Hans Popper Hepatopathology Society

Companion Meeting

March 21, 2010
Update on Steatohepatitis and Fatty Liver Disease

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Learning Objectives

- To be able to recognize the steatohepatitis pattern of injury
- To understand the spectrum of fatty liver disease in children and to recognize the distinctive zone 1 pattern of NAFLD that is more prevalent in children

Overview of Steatohepatitis

Steatohepatitis is a pattern of liver injury that may be observed in a variety of clinical situations. Although it is the pattern of injury in alcoholic hepatitis, it is more frequently encountered in situations where alcohol is not felt to be involved, so-called non-alcoholic steatohepatitis (NASH). NASH is a liver disease mainly related to states of insulin-resistance such as obesity, type II diabetes mellitus and lipodystrophy. Because obesity and diabetes are common, NASH and other forms of non-alcoholic fatty liver disease (NAFLD) have been increasingly recognized in patients with symptoms and signs of chronic liver disease. NASH may also be associated with drugs, such as amiodarone, perhexiline maleate and tamoxifen and has been associated with the acquired lipodystrophy states caused by highly-active anti-retroviral therapy. This update will review the pathology of steatohepatitis and then focus on some of the recent findings related to NAFLD and NASH in children and in patients with normal transaminase levels.

Much of the discussion that follows has been shaped by this author’s experiences as one of the pathologists of the NASH Clinical Research Network (NASH CRN). The NASH CRN is a network established by the NIDDK to study the natural history of and conduct clinical trials for adult and pediatric fatty liver disease. It has recently been re-funded for another 5 years and additional pediatric centers have been added to the network. A designated pathologist from each clinical site participates in the pathology committee for the network. All biopsies collected by the network are reviewed by the
pathology committee for features of fatty liver disease and for the NAFLD Activity Score (NAS). The presence or absence of steatohepatitis is categorized according to gestalt recognition of the pattern of disease (Table 1). A new category of “Not NAFLD” was recently added to the classification to account for cases that lack even minimal criteria to be considered as fatty liver disease. To date, more than 2000 biopsies from patients with NAFLD (adults and children) have been reviewed by the pathology committee.
NASH CRN Categorization of NAFLD for the purposes of clinical research

<table>
<thead>
<tr>
<th>NAFLD Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definite Steatohepatitis</td>
<td>The classically described features of steatohepatitis are present (see discussion below)</td>
</tr>
<tr>
<td>Borderline “Zone 3” Pattern</td>
<td>Some, but not all, of the features suggesting steatohepatitis are present; Also, cases with mild, “borderline” zone 3 injury are placed here</td>
</tr>
<tr>
<td>Borderline “Zone 1” Pattern</td>
<td>Category created to recognize a pattern of NAFLD observed in children, typically with a zone 1 distribution of fibrosis and steatosis, lacking significant ballooning or Mallory-Denk bodies</td>
</tr>
<tr>
<td>Not Steatohepatitis</td>
<td>Cases not fitting into any of the above categories are placed here.</td>
</tr>
</tbody>
</table>

The Pathology of Steatohepatitis (Figure 1)

As noted above, steatohepatitis is pattern of liver injury which still requires examination of liver tissue to recognize. Unlike some chronic liver diseases, most notably the chronic viral infections, there is no clinical test that can be performed to identify patients with NASH or distinguish NASH from other forms of NAFLD. An experienced clinician, using existing imaging technology and laboratory tests, can only make an educated guess that a patient has NASH. Therefore, if there is need to know with certainty whether or not a patient has NASH, as well as to adequately grade and stage the fatty liver disease, a liver biopsy must be performed.

Although the word steatohepatitis suggests that only steatosis and inflammation need to present to make a diagnosis of steatohepatitis, both steatosis and inflammation are relatively non-specific findings. Steatosis is present in up to 2/3rds of biopsies of chronic hepatitis C, but a much lower percentage will have evidence of steatohepatitis. Population screening studies and autopsy series have suggested that between 5 and 15% of lean individuals will have steatosis by imaging or at death In obese patients, the percent with steatosis is between 30% and 75%. Only a minority of patients with steatosis will develop
steatohepatitis and of these fewer still will progress to cirrhosis. Although the natural history of NAFLD and NASH are still incompletely understood, it is clear that those who have the histologic pattern of steatohepatitis are at much greater risk of developing end-stage liver disease.

When the full pattern of steatohepatitis is present, it should not be difficult to make the diagnosis, even in the presence of other liver diseases. There are several histologic features to note in evaluating a steatotic liver.

The **steatosis** is usually macrovesicular, but may be either purely large droplet macrovesicular steatosis or a mixture of small and large droplet steatosis. In large droplet macrovesicular steatosis, the lipid vacuole fills nearly the entire hepatocyte, pushing the nucleus to the side and deforming it. At the extreme end, these cells may look like adipocytes. Small vacuole macrovesicular steatosis occurs when there are one or more smaller vacuoles in the cytoplasm. This form of macrovesicular steatosis should be distinguished from true microvesicular steatosis, in which the hepatocyte cytoplasm is replaced by innumerable small vacuoles, giving the cell a foamy appearance. The vacuoles of small vacuole macrovesicular steatosis are usually easily distinguished from one another and are few enough in number that they could be counted. The steatosis may be distributed in a distinctly zone 3 centered pattern or evenly throughout the acinus. Rarely the steatosis may localized to zone 1 (see the discussion of pediatric NAFLD below) and as the disease progresses towards cirrhosis the steatosis may become more irregularly distributed.

Like most chronic liver disease, there is both portal and lobular **inflammation** in steatohepatitis, but the distribution differs in that the portal inflammation is generally not prominent as in chronic hepatitis or the chronic cholestatic diseases. From low magnification the portal inflammation may be inconspicuous, particularly in early stages of the disease. As the fibrosis progresses the inflammation within portal spaces and in fibrous bands becomes more prominent. The lobular inflammation consists of small foci of lymphocytes and macrophages, sometimes associated with hepatocyte dropout or acidophil bodies. Microgranulomas are frequently seen in steatohepatitis and fatty liver disease in general. Aggregates of neutrophils are rare and become prominent only if many Mallory-Denk bodies are present. The degree of lobular inflammation is usually similar to the degree seen in viral hepatitis, but can be accentuated in zone 3. One pitfall in reviewing cases of fatty liver disease is to mistake marked perivenular inflammation and fibrosis for portal inflammation and making the diagnosis of chronic hepatitis. In such cases the portal areas are often small and difficult to see from low magnification.
**Ballooning hepatocellular injury** is one of the key diagnostic features in steatohepatitis, and one which seems to cause the most diagnostic difficulty. Ballooning injury can also be seen in other liver disease, so it is not entirely specific, but in the context of NAFLD, helps to make the diagnosis of steatohepatitis. The best way to learn to recognize ballooning injury is to start with clear examples. Classic ballooned hepatocytes are two to three times the size of normal (non-steatotic) hepatocytes and they are characterized by irregularly clumped eosinophilic material suspended between regions that are optically-clear and not membrane bound. Ballooned cells may have small fat vacuoles, which should not dissuade the pathologist from making the determination that they are ballooned. If Mallory-Denk bodies are present, they are most frequently seen in large ballooned cells and conversely, finding Mallory-Denk bodies in swollen hepatocytes implies that they are ballooned. Once one has a clear idea of classic ballooning injury, then it becomes easier to identify smaller cells with similar cytoplasmic characteristics in the same biopsy and to translate the observation to biopsies where the large classic balloon cells are few in number.

Like the other features of steatohepatitis, the fibrosis also takes on a distinctive pattern. The fibrosis of steatohepatitis begins as a delicate perisinusoidal deposition of collagen in zone 3. Good quality connective tissue stains (Masson trichrome or Sirius red) should be used to assess the biopsy. Reticulin stains are not suitable for picking up early fibrosis. Pericellular fibrosis can progress to the point of being visible on the routine H&E without the development of any appreciable periportal fibrosis and may even rarely progress directly to central-central bridging fibrosis. More commonly, periportal fibrosis develops, with trapping of periportal hepatocytes by collagen and expansion of the portal areas. As bridging fibrosis develops, one can often still identify areas of perisinusoidal fibrosis, either around residual veins or as part of the bridges themselves. Because there is a tendency for steatosis and ballooning to disappear as the fibrosis progresses to cirrhosis, the retention of perisinusoidal fibrosis can be a clue to the diagnosis.

The most important aspect of this pathology is that steatohepatitis is fundamentally a zonal injury. The characteristic lesions of steatohepatitis—hepatocellular ballooning, Mallory-Denk bodies and pericellular fibrosis—are usually found in zone 3 first. When all are present, along with steatosis and spotty lobular inflammation, the diagnosis of steatohepatitis can made even in the face of other chronic liver diseases or in the background of acute liver injury. Diagnostic difficulties occur when this pattern is not clear, or when some, but not all, of the diagnostic features are present.

**Problem Cases and other Intermediate Forms of Fatty Liver Disease**
There is ongoing discussion among hepatic pathologists about how to correctly classify cases of fatty liver disease that do not fulfill all of the diagnostic criteria of steatohepatitis. At one end of the spectrum are cases that show only steatosis with or without spotty lobular inflammation and without any fibrosis or ballooning injury. These should be not be classified as steatohepatitis. There is accumulating evidence that patients with this degree of fatty liver disease do not progress to cirrhosis at the rate of those with conventional steatohepatitis so it is reasonable to separate them diagnostically.

More problematic are cases that show steatosis and fibrosis but in which no ballooning injury or Mallory-Denk bodies can be identified. Since fibrosis may result from many different kinds of liver injury and steatosis is a common finding on many liver biopsies, it may be difficult to conclude that the steatosis and fibrosis are related to a common etiology. There is still some variation in how to classify such cases. Some pathologists will classify these cases as definite forms of steatohepatitis, perhaps after excluding other liver diseases by the evidence of the clinical work-up. Others will give weight to the distribution of fibrosis and make the diagnosis of steatohepatitis when there is good evidence of perisinusoidal fibrosis. It has been our practice to use qualifying terms like “consistent with” or “suspicious for” steatohepatitis when dealing with a biopsy that has a good fibrosis pattern but lacks ballooning. If the fibrosis and steatosis follow zone 1 rather than zone 3, the pathologist should consider classifying the case as the type of fatty liver disease seen more frequently in children (described below).

The diagnosis of steatohepatitis can also be more difficult to make as the fibrosis progresses to cirrhosis. Not only do the steatosis and ballooning decrease in degree, but the distortion of liver architecture by fibrosis and nodular regeneration can cause all the zonal clues to be lost. Cirrhosis due to steatohepatitis may be indistinguishable from cirrhosis due to viral hepatitis or autoimmune hepatitis. Careful examination of the biopsy for residual veins affected by perisinusoidal fibrosis as well as for characteristic ballooning or Mallory-Denk bodies may allow one to suggest steatohepatitis as an etiology.

When a good zonal pattern of ballooning, steatosis and inflammation is present, but there is no fibrosis yet, it is our practice to make a definite diagnosis of steatohepatitis, but not all hepatic pathologists feel comfortable with this classification. However, if fibrosis is the result of injury then it follows that there must be states where the injury is present but fibrosis has not yet developed. Because the zonal injury in cases without fibrosis is often mild, making definite diagnosis of steatohepatitis difficult, the pathologist can qualify the diagnosis as described above. The NASH Clinical Research Network regularly uses a “borderline zone 3” steatohepatitis category to place cases in which the changes are mild or where the changes are incomplete, but even so, a significant minority of cases classified as definite steatohepatitis do not have any fibrosis.

Some Recent Advances in the Pathology of Steatohepatitis
A couple of studies have been published recently from the NASH CRN that focus on individual features of NAFLD. Chalasani et al. studied the distribution and severity of steatosis among adults with NAFLD. Increasing severity of steatosis was correlated with increasing degrees of lobular inflammation, but not with ballooning or Mallory-Denk bodies. Despite this, biopsies with moderate and severe steatosis were slightly more likely to be diagnosed as definite steatohepatitis. In patients with early stage fibrosis, steatosis severity was related to the presence of perisinusoidal but not periportal fibrosis. Taken together, these results suggest that the absolute amount of steatosis has only a modest effect on the development of steatohepatitis. With respect to the distribution of steatosis, a zone 3 centered distribution was more often associated with early, mild, non-fibrotic disease than panacinar or azonal distributions.

