Journal of Pathology
J Pathol 2003; **200**: 112–117.
Published online 17 February 2003 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/path.1328

Original Paper

Age-related pseudocapillarization of the human liver

Allan J McLean,[1,2]* Victoria C Cogger,[3] Guan C Chong,[2] Alessandra Warren,[3] Astrid MA Markus,[1] Jane E Dahlstrom[2] and David G Le Couteur[3]

[1]*National Ageing Research Institute and the Department of Medicine, University of Melbourne and the Royal Melbourne Hospital, Parkville, Australia*
[2]*The Canberra Hospital and Canberra Clinical School of the University of Sydney, Garran, Australia*
[3]*Centre for Education and Research on Ageing and ANZAC Research Institute, University of Sydney and Concord RG Hospital, Concord, Australia*

**Correspondence to:*
Allan J McLean, Director, National Ageing Research Institute, PO Box 31, Parkville, Victoria 3052, Australia.
E-mail:
a.mclean@nari.unimelb.edu.au

Received: 12 July 2002
Revised: 12 August 2002
Accepted: 25 November 2002

Abstract

Age-related changes in liver function are important because they may promote susceptibility to adverse drug reactions, neurotoxicity, atherosclerosis, and other important diseases in older people. Age-related changes in the rat hepatic sinusoidal endothelium, termed pseudocapillarization, have been described recently and these may contribute to hepatic impairment. The present study has examined surgical and post-mortem specimens with immunohistochemistry and transmission electron microscopy to determine whether pseudocapillarization also occurs in older humans. The age of the subject, independent of systemic disease or hepatic pathology in surgical and post-mortem samples of human liver, was associated with increased peri-sinusoidal expression of von Willebrand's factor, collagen I, collagen IV, and staining with Masson's trichrome. Electron microscopy revealed significant age-related thickening of the sinusoidal endothelium (young 165 ± 17 nm, middle age 222 ± 11 nm, older 289 ± 9 nm, $p < 0.001$) with loss of fenestrations (young 7.7 ± 0.7 per 10 μm, middle age 3.6 ± 0.5 per 10 μm, older 1.5 ± 0.4 per 10 μm, $p < 0.001$), and age-related deposition of basal lamina and collagen. In conclusion, ageing in humans is associated with morphological changes in the sinusoidal endothelium and space of Disse which are presumptively related to the ageing process and potentially represent an important link between the ageing process and disease susceptibility.

Keywords: ageing; liver; pseudocapillarization; defenestration; electron microscopy; immunohistochemistry; hepatic sinusoid

Introduction

The liver has a well-defined and pivotal role in the metabolism of drugs, disease-producing xenobiotics, and the handling of endogenous substrates such as lipids and hormones. Age-related changes in liver function will therefore have systemic effects, with potential clinical implications including adverse drug reactions [1], susceptibility to neurotoxins [2,3], and atherosclerosis [4].

Ageing in the liver is associated with reductions in mass and blood flow of the order of 30–50% [1,5–7]. These simple changes influence hepatic metabolism because clearance is variously dependent on blood flow and liver mass [1]. Nevertheless, motivated by the observation that such changes cannot fully explain the age-related impairment of hepatic drug metabolism [1], we examined the structures lying between the blood and the hepatocyte that could impose a barrier to substrate transfer, including the sinusoidal endothelium and space of Disse.

In the rat, old age was found to be associated with marked changes in the hepatic sinusoidal endothelium and extracellular space of Disse. These changes were termed pseudocapillarization because the ageing sinusoidal endothelium had become more like the capillaries seen in systemic vascular beds. Specifically, it had lost its porous liver sieve structure seen in normal young livers and had become thickened with extravascular basal lamina and collagen deposits, and there was increased expression of collagen and von Willebrand's factor (vWf) [8]. These changes in structure may impair the transfer of substrates including oxygen [8], chylomicron remnants [4], neurotoxins [3], and drugs [1,9] from sinusoidal blood into the hepatocyte.

