Serum inducible protein (IP)-10 is a disease progression-related marker for non-alcoholic fatty liver disease

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Abstract

Background: The molecular pathogenesis of non-alcoholic steatohepatitis (NASH) is not well defined. The objective of the present study was to identify disease progression-related cytokines and investigate the molecular pathogenesis of such changes in NASH.

Methods: A study population of 20 non-alcoholic fatty liver (NAFL) and 59 NASH patients diagnosed by liver biopsy and 15 healthy volunteers was recruited. The serum pro- and anti-inflammatory cytokines were measured by a multiple enzyme-linked immunosorbent assay. The hepatic mRNA expressions of cytokines were measured by real-time PCR. A monocyte cell line was stimulated with toll-like receptor (TLR) ligand under a high glucose and insulin condition, and cellular cytokine mRNA expression was quantified.

Results: One group of cytokines was higher in NAFL and NASH than in controls, while another group was higher in NASH than in NAFL and controls. The NASH-specific second group included interleukin (IL)-15 and interferon-γ-inducible protein (IP)-10. In particular, IP-10 was higher in NAFL than in controls and higher in NASH than in NAFL and controls. The sensitivity to diagnose NASH was 90%, with specificity of 50%. Insulin resistance reflecting a high glucose and insulin condition resulted in higher IP-10 mRNA expression in the monocyte cell line only with concomitant TLR-2 stimulation.

Conclusions: IP-10 is a sensitive marker of the need for liver biopsy. Insulin resistance with bacteria-related TLR-2 stimulation might induce IP-10 production from monocytes. Insulin

resistance and intestinal barrier function should be intensively controlled to prevent progression from NAFL to NASH.

Key words: non-alcoholic fatty liver disease; non-alcoholic steatohepatitis; insulin resistance, interferon-γ-inducible protein-10

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CCL2, chemokine (C-C motif) ligand 2; CK-18, cytokeratin-18; FGF, basic fibroblast growth factor; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage CSF; HCC, hepatocellular carcinoma; IFN, interferon; IL, interleukin; IP-10, interferon-γ-inducible protein-10; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; NAFL, non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PDGF-BB, platelet-derived growth factor subunit B; RANTES, regulated upon activation normal T-cell expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease that is an increasing health threat and an obesity-related disease [1]. Most patients with NAFLD show non-progressive simple fatty liver, namely non-alcoholic fatty liver (NAFL), while a subset of patients progress to chronic inflammation with liver fibrosis, namely non-alcoholic steatohepatitis (NASH). NASH is a more severe form of NAFLD that ultimately leads to cirrhosis and hepatocellular carcinoma (HCC) [2, 3]. The mechanism of NAFL progressing to NASH is not clearly understood [4, 5].

The differential diagnosis of non-progressive NAFL and NASH continues to be based on biopsy-proven pathology. Liver biopsy is expensive and time-consuming, and it is associated with some morbidity, indicating that the procedure should be performed only for patients with a risk of progressive disease [6]. Therefore, there is a need for serological markers capable of differentiating between NAFL and NASH. Many scoring systems to differentiate NAFL and NASH have been reported. The FIB-4 index and NAFLD fibrosis score might be the better markers [7]. Several reports on different populations have indicated that the measurement of CK-18 fragments is a reliable marker for this diagnosis [8], while one new report with 424 middle-aged NAFLD patients showed inadequate sensitivity (58%) and specificity (68%) for NASH diagnosis [9]. To date, these scores are insufficient to replace liver biopsy in the diagnostic process.

Immune responses and inflammation are known to be involved in metabolic diseases, such as diabetes mellitus, atherosclerosis, and NASH. Adipose tissue-derived cytokines are known to promote metabolic disease progression. In obesity, excessive numbers of proinflammatory, M1-like macrophages accumulate in adipose tissue and the liver [10]. Even in simple fatty liver, macrophage infiltration and macrophage attractant chemokine CCL2 expression are significantly increased [11]. Macrophages are an important mediator of inflammation and insulin resistance, which are the common phenomena of NAFLD. In advanced NASH, CD4 (+) and CD8 (+) T cell infiltration increases, and inflammatory cytokines, such as IL-6 or IL-8, are also increased [11]. However, a simultaneous comparison of these has not been performed.

The objective of the present study was to investigate the cytokine profile in NAFLD patients and to identify the molecular pathogenesis and biomarkers for the differential diagnosis of NAFL and NASH. Several inflammatory cytokines were upregulated in NAFLD, while interferon-γ-inducible protein-10 (IP-10, also called CXCL10) was higher in NAFL than in controls and higher in NASH than in NAFL. In addition, insulin resistance and bacterial TLR stimulation could induce IP-10 production even in an *in vitro* experimental system.

Methods

Patients

From September 2005 to April 2013, 20 patients with NAFL (NAFL group) and 59 patients

with NASH (NASH group) who were diagnosed by liver biopsy at Okayama University Hospital were enrolled. All liver specimens were assessed by two hepatologists (F.I. and T.Y.) who were blinded to study group allocation. The NAFLD diagnosis system reported by Matteoni *et al* was adopted for the differential diagnosis of NAFL and NASH [2], as recommended in several guidelines [12, 13]. The METAVIR scoring system was used to analyze the activity and stage of liver fibrosis in the patients. Twenty healthy donors were also included for serum assays. A biopsy was performed in patients with elevated serum liver transaminases more than once. The NAFL and NASH patients were diagnosed to be free of cancer and negative for hepatitis B and hepatitis C viral markers and autoantibodies. The baseline characteristics of the patients are summarized in **Table 1**.

Informed consent was obtained from each patient included in the study, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki with the approval of the Ethics Committee at Okayama University Hospital.

Blood sample collection and preparation

Fasting blood samples were collected from the patients. The serum was collected at the time of admission or at the outpatient clinic, meaning that no intervention had been performed before sample collection. The serum aliquots were stored at -30°C until subsequent analysis. The samples obtained were used to determine biochemical data, including serum cytokine levels.

Multiple cytokine assays

Measurements of multiple cytokines were performed using a BioPlex 200 System (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocols. The assay used was a Bio-Plex Pro Human Cytokine Grp 1 Panel 27-Plex, which targets IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF), eotaxin, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), interferon (IFN)-γ, IP-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor subunit B (PDGF-BB), regulated upon activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Samples were tested in duplicate, and the median values were used for further analyses.

Measurement of CK-18

Measurement of CK-18 was performed by a one-step immunoassay using an M30 Apoptosense ELISA kit (VLVbio, Sundbyberg, Sweden), according to the manufacturer's protocols.

Immunohistochemical analysis of liver biopsy samples

For infiltrated lymphocyte analysis, immunohistochemical staining of cell surface markers of CD4 (helper T cells; DAKO, Glostrup, Denmark), CD8 (cytotoxic T cells; DAKO), CD68 (monocytes; DAKO), and monocyte productive cytokine IP-10 (R&D Systems, Minneapolis, MN, USA) was performed. The expressions of these antigens were evaluated according to staining intensity and scored as follows: 0, negative; 1, weak expression; 2, moderate

expression; and 3, strong expression.

Evaluation of noninvasive NASH diagnostic tools

The APRI (AST to platelet ratio), FIB-4 (age × AST / platelet × √ALT), CK-18, and IP-10 were evaluated for the differential diagnosis of NAFL and NASH. The APRI and FIB-4 index were calculated using the original reported formulas [14, 15].

