# Secreted phosphoglucose isomerase is a novel biomarker of nonalcoholic fatty liver in mice and humans

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## Abstract

The current gold standard for diagnosis of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) is through a liver biopsy, and there is an urgent need to develop non-invasive methods for early detection. We previously demonstrated metabolic remodeling in the mouse fatty liver, which is marked by increased hepatic expression and activities of phosphoglucose isomerase (PGI) and several other glycolytic enzymes. Since PGI is actively transported out of the cell, acting as a multifunctional cytokine referred to as autocrine motility factor (AMF), we explored the possibility that PGI secreted from the fatty liver may be targeted for early detection of the silent disease. We report here that mice with NASH exhibited significantly elevated serum PGI enzyme activities compared to normal control (P < 0.005). We further confirmed the finding using serum/plasma samples (n = 73) collected from a cohort of NASH patients who were diagnosed according to Kleiner's criteria, showing a normal mean PGI of 19.5 ± 8.8 IU/L and patient mean PGI of 105.6 ± 79.9 IU/L (P < 0.005). In addition, elevated blood PGI in NASH patients coincided with increased blood L-lactate. Cell culture experiments were then conducted to delineate the PGI-lactate axis, which revealed that treatment of HepG2 cells with recombinant PGI protein stimulated glycolysis and lactate output, suggesting that the disease-induced PGI likely contributed to the increased lactate in NASH patients. Taken together, the preclinical and clinical data validate secreted PGI as a useful biomarker of the fatty liver that can be easily screened at the point of care.

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## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease afflicting ~100 million individuals in the US, and is becoming one of the top causes for liver transplantation [1]. Nonalcoholic steatohepatitis (NASH) is a progressive form of NAFLD with a major inflammatory component, and presents a significant risk factor for cirrhosis and hepatocellular carcinoma [2,3]. NASH affects an estimated 3%–6% of the US populationwith the prevalence increasing at an alarming rate [1]. Since there is often no outward sign or symptom, early detection of the fatty liver will enable the first line of treatment through a combination of healthy diet and exercise.

Our previous work on a mouse model of NASH revealed a coupling between aberrant Wnt signaling and stimulation of hepatic glycolysis, which is marked by increased expression and activities of several enzymes involved in glucose metabolism including phosphoglucose isomerase (PGI) [4]. The finding of NASH-induced PGI in the fatty liver warrants attention because while PGI catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate in the cytosol, it also functions as an extracellular trophic factor following secretion by a nonclassical mechanism [5]. The secreted form of PGI, known as autocrine motility factor (AMF), regulates cell growth, migration, and survival through interaction with its cell surface receptor AMFR/gp78 [5], raising the possibility that PGI secretion may have a pathophysiologic role in the progression of NAFLD. If induction and secretion of PGI represent an early event in the pathogenesis of NAFLD, blood PGI may be used for early detection of the silent disease. Given that there is an urgent need to develop non-invasive methods for assessment of NAFLD, the study was initiated to explore the feasibility of using secreted PGI as a biomarker.

## 2. Methods and materials

### 2.1. Animal use

The animal study was published previously [4]. In brief, twomonth old male C57BL/6 mice were fed either chow diet (control) or methionine-choline deficient diet (MCDD) for 6 weeks to induce NASH, following which tissue samples were collected for analysis. All procedures and protocols conformed to institutional guidelines for the care and use of animals in research.

### 2.2. Human subjects

This study was approved by the Children and Youth Institutional Review Board of the State University of New York at Buffalo. All NASH patients (Table 1) underwent percutaneous liver biopsy and fulfilled Kleiner's criteria on hepatic fat infiltration, inflammation, and fibrosis [6]. Insulin resistance (IR) was calculated according to the "homeostasis model assessment (HOMA)" method [7]. Enrolling only pediatric patients was an additional assurance that alcohol intake would not be a confounding factor in this study. Serum and plasma (EDTA or citrate) samples from healthy controls (<20 years of age) were purchased from ZenBio and Innovative Research. All specimens are stored at −70 °C.

