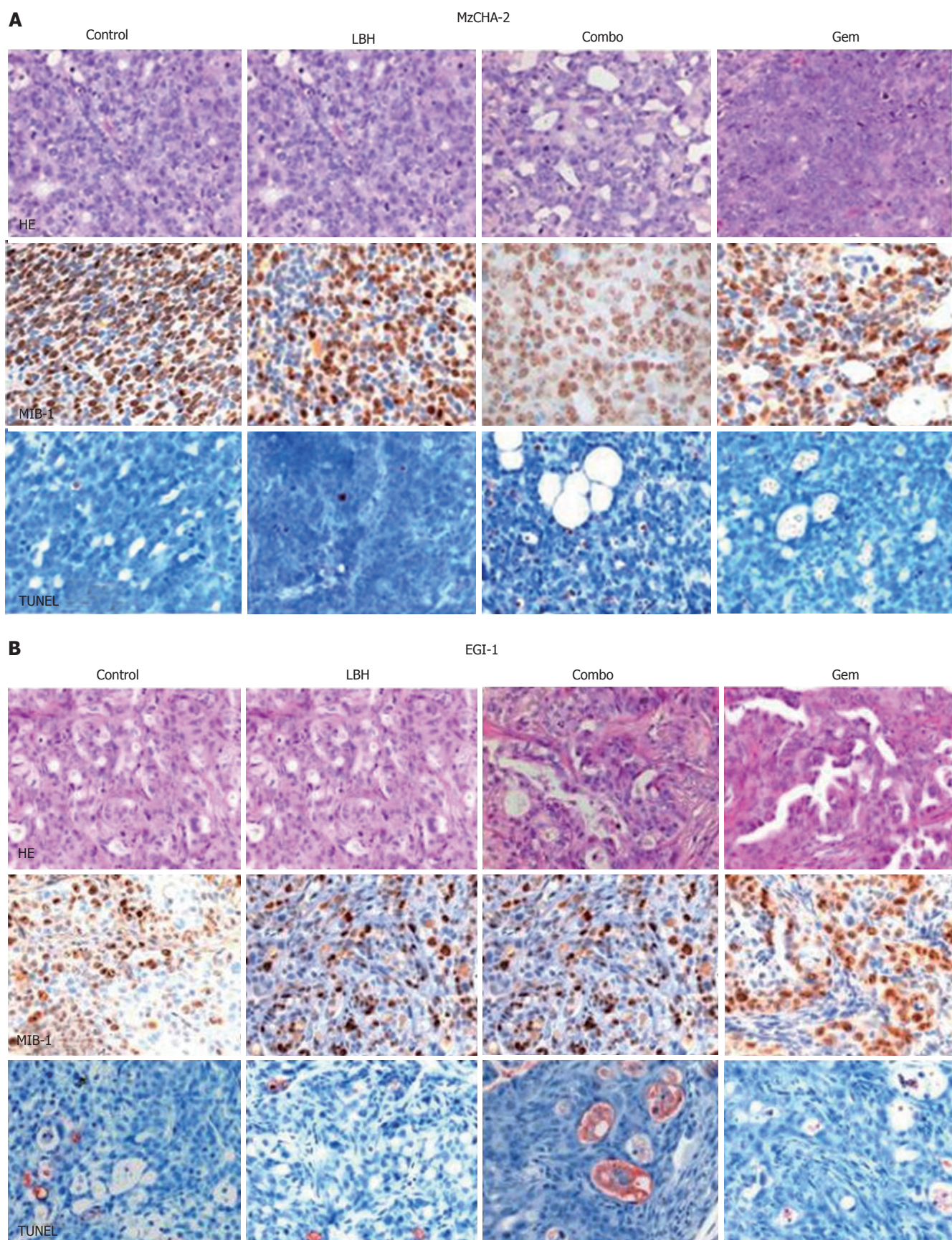


Figure 4 Hematoxylin-eosin (HE), MIB-1 (proliferation marker) and TUNEL (apoptosis marker) staining of mouse tumors (SABC, $\times 40$). **A:** cell line Mz-ChA-2; **B:** cell line EGI-1.

gemcitabine alone did not. One animal died at d 12 (EGI-1 experiment) in the COMBO group, and 2 animals died at d 22 and d 26 (Mz-ChA-2 experiment) in the NVP-

LBH589 group from significant weight loss. These three mice were replaced by back-up mice in order to guarantee equal numbers in the different groups ($n = 7$) for the

Table 3 MIB-1- and TUNEL-staining of mouse tumor specimens (mean, %)

	MZ-CHA-2		EGI-1	
	MIB-1	Apoptosis	MIB-1	Apoptosis
CTRL	85	0.1	38	4.3
GEM	77	2.2	29	6.5
LBH	62	2.9	26	5.9
COMBO	67	7.8	17	9.0

comparisons of tumor volume and tumor mass at the end of the experiment. Finally, NVP-LBH589 alone at a dose of 40 mg/kg (5 × weekly) caused diarrhea and exsiccation in the animals starting at d 16 of the experiment. We performed no further microscopic evaluation for organ damage in these animals. However, there was no macroscopic tissue damage.

In order to assess the anti-tumoral drug mechanism, paraffin sections of mouse tumors were stained with hematoxylin-eosin (HE), MIB-1 (proliferation marker) and TUNEL (apoptosis marker) (Figure 4). Treatment with NVP-LBH589, gemcitabine, and COMBO reduced proliferation (reduced MIB-1 staining) and induced apoptosis (increased TUNEL staining) (Figure 4, Table 3).

DISCUSSION

Cholangiocarcinoma (CC) is the second most common primary hepatic tumor, with increasing incidence and a high mortality^[22-24]. Tumors may occur anywhere along the biliary ductal system, and are arbitrarily defined anatomically as intra-hepatic or extra-hepatic CC as well as adenocarcinoma of the gallbladder^[25,26]. Unfortunately, the vast majority of patients with CC typically seek treatment with advanced disease, and often these patients are deemed poor candidates for curative surgery. Moreover, conventional chemotherapy and radiation therapy have not been shown to be effective in prolonging long-term survival, and although photodynamic therapy combined with stenting has been reported to be effective as palliative treatment, it is not curative^[27-30]. Thus, there is a need to develop novel therapeutic strategies for CC based on exploiting selected molecular targets that would have an impact on clinical outcome^[31,32].

One possible approach may be the use of HDACIs, perhaps in combination with conventional chemotherapy or other so called biologicals. Therefore, in our current study, we investigated the two novel cinnamic hydroxamic acid compounds NVP-LAQ824 and NVP-LBH589 for *in vitro* and *in vivo* treatment of CC. Cell-growth inhibition by NVP-LAQ824 and NVP-LBH589 was studied *in vitro* in 7 human biliary tract cancer cell lines by MTT assay. Treatment with the both compounds significantly suppressed the growth of all cancer cell lines after 3 and 6 d with a mean IC₅₀ (3d) of 0.11 and 0.05 μmol/L, and with a mean IC₅₀ (6d) of 0.05 and 0.01 μmol/L, respectively. In previous *in vitro* studies, NVP-LAQ824 exhibited a potent anti-proliferative activity against the HCT116 colon carcinoma cell line (IC₅₀ = 0.01 μmol/L), as well as against H1299 (IC₅₀ = 0.15 μmol/L) and A549 non-small cell

lung carcinoma cells (IC₅₀ = 0.15 μmol/L), DU145 (IC₅₀ = 0.018 μmol/L), PC3 prostate cancer cell lines (IC₅₀ = 0.023 μmol/L), Cal27 (IC₅₀ = 0.04 μmol/L), SCC25 (IC₅₀ = 0.095 μmol/L), SCC9 (IC₅₀ = 0.245 μmol/L), FaDu (IC₅₀ = 0.340 μmol/L) head and neck squamous carcinoma cells, and MDA-MB-435 (IC₅₀ = 0.039 μmol/L) and BT-474 (0.03 μmol/L) human breast adenocarcinoma cells after 72 h of exposure^[6,33,34]. The *in vitro* effects of NVP-LAQ824 on hematologic malignancies have been examined in several human cell lines, including AML (HL60, K562), lymphoma (Namalwa, DHL10), and multiple myeloma (IM9, 8226). Death of more than 90% of all cells was seen in all cell lines following 48 h of drug incubation, with exposures as low as 0.1 μmol/L^[35-37]. NVP-LBH589, the second investigated compound, was even more effective *in vitro* for the treatment of human chronic myeloid leukemia blast crisis K562 and LAMA-84, multiple myeloma, and acute leukemia MV4-11 cells^[5,38-40].