Human monocyte stimulation with glucose, insulin, and toll-like receptor (TLR) ligands

THP-1 cells (human acute monocytic leukemia cells), a gift from Prof. Miyake (The Institute of Medical Science, University of Tokyo), were differentiated by incubation with 10 ng/ml of PMA for 2 days and starved in 0.5% FBS and PMA for 6 hours prior to addition of high glucose and insulin. ‘Low glucose medium’ described in this paper was RPMI-based medium containing 2 g/L of glucose, and 19.5 mM of mannitol were further added to achieve the same osmolarity as high glucose medium. ‘High glucose medium’ described in this paper contained 5.5 g/L of glucose by additional 3.5 g/L glucose supplementation. Human insulin (Sigma, St. Louis, MO) was added to make a final concentration of 100 nM of insulin in ‘Insulin medium’. Insulin was supplied to standard to ‘Low glucose medium’ and ‘High glucose medium’, and a combination of mediums was made: ‘Low glucose’ medium, ‘High glucose’ medium, ‘Low glucose + insulin’ medium, and ‘High glucose + insulin’ medium. After 12-h incubation with glucose or insulin-supplied medium mentioned above, ligands of toll-like receptors (TLRs), such as peptidoglycan (PGN) as a TLR2 ligand or lipid A as a TLR 4 ligand, were added to

stimulate the differentiated THP-1 cells. THP-1 cells were stimulated with 20 μg/ml of peptidoglycan (SIGMA) or 100 ng/ml of Lipid A (Re 595) (SIGMA). These concentrations of the ligands have been found to be sufficient to induce cytokine expression in other cells [16], and thus only these concentrations of ligands were used in an attempt to stimulate THP-1 cells. It has been found that 100 nM of insulin is sufficient to induce signaling of insulin resistance *in vitro* according to several papers, as shown below [17, 18]. This concentration of insulin was tried first, but in the present model, this concentration was not enough to induce IP-10 production, and TLR ligand stimulation, such as by PGN, was found to be required to increase IP-10 production in THP-1 cells. After 6-h incubation, stimulated cells were collected and used for further cytokine mRNA examinations.

Real-time PCR for cytokine mRNA in biopsy specimens and cultured cell lines

Total RNA was reverse-transcribed into cDNA and subjected to PCR. The targeted genes mRNA and β-actin (housekeeping gene) mRNA were quantified using SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany) with specific primers on a Light cycler 480-2 (Roche Diagnostics). Each sample was run in triplicate, and the results were analyzed using the relative standard method.

Statistical analysis

Statistical comparisons were performed using JMP version 9 (SAS Institute, Cary, NC, USA). The Wilcoxon rank-sum test was used to compare continuous data, and the chi-squared test was

used to compare categorical data. For multivariate analysis, logistic regression analysis was used. The Steel-Dwass test was used for multiple group analysis. A value of $p < 0.05$ was considered significant. The accuracy of each score for the differentiation of NASH from NAFLD was assessed using receiver operator characteristic curves, reported as the area under the curve (AUC) with 95% confidence intervals (95% CI).

Results

Clinical characteristics of the patients

NASH patients were older, with lower platelet counts, higher prothrombin time international ratio (PT-INR), higher AST, and higher homeostasis model assessment as an index of insulin resistance (HOMA-IR) compared to NAFL patients (**Table 1**). All NAFL patients showed lower histological activity and fibrosis scores as NASH patients were grouped via histological analysis.

Different patterns of serum cytokine concentrations in NAFL and NASH

A total of 16 serum cytokine concentrations were not different among the healthy control, NAFL, and NASH groups. Three cytokines (group a), PDGF-BB, IL-17, and IL-5, were lower in NASH than in controls (**Fig. 1a**). Five cytokines (group b), IL-1β, IL-6, IL-8, MIP-1α, and MIP-1β, were increased in both NAFL and NASH groups (**Fig. 1b**). Three cytokines (group c), MCP-1, IL-15, and IP-10 were increased in NASH compared with controls, while IL-15 was

even higher in NASH than in NAFL, and IP-10 showed a stepwise increase from controls to NAFL and then to NASH (**Fig. 1c**).

Serum IP-10 as a non-invasive diagnostic tool for NASH

Since the multiple cytokine analysis showed that IP-10 could be a marker to differentiate among control, NAFL, and NASH groups, the diagnostic accuracy of IP-10 for NASH was evaluated (**Fig. 1d**). ROC curves of IP-10 showed it to be an effective tool to diagnose NAFLD from among all subjects, and NASH from NAFLD or all subjects. The diagnostic sensitivity and specificity of IP-10 and known tools are shown in **Table 2**. Since the FIB-4 index and APRI were designed to detect advanced fibrosis patients with F3 and F4, the specificity to differentiate NASH and NAFL was high, while it was lower for CK-18 and IP-10, at 36.8% and 50%, respectively. However, the FIB-4 index and APRI showed unsatisfactory sensitivity, at 64.4% and 71.1%, respectively. Both CK-18 and IP-10 showed high sensitivity, at 90%, while the specificity was better for IP-10 than for CK-18. The correlations between clinical characteristics and serum IP-10 were analyzed (**Table 3A**). Serum IP-10 was correlated with BMI, AST, ALT, ferritin, HOMA-IR, and CK-18, while it was negatively correlated with the platelet count. Serum IP-10 was also correlated with activity grade and fibrosis stage in liver biopsy specimens (**Table 3B**).

Evaluation of cytokine mRNA expressions in liver biopsy specimens

To evaluate the correlations between serum cytokine levels and liver mRNA expression levels,

representative cytokine mRNA expression levels were quantitatively analyzed (**Fig.2**). In group c, IL-15 mRNA levels were not different between NAFL and NASH, while MCP-1 and IP-10 mRNA levels were higher in NASH than in NAFL**,** as were serum levels.

Immunohistochemical staining of IP-10 and immune-related cells in liver biopsy specimens

To evaluate local expression of IP-10 and the invading immune-related cells, immunohistochemical staining was performed (**Fig. 3**). IP-10 was stained in portal area-invading monocytes, with more frequent positive staining in NASH than in NAFL. IP-10-positive cells were scarce, found mainly in the portal area where CD68-positive macrophages were abundant.

In vitro analysis of glucose and insulin effects on cytokine and chemokine production by monocytes

The high glucose and insulin condition increased MIP-1α (group b) expression as measured by mRNA levels, but both PGN and lipid A seemed not to strongly affect MIP-1α production (**Fig.4**). MCP-1 (group c) expression was also relatively enhanced by high glucose or PGN. On the other hand, IP-10 mRNA expression was markedly affected by PGN stimulation, and IP-10 expression was synergistically increased in combination with the high glucose and insulin condition.

Discussion

In this study, even NAFL patients showed a definite increase in several pro-inflammatory cytokines. IP-10 was the only cytokine that increased in a stepwise fashion from normal to NAFL and then to NASH. IP-10 might be a good marker of the need for liver biopsy for further evaluation, while the FIB-4 index or APRI is a good marker to detect advanced NAFLD. In *in vitro* analysis of monocyte stimulation, IP-10 production was significantly increased under insulin resistance reflecting a high glucose and insulin condition with TLR2 ligand, while MIP-1α (group b) could be increased only with high glucose and insulin. Insulin resistance and bacterial TLR2 infiltration to the liver might positively affect IP-10 production from monocytes following progression from NAFL to NASH.

