S J Forbes
Tissue Fibrosis and Remodelling Laboratory, MRC/University of Edinburgh Centre for Inflammation Research, Edinburgh, UK

Correspondence to: Dr S J Forbes, MRC/University of Edinburgh Centre for Inflammation Research, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK; stuart.forbes@ed.ac.uk

References

1 **Lyra AC**, Soares MB, da Silva LF, *et al*. Feasibility and safety of autologous bone marrow mononuclear cell transplantation in patients with advanced chronic liver disease. *World J Gastroenterol* 2007;**13**:1067–73.
2 **Thorgeirsson SS**, Grisham JW. Hematopoietic cells as hepatocyte stem cells: a critical review of the evidence. *Hepatology* 2006;**43**:2–8.
3 **Terai S**, Ishikawa T, Omori K, *et al*. Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. *Stem Cells* 2006;**24**:2292–8.
4 **Forbes SJ**, Russo F, Rey V, *et al*. Rapid communication: a significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology* 2004;**126**:955–63.
5 **Russo FP**, Alison MR, Bigger BW, *et al*. The bone marrow functionally contributes to liver fibrosis. *Gastroenterology* 2006;**130**:1807–21.
6 **Duffield JS**, Forbes SJ, Constandinou CM, *et al*. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 2005;**115**:56–65.

Inactivation of digestive proteases by deconjugated bilirubin: the possible evolutionary driving force for bilirubin or biliverdin predominance in animals

In humans and some other animals, bilirubin is excreted as the end product of haem originating from haemoglobin and other haemoproteins, and it has been the cause of jaundice and kernicterus.[1 2] The conversion of haem to bilirubin is a two-step process.[1] First, haem is converted to biliverdin by haem oxygenase. Then biliverdin is converted to bilirubin by biliverdin reductase. Interestingly, many animals take biliverdin as the end product of haem, without converting it further to bilirubin.[2] Biliverdin is water soluble and readily excreted through bile or urine.

Why the energy-consuming conversion in some animals of the innocuous biliverdin to the water-insoluble, potentially toxic bilirubin, which needs more resources for transportation and excretion, takes place has been a great puzzle.[1 2] Discovery of the antioxidant property and other properties of bilirubin has suggested that the change of biliverdin to bilirubin might be an advance in evolution.[1] However, this notion is challenged by the fact that biliverdin- or bilirubin-predominant species exist in animals at different stages of evolution, from fishes to mammals.[2] The discrepancy in bile pigments exists even in closely related species. For instance, nutria is a rodent like rats and mice, but the bile of nutria is predominantly biliverdin, in contrast to the predominant bilirubin in rats and mice.[2] Change of biliverdin to bilirubin needs only one enzyme—biliverdin reductase.[1] This enzyme exists in the oldest bacteria like cyanobacteria,[1] far before eukaryotic cells appeared on earth. So what has caused the bilirubin or biliverdin predominance in animals? Here I suggest that the inactivation of digestive proteases is the evolutionary driving force for bilirubin or biliverdin predominance.

Table 1 shows that trypsin and chymotrypsin are significantly inhibited by free bilirubin, but not conjugated bilirubin or biliverdin. The nature of this inhibition and its physiological relevance were further explored for trypsin. Figure 1 shows a Lineweaver–Burk plot of the inhibition of trypsin by free bilirubin. It indicates that the inhibition is non-competitive, suggesting that free bilirubin can inactivate the enzyme.

The effect of free bilirubin on protein digestion by trypsin was further investigated using chymotrypsinogen as the substrate. Bilirubin (10 μmol/l) showed 61% inhibition for the proteolytic activation of α-chymotrypsinogen A to chymotrypsin in a system containing 1 μg/ml trypsin and 1 μg/ml chymotrypsinogen for 30 min. Digestive proteases are inhibited by free bilirubin but not conjugated bilirubin. As bilirubin is secreted from the bile to the lumen mainly in the conjugated form,[2] the digestion of dietary proteins in the upper small intestine would proceed smoothly. Deconjugation of bilirubin by β-glucuronidase from the mucosal cells[3] would form a protective layer on the surface of the gut. A more dramatic deconjugation of bilirubin by the high amounts of β-glucuronidase from gut bacteria[3] would further cause a prompt and effective inactivation of these digestive proteases in the lower intestine. Here we can see the wonderful design of nature that turns a waste byproduct into a precious treasure. The amount of digestive proteases secreted by the pancreas largely depends on the amount of protein in the diet.[4] This would provide an explanation for the observation that bilirubin-predominant species tend to be carnivores or omnivores, while biliverdin-predominant species tend to be herbivores.[2] Large amounts of bilirubin exist in the bile of cats, dogs, opossums, armadillos, alligators, African clawed toads, bullfrogs, mudpuppies, sharks (spiny dogfish), small skates, trout, goosefish, and perch, while biliverdin is the main bile pigment of rabbits, nutrias (rodents that eat water plants), sloths (leaf eaters), birds and tilapia (fish that eat

