WHAT THE EXPERIMENTAL MODELS CAN TEACH US IN NAFLD/NASH?

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NAFLD is the most common chronic liver disease and has rapidly become an important cause of liver failure.

The worldwide "estimated" prevalence of NAFLD is 15-40%.
NAFLD do we really know what are we talking about?

Is it time to change NAFLD and NASH nomenclature?

The histological features of fatty liver, both alcoholic and non-alcoholic, range from 3–4 classes of liver disease or disease severity. The term NAFLD itself was established in the 1990s to coin a nomenclature ranging from non-alcoholic fatty liver disease (NAFLD) not only to dissect the pathologically defined subtypes of fat vacuolated in the liver but rather a more complex spectrum of disease. It was formulated in the hope of clarifying a disease process ranging from simple steatosis, steatohepatitis, cirrhosis, and hepatocellular carcinoma. However, the “D” to the acronym NAFLD was introduced as a misnomer of a more comprehensive disease process, in which fat deposition is not merely a reversible but rather an irreversible process. This was subsequently revised to the term Non-Alcoholic Steatohepatitis (NASH). This process, which is not only related to steatosis, was further defined as NASH, a process in which inflammation and fibrosis are present. Furthermore, a more specific term, NASH, was introduced to describe the disease process ranging from steatosis, steatohepatitis, cirrhosis, and hepatocellular carcinoma. However, the term NASH was introduced as a misnomer of a more comprehensive disease process, in which fat deposition is not merely a reversible but rather an irreversible process. This was subsequently revised to the term Non-Alcoholic Steatohepatitis (NASH).

TAKE HOME MESSAGE

Coordinated action is needed to achieve the important target of nomenclature, which is not merely semantic to revise the NAFLD and NASH nomenclature.

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*Stefano Bellentani, Claudio Tiribelli
NAFLD Wide Spectrum Disease

**NAFL**
- Steatosis
- Lipotoxicity
- Inflammation
- Oxidative stress

**NASH**
- Sustained Inflammation
- Hepatocyte injury
- HSC activation
- ECM remodeling
- Fibrogenesis

**Cirrhosis**
- Liver fibrosis
- Functional complications
- Sustained Inflammation
- Hepatocyte injury
- HSC activation
- ECM remodeling
- Fibrogenesis

**HCC**
- End stage liver damage
- Functional complications
- Fibrogenesis

### NAFL to NASH
- 25-30%
- 3-10%

### NASH to Cirrhosis
- 10-29%
- 10 years

### Cirrhosis to Hepatocarcinoma
- 4-27%
- 0-2.8%

**Histopathological Images**

2015 - Rosso, N.
Translational models
Available Models for the study of NAFLD

**In vivo**

**Animal models**

- **Diet-Induced**
  - Overnutrition
  - High-fat diets
  - Forced feedings
  - Hyperfagic animals

- **Genetically modified**
  - Nullizygous acyl-CoA oxidase (ACOX)
  - Methionine adenosyltransferase (MAT)-1° (MATO mice)
  - *pten* deletion
  - Animals lacking leptin (*ob*/*ob*) and leptin receptor (*db*/*db*)

**In vitro**

**Primary cell Cultures**
- Hepatocytes
- Hepatic Stellate cells
- Kupffer cells
- Endothelial Cells

**Immortalized Cell lines**
- Hepatocytes
- Hepatic Stellate cells
- Kupffer cells
- Endothelial cells

**Liver Slices**
- Multicellular system in which cell-cell and cell-ECM interactions are maintained

**Human Samples**
- Most easily available samples are biopsies from subjects undergoing bariatric surgery
Key Players

Hepatic Cell line (HuH7)

Enriched culture medium
• Oleic:Palmitic acid (2:1)
• Controlled Albumin : FFA ratio (1:4)
• Incubation time 24h

Non toxic model
Intracellular Fat accumulation
Inflammatory response
Fibrogenic cytokines
Oxidative stress
Apoptosis
The Canonical Principle of Fibrogenesis*

Liver Injury

Liver Injury

Activation of HSC

Expansion of MFB

Hepatocytes

Hepatic Stellate cells (HSC)

Myofibroblasts (MFB)

Recruitment

Fibrosis

Cirrhosis

HCC

↑ ROS

↑ Lipoapoptosis

↑ NFκB

IGF-1

PDGF

TNF-α

TGF-β

ET-1

ROS

Adipocytokines

↑ Collagen

↑ Elastin

↑ Glycoproteins

↑ Proteoglycans

↑ Hyaluronan

Activated Quiescent

Hepatic stellate cells (LX-2)

