

Biliary aminopeptidase-N and the cholesterol crystallisation defect in cholelithiasis

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Abstract

Several biliary proteins have cholesterol crystallisation promoting activity. One of these glycoproteins is aminopeptidase-N, a canalicular ectoenzyme. This study attempted to localise aminopeptidase-N along the biliary tree, to assess its concentration in a series of 98 patients subjected to abdominal surgery, 40 of them without gall stones, and to correlate its concentration with cholesterol crystal formation time of gall bladder bile. Aminopeptidase-N was isolated from purified native biliary vesicles. A specific polyclonal rabbit anti-aminopeptidase-N antibody was prepared for quantitative immunoblotting and for immunolocalisation. Tissue was obtained from liver biopsy specimens and from gall bladders removed at surgery because of gall stone disease. Aminopeptidase-N was immunolocalised to the apical membranes of hepatocytes and to the apical pole of ductular and gall bladder mucosal cells. The nucleation time of gall bladder bile was mean (SD) 4 (3) days in the gall stone group, compared with 21 (18) days in the control group ($p < 0.001$). Total absolute biliary protein and aminopeptidase-N concentrations were similar in both the control and gall stone patients. There was a reciprocal significant correlation, however, between the nucleation time and the relative aminopeptidase-N concentration ($r = -0.35$, $p < 0.01$) only in the gall stone group of patients. This study shows that this apical transmembrane ectoenzyme with cholesterol crystallisation promoting activity is present along the biliary tree and the hepatocyte. These findings support the concept that high concentrations or qualitative changes of biliary aminopeptidase-N contribute to cholesterol gall stone formation.

(Gut 1995; 37: 422-426)

Keywords: biliary cholesterol, crystallisation, aminopeptidase-N, gall stones.

The main stages of cholesterol gall stone formation are the secretion by the liver of metastable cholesterol rich phospholipid unilamellar vesicles, the aggregation of these vesicles with the appearance of microscopic cholesterol monohydrate crystals within the gall bladder and, finally, crystal growth and agglomeration to constitute a macroscopic gall stone.^{1,2} A number of biliary proteins have been reported to promote cholesterol crystal

formation (nucleation),³⁻¹³ or to inhibit this process.^{14,15} The role of these proteins, however, in the pathogenesis of cholesterol gall stone disease is still controversial.¹⁶

We have recently shown that some proto-nucleating glycoproteins have hydrophobic characteristics and are associated with native biliary vesicles, suggesting that their origin is in the liver.^{17,18} One of these vesicle associated glycoproteins was a 130 kDa glycoprotein identified as aminopeptidase-N (E.C.3.4.11.2) and localised to the canalicular membrane.¹⁹ This protein, isolated from native biliary vesicles, showed a concentration dependent cholesterol crystallisation promoting activity at concentrations usually found in hepatic bile.²⁰

This study had two objectives. Firstly, we intended to immunolocalise aminopeptidase-N to the apical pole of the epithelial cells along the biliary tree, including gall bladder mucosal cells. Secondly, we wished to assess aminopeptidase-N concentration in gall bladder bile of patients with and without gall stones and to correlate its concentration with the cholesterol crystal formation time of gall bladder bile.

Methods

Patients and bile sampling

The study was approved by the Ethical Committee for Research of the Faculty of Medicine of the Pontificia Universidad Católica de Chile. In some experiments, a fraction of the tissue removed in liver biopsy specimens were used after written informed consent from the patients. Only liver and gall bladder tissue with minor histological abnormalities were used. Gall bladder bile was collected at surgery by needle aspiration of the gall bladder.²¹ Bile was obtained from 58 patients with cholesterol gall stones (cholesterol content varied between 70% to 100% by stone weight in the patients) and from 40 patients without gall stones subjected to elective laparotomy because of intra-abdominal diseases, including mostly gastrointestinal cancer and some patients with inflammatory bowel disease. The absence of gall stones was

TABLE 1 Age and sex of 98 patients in the study

| | Control patients* | Gall stone group |
|-------------------|-------------------|------------------|
| Patients (n) | 40 | 58 |
| Age (y) | 52 (16) | 44 (15) |
| Female:male ratio | 16:24 | 46:12 |

*The control group consisted of patients subjected to laparotomy because of gastrointestinal malignancy (n=38), or inflammatory bowel disease (n=2). The gall bladder of each patient was macroscopically normal. Data presented as mean (SD).

