Pathology of Fatty Liver Disease

Role of Liver Biopsy in (Non-Alcoholic) Fatty Liver Disease

Stefan Hübscher,
Institute of Immunology & Immunotherapy, University of Birmingham
Department of Cellular Pathology, Queen Elizabeth Hospital, Birmingham

Fatty Liver Disease - Causes

Alcoholic

- Commonest cause of cirrhosis in UK
- Prevalence rising, particularly in young people and women
- Alcohol accounts for 72% of cirrhosis-related deaths in Central Europe (Rehm 2013)

Non- Alcoholic

- Primary (metabolic syndrome)*
  - Obesity, type 2 diabetes, hyperlipidaemia
- Secondary (other causes)
  - Drugs (e.g. amiodarone, perhexiline maleate, irinotecan)
  - Surgical procedures (jejunoileal bypass, biliopancreatic diversion, extensive small bowel resection, gastroplasty for morbid obesity)
  - Total parenteral nutrition

* Metabolic fatty liver disease / metabolic syndrome associated steatohepatitis (MASH) as alternative terms
(also DASH – drugs, CASH – chemotherapy, TASH – toxicant etc)

Non-Alcoholic Fatty Liver Disease

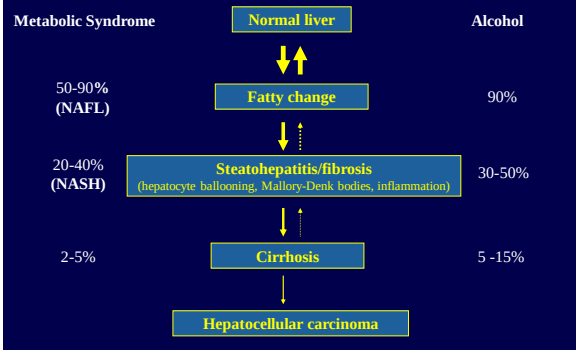
- First described in 1980
  - Ludwig et al. Non-alcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc 1980; 55(7):434-438.
- Subsequently recognition of metabolic syndrome as major risk factor
- Clinical impact of NAFLD only recognised during past 10 -15 years

NAFLD – Rising Prevalence

(Ong 2008, Ratziu 2010, Vernon 2011, Chalasani 2012, Armstrong 2014)

- Overall prevalence in Europe & US estimated at 20-30%
  - Most cases in general population have simple steatosis
  - Prevalence of NASH estimated at 3-5%
- In tertiary care centres using liver biopsy 40-60% of cases of NAFLD have features of NASH
- Now the commonest cause (40%) of newly diagnosed chronic liver disease
- Predicted to be the commonest cause of cirrhosis (and liver-related mortality)
- Only 6% of deaths in patients with NAFLD are from liver disease (versus 25% from CVS disease and 24% from neoplasia)
  - In patients with metabolic syndrome, presence of NAFLD/NASH independently increases risk of CVS disease, type 2 DM, chronic kidney disease and colorectal neoplasms

Liver Lesions In Fatty Liver Disease



NAFLD and HCC

HCC as a complication of NASH - associated cirrhosis

- Prevalence 0.35%-4.2%/year (lower than HCV-cirrhosis)

HCC arising in non-cirrhotic NAFLD

- Increasing numbers of cases reported
  - 40-65% of HCC complicating NAFLD occurred in non-cirrhotic liver (Paradis 2009, Yasui 2011, Duan 2012)

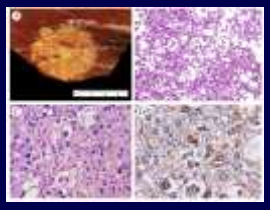
→ Metabolic syndrome as risk factor for malignancy

Clinico-pathological features of HCC in non-cirrhotic NAFLD

- Most have pre-cirrhotic fibrosis (with steatohepatitis)
- Some cases occur in patients with simple steatosis
- A few arise from adenomas (inflammatory type) (Paradis 2009)

HCC Arising in NAFLD - Different Histological Features - "Steatohepatitic HCC"

(Salomao 2010, Jain 2012, Salomao 2012, Jain 2013, Shibihara 2014)



- Present in up to 50% in NAFLD (vs < 5% in non-fatty liver disease cirrhosis)
- Associated with features of steatohepatitis in non-neoplastic liver
- May behave less aggressively than conventional HCC (tumours tend to be small and well-differentiated)

