Non-alcoholic fatty liver disease; Molecular mechanism and clinical implication

Sung-Woo Park, M.D., Ph.D.

Kangbuk Samsung Hospital, Sungkyunkwan University
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• Definition
• Epidemiology
• Diagnosis
• Pathogenesis
• Management of NAFLD
• For new drugs?
Definition of NAFLD

(a) There is evidence of hepatic steatosis, either by imaging or by histology and (>5% of hepatocytes)

(b) There are no causes for secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication or hereditary disorders
Common Causes of Secondary Hepatic Steatosis

<table>
<thead>
<tr>
<th>Macrovacular steatosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Excessive alcohol consumption</td>
</tr>
<tr>
<td>- Hepatitis C (genotype 3)</td>
</tr>
<tr>
<td>- Wilson’s disease</td>
</tr>
<tr>
<td>- Lipodystrophy</td>
</tr>
<tr>
<td>- Starvation</td>
</tr>
<tr>
<td>- Parenteral nutrition</td>
</tr>
<tr>
<td>- Abetalipoproteinemia</td>
</tr>
<tr>
<td>- Medications (e.g., amiodarone, methotrexate, tamoxifen, corticosteroids)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microvesicular steatosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Reye’s syndrome</td>
</tr>
<tr>
<td>- Medications (valproate, anti-retroviral medicines)</td>
</tr>
<tr>
<td>- Acute fatty liver of pregnancy</td>
</tr>
<tr>
<td>- HELLP syndrome</td>
</tr>
<tr>
<td>- Inborn errors of metabolism (e.g., LCAT deficiency, cholesterol ester storage disease, Wolman disease)</td>
</tr>
</tbody>
</table>
Alcohol Consumption & Definition of NAFLD

• However, the precise definition of significant alcohol consumption in patients with suspected NAFLD is uncertain.

• A recent consensus meeting concluded that, for NASH clinical trials candidate eligibility purposes, significant alcohol consumption be defined as >21 drinks per week in men(30g/d) and >14 drinks per week in women(20g/d) over a 2-year period prior to baseline liver histology.
The Spectrum of NAFLD

| Nonalcoholic Fatty Liver Disease (NAFLD) | Encompasses the entire spectrum of fatty liver disease in individuals without significant alcohol consumption, ranging from fatty liver to steatohepatitis and cirrhosis. |
| Nonalcoholic Fatty Liver (NAFL) | Presence of hepatic steatosis with no evidence of hepatocellular injury in the form of ballooning of the hepatocytes or no evidence of fibrosis. The risk of progression to cirrhosis and liver failure is minimal. |
| Nonalcoholic steatohepatitis (NASH) | Presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis. This can progress to cirrhosis, liver failure and rarely liver cancer. |
| NASH Cirrhosis | Presence of cirrhosis with current or previous histological evidence of steatosis or steatohepatitis |
| Cryptogenic Cirrhosis | Presence of cirrhosis with no obvious etiology. Patients with cryptogenic cirrhosis are heavily enriched with metabolic risk factors such as obesity and metabolic syndrome. |
| NAFLD Activity Score (NAS) | An unweighted composite of steatosis, inflammation, and ballooning scores. It is a useful tool to measure changes in liver histology in patients with NAFLD in clinical trials. |

**The Spectrum of NAFLD**

- **Fatty Liver**: Fat accumulates in the liver
- **NASH**: Fat plus inflammation and scarring
- **Cirrhosis**: Scar tissue replaces liver cells
## Risk Factors Associated with NAFLD

<table>
<thead>
<tr>
<th>Conditions with Established Association</th>
<th>Conditions with Emerging Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obesity</td>
<td>Polycystic ovary syndrome</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>Hypothyroidism</td>
</tr>
<tr>
<td>Dyslpidemia</td>
<td>Obstructive sleep apnea</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>Hypopituitarism</td>
</tr>
<tr>
<td></td>
<td>Hypogonadism</td>
</tr>
<tr>
<td></td>
<td>Pancreato-duodenal resection</td>
</tr>
</tbody>
</table>
Epidemiology
Epidemiology of NAFLD

• NAFLD is the most common cause of liver test abnormalities; the disease accounts for 70% of all cases of asymptomatic elevated transaminase enzyme levels in US adults.
• The prevalence of NAFLD was found to be 32% in a population-based study of 2,287 individuals from the US that used the highly sensitive technique of magnetic resonance spectroscopy.
• NAFLD is not restricted to adult populations; 10% of children and adolescents in the US
Epidemiology of NAFLD

- Features of the metabolic syndrome including obesity, T2DM, dyslipidemia and hypertension are strongly associated with NAFLD.
- 85% of patients with NAFLD have at least one metabolic risk factor with one-third of individuals having the metabolic syndrome itself.
- The prevalence of obesity in patients with NAFLD is reported to range between 61% and 100% and the prevalence of dyslipidemia between 27% and 67%.
- Among morbidly obese patients who undergo bariatric surgery, 90% have NAFLD.
### Epidemiology: NHANES DATA