The *in vitro* anti-tumoral drug mechanism in our study was assessed by immunoblotting for acH4 and p21^{WAF-1/CIP-1}, PARP assay, and cell cycle analysis. Treatment with both compounds was associated with hyperacetylation of nucleosomal histone H4, increased expression of p21^{WAF-1/CIP-1}, induction of PARP cleavage, and cell cycle arrest at G2/M checkpoint. Hyperacetylation of nucleosomal histones H3 and H4 has been previously reported as the primary mechanism of action of NVP-LAQ824 and NVP-LBH589 for the treatment of HCT116, A549, MDA-MB-231, BT-474, MCF-7 (human breast adenocarcinoma), HL-60, K562, MV4-11, and multiple myeloma cells^[5,6,34,35,41]. Inactivation of tumor suppressor p14/mdm-2/p53/p21^{WAF-1/CIP-1} signaling pathway is a phenomenon that occurs frequently in CC, mostly due to p53 mutations or by up-regulation of mdm-2, an inhibitor of p53^[42]. P21^{WAF-1/CIP-1} binds to the cell division kinase (CDK) 4:cyclin D complex and prevents it from phosphorylating Rb. Therefore, the release of the bound E2F transcription factor that regulates genes encoding for proteins critical to entrance into the S-phase of the cell cycle is prevented^[25]. Up-regulation of p21^{WAF-1/CIP-1} induced by NVP-LAQ824 and NVP-LBH589 has been described for BT-474, MCF-7, MDA-MB-468 (human breast adenocarcinoma), human umbilical vein endothelial cells (HUVEC), LNCaP (prostate cancer), K562, LAMA-84, MV4-11 and multiple myeloma cells^[5,34-36,38,43,44]. P21^{WAF-1/CIP-1} is likely to be transcriptionally activated by a p53-independent mechanism, since it has been shown for NVP-LAQ824 to activate the p21^{WAF-1/CIP-1} promoter^[6]. Other commonly induced tumor suppressor genes include p53, p57 and the CDK inhibitor p27^{kip1}^[34,36,38,40].

Another mechanism of *in vitro* drug action found in our study was induction of apoptosis as shown by the increase of PARP cleavage, a process mediated by active effector caspase-3. This phenomenon, induced by NVP-LAQ824 and NVP-LBH589, has been described previously for several other cell lines^[5,34-36,39,43]. Furthermore, transcriptional up-regulation of pro-apoptotic genes such as *FAS*, *TRAIL*, *BID* or *BAX* and down-regulation of anti-apoptotic genes such as *BCL-XL* or *FLIP*, although not examined in our study, may explain apoptotic cell death induced by HDACIs, besides priming of malignant cells

for innate immune effector mechanisms^[36,45-49]. Whereas a number of different mechanisms were described for HDACi-induced cell death, including the death receptor-activated caspase cascade, usually the intrinsic apoptotic pathway, mediated by mitochondrial membrane disruption, is commonly activated, which has also been shown for NVP-LAQ824 and NVP-LBH589^[38,50]. Among several genes repressed by HDACi are cyclin D1 and thymidylate synthase. The result is a G1 or G2-M cell cycle arrest and differentiation, common mechanisms of the anti-proliferative effect of HDACi. Further potential growth inhibitory mechanisms include induction of other cell cycle regulatory genes such as GADD45 α and β and up-regulation of transforming growth factor β (TGF- β) receptor signaling, leading to repression of c-myc and cell cycle arrest. Several studies, including ours, investigating NVP-LAQ824 and NVP-LBH589, could demonstrate G2-M cell cycle arrest, induced by those compounds^[6,34,44], others detected G1-arrest^[5,35,36].

Encouraged by our *in vitro* results, we decided to test the most effective drug NVP-LBH589 *in vivo* in comparison to placebo using the chimeric mouse model. At d 29 of the experiment, NVP-LBH589 significantly reduced tumor mass in comparison to placebo and potentiated the efficacy of gemcitabine. The NVP-LBH589 dose of 40 mg/kg (5 d/wk) was selected according to a study testing different intravenous doses of NVP-LAQ824 between 5 and 100 mg/kg (5 d/wk) in a similar chimeric mouse model using the human colon cancer cell line HCT116^[6]. *In vivo* data for NVP-LBH589 were only available after completion of our study, using human prostate carcinoma cell PC-3 xenografts, being able to show tumor reduction at a dose of 10 mg/kg per day^[44]. Surprisingly, weight loss of the animals, as observed in our study, was not previously reported for NVP-LAQ824^[6]. Therefore, further studies are required to examine animals microscopically for organ damage. Unfortunately, this was not done in our experiments. Since we already experienced this phenomenon in a small pilot study with 3 animals (data not shown), we decided to have the dose of NVP-LBH589 for the combination therapy with gemcitabine. The latter drug was selected since it is a commonly used drug for CC monotherapy in the palliative setting^[30]. Even with this dose reduction of NVP-LBH589, our results do strongly support an additive effect which, however, requires further investigation. In human breast cancer cell lines SKBR-3 and BT-474, NVP-LAQ824 also enhanced taxotere-, epirubicin B-, and gemcitabine-induced apoptosis *in vitro*^[34]. For head and neck squamous carcinoma cells, the combination of NVP-LAQ824 with gemcitabine was more effective *in vitro* than a combination with docetaxel, paclitaxel, or cisplatin, especially when used in the sequence of the cytotoxic agent first for 24 h, followed by 48 h of NVP-LAQ824^[33]. There might be also a synergistic *in vitro* effect of a combination of NVP-LAQ824 and VEGFR-inhibitor PTK787/ZK222584 as demonstrated for PC3 and MDA-MB-435 cells^[41]. Similar results were observed *in vitro* for a combination of NVP-LBH589 and proteasome inhibitor bortezomib^[39], NVP-LBH589 and tyrosine kinase inhibitor AMN107^[40], and NVP-LBH589 and heat-shock protein 90 (hsp90) inhibitor

17-allyl-amino-demethoxy geldanamycin (17-AAG)^[5].

Anti-tumoral drug mechanism in our *in vivo* model was assessed by TUNEL assay, and immunohistochemistry for MIB-1, which revealed increased apoptosis (TUNEL) and reduced cell proliferation (MIB-1), confirming our *in vitro* data. Surprisingly, the calculated numbers were much smaller than expected from the *in vitro* experiments.

Finally, it should be mentioned that other mechanisms of anti-tumor activity of HDACi include anti-angiogenic properties through alteration of VEGF-, HIF-1 α -, Ang-2/Tie-2-, and survivin-signaling, down-regulation of endothelial nitric oxide synthase (eNOS), suppression of tumor invasion through negative regulation of matrix metalloproteinases expression, and depletion of several oncoproteins that are normally stabilized by binding to the hsp90 multi-chaperone complexes, including bcr-abl, ErbB2, Raf-1, or AKT (HDACi may induce acetylation of hsp90 *via* inhibition of HDAC 6, thus inhibiting chaperone activity, resulting in polyubiquitination and proteasomal degradation of the hsp90 client proteins), which was not further investigated in our study^[44,51].

In conclusion, our findings suggest that NVP-LBH589 and NVP-LAQ824 are active against human biliary tract cancer *in vitro*. In addition, NVP-LBH589 demonstrated significant *in vivo* activity and potentiated the efficacy of gemcitabine. Therefore, further clinical evaluation of NVP-LBH589 alone or its combination with gemcitabine for the treatment of biliary tract cancer is recommended.

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COMMENTS

Background

Carcinoma of the biliary tree are rare tumors of the gastrointestinal tract with worldwide rising incidence for intrahepatic cholangiocarcinoma during the last several years. At present, complete resection is the only potentially curative therapy. But, most of the patients present with already advanced disease. In the palliative setting, systemic chemotherapy (chemoradiation) has not been clearly proven to prolong survival significantly.

Research frontiers

Receptor tyrosine kinase (TKs), cyclooxygenase-2 (COX-2), mammalian target of rapamycin (m-TOR), and histone deacetylase (HDAC) inhibitors are currently under preclinical and clinical evaluation as new treatment options.

Innovations and breakthroughs

In 2006, the results of a two-stage phase I and a phase II study suggested a therapeutic benefit for EGFR blockade with erlotinib and combined erbB-1/erbB-2 signaling inhibition with GW572016 (Lapatinib) in patients with biliary cancer. In 2007, a phase II study was presented examining the combination of gemcitabine, oxaliplatin and angiogenesis inhibitor bevacizumab (GEMOX-B) in unresectable or metastatic biliary tract cancer/gallbladder cancer. Early evidence of antitumor activity was seen.

Applications

The aim of our study was to investigate the *in vitro* and *in vivo* treatment with histone deacetylase inhibitors NVP-LAQ824 and NVP-LBH589 in biliary tract cancer. Our findings suggested that NVP-LBH589 > NVP-LAQ824 are active

against human biliary tract cancer *in vitro*. In addition, NVP-LBH589 demonstrated significant *in vivo* activity and potentiated the efficacy of gemcitabine.

Terminology

Histones (positively charged proteins) are the major components of chromatin. Histone acetylation and deacetylation modulate chromosome structure and regulate gene transcription. Two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), activate and repress gene expression, respectively. Aberrant HAT or HDAC activity is associated with various epithelial and hematologic cancers. HDACs may play an important role in human oncogenesis through HDAC-mediated gene silencing and interaction of HDACs with proteins involved in tumorigenesis. HDAC inhibition could potentially restore normal processes in transformed cells without affecting normal cells.

Peer review

This manuscript is original, interesting and well-written. It deserves to be published.

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Increasing dietary fiber intake in terms of kiwifruit improves constipation in Chinese patients

Annie On On Chan, Gigi Leung, Teresa Tong, Nina YH Wong

Annie On On Chan, Gigi Leung, Teresa Tong, Nina YH Wong,
Department of Medicine, The University of Hong Kong, Hong Kong, China

Supported by Zespri International Limited, New Zealand
Correspondence to: Annie On On Chan, MD, PhD, Associate Professor, Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong, China. aoochan@hku.hk
Telephone: +852-28553890 Fax: +852-28725828

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Abstract

AIM: To investigate if increased dietary fiber, in terms of kiwifruit, is effective in Chinese constipated patients.

