## Title

An enzyme-linked immunosorbent assay for measuring GPIHBP1 levels in human plasma or serum



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# An ELISA for measuring GPIHBP1 levels in human plasma or serum

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## Abstract

**BACKGROUND—**GPIHBP1, a glycosylphosphatidylinositol (GPI)-anchored protein of capillary endothelial cells, transports lipoprotein lipase to the capillary lumen and is essential for the lipolytic processing of triglyceride-rich lipoproteins.

**OBJECTIVE—**Because some GPI-anchored proteins have been detected in plasma, we tested whether GPIHBP1 is present in human blood, and whether *GPIHBP1* deficiency or a history of cardiovascular disease affected GPIHBP1 circulating levels.

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**AUTHOR CONTRIBUTIONS**

Kazuya Miyashita performed experiments, analyzed the data and contributed the scientific discussion, Isamu Fukamachi analyzed the data and contributed the scientific discussion. Manabu Nagano prepared the serum samples and provided the background and insights of the patient data, Tetsuro Ishida generated the patient cohort and contributed to scientific discussions. Junji Kobayashi contributed the scientific discussions. Tetsuro Machida performed serum analysis, Kiyomi Nakajima performed serum analysis. Masami Murakami performed ELISA analysis and contributed the scientific discussions. Michael Ploug provided important scientific insights, edited the manuscript, and generated purified proteins (including GPIHBP1) for ELISAs. Anne P. Beigneux performed experiments, analyzed the data, and wrote the manuscript. Stephen G. Young analyzed the data and wrote the manuscript. Katsuyuki Nakajima designed the experiments, analyzed the data and wrote the manuscript. All authors approved the final version of the manuscript.

**DISCLOSURES**

The authors have no financial interests to declare.

**METHODS—**We developed two monoclonal antibodies against GPIHBP1and used the antibodies to established a sandwich ELISA to measure GPIHBP1 levels in human blood.

**RESULTS—**The GPIHBP1 ELISA was linear in the 8–500 pg/ml range and allowed the quantification of GPIHBP1 in serum and in pre- and post-heparin plasma (including lipemic samples). GPIHBP1 was undetectable in the plasma of subjects with *null* mutations in *GPIHBP1*. Serum GPIHBP1 median levels were 849 pg/ml (range: 740–1014) in healthy volunteers ($n$ = 28) and 1087 pg/ml (range: 877–1371) in patients with a history of cardiovascular or metabolic disease ($n$ = 415). There was an extremely small inverse correlation between GPIHBP1 and triglyceride levels (r = 0.109; $P$ <0.0275). GPIHBP1 levels tended to be slightly higher in patients who had a major cardiovascular event after revascularization.

**CONCLUSION—**We developed an ELISA for quantifying GPIHBP1 in human blood. This assay will be useful to identify patients with *GPIHBP1* deficiency and patients with GPIHBP1 autoantibodies. The potential of plasma GPIHBP1 as a biomarker for metabolic or cardiovascular disease is yet questionable but needs additional testing.

## INTRODUCTION

Lipoprotein lipase (LPL), a triglyceride hydrolase secreted by myocytes and adipocytes, is crucial for the lipolytic processing of triglyceride-rich lipoproteins (TRLs) along capillaries.[4–6] The mechanism by which LPL reaches its site of action inside blood vessels was mysterious for decades, but the mystery was ultimately solved by Beigneux *et al.* and Davies *et al.*.[7, 8] They showed that GPIHBP1 (glycosylphosphatidylinositol-anchored HDL binding protein–1), a GPI-anchored protein of capillary endothelial cells, binds LPL in the subendothelial spaces and shuttles it across endothelial cells to its site of action in the capillary lumen. A deficiency in GPIHBP1 causes severe hypertriglyceridemia (chylomicronemia).[3, 9–13]

GPIHBP1 is a member of the Ly6/uPAR (“LU”) protein superfamily. It contains an amino-terminal acidic domain[14] and an ~80–amino acid “Ly6 domain.” The Ly6 domain contains 10 cysteines, all in a characteristic spacing pattern and all disulfide bonded, so as to create a three-finger fold.[15] Biophysical studies revealed that the Ly6 domain is largely responsible for high-affinity LPL binding to GPIHBP1, while the acidic domain has only an accessory role in LPL binding[16] and is more relevant to the stability of LPL activity.[16, 17] Consistent with these findings, a monoclonal antibody (mAb) against GPIHBP1’s Ly6 domain (RE3) was shown to block LPL binding to GPIHBP1, while a mAb against GPIHBP1’s acidic domain (RF4) did not.[2]

GPI-anchored proteins can be released from the plasma membrane by several mechanisms, for example the release of vesicles, cleavage of the polypeptide, or cleavage of the GPI anchor by GPIases such as GPI-specific phospholipase D.[18, 19] As a result, some GPI-anchored proteins can be detected in the plasma. One example is uPAR (urokinase-type plasminogen activator receptor).[20] Interestingly, the levels of uPAR in the plasma have been shown to be elevated in certain cancers, and increased uPAR levels are associated with a poor prognosis;[21] non-invasive PET-imaging reveals increased uPAR expression in the solid-tumors and their metastases.[22]

We set out to determine if GPIHBP1 is detectable in plasma or serum, and if so, to quantify the levels of GPIHBP1 in human blood. We developed two new rat mAbs against human GPIHBP1, IU-79 and IU-20, and used those antibodies to establish a sandwich immunoassay for human GPIHBP1. We used this assay to compare the GPIHBP1 blood levels in healthy subjects and in GPIHBP1-deficient subjects. We also examined GPIHBP1 levels in patients under treatment for cardiovascular or metabolic diseases.

