Quantitative analysis of gallstones in Libyan patients

Abdalla M. Jaraari1, Peela Jagannadharao1*, Trushakant N. Patil1, Abdul Hai1, Hayam A. Awamy1, Saeid O. El Saity2, Ezedin B. Abdel Kafi2, Maisoon N. El-Hemri2 and Mahmood F. Tayesh2

1Department of Biochemistry, Faculty of Medicine, Al-Arab Medical University, Benghazi, Libya; 2Department of Surgery, Faculty of Medicine, Al-Arab Medical University, Benghazi, Libya

Gallstone disease is one of the major surgical problems in the Libyan population; it is probably related to diet, especially excessive consumption of meat. The study was conducted to determine the composition of gallstones and their possible etiology in a Libyan population. The chemical composition of gallstones from 41 patients (six males and 35 females) was analyzed. The stones were classified into cholesterol, pigment, and mixed stones (MS). Cholesterol stones (CS) showed a significantly higher cholesterol content than pigment stones (PS) (p = 0.0085) though not significantly higher than MS. Their phospholipid content and inorganic phosphates were higher than in the other types of stones and oxalate content was significantly elevated in comparison with MS (p = 0.0471). In MS, the cholesterol, bile acids, and bilirubin were intermediate between cholesterol and PS, whereas triglycerides were significantly more than PS (p = 0.0004). Bilirubin (0.0001) and bile acids (p = 0.0009) were significantly higher than CS (p = 0.0001). However, they contained the lowest amounts of sodium, potassium, magnesium, and oxalate. In PS, bilirubin (p = 0.0001) was significantly higher than both groups. Bile acid content was significantly higher than CS (p = 0.0001) but not significantly more than MS. They showed the highest values of calcium, sodium, potassium, magnesium, and chlorides compared to the other types of stones. High levels of cholesterol in stones and dyslipidemia associated with mixed as well as cholesterol gallstones suggest an etiological association and efforts to reduce dietary fat among the Libyan population may lead to decreased cholesterol and mixed gallstones.

Keywords: gallstones; chemical composition; Libya; cholesterol

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Materials and methods

Gallstones from 41 patients with cholelithiasis were collected after cholecystectomy at the Department of Surgery, Seventh October Hospital, Benghazi, Libya, between March and September 2008. This study was approved by the ethics committee of the Al-Arab Medical University, Benghazi, Libya. The stones were classified into three types depending on their color and degree of hardness. Yellow and whitish stones were identified as CS, black and dark brown as pigment stones (PS), and brownish yellow or green as mixed stones (MS). Demographic data on age and sex was also collected. The stones were powdered using a mortar and dissolved in different solvents depending on the type of chemical constituent to be analyzed. To determine total cholesterol and total bilirubin, 30 mg stone powder was dissolved in 3 ml chloroform in a test tube. The tube was kept in boiling water bath for 2 min. Aliquots from these samples were used for determination of total cholesterol and total bilirubin. To determine calcium, oxalate, inorganic phosphate, magnesium, chloride, triglycerides, sodium, and potassium, 30 mg of the powdered stone was dissolved in 3 ml of HCl in a graduated 10-ml tube and the volume was made up to 10 ml with distilled water. The tubes were kept in a boiling water bath for one hour. To analyze phospholipids, 20 mg of powdered stone was dissolved in 15 ml of a 2:1 mixture of chloroform and methanol containing 1 N HCl. To measure bile acids, the stones were dissolved in chloroform–methanol (2:1) mixture. The solutions were preserved at 4°C until they were used.

