# Hypoalbuminemia following abdominal surgery leads to high serum unbound bilirubin concentrations in newborns soon after birth

Miwa, Akihiro

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# Hypoalbuminemia following abdominal surgery leads to high serum unbound bilirubin concentrations in newborns soon after birth

# 生後早期の新生児では、腹部外科手術後に低アルブミン血症による高アンバウンドビリルビン血症を引き起こす

三輪 明弘、 森岡 一朗、 久松 千恵子、 藤岡 一路、 森川 悟、
柴田 暁男、 安福 正男、 横山 直樹、 松尾 雅文

神戸大学大学院医学系研究科医科学専攻
小児科学
（指導教官:松尾雅文教授）

三輪 明弘

**Key words:** neonatal jaundice; bilirubin-induced neurologic dysfunction; bilirubin; albumin; surgical operation

**Abstract**

***Background***: The serum concentration of unbound bilirubin (UB), which is bilirubin not bound to albumin (Alb), is a better index than total bilirubin concentration (TB) for identifying infants at risk for developing bilirubin neurotoxicity. The degree to which the hypoalbuminemia following abdominal surgery in jaundiced newborns affects bilirubin binding is unknown. ***Objective***: To determine whether the lower Alb results in significantly higher UB in serum versus non-surgical patients at comparable serum TB. ***Methods***: A matched case-control study was conducted with term and late-preterm newborns. The surgery group included 15 newborns who underwent abdominal operations within 3 days after birth. Clinical and laboratory data (serum UB, TB, and Alb concentrations, UB/TB ratio, and binding constant) in the surgery group were collected and compared with those of 30 control newborns who did not undergo abdominal surgery (control group). ***Results***: Serum UB and the UB/TB ratio in the surgery group were significantly higher than those in the control group ($p<0.02$, $p<0.001$, respectively), whereas there were no significant differences in serum TB and binding constant between the groups. Serum Alb concentrations in the surgery group were significantly lower than those in the control group ($p<0.001$). When pre- and post-operative serum Alb concentrations were compared, there was a significant decrease from 3.4 g/dl to 2.7 g/dl ($p<0.001$). ***Conclusions***: Our study suggests that hypoalbuminemia following abdominal surgery causes a higher serum UB at comparable serum TB in newborns.

## Introduction

Neonatal jaundice is the result of increased bilirubin production superimposed on an immature bilirubin conjugation and excretion system in the liver. Term or late-preterm newborns transiently present with jaundice, usually resolving within 1 week after birth. However, some newborns develop pathological jaundice, resulting in high serum bilirubin concentrations (hyperbilirubinemia). With pathological neonatal jaundice, there is an increased risk for developing bilirubin-induced neurologic dysfunction (BIND), such as acute bilirubin encephalopathy, and its sequelae, kernicterus [1]. Because unbound bilirubin, which is bilirubin not bound to albumin (Alb), easily enters the brain, serum unbound bilirubin concentration (UB) has been reported to be a better index than total bilirubin concentration (TB) for identifying infants at risk for developing BIND in recent experimental and clinical studies [2-6]. Therefore, the management of newborns with hyperbilirubinemia using the measurement of serum UB has attracted much notice in recent years to prevent BIND [2].

The mass action relationship between serum UB, TB, and Alb concentrations, and the binding constant (K) is shown in an equation as follows: $[UB] = [TB] / K*([Alb] - [TB])$ [2, 7]. Alb and K are highly variable in newborns. In particular, Alb binding for bilirubin is a crucial determinant of UB, and a reduction in Alb may lead to a higher UB in serum [2, 7]. Therefore, hypoalbuminemia in newborns within 1 week after birth may carry a major risk of a high serum UB. Serum Alb concentrations are known to be significantly reduced following abdominal operations in adults [8, 9]. In newborns, hypoalbuminemia also occurs after an abdominal operation. However, there are no reports that have clarified the changes in serum Alb concentrations in newborns before and after abdominal operations soon after birth. Moreover, there are no studies about the impact of hypoalbuminemia following abdominal surgery on serum bilirubin binding in newborns soon after birth.