Co-culture
- Conditioned medium
- Transwell System
- Simultaneous co-culture
- Incubation time 24-96-144h

Cellular cross talk to progress towards Fibrosis

The interplay between hepatic stellate cells and hepatocytes in an in vitro model of NASH

Varenka J. Barbero-Becerra, Pablo J. Giraudi, Norberto C. Chávez-Tapia, Misael Uribe, Claudio Tiribelli, Natalia Rosso

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**Centro Studi Fegato (CSF) – Liver Research Center, Fondazione Italiana Fegato, Bldg Q, AREA Science Park, Basovizza Campus SS 14 km 163.5, 34149 Trieste, Italy

†Department of Medical Sciences, University of Trieste, 34100 Trieste, Italy

For hepatic stellate cell activation Hepatocyte cell-to-cell contact is necessary
Cell-to-cell interaction is necessary to fibrogenesis

The importance of the interaction between hepatocyte and hepatic stellate cells in fibrogenesis induced by fatty accumulation

Pablo J. Giraudi a, b, Varenka J. Barbero Becerra a, b, Verónica Marin a, Norberto C. Chavez-Tapia a, b, Claudio Tiribelli a, c, Natalia Rosso a, c

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b Liver Research Unit, Médica Sur Clinic & Foundation, Paseo de la Piedra 150, Col. Toribio Guerra, Tlalpan, C.P. 14000 Mexico City, Mexico
c Department of Medical Sciences, University of Trieste, 34100 Trieste, Italy

- Comparable accumulation of FFA
- HSC activation 24h in terms of gene expression
- 96h protein expression
- Regulation of ECM components
- Synthesis di collagen (96h)
- Extracellular collagen deposition (144h)
- In vitro model for the study of the complex molecular events involved in the FFA-induced fibrogenesis
In vivo model of NAFLD

An Animal Model for the Juvenile Non-Alcoholic Fatty Liver Disease and Non-Alcoholic Steatohepatitis

Veronica Marin¹, Natalia Rosso¹, Matteo Dal Ben¹, Alan Raseni², Manuela Boschelle², Cristina Degrassi³, Ivana Nemeckova⁴, Petr Nachtigal⁵, Claudio Avellini⁶, Claudio Tiribelli¹,⁶, Silvia Gazzin¹ *

RESEARCH ARTICLE | PLOS ONE | DOI:10.1371/journal.pone.0158817 | July 8, 2016

Monthly Screening
Body weight, Insulinemia and Glycaemia

Checkpoint screening
Blood tests
Histopathological analysis
Molecular analysis
Assessment of the gut permeability
Characterization of gut microbiota

✓ Obesity (BMI)
✓ WAT Hypertrophy
✓ Hepatomegaly
✓ Insulin Resistance
✓ Dyslipidemia
✓ ALT & AST increase
✓ Liver progressive fibrosis
Diagnostic Tools
Diagnosis

- Liver Biopsy is the **gold standard** for the diagnosis of NAFLD, and for the histological assessment of the liver damage.

  However for ethical reasons is impossible to perform liver biopsy to all suspected NAFLD subjects.

  The technique does not allow to perform a continuous screening

- Significant fibrosis is present in 5-10% of patients with NAFLD.

- Fibrosis determination is useful to monitor the disease progression or treatment response over the time

- To date, reliable **non invasive** diagnostic tools are missing
**Our strategy**

### In silico approach
- Analysis of protein-protein interactions in the pathological process
- Selection of candidates (found soluble in plasma)

### In vivo validation
- Plasma quantification
- Correlation with the degree of liver injury (biopsy)
- Validation as a predictor of damage and evolution of the disease

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**Table 1** Demographic and laboratory characteristics of all subjects