Brunt et al. studied the associations with portal inflammation, which was stratified in the NASH CRN as none, mild or more than mild. In adults, the higher grades of portal inflammation were associated with older age at biopsy, female sex, higher BMI and HOMA. Patients with more than mild portal inflammation were more likely to be taking medications to control diabetes, hypertension or NAFLD, but not more likely to be taking medications for obesity or hyperlipidemia. The degree of portal inflammation had no relationship to autoantibodies or transaminase levels. Histologically, severity of portal inflammation was associated with a greater degree of steatosis, ballooning and fibrosis, but curiously was not linked to lobular inflammation. With respect to fibrosis the association was dramatic: 61% of biopsies with more than mild portal inflammation had bridging fibrosis or cirrhosis, compared to 4.3% of biopsies with no portal inflammation. The authors concluded that increased portal inflammation was a marker of advanced disease in NAFLD. The mechanism for the development of portal inflammation in NAFLD is still unclear.

With respect to the diagnosis of steatohepatitis, there has been recent work on trying to better define ballooned hepatocytes with the use of immunostains. Using antibodies against cytokeratin 8/18, Lackner et al. examined the ballooned hepatocytes in a variety of liver diseases and demonstrated that such cells have significantly reduced staining compared to normal hepatocytes. Mallory-Denk bodies within such cells are strongly stained, suggesting a complete collapse of intermediate filament structure within these damaged cells. The development of specific immunostains to identify ballooned hepatocytes will be a great diagnostic aid as well as provide a more quantifiable measure of ballooning injury.

**Pediatric Non-alcoholic Fatty Liver Disease (Figure 2)**
Childhood obesity and its attendant complications of diabetes and hypertension have become a better-recognized problem in the last decade and this issue is now receiving nationwide media attention in the U.S. However, pediatric NAFLD is still a relatively unrecognized complication. It can be serious though, and has led to reported cases of hepatocellular carcinoma as well as transplantation for cirrhosis. Early on, it was recognized that, in general, there was less evidence of ballooning injury and Mallory-Denk bodies in biopsies from children than were being seen in adults. Some biopsies showed a very distinctive pattern of fibro-fatty liver disease in which most of the injury seemed to be in the periportal or zone 1 region. Biopsies showed portal fibrotic expansion or portal-portal bridging fibrosis. The steatosis was distributed either as a collar around the portal areas or in a more pan-acinar distribution. The central vein regions seemed relatively spared and lacked ballooning or significant inflammation. The steatosis showed a gradient of vacuole size with the largest vacuoles in periportal hepatocytes with smaller vacuoles in zone 3. In order to anticipate this alternate pattern of NAFLD, the NASH CRN predefined a pattern they dubbed “borderline zone 1” to acknowledge both the pattern variation and the fact that it was not clear how this novel pediatric pattern fit into the natural history of classic zone 3-centered steatohepatitis. It was also unclear whether adults also showed this pattern of injury and the pathology committee felt that the blinded case review of the NASH CRN would provide a unique opportunity to study the problem.

Cases were classified as the borderline zone 1 pattern if there was a zone 1 centricity to the injury that included both portal/periportal fibrosis and steatosis. Absence of significant zone 3 injury was an important criterion as well. Ballooning could be present or not, but when present was in zone 1 or 2 rather than clustered near central veins. Preliminary findings on the histologic variation between adults and children were reported several years ago at the AASLD and since then a paper summarizing the clinical and histologic finding in the NASH CRN pediatric cohort has been published. As noted in the initial report by Schwimmer et al., children with NAFLD are much less likely to have ballooning injury and Mallory-Denk bodies than adults and are much more likely to have a zone 1 distribution of steatosis and isolated periportal fibrosis. About a quarter of the NASH CRN children have classic zone 3 steatohepatitis that is indistinguishable from adults, but about a quarter were also classified as having the borderline zone 1 pattern, as opposed to only 1% of adults. When children with the zone 1 pattern were compared to those with the classic steatohepatitis pattern, they were younger (11.1 vs 13 years) and were at an earlier Tanner stage. They had lower triglycerides, fasting insulin levels and HOMA-IR. Hispanic ethnicity was more common among those with the zone 1 pattern (69% vs 46% Hispanic). It was suggested that the hormonal and metabolic changes that accompany puberty may play a role in the form that the fatty liver disease took. The natural history of the zone 1 pattern is still unknown.

One clue may be found in a recently published multicenter study from North American clinical centers not participating in the NASH CRN. This study examined 108 pediatric liver biopsies gathered from five centers in the U.S. and Canada. Although the terminology was somewhat different from that used in the NASH CRN, they found that 82% of the biopsies had features they felt overlapped between the classic
zone 3 pattern of steatohepatitis and the recently described zone 1 pediatric pattern. This suggests that there may be transition between the two patterns of fatty liver disease. In other respects their findings were similar to the other cohorts of children--prominent ballooning injury was only seen in 21% and Mallory-Denk bodies in 12%. In comparing the two North American cohorts, there was a significant difference in the ethnic mix of the children, with the cohort being only 30% Hispanic, while the NASH CRN cohort was 59% Hispanic. Table 2 summarizes the data from recently published series of children, including the two described above. It is clear from the biopsy studies that have been done that pediatric NAFLD can be a serious liver disease, with a significant minority of children having advanced fibrosis. Follow-up studies on these cohorts will be important to understand how quickly these children will develop cirrhosis.

Table 2: Summary of Some Recent Studies on Pediatric NAFLD

<table>
<thead>
<tr>
<th>First Author</th>
<th>Setting</th>
<th>N</th>
<th>Age Mean, Range</th>
<th>Ob/DM %</th>
<th>Zone 3 NASH %</th>
<th>Zone 1 Pattern %</th>
<th>NAS≥5 %</th>
<th>Severe Fibrosis %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patton</td>
<td>US Multicenter</td>
<td>176</td>
<td>12.4, 6-17</td>
<td>97% Ob</td>
<td>36%</td>
<td>28%</td>
<td>47%</td>
<td>14%</td>
</tr>
<tr>
<td></td>
<td>(NASH CRN)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carter-Kent</td>
<td>US/Canada Multicenter</td>
<td>108</td>
<td>12, 4-18</td>
<td>88% Ob</td>
<td>7%</td>
<td>8%</td>
<td>44%</td>
<td>20%</td>
</tr>
<tr>
<td>Manco</td>
<td>Italy Single Center</td>
<td>120</td>
<td>12.1, 3-18</td>
<td>42.5% Ob</td>
<td>Used NAS≥5</td>
<td>3%</td>
<td>32%</td>
<td>N.D.</td>
</tr>
<tr>
<td>Ko</td>
<td>Korea Single Center</td>
<td>80</td>
<td>12, 65% Ob</td>
<td></td>
<td>34%</td>
<td>44%</td>
<td></td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. = Not determined or unable to determine from data in paper

Table 3 summarizes some of the differences between these two patterns of NAFLD. The only way to distinguish which pattern is present is on biopsy, so the pathologist has an important role to play in the understanding of these diseases. As noted by Carter-Kent et al., there may be cases which are difficult to completely classify as one type or the other. Both patterns will become more difficult to distinguish as fibrosis progresses and the zonal architecture is destroyed. There are no clear differences in the amount or distribution of portal and lobular inflammation between the two types.
Table 3: Comparison of Histologic Features of Classic NASH with the Pediatric Zone 1 Pattern

<table>
<thead>
<tr>
<th>Feature</th>
<th>Classic “zone 3” NASH</th>
<th>Pediatric “zone 1” pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>Macrovesicular, in a zone 3 to panacinar distribution</td>
<td>Macrovesicular, in a zone 1 to panacinar distribution</td>
</tr>
<tr>
<td>Ballooning</td>
<td>Present, zone 3</td>
<td>Usually absent, and not in zone 3 when present</td>
</tr>
<tr>
<td>Mallory-Denk bodies</td>
<td>May be present</td>
<td>Usually absent</td>
</tr>
<tr>
<td>Early fibrosis</td>
<td>Perivenular and perisinusoidal</td>
<td>Periportal</td>
</tr>
<tr>
<td>Late fibrosis</td>
<td>Portal-central bridging frequently seen</td>
<td>Portal-portal bridging most common, with sparing of central veins</td>
</tr>
</tbody>
</table>

The Problem of NAFLD and Normal Transaminase Levels

A major issue facing physicians who are faced with the evaluation of patients with possible liver disease is distinguishing patients with NASH or hepatic fibrosis from those who have steatosis alone. There is no single biomarker test available that will distinguish these categories. Some have proposed mathematical combinations of biomarkers that improve the odds of detecting NASH, but these are largely unvalidated. MRI imaging has shown some promise in the detection of fibrosis and steatosis, but these techniques are not available yet at all institutions and remain in the arena of research. Most clinicians, and particularly clinicians who are not hepatologists, rely on sporadic determinations of transaminase levels to exclude significant liver disease. No clinical investigation may be pursued unless the transaminases remain persistently elevated or rise to some “alarm” level, like twice the upper limit of normal. A number of studies have shown that ALT is a poor discriminator in NAFLD. This test is unable to separate patients with steatosis alone from those with NASH and patients with fibrosis from those without.

A number of recent studies have evaluated the clinical and histologic changes in cohorts of patients with normal ALT. Table 4 shows some of these studies that have been drawn from clinic populations (as opposed to bariatric surgery patients). In all of these studies there is some indication for biopsy, from other abnormal laboratory tests that suggest liver disease (elevated ferritin or GGT) to hepatomegaly or other symptoms/signs of chronic liver disease. One study, that of Mofrad et al, also included patients being screened for living donor liver transplantation. One issue that must be considered in interpreting these studies is the level of ALT (or AST) used as a cutoff for the upper limit of normal (ULN). A recent study by Prati et al., has suggested that the ULN for ALT should be 30 for men and 19 for women, which
is considerably less than the ULN used in some of these studies. Nevertheless, it is clear from examination of ALT as a continuous variable that there is no value which absolutely identifies patients without fibrosis or NASH. Patients with normal transaminases and NASH tend to be older and female (consistent with varied thresholds for the ULN). Most have other risk factors for NASH, such as obesity, diabetes or metabolic syndrome. The proportion of patients with significant fibrosis or NASH in the normal enzyme population also varies widely, from only 3% in the Hong Kong cohort to between 60% and 80% in the other three. Similarly, the fraction of patients with bridging fibrosis or cirrhosis varied from 0% to 35%, although the cohort with the highest fraction of severe fibrosis also used the highest cut-offs for the ULN. Studies such as these reemphasize the necessity of a liver biopsy in order to properly diagnosis, stage and grade fatty liver disease. Although there is a huge scientific effort underway to develop non-invasive methods of diagnosis, we pathologists have a responsibility to make sure that these studies are properly grounded in careful characterization of the disease.
Table 4: Some Recent Studies of NAFLD in Adult Patients with Normal ALT (and AST)

<table>
<thead>
<tr>
<th>First Author</th>
<th>Uslusoy</th>
<th>Wong</th>
<th>Fracanzani</th>
<th>Mofrad</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Year</strong></td>
<td>2009</td>
<td>2009</td>
<td>2008</td>
<td>2003</td>
</tr>
<tr>
<td><strong>Country and Setting</strong></td>
<td>Turkey</td>
<td>Hong Kong</td>
<td>Italy</td>
<td>U.S.</td>
</tr>
<tr>
<td></td>
<td>Single center</td>
<td>2 Centers</td>
<td>Multicenter</td>
<td>Single center</td>
</tr>
<tr>
<td><strong>N (nml ALT)</strong></td>
<td>9</td>
<td>35</td>
<td>63</td>
<td>51</td>
</tr>
<tr>
<td><strong>N (whole cohort)</strong></td>
<td>34</td>
<td>173</td>
<td>458</td>
<td>101</td>
</tr>
<tr>
<td><strong>ALT ULN</strong></td>
<td>43</td>
<td>58</td>
<td>40</td>
<td>75 (men)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52 (women)</td>
</tr>
<tr>
<td><strong>AST ULN</strong></td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Characteristics of the normal cohort**

| % Female      | 89% | 63% | 25% | 69%   |
| % Obese       | 100% | 86% | 49% |       |
| % Diabetic    | 22% | 77% | 11% | 57%   |
| % Met. Syndrome | 89% | 57% | 19% |       |
| % NASH        | 78% (used NAS>5) | 3% | 59% | 69%   |
| % Bridging fibrosis or cirrhosis | 0 | 6% | 9% | 35%   |
| % Cirrhosis   | 0 | 3% | 8% | 12%   |
Fig 1. Photomicrographs from a case of steatohepatitis

Low power H&E and Masson stains showing irregular steatosis, early nodularity and early bridging fibrosis

Ballooned cells with Mallory-Denk bodies. Ballooned cells without MD bodies

Steatosis with small ballooned cells Well-developed perisinusoidal fibrosis
Figure 2: Zone 1 Injury pattern seen in some children with NAFLD

There is a clear zone 1 distribution of steatosis in parts of the biopsy

The central vein area is shows no injury, while the portal areas look scarred and contain a mild infiltrate of inflammatory cells

There was portal-portal bridging fibrosis and portal fibrotic expansion with hepatocyte trapping. There was no perisinusoidal fibrosis in this biopsy.
References (in order by author)

(1-16)


Autoimmune Liver Disease: Update for Pathologists from the Hepatologist’s Perspective

Jenny Heathcote, MD
University of Toronto

Key Points:

- AILD comprise autoimmune hepatitis, primary biliary cirrhosis and sclerosing cholangitis. Overlapping features between these 3 diseases are common.
- Liver histology is not essential to the diagnosis of AILD but when the diagnosis is uncertain, histologic confirmation helps to make a definitive diagnosis.
- Appropriate therapy enhances the outcome of AIH and PBC however no specific therapy delays progression of PSC – one of Hepatology’s greatest “unmet needs”.