The purpose of this study was to determine whether the lower serum Alb concentration occurring in newborns undergoing abdominal surgery shortly after birth is sufficient to cause higher serum UB compared with non-surgical newborns who have similar serum TB.

## Materials and Methods

*Study design and patient groups*

For this retrospective matched case-control study, we reviewed the medical charts of term and late-preterm newborn patients who were hospitalized at Kobe University Hospital between January 2006 and September 2008 with the approval of the ethical committee of Kobe University Graduate School of Medicine. Fifteen newborns born at ≥34 weeks' gestation who underwent abdominal operations within 3 days after birth (surgery group) were enrolled in the study. The 15 enrolled patients were all newborns who underwent abdominal operations at that age during the study period in our hospital. The diseases for all these newborns were congenital abnormalities of the gastrointestinal tract: four duodenal atresias, four intestinal atresias, three anorectal anomalies, two malrotations, one Hirschsprung's disease, and one esophageal atresia. The confirmed diagnosis of congenital abnormalities of the gastrointestinal tract was made by the operative findings or pathological findings of extirpated sections. All of the newborns in the surgery group were treated with some type of medication (cephazolin, cefmetazole, furosemide, famotidine, phenobarbital, and/or fentanyl citrate, but not intralipid, Alb, or fresh frozen plasma) before, during, and/or after their operations. Three of 15 newborns in the surgery group developed clinical jaundice. Pathologic causes of jaundice, such as blood group incompatibility, red blood cell enzyme abnormalities, red blood cell membrane defects, extravascular blood, polycythemia, hormonal deficiencies, or bilirubin-metabolism disorders, were not found in these patients. They also had no family history and clinical findings of hyperlipemia.

The control group consisted of 30 newborns who did not undergo any operations, including term and late-preterm newborns who were healthy or not severely sick. The control group was randomly chosen from 919 infants cared for in a level II transitional care nursery or well-baby nursery of Kobe University Hospital, and matched for gestational age, birth weight, and sex with those in the surgery group.

Clinical and laboratory data, including the serum UB, TB, direct bilirubin (DB), and Alb concentrations, UB/TB ratio, and K were compared between groups. Furthermore, pre- and post-operative serum Alb concentrations were evaluated in the surgery group.

*Measurement of the serum UB, TB, DB, and Alb concentrations*

For all newborns who are cared for in our hospital, both serum UB and TB are routinely measured after informed consent to the parents of the newborns and assessed for the management of neonatal hyperbilirubinemia. In all surgery patients, serum UB, TB, DB, and Alb concentrations were measured on same blood sample before and soon after operation, and then every 12 to 24 hours until at least 7 days after birth. In control newborns, they were measured at 0, 1, 4, 5, and 7 days after birth.

The serum TB and UB were measured by an automated UB-Analyzer (Arrows Co, Ltd, Osaka, Japan), using spectrophotometry and the glucose oxidase-peroxidase method, respectively, as previously described [10, 11]. The serum TB and UB were displayed in mg/dl (1 mg/dl = 17.1 μmol/l) and in μg/dl (1 μg/dl = 17.1 nmol/l), respectively. The serum UB was measured at a single peroxidase concentration. Serum Alb concentrations and serum DB were measured using the modified bromocresol purple method and the bilirubin oxidase method, respectively [12, 13].

*Calculation of UB/TB ratio and K*

The UB/TB ratio and K were calculated using the following equations:

UB/TB ratio = UB (μg/dl) / TB (mg/dl)

K = [TB] / [UB]*([Alb] – [TB])

The distribution of the bilirubin pool between the various compartments depends on the number of binding sites in each compartment and their affinity for binding bilirubin which is quantified by a K [2]. The UB is the free fraction of unconjugated bilirubin combined with not only Alb, but also erythrocyte membrane, lipoprotein and others in blood. Of these carriers, Alb is the main carrier for bilirubin with a high K [2].

$$\text{[free Alb]} + \text{[UB]} \underset{}{\overset{K}{\rightleftharpoons}} \text{[Alb-bound bilirubin]}$$

