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, iminostat)
  - Surgical procedures (prolonged bypass, biliopancreatic diversion, extensive small bowel resection, gastoplasty 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
- Subsequently recognition of metabolic syndrome as major risk factor
- Clinical impact of NAFLD only recognised during past 10 -15 years

Liver Lesions In Fatty Liver Disease

<table>
<thead>
<tr>
<th>Metabolic Syndrome</th>
<th>Normal liver</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-00% (NAFL)</td>
<td>Fatty change</td>
<td>90%</td>
</tr>
<tr>
<td>20-40% (NASH)</td>
<td>Steatohepatitis/fibrosis (hepatocyte ballooning, Mallory-Denk bodies, inflammation)</td>
<td>30-50%</td>
</tr>
<tr>
<td>2-5%</td>
<td>Cirrhosis</td>
<td>5 -15%</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular carcinoma</td>
<td></td>
</tr>
</tbody>
</table>

NAFLD – Rising Prevalence


- 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 neoplasia

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, Yusi 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 or adenomas (inflammatory type) (Paradis 2009)
HCC Arising in NAFLD - Different Histological Features - “Steatohepatitic HCC”

(Salamo 2010, Jain 2012, Salamal 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 (tumors 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

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

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

Methods for Assessing Presence and Severity of Steatosis

- Standard Approach for H&E stained sections (Bruns 1999, Kleiner 2005)

<table>
<thead>
<tr>
<th>% involvement</th>
<th>Severity</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;5</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>5-33</td>
<td>Mild</td>
<td>1</td>
</tr>
<tr>
<td>33-66</td>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>&gt;66</td>
<td>Severe</td>
<td>3</td>
</tr>
</tbody>
</table>

- 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

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 NASH (Soderberg 2011, Tandra 2011)
- "True" microvesicular steatosis occurred in 102/1022 (10%) of biopsies from patients with NASH (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

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

<table>
<thead>
<tr>
<th>Baseline Biopsies with NAFL</th>
<th>Median Duration of Follow-up</th>
<th>Progression to NASH</th>
<th>Progression to Bridging Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pais 2013</td>
<td>25</td>
<td>3.7 years</td>
<td>64%</td>
</tr>
<tr>
<td>McPherson 2015</td>
<td>27</td>
<td>6.6 years</td>
<td>44%</td>
</tr>
</tbody>
</table>

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

Mallory-Denk Bodies - Immunohistochemical Demonstration
(from Denk 2006, Zakrzewski 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 (Moskowitz 2011)

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+, 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

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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

1. Prevalence (in adults)

<table>
<thead>
<tr>
<th>Study</th>
<th>None</th>
<th>Mild</th>
<th>More than Mild</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brunt 2009</td>
<td>16%</td>
<td>60%</td>
<td>23%</td>
</tr>
<tr>
<td>Eshghi 2010</td>
<td>32%</td>
<td>31%</td>
<td>31%</td>
</tr>
<tr>
<td>Carotti 2015</td>
<td>13%</td>
<td>40%</td>
<td>47%</td>
</tr>
</tbody>
</table>

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

RUT: Disproportionately severe portal/periportal inflammation should still raise concerns about possible concurrent chronic liver disease (Kliewer & Brunt 2012)

Portal Changes in NAFLD - Biliary Features

- Ductular reaction
- 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)

NAFLD in Children - Differences Compared with NAFLD in Adults

- Steatosis
  - Often more severe
  - May have different distribution (panacinar or periportal)

- Other lobular changes less well developed
  - Less ballooning/Mallory's hyaline
  - Less periportal/periportal 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 82% 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)**
  - 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

- **Fibrosis Score (0-4)**
  - 1a: Zone 3  perisinusoidal (mild)
  - 1b: Zone 3  perisinusoidal (moderate)
  - 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

**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/perportal inflammation not included*

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

**Histological Grading & Staging of NASH (Kleiner System)**

<table>
<thead>
<tr>
<th>Problem/Limitation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><strong>Observer variability</strong></td>
<td>Reproducibility good for fat &amp; fibrosis, less good for inflammation &amp; ballooning</td>
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<tr>
<td><strong>Sampling variability</strong></td>
<td>Fat - reasonably uniform distribution, inflammation &amp; fibrosis more variable</td>
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| **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
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**Liver Biopsies from 976 adults in NASH Clinical Research Network studies**

<table>
<thead>
<tr>
<th>NASH Score</th>
<th>Not Steatohepatitis (n = 204)</th>
<th>Borderline Steatohepatitis (n = 183)</th>
<th>Definite Steatohepatitis (n = 589)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAS 0-4</td>
<td>134</td>
<td>118</td>
<td>176</td>
</tr>
<tr>
<td>NAS 5-8</td>
<td>17</td>
<td>52</td>
<td>407</td>
</tr>
</tbody>
</table>

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 routine histological assessment of NAFLD

**Use of Steatosis/Activity/Fibrosis (SAF) Scoring NAFLD**
(Bedossa, Hepatology 2012 & Hepatology 2014)

- **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)**

<table>
<thead>
<tr>
<th>Scoring Criteria</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Steatosis</td>
<td>0: none, 1: hepatocytes with rounded shapes and pale cytoplasm, 2: steatosis (&lt; 3x normal)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0: none, 1: zone 3 periportal (mild), 2: zone 3 periportal (moderate), 3: portal/periportal, 4: cirrhosis</td>
</tr>
<tr>
<td>Activity</td>
<td>0: none, 1: fat droplets, 2: ballooning, 3: inflammation</td>
</tr>
</tbody>
</table>

**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 (NAS vs steatosis) amongst “expert” liver pathologists (n = 8) and “non-specialist” pathologists (n = 10)
- High kappa scores achieved for individual scores of SAF
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Role of Liver Biopsy in ALD

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

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   - Interaction with other diseases
     - HCV, ALD, Iron overload

ALD vs NAFLD
(Rakha 2010, Yeh 2011, Brunt 2012, Sahney 2014, Yeh & Brunt 2014)

<table>
<thead>
<tr>
<th>More common/prominent in ALD</th>
<th>More common/prominent in NAFLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ballooning, Mallory-Denk bodies</td>
<td>Steatosis (esp in children and morbid obesity)</td>
</tr>
<tr>
<td>Lobular neutrophils (&quot;satellitosis&quot;)</td>
<td>Nuclear vacuolation of hepatocytes (70-80% of cases vs &lt;10% in ALD)</td>
</tr>
<tr>
<td>Zone 3 fibrosis</td>
<td>ALD - central necrosis/cellular necrosis/fibrosis</td>
</tr>
<tr>
<td>Hepatic vein lesions (phlebosclerosis, lymphocytic phlebitis, veno-occlusive lesions)</td>
<td>NAFLD - Nuclear vacuolation</td>
</tr>
<tr>
<td>Cholestasis</td>
<td></td>
</tr>
</tbody>
</table>

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 (Katonizadeh 2010, Moszkowicz 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

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, Koren 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 2003, 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.