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Prevalence of NAFLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1988-1994</td>
<td>5.5%</td>
</tr>
<tr>
<td>1999-2004</td>
<td>9.8%</td>
</tr>
<tr>
<td>2005-2008</td>
<td>11.0%</td>
</tr>
</tbody>
</table>

- Accounting for 47, 63 and 75% of chronic liver disease during those time periods, respectively.
  - However, it should be noted that the definition of NAFLD used in the study (elevated serum aminotransferase levels in the absence of an alternative explanation) could lead to misclassification and likely underestimated the true prevalence of NAFLD, since patients with NAFLD may have normal serum aminotransferases.
NAFLD in Asia-as common and important as in the West

• Community prevalence ranges between 20% (China), 27% (Hong Kong), and 15-45% (South Asia, South-East Asia, Korea, Japan and Taiwan).

• NAFLD is unlikely to be less severe in Asians than in other populations, but the associated obesity and diabetes pandemics have occurred more recently in Asia than in Europe and the USA, and occur with reduced degrees of adiposity.
In Korea (KBSMC)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall</th>
<th>No NAFLD</th>
<th>NAFLD, low NFS</th>
<th>NAFLD, intermediate or high NFS</th>
<th>P value for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>43,166</td>
<td>31,514</td>
<td>11,214</td>
<td>438 (26.0%)</td>
<td>(1.0%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36.8 (4.5)</td>
<td>36.5 (4.4)</td>
<td>37.3 (4.5)</td>
<td>41.9 (5.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.3 (3.1)</td>
<td>22.3 (2.6)</td>
<td>26.0 (2.6)</td>
<td>27.6 (2.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male (%)</td>
<td>62.5</td>
<td>52.4</td>
<td>89.8</td>
<td>90.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Current smoker (%)</td>
<td>30.5</td>
<td>25.6</td>
<td>44.0</td>
<td>45.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcohol intake (%)</td>
<td>23.8</td>
<td>20.4</td>
<td>32.4</td>
<td>42.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Regular exercise (%)</td>
<td>42.0</td>
<td>41.6</td>
<td>42.5</td>
<td>56.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>28.7</td>
<td>15.5</td>
<td>63.6</td>
<td>81.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>8.7</td>
<td>5.9</td>
<td>15.7</td>
<td>31.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>2.3</td>
<td>1.4</td>
<td>4.2</td>
<td>17.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Metabolic syndrome (%)</td>
<td>13.3</td>
<td>5.4</td>
<td>33.2</td>
<td>73.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Family history of diabetes (%)</td>
<td>15.6</td>
<td>14.3</td>
<td>18.9</td>
<td>23.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>112.3 (13.0)</td>
<td>110.2 (12.3)</td>
<td>117.6 (12.9)</td>
<td>121.1 (15.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>72.3 (9.3)</td>
<td>70.7 (8.8)</td>
<td>76.5 (9.1)</td>
<td>80.1 (11.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>93.3 (12.3)</td>
<td>91.5 (9.6)</td>
<td>97.5 (16.2)</td>
<td>111.1 (19.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.4 (0.4)</td>
<td>5.3 (0.4)</td>
<td>5.5 (0.6)</td>
<td>5.8 (0.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>187.2 (32.3)</td>
<td>181.6 (30.2)</td>
<td>202.6 (33.0)</td>
<td>196.3 (30.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C (mg/dl)*</td>
<td>107.4 (27.3)</td>
<td>102.6 (25.4)</td>
<td>120.9 (27.8)</td>
<td>113.0 (25.9)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

4-5 yrs follow up

NAFLD and T2DM, CVD

- 18-33% of NAFLD has IFG or T2DM
- T2DM has NAFLD 49-62%
- IR is universal in NAFLD by euglycemic clamp
- T2DM is at increased risk to progression of NAFLD to NASH and fibrosis
- Most common cause of death in NAFLD is CVD
Increased risk for diabetes in subjects with NAFLD and IFG

In 7,849 subjects without diabetes at baseline, followed up for 5 years

Table 1—HRs of incident diabetes for the NAFLD and non-NAFLD groups according to the presence of IFG and combined effect

<table>
<thead>
<tr>
<th>Categories</th>
<th>HR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFG, NAFLD</td>
<td>8.95 (6.49-12.35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IFG, non-NAFLD</td>
<td>6.79 (5.03-9.16)</td>
<td>0.035</td>
</tr>
<tr>
<td>NFG, NAFLD</td>
<td>1.39 (0.93-2.08)</td>
<td>0.121</td>
</tr>
<tr>
<td>NFG, non-NAFLD</td>
<td>1.69 (1.18-2.40)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Bae JC, Lee WY & Park SW et al. Diabetes Care, 2011
Ectopic fat accumulations lead to multi-organ dysfunction and common chronic metabolic diseases.