The relationship between serum UB, Alb, and Alb-bound bilirubin concentrations can be expressed as K = [Alb-bound bilirubin] / [free Alb]*[UB]. Because [TB] = [Alb-bound bilirubin] + [UB], and [UB] is very small quantity relative to [TB], [Alb-bound bilirubin] is almost equal to [TB]. [free Alb] is equal to [Alb] – [TB]. Therefore, the equation can be expressed as K = [TB] / [UB]*([Alb] – [TB]). For calculating K (l/μmol), TB (mg/dl), UB (μg/dl), and Alb (g/dl) were converted to μmol/l by dividing by 0.058, 58.5, and 0.006612, respectively.

*Statistical analysis*

Data are expressed as the median (range). Statistical analyses were performed with the Mann-Whitney nonparametric rank test, Chi-square test, or Fisher's exact test as appropriate. Differences were deemed statistically significant when $p<0.05$.

## Results

*Clinical characteristics in enrolled patients*

Clinical characteristics in the surgery and control groups are shown in Table 1. The control group included 30 newborns; there were 15 healthy newborns, 12 with transient tachypnea of the newborn, one with transient hypoglycemia, one with meconium aspiration syndrome with mild symptoms, and one newborn delivered from a mother with systemic lupus erythematosus. The gestational age, birth weight, gender and Apgar scores were similar in both groups. When pre-operative serum TB in the surgery group was compared with serum TB at the same age in the control group, pre-operative serum TB was a little higher than that in the control group, but there was not a significant difference. There were no significant differences in age at the maximum serum UB between the groups. All of the 15 newborn patients in the surgery group had infusion therapy, whereas approximately half of the patients in the control group had infusion therapy. Fluids with glucose and electrolytes were used for infusion therapy in both groups. No significant differences were observed in body weight changes at the age of maximum serum UB, although there were significant differences in the rates of infusion therapy between the groups.

There were no differences in gestational age, birth weight, gender, and serum UB and TB between control newborns who had infusion therapy and those who did not have therapy (data not shown). There was no significant difference in serum Alb concentrations in control newborns with infusion therapy before and after infusion therapy (3.5 g/dl [3.2 to 4.0 g/dl] vs. 3.6 g/dl [3.1 to 4.1 g/dl], p=not significant (n.s.)).

As expected, the age at beginning of feeding and age at enteral feeding at 100 ml/kg/day were significantly later in the surgery group than those in the control group ($p<0.001$, $p<0.001$, respectively). Four of 15 newborns (27%) in the surgery group and 27 of 30 newborns (90%) in the control group were receiving breast milk.

*Maximum serum UB*

The serum UB in the surgery group was significantly higher than that in the control group ($p<0.02$, Fig. 1A). The number of newborns with a serum UB greater than 1.0 μg/dl was higher (4/15, 27%) in the surgery group than that in the control group (1/30, 3%) ($p<0.05$).

*Serum TB, UB/TB ratio, and serum Alb concentrations at the age of maximum serum UB*

We compared the serum TB, UB/TB ratio, and serum Alb concentrations between the surgery and control groups at the age of maximum serum UB (Fig. 1B, C, D). No significant differences were found in serum TB between the groups. In the surgery group, the UB/TB ratio was significantly higher than that in the control group ($p<0.001$). In contrast, serum Alb concentrations in the surgery group were significantly lower than those in the control group ($p<0.001$).

*K and serum DB at the age of maximum serum UB*

No significant differences were found in K and serum DB between the groups (Table 2). Serum DB was less than 2 mg/dl in all newborns of the surgery and control groups.

*Pre- and post- operative serum Alb concentrations*

Thirteen of 15 patients in the surgery group (87%) had a reduction in post-operative serum Alb concentrations compared with pre-operative values (Fig. 2). Median serum Alb concentrations in the surgery group were significantly reduced from 3.4 g/dl (1.8 to 4.2 g/dl) pre-operation to 2.7 g/dl (2.0 to 3.2 g/dl) post-operation ($p<0.001$). However, there were no changes in median serum Alb concentrations from 0 to 5 days after birth in the control group (0 days after birth: 3.7 g/dl [3.1 to 4.4 g/dl] vs. 5 days after birth: 3.6 g/dl [3.2 to 4.1 g/dl], p=n.s.).

