

Session IV

ETIOLOGY OF NASH AND ASH

Obesity and NASH

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BACKGROUND/AIMS: Steatohepatitis (SH) is a distinctive pattern of hepatic injury that represents the necroinflammatory complication of persistent hepatic steatosis and constitutes the major pathway by which fatty liver can progress to cirrhosis. The prototypic form of SH is caused by an excessive alcohol consumption, but this entity can also occur in the absence of alcohol abuse. This latter has been termed as nonalcoholic steatohepatitis (NASH). Although recognized for many years, NASH has only recently been acknowledged as a disorder distinct from alcoholic SH. NASH must be considered as a syndrome with a multifactorial etiology, but obesity is the most consistently associated causal factor. Although exact prevalence figures are not available, NASH associated to obesity is probably more common than generally appreciated. Furthermore, risk factors of progression to advanced forms of liver disease in this particular group of NASH patients remain to be defined. Hence, the aims of this work were to determine the prevalence of NASH in a cohort of morbidly obese individuals as well as to identify pathogenic and risk factors of liver fibrosis in this selected group of NASH patients. **METHODS:** Forty-six obese patients who underwent surgical gastroplasty were studied. To identify risk factors of fibrosis, all demographics as well as clinical and histologic data were evaluated using a stepwise logistic regression analysis. Histologic grading (steatosis and inflammation) and staging (fibrosis) was performed using a modified scoring system based on a classification proposed by Brunt et al. We have defined mild NASH when the sum of steatosis and inflammation scores was equal or lower than 4, and severe NASH when it was higher than 4. We have also defined three categories of fibrosis, considering absence of fibrosis when the staging score was 0, focal fibrosis when the score was 1, and extensive fibrosis when it was equal or higher than 2. The intrahepatic immunological phenotype was assessed in all liver biopsy samples by immunohistochemistry, using a wide panel of monoclonal antibodies recognizing resting and activated mononuclear cell subsets, adhesion molecules, and nitric oxide markers. **RESULTS:** The majority of obese persons were women (65%) and the mean age*standard deviation was 41±11 years. The serum ALT level was elevated in 18 patients (39%), and an abnormal increase in alkaline phosphatase and γ -GT was observed in 6 and 12 patients, respectively. However, it is worth emphasizing that 7 out of 13 patients with severe NASH had normal serum ALT levels. Histologic findings of NASH were observed in 69.5% (32 out of 46) of obese population studied, being severe in 13 out of 32 NASH patients (41%). Regarding the stage of fibrosis, 13 out of 32 NASH patients (41%) had extensive fibrosis. Noticeable, only patients with NASH showed significant degrees of fibrosis and, more interestingly, those obese patients with severe NASH had significantly worse staging scores than those with mild NASH (sNASH: 2.4±0.8 versus mNASH: 0.8±0.5; $p < 0.001$). The age ($p = 0.02$), and the degree of steatosis ($p = 0.000002$) were independent variables positively associated with fibrosis, being the increased grade of inflammation the greatest risk factor of fibrosis (relative risk, 50.55; 95% confidence interval, 5.41 to 472.4; $p = 0.0006$). Immunohistochemical assessment of liver biopsies from NASH patients revealed that hepatocytes showed a marked upregulation of ICAM-1 expression always in close relation with either lobular or portal areas of inflammatory infiltration and necrosis. Moreover, it was interesting to note that the intrahepatic expression level of ICAM-1 was higher in the severe forms of NASH than in the mildest ones. In addition, liver-infiltrating inflammatory cells in NASH were predominantly macrophages and cytotoxic CD8+ T lymphocytes. Noticeable, the majority of CD8+ T cells coexpressed the CD69 activation molecule, and this activated cell subset was located mainly in those portal and lobular areas more severely inflamed. We have also observed that the intrahepatic immunoreactivity of endoglin, a molecule that functionally behaves as a receptor for the fibrogenic cytokine TGF- β 1, was significantly stronger and showed a more diffuse distribution pattern in NASH with extensive fibrosis (2.5±0.7) than in those with focal fibrosis (1.1±0.5, $p < 0.001$). In addition, we have found an enhanced hepatocellular expression of the inducible nitric oxide synthase (iNOS) as well as an intrahepatic abnormal accumulation of nitrotyrosine (NTY), a marker of peroxynitrite formation which is a strong oxidant agent derived from nitric oxide reaction with superoxide anion. Interestingly, a positive correlation between the expression level of both iNOS and NTY and the histological severity of NASH was clearly shown.