Diagnosis of NAFLD

- **Imaging studies**, including ultrasonography, CT and MRI, are accurate in the detection of moderate to severe hepatic steatosis.
- **Liver function tests** may be normal in most patients.
- **Proton magnetic resonance spectroscopy** accurately quantifies liver triglyceride content but is not widely available.
- **Liver biopsy** is the **gold standard** for diagnosis and is the only diagnostic tool that can accurately detect nonalcoholic steatohepatitis or fibrosis.
Initial Evaluation

• When evaluating a patient with suspected NAFLD, it is essential to exclude competing etiologies for steatosis and co-existing common chronic liver disease.

• Persistently high serum ferritin and increased iron saturation, especially in the context of homozygote or heterozygote C282Y HFE mutations may warrant a liver biopsy.

• High serum titers of autoantibodies in association with other features suggestive of autoimmune liver disease (very high aminotransferases, high globulin) should prompt a more complete work-up for autoimmune liver disease.
Non-invasive assessment of steatohepatitis and advanced fibrosis in NAFLD

- As the metabolic syndrome predicts the presence of steatohepatitis in patients with NAFLD, its presence can be used to target patients for a liver biopsy.

- NAFLD Fibrosis Score is a clinically useful tool for identifying NAFLD patients with higher likelihood of having bridging fibrosis and/or cirrhosis.

- Although serum/plasma CK18 (cytokeratin-18) is a promising biomarker for identifying steatohepatitis, it is premature to recommend in routine clinical practice.
The NAFLD fibrosis score

- Formula:
  \[-1.675 + 0.037 \times \text{age} \text{ (years)} + 0.094 \times \text{BMI} \text{ (kg/m}^2\text{)} + 1.13 \times \text{IFG/diabetes} \text{ (yes = 1, no = 0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet} \times (10^9/l) - 0.66 \times \text{albumin} \text{ (g/dl)}\]

<table>
<thead>
<tr>
<th>NAFLD Fibrosis Score</th>
<th>Probability of Fibrosis (Stage 0-2) and 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative result (&gt;0.676)</td>
<td></td>
</tr>
<tr>
<td>14.35</td>
<td>0 (0.0, 0.0)</td>
</tr>
<tr>
<td>2.71</td>
<td>10 (7, 17)</td>
</tr>
<tr>
<td>1.84</td>
<td>20 (14, 27)</td>
</tr>
<tr>
<td>1.17</td>
<td>30 (24, 37)</td>
</tr>
<tr>
<td>Indeterminate result (&lt; -1.455-&gt;0.676)</td>
<td></td>
</tr>
<tr>
<td>0.63</td>
<td>40 (34, 47)</td>
</tr>
<tr>
<td>0.12</td>
<td>50 (44, 56)</td>
</tr>
<tr>
<td>-0.37</td>
<td>60 (55, 65)</td>
</tr>
<tr>
<td>-0.92</td>
<td>70 (66, 74)</td>
</tr>
<tr>
<td>Positive result (&lt; -1.455)</td>
<td></td>
</tr>
<tr>
<td>-1.57</td>
<td>80 (76, 83)</td>
</tr>
<tr>
<td>-2.56</td>
<td>90 (87, 92)</td>
</tr>
<tr>
<td>-6.45</td>
<td>100 (99, 100)</td>
</tr>
</tbody>
</table>
When to obtain a liver biopsy in patients with NAFLD?

- Liver biopsy should be considered in patients with NAFLD who are at increased risk to have steatohepatitis and advanced fibrosis.

- The presence of metabolic syndrome and the NAFLD Fibrosis Score may be used for identifying patients who are at risk for steatohepatitis and advanced fibrosis.

- Liver biopsy should be considered in patients with suspected NAFLD in whom competing etiologies for hepatic steatosis and co-existing chronic liver diseases cannot be excluded without a liver biopsy.
Multiple Pathogenetic factors

- Insulin resistance
- Increased FFA flux to liver
- Increased De Novo lipid synthesis
- Lipotoxicity
- Chronic inflammatory change in liver
- Liver cell death and fibrosis
Pathophysiology of NAFLD, causes and consequences of which resemble those of metabolic syndrome

Adipokine dysregulation

- Excess energy
- Adiposity mass↑
- Hypoxia, impaired blood flow
- Adipocyte cell death↑
- Ceramides↑
- Cytokines↑
- Chemokines↑

Inflammation

- Macrophages
- NEFA↑

Insulin resistance

- Adiponectin↓

De novo lipogenesis

- NPC1L1
- Intestinal CM remnanat (ApoB 48)
- Triglycerides
- Impaired insulin inhibition of VLDL and glucose production
- Glucose↑ → Insulin↑
- T2DM

Visceral obesity

- Overeating, physical inactivity
- Waist↑
- ALT, AST, GGT↑
- CRP, FVII–IX, FXI–XII
- FGF21, fetuin A↑

Atherogenic Dyslipidemia

- Triglycerides↑
- HDL–C↓
- CVD

Yki-Jarvinen H. Lancet Diabetes Endocrinol, 2014
Schematic representation of the pathophysiology of NASH
Management of Patients with NAFLD
Management of Patients with NAFLD

- **Non-Pharmacological Treatment**
  - Gradual Weight reduction: Caloric restriction, Exercise
  - Stop Alcohol drinking

- **Pharmacological Treatment**
  - Insulin sensitizer: Metformin, TZD
  - Lipid lowering agent: Omega-3 fatty acids, Statin
  - Antioxidant: Vitamin E, ALA
  - Etc.: Ursodeoxycholic acid (UDCA)

- **Surgical Treatment**
  - Bariatric Surgery

- **Potential treatment target**: GLP-1, ALA, Ezetimibe,
Life style modification : most effective

- Weight loss generally reduces hepatic steatosis,

- Loss of at least 3-5% of body weight appears necessary to improve steatosis,

- Greater weight loss (up to 10%) may be needed to improve necroinflammation.