**Discussion**

The results of this study suggest that at a given serum TB, the serum UB will be higher in jaundiced, post-abdominal surgery patients if they are hypoalbuminemic. Therefore, low serum Alb concentrations following abdominal surgery require special attention when interpreting the risk of TB and TB/Alb ratio is critical in estimating serum UB unless serum UB can be measured directly.

Bowel obstructions are known to cause high serum TB via increased enterohepatic circulation [14]. It has been shown that fasting increases serum bilirubin [15]. In our study, pre- and post-operative serum TB in the surgery group was not significant higher than that at the same age in the control group (Table 1, Fig. 1). However, the total body bilirubin load in the surgery group should have increased at the age of maximum serum UB, because operated newborns have a transient decrease in peristalsis and continue to fast post operatively, and bilirubin as well as Alb, is moved into extra-vascular spaces with a fall in the vascular TB after operations. The increased total body bilirubin load after operations also may have an impact on increased serum UB. Furthermore, the location of lesion may be related to high TB [16]. When our 15 enrolled surgery patients were classified by anatomical diagnosis, serum TB of newborns with the upper bowel obstruction was higher than that of newborns with the lower bowel obstruction (data not shown). Because these data were from a small survey in our single center, further studies using a larger group of operated newborns due to congenital abnormalities of the gastrointestinal tract are needed to draw this conclusion.

In general, serum UB peaks at approximately 4 days after birth in Japanese newborns [6]. Our enrolled newborns in the surgery and control groups also reached peak concentrations of serum UB at 4 and 5 days, respectively, after birth (Table 1). The serum TB in the surgery group ordinarily would not raise concerns. However, because these newborns have relatively higher serum UB at any given serum TB, BIND may be of concern at serum TB below conventional guidelines for intervention for newborns undergoing abdominal surgery. Therefore, abdominal surgery should be included in the lists of conditions considered to place newborns at increased risk for BIND.

The UB-Analyzer occasionally shows higher values when serum DB exceeds 2 mg/dl, and lower values when there is a large quantity of vitamin C in the reaction system; its antioxidative effect interferes with bilirubin oxidation by peroxidase [6]. In all enrolled newborns in this study, serum DB was less than 2 mg/dl. None of the enrolled newborns had a large quantity of vitamin C. Therefore, we believe that the serum UB in this study was a reliable measurement.

Previous reports have shown that fasting does not cause reduction of serum Alb concentrations, even though there is a sharp decrease in Alb serum concentrations within a couple of days after an abdominal operation [9, 17]. Sixteen of 30 cases in the control group had infusion therapy for several reasons in this study. We found that newborns with infusion therapy did not have any significant differences in serum Alb concentrations before and after infusion therapy. Therefore, we concluded that infusion therapy does not contribute to large reductions in serum Alb concentrations. Our results showed clear evidence that serum Alb concentrations are reduced in newborns after abdominal operations soon after birth (Fig. 2). We found that postoperative hypoalbuminemia appears not only in adults, but also in newborns soon after birth [8]. Critical illness after major surgical stress generally alters the distribution of Alb between the intravascular and extravascular components [8, 9, 17]. Hypoalbuminemia after surgical operations is related to loss of intravascular Alb due to capillary leakage, and not hepatic dysfunction or poor nutritional status [9, 17]. Although K is often lower in sick newborns [2], we did not find a decrease in K in the surgery group.

The limitations of our study are as follows: 1) the influence of bilirubin displacers and other carriers of bilirubin has not been investigated; 2) the peroxidase method with a single peroxidase concentration using the UB-Analyzer may underestimate serum UB; and 3) this is a retrospective study with a small number of newborns who underwent abdominal operations soon after birth.