## METHODS

### MONOCLONAL ANTIBODY PREPARATION

C-DNA of human GPIHBP1 was synthesized by FASMAC (Tokyo) and expressed in CHO cells. The recombinant GPIHBP1 from transfected CHO cells was purified by anti-flag M2 column. Wister rats were immunized with the purified recombinant human GPIHBP1. Antibody titers in the plasma of the immunized rats were monitored by ELISA, and hybridomas were generated by fusing splenocytes with X63 myeloma cells. After subjecting the hybridomas to selection with azaserine and hypoxanthine, samples of medium were screened for GPIHBP1 antibodies by ELISA. 17 hybridomas were expanded and subcloned. Five monoclonal antibodies (mAbs), including mAbs IU-79 and IU-20, were isotyped with commercial kits (BD Bioscience, Tokyo). The hybridomas were adapted to serum-free medium, and the monoclonal antibodies were purified from the CHO cell culture medium on a protein G–agarose column. The antibodies were eluted from the protein G column with glycine•HCl (pH 2.5).

### IMMUNOBLOTTING

Soluble versions of wild-type human GPIHBP1, GPIHBP1-W109S, and a mutant GPIHBP1 lacking the acidic domain, all containing an amino-terminal uPAR tag (detectable with mAb R24), were expressed in *Drosophila* S2 cells.[23] Recombinant human GPIHBP1 (2.0 μg) was size-fractioned by SDS-PAGE under reducing and nonreducing conditions and transferred to a sheet of nitrocellulose. The blots were then incubated with rat mAbs IU-79 or IU-20 (5 μg/ml), followed by an IRDye-labeled donkey anti-rat IgG (Rockland, 1:2000). Western blots of nonreduced GPIHBP1 were also incubated with IRDye680-labeled mAb R24 (specific for uPAR) (1:500).[24] Blots of reduced samples were incubated with IRDye800-labeled mAb RF4 (which binds to the acidic domain of hGPIHBP1, 1:500).[2] Western blots were imaged with an infrared scanner (LI-COR).

### PREPARATION OF GPIHBP1 CALIBRATOR FOR ELISA STUDIES

A secreted version of human GPIHBP1 with an amino-terminal Flag tag was expressed in HEK-293 cells in a high-density incubator (Integra Bioscience, Switzerland). The amount of GPIHBP1 in the conditioned medium was determined by SDS-PAGE, after staining with Coomassie Brilliant Blue, against a known amount of a purified recombinant human GPIHBP1.

### GPIHBP1 SANDWICH ELISA

96-well ELISA plates were coated with 1 μg/well of mAb IU-79 overnight at 4°C. After blocking overnight at 4°C with PBS containing 1% bovine serum albumin (BSA) and 0.05%

$NaN_3$, the wells were incubated at 37°C for 60 min with serum or plasma samples. Serial dilution of the samples (1:2 to 1:256) were performed in PBS containing 1% BSA, 0.05% Tween 20, 0.05% Proclin 300, 50 μg/ml normal mouse IgG, and 5 g/l polyoxyethylene alkyl ether. After washing the plates, the wells were incubated with 0.5 μg/well of HRP-labeled mAb IU-20 Fab´ (diluted in PBS containing 1% BSA, 0.05% Tween 20, and 0.05% Proclin 300) for 30 min at 4°C. After washing, TMB substrate (Kemen-Tec) was added (50 μl/well). The reaction was stopped after 30 min by adding 50 μl of 2 M sulfuric acid. The optical density (OD) was read at 450 nm.

## A SANDWICH ELISA TO DETECT GPIHBP1–LPL COMPLEXES

96-well ELISA plates were coated with mAb 5D2 (0.5 μg/well), a mAb against human LPL,[25] at 4°C overnight. On the next day, serial dilutions of serum and plasma samples were added to the wells and incubated at 4°C overnight. After washing, the plates were incubated at 4°C for 30 min with HRP-labeled mAb IU-20. Positive controls for GPIHBP1–LPL complex were generated by co-cultivating two populations of HEK-293 cells [one transfected with an expression vector for soluble human GPIHBP1 (462 ng/ml in the medium); and a second transfected with an expression vector for human LPL (4312 ng/mL secreted in medium)].

## PREPARATION OF BLOOD SAMPLES

Blood samples were obtained according to the principles outlined in the Declaration of Helsinki, and the clinical study was approved by the Kobe University Institutional Review Board. Sera from 28 healthy volunteers were obtained at Immuno-Biological Laboratory (Fujioka, Japan). Whole blood was obtained at Kobe University Graduate School of Medicine after written informed consent from 415 Japanese patients with a history of cardiovascular disease that had been treated at the Kobe University Hospital between July 2008 and March 2014. The sera and plasma were immediately separated and stored at –80°C until analysis. We also examined de-identified archived plasma samples from patients with loss-of-function mutations in *GPIHBP1*.[1, 3, 13] Those samples had been sent to UCLA without identifiers;[1] accordingly, studies of those plasma samples were deemed exempt from institutional review board approval.