- Exercise alone in adults with NAFLD may reduce hepatic steatosis but its ability to improve other aspects of liver histology remains unknown

- Stop Alcohol use in patients with NAFLD and NASH
Pharmacological Treatment (1)

- **Metformin** has no significant effect on liver histology and is not recommended as a specific treatment for NASH.

- **Pioglitazone** can be used to treat steatohepatitis in patients with biopsy-proven NASH. However, for non-diabetic, long term safety and efficacy of pioglitazone has not been established.

- **Vitamin E** (α-tocopherol) at daily dose of 800 IU/day improves liver histology in non-diabetic adults with biopsy-proven NASH and therefore it should be considered as a first-line pharmacotherapy for this patient population.

- But vitamin E is not recommended to treat NASH in diabetic patients, NAFLD without liver biopsy, NASH cirrhosis, or cryptogenic cirrhosis until further data supporting its effectiveness become available.
Pharmacological Treatment (2)

- UDCA is **not** recommended for the treatment of NAFLD or NASH
- **omega-3 fatty acids** is **premature** to recommend
- **Statin**: Until RCTs with histological endpoints prove their efficacy, statins **should not be used to specifically treat NASH**.
Bariatric Surgery

- Bariatric surgery is not contraindicated in otherwise eligible obese individuals with NAFLD or NASH (but without established cirrhosis).

- The type, safety and efficacy of bariatric surgery in otherwise eligible obese individuals with established cirrhosis due to NAFLD are not established.

- It is premature to consider bariatric surgery as an established option to specifically treat NASH.
Pharmacological Targets

Adipokine dysregulation

- Hypoxia, impaired blood flow
- Adipocyte cell death↑
- Ceramides↑
- Cytokines↑
- Chemokines↑

Inflammation

- Macrophages
- NEFA↑
- Adiponectin↓

TZD

Overeating, physical inactivity

Waist↑

ALT, AST, GGT↑

CRP, FVII–IX, FXI–XII

FGF21, fetuin A↑

NPC1L1 inhibitors

NPC1L1

De-novo lipogenesis

VLDL

Triglycerides

Free fatty acids

Impaired insulin inhibition of VLDL and glucose production

Glucose↑ → Insulin↑

IR⇌ NAFLD ↔ DM

IR = Insulin Resistance

NPC1L1 inhibitors / Incretin

Statin, Omega-3

Atherogenic Dyslipidemia

HDL-C↓

NEFA↑

Adiponectin↓

Yki-Jarvinen H. Lancet Diabetes Endocrinol, 2014
Pharmacological Experiments

1. Thiazolidinedione: Rosiglitazone
2. GLP-1: Exenatide
3. NPC1L1 inhibitor: Ezetimibe
1. Thiazolidinediones
TZD Actions

Thiazolidinediones

Muscle

↑ Glucose Uptake

Adipose Tissue

↑ Adipogenesis
↑ Fatty Acid Uptake
↑ Lipogenesis
↑ Glucose Uptake
↑ Adiponectin
↓ TNFα

Liver

↓ Gluconeogenesis

↓ Plasma FFA

↓ Hyperglycemia

CMAJ 2005; 172 (2): 213-226
Molecular Mechanism of TZD-mediated improvement of hepatic steatosis
Hepatic-Specific Disruption of SIRT6 in Mice Results in Fatty Liver Formation Due to Enhanced Glycolysis and Triglyceride Synthesis

Hyun-Seok Kim,1,4 Culing Xiao,1,4 Rui-Hong Wang,1 Tyler Lahusen,1 Xiaoling Xu,1 Athanassios Vassilopoulos,1 Guelaguetza Guevara-Castro,2 Shenyuan Zhang,3 and Xia Deng1,*

1Genetic Epidemiology, 2Laboratory of Cellular and Molecular Biology, National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan, 3Present address: 305-701 National Institute of Health, National Institute on Aging, Bethesda, Maryland, 4The Korean National Health Insurance Corporation, Seoul, Korea, 5Department of Life and Environmental Sciences, College of Natural Sciences, Chungbuk National University, Cheongju, Chungbuk, Korea, 6Department of Biochemistry, University of California, Berkeley, California, 7Cell and Technology, Daejeon

A damage repair, neuronal riction (CR), organ metaboses, and tumorigenesis, has come from studies of Finkel et al., 2009; Jacobs and Verdin, 2007; Vaquero g et al., 2008b).