- Distinction between steatohepatitic HCC and inflammatory steatohepatitis may be difficult in liver biopsy specimens

Non-Alcoholic Fatty Liver Disease - Changing Role of Liver Biopsy

Until recently - increasingly common indication for liver biopsy

Strategies to avoid liver biopsy (non-invasive methods)

- Blood tests (single or in combination)
- Imaging studies (e.g. Ultrasound, MRI, transient elastography)

- Used to assess various components of fatty liver disease
  - mainly fat and fibrosis

Limitations of non-invasive markers of disease severity in NAFLD

1. No reliable marker for distinguishing NASH from simple steatosis
2. Less reliable than liver biopsy in assessing intermediate stages of fibrosis

Non-Alcoholic Fatty Liver Disease – Current Role of Liver Biopsy

The Diagnosis and Management of Non-alcoholic Fatty Liver Disease: Practice Guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology

Liver biopsy still regarded as "gold standard" for establishing diagnosis of NASH (versus simple steatosis) and for assessing disease severity (particularly fibrosis).

Liver biopsy also helpful in investigating patients in whom there may be co-existent causes for liver diseases (e.g. hepatitis C)

- In cases with a dual pathology, liver biopsy may help to identify the main cause of liver injury

NAFLD - Indications for Liver Biopsy
(Birmingham Liver Unit)

1. Cases where non-invasive investigations (NAFLD Fibrosis Score, Fibroscan) have produced an "indeterminate score" for fibrosis (or an unexpected score)

2. Cases where there are concerns about an additional aetiology for liver disease

Histological Assessments in NAFLD

1. Establishing the Diagnosis

2. Assessing Disease Severity
   - "Simple" Steatosis vs Steatohepatitis
   - Portal tract changes in NAFLD
   - Grading & Staging

3. Aetiological Considerations
   - NAFLD vs Other Causes of FLD (mainly alcohol)
   - Interaction with other diseases

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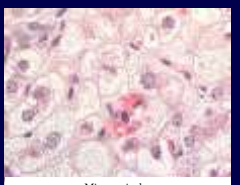
Histological Definition of Fatty Liver Disease

- Fatty change involving > 5% of hepatocytes (or parenchymal area)
- Mainly macrovesicular
- Predominantly perivenular

Hepatic Steatosis - Classification According to Droplet Size

Macrovesicular
Single large droplet, nucleus displaced to one side



Microvesicular
Numerous small droplets, nucleus remains central

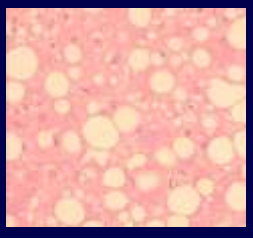
Fatty liver disease (alcoholic and non-alcoholic) mainly macrovesicular

Large droplets begin as small ones – mixed patterns of droplet size common

"Pure" microvesicular steatosis – different causes & consequences

- Disorders of mitochondrial beta -oxidation of fatty acids ("mitochondrial hepatopathies")
- Serious metabolic disturbances, including acute liver failure (e.g. Reye's syndrome, acute fatty liver of pregnancy, anti-retroviral drug toxicity)

Hepatic Steatosis - Classification According to Droplet Size



Fat droplets that are neither large nor very small. How should these be classified?

- Probably best regarded as a variant of macrovesicular steatosis
- Macrovesicular steatosis can be sub-classified into small-, medium- or large droplet forms
- "Mediovesicular steatosis" (Brunt 2012, Bedossa 2013)
  - One or more smaller droplets, easily distinguished, few enough to be counted

Assessing Fat Droplet Size in Fatty Liver Disease - Clinical Relevance

Alcoholic Liver Disease (Teli 1995)

- In patients with "pure" alcoholic fatty liver , cases with mixed droplet size had higher risk of progression to cirrhosis than those with macrovesicular steatosis only (28% vs 3%)

Recent studies in NAFLD (Soderberg 2011, Tandra 2011)

- "True" microvesicular steatosis occurred in 102/1022 (10%) of biopsies from patients with NAFLD (NASH Clinical Research Study - Tandra 2011)
- Presence associated with more severe disease (more ballooning, & inflammation, higher NAS score, more severe fibrosis) and with presence of megamitochondria
- Functional significance in mediating disease progression uncertain

