

Liver Congestion in Heart Failure Contributes to Inappropriately Increased Serum Hepcidin despite Anemia

Yukako Ohno, Haruo Hanawa,* Shuang Jiao, Yuka Hayashi, Kaori Yoshida, Tomoyasu Suzuki, Takeshi Kashimura, Hiroaki Obata, Komei Tanaka, Tohru Watanabe, Tohru Minamino

Department of Cardiovascular Biology and Medicine,

Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan

Running title: Anemia in heart failure with liver congestion

*Correspondence to: H. Hanawa, MD, Department of Cardiovascular Biology and Medicine, Niigata University Graduate School of Medical and Dental Sciences,

1-757 Asahimachi-dori, Chuo-ku Niigata 951-8510, Japan,

Phone: 81-25-227-2185, Fax: 81-25-227-0774

E-mail address: hanawa@med.niigata-u.ac.jp

Key words: anemia; heart failure; hepcidin; iron deficiency; liver congestion;

Total 6155 words

Total 239 words in abstract.

ABSTRACT

Hepcidin is a key regulator of mammalian iron metabolism and mainly produced by the liver. Hepcidin excess causes iron deficiency and anemia by inhibiting iron absorption from the intestine and iron release from macrophage stores. Anemia is frequently complicated with heart failure. In heart failure patients, the most frequent histologic appearance of liver is congestion. However, it remains unclear whether liver congestion associated with heart failure influences hepcidin production, thereby contributing to anemia and functional iron deficiency. In this study, we investigated this relationship in clinical and basic studies. In clinical studies of consecutive heart failure patients (n = 320), anemia was a common comorbidity (41%). In heart failure patients without active infection and ongoing cancer (n = 30), log-serum hepcidin concentration of patients with liver congestion was higher than those without liver congestion (p = 0.0316). Moreover, in heart failure patients with liver congestion (n = 19), the anemia was associated with the higher serum hepcidin concentrations, which is a type of anemia characterized by induction of hepcidin. Subsequently, we produced a rat model of heart failure with liver congestion by injecting monocrotaline that causes pulmonary hypertension. The monocrotaline-treated rats displayed liver congestion with increase of hepcidin expression at 4 weeks after monocrotaline injection, followed by anemia and functional iron deficiency observed at 5 weeks. We conclude that liver congestion induces hepcidin production, which may result in anemia and functional iron deficiency in some patients with heart failure.

Introduction

Anemia frequently occurs in patients with heart failure (HF) and is an independent prognostic factor for mortality (Ezekowitz et al. 2003; Hamaguchi et al. 2009). The etiology of anemia in HF patients is thought to be multifactorial. Hemodilution (Androne et al. 2003), chronic inflammation (Weiss and Goodnough 2005), renal dysfunction (Eschbach 2002), hemolysis (Kliger et al. 2013), gastrointestinal bleeding (Holleran et al. 2013), bone marrow dysfunction (Westenbrink et al. 2010), resistance to erythropoietin (van der Meer et al. 2008), hematinic deficiencies including vitamin B12, folic acid, and functional iron deficiency (FID) (Witte et al. 2004; Opasich et al. 2005) are thought to be etiological factors of anemia in HF patients. Especially, absolute and functional iron deficiency have been reported to be an important factor and a new term, cardiorenal–iron deficiency syndrome was proposed (Macdougall et al. 2012).

The central player regulating the amount of iron in the body is hepcidin (Ganz 2003). Therefore, hepcidin is postulated to play an important role in anemia and FID in HF (Matsumoto et al. 2010; Jankowska et al. 2013). Hepcidin is mainly produced in hepatocytes and hepcidin excess causes iron deficiency and anemia by inhibiting iron absorption from the intestine and iron release from macrophage stores (Ganz 2003). Therefore, we focused on pathological findings of liver in HF with anemia. The most frequent histologic appearance of liver in HF patients is congestion. We hypothesized that liver congestion in HF patients may influence expression of hepcidin and increased expression of hepcidin may contribute to anemia and FID.

Hepcidin expression is induced and regulated by multiple factors (Fleming and

Ponka 2012). Anemia and hypoxia reduce production of hepcidin (Nicolas et al. 2002), but on the other hand, increase of serum holo-transferrin and iron concentration in cells induce hepcidin expression via bone morphogenetic protein-6 (Arndt et al. 2010). Inflammation also induces hepcidin expression via interleukin-6 (Nemeth et al. 2003). Previously, we reported that a patient with severe tricuspid regurgitation with liver congestion (LC), refractory anemia and a very high serum hepcidin level recovered from anemia after valve replacement, which was accompanied by a decrease in the hepcidin and interleukin-6 levels (Suzuki et al. 2012). Moreover, we demonstrated that LC by ligation of the inferior vena cava (IVC) of rats caused inappropriately increased expression of hepcidin via bone morphogenetic protein-6 and interleukin-6 and contributed to anemia and FID (Suzuki et al. 2014). These results suggested that LC may contribute to anemia and FID of HF via inappropriately increased expression of hepcidin.

Contribution of LC to anemia and FID in HF has not been investigated. It remains unclear whether inappropriately increased expression of hepcidin occurs in HF patients with LC. In this study, we tested the hypothesis in HF patients admitted to the Cardiovascular Disease Department of Niigata University Medical and Dental Hospital. In addition, pulmonary arterial hypertension (PAH) generally leads to right HF and sometimes cause to LC (Alvarez and Mukherjee 2012). It has been reported that inappropriately raised hepcidin levels may be a causative factor for FID of patients with PAH (Rhodes et al. 2011), but the mechanisms of increased hepcidin levels in PAH are largely unknown. Therefore, to address these questions, we also studied these

interrelationships using a rat model of PAH.

Methods

Patient samples

In clinical study 1, we aimed to examine the prevalence of anemia in all HF patients admitted to the Cardiovascular Disease Department of Niigata University Medical and Dental Hospital for one year, and the relationship between markers of HF and markers of anemia in all HF patients was evaluated. Blood samples were obtained from consecutive patients (n = 320) with HF admitted between August 2013 and July 2014. HF was defined as a brain natriuretic peptide (BNP) concentration of 50 pg/mL or higher and we defined HF only based on the BNP value. Blood Hb concentration of all patients was determined. Anemia was defined as a hemoglobin (Hb) concentration below the lower level of normal (male, Hb < 13.2 g/dL; female, Hb < 10.8 g/dL), and then serum iron and unsaturated iron-binding capacity were determined in as many patients with anemia as possible. The IVC diameter, an indicator of liver congestion, was measured at the end expiratory phase by ultrasound examination.

In clinical study 2, we aimed to examine the influence of LC on serum hepcidin levels. Blood samples were obtained from patients with anemia and HF (BNP >50 pg/mL) (n = 45), who gave their consent and were admitted to the Cardiovascular Disease Department of Niigata University Medical and Dental Hospital between 2008 and 2013. The IVC diameter was measured at end expiratory phase by ultrasound examination. LC was diagnosed with IVC diameter (\geq 2.0 cm) from echography

findings of patients. Tricuspid regurgitation pressure gradient (TRPG) can be measured for 38 patients. Because anemia of chronic diseases, such as active inflammation and non-curative cancer is also caused by hepcidin (Weiss and Goodnough 2005), we excluded patients with chronic disease such as autoimmune disease, active infection, and ongoing cancer (n = 15) and analyzed the other patients (n = 30) for some analyses of serum hepcidin levels. Autoimmune disease, active infection and ongoing cancer were diagnosed with clinical finding, blood tests and imaging studies.