CONCLUSIONS: The majority of obese individuals studied (69.5%) had histological findings compatible with the diagnosis of NASH, and noticeably, the 41% of NASH patients had extensive fibrosis. Since oxidative stress as well as proinflammatory cytokines are directly implicated in the upregulation of both ICAM-1 and iNOS gene expression, the finding in the liver tissue of patients with NASH of an aberrant enhanced hepatocellular expression of these molecules provide evidence that nitric oxide-related oxidative damage and locally-released proinflammatory cytokines from intrahepatic activated T cells could play an important role in the pathogenesis of NASH in obesity.

Non-Alcoholic Steatohepatitis and Intestinal Disease

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There are two distinct clinical scenarios affecting the gastro-intestinal tract in which NASH is a predictable if not inevitable consequence - prolonged total parenteral nutrition, and jejunio-ileal bypass for obesity with retention of the excluded small-intestinal loop. 15% of patients on a home total parenteral nutrition programme had fatty liver - the great majority with NASH. Severe NASH, leading to cirrhosis, may complicate 3-10% of jejunio-ileal bypass patients. Occasionally other gastrointestinal conditions have been reported in which similar mechanisms to the bypass might be predicted - such as jejunal-diverticulosis. Whilst patients who are candidates for obesity surgery have a high chance of showing characteristics which are associated with NASH in the general population - obesity itself, hypertension, dyslipidaemia, and glucose intolerance, the same is not true for patients requiring total parenteral nutrition.

The favoured hypothesis linking all these forms of gastrointestinal disease and NASH involves bacterial overgrowth, with increased numbers of bacteria within the normally absorptive small intestine. In the jejunio-ileal bypass patient, stasis alone is enough to account for this. In the total-parenteral nutrition patients, the underlying gastro-intestinal condition with abnormal anatomy, frequently loss of the ileo-caecal valve, and a loss of normal bulk transit through the intestine may all predispose to a greater concentration of bacteria.

Animal studies in TPN-fed rats further implicate bacteria and indicate possible mechanisms. In short term experiments, both metronidazole and polymyxin administration have diminished hepatic inflammation. One plausible hypothesis suggests that increased lipopolysaccharide, via stimulation of the prototypic pro-inflammatory cytokine TNF-alpha, is a critical inflammatory mediator. TNF-alpha upregulation will induce a cascade of events that will include up-regulation of adhesion molecules and attraction of leukocytes including polymorphonuclear leukocytes into the liver. Experimental evidence for this includes a greater sensitivity to lethal doses of exogenous lipopolysaccharide in rats undergoing TPN, and improvement in histological appearances of the liver after administration of anti-TNF antibodies. However this explanation is unlikely to be more than partial, as human disease in which there is clear evidence of enhanced lipopolysaccharide absorption and increased TNF-alpha production - notably Crohn's disease, do not predispose to NASH.

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DRUGS and NASH

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Drug-induced liver disease (DILD) is a rare cause of NASH, accounting for less than 5% of cases. Likewise, NASH is a rare form of DILD, and implication of medications as causal agents in NASH is often problematic due to the more tenuous temporal relationships, including long incubation period, lack of dechallenge and rechallenge data and the high prevalence of NASH in western communities. Some agents implicated as causing cirrhosis or other chronic forms of fatty liver disorders in obese middle-aged diabetic women (methyl dopa, calcium channel blockers, estrogens) may simply be fortuitous associations with NASH among persons with the metabolic syndrome. Likewise, incrimination of occupational and environmental petrochemical solvents as a cause of NASH lacks firm evidence (1).