Methods for Assessing Presence and Severity of Steatosis
Standard Approach for H&E stained sections (Brunt 1999, Kleiner 2005)

% involvement	Severity	Grade
<5	None	0
5-33	Mild	1
33-66	Moderate	2
>66	Severe	3

- Good intra- and inter-observer reproducibility for overall grade
- Reproducibility less good for assessing finer scales of steatosis severity
- Poor correlation with fat content measured biochemically

Methods for Assessing Severity of Steatosis - Alternative Approaches
(El Badry 2009, Levene & Goldin 2012, Hall 2013, Hall 2014, Vanderbeck 2014)

Digital image analysis (H&E or Oil Red O stained sections)

- Measures surface area occupied by fat droplets
  - More accurate for quantifying steatosis
  - Correlates better with biochemical measurement of triglyceride
- Estimated "fat proportionate area" (FPA) exceeds measured FPA (Hall 2013)
  - Improved by use of guideline images (Hall 2014)
- "Supervised machine learning" may improve diagnostic accuracy of automated assessment of steatosis (versus other "white areas") (Vanderbeck 2014)

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Steatohepatitis (versus simple fatty change)

1. Presence of steatohepatitis indicates more severe disease
   - less likely to be reversible
   - more likely to progress to fibrosis or cirrhosis
2. Non-invasive techniques less reliable than liver biopsy in distinguishing simple steatosis from steatohepatitis

- Recent studies of patients with serial liver biopsies suggest that "simple" non-alcoholic steatosis may progress:

	Baseline Biopsies with NAFL	Median Duration of Follow-up	Progression to NASH	Progression to Bridging Fibrosis
Pais 2013	25	3.7 years	64%	24%
McPherson 2015	27	6.6 years	44%	22%

Steatohepatitis - Histological Features (mainly perivenular distribution)

Hepatocellular injury
- fatty change
- ballooning
- Mallory-Denk bodies
- apoptosis/necrosis

Inflammation
- neutrophil polymorphs
- other cells (e.g T lymphocytes)

Fibrosis
- perisinusoidal
- pericellular

Histopathological Diagnosis of NASH - AASLD Workshop (Sanyal 2011)
Similar to criteria previously proposed by Brunt (1999) and Neuschwander-Tetri (2003)

- > 5% steatosis, mainly macrovesicular
- lobular inflammation (polymorphs as well as mononuclear cells)
- hepatocyte ballooning, most apparent near steatotic cells

Problems With Applying AASLD Diagnostic Criteria for NASH

1. **Inflammation**
   - May be minimal/absent
   - Neutrophils rarely prominent, may not be present

→ Enlarged Kupffer cells (PAS-D+, CD 68+) may be useful (but non-specific) marker of previous inflammatory damage

2. **Ballooning**
   - What defines a ballooned hepatocyte - size, shape, cytoplasmic "clarification"? (poor observer reproducibility)

→ Use of immunostains to demonstrate small amounts of Mallory's hyaline

→ Use of connective tissue stains (HVG, Trichrome) to demonstrate foci of pericellular/perisinusoidal fibrosis

Mallory-Denk Bodies - Immunhistochemical Demonstration
(from Denk 2006, Zatloukal 2007)

K 8/18 | P62 | Ubiquitin

Co-staining for K8/18 & ubiquitin improves detection of hepatocyte injury in NAFLD
(Guy, Human Pathol 2012)

- Identifying normal-sized hepatocytes, not readily appreciated as "ballooned" in H&E sections
- Improved categorisation of cases classified as "suspicious" (borderline) for NASH
- K8/18 immunostaining also improves identification and grading of ballooning in alcoholic hepatitis (Mookerjee 2011)

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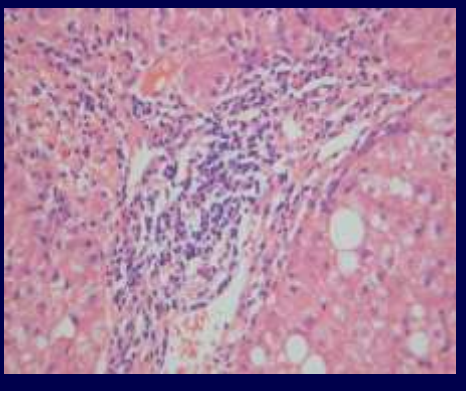
Portal/Periportal Changes in Fatty Liver Disease