1. Presumed Autoimmune Liver Diseases (AILD)

There are 3 specific AILD although one may have overlapping features of another and on occasion one may completely transform into another. This suggests that they are ‘complex diseases’ likely resulting from an untoward interaction between either the internal or external environment dictated by the genetic background of the host.

These three diseases comprise Autoimmune Hepatitis (AIH), Primary Biliary Cirrhosis (PBC) and Primary Sclerosing Cholangitis (PSC). One may have overlapping histologic and/or clinical features of another e.g. lymphoplasmacytic hepatitis invading the limiting plate in patients with overt PBC, transition from AMA positive PBC to AMA negative ANA positive AIH or concomitant or sequential AIH and PSC.

Whereas examination of liver pathology is an important (and most would consider essential) component of the diagnostic criteria for autoimmune hepatitis, most cases of PBC can be confidently diagnosed without liver biopsy, based on a positive antimitochondrial antibody (AMA) test and an elevated serum alkaline phosphatase value (ALP). In patients with chronic cholestasis who test negative for AMA, a liver biopsy is required once the large bile ducts have been shown (via MRC) to be normal. Such a patient may have AMA negative PBC. Alternatively a liver biopsy performed in such a patient may fail to reveal the typical apoptotic interlobular bile duct lesions of PBC rather sclerotic bile ducts may be seen. This finding would suggest a diagnosis of small duct sclerosing cholangitis. In patients with obvious large duct sclerosing cholangitis seen on MRC, liver biopsy is not warranted as the degree of liver fibrosis is very variable throughout the liver in PSC and thus a single biopsy may be far from being representative of the entire liver.
**Autoimmune Hepatitis (AIH):**

In 2010 it is recognized that this disease which predominantly affects women may present at any age and affect all ethnic groups. The introduction of routine screening of liver biochemistry has taught us that asymptomatic AIH is not unusual. Thus there may be a wide range of manifestations of this disease from no symptoms at all through to fulminant hepatic failure. In the elderly the disease may be diagnosed for the first time as an inactive cirrhosis with portal hypertension and its complications. There are several models for an “AIH score” only with the latest is it possible to have a high enough score without needing a liver biopsy. However, most hepatologists would feel uncomfortable making a diagnosis of AIH without a liver biopsy even though the findings on pathology are not specific. Corticosteroids ± azathioprine are the standard of care for AIH. Other diagnoses in the differential for AIH can for the most part be confirmed without a liver biopsy e.g. viral hepatitis, drug induced hepatitis and Wilson disease. Steroid treatment for these other conditions may also lead to an improvement in liver biochemistry. The essential laboratory features of AIH are a transaminitis in association with detectable ANA ± SMA, and IgG >1.5 fold elevated and compatible liver histology. Bile duct damage in the portal tracts may be noted in 15%+ and may be transient. Some patients with AIH will test positive for AMA even though there is no biochemical or histologic evidence of cholestasis. Such patients respond well to prednisone and long term follow up has not shown progression to PBC despite AMA remaining detectable over many years.

A good clinical biochemical and serologic (IgG back to normal) response to immunosuppressive therapy leads to resolution of the many histologic findings albeit at a somewhat slower rate than the clinical and biochemical manifestations.

Most patients with AIH require immunosuppressive therapy long term often life long. However once remission is induced maintenance therapy with azathioprine monotherapy is usually possible. In those few who present with a fulminant hepatitis (jaundice, coagulopathy +/- other features of liver failure) immediate liver transplant is the best option. At the other extreme those with asymptomatic AIH may also not benefit from the introduction of immunosuppressive therapy at the time of first diagnosis.

Drug induced hepatitis e.g. Minocycline may present clinically and look histologically exactly like AIH. Occasionally withdrawal of the drug alone is insufficient and steroid therapy is needed to assist resolution of liver disease although immunosuppressive therapy is unlikely to be needed long-term. Viral markers for a chronic hepatitis B,C and D should be looked for in all those suspected to have AIH. The diagnosis of Wilson disease may be hard to either confirm or refute but must be considered in all cases of presumed AIH as it is now recognized Wilson disease may become first manifest at 60 years old or older.

**Primary Biliary Cirrhosis (PBC):**
Is only as common as physicians think to test for AMA in an individual found to have an elevated ALP. The disease has been identified worldwide but is most common in women living in the Northern Hemisphere e.g. UK, Japan – where 1 in 1000 women >40 years have PBC. This disease in 2010 is most often diagnosed when asymptomatic. Jaundice is now rarely seen at presentation and a decreasing number proceed to liver transplant. This is because proceeding treatment with ursodexychocholic acid (UDCA) improves the survival of those with PBC if they have a biochemical response to UDCA (>40% fall in ALP within 1 year). Gone (at least in the developed world) are the days when patients with PBC presented with xanthoma, severe pruritus and marked jaundice with pigmentation of the skin. However pruritus may be present in the absence of jaundice (pruritus may improve with worsening of liver disease). Fatigue is the most common symptom in patients with PBC – the reason for this remains unknown.

Liver biopsy is now no longer thought essential to make a diagnosis of PBC. But in an individual with anicteric cholestasis who tests AMA negative, a liver biopsy is required. AMA negative PBC exists and clinically behaves no differently from AMA positive PBC and responds equally well to treatment with UDCA. In PBC without AMA, it is usual to detect high titre ANA (most often with a pattern of multiple nuclear dots seen on immunofluorescence) and higher IgG and lower IgM serum values than is the case for AMA positive disease. Biochemical response to UDCA is associated with halting of liver disease progression and thus is more effective in the long term in those with early disease. In those cases of PBC who are not observed to have a reduction in ALP within a year of introducing therapy with ursodeoxycholic acid (UDCA) liver biopsy may be indicated to look for a superimposed liver disease e.g. AIH, fatty liver.

PBC mostly affects women and there may be a family history going through the female line. Recently a specific genetic marker for this disease has been identified which suggests that it is the interleukin 12 pathway which is affected (1L12A, 1L12RB2 as well as STAT4 and HLA). The disease likely arises from an untoward response to an exogenous xenobiotic or infection in a genetically susceptible individual.

An interface hepatitis quite often accompanies the expansion of portal tracts where typically mainly lymphocytes invade the interlobular biliary ducts and histiocytes may be seen within granulomas which are confined to the portal tracts. Whether the presence of an additional interface hepatitis signifies a different disease i.e. AIH/PBC overlap syndrome is uncertain. However if patients with clear cut PBC have a positive AIH score it has been shown (retrospectively) that they have a worse outcome than those cases of PBC with a negative AIH score. It is not known (i.e. no RCT) if the addition of immunosuppressive therapy might improve their outcome.

**Sclerosing Cholangitis:**

It is now becoming clear that sclerosing cholangitis (typified by a pattern of stricturing and beading of the intra and/or extrahepatic biliary tree) is not always “Primary”. However, PSC
when present is most often seen in concert with inflammatory bowel disease not confined to but always involving the colon. Colitis may have been present long before the biliary disease is recognized or may develop many years after a diagnosis of PSC is made. The link between the two diseases and how this may relate to the pathogenesis of the two diseases is unclear. In any patient with anicteric cholestasis in the absence of AMA in serum a diagnosis of PSC must be suspected to be confirmed on MRC. However, “frustratingly” this chronic biliary disorder may be obvious biochemically long before any biliary changes of the large bile ducts are observed. In this situation a liver biopsy may be helpful. The finding of sclerotic “onion skin” interlobular ducts is typical of small duct sclerosing cholangitis which may in some (the minority) subsequently progress to large duct disease. To date there is no known effective treatment for PSC and thus only symptomatic therapy is appropriate. Low dose UDCA (13-15 mg/Kg) often leads to an improvement of liver biochemistry but does not improve survival. A recent trial of high dose UDCA (28-30 mg/Kg) suggested that survival was less in those randomized to the treatment arm. Currently there is no known explanation for this unexpected finding.

Similar small duct changes may also be observed in the livers of those with a secondary sclerosing cholangitis as may often be observed (but only if looked for) in patients with portal vein thrombosis. The etiology of the bile duct injury in this instance is likely ischemic. There are many other causes of ischemic damage to the biliary tree e.g. post hepatic artery thrombosis following a liver transplant, severe cholestasis, sepsis and hypotension in a patient in the ICU and in hereditary hemorrhagic telangiectasia (HHT) are just a few examples.

Only recently has the full spectrum of disease associated with autoimmune pancreatitis (AIP) been well outlined. This is a systemic inflammatory disease which heals with fibrosis which can in addition to affecting the pancreatic ducts affect the bile ducts in many ways. Formation of a pseudotumor in the head of the pancreas (often confused with pancreatic carcinoma) may cause large bile duct obstruction but in others AIP is associated with only biliary strictures (intra ± extra hepatic). Other organs may be involved:- salivary glands, retroperitoneum, lungs, kidneys to name but a few. It is essential to confirm the diagnosis; probably the most reliable is by staining for IgG4+ve plasma cells in whatever tissue specimen is available. Hence the pathologist plays a crucial role in the diagnosis of this disease which once recognized should be treated with corticosteroids, as almost universally prednisone leads to a dramatic melting of the inflammatory masses and strictures (very reassuring to the patient who has often been given a previous diagnosis of pancreatic cancer).

**Cancer in AILD:**
As with most chronic liver disease an enhanced risk of liver cancer exists, most often observed in those with advanced disease. The incidence of HCC in both AIH and PBC is very much lower than that reported in chronic viral hepatitis nevertheless regular screening via ultrasound may be appropriate in those known to be cirrhotic. Hepatocellular carcinoma may also complicate PSC but more likely is a cholangiocarcinoma. Tragically this cancer is often silent until the tumour is too large to be even operable, let alone curable. 50% of cholangiocarcinoma complicating PSC are diagnosed within the first year of diagnosis of PSC. Screening blood
tests are not very sensitive or specific (Ca 19-9 +CEA) and probably most reliable is the examination of biliary brushings for cytology via FISH. However for ERC accompanied by brushing to be contemplated there needs to be a high index of suspicion from changes seen on repeat MRC because the risk of complications particularly sepsis is 6% in those undergoing ERCP for the investigation of PSC.

**Conclusion:**

Few gastroenterologists, even hepatologists are very familiar with autoimmune liver diseases. As a consequence training in pathology likewise lacks exposure to AILD. Whereas liver biopsy in chronic viral hepatitis is mostly valuable in determining the severity of disease and is rarely helpful diagnostically – quite the opposite can be said for the pathologists input with regard to AILD. The pathologist does need to be provided with both a complete clinical history and biochemical findings to facilitate making a correct diagnosis. Although many new imaging techniques e.g. fibroscan, fibrotest, MRC have to a certain extent obviated the need for liver biopsy, if support for or confirmation of a possible diagnosis of an AILD is required, then a pathological analysis of an adequately sized liver biopsy is essential.

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Iron in the Liver: A Review for Surgical Pathologists

Hans Popper Society

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1. DEFINITIONS

The clinical and basic science research communities have made significant progress over the past several decades in understanding the causes and significance of iron accumulation in the liver. This update is designed to summarize the major advances and also to synthesize the current literature in a manner that is relevant to the practice of surgical pathology.