METHODS: 33 constipated patients and 20 healthy volunteers were recruited for a 4-wk treatment of kiwi fruit twice daily. Response during wk 1-4 was defined as an increase in complete spontaneous bowel motion (CSBM) ≥ 1 /wk. Secondary efficacy included response during wk 1-4, individual symptoms and scores of bowel habits and constipation. Responses were compared with the baseline run-in period. Colonic transit time and anorectal manometry were performed before and after treatment.

RESULTS: Responder rate was 54.5% in the constipated group. The mean CSBM increased after treatment (2.2 ± 2.6 vs 4.4 ± 4.6 , $P = 0.013$). There was also improvement in the scores for bothersomeness of constipation ($P = 0.02$), and satisfaction of bowel habit ($P = 0.001$), and decreased in days of laxative used ($P = 0.003$). There was also improvement in transit time ($P = 0.003$) and rectal sensation ($P < 0.05$). However, there was no change in the bowel symptoms or anorectal physiology in the healthy subjects.

CONCLUSION: Increasing dietary fiber intake is effective in relieving chronic constipation in Chinese population.

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Key words: Constipation; Dietary fiber; Kiwifruit; Chinese

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INTRODUCTION

Functional constipation is a common problem in clinical practice. In the Western population, the prevalence was reported to be as high as 24% in elderly persons and more commonly among women^[1]. We observed a prevalence of 14% of constipation in the Hong Kong population^[2].

In mild cases of functional constipation, it has been suggested that the treatment includes general measures like increased intake of water and dietary fiber, and use of simple laxatives. A diet with enough fiber (20-35 g each day) helps form a soft, bulky stool. Sufficient dietary fiber is also needed to promote normality in bowel movement frequency over the long term^[3,4]. Colonic transit has been shown to be related to stool weight and dietary fiber intake^[5,6]. On the other hand, there are only a few studies that looked for dietary fiber intake by patients with chronic constipation^[7-9]. In addition, in a trial with a proprietary fiber product, less than a half of the patients with self-defined constipation responded^[10]. Also, anorectal physiology was not assessed in most of these studies.

Numerous anecdotal reports have suggested that kiwifruit has laxative effects, and suggested it could be an acceptable dietary supplement, especially for the elderly who often experience constipation. It has been reported that the dietary fiber in kiwifruit is about 3.4 g/100 g, thus being a good source of dietary fiber. Rush *et al*^[11] has demonstrated the laxative effect of kiwifruit in the elderly subjects.

In the current study, we aimed at assessing if increasing dietary fiber intake could improve functional constipation. We chose kiwifruit to be the source of dietary fiber in the study. In addition to constipation symptoms, we also assessed if there was any improvement in anorectal physiology after fiber intake.

MATERIALS AND METHODS

Protocol

This is a single center, age and sex-matched, case control study performed from 31 December, 2006 to 31 March, 2007, consisting of a 2-wk baseline assessment and 4-wk treatment period. Both patients and controls were given two Zespri Kiwi fruit (1 in the morning after breakfast and 1 in the evening after dinner) throughout the four-week treatment period. The kiwifruit were of the green, 'Hayward' variety, and were supplied at an optimum ripeness for consumption. The kiwi fruit was dispatched in two batches, the first on d 0 and the second at the begin-

ning of wk 3 to ensure the freshness of the fruit. All the participants were seen by the investigator on d 0 and at the end of wk 4. Bisacodyl was given as rescue medicine and patients were instructed to take it if no bowel motion for ≥ 4 d. The subjects were asked to fill in the diary during the 2-wk baseline period, and throughout the treatment period. Anorectal physiology tests including both colonic transit and anorectal manometry were performed for both the constipated patients and healthy controls before and after the treatment with kiwi fruit. The study was performed in accordance with the Declaration of Helsinki regarding informed patient consent and institutional review board approval.

Patients and controls

Thirty-five age ≥ 18 years constipated subjects were recruited for the study. They were recruited from the Gastroenterology outpatient clinic of Department of Medicine, Queen Mary hospital, Hong Kong, after assessment by a gastroenterologist, and satisfied the following criteria: constipation for 6 mo or more, with an average of less than three complete spontaneous bowel motion (CSBM) per week; and at least one of the following occurring for more than 25% of the time: straining, passage of lumpy or hard stools, and sensation of incomplete evacuation. Complete spontaneous bowel motion referred to the feeling that defecation led to complete passage of stool rather than partial or incomplete evacuation of stool without relief of symptoms. In addition, these patients also satisfied the Chinese constipation questionnaire^[12]. Patients with a previous history of constipation predominant irritable bowel syndrome using the Rome II criteria were excluded from the present study. Additional exclusion criteria were inability to understand Chinese, constipation due to secondary causes, history of malignancy and significant systemic disease. Pregnant or breast feeding women were also excluded. Patients who failed to complete the diary or if the constipation was not confirmed by diary were excluded.

Twenty age and sex matched volunteers were recruited by posters distributed at the university campus and community. All the volunteers were screened and then assessed by the same gastroenterologist. All the subjects will be asked to maintain normal diet pattern and activity during study period.

Assessment

Assessment of constipation symptoms was done in a diary format. Patients recorded their constipation symptoms in a diary throughout the 2-wk baseline and 4-wk treatment period. On a daily basis, they recorded the symptoms of CSBM, straining score (using a 3-point score: no straining/acceptable straining/too much straining), the 7-point Bristol stool scale form (range from 1, separate hard lumps, to 7, watery with no solid pieces)^[13], and the intake of Bisacodyl as rescue medicine. Final assessment included the patients' satisfaction with bowel habits over the past week (using a 5-point ordinal scale, 0 = very satisfied, 4 = not at all satisfied), and the bothersomeness of constipation (using a 5-point ordinal scale, 0 = not at all, 4 = a very great deal).

Assessment of bowel transit time

Normal and slow transit constipation were confirmed by X-ray and colonic motility studies performed in all patients. Colonic transit time was assessed through the use of radiopaque markers, as modified from the method described by Metcalf *et al*^[14] (14). In brief, 4 sets of distinctive radiopaque markers of different shapes and size (circle on d1, semi-cylinder on d 2, dot on d 3 and cylinder on d 4) were ingested by the volunteers on 4 consecutive days. X-ray of the abdomen was taken on d 5 to assess the mouth to anal transit and segmental colon transit. Transit in the right, left, and rectosigmoid colon was calculated by adding all markers seen in these regions on d 5. Slow total colonic transit was defined as > 67 h, the mean transit plus 2 standard deviations averaged from published studies.

Anorectal manometry

The manometry catheter (Zinetics Manometric Catheter, Medtronic) had a latex balloon on its tip that could be distended with air *via* a handheld syringe, and it had 8 perfusion ports spaced 0.5 cm apart beginning 2 cm below the balloon to measure pressures. The catheter was perfused with degassed water at a rate of 0.5 mL/min by a low-compliance pump (Densleeve Manometric infusion pump-16 channel E4500). The outer diameter of the catheter was 4.5 mm. Pressures were recorded and displayed using a model (Polygraph Medtronic Functional testing Software 2.05). Pressure recordings were analyzed manually.

With the patient in the left lateral position, the manometry catheter was lubricated and inserted into the rectum. It was then pulled back in 1-cm steps, and pressures were recorded at each position while the patient was instructed to relax. The peak pressure (averaged across all 8 perfusion ports) defined anal canal resting pressure. The second perfusion port was then positioned in the high-pressure zone of the anal canal, and the rectal balloon was distended with varying volumes of air (10, 20, 30, 40, 50 mL) to determine the smallest volume of distention that elicited a rectoanal inhibitory reflex (RAIR, defined as the reflex decrease in anal canal pressure that is elicited by rectal distention). Next, the rectal balloon was inflated in 20-mL steps up to 200 mL to assess the threshold for the first sensation, sensation of urge to defecate and the maximum tolerable volume. A phosphate enema was administered approximately 30 min before the anorectal manometry and balloon defecation tests.

Statistical analysis

Primary efficacy variable: The primary efficacy variable was the responder rate for CSBM during the first 4 wk of treatment. Patients with a mean increase of CSBM ≥ 1 /wk compared with the last 14 d of baseline were defined as responders, provided that they had completed at least 7 d of treatment.

Secondary efficacy variables: These included the change from baseline in scores for individual constipated symptoms (stool form, straining scores, bothersomeness of constipation, and satisfaction of bowel habit). Days of laxatives used and percentage of patients needed laxatives were assessed.

Table 1 Demographic data and constipation symptoms of the constipated patients and healthy subjects

	Treatment group (<i>n</i> = 33)	Control group (healthy subjects) (<i>n</i> = 22)	<i>P</i> value
Sex (female)	72.70%	80%	0.74
Female to male ratio	2.67:1	04:01	
Ethnicity (Chinese)	100%	100%	
Age (yr \pm SD)	49.9 \pm 12	50.8 \pm 14	0.82
Duration of constipation (yr \pm SD)	20.3 \pm 15	-	
Complete spontaneous bowel motion per week (mean \pm SD)	1.9 \pm 1	-	
Passage of hard stool	72.70%	-	
Incomplete evacuation	84.80%	-	
Straining	90.90%	-	

Tertiary efficacy variables: These included any improvement in anorectal physiology, which included the colonic transit time, simulated defecation pressure and also the rectal sensation.