In the present study, inflammatory cytokines such as IL-1β, IL-6, neutrophil chemoattractant cytokine IL-8, and monocyte or NK cell attractant cytokines MIP-1α and MIP-1β were elevated even in NAFL, as in NASH. This is not exactly the same, but partly consistent with a previous report that indicated that IL-6 or IL-8 mRNA expression in liver biopsy specimens was not significantly different between NAFL and NASH [11]. CD68-positive macrophage infiltration and hepatic expression of macrophage chemoattractant chemokine CCL2 have been reported to be increased even in NAFL, and they have important roles in inflammatory cell recruitment and insulin resistance [10]. These changes may be considered to be an early response by fatty liver. From the present 27 cytokines that were examined, only IL-15 and IP-10 were significantly higher in NASH than in NAFL, and IP-10

showed a stepwise increase.

IP-10 is an IFN-γ-induced pro-inflammatory cytokine that recruits inflammatory cells to damaged tissue and is reported to be associated with lipotoxicity. In chronic hepatitis C, intrahepatic IP-10 levels have been reported to correlate with necroinflammatory changes and fibrosis [19]. Zhang *et al* have already reported that IP-10 could be an NAFLD biomarker, as in the present study [20]. The diagnostic power for NASH is concordant with a previous report by Zhang *et al* [20]. However, the present detailed analysis of clinical characteristics showed that higher AST and lower platelet counts were characteristics of high IP-10 patients. Given that high AST and low platelet counts are characteristics of an advanced fibrotic state, high IP-10 patients are defined as advanced fibrotic patients with NAFLD. Additionally, they showed that IP-10 could induce several key inflammatory cytokines, such as TNF-α, IL-1β, and MCP-1, and blockade of IP-10 could be protective against steatohepatitis development in the mouse model of NASH.

The differential diagnosis of NAFL and NASH is dependent on liver histology; it is not possible by non-invasive tests [21]. The present results indicated that IP-10 is a reliable, highly sensitive marker to define potential NASH patients that require liver biopsy evaluation. This pattern of markers is plausible, since CK-18 has been acknowledged as a definite NASH-defining marker. CK-18 is the major intermediate filament protein in the liver that is released into the extracellular space during cell death. Therefore, soluble CK-18 is believed to

be representative of hepatic apoptosis. The present results showed that IP-10 had sensitivity nearly equal to CK-18 and was more specific than CK-18 in distinguishing NASH from NAFL.

Insulin resistance is a NASH-related clinical phenomenon also seen in the present data [5]. The clinical characteristics of the present patients showed that NASH patients had higher insulin resistance and lower platelet counts, as well as portal hypertension. The *in vitro* analysis was performed to show why the NASH characteristics correlated with an IP-10 increase. The role of insulin resistance characterized by high glucose and insulin concentrations was evaluated in an *in vitro* analysis of cytokine production. The group b cytokines, such as MIP-1α and MIP-1β, could be increased by the high glucose plus insulin condition, indicating that they were easy to enhance in an insulin-resistant condition. The group c cytokines MCP-1 and especially IP-10 required additional TLR signal stimulation, indicating the importance of TLR stimulation in NAFL progression to NASH. PGN addition resulted in strong IP-10 expression, and the combination of high glucose and insulin resulted in a significant IP-10 mRNA increase. These data implied that insulin resistance encouraged IP-10 production during monocyte stimulation by TLR ligands, especially TLR2 ligands, and showed a synergistic effect with TLR ligands on monocyte activation. The TLRs are sensors of microbial and endogenous danger signals that are expressed and activated in innate immune cells. The key role of innate immunity in liver inflammation was demonstrated using a targeted deletion strategy of the gene responsible for innate immune receptors [22]. TLR2 is a receptor for multiple glycolipids or lipoproteins in

bacteria adhering to the cell surface of monocytes, myeloid dendritic cells, or mast cells that has been intensively investigated in NASH pathogenesis [23].

The experimental reproduction of insulin resistance and bacterial signal induction showed the mechanisms of IP-10 production. These factors should be reasonable targets to prevent NASH progression.

In conclusion, several inflammatory cytokines were upregulated even in NAFL, while IP-10 was higher in NAFL than in controls and higher in NASH than in NAFL. IP-10 might be useful as a highly sensitive biomarker in the diagnosis of NASH. IP-10 might be a good marker of the need for liver biopsy for further evaluation, while the FIB-4 index or APRI is a good marker to detect advanced NAFLD. Insulin resistance and TLR2 signaling might positively affect IP-10 production from monocytes following progression from NAFL to NASH.

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Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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Figure legends

Fig. 1. Multiple serum cytokine concentrations. (**a**) Three cytokines, IL-5, IL-17, and PDGF-BB, are reduced in NASH compared with controls (group a). (**b**) Five cytokines, IL-1β, IL-6, IL-8, MIP-1α, and MIP-1β, are elevated in both NAFL and NASH (group b). (**c**) Three cytokines, MCP-1, IL-15, and IP-10, are elevated in NASH compared with controls, while IL-15 is even higher in NASH than in NAFL, and IP-10 shows a stepwise increase from controls to NAFL and then to NASH (group c). (**d**) Receiver-operating characteristic (ROC) curves of IP-10 for diagnosing NAFLD in all subjects, NASH in all subjects, and NASH in NAFLD subjects.

NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; *$p<0.05$, †$p<0.01$

Fig. 2. Local cytokine mRNA expressions in the liver tissue of NAFL and NASH by real-time PCR. (**a**) The level of PDGF representative of group a is not different in NAFL and NASH liver specimens. (**b**) The level of MIP-1α representative of group b is not different. (c) The level of MCP-1 is higher in NASH than in NAFL. The level of IL-15 is not different. The level of IP-10 is higher in NASH than in NAFL. *$p<0.05$, †$p<0.01$

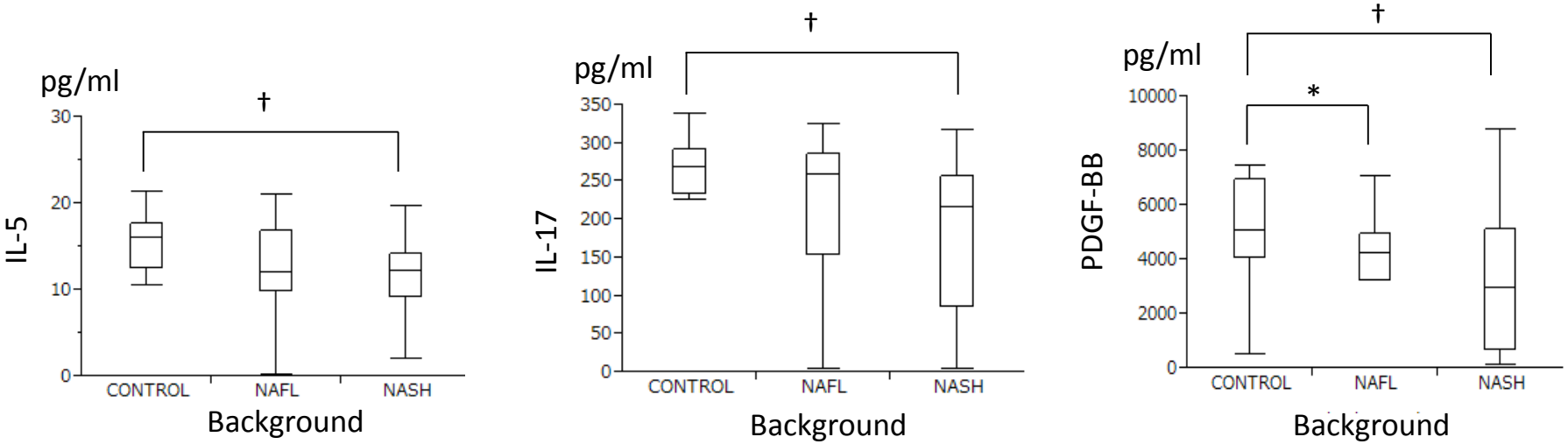
Fig. 3. Immunohistochemical staining for IP-10 in NAFL and NASH liver biopsy specimens. The average values of IP-10-positive cells in three portal areas were used for

grouping; equal or more than three IP-10-positive cells in one portal area was defined as ≥3.

