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Original Article

Serum Soluble Vascular Endothelial Growth Factor Receptor 1 as a Potential Biomarker of Hepatopulmonary Syndrome

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Abstract

Background and Aims: The results of basic research implicate the vascular endothelial growth factor (VEGF) family as a potential target of hepatopulmonary syndrome (HPS). However, the negative results of anti-angiogenetic therapy in clinical studies have highlighted the need for markers for HPS. Therefore, we aimed to determine whether VEGF family members and their receptors can be potential biomarkers for HPS through clinical and experimental studies. ***Methods:*** Clinically, patients with chronic liver disease from two medical centers were enrolled and examined for HPS. Patients were divided into HPS, intrapulmonary vascular dilation [positive contrast-enhanced echocardiography (CEE) and normal oxygenation] and CEE-negative groups. Baseline information and perioperative clinical data were compared between HPS and non-HPS patients. Serum levels of VEGF family members and their receptors were measured. In parallel, HPS rats were established by common bile duct ligation. Liver, lung and serum samples were collected for the evaluation of pathophysiologic changes, as well as the expression levels of the above factors. ***Results:*** In HPS rats, all VEGF family members and their receptors underwent significant changes; however, only soluble VEGFR1 (sFlt-1) and the sFlt-1/ placental growth factor (PLGF) ratio were changed in almost the same manner as those in HPS patients. Furthermore, through feature selection and internal and external validation, sFlt-1 and the sFlt-1/PLGF ratio were identified as the most important variables to distinguish HPS from non-HPS patients. ***Conclusions:*** Our results from animal and human studies indicate that sFlt-1 and the sFlt-1/PLGF ratio in serum are potential markers for HPS.

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Introduction

Hepatopulmonary syndrome (HPS) increases the risk of postoperative mortality and complications[1,2] and decreases quality of life,[2] with a prevalence ranging from 5% to 30%.[3] The diagnosis of HPS requires a basis of chronic liver disease (CLD), the presence of intrapulmonary vascular dilation (IPVD) determined by contrast-enhanced echocardiography (CEE), and abnormal arterial oxygenation determined by arterial blood gas (ABG) analysis.[3] CEE positivity with normal oxygenation is usually called subclinical HPS (IPVD), and CEE positivity with abnormal oxygenation is called clinical HPS.[4] IPVD seems to have a limited impact on survival or other outcomes of patients with CLD.[1,5] As the only curable treatment for HPS is liver transplantation,[6] preventing CLD patients from developing HPS is of great importance. However, only 0.45% of CLD patients are diagnosed with HPS, and the diagnostic accuracy is only 22.5%,[7] highlighting the need for specific markers in this field.

Recently, pathological pulmonary angiogenesis (PPA) has been widely accepted as one of the key mechanisms for the development of HPS.[8–10] Vascular endothelial growth factor (VEGF) family members and their receptors are also closely related to PPA in HPS.[8–11] Placental growth factor (PLGF), one of the typical members of the VEGF family, is the most promising target for HPS, and antiangiogenic therapy (sorafenib and anti-PLGF antibodies) has been shown to significantly improve abnormal oxygenation and intrapulmonary shunts in experimental animals.[8,11] Sorafenib was

Keywords: Chronic liver disease; Hepatopulmonary syndrome; Placental growth factor; Pathological pulmonary angiogenesis; sFlt-1/PLGF ratio.

Abbreviations: ABG, arterial blood gas; CBDL, common bile duct ligation; CEE, contrast-enhanced echocardiography; CLD, chronic liver disease; HPS, hepatopulmonary syndrome; IDI, integrated discrimination improvement; IPVD, intrapulmonary vascular dilation; MVD, microvessel density; NPV, negative predicted value; PLGF, placental growth factor; PPA, pathological pulmonary angiogenesis; PPV, positive predicted value; sFlt-1, soluble vascular endothelial growth factor 1; VEGF, vascular endothelial growth factor.

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shown to improve the $P_{(A-a)}$ O_2, but the effect did not reach significance, and it had a negative influence on patient quality of life.[12] Despite the poor results, there is still hope for PPA in the HPS field.[12,13]

The negative results of the aforementioned HPS translational studies indicate that the core issue in this field is the difference between the experimental models and patients, as well as the unique mechanism of HPS angiogenesis. First, common bile duct ligation (CBDL) is widely accepted as a typical HPS experimental model with high replicability, and it simulates the main pathological changes of HPS, that is, progressive hepatic injury accompanied by gas-exchange abnormalities and intrapulmonary vasodilatation.[10,14] In contrast to the high success rate of the HPS experimental model, the incidence of HPS in CLD patients is relatively low.[6] This may partially explain why some markers or targets that are effective in experimental models are not appropriate for HPS patients.[14] Second, the levels of VEGF family members and their receptors in patients with CLD are usually elevated,[15,16] but only some patients will develop HPS. This suggests that PPA may be different from angiogenesis in other situations, and specific markers or targets for HPS are needed. Therefore, we aimed to investigate whether changes in VEGF family members and their receptors are the same in experimental animals and HPS patients, and to explore whether VEGF family members and their receptors can be potential markers for HPS.

Methods

Animal model and sample collection

Male Sprague-Dawley rats (200–220 g, Army Medical University, Chongqing, China) were used in this study. An experimental HPS rat model was successfully established by CBDL as previously described.[9] Rats were housed in standard cages under standard laboratory conditions and randomly divided into three groups. All rats fasted for 12 h preoperatively. The groups were a sham group (opening the abdomen and separating the common bile duct without ligation), a CBDL 3-week group and CBDL 5-week group sacrificed 3 and 5 weeks after CBDL, Supplementary Figure 1. The ethics committee of the Army Medical University for animal care approved all protocols (AMUWEC20201230).

At the end of the experiment, the rats were sacrificed, and arterial blood from the abdominal aorta was collected. Part of the arterial blood sample was sent for ABG analysis by a standard blood gas analyzer (Radiometer ABL800 FLEX, Copenhagen, Denmark) within 15 m. The rest of the blood sample was centrifuged at 3,000 r/min for 10 m at 4°C, and then the serum supernatant was collected and stored at −80°C. After perfusion with heparinized saline, the rat livers and lungs were dissected, weighed, and photographed. Some of the tissues were used for immunohistochemistry and immunofluorescence, and others were stored at −80°C.

Pathological examination of rat liver and lung

After the tissues were fixed in 10% formalin for 24 h, they were dehydrated, embedded in paraffin, cut into 4 μm thick sections, and stained with hematoxylin and eosin (lung) or Sirius red (liver). Microphotographs of the specimens were obtained with a light microscope (BX51-PMS; Olympus, Tokyo, Japan). The degree of lung injury was evaluated in HE-stained lung sections, and the degree of liver fibrosis and the METAVIR score were evaluated in Sirius red-stained liver sections. Five randomly selected fields of each section from three different rats in each group were analyzed by two researchers blinded to the group allocation.

