Non-Invasive Assessment of NAFLD

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Disclosure Slide

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Speaker
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Non-Alcoholic Fatty Liver Disease (NAFLD)

Normal Liver

NAFLD

Steatosis “NAFL”

Fat infiltration >5% with or without mild inflammation

Steatohepatitis “NASH”

Ballooning hepatocyte degeneration, Mallory bodies, megamitochondria

Cirrhosis

Hepatocellular Carcinoma (HCC)

Risk factors for NASH include Metabolic Syndrome (Obesity, Hypertension, Dyslipidaemia & Type 2 Diabetes/Insulin resistance)

- For NAFLD, alcohol consumption <20/30g per day
- Other causes for liver dysfunction/hepatotoxins excluded

NAFLD Natural History

Risk of Death or Transplantation

“Dynamic” Steatotic/Steatohepatitic phase

“Non-Linear” Fibrotic phase
Presentation

• Symptoms
  – Usually asymptomatic – majority discovered by chance
  – Fatigue frequently present

• Often an ‘Incidental Finding’
  – Incidental abnormal LFTs
  – Incidental ‘bright liver’ on imaging
  – Incidental hepatomegaly

• Common scenarios
  – Statin monitoring
  – ‘Annual reviews’ in T2DM/Lipid/Hypertension clinics
  – Medical insurance/occupational health checks

Suspected NAFLD?...Pragmatic First Steps

1. Identify ‘Risk’
   • Metabolic Syndrome / High Prevalence Group

2. History
   • Alcohol Intake (<14/21 units/week)
   • No known pre-existing liver disease

3. Investigations
   • Liver Biochemistry (ALT, AST, etc)
   • Exclude/identify other liver diseases:
     – Negative HBV & HCV Serology
     – Negative Auto-Antibodies (ANA, AMA, SMA, LKM1, ANCA)
     – Negative Coeliac Serology
     – Normal Immunoglobulins, Ferritin, A1AT, Cu++, etc.
   • Liver Ultrasound: Increased echogenicity (Fatty Liver)
Non-Invasive Diagnosis & Risk Stratification for NAFLD

20-30% of General Population have NAFLD

Approximately 3-16% of NAFLD have NASH

Is this Steatosis or Steatohepatitis?

How much Fibrosis is there?

"Which patients should I worry about & how do I spot them?"

Routine Clinical Biochemistry (LFTs)

- NAFLD is the most common diagnosis in patients with ‘incidental’ abnormal LFTs
  - Daniel, 1999; Skelly, 2001; Pendino, 2005

- Liver enzymes may be normal in up to 80% of NAFLD patients
  - Transaminases are not a sensitive test for NAFLD/NASH.
  - Poor correlation between ALT and histology
  - ALT typically falls with advanced fibrosis
  - ALT > AST ➔ ALT < AST

- Severity of histology in NAFLD with normal LFTs no different from those with abnormal LFTs
  - Mofrad, 2003; Sorrentino, 2004; Francasi, 2008

Grade/Stage of NAFLD with normal LFTs no different from those with abnormal LFTs

Routine LFTs do not differentiate Steatosis/NASH or Stage of fibrosis
### Clinical Predictors of NASH in Patients With NAFLD

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced age*</td>
<td>Greater duration of disease</td>
</tr>
<tr>
<td>Sex</td>
<td>Postmenopausal women experience accelerated disease</td>
</tr>
<tr>
<td>Race</td>
<td>↑ Prevalence, severity in Hispanic, Asian patients; ↓ Prevalence, severity in black patients</td>
</tr>
<tr>
<td>HTN,* central obesity, dyslipidemia (↑ TG, ↓ HDL), insulin resistance/T2DM*</td>
<td>Risk increases with metabolic syndrome, 66% prevalence of bridging fibrosis if older than 50 years of age and obese or diabetic</td>
</tr>
<tr>
<td>Obstructive Sleep Apnoea</td>
<td>↑ Severity of Liver Disease</td>
</tr>
<tr>
<td>AST/ALT ratio &gt; 1, low platelets</td>
<td>Indicators of NASH cirrhosis</td>
</tr>
<tr>
<td>Persistently elevated ALT</td>
<td>Can be associated with greater risk of disease progression</td>
</tr>
</tbody>
</table>

*Strongest predictors of advanced disease, regardless of liver enzyme elevation.