1. Portal inflammation +/- interface hepatitis (chronic hepatitis-like)
2. Biliary features (resembling low-grade biliary obstruction)
3. Isolated portal fibrosis (without features of steatohepatitis)
   - Adults with morbid obesity
   - Paediatric NAFLD

Portal Inflammation in NAFLD



Portal Inflammation in NAFLD – Prevalence & Associated Features

(Brunt 2009, Rakha 2010, Carotti 2015)

1. **Prevalence (in adults)**

	None	Mild	More than Mild
Brunt 2009 (n= 728)	16%	60%	23%
Rakha 2010 (n= 214)	37%	33%	30%
Carotti 2015 (n= 52)	13%	40%	47%

2. **Associated Features**
   - Associated with steatosis severity, ballooning, lobular inflammation, advanced fibrosis and progenitor cell compartment expansion

3. **Pathogenesis & Clinical Significance**
   - Mechanism uncertain – no association with auto-antibodies
   - Predicts fibrosis progression in serial biopsies (Argo 2009)
   - May also be a feature of treated / regressed NASH

BUT: Disproportionately severe portal/peri-portal inflammation should still raise concerns about possible concurrent chronic liver disease (Kleiner & Brunt 2012)

Portal Changes in NAFLD - Biliary Features

Ductular Reaction in NAFLD

(Clouston 2005, Richardson 2007, Chiba 2011, Lieuw 2012, Skoien 2013, Aravinthan 2013, Gadd 2014)

Steatosis impairs hepatocyte replication

↓

Further hepatocyte injury triggers progenitor cell activation & ductular reaction

↓

Ductular reaction promotes periportal fibrosis

(also associated with portal inflammation – Chiba 2011, Skoien 2013, Gadd 2014)

(Gadd 2014 – portal macrophages and DR precede detectable fibrosis)

NAFLD in Children - Differences Compared with NAFLD in Adults

(Schwimmer 2005 & 2006 , Nobili 2006, Roberts 2007, Nobili 2010, Della Corte 2012, Skoien 2013)

Steatosis
- often more severe
- may have different distribution (panacinar or periportal)

Other lobular changes less well developed
- less ballooning/Mallory's hyaline
- less perisinusoidal/pericellular fibrosis

Portal/periportal changes more prominent
- more portal inflammation
- more portal fibrosis

Type 2 NAFLD (Schwimmer 2005)

Steatosis, portal inflammation and portal fibrosis (without typical features of steatohepatitis)
- Present in 62% paediatric NASH biopsies, 19% Type 1 (adult pattern), 19% mixed (type 1 & 2)
- Also referred to as "borderline, zone 1 steatohepatitis" (NASH CRN Group – Kleiner 2012)

Subsequent studies showed more frequent cases (30-80%) with mixed pattern (Carter-Kent 2009, Takahashi 2011, Skoien 2013)
- "Type 2 pattern" still more common in children than adults

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Fatty Liver Disease – Grading and Staging

Grading
- ongoing damage (fat, ballooning, inflammation)
- potential treatable

Staging
- progressive liver injury (fibrosis)
- less likely to be reversible

Semiquantitative scoring systems
- Several described
- System proposed by Kleiner et al (2005) most widely used in NAFLD
- Should be used as an adjunct to conventional reporting

NIDDK NASH Clinical Research Network - NAFLD Scoring System

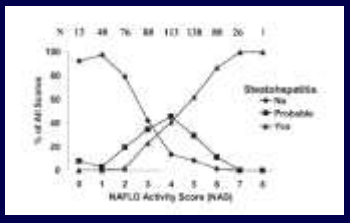
(Kleiner et al. Hepatology 2005; 41: 1313-1321)

Activity Score (0-8)	Fibrosis Score (0-4)
Steatosis (0-3) • <5%; 5-33%; 33-66%; >66% Lobular Inflammation (0-3) • <2; 2-4; >4 foci/20x Ballooning (0-2) • None, few, many/prominent	1a: Zone 3 perisinusoidal (mild) 1b: Zone 3 perisinusoidal (moderate) 1c: Portal/periportal only 2: Zone 3 & portal/periportal 3: Bridging 4: Cirrhosis

- Scoring system intended to assess disease severity, particularly in clinical trials (similar to Ishak system for HCV)
- NOT intended to establish or confirm a diagnosis of NASH

NAFLD Activity Scores in 512 Liver Biopsies from Adults with NAFLD

(Kleiner 2005)