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Accepted for publication 31 January 1995

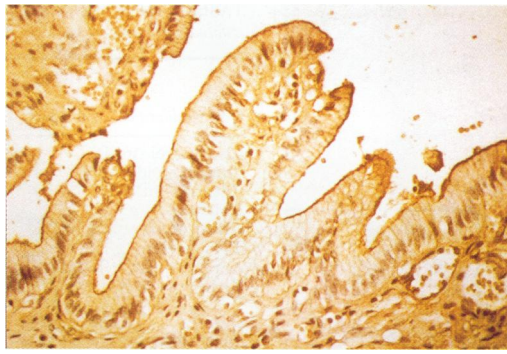


Figure 1: Microphotograph of a gall bladder section from gall bladder obtained after cholecystectomy because of gall stone disease ($\times 200$). Immunodetection of aminopeptidase-N is shown in brown on the apical pole of mucosal cells.

confirmed by intraoperative palpation of the gall bladder. Table I shows the age and sex distribution of the patients. Bile was collected in sterile tubes with 0.05% chloramphenicol, 3 mM sodium azide, 0.2 mM thymerosal, 5 mM EDTA, 1 mM PMSF, and 1 mM leupeptin as preservatives.

Laboratory methods

Aminopeptidase-N was obtained from native biliary vesicles, which were isolated from hepatic bile by ultracentrifugation and purified by column chromatography.^{17, 18} Aminopeptidase-N polyclonal antibody was prepared as previously described.^{19, 20}

The concentration of biliary aminopeptidase-N was measured by quantitative immunoblotting in gall bladder bile.^{19, 20} A rabbit anti-aminopeptidase polyclonal antibody was used as primary antibody. Absorbed immunoglobulins were detected by using protein A coupled alkaline phosphatase and the colour reaction was developed with 3-bromo-4-chloro-5-indolyl-phosphate and nitroblue tetrazolium as substrates.²⁰ Quantification was performed by densitometry of immunoblotted bile samples in comparison with previously known amounts of purified aminopeptidase-N as standards. The densitometric measurements of these standards showed a linear correlation curve within the protein concentration used in the procedure. We normalised the absolute protein and aminopeptidase-N concentration in gall bladder bile. Based on the wide physiological inter-sample variability of solute concentrations in gall bladder bile,² we determined the relative protein concentration in each bile specimen by dividing the absolute protein concentration value by its value for total lipids found in the sample.

For immunohistochemistry, liver and gall bladder samples were embedded in Paraplast. Four μm sections were mounted onto glass slides and placed in 60°C oven for one hour. After dewaxing in xylene, tissue sections were rehydrated by passing through graded ethanols. Immunohistochemistry was performed with the peroxidase-antiperoxidase method. The rehydrated sections were preincubated with methanol/H₂O₂ (9/1) for 20 minutes to block peroxidase activity. Washing steps were carried out with peroxidase-anti-

peroxidase buffer (130 mM NaCl, 3.5 mM KH₂P0₄, 10 mM Na₂P0₄, 3 mM NaN₃, and 40 mM TRIS-HCl, pH 7.6) supplemented with 0.7% carrageenan when immunoreagents were used. Firstly, the sections were incubated with the rabbit polyclonal anti-aminopeptidase diluted 1/100. Then, the samples were incubated with goat anti-rabbit IgG antiserum at 1/20 dilution for 30 minutes, and finally with rabbit peroxidase-antiperoxidase complex (dilution 1/150) for 30 minutes. After washing, sections were developed in peroxidase-antiperoxidase buffer plus 0.1% 3,3'-diaminobenzidine and 0.01% H₂O₂ for 10 minutes. Mounting followed counterstaining with haematoxylin.

Cholesterol crystal formation time

Gall bladder bile samples were processed as previously described.¹⁷ The interval between time zero and the first appearance of cholesterol crystals under polarising microscopy was designated as the cholesterol crystal formation time or nucleation time (in days) of a bile specimen. Only samples with no bacterial growth after culture were included in this study.

Chemical and enzymatic analysis

Cholesterol, phospholipids, bile salts, and biliary cholesterol saturation were determined by standard techniques used in our laboratory.^{17, 19} Total biliary proteins were assessed by a modified Lowry procedure.²² The enzymatic activity of biliary aminopeptidase was assessed with 1 mM leucine-para-nitroanilide as substrate in 25 mM imidazole-HCl buffer, pH 7.6, at 37°C.²³

Statistics

Results are presented as mean (SD). Student's *t* test was used for parametric data. Non-parametric correlations were performed by Spearman's rank correlation coefficients. The value of statistical significance was set at $p < 0.05$.