To start, some nomenclature issues: at times there have been inconsistent use of terminology related to iron in the liver, which can be somewhat confusing. For example, what is precisely meant by the term “hemochromatosis”? The term sometimes refers to any degree of tissue iron accumulation, sometimes only to those cases with sufficient iron accumulation to cause tissue damage, sometimes to those cases where the iron is only (or predominately) in the hepatocytes, and sometimes to any degree of iron accumulation as long as there is evidence of genetic mutations. Likewise, the terms “primary” and “secondary” iron accumulation are not always used in a consistent fashion. In this review, we shall adopt for practical purposes the following: the term “hemochromatosis” indicates hepatic iron accumulation in the setting of a genetic mutation, the term “siderosis” indicates hepatic iron accumulation without genetic mutations, and the terms “primary” and “secondary” will be avoided. We will also use the term “genetic non-hemochromatotic iron overload disorder” which has been proposed for a range of rare genetic disorders that lead to iron accumulation that is primarily deposited in Kupffer cells and macrophages.

Major proteins and cells involved in iron metabolism

There are many proteins and cells involved in iron metabolism that we will not be able to cover in this review. However, the major ones are listed below for quick reference.

Proteins

DMT-1: Dimetal transporter-1. Transports iron from gut lumen to enterocyte cytoplasm

Ferritin: Protein that has an enormous capacity to bind iron; located in the cell cytoplasm and a major physiological storage form of iron

Ferroportin: Transports iron out of cells into the blood stream (principally enterocytes and macrophages, also hepatocytes)

Hemojuvelin: The precise role of this membrane bound protein is not clear. However, it
appears to interact with important signaling pathways (BMP, SMAD) that have hepcidin as a downstream target. Without hemojuvelin, these signaling pathways are not able to activate hepcidin gene synthesis in a normal fashion.

**Hemosiderin:** Abnormal deposits of iron

**Transferrin:** Protein that transports iron in blood

**Cells**

**Enterocytes:** Absorption and short term storage of iron

**Hepatocytes:** Major producer of ferritin, hepcidin

Major organ for storage of iron in the form of ferritin

**Macrophages:** Main recycler of old/damage red blood cells

Major cell type for storage of iron in the form of ferritin

2. **DIETARY CONSIDERATIONS**

The recommend daily allowance (RDA) for iron is 8 mg per day for adult men and postmenopausal women and 18 mg per day for premenopausal women. The “Tolerable Upper Limit of Intake” for dietary supplementation is about 45 mg of iron per day in adults before there is gastrointestinal distress. A detailed resource on iron and dietary considerations in health and general growth and development is available free of charge from the United States Department of Agriculture (USDA) at [http://dsearch.nal.usda.gov/cgi-bin/dexpldgpi?qry1227124502;2](http://dsearch.nal.usda.gov/cgi-bin/dexpldgpi?qry1227124502;2). This report was written by the National Academy of Sciences in 2001, so it is a bit dated, but it still has a wealth of information. The latest available iron RDA, published in 2005, keeps to essentially the same RDA as noted above but with a much greater breakdown by age, gender, and caloric intake ([http://www.cnpp.usda.gov/DGAs2005Guidelines.htm](http://www.cnpp.usda.gov/DGAs2005Guidelines.htm)). Updated RDA for iron should be published by the USDA in 2011 ([http://www.cnpp.usda.gov/dietaryguidelines.htm](http://www.cnpp.usda.gov/dietaryguidelines.htm)).

Iron in the diet comes in two principal forms: heme iron from meats, including poultry and fish, and non-heme iron from grains, legumes, vegetables and fruits. Heme-iron is more readily absorbed than non-heme iron. Some fruits and vegetables that are naturally high in iron content include green
leafy vegetables such as spinach and broccoli; most dried beans such as lima beans, kidney beans, etc; dried fruit such as raisins and prunes; and citrus such as oranges, lemons, and grapefruit. Vitamin C can enhance iron absorption.

3. OVERVIEW OF NORMAL IRON METABOLISM

The normal adult body contains a total of 3-5 grams of iron. About 20 mg of iron is needed each day for normal physiological functions, largely heme synthesis, but the majority of this daily need is met through recycling of damaged and no longer properly functioning red blood cells. Because of the efficiency of this red blood cell recycling, only 1 to 2 mg per day are needed in a healthy diet, though the Recommended Daily Allowance (RDA) is somewhat higher at 8-18 mg of iron.

Iron is important in a number of metabolic processes outside of heme synthesis, including oxidative phosphorylation and DNA synthesis. Despite this, iron can be toxic at high levels and iron levels within the body are tightly regulated. The human body has no physiological way to excrete iron and regulatory mechanisms are instead focused on iron absorption from the intestine. Separate, but integrated, controls also tightly regulate blood iron levels.

Iron Absorption

Most iron is absorbed in the duodenum and proximal jejunum by a protein called DMT-1, where it is first sequestered into the cytoplasm of enterocytes. Iron can then be exported by a protein called ferroportin into the blood stream where it is carried by the protein transferrin to sites of principal usage, including the bone marrow for hemoglobin and the muscle for myoglobin. In healthy individuals, the blood contains much more transferrin protein than iron and the transferrin levels are approximately 30% saturated with iron. As blood iron levels increase, the excess transferrin proteins serve as a sort of buffer and will bind more iron to prevent excess free iron in the blood. Thus, increased serum transferrin levels can serve as a sensitive early indicator of excess iron absorption. All nucleated cells have transferrin receptors that can uptake transferrin bound iron to meet cellular needs.

Iron storage

If there is excess iron in the body, it can be incorporated into ferritin molecules for storage, largely in hepatocytes and macrophages. Ferritin is produced principally by the liver and is found in the liver cytoplasm, where it can hold up to 4500 atoms of iron per ferritin protein complex. Ferritin is typically not observed on Perls’ Prussian Blue stain, but occasionally it can be seen as a diffuse blush of blue in hepatocyte cytoplasm. The iron in ferritin can be rapidly accessed for physiological needs. If ferritin levels are excessive over a sufficiently long period of time, hemosiderin deposits can then develop.
Hemosiderin is typically granular and golden brown on H&E staining and is composed of iron and various proteins, principally degraded ferritin. The vast majority of the metal in hemosiderin is iron, but small amounts of copper and calcium can also be detected. Despite an identical H&E appearance of hemosiderin in both genetic and non-genetic causes of iron overload, there are differences in both the metallic as well as the organic components at the molecular level.\(^1\) In contrast to ferritin, the iron in hemosiderin is not as readily available for biological needs.

In sum, there are two important reservoirs of iron that can both be tapped to keep iron levels in the blood at physiologically correct levels: (1) a short term reservoir of iron stored within enterocytes and (2) a longer term reservoir of iron stored as ferritin, principally in hepatocytes and macrophages. Both reservoirs have separate but interconnected control mechanisms that serve to regulate iron flow into the blood. If they both are unable to meet the demands for iron, then anemia develops; if they have dysregulated (mutated) control mechanisms, then hemochromatosis can develop.

### 4. CONTROL OF IRON TRAFFICKING

#### Iron absorption

Iron is absorbed primarily in the duodenum and proximal jejunum. Heme-iron is taken up by the enterocytes after disassociation from globin, while non-heme iron is first reduced from a ferric to a ferrous state and then transported across the cell membrane into the enterocytes by a protein called DMT-1. There are several additional iron transport mechanisms for getting luminal iron into the enterocyte cytoplasm that we won’t be able to discuss today. Once iron is within the enterocytes, it can have several fates. If the body has sufficient iron stores, then the iron remains within the cytoplasm of the enterocytes. When the enterocyte eventually dies, the iron within the cell’s cytoplasm is lost within the fecal stream; this is a major control mechanism to prevent iron overload.

If the body needs iron, then the iron is transported out of the enterocyte by ferroportin, with some help by accessory proteins including ceruloplasmin and hephestin, and enters the blood stream where it is bound by transferrin and circulates within the blood. Individual cells have mechanisms to determine if they have sufficient iron stores within their cytoplasm to meet their needs. If not, then the cells increase their expression of transferrin receptors (there are two, conveniently named transferrin receptor 1 and transferrin receptor 2; receptor 1 is on all nucleated cells, while receptor 2 is primarily found in the liver) and take in more transferrin bound iron. Hepatocytes, with their abundant transferrin receptors, take up any excess iron which then can be stored in the form of ferritin and, in times of great excess, as hemosiderin.

#### Control of iron release from stores in the enterocytes, liver, and macrophages

When blood levels are low, iron is released from enterocytes where it has been freshly absorbed and released from hepatocytes and macrophages where it has been stored as ferritin. However, when
blood iron levels are sufficient, then iron is blocked from being released from these two compartments. **Hepcidin** is a major controller of iron metabolism: it blocks the release of iron from hepatocytes, macrophages, and enterocytes. When hepcidin levels are low, there is increased iron absorption from the gut and increased release of iron into the blood.

Hepcidin is produced mainly by hepatocytes and is an acute phase reactant. Because it is an acute phase reactant, hepcidin levels can be elevated in a variety of inflammatory and infectious conditions. In addition to inflammation, hepcidin levels are also increased by excess body iron stores and by tissue hypoxia. The main physiological role of hepcidin in healthy individuals is to lower blood iron levels by blocking transfer of iron from enterocytes to the blood and by blocking the release of iron stores from the liver and macrophages into the blood. Hepcidin accomplishes this by causing degradation of ferroportin, the protein that transports iron from enterocytes and macrophages into the blood.

Recent findings have shown the central role of hepcidin in hemochromatosis. In fact, many of the mutations that lead to hemochromatosis, whether in *HFE, HAMP, HJV, TfR2*, all lead to decreased hepcidin production or impaired hepcidin function. The lack of hepcidin first manifests as increased serum transferrin saturation levels. Later, increased serum ferritin levels are found and eventually increased serum iron levels are seen. This chronic excess of blood iron levels eventually leads to the accumulation of hemosiderin deposits in the liver and other organs.

5. MUTATIONS IN IRON RELATED GENES

"Cliff notes" version of genetic hemochromatosis

There are a number of mutations that lead to hemochromatosis. The number will probably continue to grow with time. Despite this, these conditions share a core set of common findings as listed below. I have found it very useful to understand genetic iron diseases by remembering these basic observations:

1. As noted previously, a common mechanism is that all mutations, at least in part, involve abnormally low levels or dysfunction of hepcidin
2. Most mutations are inherited—new sporadic mutations appear to very rare.
3. Most mutations are recessively inherited.
4. The clinical consequences include iron deposition in the liver, heart, joints, and endocrine organs. There is an established increased risk for hepatocellular carcinoma and possibly increased risks for cholangiocarcinoma as well as other non-hepatic malignancies.
(5) Blood findings progress in severity from elevated transferrin saturation levels, to elevated ferritin levels, to elevated iron levels.

(6) Histologically, iron is deposited primarily in hepatocytes. Classic findings on iron stains for hemochromatosis include a zone 1 distribution of iron deposits, a peri-canalicular pattern of iron deposits within the hepatocyte cytoplasm, and iron deposits in bile duct epithelial cells. These features are all fun to find, but they are neither very sensitive nor specific for hemochromatosis.

(7) Clinical management revolves around phlebotomy, which can be life saving as it can prevent the clinical sequelae listed above (No. 4). Individuals have intact erythropoiesis so tolerate phlebotomy well.

**HFE mutations**

HFE mutations were first linked to hereditary hemochromatosis in 1996. Since that time, over 37 mutations in this gene have been reported, but by far the most numerically and clinically important are C282Y and H63D mutations. C282Y mutations are strongly linked to northern European genetic ancestry, while H63D mutations have a wider ethnic distribution. 35% of those with northern European ancestry will have a HFE mutation (Table 1).

Overall, the C282Y mutation accounts for 80% to 90% of genetic hemochromatosis cases, while H63D accounts for approximately 60% of the remaining cases of genetic hemochromatosis. Other mutations, such as S65C, have also been linked to iron accumulation but these mutations are significantly less common and data on their clinicopathological significance is limited.

Gene penetrance is variable for all HFE mutations and to accommodate this, four clinical stages of the disease have been defined: genetic predisposition without abnormality, asymptomatic iron overload, iron overload with early symptoms, and iron overload with organ damage most commonly seen in the liver, heart, joints, pancreas and other endocrine organs.