Total cholesterol was estimated by a colorimetric enzymatic method (Biocon Diagnostics, Germany) (18), total bilirubin by Accurex Biomedicals (19), triglycerides by an enzymatic colorimetric method of Biocon Diagnostics (20), oxalate by the method described by Satyapal and Pundir based on colorimetric enzymatic method (21), calcium by an o-cresolphthalein complexone (OCPC) kit (Biocon Diagnostics) (22). Phospholipid and inorganic phosphate were determined according to Fiske and Subba Rao (23), magnesium by xylidyl blue (Biocon Diagnostics) (24), chloride by the method of Schoenfeld (25), sodium and potassium by flame photometry (Clinical flame photometer, Evano Electro Selenium Halsted, Essex, UK), and bile acids by the colorimetric method of Carey (26). For colorimetric procedures, we used reagents from Biocon Diagnostics, Germany, and a spectrophotometer from Labomed Inc., USA (UV-Vis-1179, RS Spectrophotometer). Statistical analyses were performed with GraphPad software (GraphPad Software Inc., USA). The unpaired t-test was used for comparison of group means. A p-value of <0.05 was considered significant.

Results

All 41 patients (six males and 35 females) had multiple gallstones. CS were bigger than MS and PS. Fourteen of the patients (34%) had MS, 16 (39%) had PS, and 11 (27%) had CS (Table 1). The incidence of gallstones was highest in age group of 41–50 years (Table 2).

CS had the highest composition of cholesterol, while MS had a high content of triglycerides and PS were comprised mostly of bilirubin (Tables 3 and 4).

Table 1. Physical properties of gallstones

|                  | Cholesterol stones | Mixed stones | Pigment stones |
|------------------|--------------------|--------------|---------------|
| **Sex**          |                    |              |               |
| Females          | 11                 | 12           | 12            |
| Males            | 0                  | 2            | 4             |
| **Shape**        |                    |              |               |
| Round            | 3                  | 4            | 6             |
| Irregular        | 8                  | 10           | 10            |
| **Color**        |                    |              |               |
| Yellow, White    | 9, 2               | 12, Greenish brown | 10 brown, 6 black |
| **Surface**      |                    |              |               |
| Smooth           | 6                  | 5            | 12            |
| Rough            | 5                  | 1            | 4             |
| **Weight (g, mean)** | 5.40             | 1.78         | 3.00          |
| **Character**    |                    |              |               |
| Soft, hard      | 9, hard            | 11, hard     | 10, hard      |
| **Size (cm, mean)** | 0.8 × 0.9         | 0.6 × 0.7    | 0.9 × 0.9     |

Table 2. Frequency of various types of gallstones according to age

| Age group (years) | CS | MS | PS | Total |
|-------------------|----|----|----|-------|
| ≤20               | 0  | 0  | 1  | 1     |
| 21-30             | 2  | 2  | 5  | 9     |
| 31-40             | 5  | 5  | 2  | 11    |
| 41-50             | 2  | 4  | 6  | 12    |
| 51-60             | 1  | 3  | 1  | 5     |
| ≥61               | 1  | 0  | 1  | 2     |
|                   | 11 | 14 | 16 | 41    |

Note: CS, cholesterol stones; MS, mixed stones; PS, pigment stones.
The concentrations of different ions also varied with the type of stone. PS had the highest concentrations of most of the ions, including calcium, sodium, potassium, magnesium, and chloride, whereas CS contained higher concentrations of phosphates and oxalates (Tables 5 and 6).

**Discussion**

**Cholesterol**

Cholesterol content was found to be highest in CS. This is because the cholesterol saturation index is more than 1 between cholesterol and bile salts (27). The finding that the highest cholesterol content was in CS reiterates that these type of stones are formed primarily because of supersaturation of cholesterol in the bile, which precipitates as a stone. The pathogenesis of cholesterol gallstones is rooted in altered lipid metabolism, e.g. hyperlipidemia type IIa and type IV give rise to a greater proportion of cholesterol relative to other bile lipids secreted from the liver into bile (28, 29). The co-existence of nucleating factors, gallbladder hypomotility (28), and mucus hyper secretion also contribute to cholesterol precipitation leading to the development of gallstones (30, 31).