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**Liver Biopsy**

- **Steatosis vs. NASH**
- **Fibrosis Stage**

**DIAGNOSIS**

**PROGNOSIS**

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Routine LFTs do not differentiate Steatosis/NASH or Stage of fibrosis
Histological Features of NAFLD & NASH

<table>
<thead>
<tr>
<th>NIDDK NASH Activity Score</th>
<th>FLIP “SAF” Score</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Category definitions</strong></td>
<td><strong>Category definitions</strong></td>
</tr>
<tr>
<td><strong>Steatosis</strong></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>-5%</td>
</tr>
<tr>
<td>2</td>
<td>5-20%</td>
</tr>
<tr>
<td>3</td>
<td>&gt;20%</td>
</tr>
<tr>
<td><strong>Fibrosis</strong></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0 fibs per ≥20 field</td>
</tr>
<tr>
<td>2</td>
<td>2-3 fibs per ≥20 field</td>
</tr>
<tr>
<td>3</td>
<td>&gt;3 fibs per ≥20 field</td>
</tr>
<tr>
<td><strong>Kleiner et al., Hepatology 2005</strong></td>
<td>4</td>
</tr>
<tr>
<td><strong>Bedossa et al., Hepatology 2012</strong></td>
<td>5</td>
</tr>
</tbody>
</table>

Is a Liver Biopsy Always Necessary in NAFLD?

- Not always required but remains necessary and useful in many cases
  - Confirm diagnosis & exclude alternative/secondary pathology
  - Stage disease
  - Stratify progression risk
- Use of Biopsy should be tailored to the individual patient
  - Marked biochemical abnormalities on LFTs
  - Diagnostic doubt
  - Non-invasive scores that are ‘High’ or ‘Indeterminate’ risk
  - Patient choice

How Can We Select Patients for Liver Biopsy?
Serum Tests to Detect NASH

- **Markers of apoptosis**
  - CK-18 fragments, Feldstein, 2009
  - CK-18 + soluble Fas, Tamimi, 2011
  - CK-18 + FGF21, Shen, 2012

- **Ferritin**, Kowdley, 2012

- **PIIINP**, Tanwar, 2013

- **NASHTest®**, Poynard, 2006
  - α2-macroglobulin, apolipoprotein A1, haptoglobin, bilirubin, GGT, ALT, serum glucose, triglycerides and cholesterol, adjusted for age, gender and BMI.

- **Palekar index**, Palekar, 2006
  - Sum of risk factors: Age >50 years, Female gender, AST >45 IU/l, BMI >30, AST/ALT Ratio >0.80, and HA >55 mcg/l

- **Shimada index**, Shimada, 2007
  - Serum adiponectin level, HOMA-IR, and serum type IV collagen 7S level.

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All require further independent validation and none are in widespread clinical use

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Blood Tests for Liver Fibrosis

<table>
<thead>
<tr>
<th>Indirect Serum Markers</th>
<th>Direct Serum Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Routine clinical blood tests</strong></td>
<td><strong>ECM components</strong></td>
</tr>
<tr>
<td>- ALT, AST</td>
<td>- Procollagen III N-peptide (PIIINP)</td>
</tr>
<tr>
<td>- Albumin, PT/INR</td>
<td>- Type IV collagen (7S domain)</td>
</tr>
<tr>
<td>- Platelet count</td>
<td>- Laminin</td>
</tr>
<tr>
<td><strong>Markers of Inflammation</strong></td>
<td>- Hyaluronic acid</td>
</tr>
<tr>
<td>- YKL-40, MCP-1</td>
<td><strong>Factors regulating Fibrogenesis and/or Fibrolysis</strong></td>
</tr>
<tr>
<td>- HsCRP</td>
<td>- Collagenases &amp; inhibitors</td>
</tr>
<tr>
<td>- Haptoglobin</td>
<td>- α2-Macroglobulin</td>
</tr>
<tr>
<td>- TNFα, IL-6, IL-8</td>
<td>- Metalloproteinases (MMPs)</td>
</tr>
<tr>
<td><strong>Markers of Apoptosis/Necrosis</strong></td>
<td>- TIMPs (TIMP-1, etc.)</td>
</tr>
<tr>
<td>- CK18 (M30/M65)</td>
<td><strong>Metabolic/Liver Function</strong></td>
</tr>
<tr>
<td><strong>Metabolic/Liver Function</strong></td>
<td>- Apolipoprotein A1</td>
</tr>
</tbody>
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Indirect and Direct Markers may be used Individually or in Combination

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'Simple Scores' for Predicting Presence of Advanced (F3/4) Fibrosis

**NAFLD Fibrosis Score**

\[-1.675 + 0.037 \times \text{Age} + 0.094 \times \text{BMI} + 1.13 \times \text{IFG/diabetes} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{Platelets} - 0.66 \times \text{Albumin}].