## A COHORT OF PATIENTS WITH A HISTORY OF CARDIOVASCULAR AND METABOLIC DISEASE

The cohort of 415 patients from the Kobe University Hospital included subjects treated for: (a) coronary artery disease, including stable angina, unstable angina, and both acute and old myocardial infarction; (b) hypertension, defined by a systolic blood pressure >140 mm Hg or a diastolic blood pressure >90 mm Hg; (c) diabetes mellitus, defined by the Japan Diabetes Society as fasting serum glucose >126 mg/dl or a hemoglobin $A1_c$ level >6.5% (National Glycohemoglobin NGSP); and (d) hypercholesterolemia, defined by the Japan Atherosclerosis Society as a serum LDL-cholesterol level >140 mg/dl. The clinical and biomedical characteristics of this cohort of patients are collected in Table 1. 192 out of these 415 patients went on to have a revascularization (coronary reperfusion and angioplasty) for coronary heart disease and were followed up at the Kobe University Hospital for up to 730 days post-revascularization.

### STATISTICAL ANALYSIS

Statistical analyses were conducted with Stat View version 5.0 (SAS Institute). A Spearman correlation coefficient analysis was used to assess associations between measurements. Results are expressed as mean (± SD); $P < 0.05$ was considered significant.

## RESULTS

### PROPERTIES OF TWO NEW MONOCLONAL ANTIBODIES AGAINST HUMAN GPIHBP1

We generated a panel of 17 rat mAbs against human GPIHBP1. We choose two mAbs with high reactivity to human GPIHBP1 by ELISA for detailed characterization. Both mAb IU-79 (IgG2A$\lambda$) and IU-20 (IgG2A$\kappa$) bound strongly and specifically to nonreduced human GPIHBP1 by western blotting (Fig. 1). They recognized full-length GPIHBP1 (both wild-type GPIHBP1 and a mutant GPIHBP1 with a W109S substitution) and a mutant GPIHBP1 lacking the amino-terminal acidic domain. IU-79 had no ability to detect reduced GPIHBP1, whereas IU-20 displayed weak reactivity against reduced GPIHBP1. These data suggested that the two mAbs may have distinct epitopes, both located within the Ly6 domain of GPIHBP1.

### DEVELOPMENT OF AN ELISA FOR HUMAN GPIHBP1

We developed a solid-phase sandwich ELISA for human GPIHBP1 using mAb IU-79 as the capture antibody and horseradish peroxidase (HRP)–labeled mAb IU-20 as the detecting antibody. The working range of this ELISA was 8–500 pg/ml when recombinant human GPIHBP1 was used as a calibrator (Fig. 2A). The analytical limit of quantification for this assay was determined according to guidelines provided by the Clinical & Laboratory Standards Institute. The limit of sensitivity for the ELISA was 3 pg/ml. A dilution test showed that the curve produced by serially diluted serum samples was parallel to the original standard curve, indicating that this assay system specifically determines the concentration of GPIHBP1 in serum (Fig. 2B).

The specificity of our ELISA was also tested by adding mAb IU-79 (during the 1$^{st}$ reaction) or IU-20 (during the 2$^{nd}$ reaction) in molar excess to either a fixed amount of recombinant human GPIHBP1 (Fig. 2C) or to human serum (Fig. 2D). An excess of mAbs IU-79 and IU-20 blocked, in a dose-dependent manner, the ability to detect GPIHBP1, both with recombinant human GPIHBP1 and human serum (Fig. 2C–D). Altogether, our results demonstrate the specificity of the ELISA for human GPIHBP1.

To assess the intra- and inter-assay variation of our ELISA, we established three quality control (QC) samples corresponding to the high, middle, and low regions of the calibration curve. We determined the intra-assay variation by 24 repeated measurements of each QC sample in a plate; we determined the inter-assay variation by assessing each QC sample across 13 different plates. The intra-assay coefficient of variation was 6.9% in the high, 7.6% in the middle, and 7.2% in the low QC samples (see Supplemental Table 1). The inter-assay coefficient of variation was 5.2% in the high, 5.2% in the middle, and 6.4% in the low QC samples (see Supplemental Table 1).

To assess the recovery rate, different concentrations of recombinant human GPIHBP1 were added to samples, and the amount of GPIHBP1 in each sample was measured by ELISA. The recovery rate was determined as the difference between the measured concentration and the theoretical concentration. When plasma samples were “spiked” with recombinant GPIHBP1, the recovery of the spiked GPIHBP1 ranged from 85 to 115% (not shown).

We also investigated the potential of free bilirubin F and C, hemoglobin, triglycerides, and rheumatoid factor to interfere with GPIHBP1 measurements. Bilirubin F and C (up to 200 mg/l) did not affect the precision of the ELISA. Similarly, hemoglobin (up to 5 g/l), triglycerides (up to 1,500 Formazin Turbidity Units), and rheumatoid factor (up to 500 IU/ml) did not significantly alter the precision of the ELISA.