In order to determine whether age-related pseudocapillarization occurs in humans, we investigated the effects of age on the sinusoidal endothelium and space of Disse in specimens of liver from persons without primary or secondary liver pathology.

Materials and methods

Specimens of human liver

Human liver specimens were collected for assessment during hepatobiliary surgery or at post-mortem. Analyses were confined to persons and livers without disease or specific organ pathology. The study was approved by the ACT Human Ethics Committee and complied

with the Declaration of Helsinki; informed written consent was given by the surgical subjects.

Light microscopy and immunohistochemistry

Liver specimens were fixed in 10% buffered formalin and embedded in paraffin wax. All specimens were examined under blinded conditions by a pathologist (JD) and only those considered to be free of disease by standard pathological criteria were included. Immunohistochemistry was used to study vWf, collagen I, collagen IV, and vimentin. Vimentin was used as a control for the quality of the tissue. Masson's trichrome stain was also performed. In total, three age groups were studied: young, 0–30 years ($n = 20$); middle age, 31–60 years ($n = 20$); and older, 61–100 years ($n = 20$).

Sections (3 μm) were mounted on slides coated with 3-amino-propyl-triethoxysilane. After deparaffinization in xylene and rehydration, endogenous peroxidase was blocked with 3% aqueous hydrogen peroxidase. Antigen retrieval was performed by either application of proteinase K (DAKO, Australia) for 2 min (vWf, collagen 1) or immersion in boiling EDTA/citrate buffer (0.01M) in a microwave for 15 min (vimentin, collagen IV). Incubation with either 10% normal horse serum or 10% normal goat serum (Vector Laboratories, CA, USA) was used to prevent non-specific staining.

Primary antibodies were mouse anti-human vWf and mouse anti-human collagen IV (DAKO, Australia), goat anti-human collagen I (Southern Biotechnology, AL, USA), and mouse anti-human vimentin (BioGenex, CA, USA). Biotinylated horse antiserum and streptavidin labelled with horseradish peroxidase (Vector Laboratories, CA, USA) were used as the detection system. Peroxidase activity was revealed using 3,3′-diaminobenzidine (Merck, Australia). Sections were counterstained in Harris haematoxylin and alcoholic ammonia, dehydrated, cleared in xylene, and coverslipped. Examination of the slides was performed separately by four investigators (AM, JD, AW, DLC). The intensity of the staining pattern was scaled between 0, +, and ++.

Electron microscopy

A total of 81 human liver specimens were obtained for electron microscopy studies (10 surgical, 71 post-mortem). After exclusion of specimens that had co-existent pathology and/or autolytic changes, six surgical specimens and 41 post-mortem specimens were examined. The post-mortem specimens were divided into three age groups: 0–30 years ($n = 9$), 31–60 years ($n = 12$), and 61–100 years ($n = 20$). The cause of death in those over 60 years was predominantly vascular (65%), whereas in subjects less than 60 years, the most common causes of death were suicide and trauma (65%). The surgical specimens studied included four from subjects between 31 and 60 years (all female) and a 73-year-old female and an 82-year-old male.

Liver tissue was fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for approximately 5 h and then embedded in Spurr's resin. Five or more blocks were prepared from each liver specimen. Blocks were sampled at random for light microscopic assessment. Two blocks per liver were finally studied, selected randomly from those satisfying requirements for quality of fixation and tissue integrity.

Fifty ultrathin (70–90 nm) sections were taken from each block for initial scanning (magnification ×8000) using a Philips CM 120 transmission microscope. A technically eligible pool of sections resulted from the low-power scanning process and ten sections were chosen at random for ultrastructural measurement from each liver. In each of the ten sections, representative fields were chosen by an operator who was unaware of the tissue category.