Results

To determine if aminopeptidase-N was present on the apical pole of bile duct and gall bladder mucosa cells and hepatocytes, we performed immunolocalisation studies, as shown in Figs 1 and 2. It can be clearly seen that the enzyme is widely distributed on the canalicular pole of hepatocytes, ductular cells (inset of Fig 2), and also throughout the apical membrane of gall bladder mucosal cells.

As Table II shows, biliary cholesterol saturation was significantly higher in the gall stone patients (130%) compared with the controls (108%). All gall stone patients had cholesterol monohydrate crystals in the sediment after ultracentrifugation. Cholesterol crystals were also seen in 10% of the control patients. As expected, the nucleation time of the gall stone group was much shorter than that of bile from

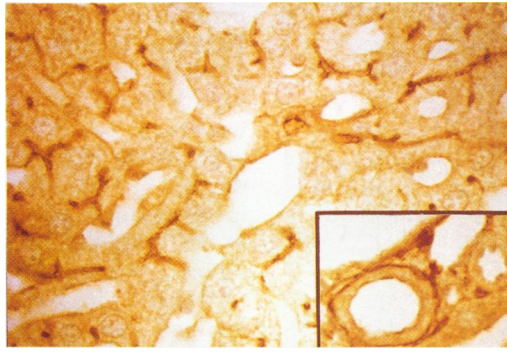


Figure 2: Microphotograph of a section from a liver biopsy specimen. Immunodetection of aminopeptidase-N is shown in brown on the canaliculi ($\times 200$) and a ductule in the inset ($\times 500$).

TABLE II Bile composition, cholesterol saturation, and nucleation time of patients with gall stones or controls

| Gall bladder bile | Control patients (n=40) | Gall stone patients (n=58) |
|--------------------------------|-------------------------|----------------------------|
| Cholesterol (mM) | 12.9 (6) | 11.5 (7) |
| Phospholipid (mM) | 35.2 (16) | 26.6 (14)* |
| Bile salts (mM) | 178 (91) | 124 (70)* |
| Total lipids (g%) | 12 (6) | 9 (5)* |
| Cholesterol saturation (%) | 109 (42) | 130 (39)* |
| Total protein (g/l) | 14.1 (8) | 15.9 (9) |
| Aminopeptidase-N (g/l) | 0.14 (0.1) | 0.13 (0.1) |
| Total protein $\times 10^2$ | 144 (150) | 214 (171)* |
| Total lipids | | |
| Aminopeptidase-N $\times 10^2$ | 1.1 (0.7) | 1.3 (1.0) |
| Total lipids | | |

Values represent mean (SD). *Shows a significant difference compared with control group ($p < 0.01$).

patients without gall stones, from 21 (18) to 4 (3) days ($p < 0.001$). Relative total biliary protein concentration was significantly higher in the gall stone patients. Aminopeptidase-N absolute and relative concentrations were similar in both groups. Multiple cholesterol gall stone patients had higher aminopeptidase-N concentration than the solitary gall stone group, but the difference was not statistically different (0.15 (19) *v* 0.12 (0.08) mg/ml). The relative concentrations of total biliary proteins and aminopeptidase-N were significantly correlated ($r = 0.42$, $p < 0.001$). A significant reciprocal correlation was only found, however, between the nucleation time and the relative biliary aminopeptidase-N concentration in the gall stone patients as shown in panel C of Fig 3 ($r = 0.35$, $p < 0.001$). In contrast, no significant correlation was found between these parameters in the control group of patients and between relative total protein concentration and nucleation time in both groups of patients. Aminopeptidase-N enzymatic activity of bile samples was similar in the control and gall stone group of patients (results not shown).