## ASSESSING GPIHBP1 LEVELS

We first measured GPIHBP1 levels in serum samples from healthy volunteers ($n$ = 28; male; 34–60 years-old). Median GPIHBP1 levels in the serum of healthy volunteers was 849 pg/ml (25–75%: 740–1014 pg/ml) (Fig. 3A). As negative controls, we included samples from a subject who was homozygous for a deletion of the entire *GPIHBP1* gene[3] and two subjects who had a GPIHBP1-C89X nonsense mutation.[1] As expected, the GPIHBP1 serum levels in the “negative control samples” (*i.e.*, samples from patients with GPIHBP1 mutations) were very low. Subjects heterozygotes for a GPIHBP1 mutation had intermediate serum GPIHBP1 levels (Fig. 3A). We found no significant difference in GPIHBP1 levels between fasting and postprandial plasma samples (see Supplemental Table 2).

To determine the effect of heparin administration on plasma levels of GPIHBP1, blood samples were drawn from healthy volunteers at 0, 15, and 240 min after an intravenous injection of heparin (30 U/kg), and plasma LPL and GPIHBP1 levels were measured by ELISA. Plasma LPL levels increased sharply after the heparin injection, whereas the plasma levels of GPIHBP1 did not increase (Fig. 3B). Rather, GPIHBP1 levels seemed to decrease (by approximately 10–20%) 15 min after heparin administration, but this apparent decrease is most likely due to the fact that measurements of GPIHBP1 levels with the ELISA tend to be lower in plasma samples (heparin or EDTA) than in serum samples.

Since LPL in tissues is largely bound to GPIHBP1 on endothelial cells,[8] we wanted to determine if an injection of heparin would release GPIHBP1–LPL complexes into the bloodstream. To detect GPIHBP1–LPL complexes, we developed a solid-phase sandwich ELISA in which plates were coated with mAb 5D2 [an LPL specific antibody].[25] The plates were then incubated with serum, EDTA-plasma samples, and post-heparin plasma samples. After washing the plates, LPL-bound GPIHBP1 was detected with HRP-labeled mAb IU-20. We used GPIHBP1–LPL complexes in the medium of HEK-293 cells as positive controls for this ELISA (see *Methods*). Although our assay easily detected GPIHBP1–LPL complexes in the medium of HEK-293 cells (see Supplemental Figure 1), we never detected GPIHBP1–LPL complexes in human serum or plasma.

## GPIHBP1 SERUM LEVELS IN SUBJECTS WITH CARDIOVASCULAR DISEASE

We measured GPIHBP1 levels in 415 de-identified archived serum samples that had been collected from patients who were being treated for cardiovascular disease (coronary heart

disease, hypertension) or metabolic disease (diabetes mellitus, hypercholesterolemia) at the Kobe University Hospital (see *Methods*). In this population, median serum GPIHBP1 levels was 1087 pg/ml (25–75%: 877–1371 pg/ml) (Table 1). We found a modest but significant ($P$ <0.001) positive correlation between GPIHBP1 and LPL levels (not shown). We found a modest inverse correlation between serum GPIHBP1 and triglyceride levels ($P$ = 0.0275) (Fig. 4A).

We examined GPIHBP1 levels in blood samples that were collected from a subset of 192 subjects before they underwent a revascularization procedure for coronary heart disease at the Kobe University Hospital. The median serum GPIHBP1 level in this group of patients was 1097 pg/ml (25–75%: 889–1347 pg/ml) somewhat higher than what we observed in 28 control subjects (849 pg/ml; 25–75%, 740–1014 pg/ml). Fifty-two of out of the 192 subjects had a major adverse cardiac event (MACE) after revascularization during follow-up, while 140 subjects remained event-free. Before revascularization, the median serum GPIHBP1 levels (1203 pg/ml; 25–75%: 998–1611 pg/ml) in the 52 subjects who went on to have a major adverse cardiac event after revascularization were slightly higher ($P$ = 0.0034) than those in the 140 subjects who remained event-free after revascularization (1053 pg/ml; 25–75%: 863–1246 pg/ml) (Fig. 4B). Plasma LPL levels were not statistically different in the two groups of patients.

## DISCUSSIONS

We report the development of a GPIHBP1 ELISA using two GPIHBP1-specific monoclonal antibodies, IU-79 and IU-20. Our GPIHBP1 ELISA is sensitive (detection limit, 3 pg/ml) and linear over a wide range of concentrations (8–500 pg/ml). The sensitivity of our ELISA is similar to that of an earlier ELISA for GPIHBP1.[1, 2] Our GPIHBP1 ELISA allowed us to quantify GPIHBP1 in serum and plasma samples, even when the samples were lipemic. The range of GPIHBP1 levels in serum was broad in a small cohort of healthy subjects (576–1,626 pg/ml) and possibly broader in patients with cardiovascular and/or metabolic disease (392–4,654 pg/ml). We found no differences in GPIHBP1 levels between pre-heparin and post-heparin plasma, or between fasted and postprandial plasma.