Serum hepcidin measurement

Serum hepcidin concentrations were determined using an enzyme-linked immunosorbent assay Kit for human hepcidin (Usen Life Science Inc., Wuhan, China). The samples were assayed in duplicates.

Ethics and Informed Consent for the Patient Study

The ethics committee of Niigata University Medical and Dental Hospital approved the study, and all patients signed an informed consent form in relation to diagnosis by blood sample.

Animal model

Male Lewis rats were obtained from Charles River, Japan (Atsugi, Kanagawa, Japan), and were maintained in our experimental animal facilities until they reached 8 weeks of age. Heart failure with liver congestion was generated using monocrotaline

(MCT) that causes pulmonary hypertension, as described in below. All animal experiments followed the guidelines for the care and use of laboratory animals published by the US National Institutes of Health and were done with the approval of Institutional Animal Care and Use Committee at the Niigata University. Rats (n = 37) were anesthetized with inhalation of isoflurane. Rats received a single subcutaneous injection of MCT (60 mg/kg; Sigma Chemical, St. Louis, MO) and were studied 28 days (4 weeks, n = 11), 35 days (5 weeks, n = 10) and 42 days (6 weeks, n = 7) after injection. Control rats received a subcutaneous injection of phosphate-buffered saline and studied (4 weeks, n = 3; 5 weeks, n = 3; 6 weeks, n = 3). All animals were euthanized by an overdose of isoflurane inhalation in a killing chamber. Death of the animals was confirmed by the absence of breathing after removal of the carcass from the chamber and exsanguinated through the heart. Livers and hearts were collected for gene expression analyses and histological analyses.

Pulse Oximetry Measurements

Rats (Control, n = 6; MCT, n = 14) were weakly anesthetized with inhalation of isoflurane and arterial blood oxygen saturation was measured immediately in the footpad of the rats with a pulse oximeter (model 9847V; Nonin Medical, Plymouth, MN) and clip on sensor (2000SL; Nonin Medical) every week.

Blood count

Rat peripheral blood samples were obtained from the right atrium. Blood Hb

concentrations were determined using the automated XE-2100 analyzer (Sysmex, Kobe, Japan).

Serum iron measurement

Rat serum iron and unsaturated iron-binding capacity were determined using an automated system H7700 (Hitachi High-Tech, Tokyo, Japan). The total iron-binding capacity was calculated as the sum of serum iron and unsaturated iron-binding capacity, and the percentage of transferrin saturation was calculated as (serum iron x100)/ total iron-binding capacity.

Histology and Berlin blue staining

Tissue samples were fixed at room temperature in 10% formalin. Samples from liver and heart were sequentially dehydrated through an alcohol series and embedded in paraffin. Four- μ m thick sections were cut, deparaffinized in xylene, and dehydrated in descending series dilutions of ethanol. Each section from liver and heart was also stained with hematoxylin and eosin (HE) stain and Berlin blue stain for iron (Berlin blue staining set; WAKO Pure Chemical, Osaka, Japan).

RNA extraction and real time RT-PCR

Total RNA was isolated from the liver tissues using Trizol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 5 μ g of total RNA with random primers and murine Moloney leukemia virus reverse transcriptase. To create the plasmids used for

the standard, rat hepcidin was amplified using the primer pairs as reported previously (Isoda et al. 2010). PCR amplified cDNAs were inserted directly into the pGEM recombinant plasmids and were isolated following transformation into *Escherichia coli* JM109-competent cells using a MagExtractor plasmid kit (Toyobo, Osaka, Japan). The absolute copy number of each mRNA was also measured by quantitative real-time RT-PCR with a LightCycler instrument (Roche Diagnostics, Tokyo, Japan) using the same primers and SYBR Premix Ex Taq (Takara, Otsu, Japan). After an initial denaturation step of 10 minutes at 95°C, a 3-step cycling procedure (denaturation at 95°C for 10 s, annealing at 62°C for 10 s and extension at 72°C for 13 s) was used for 45 cycles. The absolute copy numbers of particular transcripts were calculated by LightCycler software using a standard curve approach. Gene expressions of 5 samples in a rat liver preparation were averaged.

Statistical analysis

Statistical assessment was performed by an unpaired Student *t* test or one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. The differences were considered significant at $p < 0.05$. The data obtained from organ weight, arterial blood saturation, blood examination, quantitative RT-PCR, and enzyme-linked immunosorbent assay were expressed as mean \pm standard error of the mean (SEM) or mean \pm standard deviation. Correlations were evaluated by linear regression analysis. Data were analyzed using Pearson's correlation coefficient and Fisher's Z-transformation test.

Results

Prevalence of Anemia and Relationship between Anemia and LC in All HF Patients

Among consecutive HF patients admitted to the Cardiovascular Disease Department of Niigata University Medical and Dental Hospital, 41% had anemia (Table 1). Plasma BNP levels were significantly higher in HF patients with anemia, but IVC diameter, an indicator of liver congestion, was similar in HF patients, irrespective of anemia (Table 1). There was almost no correlation between log-plasma BNP levels and blood Hb concentrations in male (Fig. 1A) and female (Fig. 1B) HF patients. There was no significant correlation between IVC diameter and Hb concentrations in HF patients (Figs. 1D and 1E). Transferrin saturation (male, n = 40; female, n = 15) did not correlate to log-plasma BNP levels and IVC diameter in HF patients with anemia (Figs. 1C and 1F).

Serum Heparin Level in HF Patients with Anemia

We obtained 45 serum samples from patients with anemia and HF, who gave their consent and measured markers of heart failure by echocardiography. We then compared markers of heart failure, anemia and renal function between LC and non-LC patients (Table 2). Plasma BNP levels were higher in patients with LC (IVC \geq 2 cm) than those without LC (IVC $<$ 2 cm), whereas serum hepcidin levels were similar in both groups of patients. Difference of serum creatinine levels between LC and non-LC patients is

not significant.

We analyzed all patients with anemia and HF (Fig 2A). There was a weak negative correlation between log-serum hepcidin levels and Hb concentrations in LC patients, whereas there was a weak positive correlation between them in non-LC patients (Fig. 2A upper panels). On the other hand, there was no correlation between log-serum hepcidin levels and Hb concentrations in patients with high TRPG reflecting pulmonary arterial pressure and low TRPG (Fig. 2A lower panels). Subsequently, we analyzed patients (n = 30) without chronic diseases, such as active inflammation and non-curative cancer (LC, n = 1 and non-LC, n = 14 as shown in Table 2) (Figs 2B and 2C). These correlations (Fig. 2B) were similar to those in analysis of all patients (n = 45) (Fig. 2A), and, moreover, log-serum hepcidin levels were higher in those patients with LC than those without LC (All, p = 0.0316; Hb \leq 11, p = 0.0100; Hb \leq 10, p = 0.0168) (Fig. 2C left side graph). In contrast, no difference was detected in the log-serum hepcidin levels between the patients with high TRPG and those with low TRPG (Fig. 2C right side graph).