<table>
<thead>
<tr>
<th></th>
<th>MO (n = 71)</th>
<th>CTRLs (Lean) (n = 11)</th>
<th>Cirrhosis (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43 ± 12*</td>
<td>33 ± 4</td>
<td>69 ± 8***</td>
</tr>
<tr>
<td>Gender (female)</td>
<td>47 (66%)</td>
<td>6 (54%)</td>
<td>7 (50%)</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>44 ± 7***</td>
<td>23 ± 2</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>33 ± 31</td>
<td>23 ± 12</td>
<td>38 ± 21</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>24 ± 14</td>
<td>25 ± 11</td>
<td>41 ± 24</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>33 ± 24</td>
<td>25 ± 14</td>
<td>124 ± 78**</td>
</tr>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>113 ± 25*</td>
<td>93 ± 11</td>
<td>124 ± 41*</td>
</tr>
<tr>
<td>Fasting insulin (µU/mL)</td>
<td>20 ± 14</td>
<td>8 ± 5</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.3 ± 1*</td>
<td>N/A</td>
<td>7 ± 2*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>5.3 ± 4**</td>
<td>1.6 ± 0.4</td>
<td>3.7 ± 2</td>
</tr>
<tr>
<td>Diabetes</td>
<td>15 (21%)</td>
<td>0</td>
<td>8 (57%)</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>200 ± 40</td>
<td>182 ± 34</td>
<td>190 ± 54</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>141 ± 91</td>
<td>93 ± 35</td>
<td>136 ± 64</td>
</tr>
</tbody>
</table>

- Steatosis 0/1/2/3 7/29/16/19 N/A N/A
- Lobular inflammation 0/1/2 14/45/12 N/A N/A
- Balloning 0/1/2 16/18/37 N/A N/A
- Fibrosis 0/1/2/3/4 8/50/12/1/0 N/A 0/0/0/6/8
- NAFL/NASH 27 (38%)/44 (62%) N/A 60%/40%

BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; N/A, not available.

*P < .001; **P < .01 and *P < .05 were considered statistically significant vs CTRLs.
FIGURE 1  Protein-Protein Interaction biological network of the mediators involved in liver fibrosis visualized using Cytoscape open software. Individuated candidate biomarkers are highlighted in red.
Comparison of the performance of each test for the diagnosis of significant fibrosis in the MO cohort

<table>
<thead>
<tr>
<th>Biomarker/Test</th>
<th>AUROC (95% CI)</th>
<th>Cut-off</th>
<th>Sens (%)</th>
<th>Spec (%)</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF2</td>
<td>0.83 (0.70-0.92)</td>
<td>1.9</td>
<td>85.7</td>
<td>73.7</td>
<td>58.3</td>
<td>92.3</td>
</tr>
<tr>
<td>EGFR</td>
<td>0.71 (0.59-0.82)</td>
<td>102.5</td>
<td>94.4</td>
<td>52.9</td>
<td>46.2</td>
<td>95.7</td>
</tr>
<tr>
<td>EGFR/IGF2</td>
<td>0.79 (0.67-0.88)</td>
<td>58</td>
<td>73.3</td>
<td>73.6</td>
<td>54.3</td>
<td>86.6</td>
</tr>
<tr>
<td>Fibrometer</td>
<td>0.64 (0.51-0.75)</td>
<td>51</td>
<td>76.9</td>
<td>64.0</td>
<td>47.8</td>
<td>86.6</td>
</tr>
<tr>
<td>FIB-4</td>
<td>0.63 (0.50-0.74)</td>
<td>0.78</td>
<td>66.7</td>
<td>64.1</td>
<td>44.4</td>
<td>81.8</td>
</tr>
</tbody>
</table>

AUROC, Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value.
Therapeutic Options
Available drugs & targets

Dyslipidemia & Hypertension
- Statins
- Fibrates

Management of Diabetes
- Metformin
- Glucagon Like-1 and DDPIV inhibitors
- Liraglutide
- Sodium Glucose co-transporters 2 inhibitors (SGLT2)
- Thiazolidinedione (TZD)

Others
- Vitamin E
- Silymarin

Drugs in phase II/III Development
- **FXR agonists**
  - OCA, UDCA, GS-9674, LMB763, LJN452

- **Bile acids sequestrants / transport inhibitors**
  - Colesevelam, Sevelamer, ASBAT inhibitors

- **Hormone signalling**
  - FGF-21 analogues (BMS-986036)
  - FGF-19 (NGM-282)

- **Anti-inflammatory and anti-apoptotic agents**
  - Cenicriviroc, Selonsertib, VAP-1, Emricasan

- **Inhibitors of the novo lipogenesis**
  - Aramchol, Malonyl-CoA inhibitor, THR-beta agonist MGL-3196

- **Gut microbiome**
  - IMM-124e

- **Antifibrotic agents**
  - Antibody anti LOX2, LOX2 enzyme inhibitor, GR-MD-02
In vivo model of NAFLD

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RESEARCH ARTICLE  PLOS ONE | DOI:10.1371/journal.pone.0158817  July 8, 2016

Monthly Screening
Body weight, Insulinemia and Glycaemia

Checkpoint screening
Blood tests
Histopathological analysis
Molecular analysis
Assessment of the gut permeability
Characterization of gut microbiota