- A score of less than -1.455 excludes fibrosis (NPV 88-93%).
- A score of greater than 0.676 predicts fibrosis (PPV 82-90%).

**FIB-4 Score**

\[\frac{\text{Age} \times \text{AST}}{\text{Platelets} \times \sqrt{\text{ALT}}}].

- A score of less than 1.3 excludes fibrosis (NPV 95%)
- A score greater than 3.25 predicts fibrosis (PPV ~70%)

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**Comparison of the Diagnostic Performance of Simple Tests for Advanced Fibrosis (F3/F4)**

<table>
<thead>
<tr>
<th>Test</th>
<th>AUC</th>
<th>Cut-off</th>
<th>Sens (%)</th>
<th>Spec (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST/ALT ratio</td>
<td>0.83</td>
<td>0.8</td>
<td>74</td>
<td>78</td>
<td>44</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>(0.74-0.91)</td>
<td>1</td>
<td>52</td>
<td>90</td>
<td>55</td>
<td>89</td>
</tr>
<tr>
<td>APRI</td>
<td>0.67</td>
<td>1</td>
<td>27</td>
<td>89</td>
<td>37</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>(0.54-0.8)</td>
<td>2</td>
<td>89</td>
<td>44</td>
<td>27</td>
<td>95</td>
</tr>
<tr>
<td>BARD score</td>
<td>0.77</td>
<td>1.30</td>
<td>85</td>
<td>65</td>
<td>36</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>(0.68-0.87)</td>
<td>1.30</td>
<td>85</td>
<td>65</td>
<td>36</td>
<td>95</td>
</tr>
<tr>
<td>FIB-4 score</td>
<td>0.86</td>
<td>0.8</td>
<td>78</td>
<td>58</td>
<td>30</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>(0.78-0.94)</td>
<td>0.8</td>
<td>78</td>
<td>58</td>
<td>30</td>
<td>92</td>
</tr>
<tr>
<td>NAFLD fibrosis score</td>
<td>0.81</td>
<td>-1.455</td>
<td>33</td>
<td>98</td>
<td>79</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>(0.71-0.91)</td>
<td>0.676</td>
<td>33</td>
<td>98</td>
<td>79</td>
<td>86</td>
</tr>
</tbody>
</table>
NAFLD Fibrosis Score & FIB-4 Predict Long-term Outcome

Adjusted Hazard Ratio for Transplantation/Death

<table>
<thead>
<tr>
<th>NAFLD-FS</th>
<th>FIB-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate vs. Low</td>
<td>High vs. Low</td>
</tr>
<tr>
<td>4.2 [95%CI 1.3-13.8]</td>
<td>9.8 [95%CI 2.7-35.3]</td>
</tr>
<tr>
<td>Intermediate vs. Low</td>
<td>High vs. Low</td>
</tr>
<tr>
<td>2.3 [95%CI 0.8-6.6]</td>
<td>6.9 [95%CI 2.3-20.4]</td>
</tr>
</tbody>
</table>

Serum Tests Assessing Fibrosis Stage

- ‘Expanded’ lab indices
  - FibroTest*
    - Age, gGT, Bilirubin, α2-Macroglobulin, Haptoglobin, Apolipoprotein A1
    - F3/4 fibrosis: AUC 0.88 (0.82-0.92) Ratziu, 2006
  - FibroMeter*
    - Age, Weight, Glucose, ALT, AST, Ferritin, Platelets Cales, 2008
  - HepaScore
    - Age, Gender, gGT, Bilirubin, Hyaluronic acid, α2-Macroglobulin Adams, 2005