Recognition in the 1970s that perhexiline maleate was causally implicated in a spectrum of liver disease that included alcoholic hepatitis-like lesions, often with Mallory bodies and leading to cirrhosis, extended the conceptual base of steatohepatitis beyond alcoholic liver disease. This had also been recognised in 1973 in Japan for the vasodilator and antianginal agent, Coralgil (4,4'-diethylaminoethoxyhexestrol). One characteristic feature of drug-induced NASH is the ultrastructural presence of myeloid bodies - enlarged lysosomes stuffed with whorled membranous material that resembles the myelin sheath of nerves. Perhexiline maleate induced peripheral neuropathy occurs in persons who have the debrisoquine slow metabolizer phenotype, a genetic polymorphism of the cytochrome P450 gene, *CYP2D6*, which is involved with perhexiline metabolism. Together with the evidence of partial dose-dependence, this implicates drug accumulation in pathogenesis of liver disease. The drug most often implicated as causing NASH is amiodarone, an iodine-containing antiarrhythmic agent. Symptomatic liver disease occurs in 0.6-3% of patients taking amiodarone, with the risk of steatohepatitis being related to the duration of exposure (mean 21 months). Hepatic accumulation of amiodarone may be important in aetiopathogenesis of liver disease because opacification of the liver due to iodine accumulation is found in association with NASH, and progression of amiodarone-induced liver disease may continue after discontinuation of the drug. This is probably one reason why the mortality of amiodarone and perhexiline maleate induced NASH is high. Few drugs have been newly incriminated as potential causes of NASH in recent years; the only relatively convincing example is tamoxifen in which there was a temporal association of liver test abnormalities and histological NASH with drug ingestion in 3 cases (2).

Most drugs implicated as causing NASH have physicochemical (cationic amphiphilic) characteristics that favour their accumulation in lipid membranes, inhibiting phospholipases to cause phospholipidosis (which is the basis of myeloid bodies). The relationship of this storage disorder to development of NASH is not clear. Pessayre, Fromenty and colleagues have shown that perhexiline and amiodarone accumulate in mitochondria and inhibit mitochondrial β -oxidation, thereby causing steatosis (3). They may also inhibit oxidative phosphorylation, and in the longer term this could be relevant to pathogenesis of NASH by favouring electron leakage with production of hydrogen peroxide and other ROS causing lipid peroxidation (3). Thus drug-induced NASH may be a paradigm for injurious events in the more common idiopathic forms of NASH that occur in association with insulin resistance, obesity and hyperlipidemia.

Whether drugs that cause hypertriglyceridemia predispose to NASH is unclear. Finally, it should be noted that methotrexate-induced hepatic fibrosis is favored by alcohol, obesity, increasing age and diabetes, all factors associated with steatohepatitis. The possibility therefore emerges that NASH, or the genetic and metabolic factors predisposing to NASH may interact with some drugs or other toxins to promote hepatic fibrosis.

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Influence of Fat on Liver Regeneration

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Hepatic steatosis is a common problem in Western countries resulting from a variety of etiologies, such as obesity, alcohol abuse and metabolic disorders. Liver resection in patients with steatosis is associated with increased morbidity and mortality rates. Livers with more than 30% - 50% steatosis are usually not used for transplantation because of an increased risk of primary nonfunction. While there is accumulating evidence that steatotic livers tolerate ischemic injury and major tissue loss poorly, only few data are available regarding the impact of fat on this type of injury and the mechanisms involved.