Electron microscopic measurements (magnification ×17 000) of the thickness of the sinusoidal endothelial cells were made using Mitutoyo vernier dial calipers. The number of fenestrations was counted manually. Collagen deposition and basal lamina formation were assessed.

Statistics

The results given are expressed as mean ± SEM. Comparison of the three age groups (young 0–30 years, middle age 31–60 years, and old 61–100 years) was performed using ANOVA for electron microscopy data and χ^2 for immunohistochemistry data. *Post-hoc* comparisons were performed using the Bonferroni correction. Differences were considered significant when p was less than 0.05.

Results

Immunohistochemistry

The results are summarized in Table 1 and representative stains are shown in Figure 1. Vimentin staining was positive in all specimens. Significant staining (+, ++) for collagen I, collagen IV, and vWf was more frequent in the older age group. The age differences were most apparent in the peri-portal region (zone 1) region. Masson's trichrome stain also revealed perisinusoidal staining in the older livers.

Electron microscopy

Representative electron micrographs are shown in Figure 2. Transmission electron microscopic examination of the specimens from younger subjects revealed a thin sinusoidal endothelium containing fenestrations. The space of Disse showed no basal lamina and limited collagen deposits. Specimens from subjects older than

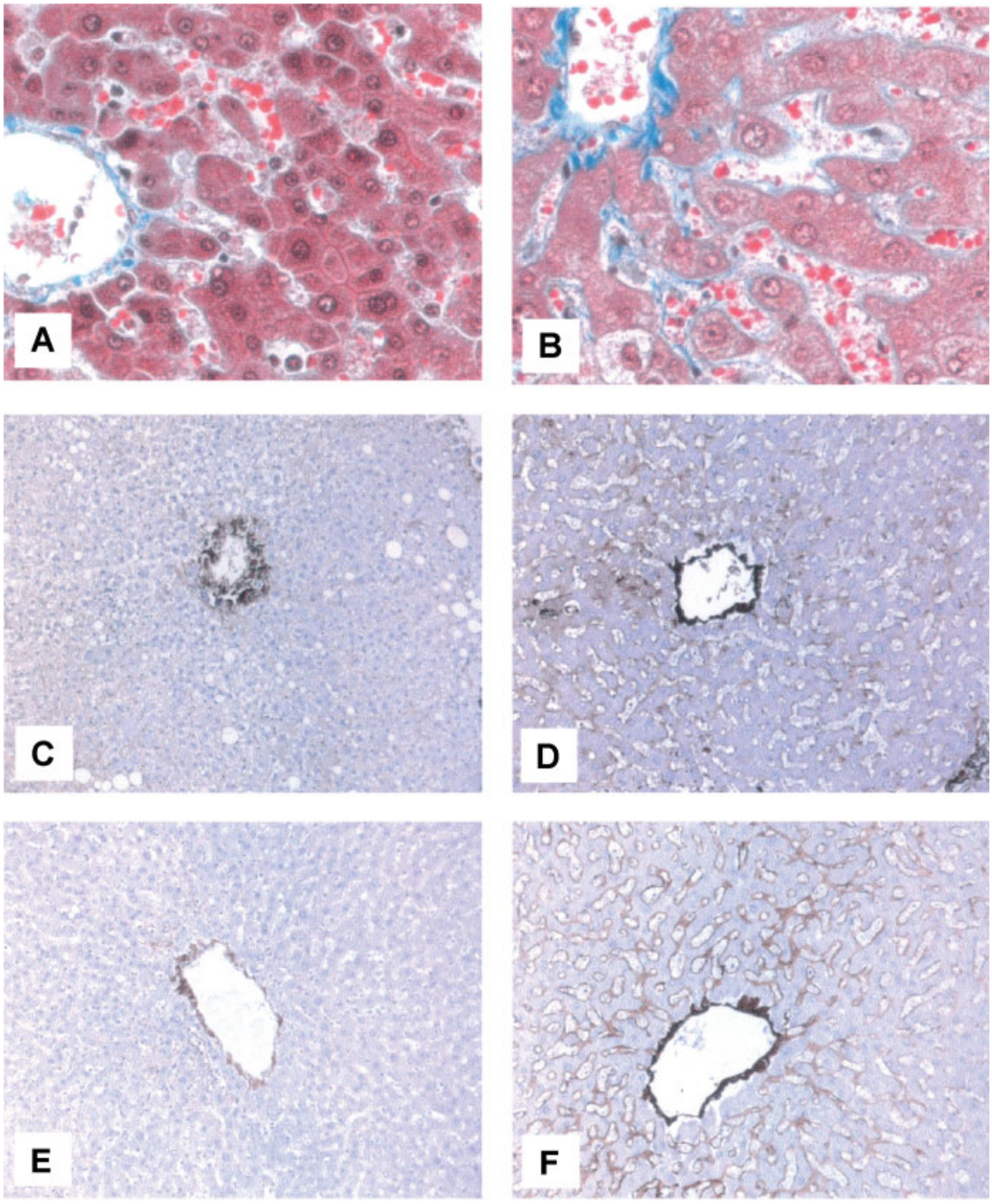