Some medications that have been reported as bilirubin displacers were used in newborns in the surgery group, such as cefazolin, cefmetazole, furosemide, and Phenobarbital [5]. Because ACTH and cortisol may increase for the stress of an operation, free fatty acids, one of the bilirubin displacers, may increase in serum. Bilirubin is combined with not only Alb, but also erythrocyte membrane, lipoprotein and others in blood. Further studies about the influence of these bilirubin displacers and other carriers of bilirubin are required to determine whether there is a significant increase in serum UB in newborns following abdominal operations soon after birth. Recently, Ahlfors et al. reported that the peroxidase method with a single peroxidase concentration increasingly underestimates serum UB as the serum UB increases [18]. In order to check the accuracy of the serum UB in samples, the re-analyses using two peroxidase concentrations should be needed. Prospective studies using a larger group of newborns with abdominal operations soon after birth are needed to confirm the conclusions of this study.

**Conclusion**

This study has shown for the first time that at any given serum TB, the serum UB may be higher in jaundiced, post-abdominal surgery patients if they are hypoalbuminemic. These findings suggest that newborns who have undergone abdominal operations soon after birth have an increased risk of developing BIND.

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**The authors have no conflicts of interest to declare.**

## References

1 American Academy of Pediatrics Subcommittee on Hyperbilirubinemia: Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. Pediatrics 2004;114:297-316.

2 Wennberg RP, Ahlfors CE, Bhutani VK, Johnson LH, Shapiro SM: Toward understanding kernicterus: a challenge to improve the management of jaundiced newborns. Pediatrics 2006;117:474-485.

3 Ahlfors CE, Parker AE: Unbound bilirubin concentration is associated with abnormal automated auditory brainstem response for jaundiced newborns. Pediatrics 2008;121:976-978.

4 Calligaris SD, Bellarosa C, Giraudi P, Wennberg RP, Ostrow JD, Tiribelli C: Cytotoxicity is predicted by unbound and not total bilirubin concentration. Pediatr Res 2007;62:576-580.

5 Amin SB: Clinical assessment of bilirubin-induced neurotoxicity in premature infants. Semin Perinatol 2004;28:340-347.

6 Lee YK, Daito Y, Katayama Y, Minami H, Negishi H: The significance of measurement of serum unbound bilirubin concentrations in high risk infants. Pediatr Int 2009; DOI: 10.1111/j.1442-200X.2009.02878.x.

7 Ahlfors CE, Wennberg RP, Ostrow JD, Tiribelli C: Unbound (free) bilirubin: improving the paradigm for evaluating neonatal jaundice. Clin Chem 2009;55:1288-1299.

8 Yuan XY, Zhang CH, He YL, Yuan YX, Cai SR, Luo NX, Zhan WH, Cui J: Is albumin administration beneficial in early stage of postoperative hypoalbuminemia following gastrointestinal surgery?: a prospective randomized controlled trial. Am J Surg 2008;196:751-755.

9 Rittler P, Jacobs R, Demmelmair H, Kuppinger D, Braun S, Koletzko B, Jauch KW, Hartl WH: Dynamics of albumin synthesis after major rectal operation. Surgery 2007;141:660-666.

10 Nakamura H, Lee Y: Microdetermination of unbound bilirubin in icteric newborn sera: an enzymatic method employing peroxidase and glucose oxidase. Clin Chim Acta 1977;79:411-417.

11 Shimabuku R, Nakamura H: Total and unbound bilirubin determination using an automated peroxidase micromethod. Kobe J Med Sci 1982;28:91-104.

12 Muramoto Y, Matsushita M, Irino T: Reduction of reaction differences between human mercaptalbumin and human nonmercaptalbumin measured by the bromcresol purple method. Clin Chim Acta 1999;289:69-78.

13 Kimura S, Iyama S, Yamaguchi Y, Hayashi S, Yanagihara T: Enzymatic assay for conjugated bilirubin (Bc) in serum using bilirubin oxidase (BOD). J Clin Lab Anal 1999;13:219-223.

14 Madan A, MacMahon JR, Stevenson DK: Neonatal hyperbilirubinemia. in William Taeusch H, Ballard RA, Gleason CA (eds): Avery's Disease of the Newborn 8th edition. Elsevier, 2005, pp 1226-1256.