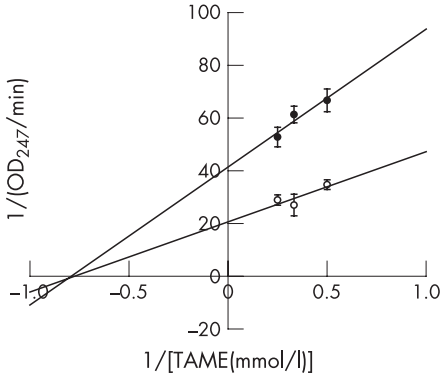

Figure 1 Lineweaver-Burk plot of the inhibition of trypsin by free bilirubin. Trypsin activity was measured using different concentrations of TAME as the substrate with (•) or without (○) 2.5 μM bilirubin. TAME, N_α-*p*-tosyl-L-arginine methyl ester.

Table 1 Effect of free bilirubin, biliverdin and conjugated bilirubin on trypsin and chymotrypsin activity

	Control (μmol/l)	Free bilirubin (μmol/l)				Biliverdin (μmol/l)	Conjugated bilirubin (μmol/l)
	0	1.25	2.5	5	10	10	10
Trypsin							
Increase of OD per min	0.0367 (0.0023)	0.0255 (0.0013)†	0.0197 (0.0018)†	0.0142 (0.0033)†	0.0102 (0.0006)†	0.0459 (0.0025)†	0.0461 (0.0007)†
Test/control	1.00	0.70	0.54	0.39	0.28	1.25	1.26
Chymotrypsin							
Increase of OD per min	0.0218 (0.0030)	0.0166 (0.0025)*	0.0121 (0.0008)†	0.0061 (0.0017)†	0.0002 (0.0006)†	0.0271 (0.0023)*	0.0237 (0.0016)
Test/control	1.00	0.76	0.56	0.28	0.01	1.24	1.09

Data are expressed as mean (SD) of quadruplicate measurements.
*$p<0.05$; †$p<0.001$ versus control.
Trypsin and chymotrypsin activities were measured using N_α-*p*-tosyl-L-arginine methyl ester (TAME) or *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate, respectively, by a method modified from that described by Hummel.[9] Free bilirubin (3 μl 50 X; Sigma, St Louis, Missouri, USA), biliverdin (Frontier Scientific, Logan, Utah, USA) or conjugated bilirubin (Frontier Scientific) was added to a 96-well ultraviolet-transparent microplate. . The final volume of the reaction solution was 150 μl, giving the final concentrations indicated in table 1. For trypsin assay, 72 μl buffer (0.046 M Tris/HCl, 0.0115 M $CaCl_2$, pH 8.1) containing 150 ng trypsin was added to the microplate by multiple pipette, followed by 75 μl of buffer containing 150 nmol TAME to start the reaction. The plate was then read at 247 nm on a microplate reader. For chymotrypsin assay, BTEE was dissolved in dimethylsulphoxide (DMSO) at 4.054 mg/ml, and then added with nine volumes of water to obtain a concentration of 1.07 mmol/l. DMSO was used instead of methanol (which was used in the original paper) because methanol greatly reduces the solubility of free bilirubin. After adding 77 μl buffer (0.080 M Tris/HCl, 0.1 M $CaCl_2$, pH 7.8) containing 150 ng chymotrypsin, the reaction was started by adding 70 μl BTEE solution. Then the plate was read at 256 nm on a microplate reader. Data were collected at 30-second intervals for 5 minutes and expressed as the increase in optical density (OD) per minute in the linear range (the first 2–3 minutes).

algae).[2] Although cattle and sheep are herbivores and their bile consists mainly of bilirubin, the activity of their hepatic biliverdin reductase was just 4–5% of that of the rats,[5] and their bile indeed contains certain amounts of biliverdin,[2] suggesting that they may be in an intermediary state of transition.