The normal liver has a unique ability to regenerate. In human, major tissue loss is restored within a few weeks, and in rodents complete regeneration is achieved within 7 days. Hepatocyte proliferation is induced by a regenerative cascade of extra- and intracellular mediators. INF α stimulates the production and release of interleukin-6 (IL-6). Binding of IL-6 to the IL-6 receptor results in intracellular phosphorylation of the Signal Transducer and Activator of Transcription Protein 3 (STAT3), which is rapidly translocated to the nucleus and binds to the promoter of immediate early genes. A second pathway involves the activation of the Ras-dependent mitogen-activated protein kinase (MAPK) cascade resulting in activation of regulatory proteins. These proteins trigger the production of DNA-synthesis proteins like PCNA.

Steatosis is associated with a significant reduction in the ability of hepatocytes to proliferate. The decreased proliferation is characterized by a failure of the fatty hepatocytes to progress from the G₀- to the G₁ phase. We recently found in a rat model of hepatic steatosis that the regenerative ability following 70% hepatectomy is initially dramatically reduced compared to normal tissue. However, while the regenerative ability improves over a few days, this decreased regenerative capacity early after liver resection was associated with a dramatic increase of mortality. Pretreatment of rIL-6, which effectively stimulates hepatocyte proliferation in normal and ischemic livers, fails to increase mitosis or improve survival in fatty livers after major tissue loss. rIL-6 induces the G₁-phase of the mitotic cycle without improving DNA synthesis. This indicates that two different blocks of mitosis are present in steatotic livers. One at the G₀-G₁-transition, rIL-6 sensitive, and a second block at the G₁/S-phase transition, rIL-6 resistant.

Future studies need to focus on better understanding of the mechanisms involved in the failure of the fatty liver to regenerate, and to identify the risk associated with various types of steatosis.

Obesity and Cryptogenic Cirrhosis

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Cryptogenic cirrhosis constitutes 10% of patients in our liver disease registry while NASH is our third most common diagnosis after hepatitis C and alcohol. Cryptogenic cirrhosis could result from a variety of diseases including occult alcohol-related disease, occult viral infection, silent autoimmune hepatitis with progression to cirrhosis or a silent metabolic disorder such as NASH in the presence of longstanding obesity or diabetes. We reported the clinical characteristics of 102 consecutive patients and examined risk factors for liver disease (Hepatology 1999;29:664-9). All available clinical and laboratory material was reviewed and telephone contact or re-interview was done to confirm any aspects of the history. Potentially significant alcohol exposure was defined as daily consumption of any amount for a year or more at anytime in the patient's life. 32 patients were excluded for inadequate data in 22 and for possible significant alcohol in 10. The remaining 70 constituted the main study group. Cirrhosis was diagnosed based on clinical and imaging findings in all and by biopsy in 52. Most of the patients were female (49 of 72, 70%) with a mean age of 63 \pm 11 years. 73% had either obesity (BMI > 31.1 male or >32.3 female) or type 2 diabetes. The mean autoimmune score was 8 \pm 2 (low likelihood) and HLA typing, performed in 25, was not suggestive of autoimmune risks. Nineteen (27%) had blood transfusions prior to the diagnosis of cirrhosis. We divided the entire group into those with prior transfusion, those with indeterminate autoimmune scores (? 10), those with only obesity and or diabetes and those with no definable risk. We could not detect major differences in this subgroup analysis although the IgG:IgA ratio was lowest (2.9) in those with only obesity or diabetes. In comparison to three control groups (non-cirrhotic NASH, non-alcoholic hepatitis C and PBC), diabetes and obesity were significantly more common in the cryptogenic cirrhosis patients compared to PBC or hepatitis C but not different from NASH. The IgG:IgA ratio was lower in cryptogenic cirrhosis compared to PBC and hepatitis C. We have since observed isolated IgA in about 25% of NASH patient and it appears to be slightly more common with more histologically advanced disease. The etiology of this abnormality is not clear. We observed a family history of unexplained liver disease in 19% of the entire cohort of cryptogenic cirrhosis patients. We have studied 8 kindreds comprised of members with both cryptogenic cirrhosis and NASH (Am J Med 2000;108:9-13). From 8 index cases, we identified 10 first degree relatives with either of these conditions. Several had been in our care and either undergone liver transplantation or died from liver-related causes. The majority of cases were female (72%) and were obese (83%) with diabetes (61%). Patterns of involvement which we observed included mother-daughter, sister-sister, sisterbrother and father-daughter.