Discussion

Aminopeptidase-N has been considered a specific marker protein for the canalicular surface of hepatocyte membranes.²⁴ We have confirmed these findings¹⁹ and this study also shows that the native biliary vesicle associated cholesterol crystallisation promotor aminopeptidase-N²⁰ is located to the canalicular membrane, as well as on the apical membrane of bile duct and gall bladder mucosal cells. The localisation of this pronucleating glycoprotein to the gall bladder mucosa and its secretion into bile raises the possibility that local membrane shedding of this epithelium may also directly contribute to cholesterol crystal formation in gall bladder bile. We have previously determined the amino terminal amino acid sequence of biliary aminopeptidase-N, which includes the cytoplasmic domain and a portion of the transmembrane hydrophobic region.²⁰ This finding is consistent with the possibility that some microdomains of the canalicular, ductular, and gall bladder mucosal cell membranes are shed into the biliary tree during bile secretion. Aminopeptidase-N activity is detected in bile

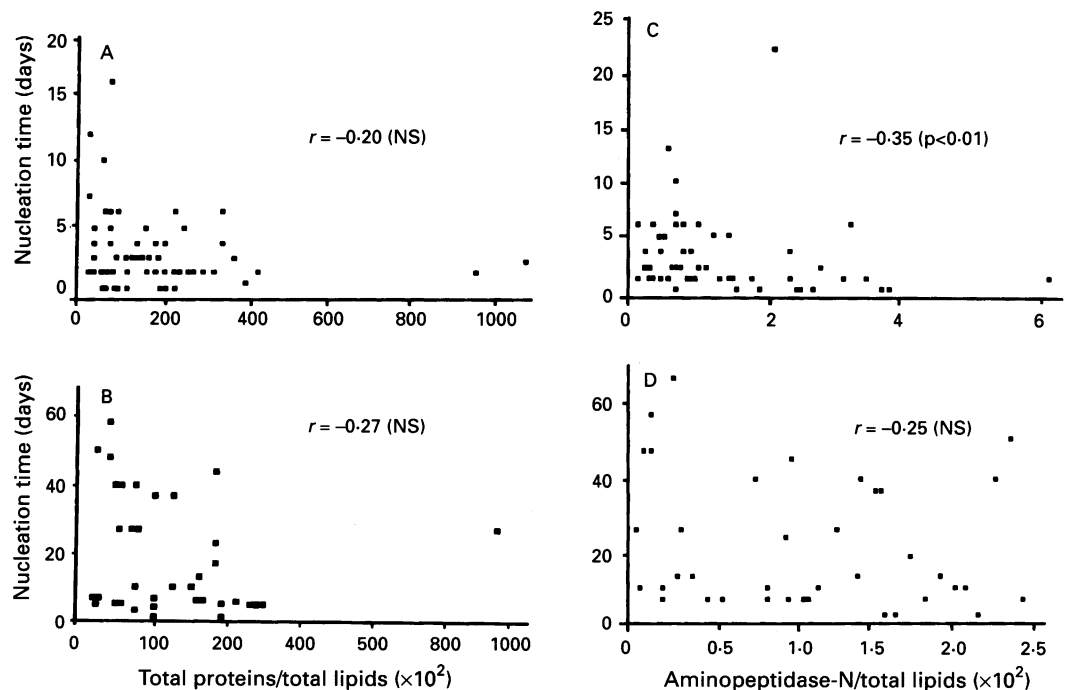


Figure 3: Relation between nucleation time, total biliary protein/total biliary lipid ratio, and aminopeptidase-N/total biliary lipid ratio in gall bladder bile. Controls patients ((B) and (D)) and gall stone patients ((A) and (C)).

supporting the hypothesis that the whole protein is released into bile during the secretory process. It has been postulated that protein-lipid interactions promoting cholesterol crystal formation probably occur after binding and aggregation of cholesterol enriched phospholipid unilamellar vesicles.² It is possible that some specific domains of aminopeptidase-N may favour the aggregation and fusion of these vesicles as a precedent step to cholesterol crystallisation. We have recently found that the hydrophilic catalytic domain of aminopeptidase-N has cholesterol crystallisation promoting activity, whereas the hydrophobic anchor has crystallisation inhibiting activity.²⁵ In contrast, gall bladder mucin, also a pronucleating protein, requires hydrophobic domains to function as a cholesterol crystallisation promoter.²⁶ These apparently contrasting results reinforce the concept that the interaction of proteins and vesicles is very complex in the process of biliary cholesterol crystal formation-inhibition. There is no consistent chemical or physicochemical pattern in the biliary proteins previously shown as promoters and inhibitors.