Statistical analysis was performed using SPSS (SPSS, Chicago, IL, USA) statistical software. Demographics for patients and controls were summarized by calculating means (SD) (or median (range)) for continuous variables (for example, age and severity score) and proportions for categorical variables (for example, sex). Comparisons were performed with the Student's *t* test for continuous variables, and with Chi square test for categorical data. Paired *t* test was used to assess the bowel habit and anorectal physiology parameters before and after treatment within the same group. All statistical tests were two-sided, and *p* value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Demographic data

There were 35 patients screened, but only 33 patients were enrolled into the study. Two of them refused to participate. The demographic data, baseline constipation symptoms of both the patients and controls are summarized in Table 1. There was no difference in the demographic data as they were purposely matched. The anorectal physiology was markedly different between the constipated patients and healthy controls. These include prolonged colonic transit time and impaired rectal sensation.

Primary efficacy

The mean CSBM per week before and after treatment in patients was 2.2 ± 2.6 vs 4.4 ± 4.6 ($P = 0.013$). The responder rate, ie mean increase of CSBM ≥ 1 /wk, was 54.5% (18 patients). Whereas in the controls, the mean CSBM per week before and after treatment was 6.5 ± 1.6 vs 7.1 ± 2.2 ($P = 0.31$). There was no difference in the responder rate between those with slow and normal transit (42.9% vs 63.2% , $P = 0.25$).

Secondary efficacy

The scores for stool form, straining, bothersomeness of constipation, satisfaction of bowel habit, and use of

laxatives before and after treatment are summarized in Table 2. The scores for bothersomeness of constipation and satisfaction of bowel habit were significantly lower after treatment. The number of days of laxatives used, and the percentage of patients needed laxatives as rescue therapy was lower after treatment with Kiwi fruit (Table 2). There was no significant change in bowel habits or satisfaction in the control group after treatment.

Tertiary efficacy

The parameters of anorectal physiology before and after treatment are summarized in Table 3. There was marked decrease in total colonic transit time, especially in the sigmoid-rectal segment, in the constipated patients after treatment. In addition, improvement in rectal sensation, in terms of the first sensation, urge sensation and the maximum tolerable volume and the rectoanal inhibitory reflex, was seen in the constipated patients after treatment. On the contrary, there was almost no significant change in the anorectal physiology parameters in the healthy subjects after treatment.

Safety and side effects

There were no side effects reported in both the patient and control groups. None of them, including the healthy subjects, reported diarrhea.

DISCUSSION

The current study demonstrated the efficacy of increasing dietary fiber in Chinese constipated patients, with significant improvement in complete spontaneous bowel motion, decrease laxatives used, decrease bothersomeness of constipation and improved satisfaction of bowel habit. More importantly, anorectal physiology was shown to improve significantly after treatment.

It has always been suggested that increasing dietary fiber might be helpful in patients with mild constipation. However, there have been few studies done on this area, and few studies assessed the improvement objectively using the criteria of CSBM, as well as anorectal physiology.

It is interesting to note that in this study, improvement was seen not only in the constipation symptoms, but also in terms of colonic transit time and rectal sensation in the constipated patients. It has been reported that gas production from fiber metabolism may limit acceptance, which is particularly true for bran^[15] and other insoluble fibers. Despite most of the fibers in kiwifruit are insoluble fiber, none of the patients or controls reported bloating or gas, or intolerance. Voderholzer *et al*^[10] reported in their study on proprietary fiber in self-defined constipation, only 20% of slow transit patients profited from fiber, whereas more than 80% of patients without identifiable cause of their complaints had a partial or complete improvement. We observed in this study that the responder rate did not differ in both groups of patients. This is probably the first study reporting an improvement in rectal sensation: the first sensation, urge and maximal tolerable volume in constipated patients after increasing fiber intake. Despite the change in anorectal physiology parameters in constipated patients, there was no significant change observed in

Table 2 Secondary efficacy variables before and after the kiwi fruit treatment in the constipated patients and healthy subjects (mean \pm SD)

	Constipated patients			Healthy subjects		
	Before treatment	After treatment	P value	Before treatment	After treatment	P value
Bristol stool scale	3.1 \pm 1.9	3.3 \pm 1.2	0.58	4.0 \pm 0.9	4.2 \pm 0.8	0.94
Straining	1.9 \pm 0.5	1.8 \pm 0.5	0.88	0.3 \pm 0.5	0.4 \pm 0.5	0.97
Bothersomeness of constipation	2.6 \pm 0.9	2.0 \pm 1.1	0.02	0.2 \pm 0.5	0.2 \pm 0.4	1.0
Satisfaction of bowel habit	2.7 \pm 0.9	1.6 \pm 1	0.001	0.3 \pm 0.5	0.2 \pm 0.5	0.97
Number of days taking laxatives (rescue therapy)/wk	2.2 \pm 2.5	0.8 \pm 1.5	0.003	0	0	-
Percentage of patients taking laxatives	60.60%	30.30%	0.013	0	0	-

Table 3 Anorectal physiology parameters before and after the kiwi fruit treatment in the constipated patients and healthy subjects

	Constipated patients			Healthy subjects		
	Before treatment	After treatment	P value	Before treatment	After treatment	P value
Transit time in right segment (h)	11.6 \pm 12	12.6 \pm 12	0.77	4.2 \pm 7	4.1 \pm 6	0.55
Transit time in left segment (h)	23.3 \pm 16	19.6 \pm 13	0.23	6.0 \pm 9	6.7 \pm 8	0.97
Transit time in sigmoid-rectal segment (h)	19.5 \pm 16	7.6 \pm 7	< 0.0001	7.2 \pm 11	3.4 \pm 5	0.045
Total colonic transit time (h)	54.5 \pm 29	39.6 \pm 22	0.003	16.8 \pm 23	14.1 \pm 14	0.23
Rectal pressure (mmHg)	26.3 \pm 18	30.4 \pm 19	0.3	23.6 \pm 19	27.2 \pm 18	0.54
Upper anal canal pressure (mmHg)	51.9 \pm 18	48.9 \pm 29	0.67	49.1 \pm 20	58.4 \pm 30	0.33
Lower anal canal pressure (mmHg)	63.9 \pm 22	67.9 \pm 25	0.44	58.4 \pm 23	74.4 \pm 27	0.025
Simulated Defecation-Push Maneuver (rectum) (mmHg)	65.8 \pm 45	90.8 \pm 55	0.019	72.2 \pm 50	88.2 \pm 50	0.31
Simulated Defecation-Push Maneuver (upper anal canal) (mmHg)	98.6 \pm 43	108.6 \pm 43	0.5	96.1 \pm 46	118.1 \pm 66	0.29
Simulated Defecation-Push maneuver (lower anal canal) (mmHg)	101.3 \pm 47	101.1 \pm 56	1.0	77.8 \pm 53	90.3 \pm 64	0.43
Rectoanal inhibitory reflex (R.A.I.R.) (mL)	17.2 \pm 7	11.3 \pm 4	0.003	12.1 \pm 4	12.2 \pm 4	1.0
First sensation (mL)	39.0 \pm 11	33.1 \pm 9	0.03	30.5 \pm 9	29.4 \pm 6	0.61
Constant sensation/urge (mL)	68.7 \pm 20	60.3 \pm 20	0.049	52.7 \pm 13	50.0 \pm 6	0.46
Maximum tolerable volume (mL)	99.6 \pm 35	85.1 \pm 21	0.031	83.2 \pm 14	78.9 \pm 14	0.07

normal healthy subjects. The reason for the difference in anorectal physiology response in the two groups is still unknown. However, Muller-Lissner in their meta-analysis did show that the improvement in transit time in constipated patients was greater than in healthy subjects^[16].

The current study aimed at assessing the effect of increasing fiber intake in constipated subjects. However, other nutrients that are present in the kiwifruit may also contribute partly to the laxative effect. The whole fruit (minus skin) was consumed, making it difficult to isolate the mechanism. One of the novel compounds in kiwifruit that has been suggested to interact in laxation is actinidin, a proteolytic enzyme belonging to the class of thiol-proteases.

It is important to note that double blinding was impossible in this study or other studies involving dietary fiber. However, the symptoms were compared to those at the baseline, and that patients themselves were their own controls. The additional age and sex matched control group was recruited to assess if there is any side effects or change in anorectal physiology in healthy subjects. The current study thus showed that dietary fiber in terms of kiwifruit is effective in Chinese patients with functional constipation, and with improvement in anorectal physiology. Further studies on the fiber intake in both constipated patients and healthy controls are warranted. In addition, studies may be needed to dissect the individual nutrient in kiwifruit, other than fiber, that may contribute to the laxative effect.

COMMENTS

Background

Dietary fiber has been suggested to improve functional constipation. However, are only a few studies that looked for dietary fiber intake by people with chronic constipation. In addition, in a trial with proprietary fiber product, less than a half of the patients with self-defined constipation responded. Also, anorectal physiology was not assessed in most of these studies.

Research frontiers

Therefore, we investigate if increased dietary fiber, in terms of kiwifruit, is effective in Chinese constipated patients. The anorectal physiology before and after the introduction of kiwifruit was assessed.

Innovations and breakthroughs

We found that responder rate to kiwi fruit was 54.5% in the constipated group. They have improvement in complete spontaneous bowel motion per week, symptom scores for constipation, as well as in colonic transit time.

Applications

Increase dietary fiber may improve functional constipation.

Peer review

This article is novel and provides an insight in the pathophysiology of constipation.