**Bile salts**

Bile salts were significantly lower in CS than in MS and PS, while bile acids were significantly higher in PS. Supersaturation of bile with calcium bilirubinate is inhibited by bile salts, which bind calcium, reducing the activity of free calcium ions. When supersaturation occurs, usually due to increased concentrations of bilirubinate anion, nucleation may be initiated by binding of calcium bilirubinate to mucin glycoproteins in bile (31, 32). Similarly, the phospholipid content was marginally higher in cholesterol than PS and MS.

**Triglycerides**

Triglyceride content was higher in MS than in the other two types of stones, but the difference was significant only compared to PS ($p=0.0004$). Triglycerides accumulate along with cholesterol salts to form gallstones. The higher content of triglycerides in MS or CS compared to PS might be due to a higher deposition of calcium salts of cholesterol and esters of fatty acids in MS and CS when compared to PS in which calcium bilirubinate is the major salt (37).

**Phospholipids**

Phospholipid content was highest in CS and lowest in MS. There were significantly more phospholipids in CS than in MS ($p=0.0080$) and PS ($p=0.0170$). This might be due to accumulation of phospholipids along with cholesterol during CS formation.

### Table 3. Concentrations of metabolites in the different types of biliary calculi

| Stone type | Cholesterol (mg/gm) | Triglycerides (mg/gm) | Bilirubin (mg/gm) | Bile acids (mg/gm) | PL (mg/gm) |
|------------|---------------------|-----------------------|------------------|--------------------|------------|
| CS         | 608 ± 173           | 51 ± 14               | 0.5 ± 0.30       | 16.5 ± 2.60        | 8.0 ± 2.70 |
| MS         | 518 ± 125           | 56 ± 10               | 2.0 ± 0.30       | 20.0 ± 2.00        | 5.7 ± 0.90 |
| PS         | 466 ± 87            | 40.5 ± 11             | 4.0 ± 0.90       | 22.0 ± 3.60        | 6.0 ± 1.40 |

Note: CS, cholesterol stones; MS, mixed stones; PS, pigment stones; PL, phospholipids. Values are the mean and standard deviation.

### Table 4. P-values for differences in chemical composition of biliary calculi shown in Table 3

| Groups        | Cholesterol | Triglycerides | Bilirubin | Bile acids | Phospholipids |
|---------------|-------------|---------------|-----------|------------|---------------|
| CS vs. MS     | 0.1472      | 0.3186        | 0.0001    | 0.0009     | 0.0080        |
| CS vs. PS     | 0.0085      | 0.0263        | 0.0001    | 0.0001     | 0.0170        |
| MS vs. PS     | 0.1983      | 0.0004        | 0.0001    | 0.8521     | 0.5101        |

Note: CS, cholesterol stones; MS, mixed stones; PS, pigment stones.
As expected, the bilirubin content was highest in PS. This has been demonstrated in many earlier studies (32). The bilirubin in these stones is mostly unconjugated because the conjugated form is water soluble and excreted through bile (38). Decreased secretion of biliary acids, increased secretion of unconjugated bilirubin into the bile, and infection of the biliary tract are the most important causative factors (39, 40).

Calcium content was highest in PS, while phosphorus was lowest. It is known that bilirubin combines with calcium to form a precipitate of calcium bilirubinate (41). Since PS have excess bilirubin, calcium forms calcium bilirubinate (42).

Phosphorus content was low in PS. The reason for this is not clear but may be related to the availability of calcium, which is more in these stones in the form of calcium bilirubinate. Phosphorus content was significantly higher in CS and MS than PS. It is likely that phosphorus may play a more important role than calcium in CS formation by forming a salt with calcium, which might be responsible for the hardness of the CS (6).

Sodium and potassium
Sodium content was higher in PS than in the other types. It was not surprising that bile acids were more in PS because an increase in sodium content facilitates excessive formation of bile salts. Potassium was also higher in PS. It is presumed that the sodium to potassium ratio will be maintained in the bile. Hence, higher sodium content is associated with higher potassium content, although the increase in the latter was not as much as that of the sodium content.