Figure 1. Light microscopy of livers from young and old livers, showing the central vein and pericentral hepatocytes. Peri-sinusoidal staining in the livers from old subjects is apparent for all stains. (A) Young liver, Masson's trichrome; (B) old liver, Masson's trichrome; (C) young liver, von Willebrand's factor; (D) old liver, von Willebrand's factor; (E) young liver, collagen IV; (F) old liver, collagen IV

30 years revealed graded thickening of the endothelium and a reduction in the number of fenestrations. There was development of a basal lamina in some of the older specimens. Although these changes were apparent in the specimens from the middle-aged subjects, the changes were most pronounced in the oldest subjects.

Stereometric analysis revealed an age-related increase in the endothelial thickness [165 ± 17 nm (0–30 years), 222 ± 11 nm (31–60 years), and 289 ± 9 nm (>60 years); $p < 0.001$]. The same pattern was seen when only the surgical specimens were analysed [218 ± 21 nm (31–60 years), 285 ± 26 nm (>60 years); $p < 0.05$].

A reduction in endothelial porosity was also found with age. The number of fenestrations per 10 µm fell from 7.7 ± 0.7 in the 0–30 years age group to 3.6 ± 0.5 in the 31–60 years age group and 1.5 ± 0.4 in those over 61 years ($p < 0.001$). Collagen was present in the space of Disse in 25% of young, 88% of middle-aged, and 65% of old subjects ($p < 0.001$). Basal lamina was not seen in the livers from young