Plasma GPIHBP1 levels are in the pg/ml range, whereas LPL levels are much higher (~30–100 ng/ml in pre-heparin plasma).[26, 27] This difference is exaggerated in post-heparin plasma, where LPL levels rise sharply but GPIHBP1 levels remain unchanged. We failed to detect GPIHBP1–LPL complexes in human serum or plasma, indicating that little of the circulating GPIHBP1 was associated with LPL. This finding aligns well with theoretical considerations based on the established binding constant for the GPIHBP1–LPL interaction ($K_D$ = 25 nM; $k_{off}$ = 0.023 $s^{-}$).[16] According to these considerations, only negligible amounts of complexes would be formed and survive in the blood, given the reported plasma levels of GPIHBP1 (0.7 ng/ml or ~0.04 nM) and LPL (68 ng/ml or ~1.4 nM). Although we did not detect GPIHBP1–LPL complexes in human serum or plasma, the possibility of GPIHBP1–LPL complexes in plasma needs to be assessed in the setting of human diseases, for example diseases associated with endothelial cell dysfunction.

A sandwich ELISA for GPIHBP1 is a useful diagnostic tool in the setting of GPIHBP1 deficiency.[1] We had no difficulty in detecting GPIHBP1 in the plasma of a hypertriglyceridemic patient who had a genetic deficiency in *LPL*, whereas GPIHBP1 levels were extremely low in hypertriglyceridemic patients who had loss-of-function mutations in *GPIHBP1* (missense, nonsense, or deletion).[1] In patients with GPIHBP1 deficiency, the plasma LPL levels were also low, reflecting markedly reduced delivery of LPL to the capillary lumen.[1]

An ELISA for GPIHBP1 was the key to discovering that GPIHBP1 autoantibodies can cause chylomicronemia.[1] The presence of GPIHBP1 autoantibodies in a plasma sample interferes with immunoassays for GPIHBP1, making it impossible to detect the endogenous GPIHBP1 in a plasma or serum sample (or even to detect recombinant GPIHBP1 after it has been spiked into the sample).

In our studies, we found a very slight inverse correlation between plasma GPIHBP1 levels and plasma triglyceride levels, and we also found slightly higher GPIHBP1 levels in patients that had undergone revascularization and subsequently had an adverse clinical event. These findings achieved statistical significance, but the clinical significance of our findings is far from being certain. The correlation of GPIHBP1 and triglyceride levels was extremely modest, and there was a very large overlap in GPIHBP1 levels in revascularization patients who had clinical events and those that did not. Additional studies, with larger cohort sizes, will be required to assess the clinical relevance of GPIHBP1 levels in patients with cardiovascular or metabolic disease. At this point, the only proven value of GPIHBP1 levels is for evaluating patients with GPIHBP1 mutations and patients with the GPIHBP1 autoantibody syndrome.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Keywords and Abbreviations

| | |
|---|---|
| **GPIHBP1** | glycosylphosphatidylinositol-anchored highdensity lipoprotein binding protein-1 |
| **TRLs** | triglyceride-rich lipoproteins |
| **LPL** | lipoprotein lipase |
| **mAb** | monoclonal antibody |
| **uPAR** | urokinase-type plasminogen activator receptor |
| **LY6** | lymphocyte antigen 6 |

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## HIGHLIGHTS

- We developed a sensitive sandwich ELISA to measure GPIHBP1 in human plasma or serum.
- The plasma GPIHBP1 levels were very low in patients harboring loss-of-function mutations in *GPIHBP1*.
- A slight trend towards higher serum GPIHBP1 levels was observed in a cohort of patients with coronary heart disease, but this finding needs to be tested in larger patient cohorts.