Immunofluorescence

The assessment of angiogenesis in the rat liver and lung was conducted on three different rats from each group. Paraffin-embedded lung and liver sections (4 μm) were dewaxed and hydrated, and antigen was repaired by EDTA. After blocking with 10% bovine serum albumin for 1 h at room temperature, the sections were incubated with anti-CD31 (ab119339, 1:100; Abcam, Cambridge, UK) antibody overnight. The next day, after washing with PBS, sections were incubated with Cy3-conjugated goat anti-mouse IgG (H+L) (115165003, 1:500; Jackson ImmunoResearch, West Grove, PA, USA) and anti-mouse secondary antibody for 1 h at room temperature. After washing with PBS three times, sections were fixed with 4′,6-diamino-2-phenylindole (DAPI) (ab104139; Abcam, Cambridge, UK) for 10 m. For each section, five randomly selected fields were observed with a fluorescence microscope (Pannoramic DESK, P-MIDI, P250; 3DHISTECH Inc, Budapest, Hungary), and the microvessel density (MVD) was calculated as the number of CD31 positive cells by Image-Pro Plus software (version 6.0; Media Cybernetics Inc, Rockville, MD, USA).

Protein extraction and quantification

Tissues were weighed to 100 mg, and lysed in RIPA buffer (P0013B; Beyotime, Beijing, China) containing 1% protease inhibitor PMSF (ST506; Beyotime, Beijing, China). The lance was blown, mixed and transferred to a 1.5 mL centrifuge tube to separate for 30 m and then centrifuged at 4°C for 15 m (13,000 r/min), and the transferred supernatant contained the extracted protein. The quantity of protein was determined by a bicinchoninic acid protein quantitative assay (23225; Thermo Scientific, Waltham, MA, USA). Finally, the concentration of all samples was adjusted to 2.5 mg/mL for ELISA.

Patients and data collection

Patients were enrolled from two centers, the First Affiliated Hospital of Army Medical University (center 1) and Sichuan Province People's Hospital of Sichuan Academy of Medical Sciences (center 2). Data for training and internal validation were collected from center 1 from October 17, 2019 to February 9, 2021. Data for external validation were collected from center 2 from September 15, 2021 to October 29, 2021. The research protocol was approved by the ethics committees of center 1 [(No: 2017(35), KY2019107)] and center 2 [No. 2021(471)], and the principal investigators were Bin Yi and Peng Li. This study was conducted according to the ethical guidelines of the Declaration of Helsinki. All participants provided written informed consent and agreed to the publication of their anonymous information.

The inclusion criteria were: (1) patients with CLD; (2) patients who were 18–65 years of age; (3) patients who underwent abdominal surgery with American Society of Anesthesiology level 2–3; (4) patients with no primary cardiopulmonary disease (ventricular septal defect, emphysema, asthma, etc.), and (5) patients who agreed to provide blood samples. The exclusion criteria were (1) severe heart, lung, and kidney disease preoperatively; and (2) forced expiratory volume (FEV1) or forced vital capacity (FVC) <70% predicted value, or FEV1/FVC<0.7. CEE and ABG analysis was performed for the diagnosis of HPS as previously described.[1] Patients were divided into three groups, CEE negative, IPVD, and HPS.

Approximately 5 mL of whole blood was obtained from

each patient, centrifuged for 10 m at 4°C at 3,000 r/min (5804R; Eppendorf, Hamburg, Germany), and stored at −80°C. Preoperative laboratory results nearest to the surgical day were collected, including AST, ALT, and albumin, among others. Postoperative extubation time, oxygen absorption time after extubation, time in the post anesthesia care unit (PACU) and postoperative pulmonary complications (PPCs) were collected without intervention.

ELISA

ELISA was used to determine the levels of VEGF family members and their receptors in rats and humans. The ELISA kits for rats were VEGF (JL21369), VEGF receptor 1(VEGFR1, JL21373), VEGFR2 (JL21374), PLGF (JL11559) and soluble VEGFR1 (sFlt-1, JL48077). The ELISA kits for humans were VEGF (JL18341), VEGFR1 (JL15314), VEGFR2 (JL46251), PLGF(JL23762) and sFlt-1 (JL13928). All ELISA kits were purchased from Shanghai Jianglai Biological Technology, China. Rat and human serum samples were diluted five times before the assay. All procedures followed the kit manufacturer's instructions. The intensity of the color was measured at an absorbance of 450 nm with a Rayto Reader (RT-6100; Rayto, Shenzhen, Guangdong, China).

Feature selection, model construction and evaluation based on machine learning

The statistical analysis was conducted on the R studio platform (version 1.4.1717). To investigate the model performance of different inputs and the importance of variables to the diagnosis or early warning of HPS, Boruta[17] and random forest (RF)[18] algorithms were applied. After data preprocessing, the data from center 1 were randomly divided into training (70%) and test (30%) datasets, while data from center 2 were used for external validation (Fig. 1A). Feature selection was conducted on the training dataset. Four different inputs were included in the analysis. Tenfold cross validation was completely repeated three times during training. Model fitting was completed by RF with the best parameter determined by cross validation. Model performance was internally and externally validated on the test and validation datasets, including AUCROC, sensitivity, specificity, positive predicted value (PPV), negative predicted value (NPV), balanced accuracy, Brier score (calibration) and integrated discrimination improvement (IDI) as shown in Supplementary File 1.

Statistical analysis

Descriptive statistics were reported means±SD or medians (interquartile range, IQR) depending to the data distribution. ELISA results outside of the ranges of [Q3+(Q3-Q1)×1.5] and [Q1-(Q3-Q1)×1.5] were determined to be outliers and were removed. Between-group comparisons were made with unpaired *t*-tests or Mann-Whitney *U* tests when appropriate. For quantitative variables, chi-square tests or Fisher's tests were used for categorical variables. Comparisons among groups were made by one-way analysis of variance or the Kruskal-Wallis H test, when appropriate. Least significance difference or Dunnett's T3 was used for pairwise comparisons based on the presence of homoscedasticity. All statistical tests were two-sided, and $p<0.05$ indicated statistical significance. The statistical analysis was performed with SPSS software for Windows, V.23.0 (IBM Corp., Armonk, NY, USA) and GraphPad PRISM (version 8.00; GraphPad Software, San Diego, California, USA).

This is an observational, consecutively enrolled study, and no existing human data could be referred to calculate the sample size. We used Cohen's f calculated by online tools (https://www.psychometrica.de/effect_size.html#transform) to measure the effect size. The effect size for the difference in serum sFlt-1 level and sFlt-1/PLGF was 0.556 and 0.497, which is greater than 0.4 indicating the sample size in the current study can provide relatively reliable results.