### 2.3. HepG2 cell culture

HepG2 cells, a human liver cancer cell line, were grown in a culture medium consisting of minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 50 μg/ml gentamycin and 0.125 μg/ml Fungizone. For PGI treatment, 50,000 cells were seeded in each well of a 24-well plate overnight, following which cells were rinsed once with Hank's balanced salt solution (HBSS) and then treated with 100 ng/ml recombinant PGI (R&D) in culture medium for 2 days. Culture medium was collected for L-lactate assay and cells were rinsed with HBSS once and stored frozen at −70 °C. Thawed cells were scraped and manually homogenized using a pipette tip in an ice-cold lysis buffer (20 mM Tris pH7.4, 0.2 mM EDTA, 100 mM NaCl, and 0.1% Triton X-100). Cell lysates were clarified by centrifugation at 13,000 rpm for 10 min at 4 °C, following which the supernatants were harvested for protein assay and enzyme activity assay.

### 2.4. Metabolic analysis

Enzyme activities of PGI and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and concentration of L-lactate present in serum and culture medium were measured using colorimetric PGI, GAPDH, and L-Lactate assay kits from Biomedical Research Service (University at Buffalo) as previously described [4,8]. The 96-well plate assays are all based on diaphorase-coupled reactions, converting p-iodonitrotetrazolium violet to formazan, which exhibits an absorption maximum at 492 nm. For lactate assay, cell culture medium and serum samples were deproteinized by protein precipitation in 25% polyethylene glycol-8000 prior to assay as described in the manual. Sample optical density was measured using a plate reader and converted to IU/L or μmol/(L•min) per manual instructions. Concentration of L-lactate was calculated from a lactate standard equation established from a set of lactate standards.

### 2.5. Statistical analysis

Comparisons between two and multiple experimental groups were made with Student's T-test and one-way ANOVA, respectively. A value of $p < 0.05$ is considered significant. Data are expressed as means ± SD.

## 3. Results

Our previous study of a mouse model of NASH demonstrated that the fatty liver exhibited a 2-fold induction in the expression and activities of several enzymes involved in glucose oxidation including PGI [4]. Recognizing that PGI is actively transported out of the cell functioning like a cytokine, we became interested in determining whether the hepatic PGI abundance profile might be recapitulated in the circulation. PGI enzyme assays on the mouse serum samples indeed revealed a 2-fold higher enzyme activity in mice with NASH (Fig. 1A). This result from the preclinical fatty liver model is in accordance with the secretory nature of PGI, and suggests the potential usefulness of blood PGI screening in diagnosing the fatty liver disease.

To gather clinical evidence along this line, we performed the PGI enzyme assay on the serum/plasma samples of a cohort of pediatric NASH patients (Table 1), who underwent

percutaneous liver biopsy and diagnosed according to Kleiner's criteria, which is a histological scoring system [6,9]. Initial assays of control serum/plasma samples from age-matched healthy donors (age 17–20 years) established the normal range for blood PGI activity: 10–40 IU/L (mean = $19.5 \pm 8.8$ IU/L). Consistent with the animal data, we found that the mean patient PGI was $105.6 \pm 79.9$ IU/L ($P < 0.005$), which is a 5-fold increase over the normal PGI (Fig. 1B). Taken together, the preclinical and clinical studies validated secreted PGI as a fatty liver disease marker.

Blood alanine aminotransferase (ALT) is routinely used to assess patient liver injury, and is the most common criterion for referral. However, patients diagnosed with NAFLD or NASH can exhibit a normal blood ALT level without showing any outward sign or symptom [10]. Indeed, we note that ~30% of the NASH patients studied here exhibited normal blood ALT (<56 U/L) with the other ~70% showing above normal ALT. Notably, regardless of their ALT profiles, both patient groups had similarly elevated blood PGI: $83.5 \pm 58.7$ IU/L for the normal ALT group and $118.3 \pm 79.6$ IU/L for the above normal ALT group (Figure C). This finding indicates that the PGI profile, but not the ALT profile, is useful for predicting the liver disease. Further, patients with mild vs. severe fibrosis or inflammation also had similarly elevated blood PGI (data not shown), indicating that secreted PGI is a sensitive diagnostic marker for early- and late-stage nonalcoholic fatty liver.