MT1 is induced, which then isome proliferator-activated (1,4) and protein.
# Sirtuins as metabolic regulator

- A family of proteins with NAD\(^+\)-dependent deacetylase and ADP-ribosyltransferase activities
- Seven mammalian sirtuins have been identified
- Suggested as metabolic regulators and potential therapeutic targets for metabolic syndrome

<table>
<thead>
<tr>
<th>Intracellular location</th>
<th>Activity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SIRT1</strong> Nucleus</td>
<td>Deacetylase</td>
<td><strong>Metabolism, inflammation</strong></td>
</tr>
<tr>
<td>SIRT2 Cytoplasm</td>
<td>Deacetylase</td>
<td>Cell cycle, tumorigenesis</td>
</tr>
<tr>
<td><strong>SIRT3</strong> Nucleus</td>
<td>Deacetylase</td>
<td><strong>Metabolism, thermogenesis, ATP production</strong></td>
</tr>
<tr>
<td>SIRT4 Mitochondria</td>
<td>ADP-ribosyl transferase</td>
<td>Insulin secretion</td>
</tr>
<tr>
<td>SIRT5 Mitochondria</td>
<td>Deacetylase</td>
<td>Urea cycle</td>
</tr>
<tr>
<td><strong>SIRT6</strong> Nucleus</td>
<td>Deacetylase ADP-ribosyl transferase</td>
<td><strong>Metabolism, DNA repair</strong></td>
</tr>
<tr>
<td>SIRT7 Nucleus</td>
<td>Unknown</td>
<td>rDNA transcription</td>
</tr>
</tbody>
</table>
Hypothesis

Rosiglitazone improves hepatic steatosis via involvement of Sirt6-AMPK pathway

Specific aim 1:
To examine whether TZDs improves hepatic steatosis and whether Sirt6-AMPK pathway is altered with TZDs treatment

Specific aim 2:
To investigate whether Sirt6 is functionally involved in the protective action of TZDs against hepatic steatosis
Specific aim 1: To examine whether rosiglitazone improves hepatic steatosis *in vivo* and whether Sirt6-AMPK pathway is altered with rosiglitazone treatment

- **Male LETO/OLETF rats**
  - 32 wks of age
  - 6 wks treatment of 4 mg/kg/day rosiglitazone via stomach gavage

  Group 1: LETO control (LETO CON)
  Group 2: LETO rosiglitazone (LETO RGZ)
  Group 3: OLETF control (OLETF CON)
  Group 4: OLETF rosiglitazone (OLETF RGZ)
The effects of rosiglitazone (RGZ) on body weights, food intake and fat pad weights

<table>
<thead>
<tr>
<th></th>
<th>LETO Control</th>
<th>LETO RGZ</th>
<th>OLETF Control</th>
<th>OLETF RGZ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (g)</td>
<td>511±14</td>
<td>515±9</td>
<td>603±23</td>
<td>611±21</td>
</tr>
<tr>
<td>Post-treatment (g)</td>
<td>522±8</td>
<td>559±17</td>
<td>530±15</td>
<td>635±43</td>
</tr>
<tr>
<td>Weight change (g)</td>
<td>12±11</td>
<td>45±16</td>
<td>−73±10</td>
<td>25±30*</td>
</tr>
<tr>
<td><strong>Food intake (g)</strong></td>
<td>23.1±0.5</td>
<td>23.4±2.3</td>
<td>39.2±0.4</td>
<td>37.9±0.6</td>
</tr>
<tr>
<td><strong>Fat pad weights (%)</strong></td>
<td>3.9±0.4</td>
<td>4.8±0.4 *</td>
<td>5.7±0.8</td>
<td>11.3±1.2*</td>
</tr>
<tr>
<td>Subcutaneous fat (%)</td>
<td>0.9±0.1</td>
<td>1.4±0.1*</td>
<td>0.9±0.2</td>
<td>1.8±0.4*</td>
</tr>
<tr>
<td>Epididymal fat (%)</td>
<td>1.3±0.2</td>
<td>1.5±0.2</td>
<td>1.4±0.1</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>Mesenteric fat (%)</td>
<td>0.6±0.0</td>
<td>0.5±0.0*</td>
<td>0.6±0.1</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td>Retroperitoneal fat (%)</td>
<td>1.2±0.1</td>
<td>1.4±0.2</td>
<td>2.9±0.4</td>
<td>6.7±0.6*</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM (n = 4–5 per group). Fat pad weights are expressed as a percentage of fasted body weight. *p<0.05 vs controls.

Rosiglitazone improves hepatic steatosis in OLETF rat livers

Scale bar, 100 μm. Original magnification, x200.
Data are means ± SEM. *P < 0.05 vs controls.

Rosiglitazone up-regulates gene expression of Sirt6 and related targets in OLETF rat livers

The expression levels were normalized with respect to those of the GAPDH gene. Data are means ± SEM. *P < 0.05 vs controls. 