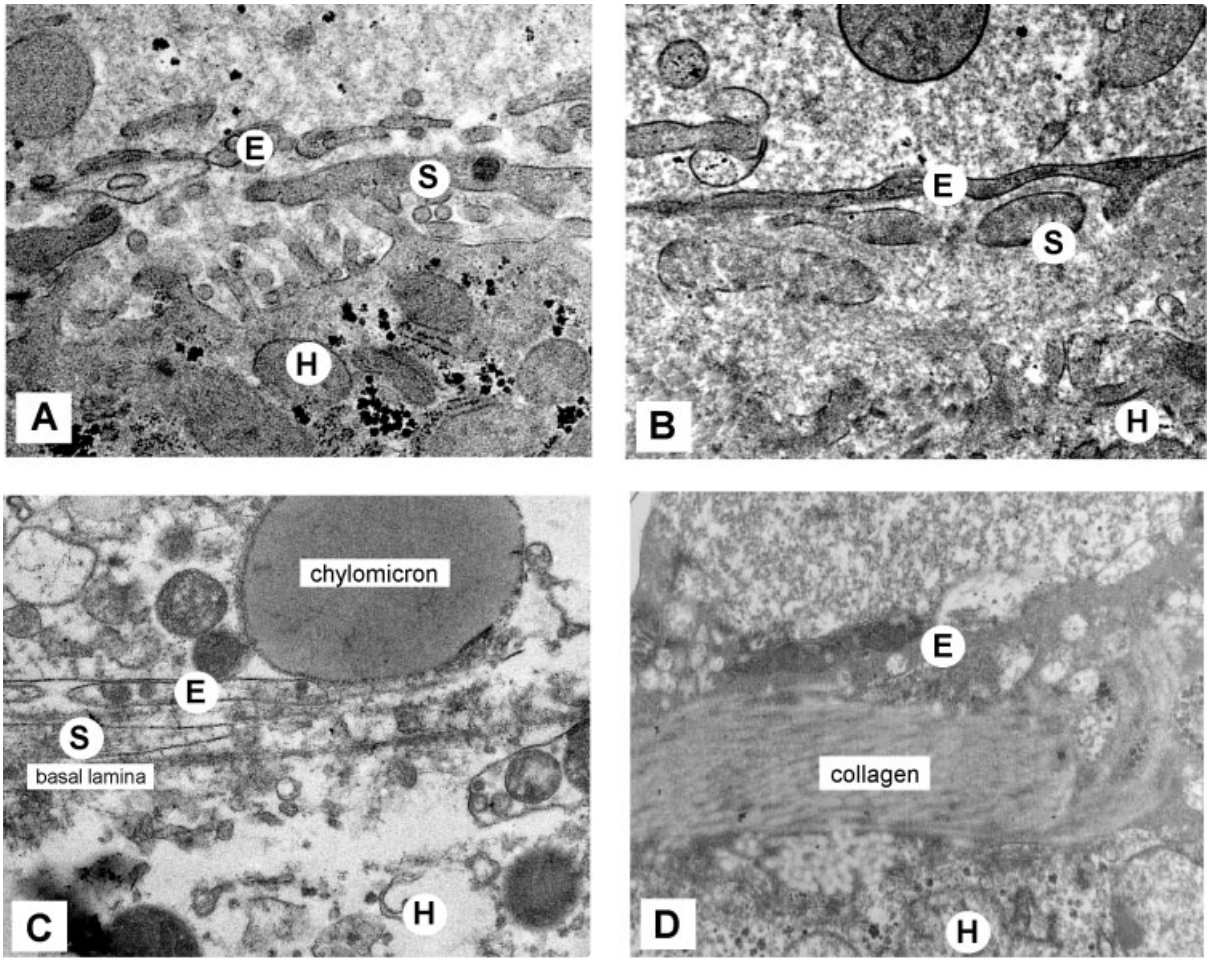

Figure 2. Transmission electron microscopy of human livers (17 000 × magnification). (A) Transmission electron micrograph of the liver from a middle-aged human. The endothelium (E), which separates the hepatocyte (H) from the sinusoidal lumen, is thin and contains fenestrations. The space of Disse, which lies between the endothelium and the hepatocyte, contains a hepatic stellate cell (S) and microvilli. (B) Transmission electron micrograph of the liver from a middle-aged subject. The endothelium is defenestrated. (C) Transmission electron micrograph of the liver from an old subject. There is basal lamina beneath the overlapping layers of sinusoidal endothelial cells. A chylomicron is lying in the sinusoidal lumen. (D) Transmission electron micrograph of the liver from an old subject. The space of Disse contains collagen and the endothelium is thickened

subjects; it was present in two livers from middle-aged subjects and four of the old subjects.

Table 1. Percentage of specimens with significant staining for immunohistochemical markers of capillarization (von Willebrand's factor, collagen I, collagen IV) and Masson's trichrome stain ($n = 20$ in each group)

	Peri-portal (zone 1)			Peri-central (zone 3)		
	Young	Middle age	Old	Young	Middle age	Old
Von Willebrand's factor	0	10	55*	0	25	95*
Collagen I	0	5	25†	15	15	25
Collagen IV	20	20	55†	25	35	65†
Masson's trichrome	0	25	25†	5	35	25

* $p < 0.0001$.
† $p < 0.05$.