Fig. 4. Monocyte production of cytokines by toll-like receptor (TLR) ligand stimulation with glucose and insulin modulation. (**a**) Group a cytokine mRNA expression in monocyte cell line THP-1 cells stimulated with TLR2 ligand peptidoglycan (PGN) or TLR4 ligand lipid A with or without high glucose and insulin. No significant changes are seen. (**b**) Group b cytokine mRNA expression. The combination of glucose and insulin, or the combination of glucose, insulin, and PGN affects MIP-1α mRNA expression. (**c-e**) Group c cytokine mRNA expression. The combination of glucose, insulin, and PGN affects IP-10 mRNA expression. The combination of glucose, insulin, and lipid A does not affect IP-10 mRNA expression. *$p<0.05$, †$p<0.01$

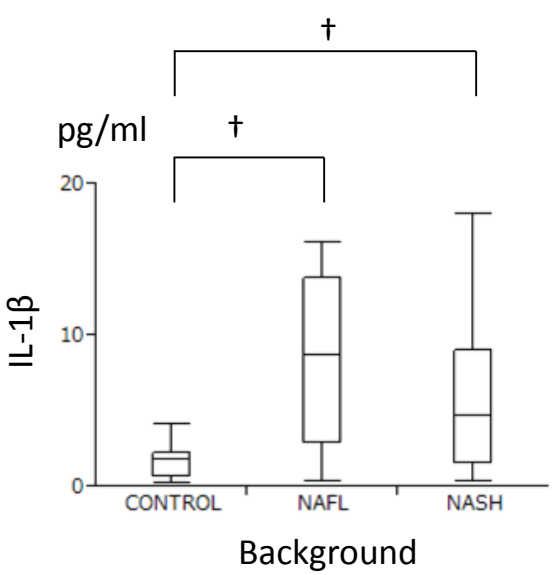
Figure 1

a (group a)



*p<0.05
†p<0.01

b (group b)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pg/ml
IL-1β
20
10
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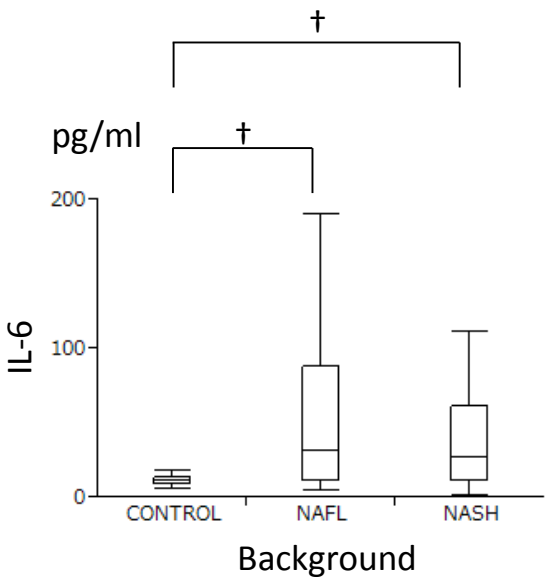
CONTROL
NAFL
NASH
Background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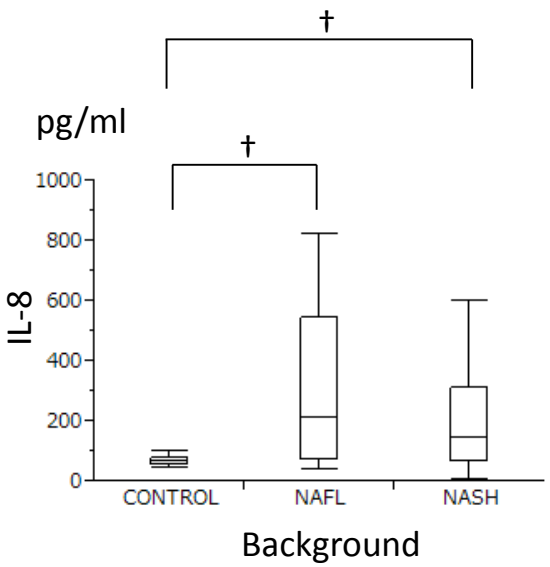
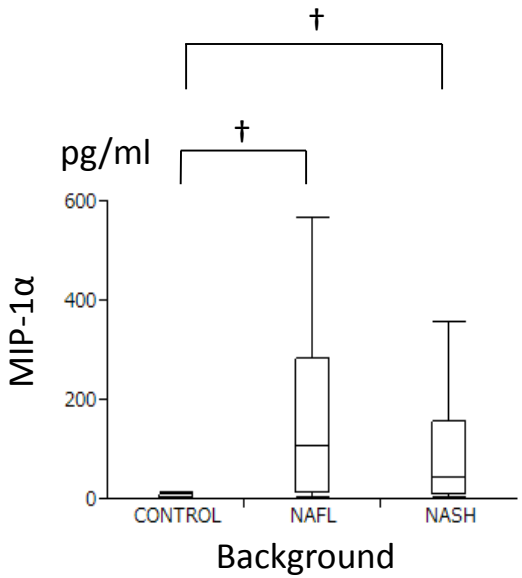
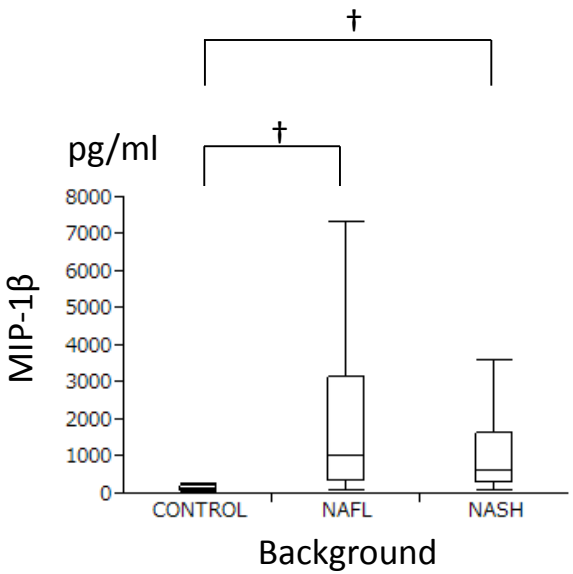
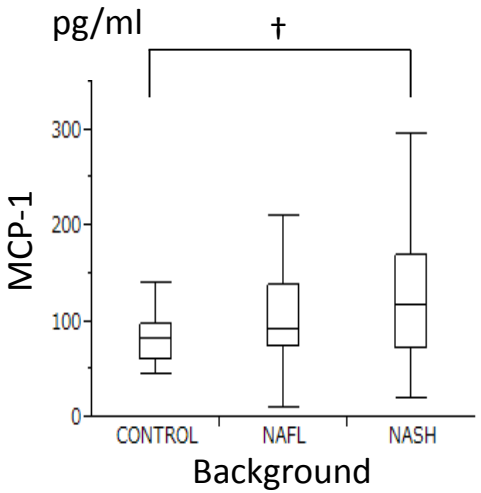
CONTROL
NAFL
NASH
Background