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S- Editor Liu Y L- Editor Alpini GD E- Editor Wang HF

RAPID COMMUNICATION

Distribution of solitary lymph nodes in primary gastric cancer: A retrospective study and clinical implications

Cai-Gang Liu, Ping Lu, Yang Lu, Feng Jin, Hui-Mian Xu, Shu-Bao Wang, Jun-Qing Chen

Cai-Gang Liu, Ping Lu, Yang Lu, Feng Jin, Hui-Mian Xu, Shu-Bao Wang, Jun-Qing Chen, Department of Surgical Oncology, First Affiliated Hospital of China Medical University, Heping, Shenyang 110001, Liaoning Province, China
Supported in part by the Gastric Cancer Laboratory of Chinese Medical University

Correspondence to: Dr. Ping Lu, Department of Surgical Oncology, First Affiliated Hospital of China Medical University, Heping, Shenyang 110001, Liaoning Province, China. luping2999@yahoo.com.cn
Telephone: +86-24-81121999 Fax: +86-24-22834060
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Abstract

AIM: To investigate the distribution pathway of metastatic lymph nodes in gastric carcinoma as a foundation for rational lymphadenectomy.

METHODS: We investigated 173 cases with solitary or single station metastatic lymph nodes (LN) from among 2476 gastric carcinoma patients. The location of metastatic LN, histological type and growth patterns were analyzed retrospectively.

RESULTS: Of 88 solitary node metastases cases, 65 were limited to perigastric nodes (N₁), while 23 showed skipping metastasis. Among 8 tumors in the upper third stomach, 3 involved right paracardial LN (station number: No.1), and one in the greater curvature was found in No.1. In the 28 middle third stomach tumors, 10 were found in LN of the lesser curvature (No.3) and 6 in LN of the left gastric artery (No.7); 5 of the 20 cases on the lesser curvature spread to No.7, while 2 of the 8 on the greater curvature metastasized to LN of the spleen hilum (No.10). Of 52 lower third stomach tumors, 13 involved in No.3 and 19 were detected in inferior pyloric LN (No.6); 9 of the 29 cases along the lesser curvature were involved in No.6.

CONCLUSION: Transversal and skipping metastases of sentinel lymph nodes (SLN) are notable, and rational lymphadenectomy should, therefore, be performed.

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Key words: Gastric cancer; Metastatic lymph node, Lymph node dissection; Rational lymphadenectomy; Sentinel lymph node

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INTRODUCTION

Lymphatic metastasis is the most important factor for prognosis of gastric carcinoma. To avoid missing positive lymph nodes, surgeons have performed an extensive radical lymphadenectomy for gastric cancer, a method which is also used when early tumors are present. As a result, patients that did not have lymph node metastasis have had to undergo operations and face potentially avoidable risks^[1,2].

As the SLN concept gains acceptance, onco-surgery researchers are optimistic that the concept may serve as a breakthrough management tool to be used in gastric cancer; however, the concept is still considered to be at an investigative stage^[3]. We studied the distribution pathway of solitary or positive lymph nodes limited to a single station to provide a foundation for undertaking rational lymphadenectomy for gastric carcinoma.

MATERIALS AND METHODS

Patients

One hundred and seventy-three patients were selected from the 2476 patients with gastric cancer for whom radical operations were performed at the first affiliated hospital of the China Medical University between 1980 and 2003. The criteria used for inclusion was: (1) D2 lymph node dissections had been performed^[4]; (2) there were greater than 15 lymph nodes, and the resected specimens had been analyzed pathologically^[5]; (3) patients with pT4 and M1 stage were excluded^[5]; (4) patients' medical records were complete. Among the 173 cases, 88 had solitary lymph metastasis and 85 involved a single station lymph node. Sixty-four of the 88 patients were male and 24 female. The average age of the patients in this group was 57.6 ± 7.2 years (range 30-80). With respect to tumor location, the tumor was found in the upper third stomach area (U) in 8 cases, in the middle third (M) in 28, and in the lower third (L) in 52. Amongst the 85 patients with

Table 1 Distribution of metastatic lymph nodes (cases)

	L tumors			M tumors			U tumors		
	SN ¹	SSN ²	P value	SN ¹	SSN ²	P value	SN ¹	SSN ²	P value
No.1	2	0	- ³	2	2	NS	3	3	NS
No.2	1	0	-	0	0	-	1	5	NS
No.3	13	11	NS ⁴	10	1	NS	1	6	NS
No.4	4	9	NS	5	1	NS	0	2	NS
No.5	4	3	NS	1	1	-	0	0	-
No.6	19	21	NS	1	2	NS	0	3	NS
No.7	4	5	NS	6	3	NS	1	1	-
No.8	3	1	-	1	0	-	1	0	-
No.9	1	0	-	0	1	-	0	1	-
No.10	0	0	-	2	0	-	0	1	-
No.11	1	0	-	0	0	-	0	1	-
No.12	0	0	-	0	1	-	0	0	-
TOTAL	52	50	-	28	12	-	8	23	-

No.2, left paracardial node; No.4, greater curvature node; No.5, superior pyloric node; No.8, common hepatic node; No.9, celiac artery node; No.11, splenic artery node; No.12, hepatoduodenal node. ¹Solitary metastatic node; ²Single station metastatic node; ³Differences of the frequency distributions between the two groups were not determined; ⁴Not significant.

single station node metastasis, 60 were male and 25 female. The average age of the patients in this group was 58.2 ± 8.3 years (range 32-76). In respect of tumor location; the tumor was in the U in 23 cases, in the M in 12, and in the L stomach areas in 50.

Reference standard

The location of the tumor, the classification of the lymph node and histological type were by the Japanese Classification of Gastric Carcinoma^[6]. For classification, the symbol "No." indicates lymph node station number and "N" indicates the lymph node group. The histological types included differentiated and undifferentiated. An "adjacent metastasis" was defined as when lymph node metastasis is limited to the tumor side of N₁; "transversal metastasis" is limited to the region of N₁ opposite the tumor; and a "skipping metastasis" indicates a lymph node metastasized outside of N₁.

Histological growth patterns included massive, nest, and diffuse types^[7].

Statistical methods

All data were analyzed using SPSS13.0 statistics software. The differences of the frequency distributions between the two groups of lymph nodes were determined by a χ^2 -test or by Fisher's exact test. A χ^2 -test was adopted in the analysis of a single factor and a P value of less than 0.05 was considered statistically significant.

RESULTS

The distribution of metastatic nodes

Among the 88 patients with a solitary metastatic lymph node, in 65 (73.9%) the lymph nodes involved were within N₁, and 23 (26.1%) were over N₁. In 8 cases the tumor was in the U location, amongst which 6 (75%) were observed to be in N₁ and 2 (25%) in N₂. In 28 cases the tumor was in the M region, amongst which 19 (67.9%) were involved

Table 2 Distribution of solitary metastatic lymph nodes according to transversal zoning (cases)

	L tumors			M tumors		U tumors	
	A ¹	B ²	C ³	A ¹	B ²	A ¹	B ²
No.1	0	2	0	1	1	1	2
No.2	0	0	1	0	0	0	1
No.3	3	9	1	0	10	0	1
No.4	2	2	0	3	2	0	1
No.5	1	2	1	0	1	0	0
No.6	7	9	3	1	0	0	0
No.7	1	2	1	1	5	0	1
No.8	0	3	0	0	1	0	1
No.9	1	0	0	0	0	0	0
No.10	0	0	0	2	0	0	0
No.11	0	0	1	0	0	0	0

¹Tumors located at the greater curvature; ²Tumors located at the lesser curvature; ³Tumors extended in a circle of the stomach.

in N₁ and 9 (32.1%) in N₂. Among the 52 patients with L region cancers, 40 were found with solitary metastatic nodes in N₁, 10 (19.2%) with nodes in N₂ and 2 (3.9%) in N₃ (Table 1).

Comparisons were also made between cases with a solitary metastasis lymph node and single station nodes. No statistically significant difference was found with respect to the distribution of metastatic lymph nodes in the U, M and L regions using a χ^2 -test or Fisher's exact test (Table 1).

Adjacent metastasis

In 7 of the 8 cases with a U region tumor, the tumor located at the side of the lesser curvature region. Amongst them, metastatic lymph nodes in 2 (28.6%) were detected within No.1. Among the 28 cases with a tumor in the M area, in 20 the tumor was observed in the lesser curvature region and in 8 in the greater curvature region. Metastatic lymph nodes in 10 of the 20 cases were found within No. 3, while 3 of the 8 were within No. 4.

Fifty-two cases had tumors in the L stomach area. Amongst these the tumors in 29 located at the lesser curvature region, 15 at the greater curvature side and 8 extended in a circle. Metastatic lymph nodes in 9 of the 29 cases were found within No.3, in 7 of the 15 within No.6, and in 3 of the 8 within No. 6 (Table 2 and Figure 1).

Transversal metastasis

There was just one case with a tumor in the U area, and solitary metastatic lymph nodes were found within No.1. Twenty-nine patients had tumors at the lesser curvature side in the L area, and 9 (31%) of them were found to have metastatic lymph nodes within No.6. Of the 15 cases with tumors at the greater curvature side, in the L stomach area, 3 (20%) were involved in No.3 (Table 2 and Figure 1).