This study shows that, although the absolute biliary aminopeptidase-N concentration is similar in control and gall stone patients, there is a highly significant reciprocal correlation between the relative aminopeptidase-N concentration and the nucleation time in patients with gall stones. The use of relative aminopeptidase-N concentrations, calculated as the protein to total lipid ratio, permitted the normalisation of this parameter by taking into account the wide variability of solute concentrations normally found in gall bladder bile. It is also important to take into account that it is difficult to select a sufficient number of control patients for this kind of comparative study in Chile, a country with a high prevalence of cholesterol gall stone disease.²³ In addition, this kind of comparative study always has the problem that measurements performed in bile obtained at a certain time do not necessarily reflect the phenomena occurred in the past during gall stone formation. The significant reciprocal correlation between relative aminopeptidase-N concentration and cholesterol crystal formation time in gall bladder bile of patients with gall stones suggests a role of this amphipathic protein in gall stone formation. It may be possible that the lack of correlation between both parameters in the control group reflects the activity of one or more cholesterol crystallisation inhibitors, which are absent or decreased in the gall stone group. Alternatively, qualitative differences of aminopeptidase-N could be responsible for the pronucleating effect in the gall stone population. In fact, biliary aminopeptidase has been found with^{20 27} and without²⁸ pronucleating activity, suggesting that there may be different forms of aminopeptidase-N in bile. These possibilities are in line with the potential pathogenic role attributed to a series of cholesterol crystal promoting biliary proteins.^{3 12 18 20}

The previous finding of *in vitro* cholesterol crystallisation promoting activity of aminopeptidase-N^{20 24} and the present correlation

between cholesterol crystal observation time and the relative aminopeptidase-N concentration in gall bladder bile of gall stone patients suggest a potential pathophysiological role of aminopeptidase-N in some patients with cholesterol gall stones. Additional studies are required to determine if quantitative or qualitative changes of biliary aminopeptidase-N, or both, have true pathogenic significance in cholesterol gall stone formation, including the comparison of gall stone patients with specific control groups and subjects with low risk of cholesterol gall stone disease and the use of experimental models of cholesterol gall stone formation. Large studies are needed to assess the predictive value of biliary aminopeptidase-N determinations in cholesterol crystal formation in humans. The concentration, relative potency, and significance of other pronucleators and inhibitors of cholesterol crystallisation must be also simultaneously assessed, before we can ascertain a specific role of aminopeptidase-N in the pathogenesis of cholesterol gall stone disease.

This study was supported by a special grant (1091 G224/TCU/CILE) of the D.G.C.S.-Ministero degli Affari Esteri d'Italia and the Istituto per la Cooperazione Universitaria, Roma, Italia. It was also partially funded by Fundación Andes and the Fondo Nacional de Desarrollo Científico y Tecnológico (0620 and 0899), Santiago, Chile.

Part of this study was presented at the Annual Meeting of the American Gastroenterological Association, Boston May 1993, and published as an abstract in *Gastroenterology* 1993; 104: A965.

- Holan KR, Holzbach RT, Hermann RE, Cooperman AM, Claffey WJ. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology* 1979; 77: 611-7.
- Strasberg SM, Harvey PRC. Biliary cholesterol transport and precipitation; introduction and overview of conference. *Hepatology* 1990; 12: 1-5.
- Burnstein MJ, Ison RG, Petrunka CN, Taylor RD, Strasberg SM. Evidence for a potent nucleating factor in the gallbladder bile of patients with cholesterol gallstone. *Gastroenterology* 1983; 85: 801-7.
- Levy PF, Smith BF, LaMont JT. Human gallbladder mucin accelerates nucleation of cholesterol in artificial bile. *Gastroenterology* 1984; 87: 270-5.
- Gallinger S, Harvey PRC, Petrunka CN, Ison RG, Strasberg SM. Biliary proteins and the nucleation defect in cholesterol cholelithiasis. *Gastroenterology* 1987; 92: 867-85.
- Groen AK, Stout JJP, Drapers JAG, Hoek FJ, Grijm R, Tytgat GN. *Hepatology* 1988; 8: 347-52.
- Groen AK, Noordam C, Drapers JAG, Egbers P, Jansen PLM, Tytgat GNJ. Isolation of a potent cholesterol nucleation-promoting activity from human gallbladder bile; role in the pathogenesis of gallstone disease. *Hepatology* 1990; 11: 525-33.
- Harvey PRC, Upadhy GA, Strasberg SM. Immunoglobulins as nucleating proteins in the gallbladder bile of patients with cholesterol gallstones. *J Biol Chem* 1991; 266: 13996-4003.
- Pattinson NR, Willis KE. Effect of phospholipase C on cholesterol solubilization in model bile: a concanavalin A-binding nucleation promoting factor from human gallbladder bile. *Gastroenterology* 1991; 101: 1339-44.
- Chijiwa K, Koga A, Yamasaki T, Shimada K, Noshiro H, Nakayama F. Fibronectin: a possible factor promoting cholesterol monohydrate crystallization in bile. *Biochim Biophys Acta* 1991; 1086: 44-8.
- Domingo N, Groen B, Lechene P, De Bruijn M, Ostrow JD, Lafont H. Anionic polypeptide fraction (APF) avidly interacts with cholesterol/lecithin vesicles and promotes crystal growth in model bile. *Gastroenterology* 1992; 102: A802.
- Abei M, Kawazak P, Nuutinen H, Langnas A, Svanvik J, Holzbach T. Isolation and characterization of a cholesterol crystallization promoter from human bile. *Gastroenterology* 1993; 104: 539-48.
- Upadhy GA, Harvey PRC, Strasberg SM. Effect of human biliary immunoglobulins on the nucleation of cholesterol. *J Biol Chem* 1993; 268: 5193-200.
- Kibe A, Holzbach RT, LaRusso NF, Mao SJT. Inhibition of cholesterol crystal formation by apolipoproteins in supersaturated model bile. *Science* 1984; 225: 514-6.
- Ohya T, Schwarzendrube J, Busch N, Gresky S, Chandler K, Takabayashi A, et al. A human glycoprotein that inhibits formation and growth of cholesterol crystals: purification and characterization. *Gastroenterology* 1993; 104: 527-38.