Individuals with C282Y mutations are at higher risk for iron accumulation than those with H63D mutations. Not surprisingly, C282Y homozygotes are at higher risk for iron accumulation than are C282Y heterozygotes. However, there is great phenotypic variation, even in individuals with C282Y homozygosity, underscoring the importance

<table>
<thead>
<tr>
<th>Genetic status</th>
<th>Population frequency</th>
</tr>
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<tbody>
<tr>
<td>C282Y heterozygote</td>
<td>9.2</td>
</tr>
<tr>
<td>C282Y homozygote</td>
<td>0.4</td>
</tr>
<tr>
<td>H63D heterozygote</td>
<td>21.6</td>
</tr>
<tr>
<td>H63D homozygote</td>
<td>2.0</td>
</tr>
<tr>
<td>C282Y/H63D compound heterozygote</td>
<td>1.8</td>
</tr>
<tr>
<td>Wild/Wild</td>
<td>65.1</td>
</tr>
</tbody>
</table>
of other factors such as polymorphisms or mutations in other genes, environmental influences, and demographics such as age and gender. For example, in a major population-based study from Australia, 203 individuals who were homozygous for C282Y mutations were followed for 12 years. Twenty-eight percent of men, but only 1% of women, developed iron-overload related diseases.\textsuperscript{8} This same research group also examined C282Y/H63D compound heterozygotes and found that only 1/82 men and none of 95 women developed iron overload related disease over a 12 year study interval.\textsuperscript{9} This and other data argues for a strong protective effect for female gender. However, this does not appear to be solely due to physiological blood loss and other gender associated polymorphisms appear likely (reviewed in Wood et. al.).\textsuperscript{10}

<table>
<thead>
<tr>
<th>Data</th>
<th>Milman et al\textsuperscript{11}</th>
<th>Niederau et al\textsuperscript{12}</th>
<th>Fargion et al\textsuperscript{13}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of deaths</td>
<td>147</td>
<td>69</td>
<td>44</td>
</tr>
<tr>
<td>Length of follow-up</td>
<td>8.5 yrs, median</td>
<td>14 yrs, mean</td>
<td>4 yrs, median</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Danish</td>
<td>German</td>
<td>Italian</td>
</tr>
<tr>
<td>Causes of Death (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cirrhosis, no cancer</td>
<td>32</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>23</td>
<td>28</td>
<td>45</td>
</tr>
<tr>
<td>Non-liver cancer</td>
<td>11</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>11</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>5</td>
<td>Not stated</td>
<td>Not stated</td>
</tr>
<tr>
<td>Respiratory disease</td>
<td>5</td>
<td>Not stated</td>
<td>Not stated</td>
</tr>
</tbody>
</table>
The mechanism by which HFE mutations lead to iron accumulation are incompletely understood. At this time there are two major theories. The first suggests that the HFE protein is critical in determining the enterocytes internal “set-point” for determining its cellular iron state. With HFE mutations, the enterocyte set-point incorrectly indicates the cell is iron-deficient, leading to increased enterocyte absorption of iron. The second theory focuses on the observation that, for incompletely understood reasons, individuals with HFE mutations have abnormally low plasma hepcidin levels. These low levels of hepcidin then lead to gradual excess iron absorption and deposition in the hepatocytes and other organ tissues. Both theories have supporting data from animal models as well as human observations, suggesting that both will be at least partially correct in the end.

Causes of death in HFE related hemochromatosis

Clinical follow-up studies have consistently identified liver de-compensation from cirrhosis as well as hepatocellular carcinoma as leading causes of death in individuals who are untreated or incompletely treated for HFE hemochromatosis (Table 2). However, there is also an increased risk for morbidity from heart failure and complications of diabetes. An increased risk for non-liver cancer has also been identified in some but not all studies. Treatment by phlebotomy can substantially lower the risk of death. A single unit of blood can safely remove 200-450 mg of iron and over a period of time, usually a year or two, phlebotomy can restore safe levels of iron within the blood.2

Liver transplantation for HFE iron overload

Overall, hereditary hemochromatosis is an uncommon indication for liver transplantation. An early study of liver transplant outcomes that examined 5,180 liver transplantations reported only 56 (1%) of the transplantations were for hemochromatosis.14 This and other early studies reported an overall decreased post transplant survival rate for patients with hereditary hemochromatosis compared to those transplanted for other causes of chronic liver disease, with major causes of mortality including infection, cardiac failure, and cancer.14-16 However, a more recent study has shown great improvement over the last decade in the survival of individuals transplanted for hemochromatosis.17 This increased survival likely reflects better patient selection and better pre and post-transplant management. Despite this, cardiovascular disease continues to be an important cause of morbidity and mortality.17

Hemojuvelin mutations (usually children/early onset). Hemojuvelin mutations are the most common cause of juvenile hemochromatosis. Nevertheless, this remains are relatively rare disease. There can be marked hepatocellular iron overload and the disease typically runs a severe clinical course.
*Hepcidin (usually children/early onset).* This rare form of genetic iron overload has marked hepatocellular iron overload and typically runs a severe clinical course. Hypogonadism and cardiac disease are also prominent clinical manifestations.

*Transferrin receptor gene 2 (usually adults/late onset).* This rare form of genetic iron overload has a variable clinical course but can have marked hepatocellular iron accumulation.

*DMT-1 mutations (usually older children).* This very rare disease has very few reported cases (about 4 to date) so data is quite limited. Children present with severe microcytic anemia. Iron accumulation is primarily in hepatocytes but biopsies can be negative for iron in very young children.

Key points on these variation mutations can be reviewed in Table 3.

*Non-hemochromatotic iron over-load disease (ie mesenchymal iron accumulation)*

Ferroportin disease is a classic example of hereditary iron overload where the iron accumulation can be predominately in Kupffer cells. In contrast to the causes of hemochromatosis discussed above, all of which have elevated transferrin saturation levels early in the disease course, transferrin saturation levels in ferroportin disease do not become elevated until much later in the disease course. Ferroportin disease also stands out for its dominant inheritance pattern. Of note, there is substantial phenotypic variability and the disease is divided into two subtypes with different disease manifestations. Several other rare forms of genetic non-hemochromatotic iron overload disorder are also listed in Table 3.
Table 3. Overview of Genetic iron diseases involving the liver

<table>
<thead>
<tr>
<th>Gene</th>
<th>AKA (some names used in the literature)</th>
<th>Chromosome</th>
<th>Transmission</th>
<th>Onset</th>
<th>Iron location</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE</td>
<td>Hemochromatosis type 1</td>
<td>6p21.3</td>
<td>Recessive</td>
<td>Late</td>
<td>Hepatocytes &gt; Kupffer cells</td>
</tr>
<tr>
<td>HJV (hemjuvelin)</td>
<td>Juvenile hemochromatosis type 2A</td>
<td>1p21</td>
<td>Recessive</td>
<td>Early</td>
<td>Hepatocytes &gt; Kupffer cells</td>
</tr>
<tr>
<td>HAMP (hepcidin)</td>
<td>Juvenile hemochromatosis type 2B</td>
<td>19q13.1</td>
<td>Recessive</td>
<td>Early</td>
<td>Hepatocytes &gt; Kupffer cells</td>
</tr>
<tr>
<td>TfR2</td>
<td>Hemochromatosis type 3</td>
<td>7q22</td>
<td>Recessive</td>
<td>Late</td>
<td>Hepatocytes &gt; Kupffer cells</td>
</tr>
<tr>
<td>SCL11A2 (DMT-1)</td>
<td>None yet</td>
<td>12q13</td>
<td>Recessive</td>
<td>Early</td>
<td>Hepatocytes &gt; Kupffer cells</td>
</tr>
<tr>
<td>SLC40A1 (ferroportin)</td>
<td>Ferroportin disease type B</td>
<td>2q32</td>
<td>Dominant</td>
<td>Late</td>
<td>Hepatocytes &gt; Kupffer cells</td>
</tr>
</tbody>
</table>

Diseases with iron deposited primarily in mesenchymal cells

| SLC40A1 (ferroportin) | Ferroportin disease type A (hemochromatosis type 4) | 2q32       | Dominant     | Late  | Kupffer cells > hepatocytes    |
| Tf (Transferrin)      | Hypotransferrinemia                          | 3q21       | Recessive    | Early | Kupffer cells > hepatocytes    |
| CP (Ceruloplasmin)    | Hypoceruloplasminemia                        | 3q23-35    | Recessive    | Late  |                               |

Links to other chronic diseases
There are complex genetic, environmental, and dietary variables that determine the penetrance of disease in individuals with mutations in iron metabolism genes such as HFE. Thus, it is not surprising that other chronic liver diseases have been linked to iron overload and/or HFE mutations. As discussed in more detail in section 8, certain diseases such as alpha-1-antitrypsin and cryptogenic cirrhosis (many of which are now presumably NAFLD related) can show marked iron accumulation, with iron levels that equal those of genetic hemochromatosis. Whether or not such cases are enriched for HFE mutations is unclear and will require future studies, but it seems biologically plausible for one HFE mutation to predispose to iron accumulation, and for that predisposition in turn to become increasingly penetrant in the setting of a second significant liver disease.

The relationship between disease severity and the presence of iron accumulation and/or the presence of HFE mutations has been investigated by numerous studies for many of the major chronic liver diseases, including chronic viral hepatitis C and B, alcohol related liver disease, and non-alcoholic fatty liver disease. While the data is substantially mixed, there is evidence to support an association between more severe disease and excess iron accumulation in all of these chronic liver diseases. The many negative studies highlight the difficulty of identifying what is most likely a modest impact for iron in the very complex setting of clinical cohort studies where it is very difficult to adequately control for all of the factors that have been reported to influence iron status.

6. DETECTION OF IRON IN THE LIVER

Significance

The normal adult liver has between 10 to 36 μmol iron/g dry weight of liver. Iron in the range of 400 μmol and above can cause cirrhosis; lower levels of iron may also be relevant to fibrosis progression if there are concurrent diseases.

Iron stains

The major histochemical stain used to detect iron in the liver is Perls’ Prussian Blue (note that the most correct spelling is Perls or Perls’ Prussian Blue, not Perl’s Prussian Blue). This stain is named after Max Perls, a German pathologist who first suggested the stain. The basic chemistry of Perls’ Prussian Blue is that iron in the ferric state will react with hydrochloric acid to form ferric ferrocyanide, an insoluble blue compound (Prussian Blue) that can be seen histologically. The distribution and density of blue staining correlates, albeit imperfectly, with tissue iron concentrations. The stain is not as sensitive for very low levels of iron but is easier and more reproducible than other methods such as the Tirmann-Schmeltzers method, which can identify both ferric and ferrous forms of iron.

Ferritin: Normally no ferritin will be seen. However, in cases of elevated serum ferritin levels, ferritin may be seen as a light, diffuse, blue blush of the hepatocyte or Kupffer cell cytoplasm.
**Hemosiderin**: Hemosiderin can be seen as brown granular deposits on H&E stains and as a bright blue granular staining on iron stain. Residual brown granular material is often seen on iron stain and represents lipofuscin in most cases.

**Iron grading systems**

There are many iron grading systems that have been proposed over the years. They vary considerably in their approach: some are based on zonation of iron distribution, some on the lowest magnification that discernable granules can be seen, some on the percent of hepatocytes positive for iron. There is a nice summary of these iron grading systems available on line at [http://tpis1.upmc.com:81/tpis/dlp/DLPHome.html](http://tpis1.upmc.com:81/tpis/dlp/DLPHome.html), then click on the Chapter 9 and find Table 9-3. This book chapter is somewhat dated and does not cover several newer systems, but is still very useful. The system by Turlin et. al.\(^\text{18}\) has the advantage of having been validated, but it is too complex to be readily adopted for routine diagnostic use.