Rosiglitazone activates LKB1-AMPK pathway in OLETF rat livers

Data are means ± SEM. *P < 0.05 vs controls.

Specific aim 2: To investigate whether Sirt6-AMPK pathway is involved in the protective action of rosiglitazone against hepatic steatosis

- RNAi experiment to induce knockdown of Sirt6

- AML12 mouse hepatocytes
  - Hepatocyte steatosis was induced with FFA treatment for 48 h
  - FFA and/or RGZ was treated for additional 24 h

Group 1: Control
Group 2: RGZ (10 µM)
Group 3: FFA (250 µM palmitic acid)
Group 4: FFA+RGZ
Group 5: Sirt6 RNAi
Group 6: Sirt6 RNAi+FFA
Group 7: Sirt6 RNAi+FFA+RGZ
Sirt6 knockdown suppressed the effects of rosiglitazone to regulate gene expression of Sirt6 and related targets in AML12 mouse hepatocytes.

Data are means ± SEM. *P < 0.05 vs control (CON; white bar), †P < 0.05 vs FFA alone.
Sirt6 knockdown diminished the effects of rosiglitazone to reduce hepatocyte lipid accumulation in AML12 mouse hepatocytes.

Data are means ± SEM. *P < 0.05 vs control (CON; white bar), †P < 0.05 vs FFA alone.

The effect of rosiglitazone on LKB1-AMPK pathway was abolished by knockdown of Sirt6 in AML12 mouse hepatocytes.

Rosiglitazone (RGZ) alters Sirt1 and Sirt1 deacetylase activity in the livers (in vivo)

[Graph A: Relative Sirt1 mRNA expression in liver
- CON, RGZ in LETO
- CON, RGZ in OLETF]

[Graph B: Liver Sirt1 deacetylase activity (% LETO CON)
- CON, RGZ in LETO
- CON, RGZ in OLETF]
The effect of rosiglitazone (RGZ) on LKB1-AMPK pathway

Unpublished data
Summary

- TZDs improved hepatic steatosis,

- TZDs’ action is likely mediated by the activation of the Sirt1/6-AMPK pathway,

- Sirt 1 and Sirt6 may be compensatory but without synergistic effects

- These results are suggesting sirtuins as a therapeutic target for hepatic steatosis and its related diseases.
Effect of TZDs on adipose tissue redistribution
Biochemical data after 5 weeks of treatment

<table>
<thead>
<tr>
<th></th>
<th>RGZ-untreated LETO</th>
<th>RGZ-treated LETO</th>
<th>RGZ-untreated OLETF</th>
<th>RGZ-treated OLETF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>116.3 ± 11.8</td>
<td>113.3 ± 14.8</td>
<td>201.6 ± 33.9*</td>
<td>135.4 ± 18.5‡</td>
</tr>
<tr>
<td>Fasting insulin (µIU/mL)</td>
<td>10.5 ± 2.9</td>
<td>6.8 ± 4.5</td>
<td>21.1 ± 5.2†</td>
<td>7.0 ± 2.4§</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>92.8 ± 17.4</td>
<td>89.5 ± 46.6</td>
<td>356 ± 111.5†</td>
<td>107.4 ± 30.8§</td>
</tr>
<tr>
<td>FFA (µEq/L)</td>
<td>279.3 ± 40.3</td>
<td>242.5 ± 50.7</td>
<td>627.2 ± 172.6†</td>
<td>338 ± 97.1‡</td>
</tr>
</tbody>
</table>

Data are mean ± SD.

* P < .05 vs RGZ-untreated LETO rats.
† P < .01 vs RGZ-untreated LETO rats.
‡ P < .05 vs RGZ-untreated OLETF rats.
§ P < .01 vs RGZ-untreated OLETF rats.
### Summary

- **Lipid storage**
  - Glycerol
  - Glycerol kinase, PEPCK
  - Aquaporin adipose

- **Fatty acid**
  - Via uptake from lipoproteins by the action of LPL
  - Via lipolysis from stored triglycerides by HSL
  - Via de novo lipid synthesis
    - Acetyl-CoA carboxylase (ACC-\(\alpha\))
    - Fatty acid synthase (FAS)
    - Stearoyl-CoA Desaturase-1 (SCD-1)

- **Fatty acid Transport protein**

- **Glucose**
  - GLUT4

- **Energy expenditure**
  - CPT-1

---

**[ In Adipocyte ]**

<table>
<thead>
<tr>
<th></th>
<th>Subcutaneous</th>
<th>Visceral</th>
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</thead>
<tbody>
<tr>
<td><strong>TG synthesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol substrate</td>
<td>↑→</td>
<td>➔</td>
</tr>
<tr>
<td>FFA</td>
<td>↑</td>
<td>➔</td>
</tr>
<tr>
<td>✓Lipolysis gene</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>✓Fatty acid Transport protein</td>
<td>↑→</td>
<td>➔</td>
</tr>
<tr>
<td>✓De novo synthesis</td>
<td>↑→</td>
<td>➔</td>
</tr>
<tr>
<td><strong>β-oxidation</strong></td>
<td>➔</td>
<td>↑</td>
</tr>
</tbody>
</table>