Organ Weight and Histologic Findings

The heart weight/body weight ratio of MCT-treated rats gradually increased and the ratios of MCT-treated rats were significantly higher than that of control rats at all time points (4 weeks, n = 3; 5 weeks, n = 3; 6 weeks, n = 3) (Fig. 3A). Marked right ventricular dilatation and hypertrophy of MCT-treated rats caused by pulmonary hypertension (PH) were observed from 4 wks to 6 wks (Fig. 4 left side panels).

Microscopic examinations of livers of MCT-treated rats from 4 weeks to 6 weeks showed red blood cell accumulation in the sinusoidal spaces around the central vein (Fig. 4 middle panels) and several hemosiderin-loaded macrophages in Berlin blue stained sections (Fig. 4 right side panels), namely LC. The liver weight/body weight ratio of MCT-treated rats was increased by LC at 4 weeks. However, the ratio gradually decreased from 5 weeks to 6 weeks (Fig. 3B).

Arterial Blood Oxygen Saturation

Arterial blood oxygen saturation levels of control rats were maintained at over 96%. But those of MCT-treated rats gradually decreased to 82% at 6 weeks (Fig. 5).

Anemia and Serum Iron in MCT-Treated Rats

Hb and serum iron concentration and transferrin saturation of MCT-treated rats decreased at 4 - 5 weeks (Figs. 6A, 6B, and 6C). However, Hb and serum iron concentration and transferrin saturation of MCT-treated rats recovered at 6 wks.

Expression of Hepcidin

The expression levels of hepcidin in livers of MCT-treated rats at 4 weeks were significantly higher than those in control rats and those in livers of MCT-treated rats at 5 weeks were similar to levels of control rats despite anemia and FID (Fig. 6D and squares and lozenges in Fig. 7). On the other hand, those at 6 weeks further decreased despite recovery of anemia and FID (Fig. 6D and triangles in Fig 7).

Discussion

The most common form of anemia is iron deficiency anemia, which is characterized by a reduction of hepcidin (such as iron deficiency anemia, and bleeding anemia). In contrast, the anemia of chronic disease is characterized by induction of hepcidin, which causes a degradation of ferroportin channels (De Domenico et al. 2007). Consequently, iron cannot be absorbed from the intestine, and is trapped within iron storage sites such as macrophages and hepatocytes.

Anemia and hepcidin in HF patients

Previous studies reported that 17 - 57% of patients admitted with HF had anemia (Horwich et al. 2002; Ezekowitz et al. 2003; Hamaguchi et al. 2009; Caira et al. 2013). In the present clinical study, the observed rate of anemia in consecutive HF patients admitted to the Cardiovascular Disease Department of Niigata University Medical and Dental Hospital was 41%, which agreed with the previous reports. As previously reported (Horwich et al. 2002; Tsuji et al. 2004), plasma BNP levels of HF patients with anemia were significantly higher than those without anemia. Anemia independently predicts substantially increased risks of death and hospitalization in HF (Mozaffarian et al. 2003; Go et al. 2006). These data show that clinicians should attend to anemia in medical care of HF patients as is widely proposed.

Firstly, we evaluated the relation between anemia and LC in all HF patients with anemia. But in this study, the IVC diameter, an indicator of liver congestion, of HF

patients with anemia were not significantly larger than those without anemia, and Hb concentrations were not correlated to the severity of LC. These results suggest that LC-associated anemia may occur in a minority of cases with anemia.

Secondly, we evaluated the influence of LC on the serum hepcidin level that may reflect hepcidin production. Hb concentrations correlated positively with serum hepcidin levels in patients without LC. This phenomenon has also been observed in children with iron deficiency (Choi et al. 2012), the most common form of iron deficiency anemia, which is characterized by a reduction of hepcidin. In contrast, Hb concentrations in LC patients correlated negatively with hepcidin levels, which is a type of anemia characterized by induction of hepcidin. Moreover, because hepcidin is induced by chronic disease including infections, malignancies, and rheumatologic disorders (Weinstein et al. 2002; Weiss and Goodnough 2005; Maes et al. 2010), we analyzed serum hepcidin levels in HF and anemia patients without chronic disease. In this series of HF patients without chronic disease, serum hepcidin levels were higher in LC patients than those in patients without LC; namely, hepcidin levels of LC patients did not decrease despite low Hb levels. These results suggest inappropriately increased hepcidin production in LC patients.

Anemia, FID and hepcidin expression in MCT-Treated PAH Rats

Histological findings of liver in MCT-treated rats at 4 – 6 weeks showed congestion. In MCT-treated rats at 4 – 5 weeks, Hb and serum iron concentration and transferrin saturation were decreased and the expression levels of hepcidin in livers were not

decreased despite anemia and FID. These findings were similar to those of rats with LC by ligating the IVC as our previously reported (Suzuki et al. 2014) and suggest inappropriately increased expression of hepcidin, that is, a form of anemia characterized by an induction of hepcidin. On the other hand, MCT-treated rats at 6 weeks were quite different from rats with LC by ligating the IVC (Suzuki et al. 2014). Liver weight/body weight ratio in LC rats in which the IVC was ligated was persistently high and with severe continuing LC throughout the course of ligation, while, in contrast, liver weight/body weight ratio in MCT-treated rats at 6 weeks decreased and liver atrophy was observed. We surmised that the liver atrophy observed in this model was due to the unusual and severe hypoxemia and low cardiac output (Denis et al. 2004; Farahani et al. 2008). Previous studies have reported that hypoxia reduced hepcidin expression in hepatocytes (Nicolas et al. 2002). Moreover, the low cardiac output causes hypoxia in several organs and tissues (Fahey and Lister 1989); therefore, the low cardiac output may regulate hepcidin expression in a similar way as hypoxemia. In fact, hepcidin expression of MCT-treated rats at 6 weeks decreased; therefore, the decrease is likely due to hypoxemia and low cardiac output.

The most common findings of liver in clinical right HF patients are not atrophy but congestion. PAH, mitral stenosis, tricuspid regurgitation, cor pulmonale, constrictive pericarditis, cardiac amyloidosis, and some types of congenital heart disease, etc., is not consistent with liver atrophy but LC (Alvarez and Mukherjee 2012). Right HF with liver atrophy is rare (Greenberg and Kahn 2012). Our previous reported case of tricuspid regurgitation (Suzuki et al. 2012) also had persistent LC, refractory anemia,

and a very high serum hepcidin level, but did not have severe hypoxemia and the low cardiac output. In such a case with persistent LC, inappropriate increased hepcidin expression seems to be caused by LC and results in anemia and FID.