Studies have also shown that bile pigments change from bilirubin to biliverdin in fishes after starvation.[6] This further suggests bilirubin is only produced as necessary, which depends in some way on the feeding activities. On the other hand, the energy-consuming conversion of biliverdin to bilirubin suggests that bilirubin may have an important role for the body. An impairment in inactivation of digestive proteases by deconjugated bilirubin may have a causative role in diseases such as inflammatory bowel disease.[7] It would also provide a possible explanation as to why chronic blockage of bile flow in diseases such as primary sclerosing cholangitis is often accompanied by gut damage like inflammatory bowel disease.[8] These areas require further investigation.

Xiaofa Qin

Correspondence to: Dr X Qin, Department of Surgery, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103, USA; qinxi@umdnj.edu

doi: 10.1136/gut.2007.132076

References

1 **McDonagh AF**. Turning green to gold. *Nat Struct Biol* 2001;**8**:198–200.
2 **Cornelius CE**. Comparative bile pigment metabolism in vertebrates. In: Ostrow JD, eds. *Bile pigments and jaundice: molecular, metabolic, and medical aspects*. New York: Dekker, 1986:601–47.
3 **Rod TO**, Midtvedt T. Origin of intestinal beta-glucuronidase in germfree, monocontaminated and conventional rats. *Acta Pathol Microbiol Scand [B]* 1977;**85**:271–6.
4 **Howard F**, Yudkin J. Effect of dietary change upon the amylase and trypsin activities of the rat pancreas. *Br J Nutr* 1963;**17**:281–94.
5 **George JW**, Nulk K, Weiss A, *et al*. Biliverdin reductase-activity in cattle, sheep, rabbits and rats. *Int J Biochem* 1989;**21**:477–81.
6 **Fang LS**. Study on the heme catabolism of fish. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 1987;**88**:667–73.
7 **Qin XF**. Impaired inactivation of digestive proteases by deconjugated bilirubin: the possible mechanism for inflammatory bowel disease. *Med Hypoth* 2002;**59**:159–63.
8 **Qin X**. Primary sclerosing cholangitis and inflammatory bowel disease: where is the link? *Am J Gastroenterol* 2007;**102**:1332–3.
9 **Hummel BC**. A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. *Can J Biochem Physiol* 1959;**37**:1393–9.

E/A ratio alone cannot reliably diagnose diastolic dysfunction in the assessment before and after TIPS

We congratulate Cazzaniga *et al* on their study focusing on the heart (*Gut* 2007;**56**:869–75), an often neglected organ when assessing a patient with cirrhosis. The cardiovascular changes in cirrhosis are complex and measurement of the ratio between the early maximal ventricular filling velocity and the late filling velocity (E/A ratio) after the insertion of a transjugular intrahepatic portosystemic shunt (TIPS) may provide a powerful tool for identifying a poorly responding subgroup of patients.

However, we have certain observations about the study and its conclusions. Specifically, whether it is possible to diagnose diastolic dysfunction (DD) using the E/A ratio alone, given its dependence on loading conditions and age.

The use of echocardiography is the cornerstone of a comprehensive evaluation of DD. However, it is operator dependent and prone to interobserver variation and bias. Lack of an independent observer to verify the measured calculations weakens this study.

Loading conditions are of critical importance when interpreting diastolic function based on the E/A ratio. Any increase in preload will cause an increase in left atrial pressure and hence the early velocity of ventricular filling.

In the above study, all patients showed an increase in E/A ratio, corroborating the view that the E/A ratio changes with an increase in preload (left ventricular end-diastolic volume). Even in the subgroup whose E/A ratio was <1 at 28 days (group 1), the E/A ratio rose to 0.97 (11), which is probably normal and expected for that age group (65 years).

With increasing age there is a physiological decrease in E/A ratio.[1] Although the difference between the groups' ages (65 vs 59) was not statistically significant, this might have been owing to the small sample size.

More relevant to the outcome, may have been the drop in the left ventricular ejection fraction in the group with the poor outcome, despite the increased preload. Hence, rather than DD we could interpret the data as unmasking a deterioration in systolic function.

The assertions about DD might have been made clearer had more ''load-independent'' methods for assessing DD been used.