Discussion

Old age in humans was found to be associated with marked structural and immunohistochemical changes in the sinusoidal endothelium and space of Disse in human livers assessed as normal by normal macroscopic and light microscopic pathological criteria, ie free of specific pathology and significant post-mortem autolytic effect. The changes are qualitatively the same as those previously reported in the liver of the rat as part of the newly described age-related change termed pseudocapillarization [7,8].

On electron microscopy, old age was associated with thickening of the sinusoidal endothelium, loss of fenestrations, development of basal lamina, and extravascular deposition of collagen. Immunohistochemical analysis revealed an age-related increase in the expression of von Willebrand's factor and collagen. These antigens are not expressed to any extent in the normal liver but are up-regulated in hepatic

fibrosis, cirrhosis, and the ageing rat liver [8]. Overall, the age-related changes that occur in the human liver are very similar to those reported previously in the rat liver. Recently, it has been reported that ageing in the human lung is associated with increased expression of vWf in all blood vessels and increased expression of another endothelial marker, CD34, in veins and arteries [10].

There are a number of limitations on our data from humans. First, satisfactory scanning electron microscopic analysis of the liver tissue was not technically possible, so we could not apply the best quantitative approach to measuring defenestration of the sinusoidal endothelium. This method can usually be performed only when the liver has been perfused with fixative, which is technically prohibitive in human liver samples. Nevertheless, transmission electron microscopy did show a statistically significant reduction in the number of fenestrations. Second, while the results from the surgical specimens were completely congruent with the results from post-mortem specimens and there were surgical samples across the age range, the bulk of our samples were from post-mortem specimens, raising the question of the possible contribution of post-mortem change to the findings in liver. To overcome this problem, we excluded from analyses all post-mortem samples with significant evidence of autolysis. Importantly, immunohistochemical results are not influenced by these considerations and the development of basal lamina and collagen are clearly not post-mortem artefacts. Accordingly, the human changes of pseudocapillarization appear to be age-related rather than an artefact of the tissue source.

There is always the inherent problem in the field of ageing biology of separating disease processes from the ageing process itself [11]. Recognizing this, we carried out an analysis of whether there were systematic differences between the results of patients who died from violence or vascular disease. Although the numbers were small for the older group, the age-related trends were the same. Thus, the conclusion remains that age is associated with changes in hepatic sinusoidal endothelium, apparently independent of underlying pathology. It becomes a semantic issue as to whether the changes represent some occult pathological process that has not been previously described. Regardless of putative pathogenic mechanisms, the mechanisms of change underlying hepatic pseudocapillarization are likely to represent processes inherent in human ageing.

These changes of pseudocapillarization have important implications, whether secondary to an age-related pathological process or intrinsic to ageing. At a minimum, it is important that pathologists are aware that microvascular abnormalities are frequent in livers from older people. However, we believe that there are systemic functional and pathological implications. Thickening of the endothelium and deposition of extravascular collagen might impair the transfer by diffusion of substrates such as oxygen [8] and drugs [9]. In fact, there is evidence for intrahepatocytic hypoxia in aged rats [8] and in hepatic cirrhosis, where similar changes also occur in the endothelium [12–14]. These changes will impair the hepatic handling of drugs, xenobiotics, and endogenous substrates, thus predisposing older people to adverse drug reactions and diseases with toxic pathogenesis [2,3]. Fenestrations are also required for the passage of chylomicron remnants from the blood into the space of Disse for binding LDL receptors [15]. Loss of fenestrations with age will impede the uptake of chylomicron remnants, contributing to hyperlipidaemia and vascular disease [4].