Results

Changes in VEGF family members and their receptors in various tissues of HPS rats

CBDL is a widely accepted model for HPS basic research, and the CBDL 5w rats were identified as HPS rats. Compared with sham rats, the liver developed cirrhosis as evidenced by Sirius red staining and increased METAVIR scores over time after CBDL (Fig. 2A, B and Supplementary Table 1). The pathophysiological changes in HPS lungs manifested as lung surface necrotic lesions and petechiae, increased lung injury scores, decreased PaO_2 and increased $P_{(A-a)}O_2$ (Fig. 2A–C). As shown in Figure 2D, the MVD of the liver and lung increased significantly three weeks after CBDL. In addition, there were also statistically significant differences in liver and lung MVD between CBDL 3w and HPS rats.

As shown in Figure 2E, compared with those in the sham group, the levels of VEGF, VEGFR1, VEGFR2, PLGF and sFlt-1 in the serum, liver and lung were significantly increased in CBDL 3w and 5w groups. However, changes in PLGF, sFlt-1 and the sFlt-1/PLGF ratio were not completely consistent in the serum, liver and lung between the CBDL 3w and HPS groups. The levels of sFlt-1 and the sFlt-1/PLGF in the liver and lung were significantly decreased between the CBDL 3w and HPS groups, however, which were not significant in the serum. In contrast, changes in PLGF rather than sFlt-1 and the sFlt-1/PLGF ratio in serum were significant between the CBDL 3w and HPS groups. Interestingly, despite the increase in sFlt-1 and PLGF in the HPS group, the sFlt-1/PLGF ratio in the liver and lung of the HPS group was significantly decreased, and even lower than that in the sham group, thereby potentially revealing a key role of the signal imbalance between pro- and anti-angiogenetic factors in this process. Furthermore, referring to the sham group, the trend of the sFlt-1/PLGF ratio in serum (increased) was opposite to that in the liver and lung (decreased) in the HPS group.

Baseline information and postoperative recovery in human data

In total, 105 patients from center 1 and 27 patients from center 2 were analyzed, including 41 and 7 HPS patients, 31 and 4 IPVD patients, and 33 and 16 CEE-negative patients, respectively (Supplementary Fig. 2). Additionally, we also included six healthy volunteers without liver disease (Supplementary Table 2). Other than PaO_2 and $P_{(A-a)}O_2$, there were almost no other significant differences in basic information between HPS and non-HPS patients (combination of CEE-negative and IPVD patients) (Table 1 and Supplementary Table 3). Compared with non-HPS patients, HPS patients stayed statistically longer in the PACU and had a higher incidence of PPCs and pleural effusions (PPCs: 73.2% vs. 53.1%, $p=0.040$; pleural effusions: 73.2% vs. 51.6%, $p=0.027$) (Table 1).

Changes in serum levels of the VEGF family members and their receptors in patients

As shown in Figure 3, except for VEGFR2, statistically significant differences were found in the serum levels of the VEGF

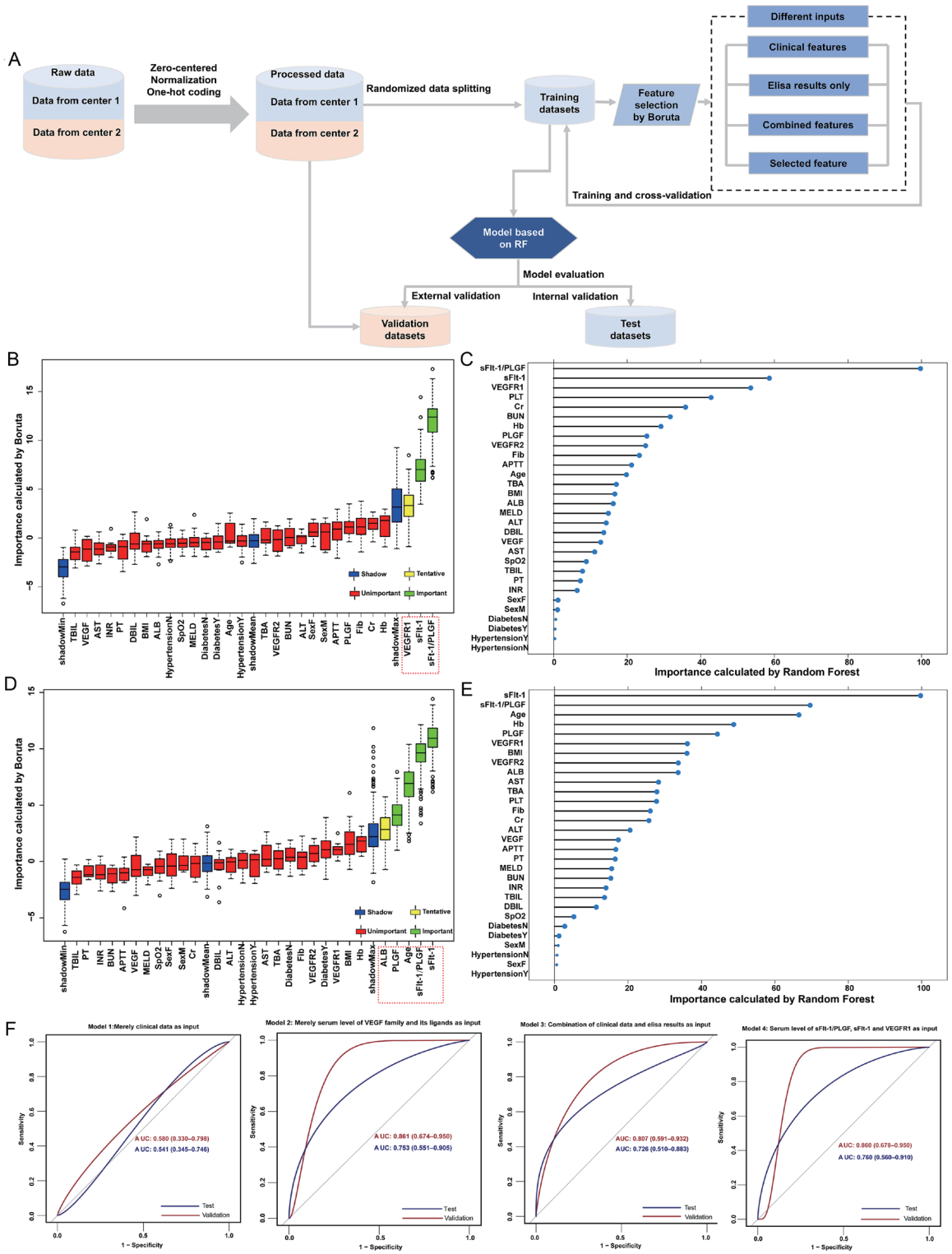

Fig. 1. Machine learning procedure, feature selection results and model performance based on four different inputs. (A) Machine learning procedure in this study. The results of feature selection by Boruta for distinguishing HPS and non-HPS (B) or HPS, CEE negative and IPVD (D); The rectangular box with the red dotted line is for the selected variable for model construction in the current study. The results of feature selection by RF for distinguishing HPS and non-HPS (C) or HPS, CEE-negative and IPVD (E). (F) The ROC curves on the test on the test (internal) and validation (external) based on four different inputs. The AUCROC was smoothed so that a bit different from Table 2. APTT, activated coagulation time of whole blood; BUN, blood urea nitrogen; Cr, creatinine; DBIL, direct bilirubin; Fib, fibrinogen; Hb, hemoglobin; INR, international normalized ratio; MELD, model of end-stage liver disease; PLGF, placental growth factor; PLT, platelet; PT, prothrombin time; sFlt-1, soluble vascular endothelial growth factor 1; SpO_2, pulse oxygen saturation; TBA, total bile acid; TBIL, total bilirubin; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