On the other hand, findings of MCT-treated rats at 6 weeks suggest that hypoxemia and the low cardiac output observed in HF patients are also important players in iron metabolism. Clinical PAH patients with hypoxemia are often treated by oxygen inhalation (Palmisano et al. 1990), and congenital heart disease with right-to-left shunt flow often causes severe hypoxemia (Webb et al. 2012) and severe left HF with lung congestion (Gheorghiade et al. 2012) leading to hypoxemia and the low cardiac output. Hypoxemia results in secondary polycythemia (Webb et al. 2012) and increased iron absorption (Simpson 1996; Mastrogiannaki et al. 2013). In this study, MCT-treated rats showed severe hypoxemia at 6 weeks and the anemia and FID later recovered unlike LC rats in which the IVC was ligated. Anyway, in HF patients, various factors influence hepcidin expression, such as LC, hypoxemia, and low cardiac output; these factors should be also considered for understanding iron metabolism.

In conclusion, LC in parts of HF with anemia induces hepcidin production and may results in anemia and FID, whereas hypoxemia and low cardiac output in HF may also influence hepcidin expression. Recently, the effects of intravenous iron therapy for anemia and iron deficiency of HF patients have been studied (Anker et al. 2009a; Anker et al. 2009b). Other investigators have been developing anti-hepcidin drugs (Cooke et al. 2013; Schwoebel et al. 2013). Future studies are needed to classify anemia and FID of HF patients based on etiology and to establish proper therapies for anemia and FID of

HF patients.

Study Limitations

A limitation of this study is that MCT-treated rat model is not a typical model of clinical right HF patients with LC. Therefore, this study could not investigate anemia and FID of right HF patients with persistent LC. This study to compare serum hepcidin levels of LC patients with those of non-LC patients was conducted in a limited number of HF patients with anemia. Additional studies are needed to evaluate other models of right HF with LC and confirm the results in a large number of HF patients with anemia.

Acknowledgments

This study was supported in part by a Grant for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (no. 25461047).

Conflict of Interest

The authors declare no conflict of interest.

References

- Alvarez, A.M. & Mukherjee, D. (2012) Liver abnormalities in cardiac diseases and heart failure. *Int. J. Angiol.*, **20**, 135-142.
- Androne, A.S., Katz, S.D., Lund, L., LaManca, J., Hudaihed, A., Hryniewicz, K. & Mancini, D.M. (2003) Hemodilution is common in patients with advanced heart failure. *Circulation*, **107**, 226-229.
- Anker, S.D., Colet, J.C., Filippatos, G., Willenheimer, R., Dickstein, K., Drexler, H., Luscher, T.F., Mori, C., von Eisenhart Rothe, B., Pocock, S., Poole-Wilson, P.A., Ponikowski, P., committees, F.-H. & investigators (2009a) Rationale and design of Ferinject assessment in patients with IRon deficiency and chronic Heart Failure (FAIR-HF) study: a randomized, placebo-controlled study of intravenous iron supplementation in patients with and without anaemia. *Eur. J. Heart Fail.*, **11**, 1084-1091.
- Anker, S.D., Comin Colet, J., Filippatos, G., Willenheimer, R., Dickstein, K., Drexler, H., Luscher, T.F., Bart, B., Banasiak, W., Niegowska, J., Kirwan, B.A., Mori, C., von Eisenhart Rothe, B., Pocock, S.J., Poole-Wilson, P.A., et al. (2009b) Ferric carboxymaltose in patients with heart failure and iron deficiency. *N. Engl. J. Med.*, **361**, 2436-2448.
- Arndt, S., Maegdefrau, U., Dorn, C., Schardt, K., Hellerbrand, C. & Bosserhoff, A.K. (2010) Iron-induced expression of bone morphogenic protein 6 in intestinal cells is the main regulator of hepatic hepcidin expression in vivo. *Gastroenterology*,

138, 372-382.

- Caira, C., Ansalone, G., Mancone, M., Canali, E., Pagliaro, M., Fratarcangeli, L., Aznaran, C.A., Gatto, M.C., Di Pietro, R. & Fedele, F. (2013) Heart failure and iron deficiency anemia in Italy: results from CARMES-1 registry. *Future Cardiol.*, **9**, 437-444.
- Choi, H.S., Song, S.H., Lee, J.H., Kim, H.J. & Yang, H.R. (2012) Serum hepcidin levels and iron parameters in children with iron deficiency. *Korean J. Hematol.*, **47**, 286-292.
- Cooke, K.S., Hinkle, B., Salimi-Moosavi, H., Foltz, I., King, C., Rathanaswami, P., Winters, A., Steavenson, S., Begley, C.G., Molineux, G. & Sasu, B.J. (2013) A fully human anti-hepcidin antibody modulates iron metabolism in both mice and nonhuman primates. *Blood*, **122**, 3054-3061.
- De Domenico, I., Ward, D.M. & Kaplan, J. (2007) Hepcidin regulation: ironing out the details. *J. Clin. Invest.*, **117**, 1755-1758.
- Denis, C., De Kerguenec, C., Bernuau, J., Beauvais, F. & Cohen Solal, A. (2004) Acute hypoxic hepatitis ('liver shock'): still a frequently overlooked cardiological diagnosis. *Eur. J. Heart Fail.*, **6**, 561-565.
- Eschbach, J.W. (2002) Anemia management in chronic kidney disease: role of factors affecting epoetin responsiveness. *J Am Soc Nephrol*, **13**, 1412-1414.
- Ezekowitz, J.A., McAlister, F.A. & Armstrong, P.W. (2003) Anemia is common in heart failure and is associated with poor outcomes: insights from a cohort of 12 065 patients with new-onset heart failure. *Circulation*, **107**, 223-225.

- Fahey, J.T. & Lister, G. (1989) Response to low cardiac output: developmental differences in metabolism during oxygen deficit and recovery in lambs. *Pediatr. Res.*, **26**, 180-187.
- Farahani, R., Kanaan, A., Gavrialov, O., Brunnert, S., Douglas, R.M., Morcillo, P. & Haddad, G.G. (2008) Differential effects of chronic intermittent and chronic constant hypoxia on postnatal growth and development. *Pediatr. Pulmonol.*, **43**, 20-28.
- Fleming, R.E. & Ponka, P. (2012) Iron overload in human disease. *N. Engl. J. Med.*, **366**, 348-359.
- Ganz, T. (2003) Heparin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood*, **102**, 783-788.
- Gheorghiade, M., Filippatos, G. & Felker, G.M. (2012) Diagnosis and management of acute heart failure syndromes. In *Braunwald's Heart Disease*, 9th ed., edited by Bonow, R.O., Mann, D.L., Zipes, D.P., Libby, P. & Braunwald, E. Elsevier, Philadelphia, PA, pp. 517-542.
- Go, A.S., Yang, J., Ackerson, L.M., Lepper, K., Robbins, S., Massie, B.M. & Shlipak, M.G. (2006) Hemoglobin level, chronic kidney disease, and the risks of death and hospitalization in adults with chronic heart failure: the Anemia in Chronic Heart Failure: Outcomes and Resource Utilization (ANCHOR) Study. *Circulation*, **113**, 2713-2723.
- Greenberg, B. & Kahn, A.M. (2012) Clinical assessment of heart failure. In *Braunwald's Heart Disease*, 9th ed., edited by Bonow, R.O., Mann, D.L., Zipes,