In conclusion, in our study of humans whose livers were free of recognized pathology, age was associated with the development of abnormalities in the hepatic sinusoidal endothelium and space of Disse that are very similar to those reported in the rat. These changes are presumptively related to the ageing process rather than recognized pathological processes and may promote age-related susceptibility to adverse drug reactions, atherosclerosis, and other diseases in older humans where the pathogenesis may be linked to impaired hepatic detoxification or metabolism.

Acknowledgements

We gratefully acknowledge the assistance of Dr Alex Fisher, Professor Peter Herdson, and Dr Sanjiv Jain (collection and processing of surgical specimens); Professor Robin Fraser (analysis of electron microscopy results); and Dr Annick Ansselin (electron microscopy methods). This study was funded by grants from the National Health and Medical Research Council of Australia and the Department of Veterans' Affairs, with additional support from the University of Sydney SESQUI Grant, Ageing and Alzheimer's Research Foundation, and the Private Practice Trust Fund, Canberra Hospital.

References

1. Le Couteur DG, McLean AJ. The aging liver: drug clearance and an oxygen diffusion barrier hypothesis. *Clin Pharmacokinet* 1998; **34**: 359–373.
2. Le Couteur DG, Muller M, Yang MC, Mellick GD, McLean AJ. Age–environment and gene–environment interactions in the pathogenesis of Parkinson's disease. *Rev Environ Health* 2002; **17**: 51–65.
3. Yang MC, McLean AJ, Le Couteur DG. Age-related alteration in hepatic disposition of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and pesticides. *Pharmacol Toxicol* 2002; **90**: 203–207.
4. Le Couteur DG, Fraser R, Cogger VC, McLean AJ. Hepatic pseudocapillarisation and atherosclerosis in ageing. *Lancet* 2002; **359**: 1612–1615.
5. Schmucker DL. Aging and the liver: an update. *J Gerontol* 1998; **53A**: B315–B320.
6. Wynne HA, Cope LH, Mutch E, Rawlins MD, Woodhouse KW, James OFW. The effect of age upon liver volume and apparent liver blood flow in healthy man. *Hepatology* 1989; **9**: 297–301.
7. Jansen PL. Liver disease in the elderly. *Best Pract Res Clin Gastroenterol* 2002; **16**: 149–158.
8. Le Couteur DG, Cogger VC, Markus AM, *et al*. Pseudocapillarization and associated energy limitation in the aged rat liver. *Hepatology* 2001; **33**: 537–543.
9. Fenyves D, Gariepy L, Villeneuve JP. Clearance by the liver in cirrhosis: I. Relationship between propranolol metabolism *in vitro*

and its extraction by the perfused liver in the rat. *Hepatology* 1993; **17**: 301–306.
10. Muller AM, Skrzynski C, Nesslinger M, Skipka G, Muller KM. Correlation of age with *in vivo* expression of endothelial markers. *Exp Gerontol* 2002; **37**: 713–719.
11. Lipman RD, Dallal GE, Bronson RT. Lesion biomarkers of aging in the B6C3F1 hybrid mice. *J Gerontol* 1999; **54A**: B466–B477.
12. Harvey PJ, Le Couteur DG, McLean AJ. Hepatic oxygenation supplementation as therapy in cirrhotic liver disease. *Gastroenterology* 1999; **117**: 1025–1029.
13. Le Couteur DG, Hickey H, Harvey P, Gready J, McLean AJ. Hepatic artery flow and propranolol metabolism in the perfused cirrhotic rat liver. *J Pharmacol Exp Ther* 1999; **289**: 1553–1558.
14. McLean AJ, Morgan DJ. Clinical pharmacokinetics in patients with liver disease. *Clin Pharmacokinet* 1991; **21**: 42–69.
15. Fraser R, Dobbs BR, Rogers GW. Lipoproteins and the liver sieve: the role of fenestrated sinusoidal endothelium in lipoprotein metabolism, atherosclerosis, and cirrhosis. *Hepatology* 1995; **21**: 863–874.