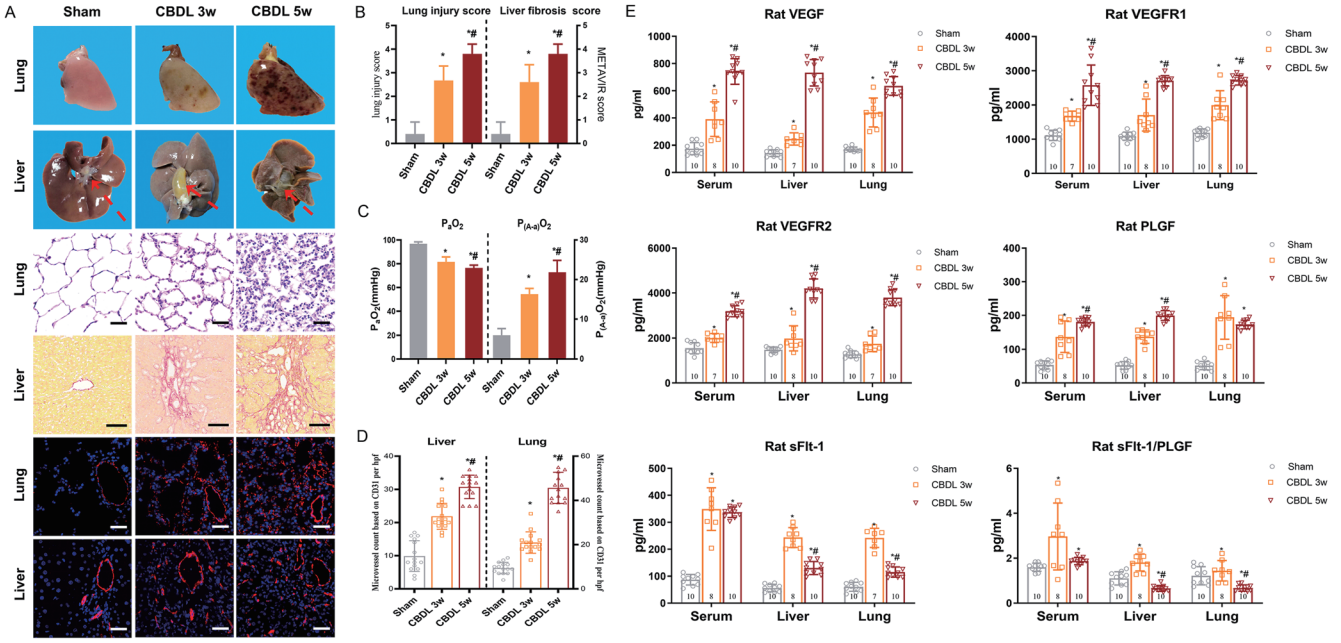

Fig. 2. Pathophysiologic changes and levels of the VEGF family members and their receptors after CBDL. (A) The pathological changes and angiogenesis of the liver and lung after CBDL. (B) The lung injury score (based on HE) and level of liver fibrosis (based on Sirius red) after CBDL. (C) Changes in PaO_2 and $P_{(A-a)}O_2$ after CBDL by ABG analysis. (D) MVD of the liver and lung after CBDL. (E) Expression levels of VEGF, VEGFR1, VEGFR2, PLGF, sFlt-1 and the sFlt-1/PLGF ratio in rat serum, liver, and lung (shown on the *X*-axis) after CBDL. Data are means±SD, and the sample size is shown at the bottom of the box. Scale bar=50 μm. Red arrows indicate the common bile duct to show the effects of our surgery. *Compared with the sham group, $p<0.05$, #Compared with the CBDL 3w group, $p<0.05$. CBDL, common bile duct ligation; HE, hematoxylin; MVD, microvascular density; PLGF, placental growth factor; sFlt-1, soluble vascular endothelial growth factor 1; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

family members and their receptors among CEE-negative, IPVD and HPS patients. However, only the levels of sFlt-1 and the sFlt-1/PLGF ratio significantly changed for different HPS statuses. Interestingly, the level of sFlt-1 was initially increased in IPVD patients and decreased in HPS patients (CEE-negative vs. IPVD vs. HPS: 277.4 pg/mL vs. 330.0 pg/mL vs. 244.0 pg/mL, $p<0.001$). The sFlt-1/PLGF ratio in HPS patients was significantly lower than that in CEE-negative and IPVD patients (CEE-negative vs. IPVD vs. HPS: 6.22 vs. 7.4 vs. 5.2, $p<0.001$).

Inconsistent changes in levels of the VEGF family members and their receptors between patients and rats

The levels of VEGF, VEGFR1, VEGFR2, PLGF and sFlt-1 in HPS patients and rats were significantly increased compared with those in healthy controls and sham rats (Fig. 4). The levels of VEGF, VEGFR1 and VEGFR2 were significantly increased in HPS rats compared with sham and CBDL 3w rats; however, these results were inconsistent with patient data. Interestingly, the fold change in the sFlt-1/PLGF ratio in HPS patients was more similar to that in the liver and lung of HPS rats than that in serum (Fig. 4). In summary, the changes in the VEGF family members and their receptors in HPS patients were quite different from those in experimental rats, while the changes in sFlt-1 and the sFlt-1/PLGF ratio were similar to those in rat liver and lung.

sFlt-1 and the sFlt-1/PLGF ratio as the most important variables for HPS

The inconsistency of the VEGF family members and their receptors between patients and rats allowed us to further use clinical variables and ELISA results (Supplementary Table 4) to study whether the VEGF family members and their receptors might contribute to the diagnosis of HPS. sFlt-1 and the sFlt-1/PLGF ratio were the two most important variables for discriminating between HPS and non-HPS patients (Fig. 1B, C and Supplementary Tables 5 and 6) and between clinical and subclinical HPS patients (Fig. 1D, E and Supplementary Tables 7 and 8). Model performance for discriminating between HPS and non-HPS patients with different inputs are shown in Table 2. Taking the results of internal and external validation into consideration, the discrimination ability and calibration of the model with clinical variables as input was much poorer than that of the other three models, indicating the importance of specific biomarkers. ELISA results as input yielded the best model performance (AUCROC larger than 0.75, sensitivity and specificity higher than 70%, and a negative IDI when comparing the improvement of other models with the ELISA result model), but the cost was higher. When using the variables selected by Boruta (sFlt-1, the sFlt-1/PLGF ratio and VEGFR1) as input, the model performance was similar to the ELISA result model (IDI on the test dataset: 0.9%, $p=0.870$; IDI on the validation dataset: −2.0%, $p=0.666$) but with fewer variables and costs (Table 2 and Fig. 1F). The analysis strongly supported that sFlt-1 and the sFlt-1/PLGF ratio were promising biomarkers for the diagnosis and early warning of HPS.