Since obesity, dyslipidemia, and type 2 diabetes mellitus are the most common metabolic risk factors associated with NAFLD [2], we used linear regression analysis to evaluate blood PGI activity in relation to additional patient characteristics. Fig. 2 shows that blood PGI does not appear to vary considerably by patient age ($R^2 = 0.0035$), nor does it change appreciably between early and severe insulin resistance as assessed by HOMA ($R^2 = 0.0224$). Nonetheless, patients with higher body mass index ($R^2 = 0.0951$) or hypertriglyceridemia ($R^2 = 0.0642$) tend to exhibit a slightly higher blood PGI profile.

Secreted PGI, or AMF, is known to stimulate tumor cell metastasis driven by an increased glycolytic flux [11], which can lead to an increased production of the byproduct L-lactate. The finding that the NASH patients have prominently elevated levels of blood PGI (Fig. 1B) prompted us to measure the level of blood lactate. Consistent with this notion, Fig. 3A reveals a statistically significant increase in patient serum lactate ($P < 0.05$). To further dissect the PGI-lactate axis, we delved into cell culture experiments using HepG2 cells, a human liver cancer cell line. Cells were treated with recombinant PGI protein for 2 days, after which cell lysates were prepared for enzyme activity assays and culture media were collected for lactate assay. Fig. 3B shows that HepG2 cell lysates exhibited significantly enhanced glucokinase activity upon PGI treatment ($P < 0.05$). This stimulatory effect of PGI on glucokinase was accompanied by a significantly increased lactate output as shown in Fig. 3C ($P < 0.01$). Thus, the fatty liver-derived PGI likely contributes to the increase in patient serum lactate, which provides additional validation for the role and diagnostic use of secreted PGI.

## 4. Discussion

The current study provides evidence, both preclinical and clinical, that validates the use of secreted PGI for detection of nonalcoholic fatty liver, which often exhibits no outward symptom. Secreted PGI (or AMF) signaling through its cognate receptor AMFR further stimulates glycolysis, resulting in elevated blood lactate. The induced PGI-lactate axis as demonstrated here is a notable pathophysiologic feature of the fatty liver that may represent a unique component of the metabolic syndrome. Although liver biopsy remains the gold standard for disease diagnosis, the simple assay of blood PGI enzyme activity aided by blood lactate analysis adds to the armamentarium currently available for NAFLD management.

Many blood diagnostic tests used in the clinic are directed towards secreted proteins, highlighting the importance of this family of proteins in laboratory medicine. PGI is among the many moonlighting proteins identified in recent years that can function in multiple cellular contexts [5]. The secreted form of PGI regulates multiple aspects of cell function through interaction with its receptor AMFR/gp78. Additional names exist for AMF, including neuroleukin [12] and maturation factor [13], which attests to the functional versatility of extracellular PGI. Thus, it is likely that increased blood PGI activity may also be associated with other morbidities. For instance, blood PGI level has been shown to be helpful for diagnosis or prognosis of certain types of cancer and rheumatoid arthritis [14,15]. Whether secreted PGI may be of value in detection of alcoholic fatty liver disease remains to be determined because the current study only encompassed NAFLD.

The best practice in clinical diagnostic relies on multiple but complementary approaches, such as measuring serum biomarkers and liver stiffness using ultrasound- or magnetic resonance-based elastography techniques [16]. Multiple blood chemistries are routinely performed to assess hepatic function and injury. Increased blood ALT can be associated with liver disease, myocardial infarction, myopathy, and many other conditions. On the other hand, normal blood ALT results do not exclude the presence of a liver disease [10], as also revealed from the current study showing that ~30% of the NASH patients exhibited normal ALT but above normal blood PGI, making secreted PGI a superior biomarker of NAFLD. Unlike ALT, a rise in blood PGI is not predicated on liver injury because cytosolic PGI is actively transported out of the fatty liver and readily detectable in the circulation.