Magnesium
Magnesium was higher in PS and CS than MS. This is in conformity with observations made by Chandran et al. (13).

Oxalates
Since the higher oxalate content in CS is likely to be associated with higher magnesium content, the formation of magnesium oxalate may be responsible for the hardness of PS.

The Libyan population seems to be more susceptible to cholelithiasis when compared with other countries according to earlier studies in Libya (2, 3). The observation that CS are the most prevalent type of gallstones is in agreement with studies performed in northern India (6, 7, 11), Japan (14), and Singapore (15), but differs from sub-Saharan countries and southern India, where PS are more prevalent, with a higher bilirubin and calcium content and lower cholesterol content when compared with stone composition in Libya (43, 44).

The potential reasons for stone formation may be attributed to (a) dietary factors, (b) multiple pregnancies with less spacing between pregnancies, (c) metabolic syndrome, (d) familial, and (e) ethnic (45–47). It is known that foods that are rich in lipids result in development of hyperlipidemias and subsequent increase in all lipid constituents. There is no corresponding

Table 5. Concentrations of different ions in gallstones (mg/gm powder)

| Type | Ca²⁺ | PO₄⁻ | Na⁺ | K⁺ | Mg²⁺ | Cl⁻ | Oxalate |
|------|------|------|-----|----|------|-----|--------|
| CS   | 11.4±2.40 | 14.0±1.60 | 1.4±0.50 | 0.5±0.17 | 8.5±1.80 | 19.5±1.50 | 7.0±1.18 |
| MS   | 18.0±4.70 | 10.5±1.90 | 1.15±0.18 | 0.32±0.06 | 7.75±0.90 | 21.5±1.90 | 6.0±1.20 |
| PS   | 21.75±3.60 | 9.0±1.50 | 3.5±0.73 | 0.65±0.17 | 10.8±1.25 | 33.5±3.60 | 6.5±0.90 |

Note: CS, cholesterol stones; MS, mixed stones; PS, pigment stones. Values are the mean and standard deviation.

Table 6. P-values for differences in composition of various ions of gallstones shown in Table 5

| Comparison | Ca²⁺ | PO₄⁻ | Na⁺ | K⁺ | Mg²⁺ | Cl⁻ | Oxalate |
|------------|------|------|-----|----|------|-----|--------|
| CS vs. MS  | 0.0002 | 0.0001 | 0.1044 | 0.0016 | 0.1952 | 0.0080 | 0.0471 |
| CS vs. PS  | 0.0001 | 0.0001 | 0.0001 | 0.0291 | 0.0005 | 0.0001 | 0.2140 |
| MS vs. PS  | 0.0218 | 0.0248 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.2105 |

Note: CS, cholesterol stones; MS, mixed stones; PS, pigment stones.
increase in substances such as bile salts and phospholipids, which are responsible for solubilization of cholesterol and other biliary constituents. Consequently, there is a precipitation of cholesterol and bilirubin leading to stone formation (40).

Comparison of the chemical composition of gallstones in Libyan individuals with those in other countries is summarized in Table 7. Though cholesterol is a major component of gallstones, the composition of gallstones varies from country to country and region to region. One study in sub-Saharan Africa showed that majority of stones were of the pigment type, in which the contribution of cholesterol is less than in other stones (43). Similarly, a study in southern India reported predominance of pigment and mixed gallstones with reduced cholesterol and increased bilirubin and calcium concentrations in the stones (44).

CS are more prevalent than PS in north India, Australia, Bolivia, Germany, England, Kuwait, USA, Sweden, and South Africa (48–51). Although all types of gallstones are prevalent among the Libyan population, it is interesting to note that cholesterol is the major component of all stones.