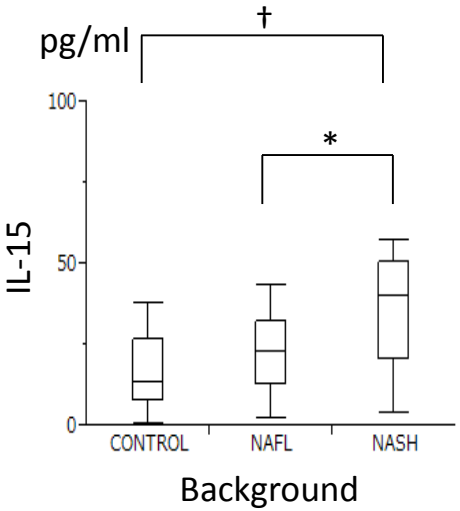

†
†
pg/ml
IL-8
1000
800
600
400
200
0
CONTROL
NAFL
NASH
Background

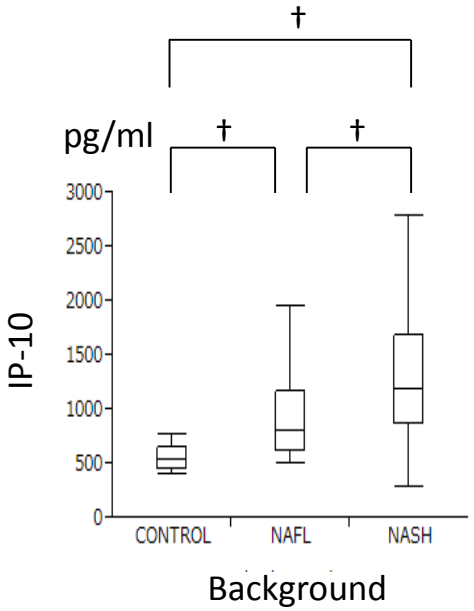

†
†
pg/ml
MIP-1α
600
400
200
0
CONTROL
NAFL
NASH
Background


†
†
pg/ml
MIP-1β
8000
7000
6000
5000
4000
3000
2000
1000
0
CONTROL
NAFL
NASH
Background

c (group c)


pg/ml
†
MCP-1
300
200
100
0
CONTROL
NAFL
NASH
Background

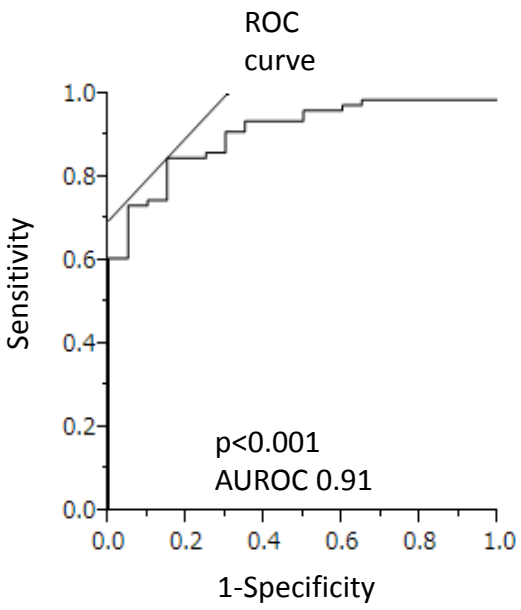

pg/ml
†
*
IL-15
100
50
0
CONTROL
NAFL
NASH
Background


†
pg/ml
†
†
IP-10
3000
2500
2000
1500
1000
500
0
CONTROL
NAFL
NASH
Background

d

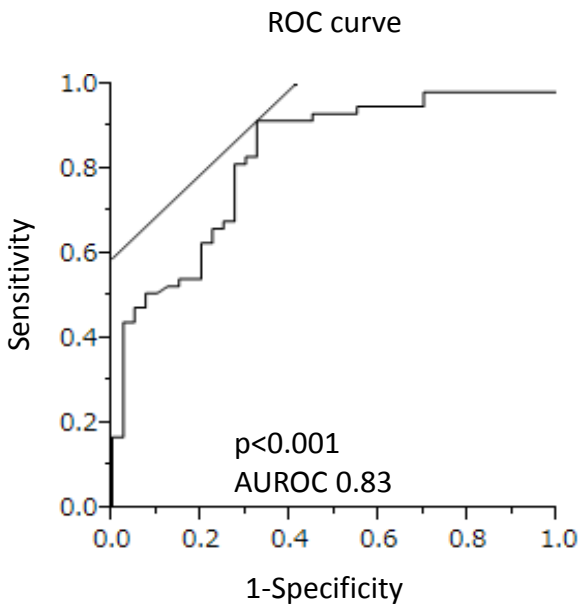
A

Diagnosis of NAFLD in all subjects



B

Diagnosis of NASH in all subjects



C

Diagnosis of NASH in NAFLD patients

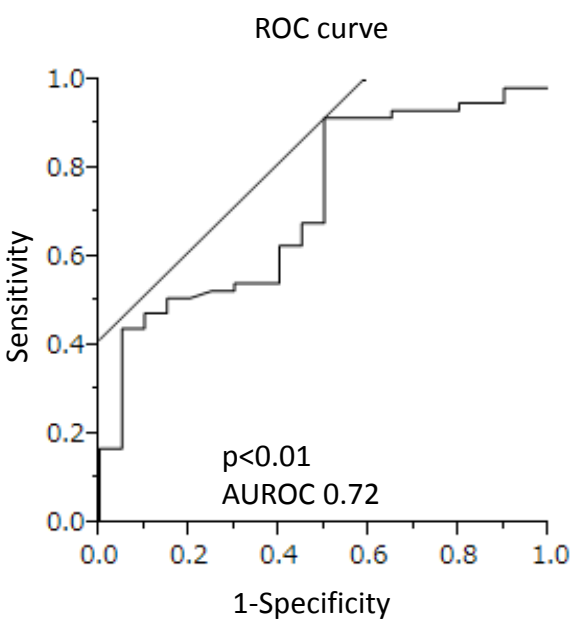
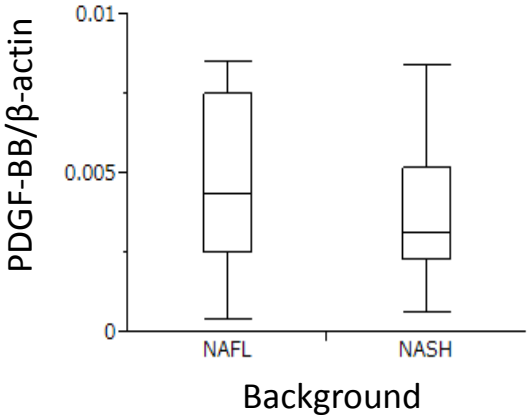
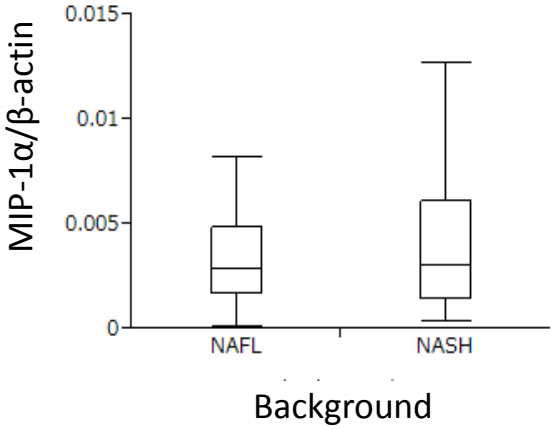