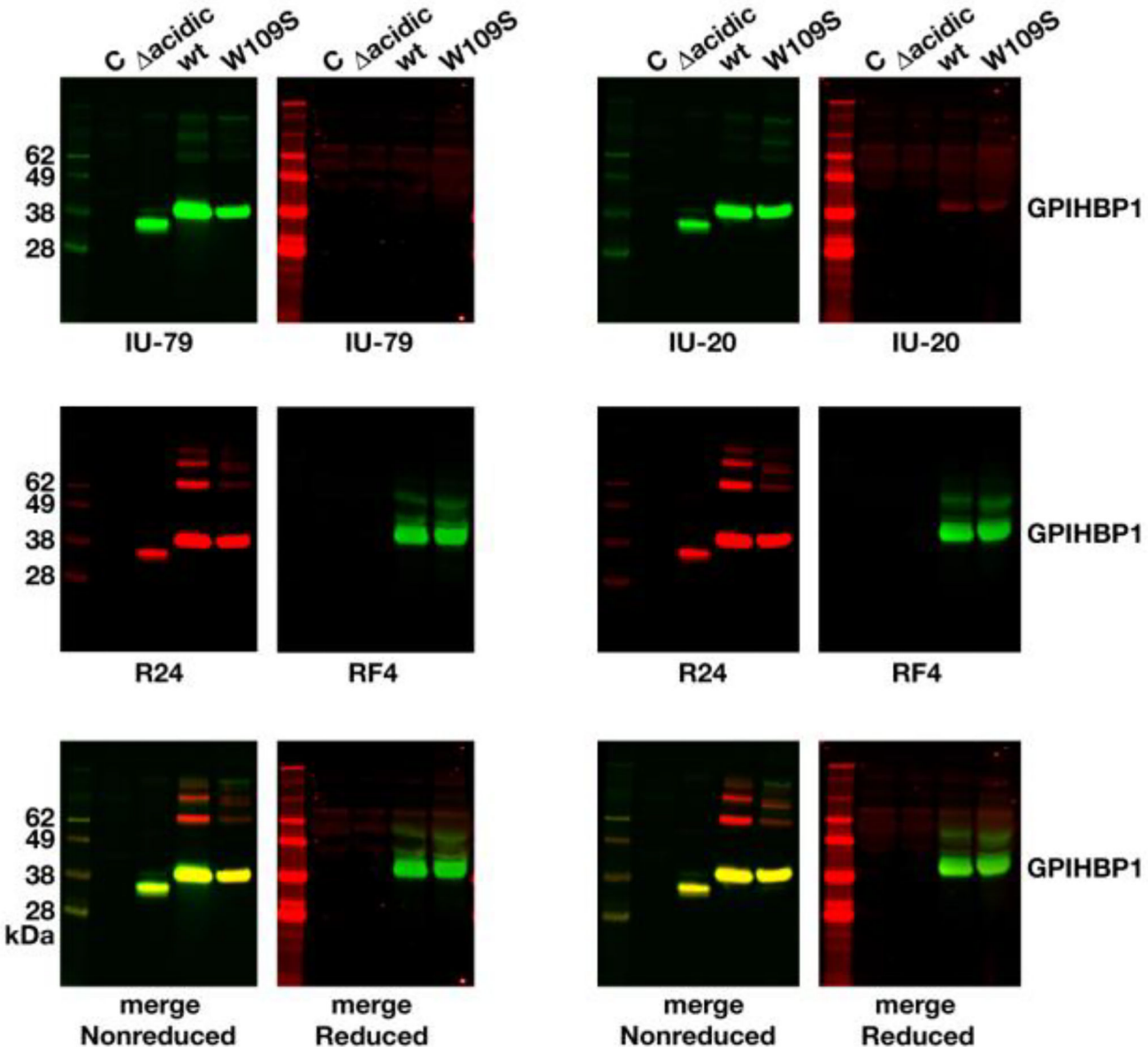


**Fig. 1. mAbs IU-79 and IU-20 bind to the LY6 domain of human GPIHBP1**

uPAR-tagged versions of wild-type GPIHBP1 (wt), GPIHBP1-W109S, and a mutant GPIHBP1 lacking the acidic domain (Δacidic) were produced in *Drosophila* S2 cells, size-fractioned by SDS-PAGE under reducing and nonreducing conditions, and then transferred to a nitrocellulose membrane. Western blots show that both mAbs IU-79 and IU-20 (top row, *green*) bound avidly to nonreduced GPIHBP1, including the mutant GPIHBP1 lacking the acidic domain. Both mAbs also exhibited weak reactivity with GPIHBP1 multimers (found in the setting of GPIHBP1 overexpression in *Drosophila S2* cells). mAb IU-20 displayed a week affinity for reduced GPIHBP1 (top row, *red*), while mAb IU-79 did not react with reduced GPIHBP1. As expected, RF4, a mAb against the acidic domain of human GPIHBP1, did not detect the mutant GPIHBP1 lacking the acidic domain (middle row,

*green*). mAb R24, specific for the uPAR tag, was used as a loading control. C, medium from *Drosophila* S2 cells that do not express GPIHBP1.