---

*Kang JG, Park CY, Ihm SH & Park SW Metabolism 2010;59(1):46-53*
Hypothesis in adipocytes

- Depot specificity of lipid storage & energy expenditure gene regulation
Co-culture of 3T3-L1 adipocyte and AML12 hepatocyte

Figure 1
Different modes of co-culture using ThinCert™ cell culture inserts. Two cell populations that are co-cultivated in different compartments (insert and well) stay physically separated, but may communicate via paracrine signalling through the pores of the membrane (A, B). Alternatively, both cell populations may be co-cultivated in the upper compartment (insert), thus allowing extensive and direct cell-cell interactions (C).

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 8</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell seeding</td>
<td>MDI media</td>
<td>Insulin media</td>
<td>Complete media</td>
<td>Serum-free media (starvation)</td>
<td>Treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FFA</td>
<td>FFA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TZD</td>
<td>TZD</td>
</tr>
<tr>
<td>Sample collection</td>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FFA
TZD
RGZ improves hepatocyte steatosis (in vitro)

Mean ± SD, *p<0.05, #p<0.05 vs. control
Liver

AMPK → LKB1 → Sirtuins

β-oxidation → FA synthesis → Lipid content

Lipid uptake → TG synthesis → FFA TG

How sirtuins-AMPK pathway works?
1. Phosphorylation by AMPK
2. Deacetylation by sirtuins
3. Sirtuins act as transcription factor for PGC-1α/FOXO-1 and other target genes

Lipid Storage < Expenditure
Visceral AT

Lipid Storage > Expenditure
Subcutaneous AT
2. Glucagon Like Peptide-1 (GLP-1)
GLP-1 Actions

- Heart: Cardioprotection, Cardiac function
- Brain: Neuroprotection, Appetite
- Intestine: GLP-1, Insulin sensitivity
- Liver: Glucose production
- Pancreas: Insulin secretion, Glucagon secretion, Insulin biosynthesis, β-cell proliferation, β-cell apoptosis
- Stomach: Gastric emptying
- Adipose tissue: Glucose uptake and storage

Lancet 2006;368(9548):1696-705
Exedin-4 alters body weight, food intake and serum lipid levels in HF-induced obese mice

* p<0.05, ** p<0.01 compared with control
# p<0.05, ## p<0.01 compared with HF group
Exendin-4 improves hepatic steatosis & NAFLD

Liver Tissue Weight

* p<0.05, ** p<0.01 compared with control
# p<0.05, ## p<0.01 compared with HF group

Influence of Exendin-4 on expression of Sirt1 and other related targets in mouse livers

* p<0.05, ** p<0.01 compared with control
# p<0.05, ## p<0.01 compared with HF group

Influence of Exendin-4 on mRNA expression involved in fatty acid oxidation and lipogenesis

Fatty acid oxidation

Lipogenesis

* p<0.05, ** p<0.01 compared with control
# p<0.05, ## p<0.01 compared with HF group

Effects of Ex-4 on PA-induced fat accumulation in human hepatocytes

Oil Red O Staining

TG Level

* p<0.05, ** p<0.01 compared with control
# p<0.05, ## p<0.01 compared with HF group

Influence of Ex-4 on GLP-1R, Sirt1 and AMPK expression in HepG2 and Huh7 cells

* p<0.05, ** p<0.01 compared with control
# p<0.05 compared with PA

Effects of exendin-4 on Sirt1-AMPK pathway

NAM (nicotinamide); SIRT1 inhibitor
CC (compound C); AMPK inhibitor

• p<0.05, ** p<0.01 compared with control,
• # p<0.05, ## p<0.01 compared with PA,
• † p<0.05, †† p<0.01 compared with Ex-4

FGF21

- FGF21 exerts diverse pharmacological effects on glucose and lipid metabolism, ketogenesis, and growth hormone signaling in hepatocytes in mice.

- FGF21 is expressed in the liver, pancreas, white adipose tissue, and muscle.

- FGF21 is predominantly produced in the liver, where it enhances hepatic fat oxidation.

- FGF21 has a specific affinity for FGFR1 but can also act through different isoforms such as FGFR2 and FGFR4.