**Conclusion**

An interesting finding of our study is that although PS was the most common type of gallstones, cholesterol seemed to be the major component in all types of stones. High cholesterol content in CS especially suggests supersaturation of cholesterol in bile consequent to dyslipidemia (excessive cholesterol and altered lipid metabolism) is an etiological factor. Higher triglyceride content in MS also suggests that dyslipidemic changes contribute to etiology. Our findings suggest that dyslipidemia consequent to high intake of fats by Libyan population may be responsible for gallstones, and dietary modification might reduce the incidence of gallstones. Further, considering that cholesterol levels in the gallstones mirrors the serum cholesterol levels, health issues associated with increased cholesterol levels, such as cardiovascular diseases, might be associated. However, larger randomized studies are required to study this association and to confirm these observations.

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**References**

1. Schafmayer C, Hartleb J, Tepel J, Albers S, Freitag S, Volzke H, et al. Predictors of gallstone composition in 1025 symptomatic gallstones from Northern Germany. BMC Gastroenterol. 2006; 6: 36.
2. Elmehdawi RR, Elmajberi SJ, Elramli A. Prevalence of gallbladder stones among type 2 diabetic patients in Benghazi, Libya. A case control study. Libyan J Med. 2009; 4: 27–30.
3. Nasser E, Issa A, Rajab E, Ali G. Prevalence of gallbladder stones in post cholecystectomy in diabetic patients. JMJ 2007; 7: 42–44.
4. Baruni A, Gupta A, Zerdeh A. Vomiting of gall stones – a case report. Gypsyounis Med J. 1992; 15: 90–1.
5. Raha PK, Sengupta KP, Aikat BK. X-ray diffraction analysis of gallstones. Indian J Med Res. 1966; 54: 729–34.
6. Udupa KN, Chansouria JPN, Gode JD, Gupta S. Studies on etiology of gallstone. Indian J Surg. 1968; 68: 120–8.
7. Tyagi SP, Tyagi N, Maheshwari V, Ashraf SM, Sahoo P. Morphological changes in diseased gall bladder: a study of 415 cholecystectomies at Aligarh. J Indian Med Assoc. 1992; 90: 178–81.