There are several biomarkers that have been investigated to predict the fatty liver disease. NASH patients may exhibit increased levels of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [17]. However, neither TNF-α nor IL-6 is specific to liver inflammation. Although elevated serum levels of C-reactive protein (CRP) correlate with the observed inflammatory status in NASH, CRP does not exhibit NASH specific inflammatory changes [18]. Circulating levels of cytokeratin 18 (CK18) fragment are a marker of hepatocyte apoptosis, but the accuracy of the blood test is modest at best [19].

In summary, our data indicate that the PGI enzyme activity assay is useful for detecting nonalcoholic fatty liver even in the absence of liver injury. The PGI assay can be further

aided by blood lactate analysis to confirm pathophysiologic activation of the PGI-lactate axis in the fatty liver. It should be noted that the PGI assay reported here is technically unique because it is based on enzyme activity, which measures the presence of "functional" protein. This feature of the PGI activity assay differs from the traditional antibody-based assay, which can detect both the intact and degraded forms of a protein, potentially generating ambiguous test results and confounding data interpretation. Given that NAFLD is the most common chronic liver disease in the US, early detection using secreted PGI as a biomarker can represent a vital part of the disease prevention and treatment strategies.

## Acknowledgments

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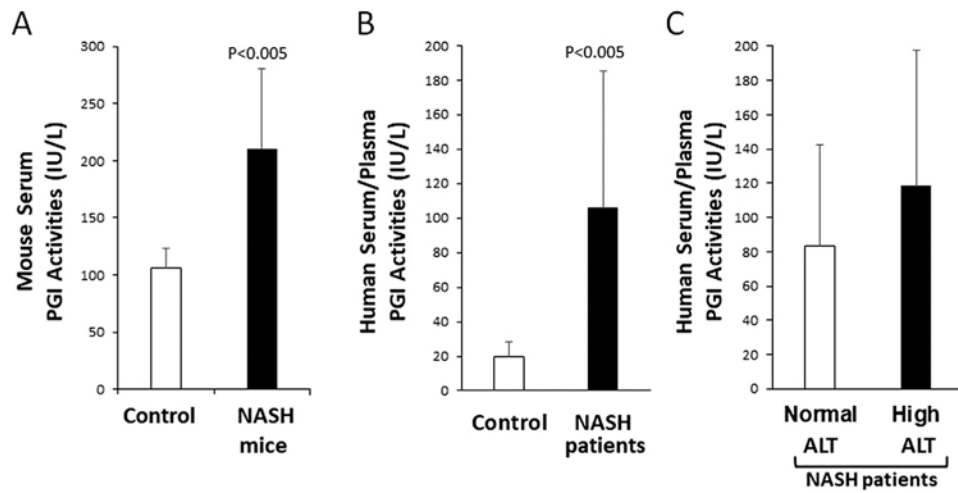