Is one system clearly the best? Probably not, but I personally use a schema (Table 4) based on the percent of hepatocytes positive for iron, similar to that described by LeSage et. al.\(^\text{19}\) For routine diagnostic purposes, I include the descriptor (e.g. “mild” etc) in the pathology report but do not routinely provide the corresponding numerical grade. I believe that this simple-to-use classification system provides sufficient clinical information for patient care. But there are many reasonable alternatives to consider if you prefer a different approach. A modified Scheuer’s system (shown in Table 5) is also a very useful and popular system. If employed, separate numbers should be given for hepatocellular and the reticuloendothelial iron.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
<th>Hepatocytes</th>
<th>Lobular Kupffer cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>1</td>
<td>Minimal</td>
<td>&lt; 5%</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>2</td>
<td>Mild</td>
<td>5-30%</td>
<td>5-30%</td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>31-60%</td>
<td>31-60%</td>
</tr>
<tr>
<td>4</td>
<td>Marked</td>
<td>&gt;60%</td>
<td>&gt;60%</td>
</tr>
</tbody>
</table>

Table 4. My iron scoring system (similar to that of LeSage)\(^\text{19}\)

Note: For studies, I also record the zonal pattern of iron and whether the distribution is homogenous. For some studies, I also record endothelial iron and portal macrophage iron.
Table 5. Modified Scheuer's

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Iron granules absent or</td>
</tr>
<tr>
<td></td>
<td>Iron granules barely seen at 400X</td>
</tr>
<tr>
<td>1</td>
<td>Iron granules resolved at 250X</td>
</tr>
<tr>
<td>2</td>
<td>Iron granules resolved at 100X</td>
</tr>
<tr>
<td>3</td>
<td>Iron granules resolved at 25X</td>
</tr>
<tr>
<td>4</td>
<td>Iron deposits resolved at 10X or</td>
</tr>
<tr>
<td></td>
<td>Iron deposits visible without magnification</td>
</tr>
</tbody>
</table>

Quantitative measurement of hepatic iron concentrations

As noted previously, the normal adult liver has between 10 to 36 μmol iron/g dry weight of liver. Hepatic iron concentrations measured in fresh liver tissues or in paraffin embedded tissues are equivalent. Thus, paraffin embedded tissues are preferred over fresh tissues in most cases because it allows direct visualization of the tissue and assures the tissue is representative. This prevents submission of tissue that is largely composed of collapsed/fibrotic stroma or a nodule that is either unusually high or low in stainable iron compared to the rest of the tissue. Excess iron accumulation has been classified as mild (up to 150 μmol iron/g dry weight of liver), moderate (151-300), and marked (>301). Iron levels greater than 400 μmol are the most strongly associated with cirrhosis, but lower levels of iron also contribute to fibrosis progression in the setting of other liver diseases.

Hepatic Iron Index

Historically, the hepatic iron index was calculated as an aide to interpreting quantitative tissue iron levels. The hepatic iron index adjusts the total iron concentration for age, based on the observation that hepatic iron concentrations tend to increase steadily with age in individuals with genetic hemochromatosis, but not in individuals with “secondary” iron overload. In a non-cirrhotic liver, a hepatic iron index greater than 1.9 was considered suggestive of genetic hemochromatosis. Given the advances in understanding the causes of hemochromatosis and the readily available genetic testing for HFE mutations in many parts of the world, the diagnostic role of the hepatic iron index has diminished in
importance, but direct measurement of hepatic iron concentrations remain useful in guiding therapy and we still get many requests for blocks to be submitted for quantitative iron analysis.

**Non-invasive measurements of hepatic iron**

MRI based imaging studies have advanced in recent years to the point that they can reasonably assess iron accumulation and can also distinguish hepatic from reticuloendothelial iron deposits. There have been multiple validation studies and MRI has established for itself an important role in measuring iron in the liver. Recent expert opinion review articles on hemochromatosis have highlighted the changing role of the biopsy in managing patients with HFE hemochromatosis. Biopsies continue to be important in determining the fibrosis stage and to search for any associated lesions (eg evaluation of mass lesion). However, some experts foresee a further diminution of the role for liver biopsies with the advent of non-invasive markers of liver fibrosis.

7. **HISTOLOGICAL FINDINGS**

In genetic hemochromatosis, iron classically accumulates initially within zone 1 hepatocytes. A clear gradient in the amount of iron between zone 1 and zone 3 hepatocytes can often be seen, even with advanced iron accumulation. In addition, the iron distribution often has a distinctive clustering around the bile canaliculi. With time, injury and death of hepatocytes will lead to a redistribution of iron into Kupffer cells and portal macrophages. However, a zone 1 distribution of iron can be seen in other non-hemochromatosis conditions, particularly once a liver is cirrhotic, and a diagnosis of hemochromatosis should not be based on recognizing a zonal pattern alone. My personal opinion is that the zone 1 predominate pattern of iron deposition can be seen whenever there is dysregulation of hepcidin, either through mutations or through reduced hepcidin production from other causes.

Iron can also be seen in biliary epithelium on iron stain. First, iron is commonly seen in proliferating bile ductules in areas of subacute parenchymal collapse in cirrhotic or non-cirrhotic livers. This finding appears to have no association with hemochromatosis. Iron can also be deposited in the epithelium of the bile duct proper. In my experience, this pattern of iron deposition tracks better with the overall severity of iron deposition within the liver and less so with HFE mutations per se. However, there is very little data that examines this specific question.

With iron overload due to transfusion dependent anemias and similar causes, iron is classically first deposited in Kupffer cells and with time there is involvement of the hepatocytes. However, in practice most cases show a mixed hepatocellular and Kupffer cell iron staining pattern.

Iron can also be seen in some cases either exclusively in portal endothelial cells or in a combination of endothelial, hepatocyte, and Kupffer cell iron accumulation. At this time, there has not been any specific linkage of endothelial iron accumulation to a disease process or genetic mutation. In
one study, endothelial iron positivity was linked to decreased interferon response in individuals with chronic hepatitis C infection.\textsuperscript{22}

8. CLINICOPATHOLOGICAL SIGNIFICANCE OF IRON OVERLOAD

Iron in explanted livers

Iron can accumulate in cirrhotic livers of individuals who do not have clinical findings of genetic hemochromatosis. In a classic study by Ludwig et. al., iron stains were positive in 32\% of 447 liver explants with varying underlying liver diseases. For those diseases with at least 5 cases in this study, the proportions of cases with any degree of positivity by iron stain were as follows: hereditary hemochromatosis (100\%), cryptogenic cirrhosis (65\%), alcohol cirrhosis (63\%) chronic hepatitis B cirrhosis (65\%), A1AT cirrhosis (56\%), chronic hepatitis C cirrhosis (42\%), primary biliary cirrhosis (10\%), and primary sclerosing cholangitis (7\%).

In this same study, the number of cases with a hepatic iron index of greater than 1.9 were as follows: HH (100\%), A1AT (28\%), cryptogenic cirrhosis (19\%), alcohol cirrhosis (14\%), chronic hepatitis B cirrhosis (18\%), chronic hepatitis C cirrhosis (7\%), primary biliary cirrhosis (1\%), and cirrhosis from primary sclerosing cholangitis (1\%). This and other data sets document that other diseases can have iron deposition within the liver and that in alpha-1-antitrypsin deficiency and in cryptogenic cirrhotic livers, 20\% or more of cases can have hepatic iron indexes greater than 1.9. Another important observation from these data is that biliary cirrhosis is only rarely associated with iron overload.

An important study by Kowdley et al.\textsuperscript{23} found that patients with significant hepatic iron accumulation had decreased survival following transplantation regardless of whether they had an HFE mutation. The reason(s) for this are unclear, but at least in a subset of these individuals, there can be significant extrahepatic stores of iron at the time of transplantation, often which are clinically unrecognized.\textsuperscript{24} The stress of surgery or other post transplant factors may then place this group of patients at increased risk for heart failure.

When examining an explanted liver with iron overload, any foci (even smaller subcentimeter foci) with decreased iron deposition should be targeted for sectioning to evaluate for carcinoma. These “iron free foci” are often associated with dysplastic nodules or with frank carcinoma. They can rarely be seen on needle biopsies also, and, when present, should be indicated in the report as well as fully evaluated for malignancy.

Iron in donor liver biopsies
There is very little data on the relevance of iron levels in donor livers (an interesting area that hopefully will get more attention in the future). One study is available that looked at the significance of donor iron for subsequent fibrosis progression in individuals transplanted for chronic HCV. Counter-intuitively, they found a link between female gender, pre-transplant iron content, and risk for fibrosis progression.\(^{25}\)

**Iron in liver tissues with chronic hepatitis C Virus infection**

Iron deposits, including both hepatocellular as well as reticuloendothelial, are seen in liver biopsies of approximately 5 to 48% of individuals with chronic HCV.\(^{26-31}\) Overall, the median is approximately 30% for these studies and the variation presumably reflects differences in gender, viral genotypes, and the proportion of cirrhotics in the cohort. Livers with genotype 3 infection tend to have more hepatocellular iron than other genotypes.\(^{29}\) In the majority of cases, the iron deposits are mild, occasionally moderate, and only very rarely severe.

A large body of literature has been published on the question of the significance of HFE mutations in chronic HCV. Some of the larger studies are summarized for you in Table 6 on the following page. Unfortunately, despite all of the work, the literature is substantially mixed on the question of whether HFE mutations increase the risk for fibrosis progression. This current state of confusion likely reflects the many different study populations, study designs, as well as variable penetration of genetic hemochromatosis. Many studies also do not adequately control for potentially confounding variables such as gender, viral genotypes, duration of HCV infection, etc. Nevertheless, one reasonable way to synthesize the data is as follows: (1) individuals with chronic HCV do not have an increased risk for HFE mutations;\(^{26,32-34}\) (2) once an individual has chronic HCV infection, HFE mutations may increase the rate of fibrosis progression\(^ {35}\) and the presence of HFE mutations is associated with higher fibrosis stages in many\(^ {26,32-37}\) but not all studies.\(^ {27,28}\) The strength of the association between HFE mutations and fibrosis has been measured by both relative risks, where a relative risk of 4.6 has been reported,\(^ {34}\) as well as odds ratio, where odds ratios for C282Y heterozygosity has been reported ranging from 2.5 to 30.\(^ {26,32,35}\) Overall, C282Y alleles appear to have a stronger risk for fibrosis than H63D alleles.\(^ {35}\) With a sufficiently long duration of chronic HCV infection, the risk of cirrhosis is high regardless of HFE mutational status and the effect of HFE mutations may be harder to discern.\(^ {35}\)

Interestingly, for unclear reasons, HFE mutations have also been linked to increased inflammation on liver biopsy in some studies.\(^ {33,34}\) Despite the observations linking HFE mutations to increased fibrosis and less consistently to increased inflammation, HFE mutation status has typically not been associated with increased iron deposits by histochemical analysis.\(^ {28,33,34}\) As an exception, H63D, but not C282Y mutations, were associated with increased hepatic iron concentrations in one study.\(^ {35}\)

Most of the data discussed above is from studies that looked at HFE mutations. The question then naturally arises of the meaning of mild to moderate iron deposits in individuals with chronic HCV.
who lack HFE mutations. Unfortunately, the data is no clearer on this point than it is for HFE mutations and the same “take home message” as above appears to apply: most likely there is either no role or a very limited role for minimal or very mild iron on a liver biopsy in terms of fibrosis progression; for moderate iron there is likely a modest role. For marked iron accumulation, a role in fibrosis progression seems likely even it has not yet been specifically demonstrated.
<table>
<thead>
<tr>
<th>Study</th>
<th>Study design, tissue</th>
<th>N</th>
<th>Study location</th>
<th>Demographics Mean age (yrs); gender</th>
<th>Associations with HFE mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tung</td>
<td>Cross sectional, biopsies and explants</td>
<td>316</td>
<td>USA</td>
<td>46; 71%M</td>
<td>Serum: C282Y: no associations</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serum: H63D: increased iron, TIBC, tran sat, ferritin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: C282Y: odds ratio 30 for advanced fibrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: H63D: odds ratio 22 for advanced fibrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: any mutation: odds ratio 18 for advanced fibrosis</td>
</tr>
<tr>
<td>Geier et al</td>
<td>Cross sectional, consecutive biopsies</td>
<td>166</td>
<td>Germany</td>
<td>42; 60%M</td>
<td>Serum: C282Y: increased iron, ALT, AST,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serum: H63D: increased iron, tran sat, ferritin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: C282Y: increased inflammation, fibrosis. <em>Not iron stain</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: H63D: increased fibrosis. <em>Not inflammation, Not iron stain</em></td>
</tr>
<tr>
<td>Gehrke</td>
<td>Cross sectional, biopsies</td>
<td>256</td>
<td>Germany</td>
<td>42, 63%M</td>
<td>Serum: C282Y: increased ferritin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serum: H63D: increased ferritin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: C282Y: odds ratio 2.5 for advanced fibrosis, increased stainable iron</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: any association with fibrosis</td>
</tr>
<tr>
<td>Erhardt</td>
<td>Cross sectional, biopsies</td>
<td>401</td>
<td>Germany</td>
<td>48, 60%M</td>
<td>Serum: C282Y: increased ferritin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(217 biopsied)</td>
<td></td>
<td></td>
<td>Serum: H63D: increased ferritin, increased trans sat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: C282Y: increased fibrosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: H63D: increased fibrosis.</td>
</tr>
<tr>
<td>Thorburn</td>
<td>Cross sectional, consecutive biopsies</td>
<td>164</td>
<td>United Kingdom</td>
<td>36, 63%M</td>
<td>Blood: no associations</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: no associations</td>
</tr>
<tr>
<td>Valenti</td>
<td>Cross sectional, consecutive biopsies</td>
<td>143</td>
<td>Italy</td>
<td>50, 60%M</td>
<td>Serum: mutation data combined: associated with increased ferritin and tran sat</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver: no associations</td>
</tr>
</tbody>
</table>
Negro\textsuperscript{28} Cross sectional, biopsies 120 Switzerland 42, 67\%M Liver: no association with inflammation or fibrosis