Discussion

This study had two main novel findings. First, changes in sFlt-1 and the sFlt-1/PLGF ratio were significant in patients and experimental rats and are potential biomarkers for HPS, with further validation. Second, PPA was indeed one of the main mechanisms controlling HPS development in both pa-

Table 1. Comparisons of baseline information and postoperative recovery in HPS and non-HPS patients

	Non-HPS, *n*=64			HPS, *n*=41	Test statistics	*p*
	CEE neg, *n*=33	IPVD, *n*=31	In total, *n*=64			
Baseline information						
Age in years	54.8±11.5	47.7±10.6	51.4±11.5	52.7±8.2	−0.665	0.508
BMI in kg/m^2	24.3±3.2	22.8±2.4	23. 6±2.9	23.9±3.3	−0.494	0.622
Sex as M/F	26/7	29/2	55/9	30/11	2.642	0.104
Hypertension, *n* (%)	6 (18.2%)	3 (9.7%)	9 (14.1%)	5 (12.2%)	0.075	0.784
Cirrhosis, *n* (%)	9 (27.3%)	10 (32.2%)	19 (29.7%)	10 (24.4%)	0.351	0.544
Portal hypertension, *n* (%)	8 (24.2%)	5 (16.1%)	13 (20.3%)	7 (17.0%)	0.170	0.680
Diabetes, *n* (%)	7 (21.2%)	1 (3.2%)	8 (12.5%)	3 (7.3%)	0.716	0.398
Nephropathy, *n* (%)	1 (3.0%)	2 (6.5%)	3 (4.7%)	5 (12.2%)	2.001	0.257
Drinking, *n* (%)	11 (33.3%)	14 (45.2%)	25 (39.1%)	16 (39.0%)	<0.001	0.997
Smoking, *n* (%)	11 (33.3%)	12 (38.7%)	23 (35.9%)	19 (46.3%)	1.127	0.288
SpO_2, %	97.0 (96.0–98.0)	98.00 (96.0–98.0)	97.5 (96.0–98.0)	97.0 (96.0–98.0)	−1.366	0.172
PaO_2 in mmHg	87.7±6.6	93.1±8.2	90.3±7.8	80.3±5.8*	7.060	<0.001
$P_{(A-a)}O_2$ in mmHg	12.7±4.9	9.4±3.8	11.1±4.7	22.6±5.2*	11.705	<0.001
MELD	3.4 (1.6–6.6)	5.1 (2.7–6.6)	4.4 (2.4–6.6)	3.7 (1.3–5.5)	−1.071	0.284
TBA in μmol/L	5.8 (2.9–14.1)	5.0 (2.2–15.2)	5.7 (2.8–14.3)	7.3 (3.7–14.0)	1.077	0.281
TBIL in μmol/L	15.7 (12.5–20.9)	15.0 (12.5–20.8)	15.5 (12.6–20.6)	15.3 (11.4–22.5)	−0.177	0.859
DBIL in μmol/L	3.0 (2.5–4.3)	2.9 (2.3–5.3)	3.0 (2.4–4.8)	3.4 (2.4–5.5)	0.683	0.494
Albumin in g/L	40.3 (39.1–42.6)	40.8 (36.6–45.2)	40.4 (38.0–43.4)	40.4 (37.5–43.3)	−0.526	0.599
AST in U/L	28.2 (22.2–36.7)	33.9 (27.0–59.7)	30.4 (23.0–46.5)	31.1 (24.4–53.2)	0.621	0.535
ALT in U/L	28.8 (17.5–39.2)	34.8 (21.5–54.0)	31.4 (20.6–46.0)	29.0 (20.8–51.7)	0.204	0.839
BUN in μmol/L	5.2±1.4	5.4±1.3	5.3±1.4	5.1±1.4	0.784	0.435
Creatinine in μmol/L	69.8±13.6	77.1±11.1	73.3±12.9	68.9±14.8	1.635	0.105
Hemoglobin in g/L	146.0 (129.0–160.0)	147.0 (138.0–154.0)	146.0 (133.0–157.0)	137.0 (121.5–152.0)	−1.896	0.058
Platelet as 10^9/L	165.4±80.2	147.0±69.4	156.5±75.1	133.7±78.2	1.490	0.139
APTT in s	28.0 (26.3–30.2)	28.3 (26.7–29.4)	28.3(26.3–30.0)	27.8 (26.9–29.2)	−0.187	0.851
Prothrombin time in s	11.4 (10.8–11.9)	11.7(10.9–12.5)	11.4 (10.8–11.9)	11.6 (11.0–12.3)	0.871	0.384
Fibrinogen in g/L	2.5 (2.2–3.2)	2.4 (2.0–3.4)	2.5 (2.1–3.2)	2.3 (1.9–2.6)	−1.636	0.102
INR	1.0 (0.9–1.0)	1.0 (0.9–1.1)	1.0 (0.9–1.0)	1.0 (1.0–1.1)	0.835	0.404
Postoperative recovery						
Post-operative extubation time in min	34.0 (15.0–48.0)	33.0 (17.0–50.0)	33.5 (16.5–47.3)	37.0 (19.5–69.0)	1.469	0.142

(continued)