Figure 2

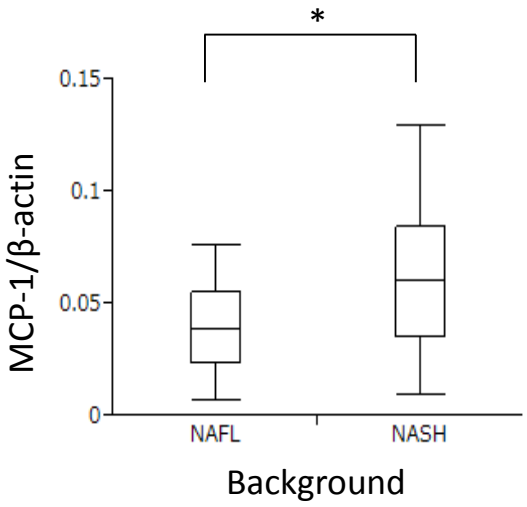
a (group a)

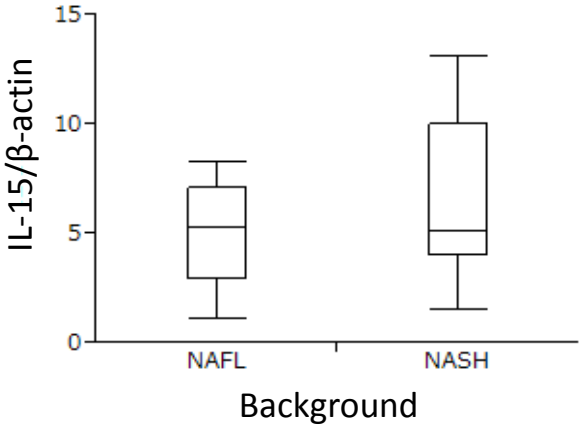


b (group b)



c (group c)





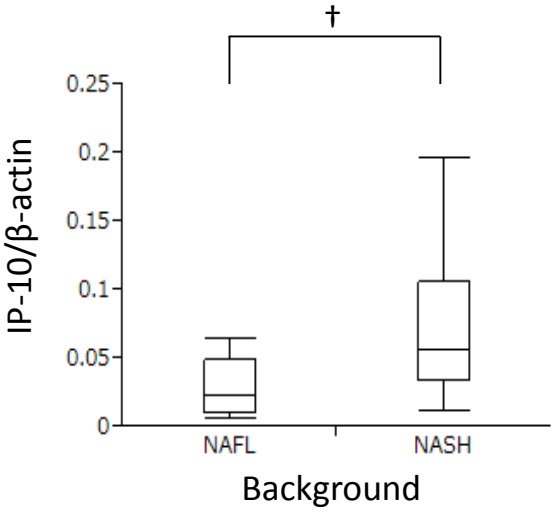
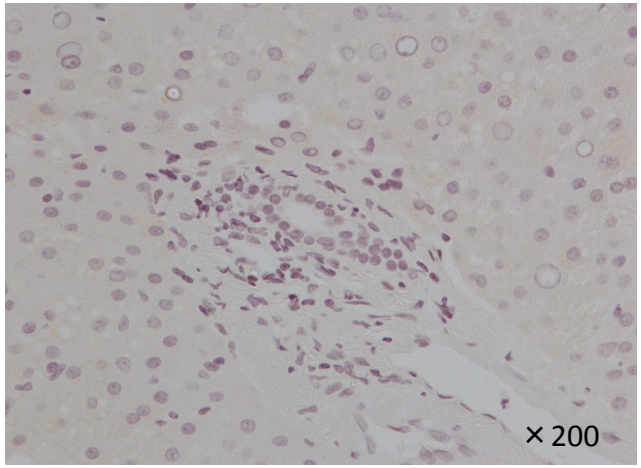
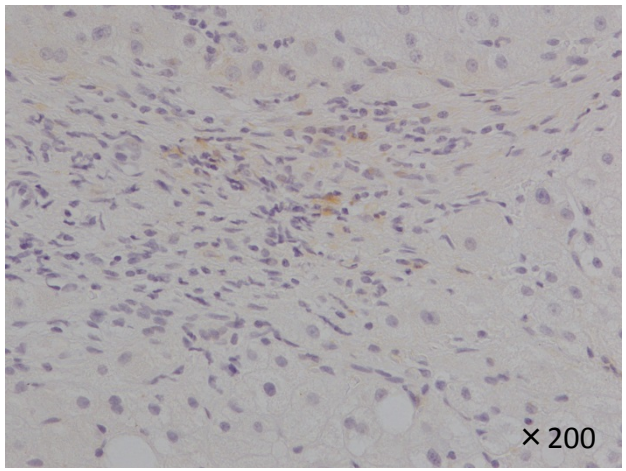