We observed that most cases of cryptogenic cirrhosis are older, obese and often diabetic females. We suspect that they developed cirrhosis from silent NASH which progressed over many years or decades to cirrhosis. As has been previously described, the liver histology loses characteristic markers as NASH progresses. Relatively low IgG:IgA ratios seem to be a residual marker of this process. Familial clustering of NASH and cryptogenic cirrhosis is common - it is often in association with obesity and type 2 diabetes. This suggests a genetic predisposition which could be based on inherited risks for obesity or diabetes. However, the apparent low frequency of NASH in African American females, a group with a very high prevalence of obesity and diabetes but who constitute less than 1% of either our NASH or cryptogenic patients indicates a risk apart from either diabetes or obesity. Mitochondrial inheritance is an attractive possibility which could also relate to what we think is ethnic clustering among European-Americans. However, the familial patterns do not indicate a uniformly typical mitochondrial inheritance which is usually maternal as two of the kindreds we described were related along paternal lines.

Diet and Alcoholic Steatohepatitis (ASH)

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Nutritional deficiencies are common in patients with alcoholic liver disease, and are best documented in indigent alcoholics admitted to municipal hospitals. While 68% of indigent alcoholics had a poor dietary intake with ingestion of less than one meal per day, employed alcoholic patients with more subtle nutritional deficiencies had a similar non-alcohol calorie intake than control subjects. Total calorie intake in ASH patients admitted to the hospital was a similar to that of control subjects. Alcohol, however, accounted for 50% of the calories in the patients as compared with 15% in the controls, while the intake of protein, carbohydrate and fat was decreased in the patients as compared with the controls. Furthermore patients with severe ASH had the lowest protein intake and the lowest intake of non-alcohol calories. While decreased dietary intake is the principal cause of nutritional deficiencies, additional factors that contribute to malnutrition are decreased intestinal absorption, decreased hepatic storage, abnormal metabolism, increased losses and increased requirements of nutrients. An association between the degree of protein-calorie malnutrition and both the morbidity and mortality of patients with ASH is well documented. Achievement of a positive nitrogen balance by oral (and or enteral) or parenteral routes by administration of casein protein hydrolysates or aminoacids respectively has been found to improve clinical status, encephalopathy, and laboratory values in most cases of ASH, and reduced mortality in a few studies. On the other hand obesity has been associated with increased fatty infiltration and worse histological changes (necrosis and fibrosis) in the liver and was found to be an independent risk factor for ASH. The degree of fatty infiltration is a risk factor for the subsequent development of alcoholic cirrhosis. Rats fed ethanol rich in polyunsaturated fatty acids (fish oil) develop the most severe hepatic injury. This injury is most likely related to increased induction of cytochrome P450 2E1 resulting in increased formation of oxygen radicals and to the greater susceptibility of polyunsaturated fatty acids to lipid peroxidation. In summary nutritional deficiencies particularly of protein with a

negative nitrogen balance are associated with increased morbidity and mortality in ASH, while obesity is associated with a greater severity of ASH and progression to cirrhosis. Both extremes require that nutritional management accompany sobriety.

Gender and Alcoholic Steatohepatitis

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Epidemiological studies have indicated that women tend to develop significant alcoholic liver disease (alcoholic hepatitis and cirrhosis) after fewer years of drinking and lower total intake of ethanol than men. Several possible explanations have been put forward. Some studies reported that women metabolized alcohol faster than men. This may result from the higher fat composition of women, which results in a lower volume of distribution of ethanol. A recent study showed that alcohol metabolism measured by IV alcohol administration and "alcohol clamping" was the same in men and women when expressed per liter of liver mass. Despite this, women are reported to have higher blood acetaldehyde after a dose of ethanol, which could be hepatotoxic. Women are also likely to have higher blood acetate levels, since much acetate is cleared by oxidation by muscle, and the muscle mass of women is considerably lower than that of men.