- Cases with NAS 0-2 mostly diagnosed as "not NASH"
- Cases with NAS 5-8 mostly diagnosed as "NASH"

- NAS ≥ 5 has subsequently been used to establish diagnosis of NASH, both in clinical trials and in routine practice

Nonalcoholic Fatty Liver Disease (NAFLD) Activity Score and the Histopathologic Diagnosis in NAFLD: Distinct Clinicopathologic Meanings

Liver Biopsies from 976 adults in NASH Clinical Research Network studies

	Not Steatohepatitis (n = 204)	Borderline Steatohepatitis (n= 183)	Definite Steatohepatitis (n = 543)
NAS 0-4	194	131	136
NAS 5-8	14	52	407

NAS Score ≥ 5 present in:
- 75% of biopsies with definite NASH
- 28% of biopsies with borderline NASH
- 7% of biopsies with not NASH

Conclusions:
1. NAS should only be used as an adjunct to the conventional morphological diagnosis of NASH
2. NAS more useful for clinical trials than in routine histological assessment of NAFLD

Histological Grading & Staging of NASH (Kleiner System) Problems and Limitations

Observer variability	Reproducibility good for fat & fibrosis Reproducibility less good for inflammation & ballooning
Sampling variability	Fat - reasonably uniform distribution Inflammation & fibrosis more variable
Uncertain significance of individual NAS Features or overall NAS Score	Importance of steatosis severity uncertain: • No longer regarded as "first hit" in pathogenesis of NASH • May be a protective mechanism (Neuschwander-Tetri 2010) Portal/periportal inflammation not included*

* Portal inflammation (0-2) incorporated into a recently proposed system for scoring paediatric NAFLD (Alkhouri 2012)

Use of Steatosis/Activity/Fibrosis (SAF) Scoring NAFLD

(Bedossa, Hepatology 2012 & Hepatology 2014)

Steatosis, Activity and Fibrosis scored separately
- Steatosis (0-3), Fibrosis (0-4) scored as per NASH-CRN (Kleiner 2005)
- Activity Score (0-4) = combined score for ballooning (0-2) and inflammation (0-2)

Ballooning	Inflammation
0 = none 1 = hepatocytes with rounded shape and pale cytoplasm 2 = same as grade 1 with enlarged hepatocytes (> 2x normal)	0 = none 1 = ≤ 2 foci per 20x field 2 = > 2 foci per 20x field

Diagnostic Algorithm for NASH
- Activity score ≥ 2 (at least 1 for ballooning **and** inflammation)
- Good correlation with initial diagnosis of NASH
- Improved inter/intraobserver agreement for the classification of NAFLD (NASH vs steatosis) amongst "expert" liver pathologists (n =6) and "non-specialist" pathologists (n=10)
- High kappa scores achieved for individual scores of SAF

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ALD vs NAFLD

(Rakha 2010, Yeh 2011, Brunt 2012 , Sakhuja 2014, Yeh & Brunt 2014)

More common/prominent in ALD	More common/prominent in NAFLD
Ballooning, Mallory-Denk bodies	Steatosis
Lobular neutrophils ("satellitosis")	(esp in children and morbid obesity)
Zone 3 fibrosis	
Hepatic vein lesions (phlebosclerosis, lymphocytic phlebitis, veno-occlusive lesions)	Nuclear vacuolation of hepatocytes
Cholestasis	(70-80% of cases vs <10% in ALD)
Portal neutrophils, cholangiolitis	

Severe alcoholic (steato)hepatitis

ALD - central sclerosing hyaline necrosis/fibrosis

NAFLD
Nuclear vacuolation

Role of Liver Biopsy in ALD

(AASLD Guidelines – O'Shea 2010, EASL Guidelines - Mathurin 2012)

- Not required to establish a routine diagnosis
- May be helpful in cases where there is diagnostic uncertainty (including the possibility of dual pathology)

Role of Liver Biopsy in ALD
Assessing Disease Severity - Relevance for Prognosis (and Treatment)

1. **Chronic ALD**
   - Steatosis severity, mixed droplet steatosis, alcoholic hepatitis, extent of hepatocyte ballooning, fibrosis severity predict poor outcome
   - Presence of megamitochondria associated with better outcome