Figure 3

NAFL



NASH



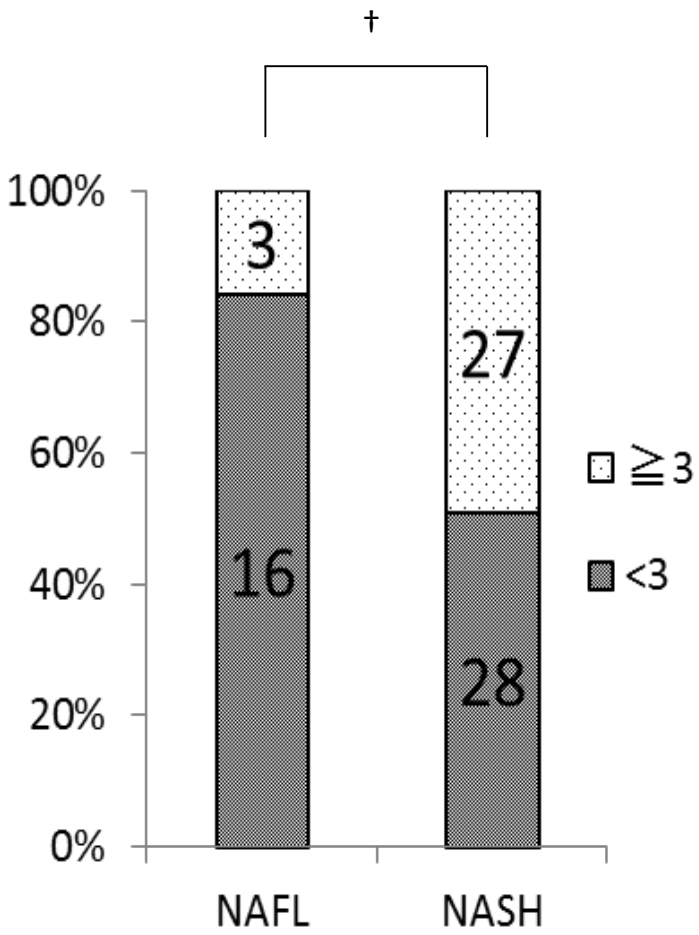
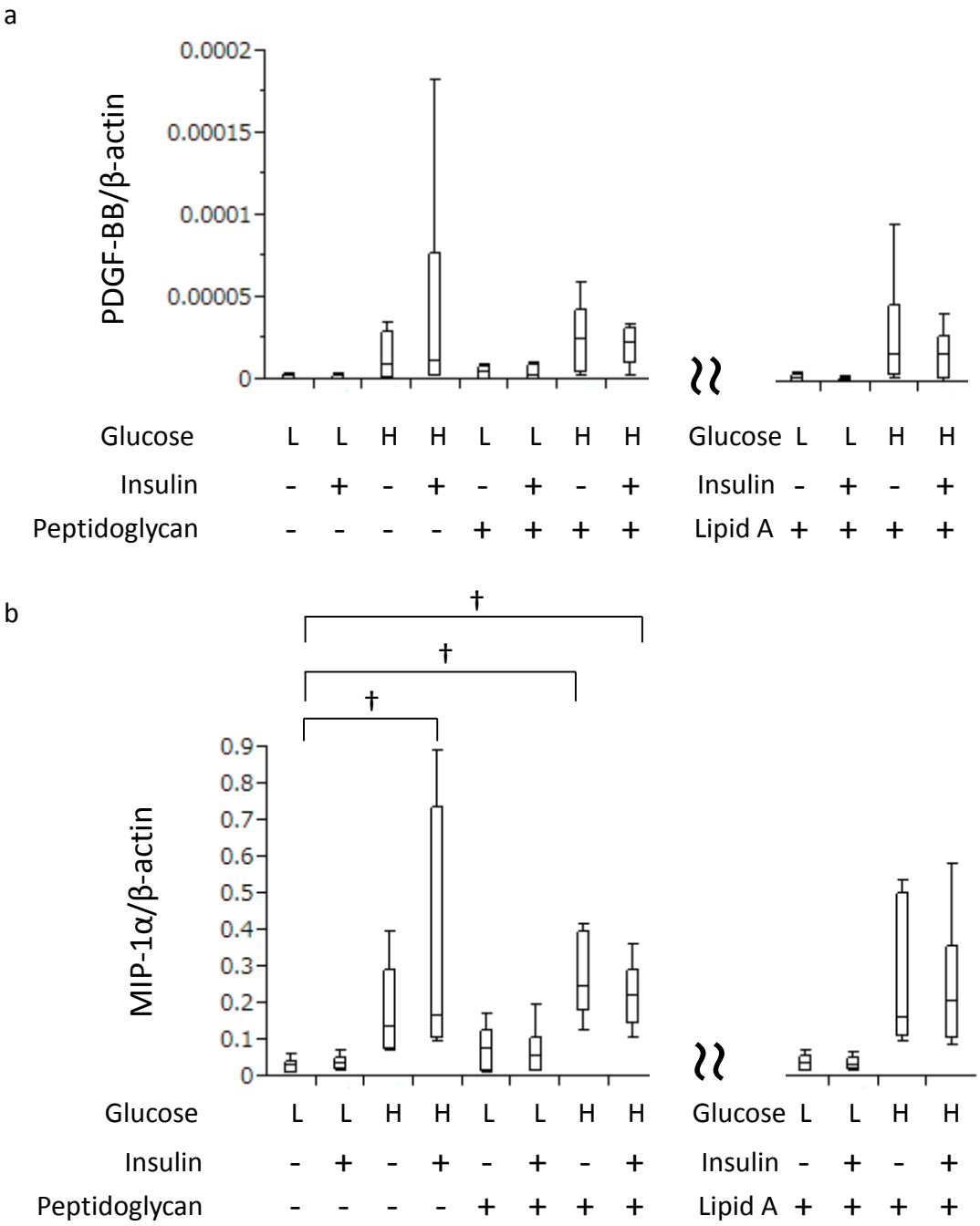
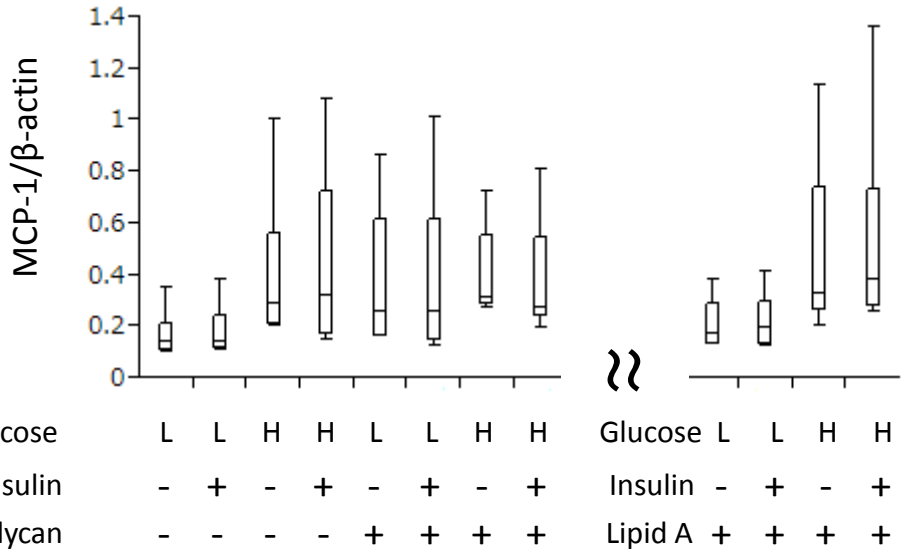


Figure 4

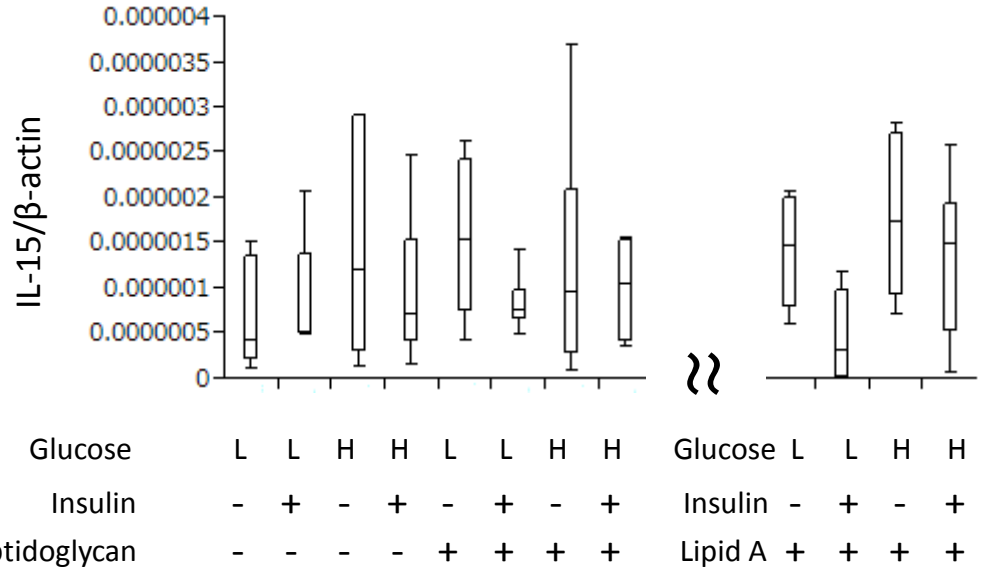


*p<0.05
†p<0.01

c


MCP-1/β-actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Glucose L L H H L L H H Glucose L L H H
Insulin - + - + - + - + Insulin - + - +
Peptidoglycan - - - - + + + + Lipid A + + + +