Characterization of the juvenile model

 HFHC diet

♂ ♀
(P21) 3 weeks

T0

T1 4 weeks

T2 8 weeks

T3 12 weeks

T4 16 weeks

☑ Obesity (BMI)
☑ WAT Hypertrophy
☑ Hepatomegaly
☑ Insulin Resistance
☑ Dyslipidemia
☑ ALT & AST increase
☑ Liver progressive fibrosis
**In vivo model of NAFLD – Therapeutic strategy**

**nutrients**

**Article**

Effects of Oral Administration of Silymarin in a Juvenile Murine Model of Non-alcoholic Steatohepatitis

Veronica Marin 1, Silvia Gazzin 1, Sabrina E. Gambaro 1, Matteo Dal Ben 1, Sonia Calligaris 2, Monica Anese 2, Alan Raseni 3, Claudio Avellini 4, Pablo J. Giraudi 1, Claudio Tiribelli 1 and Natalia Rosso 1,2

**Parameters under study**

- Changes in Body, Adipose Tissue and Liver Weight
- Glucose homeostasis
- Dyslipidemia (Cholesterol, LDL, HDL)
- AST/ALT
- Liver histology (steatosis, inflammation and fibrosis)
- Molecular analysis
- Oxidative stress
- Apoptosis

**In vivo model of NAFLD – Therapeutic strategy**

- **CTRL diet**
- **HFHC diet**
- **Diet Switch**
- **Parameters under study**
  - Changes in Body, Adipose Tissue and Liver Weight
  - Glucose homeostasis
  - Dyslipidemia (Cholesterol, LDL, HDL)
  - AST/ALT
  - Liver histology (steatosis, inflammation and fibrosis)
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<tr>
<th>CTRL</th>
<th>HFHC</th>
<th>HFHC + SIL</th>
<th>HFHC → CTRL</th>
<th>HFHC → CTRL + SIL</th>
</tr>
</thead>
</table>

**H&E**

- A
- B
- C
- D
- E

**Gomori**

- F
- G
- H
- I
- J

**Sirius red/ fast green**

- K
- L
- M
- N
- O

**U**

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<th></th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
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**Note:**

- The images (A to T) represent histological sections under different staining methods.
- The graph (U) shows the collagen/mg protein levels for each group.
NAFLD treatment

Is limited by the complex nature and the rather high individual variability

To date, none of the therapeutic approaches have provided a real, long-lasting benefit.

Lifestyle changes & Weight loss

Remain the cornerstone in the management of this disease

Aerobic activities as well as resistance training are equally efficient in reducing visceral fat and liver steatosis, even if weight loss is not achieved

Low COMPLIANCE
Conclusions
NAFLD: do we really know what are we talking about?
How far are we from a cure?

- We name the disease, stating **what it is not**

- There is consensus about the **complexity of the pathophysiology**
  “More than one organ/cell/pathway is involved” **However, the single mechanism has not been fully understood**

- **Obesity** is tightly related to NAFLD, **however we are unable to perform an accurate diagnosis in this population**

- **Reliable experimental models** need to be used **to define the disease and possible treatment(s)**

- **Efficient therapy** is currently unavailable, and the best options still relies on **life style changes**
Thank you for your attention
Improvement of plasmatic levels after bariatric surgery

**IGF2**

- **p<0.01** T0 vs T12M
- **p<0.001** T0 vsCtrls

**EGFR**

- *p<0.05** T0 vs T6M
- **p<0.001** T0 vs T12M
- ***p<0.001** T0 vsCtrls

2017 - Giraudi PJ et al- in preparation
Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease.

Ludwig J, Viggiano TR, McGill DB, Oh BJ.

Abstract
Nonalcoholic steatohepatitis is a poorly understood and hitherto unnamed liver disease that histologically mimics alcoholic hepatitis and that also may progress to cirrhosis. Described here are findings in 20 patients with nonalcoholic steatohepatitis of unknown cause. The biopsy specimens were characterized by the presence of striking fatty changes with evidence of lobular hepatitis, focal necroses with mixed inflammatory infiltrates, and, in most instances, Mallory bodies; Evidence of fibrosis was found in most specimens, and cirrhosis was diagnosed in biopsy tissue from three patients. The disease was more common in women. Most patients were moderately obese, and many had obesity-associated diseases, such as diabetes mellitus and cholelithiasis. Presence of hepatomegaly and mild abnormalities of liver function were common clinical findings. Currently, we know of no effective therapy.