- FGF-21/FGF-21 receptor interaction and activation is determined by beta-Klotho. (J Cell Physiol. 2008 Apr;215(1):1-7.)
## Results

<table>
<thead>
<tr>
<th>Variable</th>
<th>Con</th>
<th>HF</th>
<th>HF+Ex-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>5.78±0.49</td>
<td>21.1±0.72</td>
<td>18±1.01</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.13±0.048</td>
<td>1.70±0.10</td>
<td>1.44±0.07</td>
</tr>
<tr>
<td>Hepatic triglycerides (mg/g of protein)</td>
<td>48.44±2.69</td>
<td>322.72±9.74</td>
<td>231.33±7.79</td>
</tr>
</tbody>
</table>

The results are reported as means ± standard error for 8 mice. HF=high fat diet; Ex-4=exendin-4. *p*<0.05, ***p*<0.001 compared with control and †p*<0.05, ††††p*<0.001 compared with a HF (ANOVA).
Exendin-4 increases the expression of FGF21 and its receptors in vivo and in vitro

Metabolism (in press, 2014)
Relationship between FGF21 and hepatic lipid accumulation

A

Vehicle | Ex-4 | FGF21

Con | PA

Metabolism (in press, 2014)
Relationship between FGF21 and hepatic lipid accumulation

B

Intracellular TG content (relative to control)

C

FGF21/β-actin mRNA

D

FGFR1/β-actin mRNA

FGF21/β-actin mRNA

PPARα/β-actin mRNA

Con
Ex-4
Ex-4 + FGFR1 siRNA
Con siRNA

Metabolism (in press, 2014)
Regulation of FGF21 by SIRT1 in HepG2 cells

A

Fold change in mRNA expression

** SIRT1
** FGF21

B

Fold change in mRNA expression

** SIRT6
** FGF21

C

Fold change in mRNA expression

** FGF21
** SIRT1
** SIRT6

Metabolism (in press, 2014)
3. NPC1L1 inhibition
NPC1L1 (Niemann-Pick C1-Like 1) Intestinal & hepatic

Park SW. Diabetes Metab J 2013: 37(4): 240-8
Tissue and Species Specific NPC1L1 Expression

Graph showing relative levels of NPC1L1 mRNA in various tissues and segments of the rat small intestine.
Ezetimibe: NPC1L1 Inhibitor
(Cholesterol Absorption Inhibitor)

- **Discovery:** Ezetimibe was discovered as an active and potent metabolite of Schering-Plough's SCH48461 substance.
Effects of Ezetimibe on body weight and daily food intake in OLETF Rats

**: p<0.01 compared to LETO Control

Ezetimibe improves serum metabolic parameters in OLETF Rats

Serum Glucose

Serum Insulin

Serum Cholesterol

Serum TG

Serum active GLP-1

Serum DPP4 Activity

Ezetimibe improves hepatic steatosis & NAFLD

**Tissue Weight (% BW)**

- Control
- Ezetimibe

**Liver Cholesterol**

- Control
- Ezetimibe

**Liver TG**

- Control
- Ezetimibe

Unpublished data

*p<0.05, ** p<0.01 compared to controls
Ezetimibe increases autophagy markers in OLETF liver tissue

A

mRNA expression (Fold Change)

LETO Con OLETF Con OLETF Ez

ATG5

ATG6

ATG7

B

Protein Expression (Fold change)

LETO Con OLETF Con OLETF Ez

ATG5

ATG6

LC3-I

LC3-II

α-tubulin

* p<0.05, ** p<0.01 compared to controls

Unpublished data
Ezetimibe attenuates PA-induced TG accumulation and induces autophagy

A

B

C

Unpublished data
Ezetimibe increases autophagosome formation in human hepatoma cell line

Unpublished data
Ezetimibe increases autophagic flux in human hepatoma cell line

Unpublished data

†, p<0.05 and ††, p<0.01, compared to control in the absence of PA and BAF; ##, p<0.01, compared to control in the absence of PA and presence of BAF; **, p<0.01, compared to same PA, BAF treated controls
Summary

• Ezetimibe treatment attenuates hepatic fat accumulation and improves hyperglycemia.

• Ezetimibe increases the autophagy flux, which might be associated with the improvement in metabolic characteristics.

• These findings suggest the new possible target of ezetimibe actions and potential intervention with ezetimibe for the treatment of hepatic steatosis.
In Summary

Adipokine dysregulation

- Hypoxia, impaired blood flow
- Adipocyte cell death
- Ceramides
- Cytokines
- Chemokines

Inflammation

- Macrophages
- NEFA
- Adiponectin

TZD

- Excess energy
- Adipose mass

NPC1L1 inhibitors

- De-novo lipogenesis
- VLDL
- Triglycerides

NPC1L1

- Impaired insulin inhibition of VLDL and glucose production
- Glucose
- Insulin

IR ⇔ NAFLD ⇔ DM

Overeating, physical inactivity

- Waist
- ALT, AST, GGT
- CRP, FVII–IX, FXI–XII
- FGF21, fetuin A

NPC1L1 inhibitors /

Incretin

- Statin, Omega–3

Atherogenic Dyslipidemia

- Triglycerides
- HDL–C

Yki-Jarvinen H. Lancet Diabetes Endocrinol, 2014
Conclusion

• NAFLD is not a single disease, but rather ectopic fat accumulation of liver manifestation.

• NAFLD is a risk factor for DM, CVD and carries atherogenic potential.

• Insulin resistance and other inflammation, oxidative stress resulting in NASH and cirrhosis may develop into a progressive disease and early intervention is necessary.

• For treatment, lifestyle modification is the most effective and proven intervention method.

• However, it is necessary to continue to find treatment effects for NAFLD.

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