2. **Acute Alcoholic (Steato)hepatitis** (Katoonizadeh 2010, Mookerjee 2011, Spahr 2011, Altamirano 2014)
   - ***Predictors of early mortality***
     - Cholestasis (ductular/canalicular/intraparenchymal) –also predicts sepsis
     - Mallory-Denk bodies
     - Severe fibrosis
   - ***Features associated with better outcome***
     - Polymorphs - but other studies suggest worse prognosis (O'Shea 2011)
       - also predict good response to treatment with corticosteroids (Mathurin 1996)
     - Megamitochondria

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     - HCV, ALD, Iron overload

Interactions Between HCV and NAFLD

(Eslam 2011, Hubscher 2011, Bugianesi 2012)

Steatosis frequently present in biopsies from HCV+ patients (40-86%)

Two main pathways for HCV-induced steatosis:

1. Viral (genotype 3) - steatosis severity correlates with HCV RNA levels
2. Metabolic (other genotypes) - steatosis severity associated with insulin resistance (HCV infection promotes several mechanisms leading to insulin resistance – e.g. insulin signalling, glucose uptake, cytokine production)

Viral eradication results in improvement of steatosis (HCV-genotype 3) and insulin resistance (HCV- genotype 1)

Both pathways can lead to the development of steatohepatitis

Interactions Between HCV and NAFLD
(Eslam 2011, Hubscher 2011, Bugianesi 2012)

Clinical Relevance of Steatosis and Insulin Resistance

1. **Prognosis**
- Increased risk for fibrosis progression and development of HCC

2. **Treatment**
- Predict poor response to treatment with interferon and ribavirin (may not apply to newer anti-viral agents – e.g. Protease inhibitors)

- Recent data suggest that insulin resistance (rather than steatosis) is the main factor determining fibrogenesis, carcinogenesis and therapeutic responses
- Role of HCV as an independent risk factor for insulin resistance has recently been challenged (Ruhl 2014)

Interaction between NAFLD and Alcoholic Liver Disease

- Diagnosis of NAFLD requires absence of significant alcohol consumption (< 20g/day in women, < 30g/day in men)
- In patients with presumed NAFLD , modest alcohol consumption (< 20g/day) associated with a reduced frequency of steatohepatitis and severity of fibrosis (Dunn 2012, Kwon 2014)
- Heavy alcohol consumption (including "binge drinking") associated with increased risk of fibrosis progression (Ekstedt 2009, Stepanova 2010)
- Obesity is an important risk factor for progression to cirrhosis in heavy alcohol drinkers (Mathurin 2012)
- "Until further data from rigorous prospective studies become available, people with NAFLD should avoid alcohol of any type or amount" (Liangpunsakul & Chalasani 2012)

Interaction between NAFLD and Iron Overload
(Corradini 2012, Dongiovanni 2012)

- Mild siderosis (hepatocellular and non-parenchymal) common in NAFLD
  - Insulin resistance important in pathogenesis ("dysmetabolic iron overload syndrome") – prevalence of DIOS in NAFLD is 20-30%
- Hepatic iron overload also promotes insulin resistance
  - Insulin resistance reversed by iron depletion
- Siderosis in hepatocytes and reticulo-endothelial cells both associated with more severe fibrosis in NAFLD (Valenti 2010, Nelson 2011)
- Siderosis also implicated in the pathogenesis of HCC in NAFLD (Sorrentino 2009)
- In patients with haemochromatosis (C282Y homozygotes), steatosis and diabetes implicated in fibrosis progression (Powell 2005, Wood 2012)

Role of Liver Biopsy in Fatty Liver Disease – Summary and Conclusions

1. Most cases of fatty liver disease (NAFLD and ALD) are diagnosed on the basis of clinical history and results of non-invasive investigations
2. In patients with NAFLD, liver biopsy may be helpful in assessing disease severity in cases where non-invasive investigations have provided unexpected or inconclusive findings.
3. Liver biopsy remains the "gold standard" for distinguishing "simple" steatosis from steatohepatitis.
4. Assessment of portal /periportal changes (inflammation, ductular reaction and fibrosis) may provide novel insights into disease progression in NAFLD.
5. Recent studies suggest that liver biopsy may provide information relevant for prognosis and management in cases of severe alcoholic hepatitis.
6. In cases where a dual pathology is suspected (e.g. HCV and NAFLD), liver biopsy is useful to confirm the diagnosis and may help to identify the predominant cause of liver injury