- D.P., Libby, P. & Braunwald, E. Elsevier, Philadelphia, PA, pp. 505-516.
- Hamaguchi, S., Tsuchihashi-Makaya, M., Kinugawa, S., Yokota, T., Takeshita, A., Yokoshiki, H., Tsutsui, H. & Investigators, J.-C. (2009) Anemia is an independent predictor of long-term adverse outcomes in patients hospitalized with heart failure in Japan. A report from the Japanese Cardiac Registry of Heart Failure in Cardiology (JCARE-CARD). *Circ. J.*, **73**, 1901-1908.
- Holleran, G., Hall, B., Hussey, M. & McNamara, D. (2013) Small bowel angiodysplasia and novel disease associations: a cohort study. *Scand J Gastroenterol*, **48**, 433-438.
- Horwich, T.B., Fonarow, G.C., Hamilton, M.A., MacLellan, W.R. & Borenstein, J. (2002) Anemia is associated with worse symptoms, greater impairment in functional capacity and a significant increase in mortality in patients with advanced heart failure. *J. Am. Coll. Cardiol.*, **39**, 1780-1786.
- Isoda, M., Hanawa, H., Watanabe, R., Yoshida, T., Toba, K., Yoshida, K., Kojima, M., Otaki, K., Hao, K., Ding, L., Tanaka, K., Takayama, T., Kato, K., Okura, Y., Kodama, M., et al. (2010) Expression of the peptide hormone hepcidin increases in cardiomyocytes under myocarditis and myocardial infarction. *J. Nutr. Biochem.*, **21**, 749-756.
- Jankowska, E.A., Malyszko, J., Ardehali, H., Koc-Zorawska, E., Banasiak, W., von Haehling, S., Macdougall, I.C., Weiss, G., McMurray, J.J., Anker, S.D., Gheorghiade, M. & Ponikowski, P. (2013) Iron status in patients with chronic heart failure. *Eur. Heart J.*, **34**, 827-834.

- Kliger, C., Eiros, R., Isasti, G., Einhorn, B., Jelnin, V., Cohen, H., Kronzon, I., Perk, G., Fontana, G.P. & Ruiz, C.E. (2013) Review of surgical prosthetic paravalvular leaks: diagnosis and catheter-based closure. *Eur Heart J*, **34**, 638-649.
- Macdougall, I.C., Canaud, B., de Francisco, A.L., Filippatos, G., Ponikowski, P., Silverberg, D., van Veldhuisen, D.J. & Anker, S.D. (2012) Beyond the cardiorenal anaemia syndrome: recognizing the role of iron deficiency. *Eur. J. Heart Fail.*, **14**, 882-886.
- Maes, K., Nemeth, E., Roodman, G.D., Huston, A., Esteve, F., Freytes, C., Callander, N., Katodritou, E., Tussing-Humphreys, L., Rivera, S., Vanderkerken, K., Lichtenstein, A. & Ganz, T. (2010) In anemia of multiple myeloma, hepcidin is induced by increased bone morphogenetic protein 2. *Blood*, **116**, 3635-3644.
- Mastrogiannaki, M., Matak, P. & Peyssonnaud, C. (2013) The gut in iron homeostasis: role of HIF-2 under normal and pathological conditions. *Blood*, **122**, 885-892.
- Matsumoto, M., Tsujino, T., Lee-Kawabata, M., Naito, Y., Akahori, H., Sakoda, T., Ohyanagi, M., Tomosugi, N. & Masuyama, T. (2010) Iron regulatory hormone hepcidin decreases in chronic heart failure patients with anemia. *Circ. J.*, **74**, 301-306.
- Mozaffarian, D., Nye, R. & Levy, W.C. (2003) Anemia predicts mortality in severe heart failure: the prospective randomized amlodipine survival evaluation (PRAISE). *J. Am. Coll. Cardiol.*, **41**, 1933-1939.
- Nemeth, E., Valore, E.V., Territo, M., Schiller, G., Lichtenstein, A. & Ganz, T. (2003) Heparin, a putative mediator of anemia of inflammation, is a type II

- acute-phase protein. *Blood*, **101**, 2461-2463.
- Nicolas, G., Chauvet, C., Viatte, L., Danan, J.L., Bigard, X., Devaux, I., Beaumont, C., Kahn, A. & Vaulont, S. (2002) The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *J. Clin. Invest.*, **110**, 1037-1044.
- Opasich, C., Cazzola, M., Scelsi, L., De Feo, S., Bosimini, E., Lagioia, R., Febo, O., Ferrari, R., Fucili, A., Moratti, R., Tramarin, R. & Tavazzi, L. (2005) Blunted erythropoietin production and defective iron supply for erythropoiesis as major causes of anaemia in patients with chronic heart failure. *Eur. Heart J.*, **26**, 2232-2237.
- Palmisano, J.M., Martin, J.M., Krauzowicz, B.A., Truman, K.H. & Meliones, J.N. (1990) Effects of supplemental oxygen administration in an infant with pulmonary artery hypertension. *Heart Lung*, **19**, 627-630.
- Rhodes, C.J., Howard, L.S., Busbridge, M., Ashby, D., Kondili, E., Gibbs, J.S., Wharton, J. & Wilkins, M.R. (2011) Iron deficiency and raised hepcidin in idiopathic pulmonary arterial hypertension: clinical prevalence, outcomes, and mechanistic insights. *J. Am. Coll. Cardiol.*, **58**, 300-309.
- Schwoebel, F., van Eijk, L.T., Zboralski, D., Sell, S., Buchner, K., Maasch, C., Purschke, W.G., Humphrey, M., Zollner, S., Eulberg, D., Morich, F., Pickkers, P. & Klussmann, S. (2013) The effects of the anti-hepcidin Spiegelmer NOX-H94 on inflammation-induced anemia in cynomolgus monkeys. *Blood*, **121**, 2311-2315.

- Simpson, R.J. (1996) Dietary iron levels and hypoxia independently affect iron absorption in mice. *J. Nutr.*, **126**, 1858-1864.
- Suzuki, T., Hanawa, H., Ding, L., Ito, M., Kashimura, T., Obata, H., Hu, Y.H., Jiao, S., Namura, O. & Kodama, M. (2012) Improvement of anemia with decreasing hepcidin levels following valve replacement for severe tricuspid regurgitation. *Eur. J. Haematol.*, **88**, 551-552.
- Suzuki, T., Hanawa, H., Jiao, S., Ohno, Y., Hayashi, Y., Yoshida, K., Kashimura, T., Obata, H. & Minamino, T. (2014) Inappropriate expression of hepcidin by liver congestion contributes to anemia and relative iron deficiency. *J. Card. Fail.*, **20**, 268-277.
- Tsuji, H., Nishino, N., Kimura, Y., Yamada, K., Nukui, M., Yamamoto, S., Iwasaka, T. & Takahashi, H. (2004) Haemoglobin level influences plasma brain natriuretic peptide concentration. *Acta Cardiol.*, **59**, 527-531.
- van der Meer, P., Lok, D.J., Januzzi, J.L., de la Porte, P.W., Lipsic, E., van Wijngaarden, J., Voors, A.A., van Gilst, W.H. & van Veldhuisen, D.J. (2008) Adequacy of endogenous erythropoietin levels and mortality in anaemic heart failure patients. *Eur. Heart J.*, **29**, 1510-1515.
- Webb, G.D., Smallhorn, J.F., Therrien, J. & Redington, A.N. (2012) Congenital heart disease. In *Braunwald's Heart Disease*, 9th ed., edited by Bonow, R.O., Mann, D.L., Zipes, D.P., Libby, P. & Braunwald, E. Elsevier, Philadelphia, PA, pp. 1411-1467.
- Weinstein, D.A., Roy, C.N., Fleming, M.D., Loda, M.F., Wolfsdorf, J.I. & Andrews,