- 16 Harvey PRC, Strasberg SM. Will the real cholesterol-nucleating and -antinucleating proteins please stand up? *Gastroenterology* 1993; **104**: 646-50.
- 17 Miquel JF, Rigotti A, Rojas E, Brandan E, Nervi F. Isolation and purification of human biliary vesicles with potent cholesterol nucleation promoting activity. *Clin Sci* 1992; **82**: 175-80.
- 18 Miquel JF, Núñez L, Rigotti A, Amigo L, Brandan E, Nervi F. Isolation and partial characterization of cholesterol pronucleating hydrophobic glycoproteins associated with native biliary vesicles. *FEBS Lett* 1993; **318**: 45-9.
- 19 Rigotti A, Núñez L, Amigo L, Puglielli L, Garrido J, Santos M, *et al.* Biliary lipid secretion: immunolocalization and identification of a protein associated with lamellar cholesterol carriers in supersaturated rat and human bile. *J Lipid Res* 1993; **34**: 1883-94.
- 20 Núñez L, Amigo L, Rigotti A, Puglielli L, Mingrone G, Greco AV, *et al.* Cholesterol crystallization-promoting activity of aminopeptidase-N isolated from the vesicular carrier of biliary lipids. *FEBS Lett* 1993; **329**: 24-8.
- 21 Gollish SH, Burnstein MJ, Ilson RG, Petrunka CN, Strasberg SM. Biliary proteins and the nucleation factor in the gallbladder bile of patients with cholesterol gallstones. *Gut* 1983; **24**: 836-44.
- 22 Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 1978; **87**: 206-10.
- 23 Nervi F, Duarte I, Gómez G, Rodríguez G, Del Pino G, Ferreiro O, *et al.* Frequency of gallbladder cancer in Chile, a high risk area. *Int J Cancer* 1988; **41**: 657-60.
- 24 Roman LM, Hubbard A. A domain-specific marker for the hepatocyte plasma membranes: localization of leucine aminopeptidase to the bile canalicular domain. *J Cell Biol* 1984; **96**: 1548-58.
- 25 Amigo L, Rigotti A, Núñez L, Puglielli L, George-Nascimento C, Nervi F. Biliary cholesterol-crystallization influencing activity of different domains of aminopeptidase-N. *Gastroenterology* 1994; **107**: A127.
- 26 Smith BF, LaMont JT. Hydrophobic binding properties of bovine gallbladder mucin. *J Biol Chem* 1984; **259**: 12170-7.
- 27 Offner G, Gong S, Afdahl HN. Identification of a 130-kilodalton human biliary concanavalin-A binding protein as aminopeptidase-N. *Gastroenterology* 1994; **106**: 755-62.
- 28 Abei M, Schwarzendrube J, Nuutinen H, Broughan TA, Kawerzak O, Williams C, *et al.* Cholesterol crystallization-promoters, -acid glycoprotein, phospholipase C and aminopeptidase-N. *J Lipid Res* 1993; **34**: 1141-8.