- ‘Direct’ fibrosis markers
  - Procollagen III N-Peptide (PIIINP) Tanwar, 2013
  - The ELF-test®
    - HA, PIIINP, TIMP1
    - F3/4 fibrosis: AUC 0.90 (0.84-0.96) Rosenberg, 2004; Guha, 2008
  - Neo-Epitope “Protein Finger Print®” tests

Further independent validation in NAFLD cohorts needed before widespread clinical use
Equilibrium of ECM Turnover

Connective Tissue Equilibrium

Fibrolysis Fibrogenesis

↑ ECM Degradation
- Collagenases & inhibitors
- TIMPs (TIMP-1, etc.)
- C3M
- Pro-C3/C5

↑ ECM Formation
- PIIINP
- Hyaluronic Acid
- Type IV collagen (7S)
- Metalloproteinases (MMPs)
- C3M

If validated, Direct Markers may better reflect the dynamics of matrix turnover and so could be useful not only as diagnostics but for monitoring response to treatment.

Adapted from Karsdal et al. Am J Physiol 2015

Validation of Terminal Peptide of Procollagen III for the Detection and Assessment of Nonalcoholic Steatohepatitis in Patients With Nonalcoholic Fatty Liver Disease

n=172 histologically characterised NAFLD patients

PIIINP > 11ng/ml: PPV 74-100%; NPV 89-93% for advanced fibrosis

PIIINP discriminates between NAFL and “NASH OR advanced fibrosis”
Performance of Enhanced Liver Fibrosis (ELF®) Test in NAFLD

Combined 3 direct markers of fibrosis:
- Procollagen III N-terminal peptide (PIIINP)
- Hyaluronic acid (HA)
- Tissue inhibitor of metaloproteinase 1 (TIMP1)

**ELF Algorithm**
\[ DS = -7.412 + (\ln(HA)*0.681) + (\ln(PIIINP)*0.775) + (\ln(TIMP1)*0.494). \]

**ELF Test Predicts Long-term Outcome**

<table>
<thead>
<tr>
<th>ELF Test</th>
<th>Adjusted Hazard Ratio for Transplantation/Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELF F1 vs. ELF F3</td>
<td>20 [95%CI 5.4-71.0]</td>
</tr>
<tr>
<td>ELF F1 vs. ELF F4</td>
<td>76 [95%CI 17.6-325]</td>
</tr>
</tbody>
</table>

n=457 Mixed aetiology cohort, 0-9 years follow-up
*Most HCV, only 10% cases NAFLD*
Protease Generated Neo-Epitopes as Novel Fibrosis Biomarkers

Protease Mediated Connective Tissue Destruction

Pathology Dependent Protease Enzyme

Specific degradation site

Signature Protein

Protein Fragments (neo-epitopes)

• C5M (MMP-mediated type V collagen degradation)
• C6M (MMP-mediated type VI collagen degradation)
• Pro-C3 (released by ADAMTS2 during Type III collagen formation)
• P4NFS (Type IV collagen formation)
• Pro-C5 or P5CP (Type V collagen formation)
• Pro-C6 or PVINP (Type VI collagen formation)

Pro-C3 is diagnostic as well as able to predict fibrosis progression in HCV (Nielson et al, 2015)
Pro-C3 is a marker of fibrogenesis and can be used in the identification of patients with progressive disease and monitoring response to antifibrotic therapies (Nielson et al, 2016)

A signature protein from a given tissue combined with a pathology dependent protease provides a unique protein degradation site, which is the biomarker target

Imaging Based Modalities

Routine imaging (Ultrasound, CT, MRI)
• Can not differentiate steatosis vs. steatohepatitis
• Can not identify fibrosis in the absence of advanced cirrhosis.

Steatosis
– Controlled Attenuation Parameter (CAP®)
– MRI-PDFF

Steatohepatitis
– Experimental techniques, e.g. LMS®

Fibrosis
– Transient elastography (FibroScan®)
– Acoustic radiation ARFI
– Real time elastography
– MR elastography

Yoneda 2008, Wong 2010, Myers 2011
Palmeri 2011, Bota 2014
Ochi 2012
Loomba 2014
Approximately 10% of subjects were excluded due to failure to acquire 10 successful FibroScan® measurements.

Failure Rate → 25.5% if BMI ≥ 30 and 2.6% if BMI 25-30.