Another possible contributor may be the higher prevalence of obesity in women. Overweight has been implicated as an independent risk factor for steatosis, alcoholic hepatitis, and cirrhosis, along with female gender. While body mass index (BMI) tends to fall with increased alcohol consumption in women, there do not appear to be studies of the prevalence of overweight in alcoholic women with and without cirrhosis. In animals, obesity renders the liver much more sensitive to endotoxin, particularly in females.

Estrogen appears to increase the sensitivity of the liver to alcohol in rodents. Female rats develop more severe liver injury than males in the Irukumoto-French model. Males are made more susceptible to liver injury by administering estrogen. Estrogen increases gut permeability to endotoxin, resulting in endotoxin levels that are higher in female rats given alcohol than in males. Estrogen also induces the lipopolysaccharide receptor (CD14) on Kupffer cells, and thus the Kupffer cells more activated in female rats fed alcohol. Other differences between male and female rats include more pronounced central hypoxia, less induction of fatty acid binding protein or fatty acid omega oxidation, and more marked activation of NF- κ B in females fed alcohol. Thus, many of the factors implicated in liver injury (acetaldehyde, endotoxin stimulation of the Kupffer cells, central hypoxia, and elevated free fatty acids in the liver) are more severely affected in female rats than in males.

Extra-intrahepatic Pathophysiologic Abnormalities in NASH

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The pathogenesis of nonalcoholic steatohepatitis (NASH) is unknown. We tested the hypothesis that NASH was associated with two defects: (1) peripheral insulin resistance which increased lipolysis, delivery of free fatty acids (FFA) to the liver, and hepatic fatty acid β oxidation thereby creating oxidative stress and (2) an abnormality within the hepatocytes which might render them more susceptible to injury from oxidative stress. This was tested by evaluation of: (1) insulin resistance by a two-step hyperinsulinemic (10 and 40 mU/m²/min) euglycemic clamp, (2) insulin effects on lipolysis by enrichment of (U-¹³C)-glycerol, (3) frequency and severity of structural defects in hepatocyte mitochondria in vivo, (4) fatty acid β oxidation from serum [β -OH butyrate], release of water-soluble radioactivity from ³H-palmitate by cultured fibroblasts and urinary dicarboxylic acid excretion, and (5) hepatic lipid peroxidation by immunohistochemical staining for 3-nitrotyrosine (3-NT). Subjects with NASH (n= 6-10 for different studies) were compared to those with fatty liver (n=6) or normal controls (n=6). NASH and fatty liver were both associated with insulin resistance with mean glucose infusion rates (normal:fatty liver: NASH) of: step 1: 3.6:1.6:0.9 and step 2: 9.5:7.7:4.5 (p < 0.03 for both steps). While baseline rates of glycerol appearance were higher in those with NASH compared to fatty liver (means: 14.6 vs 21.6 μ mol/kg/min, p < 0.05), neither group significantly suppressed glycerol appearance at insulin infusion rates of 10 mU/m²/min. NASH was associated with loss of mitochondrial cristae and paracrystalline inclusions in 9110 subjects compared to 016 subjects with fatty liver. However, no evidence of a generalized defect in fatty acid β oxidation were noted in any group. Also, mean [β -OH butyrate] were highest in those with NASH (means: 90 vs 110 vs 160 pM, p < 0.04). Increased staining for 3-NT was present in fatty liver and even greater staining was seen in NASH. These data indicate that peripheral insulin resistance, increased fatty acid β oxidation and hepatic oxidative stress are present in both fatty liver and NASH but NASH alone is associated with mitochondrial structural defects.