Colour M-mode echocardiography using the early diastolic flow propagation velocity as the blood flows from the mitral valve to the apex,[2] or mitral annular Doppler tissue imaging measurements of the myocardium during the cardiac cycle[3] are two load-independent methods that can be used, in conjunction with the E/A ratio, to better characterise DD.

In summary, the importance of cardiac dysfunction in patients undergoing TIPS is significant. Although Cazzaniga *et al* may show that the E/A ratio after TIPS is possibly a powerful indicator of prognosis, it cannot be reliably used to diagnose diastolic dysfunction on its own. Load-independent methods such as colour M-mode echocardiography, or Doppler tissue imaging, may help to clarify the contribution of diastolic dysfunction in cirrhosis and may improve patient selection for TIPS.

R D Abeles, D L Shawcross, G Auzinger
Institute of Liver Studies, King's College Hospital London, UK

Correspondence to: Dr G Auzinger, Institute of Liver Studies, King's College Hospital London, Denmark Hill, London SE5 9RS, UK; Georg.Auzinger@kch.nhs.uk

References

1 **McCullough, Khandelwal AK, McKinnon JE, et al**. Outcomes and prognostic factors of systolic as compared to diastolic heart failure in urban America. *Congest Heart Fail* 2005;**11**:6–11.
2 **Chinnaiyan KM**, Alexander D, Maddens M, *et al*. Curriculum in cardiology: Integrated diagnosis and management of diastolic heart failure. *Am Heart J* 2007;**153**:189–200.
3 **Nagueh SF**, Middleton KJ, Kopelen HA, *et al*. Doppler tissue imaging: a non-invasive technique for evaluation of left ventricular relaxation and estimation of filling pressures. *J Am Coll Cardiol* 1997;**30**:1527–33.

Authors' reply

We thank Dr Abeles and colleagues for their comments on our recent paper on the predictive value of E/A ratio in cirrhotic patients with a transjugular intrahepatic portosystemic shunt (TIPS), which gives us the opportunity to extend the discussion on some particular aspects.

We agree that echocardiographic measurement of the E/A ratio can be an imperfect tool for diagnosing diastolic dysfunction compared with new more sophisticated methods such as Doppler tissue imaging. However, when we started our study this new methodology was not available in our centre. Nevertheless, almost all previous studies on cirrhotic cardiomyopathy have used E/A ratio, deceleration time and/or isovolumic relaxation time to diagnose diastolic dysfunction.[1–3] Moreover, Abeles observes that echocardiographic calculations were not verified by an independent observer to avoid operator bias. However, this risk was limited in our study because there was just one echocardiographic operator who was unaware of the clinical conditions of the patients.

The most important concern of Abeles and colleagues is that interpretation of the E/A ratio is affected by two important variables: loading conditions and age. For the first variable, one should take into account that patients with advanced cirrhosis have central hypovolaemia owing to a redistribution of the blood volume from the central to the splanchnic bed, and that TIPS insertion markedly increases the blood volume return to the heart and often corrects central hypovolaemia.[4] This is confirmed by our data, which show a significant increase of the average value of the left ventricular end diastolic volume (LVEDV) after TIPS in both groups of patients. Therefore, measuring the E/A ratio in patients with TIPS may lead to a wrong diagnosis of normal diastolic function (pseudonormalisation effect) but is less likely to lead to a wrong diagnosis of diastolic dysfunction. Abeles and colleagues affirm that the E/A ratio increased after TIPS in all patients, but this is incorrect as the E/A ratio decreased or remained unchanged in 11 of 32 patients. Indeed, we have already reported that TIPS in cirrhotic patients may be able to distinguish between patients who have a true diastolic dysfunction due to ventricular stiffness (those whose E/A ratio did not rise or decrease after TIPS) and those who have an E/A ratio <1 because of circulatory underfilling and an excessively low preload (those whose E/A ratio increases after TIPS).

We also agree that age must be taken into account when interpreting E/A ratios, because they tend to reduce with aging. In our study, however, the average age was not significantly different between the two groups of patients with low or normal E/A ratio after TIPS insertion. In addition, although we observed a marginal inverse correlation between the E/A ratio and age ($p = 0.1$), age was not associated with death at univariate analysis, making it unlikely that the predictive value of the E/A ratio was dependent on aging.

The hypothesis that a deterioration of systolic function rather than of diastolic