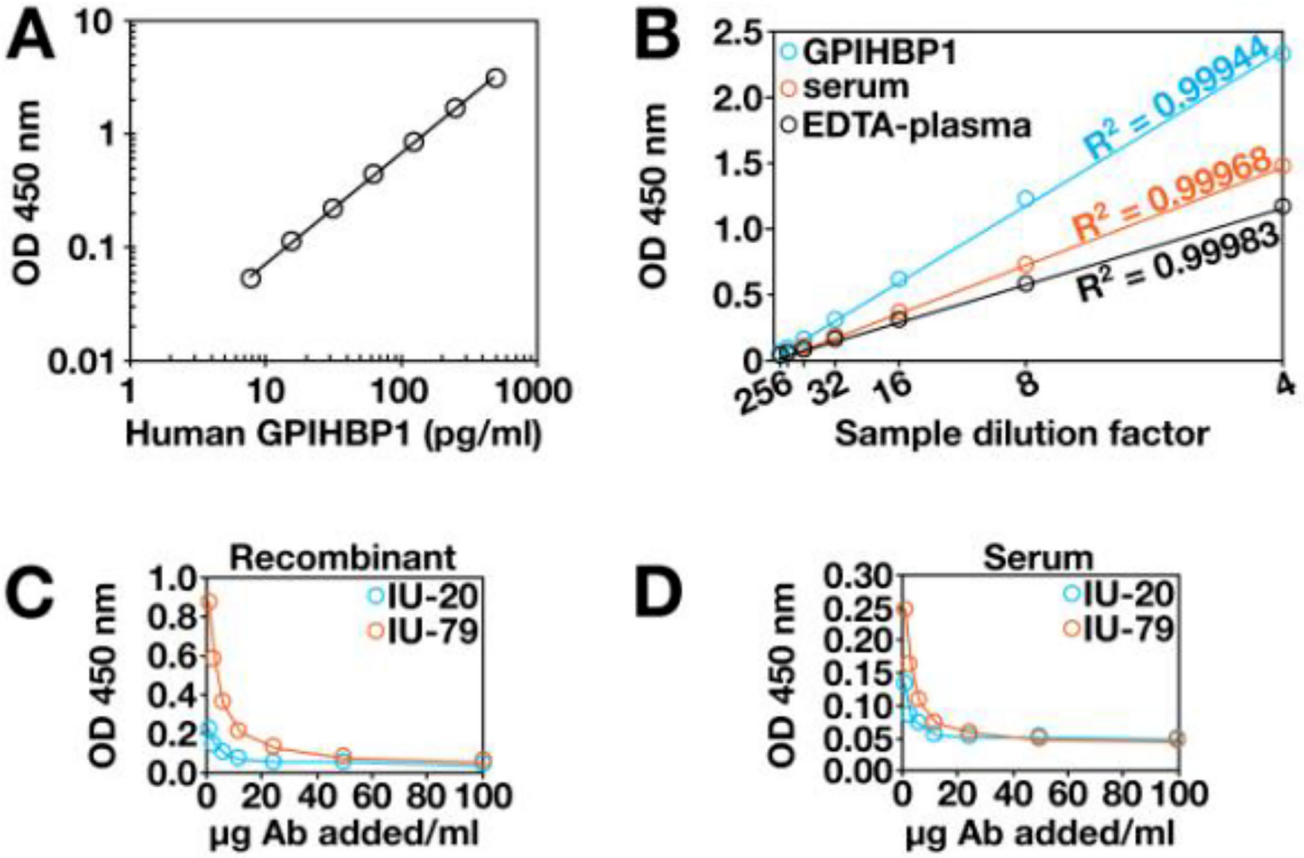


**Fig. 2. Linearity and specificity of the GPIHBP1 ELISA**

(**A**) Log-log plot of a representative standard curve of recombinant human GPIHBP1 ranging from 8–500 pg/ml. (**B**) Plot showing the linearity of the GPIHBP1 ELISA over a wide range (from 1:4 to 1:256) of serial 1:2 dilutions of serum (orange line), EDTA-plasma (black line), and recombinant human GPIHBP1 (blue line). The 1:4 dilution corresponds to 125 pg/ml concentration of recombinant human GPIHBP1. (**C–D**) The specificity of the ELISA was tested by adding mAb IU-79 (orange line) in molar excess to the recombinant human GPIHBP1 (**C**) and to human serum (**D**), and by adding mAb IU-20 (blue line) in molar excess during the incubation with HRP-labeled IU-20 (**C–D**).

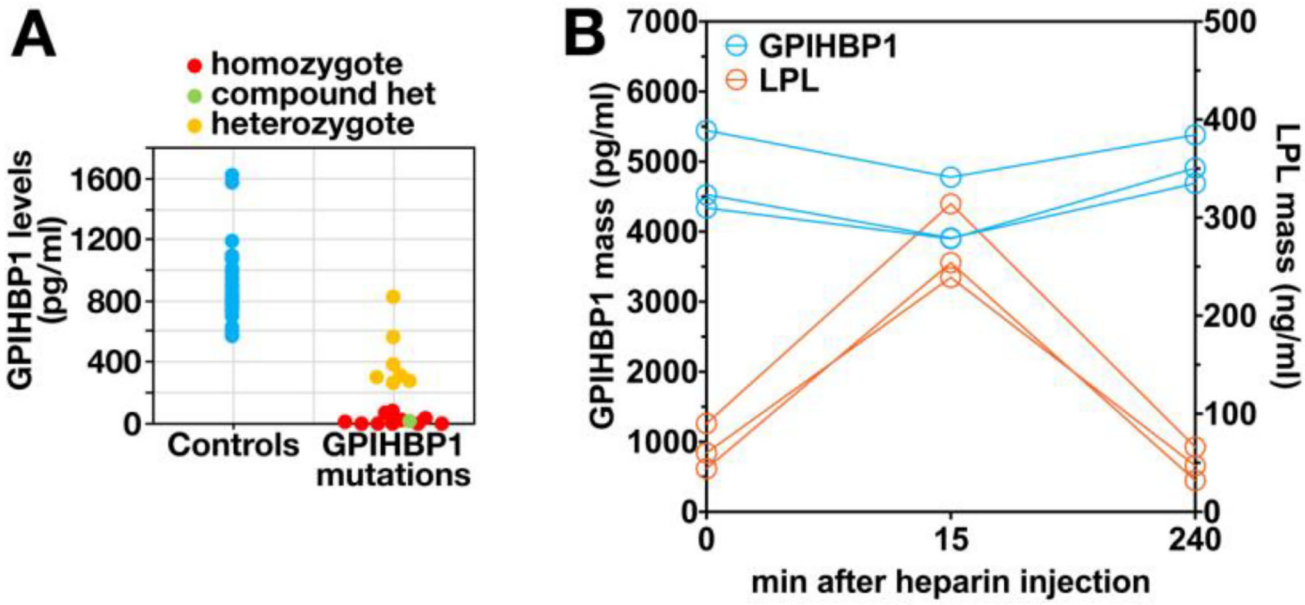


**Fig. 3. Blood GPIHBP1 levels in healthy volunteers**

(**A**) Plot showing the range of GPIHBP1 serum levels in 28 healthy volunteers (males, age 34–60). Subjects with *GPIHBP1* mutations (missense,[1, 2] nonsense,[1, 2] or deletion[3]) were included for comparison. As expected, GPIHBP1 levels in subjects with *GPIHBP1* deficiency were extremely low when compared to healthy volunteers (controls). (**B**) Plot showing plasma GPIHBP1 and LPL levels after an intravenous injection of heparin in three healthy volunteers. Blood was withdrawn at 0, 15, and 240 min after an intravenous injection of heparin (30 U/kg). LPL levels (orange) rose sharply after the heparin injection, while GPIHBP1 levels (blue) did not.