Table 1. *(continued)*

	Non-HPS, *n*=64			HPS, *n*=41	Test statistics	*p*
	CEE neg, *n*=33	IPVD, *n*=31	In total, *n*=64			
Staying time of PACU in min	86.0 (53.0–114.0)	91.0 (68.0–110.0)	88.0 (68.0–113.0)	98.0 (84.5–132.5)*	2.344	0.019
Oxygen absorption time after extubation in min	23.0 (21.0–39.0)	20.0 (13.0–32.5)	23.0 (15.0–34.8)	24.0 (17.0–32.0)	0.211	0.833
Length of stay in days	15.0 (10.0–18.0)	17.0 (13.5–20.0)	16.0 (11.0–18.8)	17.0 (13.0–20. 0)	1.114	0.265
Total costs in yuan	55,259.29±20,988.93	65,750.58±15,398.14	60,303.18±19,083.48	57,854.98±13,669.80	0.714	0.477
PPCs, *n* (%)	18 (54.5%)	16 (51.6%)	34 (53.1%)	30(73.2%) *	4.219	0.040
Pneumonia, *n* (%)	9 (27.2%)	7 (22.6%)	16 (25.0%)	14 (34.1%)	1.024	0.311
Pulmonary atelectasis, *n* (%)	8 (24.2%)	7 (22.6%)	15 (23.4%)	8 (19.5%)	0.255	0.635
Pleural effusions, *n* (%)	17 (51.5%)	16 (51.6%)	33 (51.6%)	30 (73.2%)	4.862	0.027

Mean±standard deviation presented for normally distributed continuous variables, whereas median (IQR) was given to those with non-normally distributed continuous variable. Unless otherwise stated, n is as indicated in the column headings. Additional explanation: When analyzing the first five variables (post-operative extubation time, staying time of PACU, oxygen absorption time after extubation, hospitalization days, hospital costs), we excluded patients underwent liver transplantation (n= 11), so that 94 patients were analyzed. However, due to lacking data of post-operative extubation time, staying time of PACU, oxygen absorption time after extubation in 5 cases, only 89 cases were analyzed for the above three variables (Non-HPS: n=52, HPS: n=37). *, compared with non-HPS, p<0.05. APTT, activated coagulation time of whole blood; BUN, blood urea nitrogen; DBIL, direct bilirubin; INR, international normalized ratio; MELD, model of end-stage liver disease; PaO_2, arterial oxygen partial pressure; $P_{(A-a)}O_2$, alveolar-arterial oxygen difference; PACU, Post-anesthesia care unit; PPCs, postoperative pulmonary complications; TBA, total bile acid; TBIL, total bilirubin.

tients and animals; however, changes in the VEGF family members and their receptors were partially inconsistent between them.

VEGF-A (usually called VEGF) is a trophic factor for healthy blood vessels, but in liver cirrhosis and cancers, it is also overexpressed both in patients and experimental animals.[19] PLGF has key roles in pathological angiogenesis and is a promising target for pathological angiogenesis.[11,15] VEGF can bind to both VEGFR1 and VEGFR2, while PLGF can only bind to VEGFR1. VEGFR2 is responsible for inducing angiogenesis and increasing vascular permeability,[20] while VEGFR1 acts as a decoy receptor to control blood vessel growth and morphogenesis.[21] sFlt-1 is an endogenous anti-angiogenetic factor that can bind to VEGF and PLGF to prevent membrane receptor activation, and its dysregulation has been associated with different pathological processes. For example, in patients with sepsis and cirrhosis, elevated serum sFlt-1 levels have been found to be correlated with worse outcomes.[22] Moreover, abnormal sFlt-1/PLGF or sFlt-1/VEGF ratios were also correlated with the prognostic factors of malignant tumors.[19,23]

As with previously published works,[8,10,11] our results showed that as MVD increased in the liver and lung, VEGF, VEGFR2, VEGFR1 and PLGF in the serum, liver and lung increased. The levels of sFlt-1 in serum, liver and lung were significantly increased after CBDL; however, compared with the CBDL 3w group, HPS rats presented a slight reduction in these levels, which is consistent with a previous report (Fig. 1E).[11] Strikingly, we revealed for the first time that along with the increased MVD, the sFlt-1/PLGF ratio in the liver and lung of HPS rats was decreased. Serum levels of VEGF, sFlt-1 and PLGF have been reported to be elevated in patients with cirrhosis[15,16] and pulmonary hypertension,[24,25] but there were no specific data for HPS. The factors that exhibited significantly changed levels in CBDL rats but not in humans may be related to the difference between the high success rate of the HPS model and the relatively low incidence of HPS in patients. Interestingly, only the level of sFlt-1 and the sFlt-1/PLGF ratio in the three groups changed significantly in the same manner as those in HPS rats, which suggested that serum sFlt-1 and the sFlt-1/PLGF ratio may be potential markers of HPS.

Several teams have tried to identify markers that aid in HPS diagnosis according to recognized mechanisms such as vascular tone,[26] endothelial dysfunction,[27,28] and PPA;[9,29] however, these clinical studies were not sufficiently effective. Endothelin-1 seems to be able to discriminate between HPS and non-HPS patients but with low PPV (53.8%).[28] Moreover, endothelin-1 levels cannot discriminate between clinical and subclinical HPS patients.[27] Although the discrimination of serum vWF is relatively satisfactory, the specificity is only 54%.[30] ICAM-3 and VCAM-1 are considered good predictors of HPS diagnosis, but there is insufficient detailed information on their clinical application.[29] In summary, most of the research in this area has been conducted on a small sample size of HPS patients; furthermore, there are few studies on discriminating among CEE negative, IPVD and HPS patients. Here, our study demonstrates that changes in serum sFlt-1 level and the sFlt-1/PLGF ratio exhibit the same pattern in humans and rats, supporting these changes as potential markers of HPS. Interestingly, in addition to the importance of sFlt-1 and the sFlt-1/PLGF ratio for liver diseases, it has been found that the serum levels of sFlt-1 and PLGF are negatively correlated with respiratory function,[24] further supporting sFlt-1 and the sFlt-1/PLGF ratio as promising markers for pulmonary diseases. Herein, we also found that the correlations between the serum level of PLGF, sFlt-1, the sFlt-1/PLGF ratio and PaO_2 were −0.237, 0.336 and 0.363