Skipping metastasis

Among the 8 cases with tumors in the U area of the lesser curvature region, one solitary metastatic lymph node (14.3% of total) was observed within No.7, and another node (14.3%) was found within No.8. In the M area, 5 (25%) of the 20 patients who had tumors at the lesser curvature

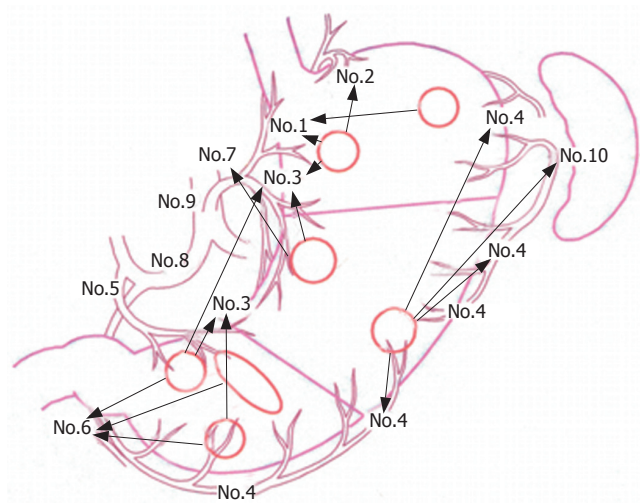


Figure 1 The metastatic pathway of SLN. Tumors are identified by circles and tumors extend in a circle in the lower third stomach.

Table 3 Histological type and skipping metastasis (cases)

	Group N ₁	Group > N ₁	P value
L tumors			0.192
Differentiated	22	4	
Undifferentiated	18	8	
M tumors			0.299
Differentiated	3	3	
Undifferentiated	16	6	
U tumors			1.000
Differentiated	3	1	
Undifferentiated	3	1	
Total			0.489
Differentiated	28	8	
Undifferentiated	37	15	

side had metastatic lymph nodes within No.7, while a metastatic node was detected within No.10 in 2 (25%) of the 8 patients with a tumor in the greater curvature side. Among the 29 cases with a tumor in area L, the number of lymph node metastases involved in No.1, No.7 and No.8 were 2 (6.9%), 2 (6.9%) and 3 (10.3%), respectively (Table 2 and Figure 1).

Relationships among histological type, growth pattern, and distribution pattern of solitary lymph nodes

Comparing the 65 patients with metastasis within N₁ and the 23 with a skipping metastasis, no statistical significance was found between histological type and the growth patterns of the two groups (Tables 3 and 4). Also, there was no significant difference between the adjacent metastasis group (39) and the transversal metastasis group (20) (Tables 5 and 6).

DISCUSSION

The distribution pattern of solitary metastatic nodes

There is little clinical literature available on the lymphatic routes of the stomach^[8-10]. After analyzing 51 cases, Kosaka *et al*^[11] reported that 44 gastric carcinomas with solitary lymph node metastasis were involved within N₁. Ichikura

Table 4 Histological growth pattern and skipping metastasis (cases)

	Group N ₁	Group > N ₁	P values
L tumors			0.087
Massive	8	3	
Nest	21	3	
Diffuse	11	6	
M tumors			0.456
Massive	2	2	
Nest	8	2	
Diffuse	9	5	
U tumors			0.645
Massive	1	1	
Nest	2	0	
Diffuse	3	1	
TOTAL			0.051
Massive	11	6	
Nest	31	5	
Diffuse	23	12	

Table 5 Histological type and transversal metastasis (cases)

	TN ¹	AN ²	P values
L tumors			0.793
Differentiated	8	11	
Undifferentiated	7	8	
M tumors			0.426
Differentiated	0	3	
Undifferentiated	3	13	
U tumors			0.114
Differentiated	0	3	
Undifferentiated	2	1	
Total			0.793
Differentiated	8	17	
Undifferentiated	12	22	

¹Transversal metastatic nodes; ²Adjacent metastatic nodes.

et al^[12] analyzed 69 cases with solitary lymph node metastasis, and found that in 6 of the 28 cases that had a lesion along the lesser curvature region, lymph node metastasis was observed along the greater curvature region. Kikuchi^[13] reported that skipping metastasis occurred in 14% of their patients with a solitary lymph node.

We investigated 173 cases with a solitary or single station metastatic lymph node from 2476 gastric cancer patients. First, 74.2% of the solitary metastasis lymph nodes were limited to within N₁. The lesion found in each region had one or more adjacent lymph node stations where a metastasis was more frequently found. These lymph node stations were relatively certain for the lesion in each region. Second, the frequency of transversal lymph node metastases, referring to the lesion in each region, was also relatively high. For example, with reference to a lesion in the L region, 31% of patients with a primary tumor on the lesser curvature area involved No.6, while 20% with a lesion on the greater curvature involved No.3. Third, the frequency of skipping metastases in our study was 25.8%. The frequencies of metastases in N₂ in regions U, M and L were 25%, 32.1% and 19.2%, respectively. We noted that the frequency of skipping metastasis in region M was 25%, that is, 25% of the lesions at the lesser curvature side were

Table 6 Histological growth pattern and transversal metastasis (cases)

	TN ¹	AN ²	P values
L tumors			0.513
Massive	6	2	
Nest	5	11	
Diffuse	4	6	
M tumors			0.453
Massive	0	2	
Nest	2	6	
Diffuse	1	8	
U tumors			0.687
Massive	1	0	
Nest	1	1	
Diffuse	1	2	
TOTAL			0.863
Massive	7	4	
Nest	8	18	
Diffuse	6	16	

¹Transversal metastatic nodes; ²Adjacent metastatic nodes.

detected in No.7 and No.8, and 25% of the lesions at the greater curvature were found in No.10.

From the above results, it is apparent that the distribution patterns of solitary nodes in gastric carcinoma are basically adjacent metastases; however, transversal and skipping metastasis were also found. The histological type and growth patterns did not influence the distribution of solitary metastatic nodes.

The first possible nodes of metastasis along the route of lymphatic drainage from the primary lesion should be SLN^[14]. However, because of the multidirectional and complicated lymphatic flow from the GI tract, when using methods that inject dyes or radioactive tracers, there is likely to be some bias in the description of the distribution pathway of SLN in gastric carcinoma^[15-17]. By analyzing the location of solitary metastatic lymph nodes, the distribution pathway of SLN in gastric carcinoma can be accurately assessed.

Reasons for skipping metastasis

After analyzing the clinical records of 51 patients with solitary lymph node metastasis, Kosaka *et al.*^[11] reported 7 cases of lymph node metastases in N₂-N₃ without being in N₁. Kosaka *et al.*^[11] suggested that the following could have a role in skipping metastasis: (1) occult metastases may remain unseen in a routine histopathological examination; (2) there may be a great number of lymphatic routes in the minor omentum; and (3) there may have been only a few perigastric nodes in those cases.

In this study, transversal and skipping metastasis were found to be notable. However, to date the reasons for the occurrence of skipping metastasis remain poorly understood. Chen *et al.*^[18] studied the dynamic role of stomach lymphatic flow from 138 infant corpses using 20% Prussian Blue Chloroform Solution as lymphatic dye. They reported that in 40 of 41 cases in which the drainage pointed at the greater curvature of the corpus gastricum, the lymphatic channel flowed directly to No.10. Therefore, with reference to lesions in a certain region, the so-called “skipping metastasis” of a SLN may be the first lymph

node in the lymphatic drainage system. In cases where metastasis first occurs in N₂ or N₃, the function of N₂ or N₃ is considered to be the same as N₁^[14].

Clinical implications

In the past 23 years, 2476 patients with gastric cancer were treated at our hospital. Among them, for the 728 patients without metastatic lymph nodes an extended D1 dissection was also performed, and as a result some cases had complications connected with the dissection. A great achievement of gastric surgeons in the last century, one that deserves unequivocal respect, has been to establish radical surgery with extensive lymph node dissection for gastric cancer. However, we now need to proceed to another stage by improving post-operative function and quality of life after gastric cancer surgery without impairing long-term outcomes^[19,20]. The concept of a “minimally invasive, curative, safe operation” has gradually gained acceptance^[21,22]. Here, we have attempted to discover the distribution pathway of SLN in order to provide clinical data for rational lymphadenectomy.

Based on the study we suggest that: (1) for patients with U area cancer at the lesser curvature region, No.7 and No.8 should be treated in the same way as N₁; (2) for patients with cancer in the M area in the lesser curvature region, No.7 should be treated in the same way as N₁; (3) for patients with cancer in the M area at the greater curvature region, No.10 should be inspected more carefully, although No.10 can be regarded as the same as N₃ for an M area cancer^[6]; further, if No.10 is questionable, a resection of the spleen should be undertaken^[4]; and 4) for patients with cancer in the L area at the lesser curvature region, No.1, No.7 and No.8 should be inspected more carefully.

COMMENTS

Background

The first possible nodes of metastasis along the route of lymphatic drainage from the primary lesion should be a sentinel lymph node (SLN). If a SLN is negative, patients can be considered to be without lymph node metastasis, and should not have to endure possible operations and face avoidable risks. The SLN concept is gaining greater acceptance, and thus clinicians and researchers can be optimistic that it may serve as a breakthrough management tool for use in gastric cancer.

Research frontiers

Many clinicians and researchers are presently undertaking studies of the distribution pathway of SLN in gastric carcinoma. However, because of the multidirectional and complicated lymphatic flow from the GI tract, there is likely to be some bias in the description of the distribution pathway of SLN in gastric carcinoma when using methods that inject dyes or radioactive tracers. The first possible sites of metastasis along the route of lymphatic drainage from the primary lesion are known as SLN. Therefore, a solitary metastatic lymph node could be regarded as SLN in gastric carcinoma. Some onco-surgery scholars have attempted to assess the distribution pathway of SLN in gastric carcinoma by analyzing the location of solitary metastatic lymph nodes.