Martinelli Cross sectional, biopsies 135 (102 biopsied) Brazil 36; 100\%M Serum: C282Y: increased iron
Serum: H63D: increased iron, tran sat Liver: mutations data combined: increased inflammation, increased fibrosis; \textit{Not iron.}

\textsuperscript{2}Median age

Of note, there is only limited longitudinal data or paired biopsy studies that examine the role of iron in fibrosis progression and it is hoped that future studies will permit a more accurate and nuanced understanding of the role of iron in fibrosis progression. One of the few paired biopsy studies that specifically analyzed the role for iron found no association with fibrosis progression in 214 individuals, but the time interval between biopsies was only 2.5 years, which limits the findings general applicability.\textsuperscript{38}

Mutations in the TFR1 gene have also been investigated by several groups in the context of chronic HCV infection, but no relationship to the severity of disease has been found.\textsuperscript{26,37} TFR2 mutations appear to be very rare and data is limited.\textsuperscript{31,34}

\textit{Iron in non-alcoholic fatty liver disease (NAFLD)}

Iron deposition in NAFLD is common, with about 30 to 40\% of liver biopsies showing iron accumulation. As with chronic viral hepatitis, in most cases the siderosis is mild and may involve either or both of the hepatic and Kupffer cell compartments. Moderate iron accumulation is much less common and marked iron accumulation is rare. The role of iron in fibrosis progression is even less clear than with chronic HCV. In one of the first studies to address this question, George et. al. found in a study of 51 patients that increased iron on Perls iron stain was associated with increased fibrosis, with a relative risk of 5.5.\textsuperscript{39} However, several other studies have not been able to identify an increased risk for fibrosis in cases of siderosis and NAFLD.\textsuperscript{40,41} This topic has been recently reviewed in detail by Sumida et. al.\textsuperscript{42}

\textit{Iron overload and Liver Carcinoma}

There is a high risk for hepatocellular carcinoma in individuals with genetic hemochromatosis and marked iron accumulation. The risk increases further with the combination of iron accumulation and cirrhosis, but hepatocellular carcinomas can arise even in non-cirrhotic livers. The risk was
previously estimated to be extremely high but more recent data suggests a lower, but still elevated, risk. Precursor lesions include iron free foci. Most liver carcinomas in genetic hemochromatosis are hepatocellular carcinomas, but intrahepatic cholangiocarcinomas have also been reported.

9. ACQUIRED IRON OVERLOAD

The topic of siderosis in either the hepatic or Kupffer cell compartment as an acquired condition is usually considered under the notion of “secondary iron overload”. This classification approach, of primary versus secondary iron accumulation, has historically been very useful as a tool in classifying iron accumulation, but it has not been seriously updated to match the current state of knowledge with the various new genetic mutations. Nevertheless, several broad categories deserve brief consideration below. While the classic description of iron deposition in these conditions is that of an exclusive or predominant Kupffer cell or macrophage pattern, in actual practice a mixed pattern of Kupffer cell and hepatocellular iron accumulation is almost always seen.

Hematological disorders

In routine surgical pathology practice, it is fairly common to see hepatic siderosis in liver biopsies of patients with various hematological disorders including sickle cell disease, thalessemia, etc. Liver biopsies in individuals with bone marrow transplants also commonly show excess iron accumulation.

Anemia of chronic disease

Since hepcidin is an acute phase reactant, chronic inflammatory conditions can lead to mild siderosis that involves primarily Kuppfer cells and to lesser extent hepatic iron accumulation.

Excess iron intake

Hepatic siderosis secondary to excess dietary intake is unusual, but rare cases do occur. Almost always such cases are seen in the setting of dietary/vitamin supplements. It is much more common to see mild siderosis in individuals who have had multiple blood transfusions.

Chronic liver disease

As discussed in more detail above, chronic viral hepatitis and chronic fatty liver disease often have mild siderosis involving both the Kupffer cells and the hepatocytes. Both individuals with and
without HFE mutations may be affected. The mechanism varies with the underlying liver disease, but as an example, alcohol has been shown to inhibit hepcidin expression. Other studies have also suggested that non-alcoholic fatty liver disease may be associated with a relative hepcidin resistance state.

10. SUMMARY

- Many new mutations leading to iron overload have been reported.
- Those that lead to hepatic iron accumulation all have a shared mechanism through their impact on the levels of hepcidin.
- Hepcidin blocks iron absorption from the gut and blocks iron release from hepatocytes and macrophages/Kupffer cells.
- Recent data indicates improvement in patient survival after liver transplantation for HFE, but cardiac disease continues to be a cause of morbidity and mortality.
- Marked iron overload in an explanted liver can be clinically important, even if HFE mutations are negative.
- Individuals with genetic hemochromatosis have an increased risk for hepatocellular carcinoma and possibly for cholangiocarcinoma and other non-liver malignancies.
- The role of HFE mutations in disease progression in chronic HCV and non-alcoholic fatty liver disease is controversial: there appears overall to be a modest affect on fibrosis progression.
APPENDIX. FREQUENTLY ASKED QUESTIONS ABOUT IRON

For a biopsy performed to stage and grade chronic viral hepatitis, what is the significance of iron on the iron stain?

A. The data on this question is quite mixed. Nevertheless, a reasonable distillation of the data is as follows: iron accumulation most likely has a small but measurable impact on fibrosis progression. However, other known risk factors for fibrosis progression, such as viral genotype, duration of infection, gender, etc, appear to have a stronger and more consistent impact on fibrosis than iron accumulation. The risk for fibrosis progression and iron accumulation can most likely be further stratified by the extent of iron accumulation, with marked iron have the greatest risk.

I have a liver biopsy where the only iron is present in endothelial cells. What does this mean?

A. While not common, this can be seen in a small proportion of cases, especially if iron stains are carefully examined at higher magnifications. One study reported that individuals with chronic HCV and endothelial iron had lower responses to interferon therapy, but this study has not been replicated and there is no established clinical significance at this time.

Is an iron stain necessary as part of the “standard of care” for evaluating a liver biopsy?

A. I am aware of no evidence based data on this point. I do a routine iron stain in my practice. I suspect that most pathology practices also include an iron stain as part of the routine evaluation of liver biopsies. It seems likely that an approach based on ad hoc ordering of iron stains after examining the H&E stain would be unlikely to miss cases with moderate or marked iron accumulation. Many cases with minimal or focal mild iron would be missed I suspect, but since the clinical relevance of these lower grades of iron is not well established, it would seem unlikely to materially impact patient care.

In an explanted liver, what is the significance of findings moderate or marked grades of hepatocellular iron in the situation where there is already a known cause of the liver disease, such as chronic hepatitis C?
A. Moderate or marked iron accumulation carries an increased risk of having an HFE mutation. However, many cases with marked iron, including those with biliary epithelial iron accumulation as well as those with hepatic iron indexes of greater than 1.9, will not have HFE genetic mutations. Because of this, genetic testing is required if the patient/clinical team wants to determine the status of the HFE gene.

Some patients with marked iron accumulation in their explanted livers can also have systemic iron overloading, even if HFE mutational studies are negative. These individuals have an increased risk of cardiac iron deposition and some can develop significant cardiac disease post transplantation.

*The iron stain shows a diffuse light cytoplasmic staining of the hepatocytes. What does this mean?*

A. Typically this is ferritin and is more commonly seen in cases with elevated serum ferritin levels. Ferritin can also be seen in macrophages.

*Do I need to formally grade the iron in liver specimens in routine practice of surgical pathology?*

A. It is prudent patient care to provide information on the amount of iron accumulation in the hepatocellular and Kupffer cell compartments that is sufficiently detailed to be clinically actionable when appropriate. A description is sufficient for this purpose and there is no data to support an additional need to provide a formal number based on a specific scoring system.

However, if the pathologists or clinicians prefer a formal numerical assessment, that is fine. Sufficient scoring system detail should then be provided to allow a reader of the report to determine what the numbers mean (and should be in the body of the report; a statement that the grading system is “on file” or “available on request” is suboptimal). As an example, a statement of the sort “iron grade 2” is in itself fairly useless and is strongly discouraged as neither the magnitude of the scale nor the location of the iron is apparent from this statement.
What is the best grading system for evaluating histological iron accumulation?

A. There are many adequate grading systems. They can be very useful in research studies and the specific system can be chosen based on the goals of the study. Please see Tables 4 and 5 for two useful approaches.

Does iron in the bile duct epithelium have special significance? How about in the bile ductules?

A. Iron in the bile duct epithelium is typically a marker of heavy iron accumulation, but it is not a marker of HFE mutations per se.

Iron in proliferating bile ductules can be seen particularly in areas of parenchymal collapse, even in livers with only modest iron accumulation, and does not indicate HFE mutations are present.

References


Diagnosis of well-differentiated hepatocellular lesions: role of immunohistochemistry and other ancillary techniques

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The most common problems encountered in the differential diagnosis of hepatocellular lesions are: (a) adenoma vs well-differentiated hepatocellular carcinoma (HCC) in non-cirrhotic liver, (b) focal nodular hyperplasia (FNH) vs adenoma in non-cirrhotic liver, and (c) HCC vs high grade dysplastic nodules and other non-neoplastic nodules in cirrhotic liver. This discussion provides an update of immunohistochemistry and other techniques that aid in this differential diagnosis.

A. ADENOMA vs WELL-DIFFERENTIATED HEPATOCELLULAR CARCINOMA

HCC is distinguished from hepatic adenoma based on presence of wide cell plates (>3 cells thick), prominent acinar pattern, small cell change, cytologic atypia, mitotic activity, vascular invasion, absence of Kupffer cells and loss of reticulin network. However, some or most of these features are often not present in well-differentiated HCC. On the other hand, atypical features like nuclear atypia and acinar architecture can be focally present in hepatic adenomas. It has been shown that tumors that morphologically resemble adenoma can recur and metastasize, especially in males and patients over 50 years (1). The natural history and management for hepatic adenoma and HCC are different and hence it is crucial to make this distinction. The following approaches have been used in recent years to distinguish adenoma from HCC or identify adenomas that are at high risk for progression to HCC.

(1) Chromosomal analysis

HCCs show a consistent pattern of chromosomal gains and losses (2,3). The most prominent changes are gains of part or entire chromosome arms 8q (49-81%), 1q (60-79%) and 7q (40-64%), and loss of 16q (36-65%). Other common abnormalities include overrepresentation at sites Xq and 5p, and losses at 4q, 8p, 13q, 16q and 17p. These abnormalities are observed in >80% of well-differentiated HCC, but have not been observed in adenomas in women of reproductive age group.

Cytogenetic analysis can be done on paraffin-embedded tissue by comparative genomic hybridization (CGH) or fluorescence in situ hybridization (FISH). CGH is a cumbersome procedure and not presently suitable for routine diagnostic purposes. On the other hand, FISH can be easily performed on slides obtained from paraffin blocks. Gains of chromosomes 1q and 8q, the two most common abnormalities in well-differentiated HCC have been successfully used to distinguish adenoma and HCC in several studies (4-6). In one study, gains of 1q and 8q were frequently seen in adenoma-like neoplasms in men and patients over 50 years, but not in adenomas in women between 15 and 50 years (6). Some of the former also recurring to metastasized indicating that at least some of these adenoma-like neoplasms in men or patients over 50 years of age may represent well-differentiated HCC.

(2) Gene expression
Wang et al studied the expression of several thousand genes in hepatic adenoma and HCC, and showed that 53 genes were differentially expressed (7). Some of the genes were further validated by reverse transcriptase PCR and immunohistochemistry including insulin growth factor-II, clusterin, estrogen receptor and PCNA. However, this technique and the genes identified by it have not been studied more widely and its utility in needle biopsies remains to be established.