- N.C. (2002) Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. *Blood*, **100**, 3776-3781.
- Weiss, G. & Goodnough, L.T. (2005) Anemia of chronic disease. *N. Engl. J. Med.*, **352**, 1011-1023.
- Westenbrink, B.D., Voors, A.A., de Boer, R.A., Schuringa, J.J., Klinkenberg, T., van der Harst, P., Vellenga, E., van Veldhuisen, D.J. & van Gilst, W.H. (2010) Bone marrow dysfunction in chronic heart failure patients. *Eur J Heart Fail*, **12**, 676-684.
- Witte, K.K., Desilva, R., Chattopadhyay, S., Ghosh, J., Cleland, J.G. & Clark, A.L. (2004) Are hematinic deficiencies the cause of anemia in chronic heart failure? *Am. Heart J.*, **147**, 924-930.

Figure legends

Fig. 1

Correlation among markers of HF and markers of anemia.

Characterization of HF patients is provided in Table 1. (A) Correlation between log-plasma BNP levels and Hb concentration in male patients. (B) Correlation between log-plasma BNP levels and Hb concentration in female patients. (C) Correlation between log-plasma BNP levels and transferrin saturation. (D) Correlation between IVC diameter and Hb concentration in male patients. (E) Correlation between IVC diameter and Hb concentration in female patients. (F) Correlation between IVC diameter and transferrin saturation.

Fig. 2

Serum hepcidin concentration in patients with HF.

Characterization of HF patients with anemia is provided in Table 2. (A) Correlation between log-serum hepcidin levels and Hb concentrations in all HF patients with anemia (LC, n = 19; non-LC, n = 26) (TRPG \geq 30 mmHg, n = 18; TRPG < 30 mmHg, n = 20). Upper panels, difference between LC (IVC \geq 2 cm) (left) and non-LC (IVC < 2 cm) (right) patients; and lower panels, difference between TRPG \geq 30 mmHg (left) and TRPG < 30 mmHg (right) patients. (B) Correlation between log-serum hepcidin levels and Hb concentrations in patients without chronic disease (LC, n = 18; non-LC, n = 12) (TRPG \geq 30 mmHg, n = 13; TRPG < 30 mmHg, n = 13). Upper panels, difference between LC (IVC \geq 2 cm) (left) and non-LC (IVC < 2 cm) (right) patients;

and lower panels, difference between TRPG \geq 30 mmHg (left) and TRPG $<$ 30 mmHg (right) patients. (C) Serum hepcidin concentration in HF patients without chronic disease defined by Hb levels. Left panel, difference between LC and non-LC patients; and right panel, difference between TRPG \geq 30 mmHg and TRPG $<$ 30 mmHg patients. Error bars represent SEM. Statistical analysis was performed by an unpaired Student's t test. * $p < 0.05$.

Fig. 3

Organ weight of MCT-treated rats.

(A) Heart weight/body weight ratio. (B) Liver weight/body weight ratio. Error bars represent SEM. Statistical analysis was performed by one-way ANOVA and Bonferroni's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Control rats (n = 9) were injected with phosphate buffered saline (4 weeks, n = 3; 5 weeks, n = 3; 6 weeks, n = 3).

Fig. 4

Microscopic finding.

Tissue sections were stained with HE and Berlin blue for iron. Left panels, Sections stained with HE from hearts of a control rat at 4 weeks and MCT-treated rats; Middle panels, Sections stained with HE from livers of a control rat at 4 weeks and MCT-treated rats; Right panels, Sections stained with Berlin blue from livers of a control rat at 4 weeks and MCT-treated rats. Arrow heads indicate hemosiderin-loaded

cells, which were faintly stained by Berlin-blue. LV, left ventricle; RV, right ventricle.

Fig. 5

Arterial blood oxygen saturation.

Open circles represent control rats and open squares represent MCT-treated rats. Error bars represent standard deviation. Statistical analysis was performed by an unpaired Student's t test. * $p < 0.05$ vs control rats, *** $p < 0.001$ vs control rats.

Fig. 6

Anemia and iron markers in control rats and MCT-treated rats.

(A) Blood Hb concentration. (B) Serum iron concentration. (C) Transferring saturation. (D) Hpcidin mRNA of liver. Hpcidin expressions of 5 samples in a rat liver preparation were averaged. Error bars represent SEM. Statistical analysis was performed by one-way ANOVA and Bonferroni's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Control rats ($n = 9$) were injected with phosphate buffered saline (4 weeks, $n = 3$; 5 weeks, $n = 3$; 6 weeks, $n = 3$).

Fig. 7

Relationship between Hb concentration and hepcidin expression.

Relationship between Hb concentration and log-hepcidin expression levels in control rats (circles) and MCT-treated rats at 4 weeks (squares), 5 weeks (lozenges) and 6 weeks (triangles). Solid symbols indicate mean levels in each group. Error bars

represent SEM.

**Table 1. Characterization of heart failure patients
(Clinical study 1)**

	Anemia n=131 (41%)	non-anemia n=189 (59%)	p Value
Age yrs	72.2 ± 11.0	64.1 ± 13.2	<0.0001
Sex			
female	28 (28%)	71 (72%)	
male	103 (47%)	118 (53%)	
BNP (pg/mL)	743 ± 1091	483 ± 774	0.013
IVC diameter * (cm)	1.64 ± 0.55	1.54 ± 0.52	0.145

Values are mean ± SD or n (%).

Consecutive patients with heart failure (BNP >50 pg/mL) were admitted to the Cardiovascular Disease Department of Niigata University Medical and Dental Hospital between August 2013 and July 2014.

Anemia was defined as a hemoglobin concentration below normal levels (male, Hb<13.2 g/dL; female, Hb<10.8 g/dL)

* IVC diameter of 255 patients (Male 173, Female 82; Anemia 99, Non-anemia 156) were measured.