Innovations and breakthroughs

Based on a large sample study, we described the distribution pathway of solitary metastatic lymph nodes and put forward concrete suggestions for lymph node dissection in gastric cancer.

Applications

We attempted to discover the distribution pathway of solitary metastatic lymph nodes in gastric cancer. Our results provide clinical data for rational lymphadenectomy and for experimental study of SLN.

Terminology

Minimally invasive surgery (MIS) is well recognized; however, rational lymphadenectomy operations are more focused on in this study. MIS is more about treatment of early gastric cancer, while rational lymphadenectomy is more about advanced gastric cancer.

Peer review

This manuscript presents a good overview of the distribution of lymph node metastasis in gastric cancer and should be of interest to GI researchers and clinicians.

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Gastric adenocarcinoma with features of endodermal sinus tumor

Malvinderjit Singh, Mukul Arya, Sury Anand, Nan Sandar

Malvinderjit Singh, Mukul Arya, Sury Anand, Nan Sandar, Department of Gastroenterology, The Brooklyn Hospital Center, Department of Gastroenterology, Wyckoff Heights Medical Center, Brooklyn, NY 11201, United States

Correspondence to: Malvinderjit Singh, MD, The Brooklyn Hospital Center, Division of Gastroenterology, 121 Dekalb Avenue, Brooklyn, NY 11201, United States. malisingh@hotmail.com

Telephone: +1-718-2506945 Fax: +1-718-2506489

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Abstract

Extragenadal germ cell tumors are rare. The most common sites for EGGCTs are in midline locations such as the mediastinum, retroperitoneum and pineal gland. These tumors rarely present in the stomach. We describe here a case where a middle aged man presented with typical symptoms of gastric cancer. After extensive workup, which included blood work, CT abdomen scan, upper endoscopy, and endoscopic ultrasound, the patient was diagnosed with gastric cancer. However, due to very high blood levels of alpha-fetoprotein, the specimen was sent for special histochemical staining, which demonstrated that the tumor had features of both adenocarcinoma and endodermal sinus tumor. This is a very aggressive tumor with a very poor prognosis.

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Key words: Extragenadal germ cell tumor; Endodermal sinus tumor; Seminoma; Non-seminoma; Adenocarcinoma; Mediastinum; Retroperitoneum; Metastasis; Alpha fetoprotein

Singh M, Arya M, Anand S, Sandar N. Gastric adenocarcinoma with features of endodermal sinus tumor. *World J Gastroenterol* 2007; 13(35): 4781-4783

<http://www.wjgnet.com/1007-9327/13/4781.asp>

INTRODUCTION

Extragenadal germ cell tumors are rare, accounting for only 1% to 4% of all germ cell tumors. Ninety-five percent of all testicular tumors are germ cell tumors. These tumors originate in the sperm forming cells in the testicles (the male gonads) or egg producing cells in the ovary (female gonads). On rare occasions, however, germ cell tumors

develop elsewhere in the body without any evidence of cancer in the testes. When this happens, they are referred to as extragonadal germ cell tumors (EGGCTs) (from the Testicular Cancer Resource Center)^[1].

At approximately 4 to 6 wk of embryonic development, the germ cells migrate into the embryo where they populate the developing testes or ovaries. If these cells miss their destination, they are likely to come to rest in one of a number of midline sites in the body. Extragonadal tumors arise when these cells become cancerous.

EGGCTs can be either benign or malignant and the malignant tumors can be either seminoma or non-seminoma. Extragonadal tumors are much more common in females. Malignant extragonadal tumors are much more common in men.

Extragenadal tumors can arise anywhere in the body. But, the majority are found in 3 common sites: anterior mediastinum, the retroperitoneum, and pineal gland in the brain. EGGCTs are aggressive and are usually seen in young adults. The treatment and prognosis of the disease depends on a variety of factors including the type of cancer, the tumor location, and the size of the tumor.

CASE REPORT

A 67-year-old Hispanic male presented to our institution with a complaint of epigastric pain radiating to the right upper quadrant, 8/10 in intensity, and it worsened with eating for 2 to 3 mo. The patient also complained of a weight loss of 10 pounds over the past month and vomiting for 2 d. He denied melena, hematochezia, and hematemesis. Past medical history was only significant for hyperlipidemia. The patient smoked 1 pack of cigarettes for 40 years, but denied use of alcohol. Family history was only significant for diabetes and hypertension. On physical examination, the patient felt a slight pain on deep palpation in the epigastrium with voluntary guarding. The remainder of his physical examination, including genital examination, was normal. Initial blood work obtained in the emergency room showed iron deficiency anemia, and elevated AST, ALT, and alkaline phosphatase. Tumor markers were sent and his serum alpha-fetoprotein was very high (4178; normal 0-1.8). Beta-HCG was normal. A CT scan of the abdomen showed gastric wall thickening and several 2 cm lymph nodes in mesenteric fat inferior to the stomach and liver extensively replaced with metastatic disease (Figure 1). An upper endoscopy, and endoscopic ultrasound were obtained (Figures 2 and 3). Endoscopy showed a large 8-10 cm exophytic-ulcerating lesion along the greater

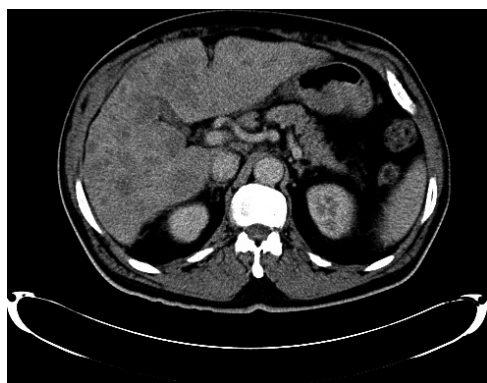


Figure 1: CT scan of the abdomen shows gastric wall thickening, several lymph nodes, and liver extensively replaced with metastatic disease.

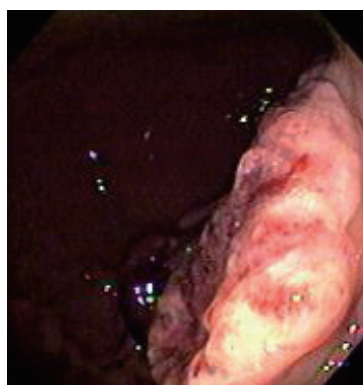


Figure 2: Upper Endoscopy shows 8-10 cm exophytic-ulcerating lesion along the greater curvature, extending from mid body to antrum

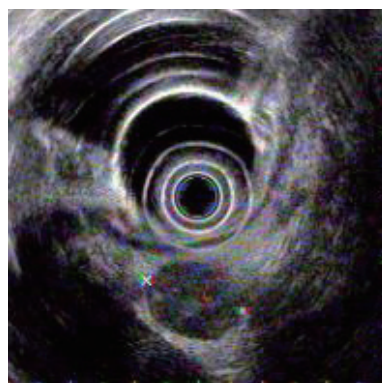


Figure 3: EUS showing a mass extending through all layers of the stomach.

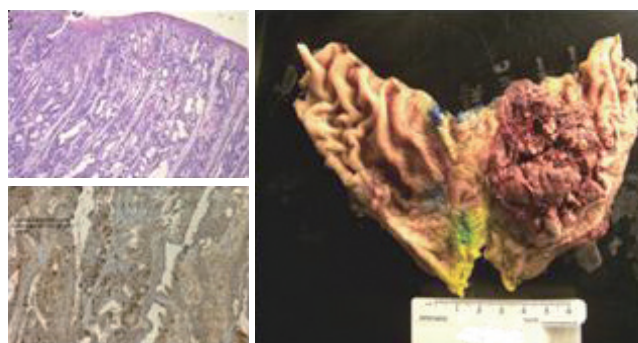


Figure 4: Immunohistochemical stains for alpha-fetoprotein strongly reactive with tumor cells.

curvature, extending from mid body to the antrum. A tissue biopsy was obtained. EUS demonstrated a mass extending through all layers of the stomach. Tissue biopsy obtained *via* EGD demonstrated invasive adenocarcinoma, which was poorly differentiated, and there were no *H Pylori* organisms. In view of the patient's very high serum alpha fetoprotein, a mixed component of extragonadal germ cell and adenocarcinoma was suspected, and the biopsy specimen was sent for special immunohistochemical staining (Figure 4). The immunohistochemical stains for alpha-fetoprotein strongly reacted with the tumor cells. Synaptophysin was focally positive and chromogranin was negative. These findings support the diagnosis of gastric adenocarcinoma, which was moderate to poorly differentiated, with features of endodermal sinus tumor and endocrine type cells.

The patient underwent exploratory laparotomy with partial gastric resection. The patient also received platinum based chemotherapy with Platinol and Taxol. He had a very eventful 2 mo of hospital course with multiple infections, upper gastrointestinal bleeding and eventual respiratory failure and death.

DISCUSSION

Extragonadal germ cell tumors are rare, representing 1% to 4% of all germ cell tumors. Seminomas account for 30% to 40% of these tumors, and non-seminomas account for 60% to 70%. The most common site of EGGCTs is the mediastinum (50%-70%) followed by the retro

peritoneum (30%-40%), the pineal gland (5%), and the sacrococcygeal area (less than 5%). In children, benign and malignant EGGCTs occur equally in males and females. In adults, only benign EGGCTs (teratomas) occur at equal frequency in both sexes; more than 90% of malignant EGGCTs occur in males^[2].