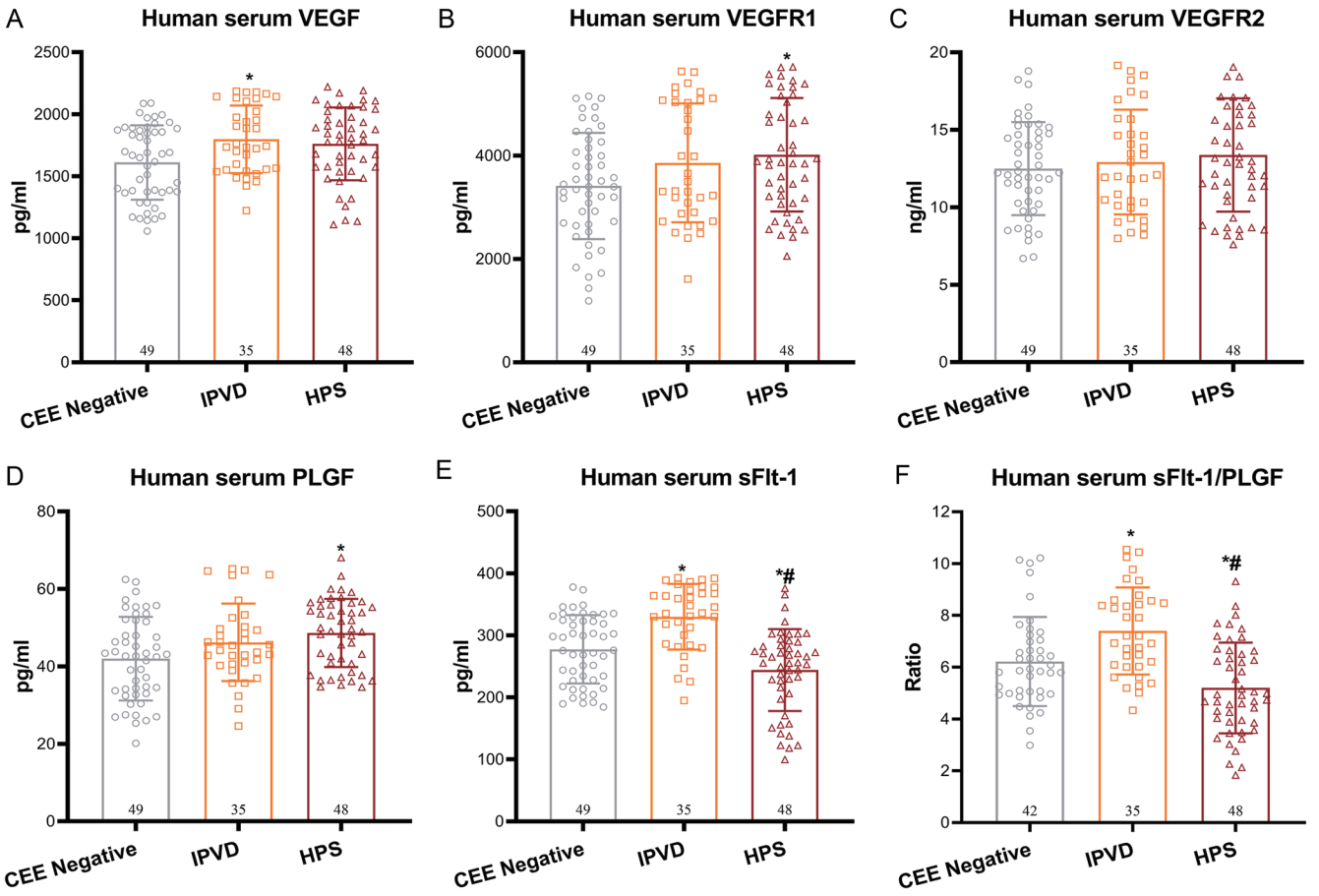

Fig. 3. Serum levels of VEGF (A), VEGFR1 (B), VEGFR2 (C), PLGF (D), sFlt-1 (E), and the sFlt-1/PLGF ratio (F) in patients with chronic liver disease. Data are means±SD, and the sample size is shown at the bottom of the box. *Compared with CEE-negative group, $p<0.05$, #Compared with IPVD group, $p<0.05$. CEE, contrast-enhanced echocardiography; CLD, chronic liver disease; HPS, hepatopulmonary syndrome; IPVD, intrapulmonary vascular dilation; PLGF, placental growth factor; sFlt-1, soluble vascular endothelial growth factor 1; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

($p<0.05$), respectively (Supplementary Table 9). Meanwhile, the correlations between the serum level of PLGF, sFlt-1, the sFlt-1/PLGF ratio and $P_{(A-a)}O_2$ were 0.186, −0.395 and −0.371 ($p<0.05$), respectively (Supplementary Table 10). In the current study, random forest and Boruta algorithms instead of logistics regression were used because they are capable of dealing with nonlinear, complex data and are less likely to experience overfitting. Through feature selection, we showed that serum sFlt-1 level and the sFlt-1/PLGF ratio were the most important features for HPS (Supplementary Tables 5–8). Even when distinguishing among HPS, CEE-negative and IPVD patients, the AUCROC on the external validation datasets was larger than 0.75 (Supplementary Table 10). To our knowledge, this is the first work to report serum sFlt-1 level and the sFlt-1/PLGF ratio as important variables for discriminating between HPS and non-HPS (or CEE-negative and IPVD) patients.

As previously described, the negative results from the first attempt at anti-angiogenetic therapy in HPS may be related to the duration and dose of sorafenib, timely enrolment of patients, and small sample size.[12,13] There were no significant differences in VEGF and VEGFR2 between CEE-negative and HPS patients in this study, thus explaining the failure of clinical application of sorafenib, which targets the above-mentioned factors.[12] Our results suggested that monitoring serum sFlt-1 level and the sFlt-1/PLGF ratio might be beneficial to the early warning and diagnosis of HPS.

Our study has some limitations. First, we only reported an interesting phenomenon, that is, serum sFlt-1 and the sFlt-1/PLGF ratio may be potentially useful for early warning and the diagnosis of HPS; however, no intervention studies have been conducted. Second, this is a prospective cross-sectional study so we only observed the levels of angiogenesis-associated factors at a certain moment. The results are not sufficient to clarify the causal relationship between angiogenetic factors and HPS. Third, although we have internally and externally validated the model performance distinguishing between HPS and CLD patients to prove the generalization, the sample size is slightly limited. A larger cohort and long-term follow-up should be carried out to determine the causal relationship between serum sFlt-1 level, the sFlt-1/PLGF ratio and the development of HPS.

Conclusions

Our results provide compelling evidence that the serum sFlt-1 level and the sFlt-1/PLGF ratio are related to the development of HPS. Furthermore, serum sFlt-1 levels and the sFlt-1/PLGF ratio are potential markers for early warning, diagnosis, and management of HPS. The results support the necessity for larger, prospective, randomized studies to expand these preliminary observations.