**Table 2. Characterization of heart failure patients with anemia
(Clinical study 2)**

	LC n=19 (42%)	non-LC n=26 (58%)	p Value
Age yrs	63.7 ± 12.5	68.2 ± 17.4	0.343
Sex			
female	6 (32%)	7 (27%)	
male	13 (68%)	19 (73%)	
Basic cardiac disease			
Valvular heart disease	8 (42%)	4 (15%)	
Congenital heart disease	2 (11%)		
Dilated cardiomyopathy	5 (26%)	5 (19%)	
Hypertrophic cardiomyopathy	1 (5%)	1 (4%)	
Cardiac amyloidosis	1 (5%)		
Ischemic heart disease		4 (15%)	
Hypertensive heart disease		4 (15%)	
Atrial fibrillation		1 (4%)	
Idiopathic ventricular tachycardia		1 (4%)	
Pulmonary hypertension	2 (11%)	3 (12%)	
Cardiac tumor		1 (4%)	
Cardiac tamponade		1 (4%)	
Pericarditis		1 (4%)	
Hb (g/dL)	9.8 ± 1.3	10.5 ± 1.3	0.091
Cre (mg/dL)	2.0 ± 1.7	2.4 ± 3.2	0.597
BNP (pg/mL)	1138 ± 1560	478 ± 486	0.048
ferritin * (ng/mL)	196 ± 246	297 ± 481	0.409
hepcidin (ng/mL)	169 ± 196	166 ± 264	0.9731
IVC diameter (cm)	2.43 ± 0.28	1.37 ± 0.36	<0.0001
Chronic diseases such as active inflammation and non-curative cancer	1 (5%)	14 (54%)	

Values are mean ± SD or n (%).

Consenting patients with heart failure (BNP >50 pg/mL) and anemia were admitted to the Cardiovascular Disease Department of Niigata University Medical and Dental Hospital between 2008 and 2013.

Liver congestion (LC) was diagnosed by IVC diameter (>2.0 cm) or clinical findings.

* Ferritin was measured for 42 patients (Male 30, Female 12; LC 19, non-LC 23)