d


IL-15/β-actin
0.000004
0.0000035
0.000003
0.0000025
0.000002
0.0000015
0.000001
0.0000005
0
Glucose L L H H L L H H Glucose L L H H
Insulin - + - + - + - + Insulin - + - +
Peptidoglycan - - - - + + + + Lipid A + + + +

e

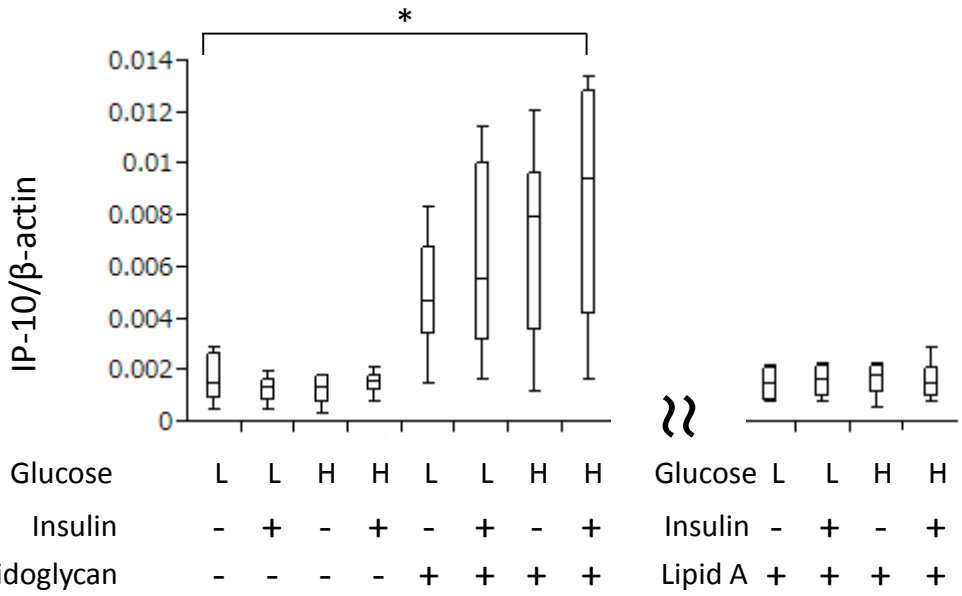

*
IP-10/β-actin
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Glucose L L H H L L H H
Insulin - + - + - + - +
Peptidoglycan - - - - + + + +
Glucose L L H H
Insulin - + - +
Lipid A + + + +

Table 1. Patients' baseline characteristics

	Control (n=20)	NAFL (n=20)	NASH (n=59)	p
Age (y)	47 (36-52)	45 (19-65)	57 (17-78)	0.04
Sex (M/F)	11/9	10/10	25/34	0.55
BMI (kg/m^2)	21.7 (19.6-22.2)	25.7 (14.3-36.1)	27.4 (16.7-40.7)	0.38
Steatosis (<30%/30 - 60%/>60%)	–	4/13/3	20/28/11	0.37
Activity grade (METAVIR score) (A0~1/A2~3)	–	20/0	31/28	<0.01
Fibrosis stage (METAVIR score) (F0~2/F3~4)	–	20/0	29/30	<0.01
Platelets (×10^4/μl)	23.7 (20.5-31.5)	25.4 (10.1-34.1)	21 (6.7-79.1)	0.01
PT-INR	1.01 (0.91-1.03)	0.91 (0.85-1.15)	0.98 (0.83-1.47)	<0.01
AST (IU/l)	22 (17-25)	36 (14-81)	61 (21-201)	<0.01
ALT (IU/l)	23 (14-41)	56 (16-128)	75 (14-452)	0.13
γ-GTP (IU/l)	28 (13-51)	67 (15-264)	85 (20-1059)	0.38
T-Cho (mg/dl)	204 (161-274)	198 (116-308)	186 (102-354)	0.55
HDL-Cho (mg/dl)	58 (43-76)	56 (34-76)	49 (30-118)	0.53
TG (mg/dl)	84 (51-422)	171 (70-687)	152 (32-392)	0.34
HbA1c (%)	5.4 (5-5.5)	5.6 (4.8-8.7)	5.6 (3.3-10.5)	0.52
HOMA-IR	1.3 (0.6-3.7)	1.8 (0.6-47.6)	3.6 (1.3-30.9)	<0.01
Ferritin (ng/ml)	119 (58-367)	188.7 (17-1023)	249.6 (15.6-3068)	0.35

Table 2. Diagnostic sensitivity and specificity of IP-10 and known tools to differentiate NASH from NAFL

	Cut-off	Sensitivity (%)	Specificity (%)	AUC
Fib-4 index	1.7	64.4	90	0.78
APRI	0.7	71.1	85	0.83
CK18	130 U/l	92.3	36.8	0.62
IP-10	700 pg/dl	91.5	50	0.72

Table 3.

A. Correlations between clinical characteristics and serum IP-10

	IP-10 < 700 pg/ ml	IP-10 ≥ 700 pg/ ml	Univariate p	Multivariate p
BMI (kg/m^2), median (interquartile range)	24.9 (22-27.6)	26.7 (24-29.2)	0.048	0.38
Steatosis (<30/30 - 60/>60)	4/11/1	20/30/13	0.21	
Platelets (×10^4/μl), median (interquartile range)	25.6 (21.8-28.3)	22.1 (14.8-25.3)	0.02	0.047
PT-INR, median (interquartile range)	0.96 (0.91-1.02)	0.96 (0.91-1.01)	0.98	
AST (IU/l), median (interquartile range)	25 (22-35)	60 (45-82)	<0.01	0.01
ALT (IU/l), median (interquartile range)	33 (23-61)	78 (47-116)	<0.01	0.92
γ-GTP (IU/l), median (interquartile range)	51 (25-134)	71 (44-123)	0.053	
T-Cho (mg/dl), median (interquartile range)	198 (168-215)	189 (167-226)	0.8	
HDL-Cho (mg/dl), median (interquartile range)	57 (48-67)	49 (43-60)	0.12	
TG (mg/dl), median (interquartile range)	142 (86-196)	152 (106-195)	0.69	
HbA1c (%), median (interquartile range)	5.4 (5.1-5.9)	5.6 (5.3-6)	0.38	
HOMA-IR, median (interquartile range)	1.6 (1.3-2.7)	3.5 (2.5-5.8)	<0.01	0.08
Ferritin (ng/ml), median (interquartile range)	158.3 (86.6-252.5)	249.6 (143.8-417.8)	0.02	0.96
CK18 (U/l), median (interquartile range)	123.2 (97.6-234.1)	306.3 (198.4-561.8)	<0.01	0.13

B. Correlations between activity grade/fibrosis stage and serum IP-10

	IP-10 < 700 pg/ ml	IP-10 ≥ 700 pg/ ml	Univariate p
activity grade (1/2-3)	14/2	37/26	0.03
fibrosis stage (1-2/3-4)	14/2	35/28	0.02