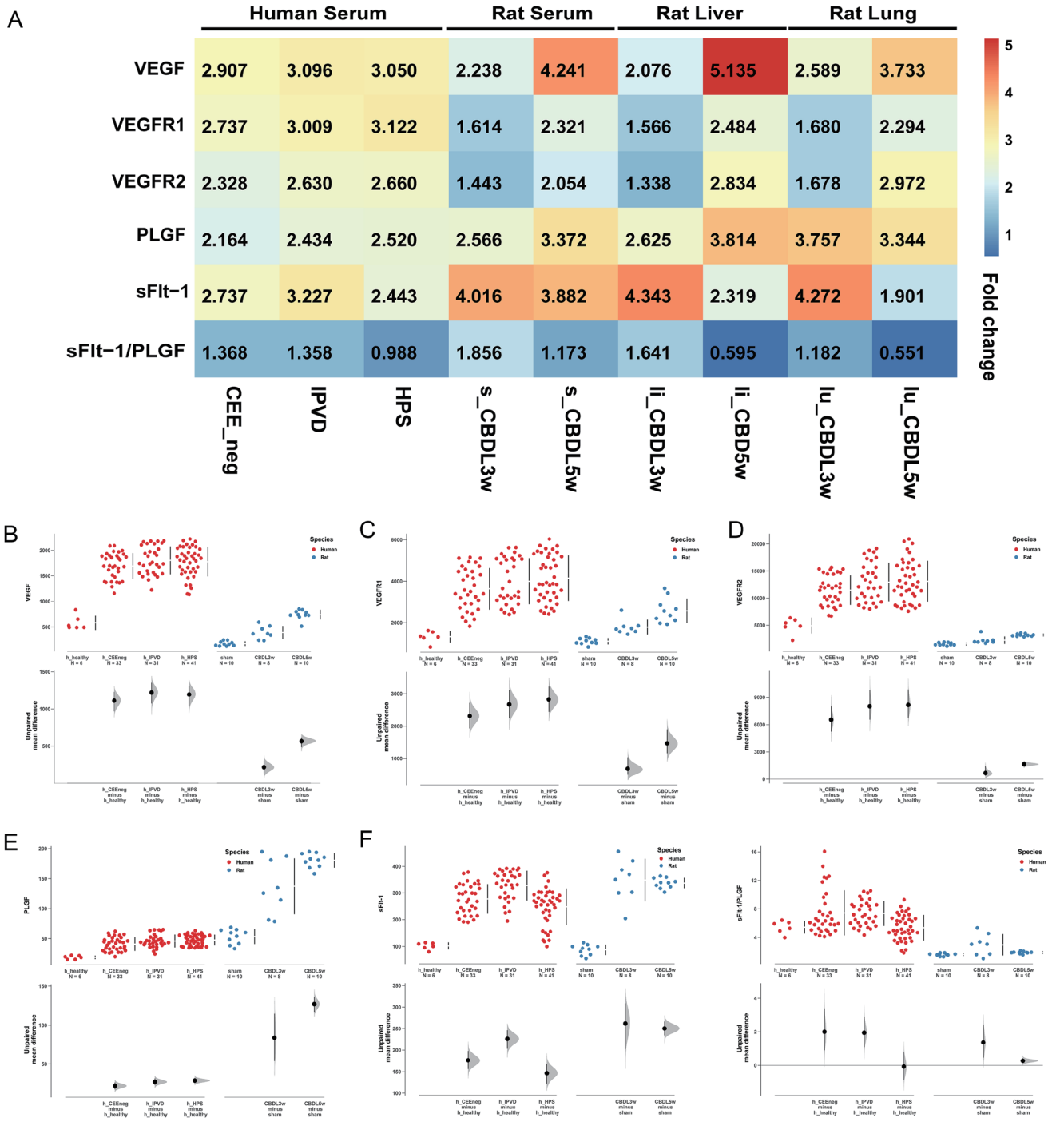

Fig. 4. Inconsistent changes in the levels of VEGF family members and their receptors between patients and experimental rats. (A) Fold change in the expression level of the VEGF family members and their receptors in patients (healthy group as baseline) and rats (sham group as baseline) presented as a heatmap. Gardner-Altman Estimation plot of VEGF (B), VEGFR1 (C), VEGFR2 (D), PLGF (E), sFlt-1 and the sFlt-1/PLGF ratio (F) in human (the healthy group as baseline) and rat serum (the sham group as baseline) was demonstrated by an estimation diagram. CBDL, common bile duct ligation; CEE, contrast-enhanced echocardiography; h, human; HPS, hepatopulmonary syndrome; IPVD, intrapulmonary vascular dilation; li, liver; lu, lung; neg, negative; PLGF, placental growth factor; s, serum; sFlt-1, soluble vascular endothelial growth factor 1; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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Table 2. Model performance on test (n=31) and validation datasets (n=27) for discriminating HPS and non-HPS based on clinical data and serum level of the VEGF family members and their receptors

Datasets	AUCROC	Sens	Spec	PPV	NPV	Balanced acc	Brier score	IDI	*p*
Model 1: Merely clinical data as input									
Test	0.548 (0.332–0.765)	16.7%	78.9%	33.3%	60.0%	47.8%	0.246	−26.2% (−48.5% – −4.0%)	0.021
Validation	0.589 (0.321–0.858)	14.3%	65.0%	12.5%	68.4%	39.6%	0.245	−42.6% (−65.3% – −19.9%)	<0.001
Model 2: Merely serum level of The VEGF family members and their receptors as input									
Test	0.761 (0.575–0.947)	75.0%	79.0%	69.2%	83.3%	77.0%	0.190	/	/
Validation	0.864 (0.724–1.000)	71.4%	85.0%	62.5%	89.5%	78.2%	0.143	/	/
Model 3: Combination of clinical data and serum level of The VEGF family members and their receptors as input									
Test	0.737 (0.539–0.9359)	58.3%	73.7%	58.3%	73.7%	66.0%	0.198	−12.2% (−24.9%–0.5%)	0.060
Validation	0.843 (0.689–0.997)	57.1%	85.0%	57.1%	85.0%	71.1%	0.166	−18.4% (−32.9% – −3.9%)	0.013
Model 4: sFlt-1, the sFlt-1/PLGF ratio and VEGFR1 selected by Boruta as input									
Test	0.776 (0.603–0.950)	50.0%	84.2%	66.7%	72.7%	67.1%	0.196	0.9% (−9.6%–11.4%)	0.870
Validation	0.857 (0.711–1.000)	85.7%	85.0%	66.7%	94.4%	85.4%	0.150	−2.0% (11.2%–7.2%)	0.666

IDI was compared between model 2 and the other three models. acc, accuracy; IDI, integrated discrimination improvement; NPV, negative predicted value; PLGF, placental growth factor; PPV, positive predicted value; Sens, sensitivity; sFlt-1, soluble vascular endothelial growth factor 1; Spec, specificity; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Contributed to the study conception and design (BY, ZYX), contributed to acquisition of data (XFW, DDW, HL, XYH, JQG), analyzed and interpreted the data (YJL, PL, XS, ALS), contributed to technical support (WFY, LQY, XBW, KB), contributed to material support (YZS, JHL, JL, CYY), wrote the original manuscript (YL, PL, DDW), revised the manuscript (YJL, PL, XFW), and obtained funding (BY, YJL, PL, XFW). All authors read and approved the final manuscript.

Ethical statement

The research protocol was approved by the ethics committees of the Army Medical University for animal care (AMUWEC20201230), the First Affiliated Hospital of Army Medical University [(No: 2017(35), KY2019107)] and Sichuan Province People's Hospital of Sichuan Academy of Medical Sciences [No.2021(471)].

Data sharing statement

Data used for this manuscript are available on request.

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