Only a minority (28.5%) of patients in the study had a BMI >30 vs 67% of the population.
A Tractable & Pragmatic Approach Using Readily Available Tests
Calculate FIB-4 Fibrosis Score

Age <65: FIB-4 <1.3
Age >65: FIB-4 <2.0

Low risk (NPV >90%)

More than 2.67

High risk (PPV >70%)

Indeterminate

Refer to Secondary Care

Transient Elastography (Fibroscan)

M probe < 7.9 kPa
XL probe < 7.2 kPa

M probe 7.9 – 9.6 kPa
XL probe 7.2 – 9.3 kPa

M probe > 9.6 kPa
XL probe > 9.3 kPa

Advanced [F3-F4]
Fibrosis Excluded (NPV 89-97%)

Advanced [F3-F4]
Fibrosis Likely (PPV 71-72%)

Indeterminate

Liver Biopsy

Fibrosis F0-1

Fibrosis F2-3

Cirrhosis F4

Manage in Primary Care, Lifestyle Advice, Address CVD Risks, Recalculate FIB-4 3-5 years.

Lifestyle Advice, Address CVD Risks, NAFLD Directed Therapy, HCC & Variceal Surveillance

Suspected NAFLD

Features of the Metabolic Syndrome, radiological evidence of steatosis and/or abnormal liver biochemistry, elevated FLI, alternative diagnoses excluded.
Advances in the fields of genomics, epigenetics, proteomics and metabolomics/lipidomics have led to a rapid expansion in the discovery of potential biomarkers. Although promising, many are highly experimental and require robust independent validation.
Metabolomic Profiling for Diagnosis of NASH

Serum metabolomic profile reflects hepatic metabolism

Discovery and validation of test based on twenty-eight serum triglycerides

<table>
<thead>
<tr>
<th>Test cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAFLD vs. Normal Liver</td>
<td>NAFLD vs. Normal Liver</td>
</tr>
<tr>
<td>Test (N)</td>
<td>467</td>
</tr>
<tr>
<td>Validation (N)</td>
<td>111</td>
</tr>
</tbody>
</table>

Steatosis (NAFL) vs. NASH

<table>
<thead>
<tr>
<th>Test cohort</th>
<th>Validation cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test (N)</td>
<td>377</td>
</tr>
<tr>
<td>Validation (N)</td>
<td>95</td>
</tr>
</tbody>
</table>

MicroRNAs as Potential Biomarkers for Hepatic Fibrosis

- Highly conserved.
- Small, 18-25 nucleotide, non-coding RNAs.
- Binds mRNA 3'-UTR repressing translation by destabilizing mRNA/silencing translation
- Regulate gene expression at post-transcription - translational level.

miRNAs circulating in:
- Protein complexes (Argo-2)
- Lipoproteins
- Microvesicles
- Exosomes

Szabo & Bala, Nat Rev Gastroenterol Hepatol, 2013: 10, 542
miRNAs Discriminating Patients “To-Be-Treated” vs. “Not To-Be-Treated”

“To-Be-Treated” = Histological Features of NASH & Moderate/Advanced Fibrosis (NAS ≥4 & Fibrosis Stage ≥F2)

Circulating levels of 2,083 miRNAs measured by HTG-EdgeSeq-NGS and confirmed by RT-qPCR.

Algorithm containing:
- a2-Macroglobulin, miR34a, YKL-40 and HbA1c

Harrison et al, Poster presentation, EASL 2017
Conclusions

- NAFLD is highly prevalent, largely asymptomatic disease characterised by substantial inter-patient variability in disease severity and outcome.
- Serum based biomarkers may be considered in two distinct groups, although in practice these are often combined into biomarker panels:
  - Indirect Serum Markers.
  - Direct Serum Markers.
- Imaging techniques also offer potential benefits for both clinical and trial use.
- At present, the staged application of available ‘simple panel’ biomarkers (NFS, FIB4) followed by a second non-invasive test (e.g. Fibroscan, MRE) helps to rule out cases that are unlikely to have significant disease.
- There is an urgent need for more sensitive and specific, independently validated and qualified biomarkers for use in NAFLD. Promising experimental biomarkers include novel direct biomarkers related to ECM turnover, Metabolomic profiling, miRNAs and DNA methylation signatures.