**Fig. 1. Blood PGI activity profiles in mice and humans.**
A 20-min PGI activity assay was conducted using 5 μl of mouse and human serum/plasma. (A) Serum PGI activities of control mice (n = 4) and those with NASH (n = 4). (B) Serum/plasma PGI activities of healthy donors (control n = 8) and NASH patients (n = 73). (C) Serum/plasma PGI activities of NASH patients exhibiting normal ALT (<56 U/L; n = 18) and abnormally high ALT levels (n = 41). Statistical significance is indicated where relevant.
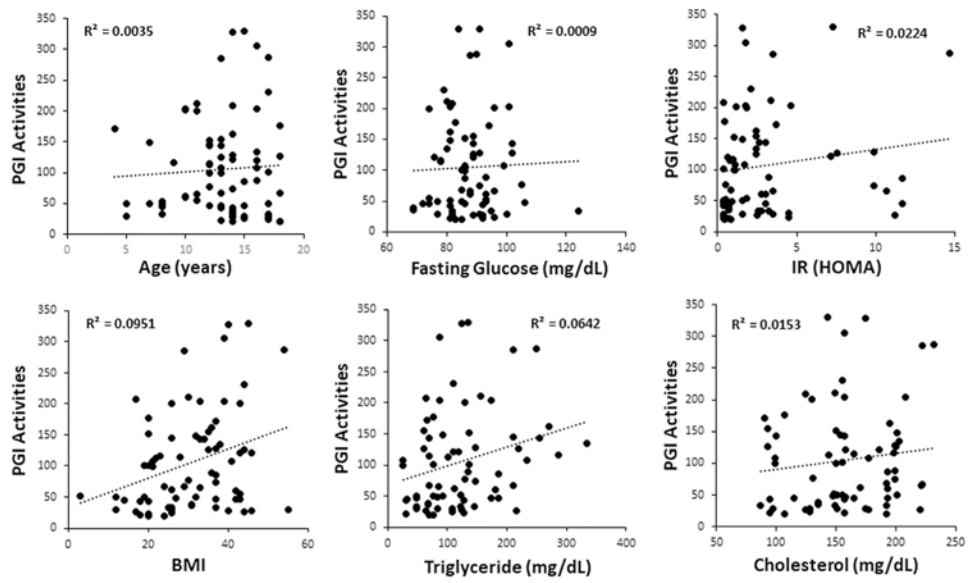
**Fig. 2. Linear regression analysis of NASH patient characteristics and blood PGI activity.**
Patient age, fasting glucose, insulin resistance (HOMA), BMI, triglyceride, and cholesterol
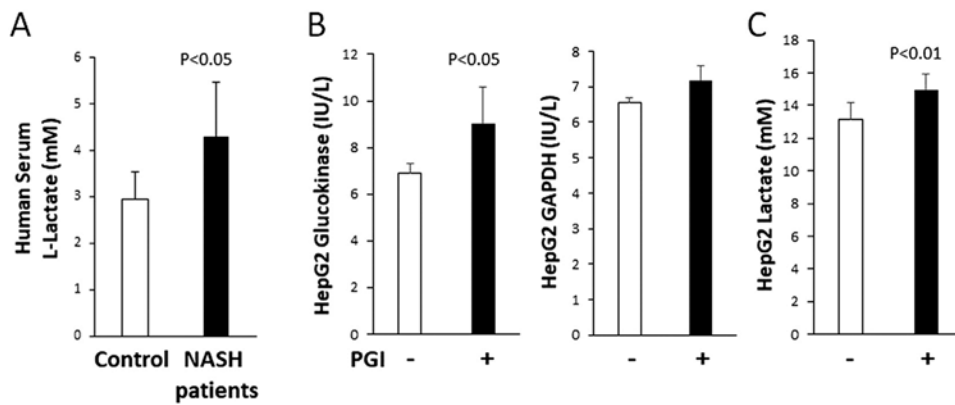are each compared against blood PGI activities. The R-squared value is indicated in each
panel.

**Fig. 3. Analysis of the PGI-lactate axis.**

(A) Serum L-lactate levels (mM) of healthy donors (n = 5) and NASH patients (n = 14). A 30-min lactate assay was performed using deproteinized serum samples after a 10-fold dilution with $dH_2O$. (B) HepG2 cells were treated with 100 ng/ml recombinant PGI protein for 3 days, following which cell lysates were prepared. Five µg proteins of each cell lysate were used to assay the enzyme activities of glucokinase and GAPDH. (C) Cell culture medium was harvested after 3 days. Culture medium L-lactate concentrations were determined using deproteinized medium samples after a 10-fold dilution with $dH_2O$. Statistical significance is indicated where relevant.

**Table 1**

NASH patient characteristics.

| Age (year) | 4–18 |
|---|---|
| Gender (F/M) | 27/32 |
| BMI | 3–55 |
| ALT (U/L) | 9–231 |
| AST (U/L) | 17–126 |
| IR (HOMA) | 0.37–14.67 |
| Cholesterol (mg/dL) | 90–232 |
| Triglyceride (mg/dL) | 26–334 |