Fig. 1

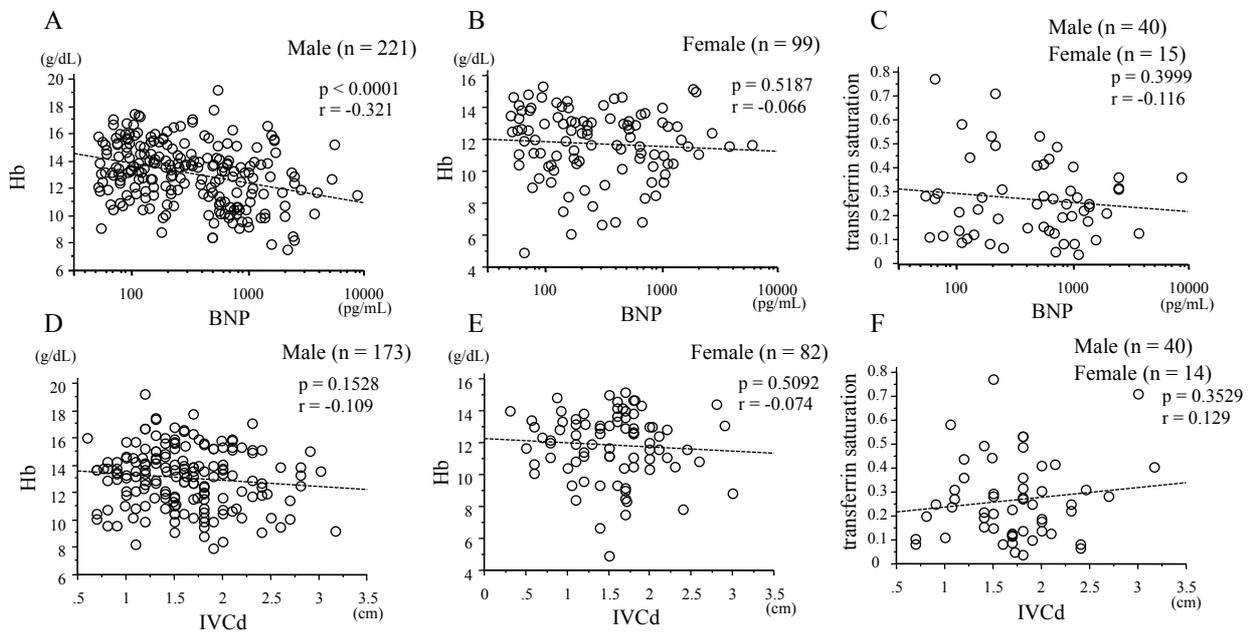


Fig. 2

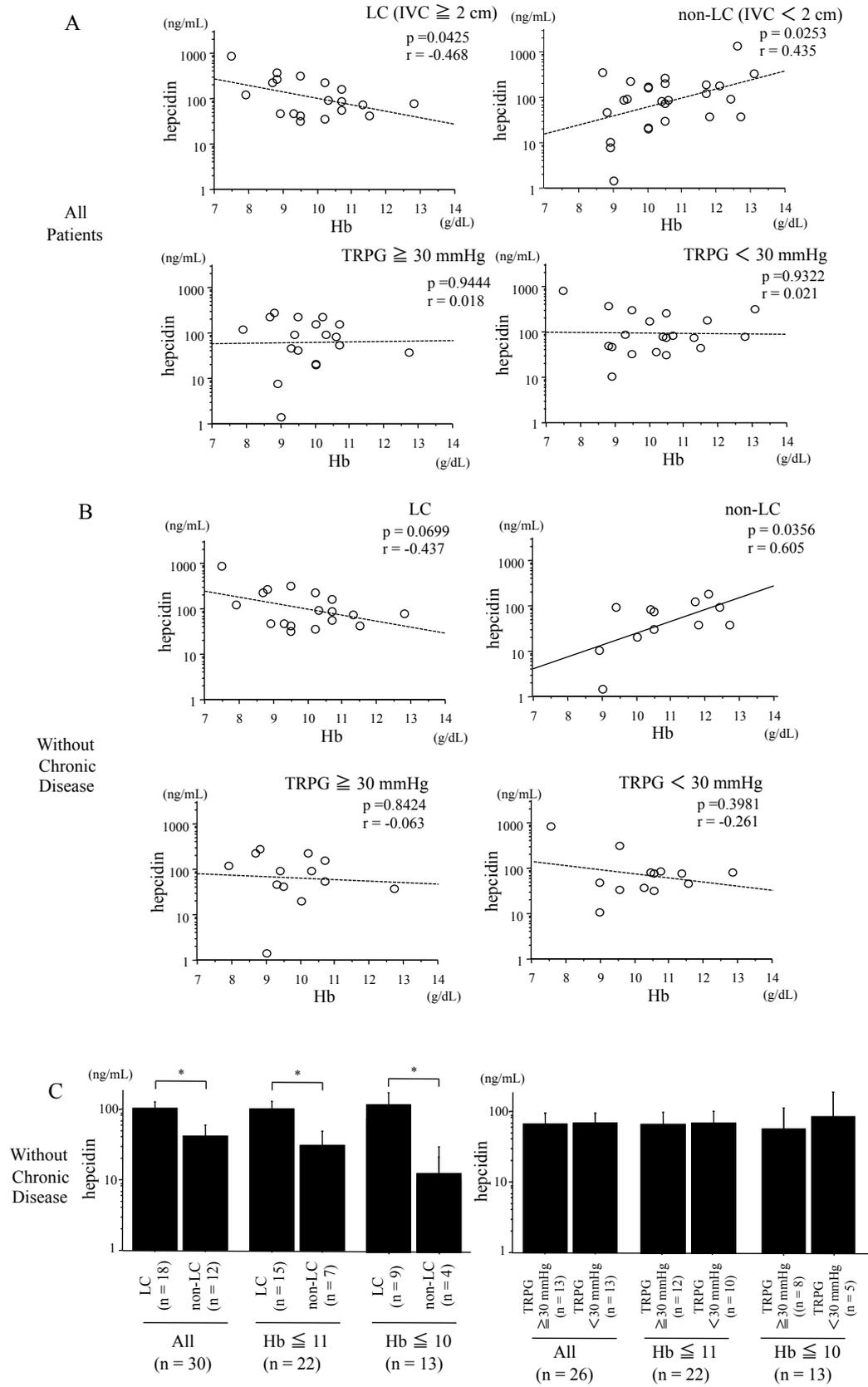


Fig. 3

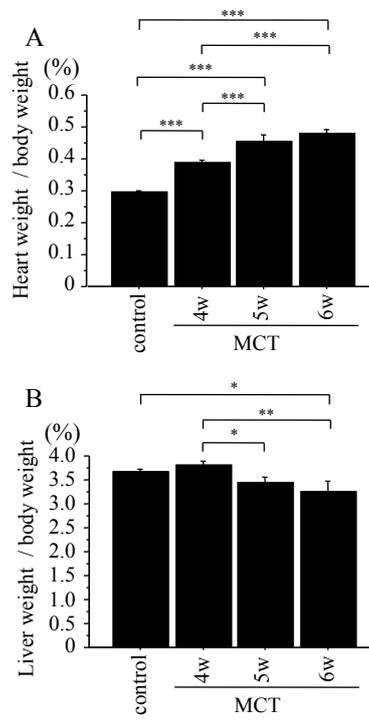


Fig. 4

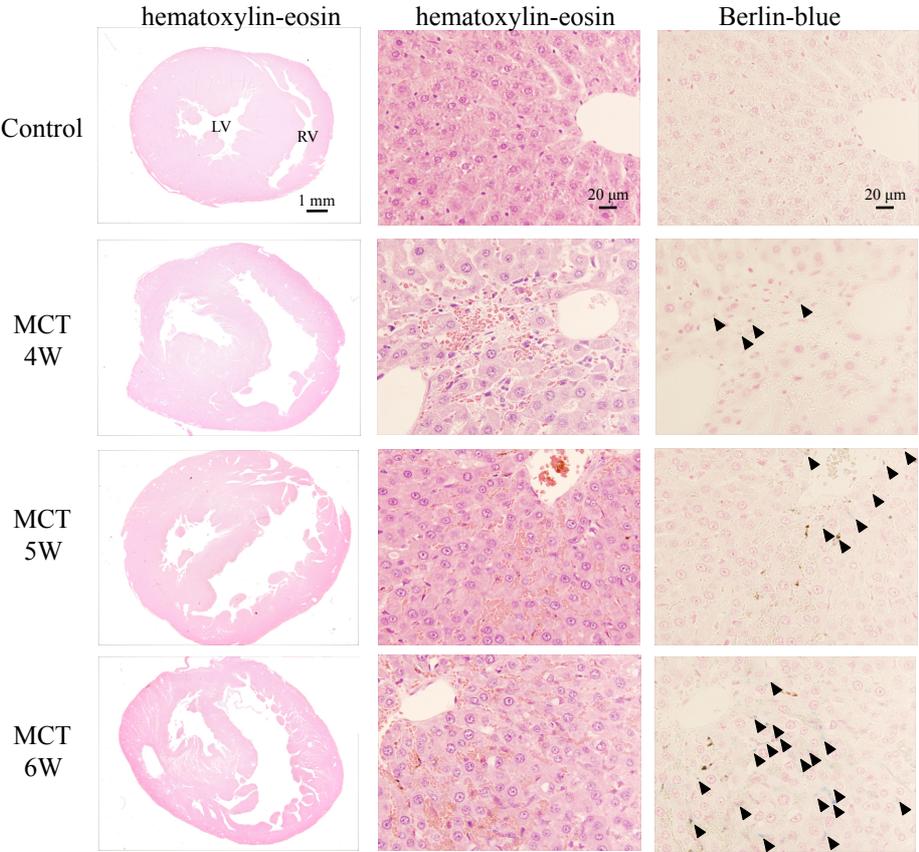


Fig. 5

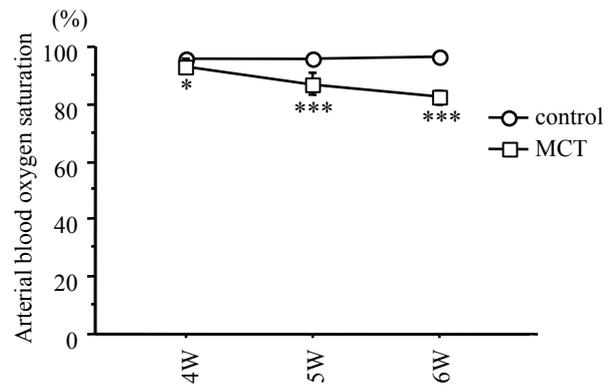


Fig. 6

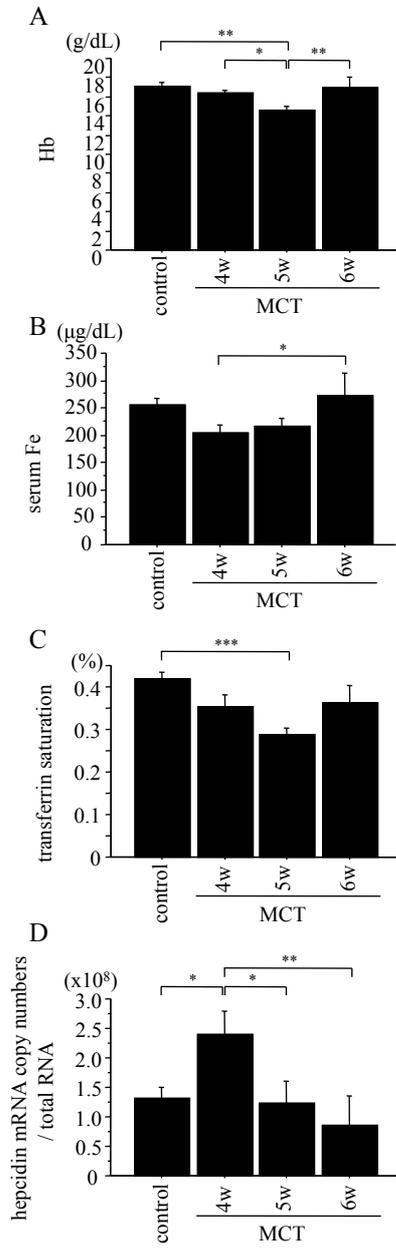


Fig. 7