**Table 7. Percentage of chemical composition of gallstones from various countries**

| Country and reference | No. of stones | Cholesterol (%) | Triglycerides (%) | Bilirubin (%) | Calcium (%) | PO₄ / Mg, Oxalates, Na⁺, K⁺, and Cl⁻ (%) |
|-----------------------|--------------|----------------|-----------------|----------------|-------------|----------------------------------------|
| Australia (49)         | 5            | 93             |                 |                |             | 3.5                                    |
| England (51)           | 11           | 66             |                 |                |             | 17                                     |
| Germany (1)            | 1,025        | 93.3           | 5.5             | 4.8            |             |                                        |
| India (13)             | 200          | 56.6           | 5.7             | 0.3            | 2.2         | 35.2                                   |
| Kuwait (51)            | 10           | 60             |                 |                | 37          | 3                                      |
| South Africa (51)      | 11           | 66             |                 |                | 29          | 5                                      |
| Sweden (51)            | 27           | 94             |                 |                | 4           | 2                                      |
| USA (51)               | 42           | 88             |                 |                | 9           | 3                                      |
| Bolivia (50)           | 66           | 93.3           |                 |                | 4.1         |                                        |
| Libya (present study)  | 41           | 53             | 4.9             | 2.16           | 1.70        | 38.4                                   |
8. Bansal SK, Gupta SK, Bansal A, Rajput VS, Joshi LD. Chemical composition of biliary calculi from Kanpur region. Indian J Clin Biochem. 1992; 7: 27–9.
9. Bhansali SK. Cholelithostomy. Indian J Surg. 1979; 41: 485–91.
10. Singh A, Bagga SPS, Jindal VP, Singh K, Rao SS. Gall bladder disease: an analytic report of 250 cases. J Indian Med Assoc. 1989; 87: 253–6.
11. Verma GR, Pandey AK, Bose SM, Prasad R. Study of serum calcium and trace elements in chronic cholelithiasis. ANZ J Surg. 2002; 72: 596–9.
12. Pandir CS, Chaudhary R, Rani K, Chandran P, Kumari M, Garg P. Chemical analysis of biliary calculi in Haryana. Indian J Surg. 2001; 63: 370–3.
13. Chandran P, Kuchhal NK, Garg P, Pandir CS. An extended chemical analysis of gallstone. Indian J Clin Biochem. 2007; 22: 145–50.
14. Nakayama F. Quantitative microanalysis of gallstones. J Lab Clin Med. 1968; 72: 602–11.
15. Ti JK, Yuen R. Chemical composition of biliary calculi in relation to pattern of biliary disease in Singapore. Br J Surg. 1985; 72: 556–8.
16. Tandon RK, Saraya A, Paul S, Kapur BM. Dietary habits of gallstone patients in Northern India. J Clin Gastroenterol. 1996; 22: 23–7.
17. Jonnalagadda SS, Trautwein EA, Hayes KC. Dietary fats rich in saturated fatty acids (12:0, 14:0, and 16:0) enhance gallstone formation relative to monounsaturated fat (18:1) in cholesterol-fed hamsters. Lipids 1995; 30: 415–24.
18. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem. 1974; 20: 470–5.
19. Gambino SR. In: Metter S, editor. Standard methods of clinical chemistry. vol. 5. New York, NY: Academic Press; 1965. p. 55.
20. Buccolo G, David H. Quantitative determination of serum triglycerides by the use of enzymes. Clin Chem. 1973; 20: 470–5.
21. Pandir CS. Purification and properties of an oxalate oxidase from leaves of grain sorghum hybrid. Biochim Biophys Acta. 1993; 1161: 1–5.
22. Young DS, Pestaner LC, Gibberman V. Effects of drugs on clinical laboratory tests. Clin Chem. 1975; 21: 1D–432D.
23. Fiske CH, Subba Row Y. The colorimetric determination of phosphorus. J Biol Chem. 1925; 66: 375–400.
24. Elharrati V, Baadenhuijsen H, Brenna S, Browne M, Garcia-Beltran L, Hellsing K, et al. Results of multicenter evaluation of reagents for determination of sodium, potassium and chloride ions using enzyme activation. Wien Klin Wochenschr Suppl. 1992; 192: 12–21.
25. Schoenfeld RG, Lewellen CJ. A colorimetric method for determination of serum chloride. Clin Chem. 1964; 10: 533–9.
26. Carey JB. The serum trihydroxy-dihydroxy acid bile ratio in liver and biliary tract disease. J Clin Invest. 1958; 17: 1494–502.
27. Smith JL, Nathanson LK, Riottot M. Effect of statins on biliary lipids and cholesterol gallstones. J Für Kardiologie. 2002; 9: 295–8.
28. Saraya A, Irshad M, Gandhi BM, Tandon RK. Plasma lipid profile in gallstone patients from North India. Trop Gastroenterol. 1995; 16: 16–21.
29. Apstein MD, Carey MC. Pathogenesis of cholesterol gallstones: a parsimonious hypothesis. Eur J Clin Invest. 1996; 26: 343–52.
30. Portincasa P, Di Cauia A, Vendemiale G, Palmieri V, Moschetta A, Vanberge-Henegouwen GP, et al. Gallbladder motility and cholesterol crystallization in bile from patients with pigment and cholesterol gallstones. Eur J Clin Invest. 2000; 30: 317–24.
31. Lamont T. Mucin glycoprotein content of human pigment stone. Hepatology 1983; 3: 372–82.

*Peela Jagannadharao
Department of Biochemistry
Faculty of Medicine
Al-Arab Medical University
Benghazi, Libya,
Email: pjqagannadharao@hotmail.com