Symptoms vary depending on the site and the size of the tumor. A complete physical examination is required. Workup of EGGCTs begins with lab studies with the tumor markers alpha-fetoprotein and beta-HCG. These tumor markers provide diagnostic, staging and prognostic information. Pure seminomas and pure choriocarcinomas do not produce AFP. A CT scan of the chest, abdomen, and pelvis should be obtained. Treatment consists of surgery and chemotherapy. A classification system has been developed by the International Germ Cell Collaborative Group (IGCCG). This system categorizes tumors on the basis of histologic type, localization of metastases, and initial levels of serum AFP, beta-HCG, and LDH. Our patient who had very high levels of AFP and liver metastases expired within two months of the diagnosis^[3].

As mentioned earlier, these tumors generally present at an early age. However, few cases of EGGCTs in the elderly have been described in the literature^[4]. A case of yolk sac tumor with adenocarcinoma has been described in a 56-year-old female^[5]. On even rarer occasions, a gastric adenocarcinoma can present with a combination of these tumors. A case with such a tumor has been described in a Korean pathology journal where gastric adenocarcinoma was mixed with gastric choriocarcinoma and endodermal sinus tumor^[5].

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LETTERS TO THE EDITOR

Can *Campylobacter jejuni* play a role in development of celiac disease? A hypothesis

Behnam Sabayan, Farzaneh Foroughinia, Mohammad Hadi Imanieh

Behnam Sabayan, Farzaneh Foroughinia, Student Research Center of Shiraz University of Medical Sciences, Shiraz, Iran
Mohammad Hadi Imanieh, Gastrohepatology Research Center, Department of Pediatrics, Shiraz University of Medical Sciences, Shiraz, Iran

Correspondence to: Behnam Sabayan, Student Research Center of Shiraz University of Medical Sciences, Shiraz, Iran. b.sabayan@gmail.com

Telephone: +98-91-73160886 Fax: +98-71-12338007

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Abstract

Celiac disease (CD) is an entropathy with malabsorptive condition in which an allergic reaction to the cereal grain-protein (gluten) causes small intestine mucosal injury. CD is a multifactorial disorder in which both genetic and environmental factors contribute to the disease development. Mechanisms have been described to explain the pathology of CD. T cells specific for multiple gluten peptides are found in virtually all patients. Generation of such a broad T cell response may be a prerequisite for disease development. CD is associated with multiple extraintestinal presentations, including neurological deficits. Recent studies have shown a significant correlation between anti-ganglioside antibodies and neurological disorders in patients with underlying CD. Gangliosides are glycosphingolipids which are abundant in nervous system and in other tissues including gastrointestinal tract. It is not known what triggers the release of anti-ganglioside antibodies in people with gluten sensitivity. But, the mechanism is likely to involve the intestinal immune system response to ingested gliadin, a component of wheat gluten. Studies showed that mechanisms different from gluten exposure may be implicated in antibody formation, and other environmental factors may also exist. In addition, considering the fact that genetic predisposition dysregulating mucosal immune responses in the presence of certain environmental triggers like gastrointestinal infections may be strong etiological factors for developing chronic intestinal inflammation including CD, the hypothesis raised in our mind that antiganglioside antibody formation in CD may play a role not only in development of neurological complications in celiac patients, but also in development of CD itself. As presence of *Campylobacter jejuni* in other diseases with antigangliosides antibody formation has been established, we propose the possible role of *Campylobacter jejuni* in development of CD in association

with other genetic and environmental factors by the mechanism that molecular mimicry of gangliosides-like epitopes common to both lipo-polysaccharide coats of certain strains of *Campylobacter jejuni* and gangliosides in cell structure of gastrointestinal mucosa may cause an autoimmune response and consequently lead to atrophy and degeneration of mucosa possibly by apoptosis.

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Key words: Celiac disease; Gangliosides; *Campylobacter jejuni*; Molecular mimicry

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TO THE EDITOR

Celiac disease (CD) is an entropathy with malabsorptive condition in which an allergic reaction to the cereal grain-protein (gluten) causes small intestine mucosal injury. CD is a multifactorial disorder in which both genetic and environmental factors contribute to the disease development^[1]. Most of CD patients carry human leukocyte antigens (HLA)-DQ2 or HLA-DQ8. But, this genetic predisposition cannot fully explain the pathogenesis of CD, as CD just develops in minority of HLA-DQ2 and HLA-DQ8 positive individuals^[2]. Several mechanisms have been described to explain the pathology of CD. Gluten proteins have several unique factors that contribute to their immunogenic properties. They are extremely rich in amino acid proline and glutamine. Due to high proline content, gluten is highly resistant to proteolytic degradation within the gastrointestinal tract. Moreover, high glutamine content makes gluten a good substrate for tissue transglutaminase (Ttg), which can convert glutamine into negatively charged glutamic acid. Such modified gluten peptides can bind to HLA-DQ8 and subsequently cause T cell response. T cells specific for multiple gluten peptides are found in virtually all patients. Generation of such a broad T cell response may be a prerequisite for disease development^[3]. CD is associated with multiple extraintestinal presentations, including bone disease, endocrine disorders and neurological deficits^[4].

Neurological disorders and CD

Limited neurological disorders are recognized in association with CD. But, their spectrum becomes wider as complications of prediagnosed CD and/or as an initial manifestation of CD. Neurological disorders include cerebellar ataxia, peripheral neuropathy, epilepsy, dementia, migraine, encephalopathy and Guillain-Barre-like syndrome. Vitamin deficiency due to malabsorption was a first described as the etiology of neurological manifestations. But, it could not explain neurological disorders in patients with normal level of vitamins or in individuals with vitamin deficiency, but without neurological syndromes^[5]. Recent studies have shown a significant correlation between anti-ganglioside antibodies, and neurological disorders in patients with underlying CD. Gangliosides are glycosphingolipids which are abundant in the nervous system and in other tissues including gastrointestinal tract^[6]. It is not known what triggers the release of anti-ganglioside antibodies in people with gluten sensitivity. But, the mechanism is likely to involve the intestinal immune system response to ingested gliadin, a component of wheat gluten. Two mechanisms have been postulated for the release of anti-ganglioside antibodies: one is the presence of ganglioside-like epitopes in gliadin and the other is the potential for complex formation between gliadin and GM1 ganglioside. One study evaluated the feasibility of these two mechanisms, and found that certain gliadin species are glycosylated. But, they do not appear to carry GM1-like carbohydrate moieties^[7]. In contrast, *in vivo* formation of gliadin-GM1 complexes is probably feasible, since abundant GM1 is found in gut epithelial cells^[7].

It was reported that antibody titer is reversed in some patients after gluten-free diet, whereas it increases in other patients after such a diet^[8], suggesting that mechanisms different from gluten exposure may be implicated in antibody formation, and other environmental factors may exist.

Hypothesis

The above findings, and the fact that a genetic predisposition dysregulates mucosal immune responses in the presence of certain environmental factors such as gastrointestinal infections are strong etiological factors for development of chronic intestinal inflammation including CD (We can define the hypothesis in our mind that anti-ganglioside antibody formation in CD may play a role not only in developing neurological complications of celiac patients, but also in developing CD itself).

Among disorders associated with anti-ganglioside antibody formation, we focused on an autoimmune disorder with some neurological presentations like CD, and Guillain-Barre syndrome (GBS). In GBS a preceding infection may trigger an autoimmune response through

molecular mimicry in which the host generates an immune response to an infectious organism which shares ganglioside-like epitope with the host's peripheral nervous system. Among bacterial organisms which have a role in development of GBS, *Campylobacter jejuni* has been best studied, showing that about 25% of patients with GBS have a recent *Campylobacter jejuni* infection. Now, it is well established that lipo-oligosaccharide located in the wall of *Campylobacter jejuni* cross-reacts with ganglioside in axonal membrane of neurons.

We proposed a possible role of *Campylobacter jejuni* in development of CD in association with other genetic and environmental factors by the mechanism that molecular mimicry of gangliosides-like epitopes common to both lipo-polysaccharide coats of certain strains of *Campylobacter jejuni* and gangliosides in cell structure of gastrointestinal mucosa may cause an autoimmune response, and consequently lead to atrophy and degeneration of mucosa damage possibly by apoptosis in a manner similar to nerve tissue injury in GBS. The proposed mechanism can also explain the presence of neurological manifestations of CD.

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Mark D Gorrell, PhD, Professor

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Frank A Anania, Professor

Emory University School of Medicine, Division of Digestive Diseases, 615 Michael Street, Room 255 Whitehead Biomedical Research Building, Atlanta, GA 30322, United States

Stefano Fiorucci, MD

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Katri Maria Kaukinen, MD, PhD

Medical School, FIN-33014 University of Tampere, Tampere, Finland

Kyuichi Tanikawa, Professor

International Institute for Liver Research, 1-1 Hyakunen Kouen, Kurume 839-0864, Japan

Khalid Ahnini Tazi, PhD, Assistant Professor

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Marc Basson, MD, PhD, MBA

Chief of Surgery, John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Takuji Torimura, MD

Second Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume City, Fukuoka 830-0011, Japan

Thomas Langmann, Associate Professor

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Michael E Zenilman, MD

Clarence and Mary Dennis Professor and Chairman, Department of Surgery, SUNY Downstate Medical Center, Box 40, 450 Clarkson Avenue, Brooklyn, NY 11202, United States

Deborah L Diamond, PhD

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Bijan Eghtesad, PhD, Associate Professor

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Philip Rosenthal, MD

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