(3) Immunohistochemistry

(a) Glypican-3
Glypican-3 (GPC-3) is a membrane anchored heparin sulfate proteoglycan normally expressed in fetal liver and placenta, but not in normal adult liver. It is an oncofetal antigen that is a reliable serum and histochemical marker for hepatocellular carcinoma. GPC-3 expression has been reported in 70-90% of HCCs in most studies (8-12). Most of the hepatocellular markers used for the diagnosis of HCC like Hep Par 1 and polyclonal CEA are expressed both in benign and malignant hepatocytes. Expression of GPC-3, however, has not been observed in benign hepatocellular lesions by in situ hybridization or immunohistochemistry. However, the sensitivity of GPC-3 in well-differentiated HCC is around 50%, and is likely to be lower in needle biopsies. There are anecdotal observations of GPC-3 expression in histologically typical adenomas in young women. Although the expression of GPC-3 would strongly favor HCC, larger series with follow-up information are necessary to fully establish the utility of GPC-3 in this setting.

(b) Beta-catenin
The Wnt signaling pathway plays an important role in cell adhesion and cell proliferation. Beta-catenin, a key component of this pathway is predominantly bound to cell membranes in normal cells. Mutations in beta-catenin or abnormalities in other components of this pathway can lead to nuclear translocation of beta-catenin that can be demonstrated by immunohistochemistry. Beta-catenin mutations occur in around 20% of HCC, but have been reported in up to 40% in HCC arising in chronic hepatitis C (13).

Recent studies have shown that liver tumors that morphologically resemble adenomas and have nuclear localization of beta-catenin are at higher risk of transformation to HCC (14,15). These tumors often occur in men and show atypical features like acinar architecture and cytological abnormalities. Tumors with borderline features of adenoma and HCC often show nuclear translocation of beta-catenin. As described above, some adenoma-like neoplasms in men and patients over 50 years of age show chromosomal changes typical of HCC. These tumors often show nuclear localization of beta-catenin (16). Hence beta-catenin mutations or nuclear expression of beta-catenin in a hepatocellular neoplasm signifies a high-risk adenoma or an adenoma-like neoplasm that represents an extremely well-differentiated HCC.

(c) Glutamine synthetase
The nuclear translocation of beta-catenin as described above leads to activation of several transcription factors leading to increased expression of several genes that play a key role in cell proliferation. Glutamine synthetase (GS) is one of the genes that is upregulated as a result of nuclear translocation of beta-catenin. Hence GS shows strong and diffuse expression in tumors with beta-catenin mutations. GS is an enzyme that helps in the conversion of glutamine.

In normal liver, GS expression is seen in pericentral hepatocytes, but not by midzonal or periportal hepatocytes. In most hepatic adenomas, GS is negative, localized to the pericentral areas or shows patchy expression with no distinct pattern. In adenoma-like neoplasms and in HCC with beta-catenin
abnormalities, strong and diffuse GS expression in seen in tumor cells (15,16). The significance of strong and diffuse expression is the same as beta-catenin nuclear translocation. In some cases, strong and diffuse GS expression is seen in the absence of nuclear staining of beta-catenin. The reason for this is not clear, but it is likely that these tumors have abnormalities in the Wnt signaling pathway.

B. FNH vs. HEPATIC ADENOMA

The diagnosis of FNH can be achieved by imaging in >70% of cases. Liver biopsy is obtained in cases with atypical features on imaging. The distinction between FNH and adenoma can be challenging on liver biopsies. The presence of scar, nodular architecture, prominent ductular reaction and aberrant arterioles favors FNH. However, the telangiectatic (or variant) adenoma can show ductular reaction and can be especially difficult to separate from FNH. Some recent studies have indicated that immunohistochemistry can be helpful in this regard.

(a) Glutamine synthetase
Immunohistochemistry with glutamine synthetase (GS) demonstrates a characteristic ‘map-like’ pattern of staining in FNH. Large groups of hepatocytes are positive in a relatively continuous anastomosing fashion, often surrounding hepatic veins, whereas GS is not expressed in hepatocytes close to fibrotic bands containing arteries and ductules (17). This staining pattern has been described as very specific for FNH, and is seen in all cases irrespective of size or atypical features. In contrast, most hepatic adenomas are largely negative or show GS staining of a few pericentral, peripheral or scattered hepatocytes (16,17). As described above, adenomas with beta-catenin mutations show diffuse and strong GS expression. This pattern is different from the map-like staining seen in FNH.

(b) Beta-catenin
Mutations in beta-catenin results in nuclear staining in a subset of adenomas as described above. These adenomas show diffuse cytoplasmic staining with GS. Beta-catenin mutations have not been observed in FNH (18,19).

(c) Serum amyloid associated protein
Adenomas with telangiectatic (variant) features show sinusoidal dilatation, ductular reaction and inflammation, and hence resemble FNH. Most telangiectatic (variant) adenomas show strong and diffuse expression of serum amyloid associated (SAA) protein (15,16). Staining with SAA is not seen in FNH.

(d) Cytokeratin 7
In adenomas, CK7 highlights singly scattered or small aggregates of hepatocytes (20,21). The small and intermediate sized hepatocytes show the strongest CK7 expression, while the large mature hepatocytes show no or weak staining. Although bile ducts are typically absent in HA, occasional ductules can be identified with CK7. In contrast, CK7 highlights the ductular reaction in FNH and the hepatocytes are negative or show mild and focal staining. CK7 may not be helpful in the diagnosis of telangiectatic (variant) adenoma as the pattern of staining can overlap with FNH and conventional adenoma (20).

C. DYSPLASTIC NODULES vs EARLY HCC IN CIRRHOTIC LIVER

Small HCC are defined as tumors that are <2 cm. Based on work emanating from Japan in the last few years, small HCCs are divided into two distinct groups (22-24):
(1) Early HCC (vaguely nodular HCC, early well-differentiated HCC) characterized by a vaguely nodular gross appearance. These are extremely well-differentiated and are difficult to distinguish from high-grade dysplastic nodules.

(2) Progressed HCC characterized by a distinctly nodular pattern, easily identifiable thick cell plates. The diagnosis is often obvious in these cases.

Most hepatocellular nodules >2 cm in cirrhotic liver are HCC and can be diagnosed by typical imaging characteristics of arterial hypervascularity and venous washout on triphasic CT or MRI (25-27). Therapeutic approaches like resection and ablation are pursued in these cases based on clinical and radiological features and there is no need for a liver biopsy. Biopsy confirmation is necessary only if imaging features are not typical.

For hepatocellular nodules <2 cm, it is thought that the majority are HCC. However, HCCs <2 cm are often hypovascular and cannot be reliably diagnosed by imaging. Definite diagnosis of smaller nodular lesions is clinically significant for several reasons (23,25,27):
(1) Small HCCs have lower propensity for vascular invasion and offer a high probability of cure.
(2) Allows definite therapy like resection or ablation.
(3) Small HCCs are more amenable to ablation or resection. Complete ablation is more likely to be achieved in small lesions. As per the AASLD Practice Guidelines Committee (27), it is equally important not to apply invasive treatment to lesions that do not have any malignant potential as up to 50% of these nodules can regress spontaneously.
(4) Definite diagnosis of HCC enables patients to receive priority for transplantation.

Since imaging is not reliable, liver biopsy is the only modality that can achieve a reliable diagnosis. Most of the criteria of distinction between high grade dysplastic nodule (HGDN) and well-differentiated HCC (WD-HCC) are quantitative and hence very subjective. The presence of uniformly thick (>3 cells) plates, prominent pseudoglands and loss of reticulin favor HCC. However, these features are not present in all cases of HCC and are more likely to be absent in early HCC. In addition, similar features are focally present in HGDN. Vascular invasion is diagnostic of HCC, but is typically not seen in early HCC. The most reliable morphological criterion that distinguishes early HCC from HGDN is stromal invasion. However, this feature is difficult to assess in biopsies. The following approaches have been used to distinguish HGDN and early HCC.

1. Immunohistochemistry

   (a) Cytokeratin 7
   Stromal invasion into portal tracts, fibrous septa or adjacent parenchyma is considered diagnostic of HCC (22-25). In the setting of chronic liver disease or cirrhosis, small groups of hepatocytes (referred to as hepatocyte buds) can be surrounded by fibrous septa and has to be distinguished from true stromal invasion. The intraseptal hepatocyte buds are contiguous with ductular reaction, and is indicative of regeneration from intrabiliary progenitors (28). This regenerative ductular reaction can be highlighted by CK7 around noninvasive nodules. In areas of stromal invasion, ductular reaction is at least focally absent. Small nodular HCC has more extensive loss of CK7+ ductular reaction compared to early vaguely nodular HCC (28).

   CK7+ ductular reaction is prominent in most cases of regenerative nodules and HGDN. A vast majority of HGDN show ductular reaction around >50% of the circumference of the nodule, but overlap with HCC can be seen in 5-10% of cases (28). CK19 can also be used to highlight the ductular reaction (29).
(b) **CD34**
Most HGDNs show focal staining at the periphery of the nodule. Early HCC (vaguely nodular HCC) typically shows multifocal sinusoidal CD34, while diffuse sinusoidal staining is typical of nodular (progressed) HCC.

(c) **Heat shock protein 70**
Heat shock proteins (HSP) are highly conserved proteins that are expressed in stressful conditions and play an important role in protein homeostasis, regulation of cell cycle progression and apoptosis. Different classes of HSPs are designated by their molecular weight. HSP70 is a potent anti-apoptotic and its overexpression allows cell survival. In a gene expression study comparing early HCC with other hepatocellular nodules, a set of 95 genes provided the molecular signature that distinguishes early HCC from noncancerous tissue (30). Of these, the most abundantly upregulated gene in early HCC was HSP70.

HSP was closely correlated with pathologic parameters of tumor progression like large size, vascular invasion, advanced stage and high Ki-67 index in some (31) but not all studies (32). HSP70 is immunohistochemically expressed in up to 80% of early HCC in resection specimens, but less than 50% of cases on biopsy (32-35). While regenerative cirrhotic nodules are negative, HSP70 expression has been seen in 5-10% of HGDN.

(d) **Glypican-3**
As described in the earlier section, glypican-3 (GPC-3) is an oncofetal antigen that is expressed in HCC but not in benign hepatocellular nodules. The sensitivity of 60-70% has been reported for GPC-3 in early HCC, but is likely to be lower in biopsies. The expression of GPC-3 in HGDN is highly variable across studies ranging from 7-22% (9,10,33) in most series and 75% in one study (11). Other pitfalls in diagnosis include patchy GPC-3 expression in hepatocytes in cases with high necroinflammatory activity (36) and in 4% of regenerative cirrhotic nodules (12).

(e) **Glutamine synthetase**
As described in the earlier section, diffuse cytoplasmic expression of glutamine synthetase (GS) correlates with mutations in beta-catenin. Upregulation of GS mRNA, protein and activity has been described in human HCC with stepwise increase from precancerous to early and advanced HCC (37). GS expression has been reported in 13-70% of early HCC (33,34). GS expression can be seen in 10-15% of HGDN. In contrast to diffuse expression (>50% tumor cells) in HCC, GS expression in HGDN is typically focal with involvement of <50% of tumor cells (34).

(f) **Combined use of HSP70, GPC-3 and GS**
Two studies from the same group, one based on resections and the other on biopsies, have evaluated the utility of the combined use of these three markers for diagnosis of HCC (33,35). The specificity and sensitivity for diagnosis of HCC was 100% and 57% respectively when two of the three markers are positive. All three stains are negative in >70% of HGDN compared to 3% of early HCC in resection specimens, while all three are positive in 43% of early HCC compared to none of the HGDN.

(g) **Cyclase associated protein 2**
This protein is plays a role downstream of the ras pathway and is involved in cytoskeletal function through binding with actin. Cyclase associated protein 2 (CAP2) is strongly expressed in smooth muscle, but is negative in normal liver (34). upregulated in a stepwise manner in multistage
hepatocarcinogenesis (38). Regenerative nodules are usually negative, but can show peripheral expression. Most HGDN are negative or focally positive. CAP2 expression has been observed in 40% of early HCC in more than 70% of tumor cells (34). The tumor cells in areas of stromal invasion are often positive.

2. Gene expression

Differential expression of genes has been demonstrated by quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) in HGDN and early HCC (39,40). In one study, 12 genes were differentially expressed in early HCCs compared with dysplastic nodules. Of these, 3-gene set including GPC3, LYVE1, and survivin had a discriminative accuracy of 94% (40).

References:


