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# Ultrastructural and cytochemical changes in the liver of primary biliary cirrhosis patients

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**Key words:** human liver, ultrastructure, cytochemistry

**ABSTRACT:** The aim of this paper is to establish whether there are cytochemical or ultrastructural alterations in the hepatocytes of patients with primary biliary cirrhosis (PBC) at stages I and II compared with the biopsies from individuals with normal liver. Cytochemical technique with ATP as substrate, transmission electron microscopy (TEM) and freeze fracture were used for the studies.

In the normal liver biopsies the ultrastructural cytochemical localization of the enzymatic activity was clearly shown in the bile canaliculi. In the PBC biopsies, the enzymatic activity is increased in the bile canaliculi and is also present in the lateral membranes of the hepatocyte.

TEM of the lateral surface of the hepatocyte in normal livers showed a smooth surface without microvilli but in PBC livers a large number of microvilli were seen in the lateral membranes. The Golgi apparatus in these patients was localized not only near the canaliculi (normal livers) but also in front of the microvilli. Freeze-fracture showed normal features in the bile canaliculus junctions of the PBC patients.

We suggest that the localization of the enzymatic reaction, microvilli and Golgi apparatus at the PBC hepatocyte lateral membranes may represent a compensatory mechanism for derivation of bile flow and other components from the hepatocyte to the intercellular space.

## Introduction

Hepatobiliary transport processes are essential for the production of bile. Both hepatocytes and cholangiocytes participate in hepatobiliary transport. The human liver secretes approximately 600 to 800 ml of bile per day. Bile is primarily formed by hepatocytes and is then modified downstream by admixture of ductular bile as well as by reabsortive mechanisms. A fraction of this hepatic bile is concentrated in the gallbladder and is then delivered at intervals into the duodenum. Bile plays a central role in a broad range of physiologic function including intestinal absorption of nutrients, excretion of endobiotics and xenobiotics, and maintenance of intestinal immunity. Hepatocytes have polarized transport systems from the sinusoidal and basolateral domains to the canalicular (apical) domain (Alvaro *et al*., 2000). This is a very complex process and it is regulated by multiple transporters at the basolateral and apical membranes.

The basolateral hepatocyte membrane expresses the predominant bile salt uptake system, the Na+-taurocholate cotransporting polypeptide (NTCP) (Baiocchi *et al*., 1999; Bolder *et al*., 1997; Dickson *et al*., 1979), as well as representatives of the organic anion transporting proteins (OATPs) (Eckhardt *et al*., 1999), the organic cation transporters (OCTs) (Giacomini, 1997), and the or-

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ganic anion transporters (OATs) (Kullack-Ublick *et al*., 1995, 1996, 1997). Basolateral efflux of organic anions is induced via the multidrug resistance proteins MRP1, MRP3, MRP5 and MRP6. MRP3 is ATP-dependent, and is upregulated in cholestasis (König *et al*., 1999). Efflux of bile salts, glutathione and glucuronic acid conjugates across the canalicular membrane is mediated by the bile salt export pump (BSEP) (Gerloff *et al*., 1998; Suchy *et al*., 1997; Stieger *et al*., 1992) and by MRP2 (Paulusma and Oude Elferink, 1997; Keppler and Köning, 1997; Jedlitschky *et al*., 1996). Both systems are ATP-dependent. The multidrug resistance gene product MDR3 (Smith *et al*., 1994; van Helvoort *et al*., 1996; Jacquemin *et al*., 2001) is critical for biliary phospholipid secretion, whereas MDR1, (Kullack-Ublick *et al*., 2000) is involved in the transport of cytotoxic cations. Chloride/bicarbonate exchange is mediated by the anion exchanger 2 (AE2), (Kullack-Ublick *et al*., 2000). Ductular bile is modified by chloride channels such as the cystic fibrosis transmembrane conductance regulator (CFTR) and by AE2 (Baiocchi *et al*., 1999).

The canalicular secretion of bile salts mediated by the "bile salt export pump" (BSEP) and the secretion of phospholipid mediated by MDR3 are entirely ATP-dependent.

Alterations of some of these transporters have been observed in diverse cholestatic diseases (Jacquemin *et al*. 1999; Leevy *et al*., 1997 and Bossard *et al*., 1993).

Primary biliary cirrhosis (PBC) is an immune-mediated chronic cholestatic liver disease characterized by progressive destruction of the intrahepatic bile ducts leading to cirrhosis. Virtually all patients with PBC have antibodies reactive to mitochondrial antigens (AMA). Their principal target is the $E_2$ subunit of the pyruvate dehydrogenase complex (P DC- $E_2$) (Mackay and Gershwin, 1998).

The aim of this paper is to establish whether there are cytochemical or ultrastructural alterations in the hepatocytes of patients with primary biliary cirrhosis at stages I and II compared with the biopsies from individuals with normal liver.

## Material and Methods

### *Cytochemistry*

Normal and PBC liver biopsies (stages I-II, n = 10), obtained to rule out concomitant hepatic pathology according to ethical rules, were used for the studies. The ultrastructural cytochemical method of Puvion *et al*. (1976) was used to detect the ATP dependent reaction. Two cm long and 2 mm thick cylindrical liver biopsies were fixed for 15 min in 0.25% glutaraldehyde in 0.1M cacodylate buffer pH7.2, washed in the same buffer and sectioned with a razor blade in 1.2 mm thick sections. These sections were incubated for 1 h at 37°C in the reaction medium consisting of 10 mM MgCl, 1 mM ATP (Sigma St Louis Mo) and 3.6 mM Pb $(NO_3)_2$ in 0.08 M. Tris-maleate buffer pH 7.2. After incubation, the sections were washed with this buffer, postfixed for 60 min in 2.5% glutaraldehyde in cacodylate buffer, washed overnight. Then, samples were fixed in 1% osmiun tetroxide and processed as described below for electron microscopy.

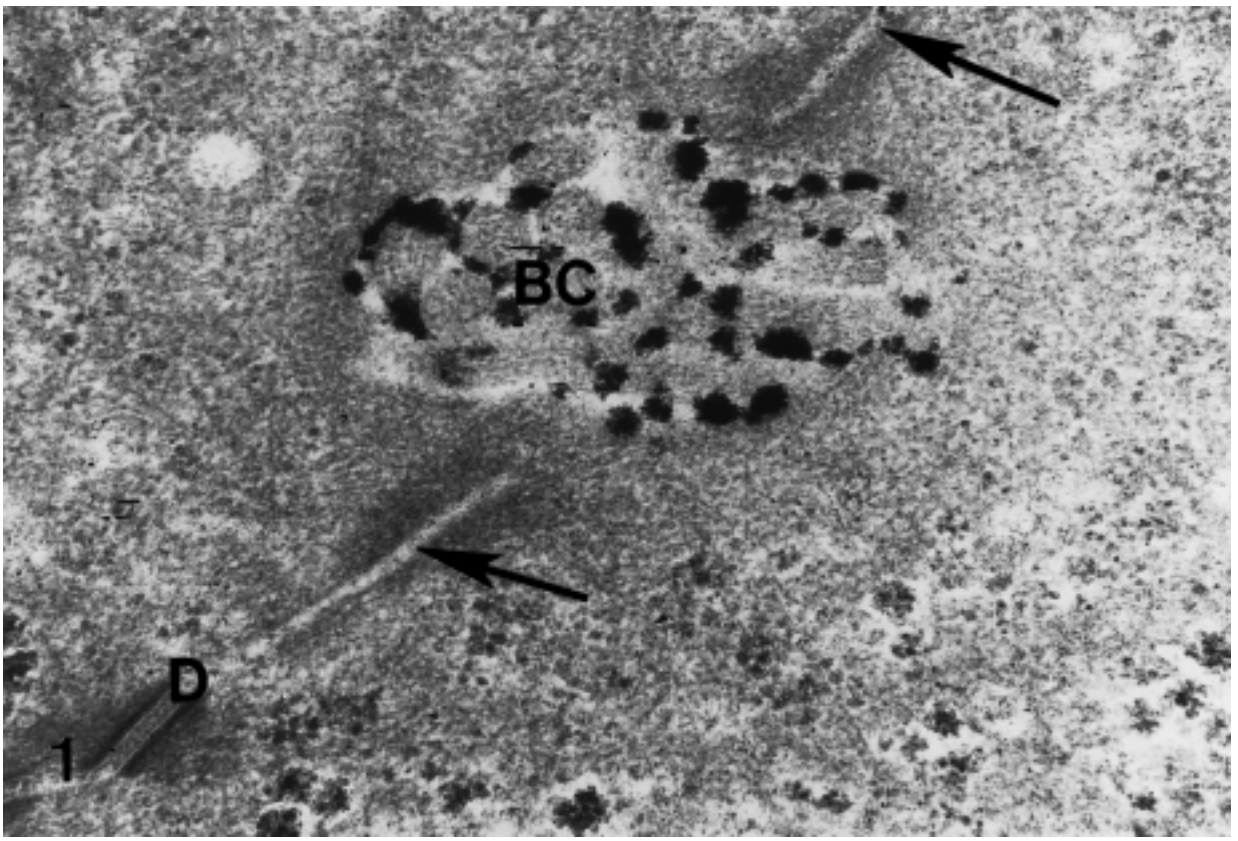
### *Transmission Electron Microscopy*

Liver sections (1-2 mm thick) were fixed in 5% glutaraldehyde in 0.1M cacodylate buffer pH 7.4, refixed in 1% osmium tetroxide, dehydrated in ethanol and acetone, and embedded in Epon. The ultrathin sections were mounted on grids, stained with uranyl-lead and examined in a Siemens Elmiskop IA electron microscope.

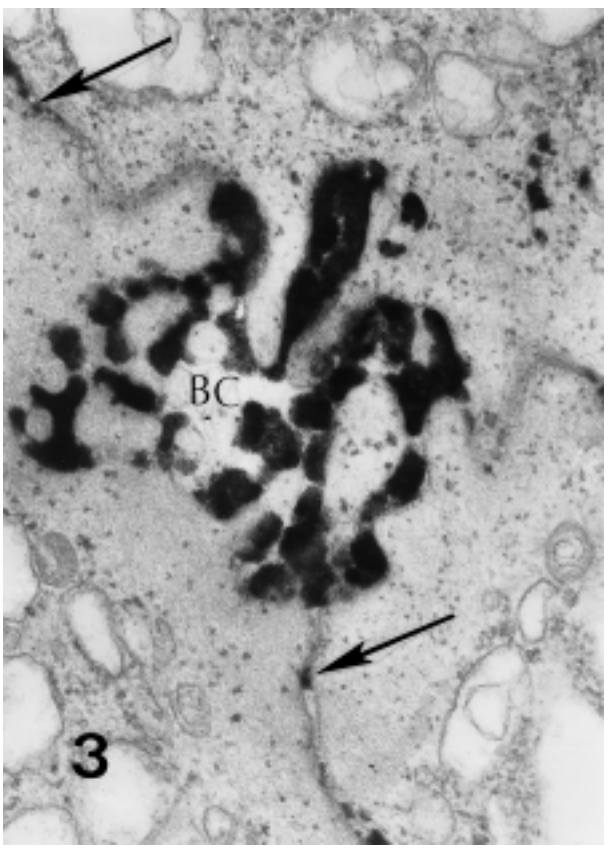
**FIGURE 1.** Normal liver. Cytochemical reaction is localized in bile canaliculus (BC) and absent in the intercellular space and intermediate junction (arrows). Desmosome (D). X 33,500

**FIGURE 2.** Liver with PBC. Cytochemical reaction is localized in the intercellular space lateral wall of the hepatocytes. X 26,500

**FIGURE 3.** Liver with PBC. Cytochemical reaction is localized in both bile canaliculi (BC) and lateral wall of hepatocytes (arrow). X 46,500


BC
D
1


2


BC
3

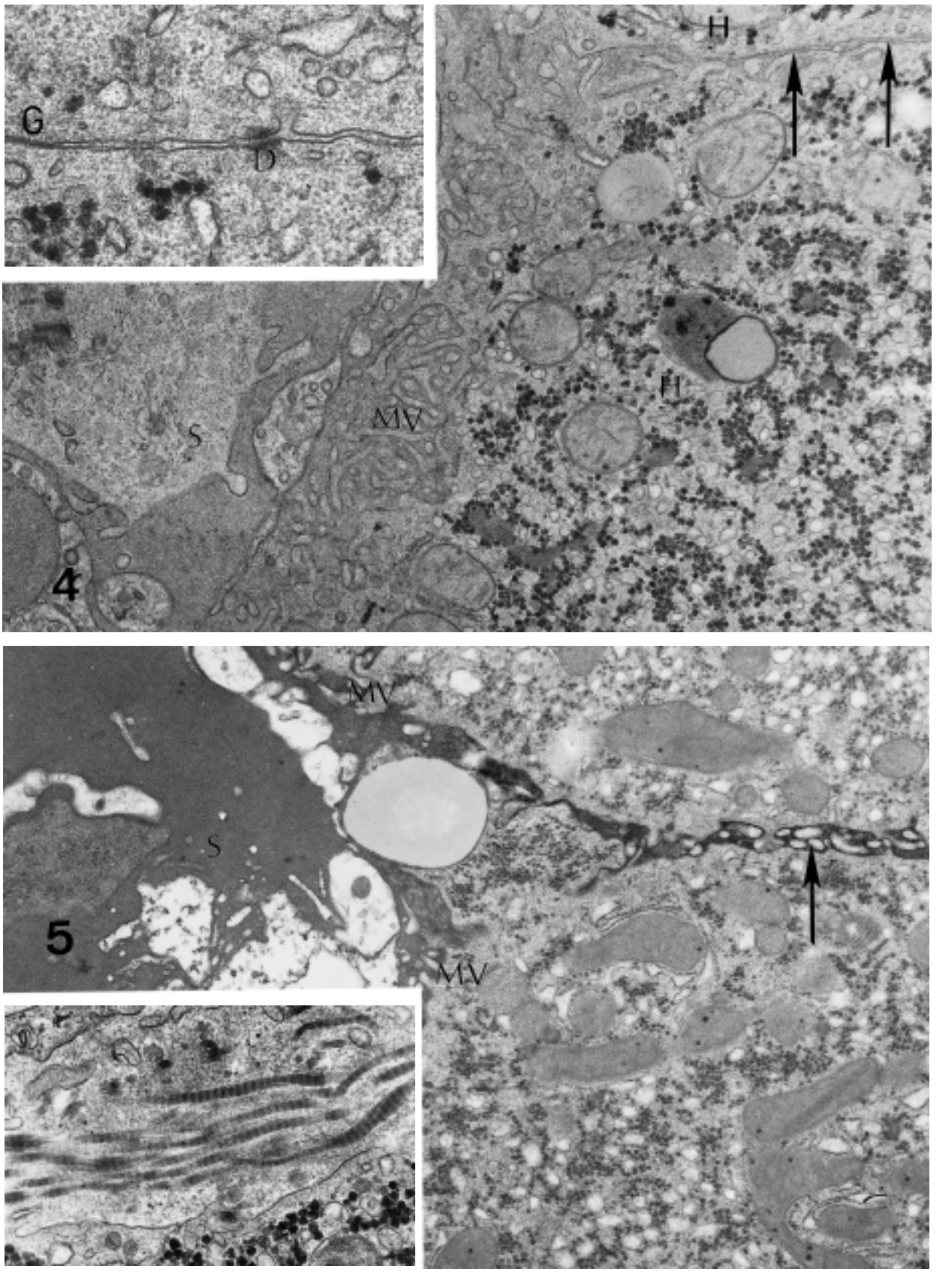
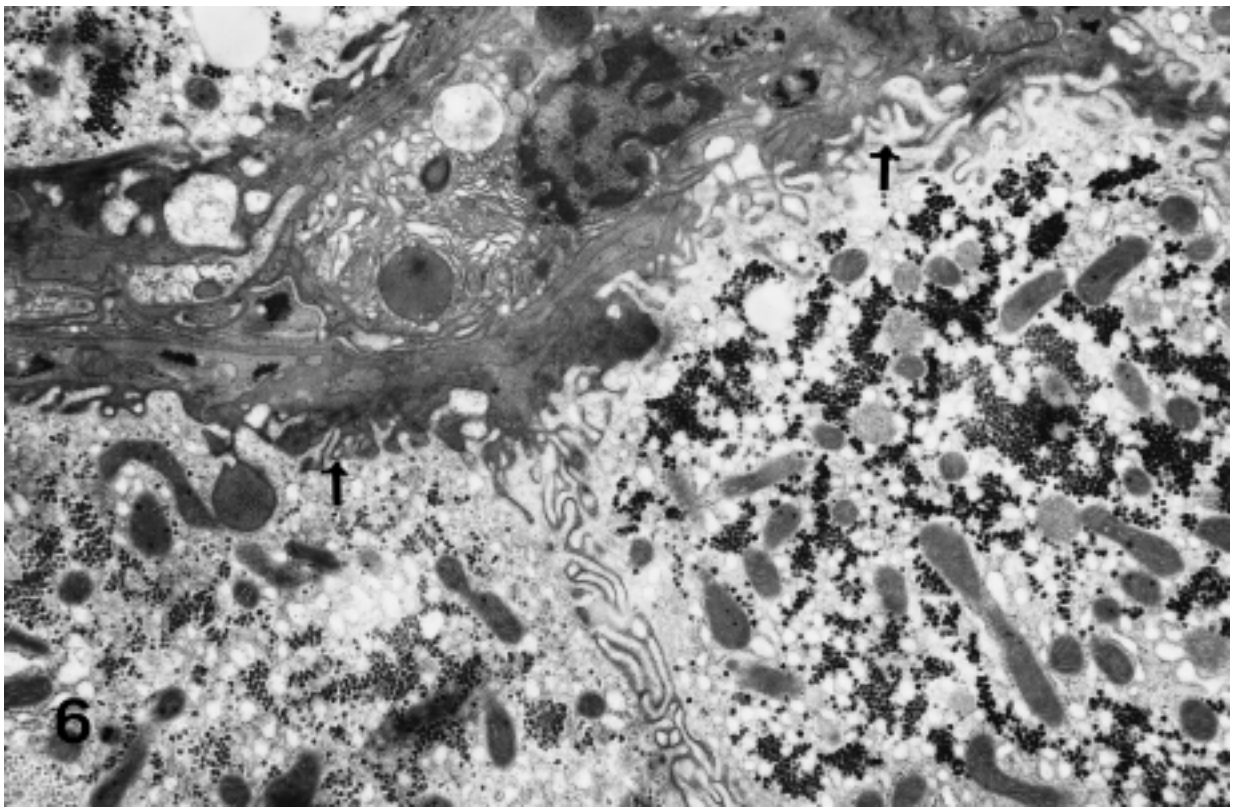



**FIGURE 4.** Normal liver. Part of a sinusoid (S) and numerous microvilli in the space of Disse (MV) of two hepatocytes (H), note the smooth lateral walls (arrows). X 22,800. Inset: Portion of the smooth lateral walls of two hepatocytes. Gap junction (G). Desmosome (D). X 35,000

**FIGURE 5.** Liver with PBC. Part of sinusoid (S). Few microvilli emerge to the space of Disse from the adjacent hepatocyte surface (MV). The intercellular space (arrow) shows the presence of microvilli. X 10,000. Inset: Collagen fibers are present in the space of Disse. X 20,000



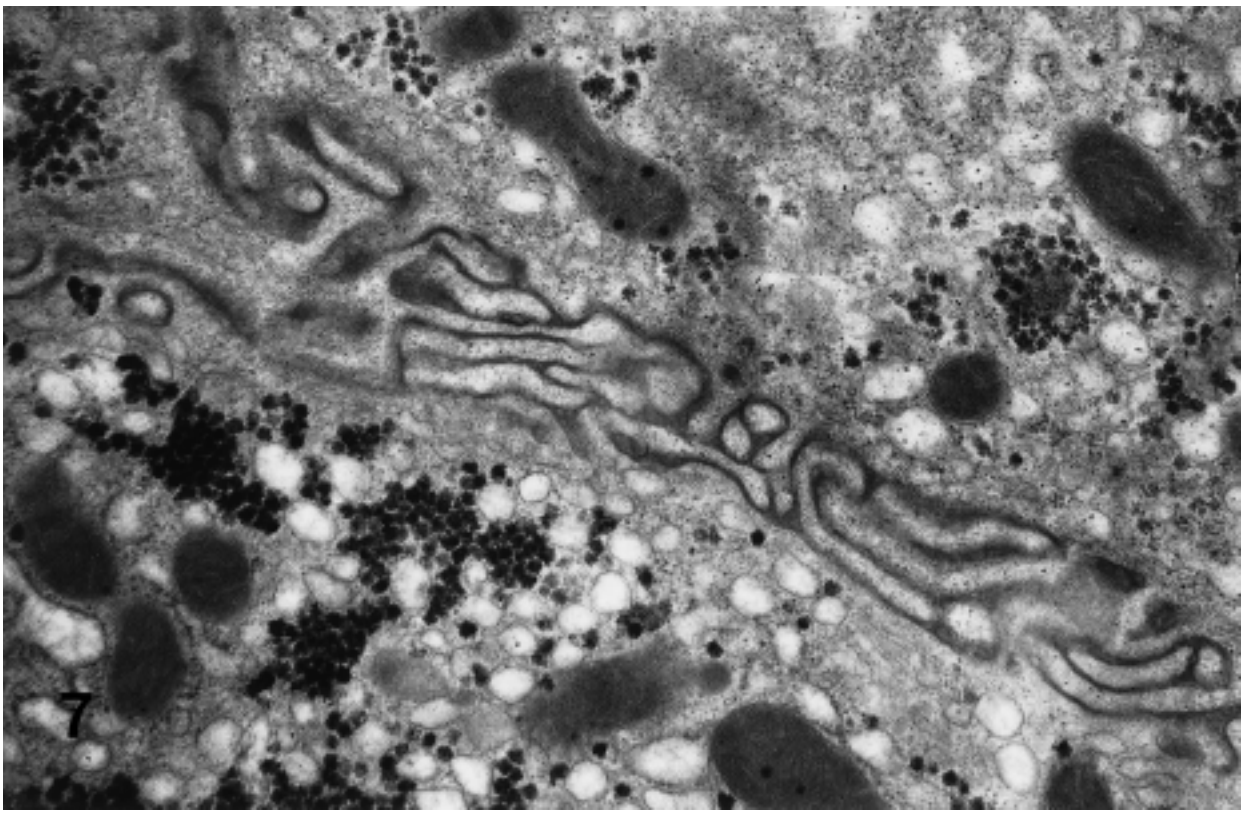




**FIGURE 6.** Liver with PBC. The hepatocyte surface adjacent to the sinusoid shows dissorganized and few microvilli (arrows). The lateral intercellular membranes present numerous microvilli. X 5,000
**FIGURE 7.** Liver with PBC. The same biopsy as Fig. 6 with higher magnification. Numerous microvilli are seen in the lateral membranes between two hepatocytes. X 25,000

*Freeze Fracture Technique*

Liver biopsy sections (2-3 mm thick) were fixed for 4 h in 5% glutaraldehyde in 0.1M cacodylate buffer pH 7.4, washed in this buffer, treated with 30% glycerol in the same buffer (3 changes of 30 min), mounted on gold discs and frozen in freon and liquid nitrogen. Then the samples were fractured at -104°C in a Balzer equipment with platinum and carbon evaporation at an angle of 45° and 90°, respectively. Replicas were mounted on copper grids without membrane and studied in the Siemens Elmiskop IA electron microscope.

## Results

In the normal liver biopsies the ultrastructural cytochemical localization of the enzymatic activity is clearly shown in the bile canaliculi. No activity is observed in the lateral cell membrane (Fig. 1).

In the PBC biopsies, the enzymatic activity is increased in the bile canaliculi and is also present in significant amounts in the lateral membranes of the hepatocyte (Figs. 2 and 3).

The ultrastructure of the hepatocyte surface in the normal biopsies, facing the sinusoid in the space of Disse, shows numerous microvilli (Fig. 4). In the PBC biopsies the same region of the hepatocyte surface displays less and more disorganized microvilli (Fig. 5). Numerous collagen fibers are seen between the hepatocyte and the sinusoid cells in the PBC biopsies (Fig. 5 inset).

Concerning the intercellular space in all the biopsies of the PBC patients, abundant microvilli are seen to emerge from the lateral membranes of the hepatocytes (Figs. 5, 6, 7). These microvilli are not seen in the normal liver biopsies (Fig. 4).

The cell junctions are well preserved in all the biopsies as shown by comparing Figures 1 and 8 (normal) with Figures 9 and 10 (PBC). The bile canaliculi well sealed at both sides by the tight junctions are clearly seen in freeze-fracture replicas of PBC biopsies (Figs. 11-12).

Desmosomes are present and appear normal.

In the normal liver hepatocyte the Golgi apparatus is found lying mainly in front of the bile canaliculus (Fig. 8), whereas in PBC livers it is observed not only at this level but also near the microvilli of the lateral membranes (Fig. 9).

## Discussion

Primary biliary cirrhosis is a disease of probable autoimmune aetiology, (Dickson *et al*., 1979), which alters the structure of the median size ducts at its first stage. The earliest biochemical alterations are the increase of cholestatic enzymes such as alkaline phosphatase (APh), 5= Nucleotidase (5NU) and Gamma - glutamyl transpeptidase (γGT).

The antimitochondrial antibodies, anti M2, are positive in the 90-95% of the cases (Migliaccio *et al*., 1998).

What functional or structural alterations exist in those first stages in the liver of patients with primary biliary cirrhosis which determine the elevation of cholestatic enzymes?

We have observed that the bile canaliculi in the first stages of PBC present a normal structure through the transmission electron microscopy and well sealed gap junctions by freeze fracture. Desmosomes, tight junctions and gap junctions are normal.

In the ultrastructure we observed very important modifications in the basolateral membranes. At the sinusoidal domain, there was a marked decrease of the microvilli and we found numerous collagen fibers.

In cholestasis the NTCP (Bolder *et al*., 1997; Green *et al*., 1996, 1997) is reduced. When cholestasis disappears, this transporter normalizes. Probably, the decrease of microvilli at sinusoidal level observed in these patients is due to the reduction of NTCP to prevent the uptake of bile salts that could cause a greater damage to the hepatocyte.

Patients with PBC (stages I and II) showed abundant microvilli in the lateral membranes of the cells which do not exist in the biopsies of normal livers. The cytochemical method used showed a great enzymatic activity of ATPase in the canaliculus and in the lateral membranes of the hepatocytes, a finding that did not appear in the biopsies of normal livers.

An elevation of MRP3 in the lateral membranes has been shown in Dubin-Johnson Syndrome (DJS) (König *et al*., 1999), possibly to compensate the canalicular decrease of MRP2 (Kartenbbeck *et al*., 1996; Trauner *et al*., 1997) present in DJS. In PBC (stages I and II) there is an increase of MRP3 in the lateral membranes of the hepatocyte probably as a compensating mechanism (König *et al*., 1999). The family of the MRP transporters is ATP-dependent.

Our finding of great ATP-dependent enzymatic activity in the lateral membranes and the existence of microvilli in the same place, is consisting with this phenomenon.

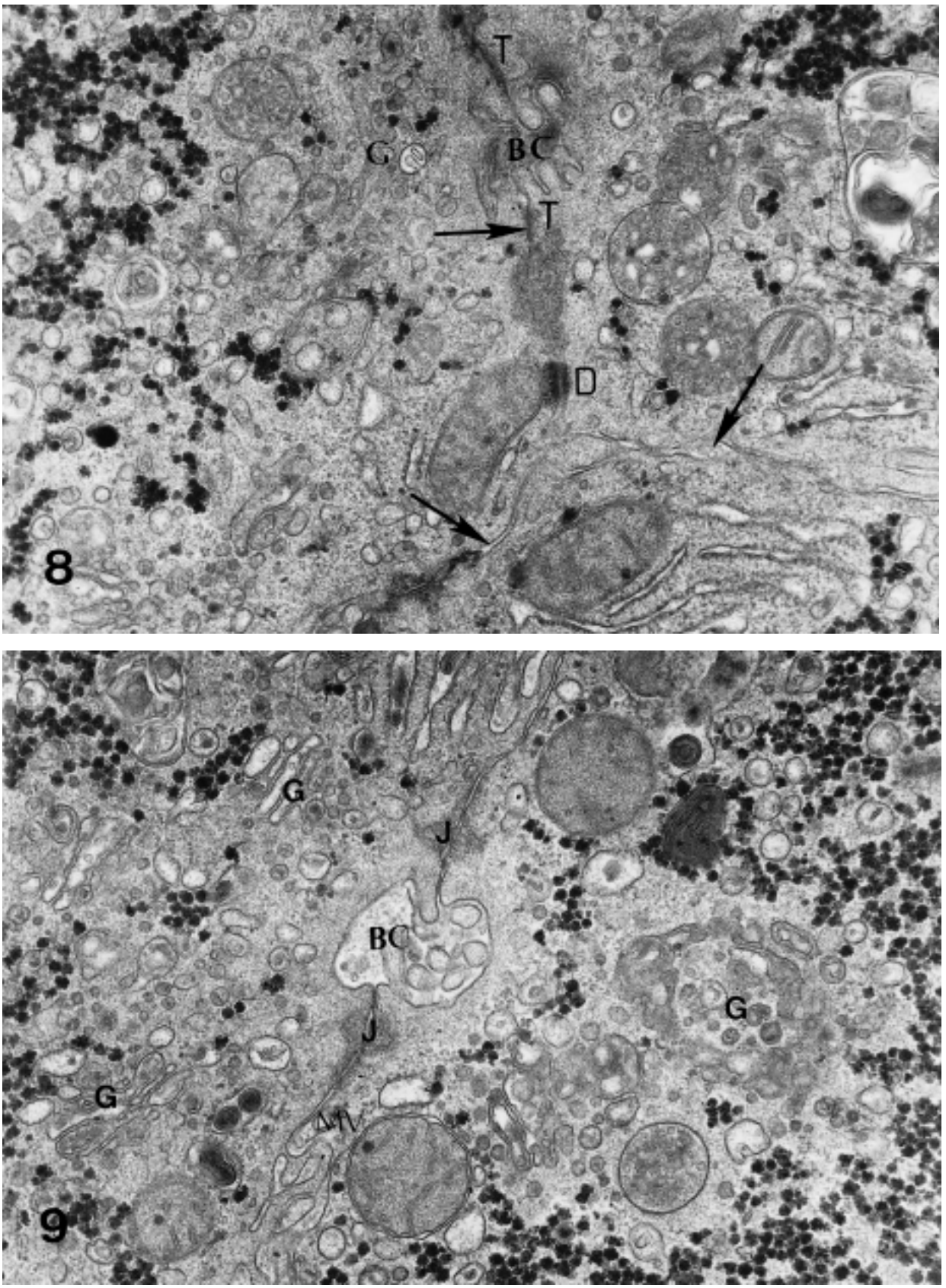
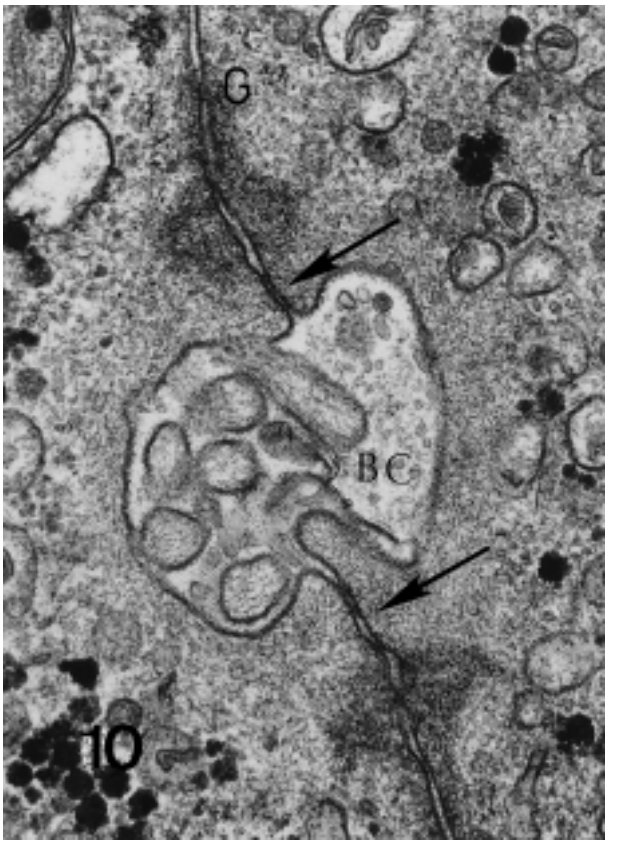



**FIGURE 8.** Normal liver. Smooth intercellular space between three hepatocytes (arrow). Golgi (G) membranes appear in front the bile canaliculus (BC). Desmosome (D). Tight junction (T). X 10,000

**FIGURE 9.** Liver with PBC. Portion of two hepatocytes. Bile canaliculus (BC) and adjacent intercellular space closed by intermediate junction (J) and with microvilli (MV). Note the presence of Golgi complexes (G) close to the lateral membranes. X 23,300



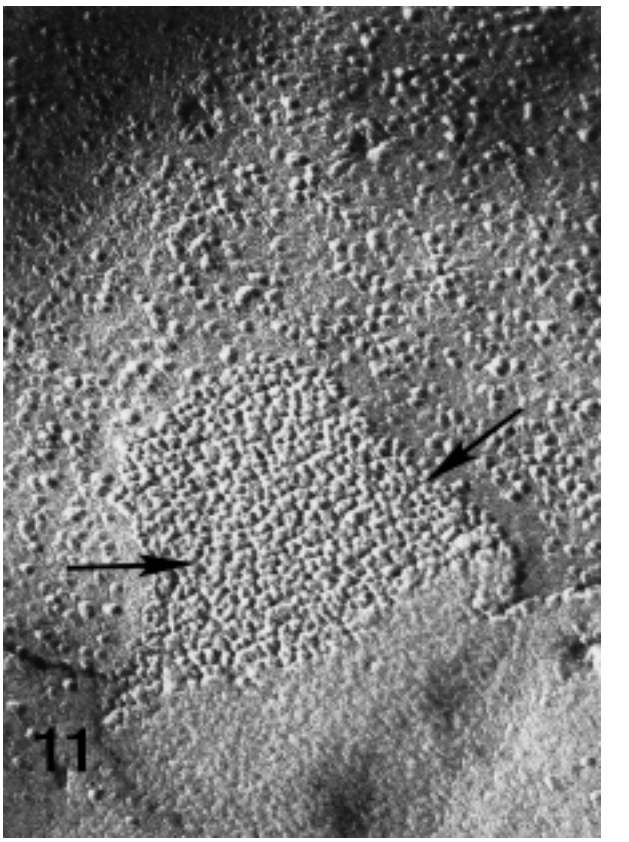





**FIGURE 10.** Liver with PBC. Higher magnification of bile canaliculus (BC) and the adjacent junctional complexes. Tight junction (arrow) and gap junction (G). X 40,000.
**FIGURE 11.** Liver with PBC. Freeze fracture replica, Gap junction (arrows). X 68,000.
**FIGURE 12.** Liver with PBC. Freeze fracture replica. Bile canaliculus (BC). Tight junctions rows of particles in the "P" face of the cell membrane (arrows) and in the "E" face (double arrows). X 60,000.

A reduction of the expression of AE2 in the hepatocytes was demonstrated by Medina *et al.* (1997) in patients with PBC. These authors also proved its rise after UDCA treatment.

Alvaro *et al.* (2000), demonstrated that the chronic elevation of alkaline phosphatase produces a decline of the bile flow and also of the bicarbonate excretion. It would be interesting to determine whether the findings of Medina *et al.* (1997) are due to the increase of alkaline phosphatase observed in patients with primary biliary cirrhosis or they are a primary alteration in this disease.

In the biopsies of patients with PBC (stages I and II) we have observed that the Golgi apparatus is localized not only in the apical (biliary) domain but also in the lateral membranes. This can be interpreted as an increment of the bile excretion in the lateral membranes. The bile canaliculus has normal structure and shows a marked increase of enzymatic activity as a proof of an incremented excretion.

In conclusion, we believe that in the first stages of primary biliary cirrhosis there are compensating phenomena in the hepatocyte which tries to increase the bile flow modified by the alteration of the median size ducts. These are the increment of excretion in the apical domain and the appearance of microvilli and enzymatic activity in the lateral membranes.

In PBC there is a reduction of NTCP at the sinusoidal domain, probably to prevent the uptake of bile salts. The decrease of microvilli observed at the sinusoidal domain agrees with the functional alteration.We consider that further investigations are needed to confirm these findings and to provide better therapeutic possibilities.

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