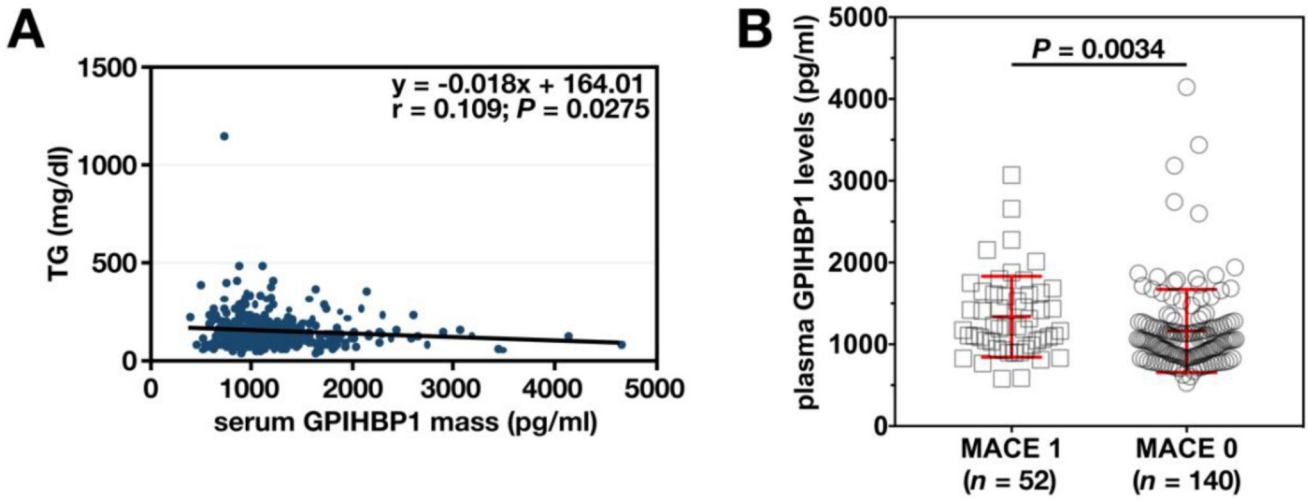


**Fig. 4. Blood GPIHBP1 levels in subjects with a history of cardiovascular or metabolic disease**

(**A**) Plot depicting the relationship between serum triglyceride levels and serum GPIHBP1 levels in 415 patients from Kobe University hospital who had been followed for cardiovascular or metabolic disease. TG, triglycerides. (**B**) Scatter plot showing the distribution of serum GPIHBP1 levels in subjects who had a major adverse cardiac event after revascularization (MACE 1; $n = 52$) and those who remained free of major adverse cardiac events after revascularization (MACE 0; $n = 140$). All blood samples were collected prior to the revascularization procedure. The median serum GPIHBP1 levels in the entire cohort of 192 patients was 1097 pg/ml (25–75%: 889–1347 pg/ml). The median GPIHBP1 serum level (1203 pg/ml) was slightly higher in the 52 patients who had a major adverse event after revascularization than in 140 patients who did not have an adverse event (1053 pg/ml) ($P = 0.0034$).

## Table 1

Clinical and biochemical characteristics of the cohort of 415 patients treated at the Kobe University hospital for a history of cardiovascular or metabolic disease. Values are expressed as mean ± SD or as a percentage (%).

| | **All patients (*n* = 415)** |
|---|---|
| **Age (years)** | 67.9 ± 10.5 |
| **Gender (% male)** | 80.7 |
| **Body mass index (kg/m$^2$)** | 24.6 ± 3.5 |
| **Current smoking** | 17.8 |
| **GPIHBP1 (pg/ml)** | 1,202 ± 514 |
| **LPL (ng/ml)** | 69.0 ± 23.2 |
| **HTGL (ng/ml)** | 52.4 ± 22.4 |
| **EL (ng/ml)** | 261 ± 172 |
| **Triglycerides (mg/dl)** | 142.3 ± 85.6 |
| **LDL-TG (mg/dl)** | 36.9 ± 18.4 |
| **HDL-TG (mg/dl)** | 14.0 ± 9.8 |
| **VLDL-TG (mg/dl)** | 87.1 ± 62.9 |
| **CM-TG (mg/dl)** | 3.6 ± 2.6 |
| **Total cholesterol (mg/dl)** | 165.1 ± 38.2 |
| **LDL-cholesterol (mg/dl)** | 93.4 ± 30.3 |
| **HDL-cholesterol (mg/dl)** | 44.3 ± 13.7 |
| **Hypertension (%)** | 87.7 |
| **Diabetes mellitus (%)** | 52.5 |
| **Dyslipidemia (%)** | 85.1 |

HTGL, hepatic triglyceride lipase; EL, endothelial lipase; TG, triglycerides; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; CM, chylomicron.