15 Meyer BH, Scholtz HE, Schall R, Muller FO, Hundt HK, Maree JS: The effect of fasting on total serum bilirubin concentrations. Br J Clin Pharmacol 1995;39:169-171.

16 Tan KL, Lim SC: Phototherapy for neonatal jaundice in infants with intestinal obstruction. J Pediatr 1983;103:471-473.

17 Nicholson JP, Wolmarans MR, Park GR: The role of albumin in critical illness. Br J Anaesth 2000;85:599-610.

18 Ahlfors CE, Vreman HJ, Wong RJ, Bender GJ, Oh W, Morris BH, Stevenson DK: Effects of sample dilution, peroxidase concentration, and chloride ion on the measurement of unbound bilirubin in premature newborns. Clin Biochem 2007;40:261-267.

**Figure legends**

Fig. 1. Comparison of maximum serum UB (A), and serum TB (B), UB/TB ratio (C), and serum Alb concentrations (D) at the age of maximum serum UB between the surgery and control groups. Serum UB (μg/dl, 1 μg/dl = 17.1 nmol/l), serum TB (mg/dl, 1 mg/dl = 17.1 μmol/l), UB/TB ratio, and serum Alb concentrations (g/dl) in each group are expressed as the median (range). Serum UB in the surgery group was significantly higher than that in the control group ($p<0.02$). In the surgery group, the UB/TB ratio, but not serum TB, was significantly higher than that in the control group (serum TB: p=n.s., UB/TB ratio: $p<0.001$). In contrast, serum Alb concentrations were significantly lower in the surgery group than those in the control group ($p<0.001$). *UB*, unbound bilirubin concentration; *TB*, total bilirubin concentration; *UB/TB ratio*, unbound bilirubin concentration / total bilirubin concentration; *Alb*, albumin; *n.s.*, not significant.

Fig. 2. Changes in pre- and post- operative serum Alb concentrations in the surgery group (n=15). Thirteen of 15 patients (87%) had decreased serum Alb concentrations. Serum Alb concentrations after the operation were significantly decreased compared with those before the operation ($p<0.001$). *Alb*, albumin.

Table 1. Clinical characteristics in the surgery and control groups.

| | | Surgery n=15 | Control n=30 | P value |
|---|---|---|---|---|
| Gestational age (weeks) | | 37 (34-41) | 38 (35-41) | n.s. |
| Birth weight (g) | | 2,740 (2,106-3,772) | 2,961 (2,112-3,676) | n.s. |
| Apgar score | 1 min | 8 (5-9) | 8 (5-9) | n.s. |
| | 5 min | 9 (7-10) | 9 (7-10) | n.s. |
| Male (%) | | 7 (47%) | 14 (47%) | n.s. |
| Serum TB before the age of operation (mg/dl) | | 6.4 (2.0-13.6) | 5.2 (1.8-11.7)* | n.s. |
| Age at operation (days) | | 2 (0-3) | — | |
| Age at maximum UB in serum (days) | | 4 (2-7) | 5 (3-7) | n.s. |
| Infusion therapy (%) | | 15 (100%) | 16 (53%) | < 0.01 |
| Body weight changes at the age of maximum serum UB (%) | | -6.0 (-12.2 to +7.2) | -4.8 (-9.2 to +1.8) | n.s. |
| Age at beginning of feeding (days) | | 6 (4-39) | 0 (0-3) | < 0.001 |
| Age at enteral feeding at 100 ml/kg/day (days) | | 10 (7-111) | 4 (2-7) | < 0.001 |

Data are expressed as median (range) or number (percent); * Serum TB at the same age of the surgery group (1 mg/dl = 17.1 μmol/l); *UB*, unbound bilirubin concentration; *TB*, total bilirubin concentration; *n.s.*, not significant

Table 2. K and serum DB at the age of maximum serum UB in the surgery and control groups.

| | Surgery n=15 | Control n=30 | P value |
|---|---|---|---|
| K (l/μmol) | 88.9 (35.3-172.2) | 84.6 (53.4-123.6) | n.s. |
| Serum DB (mg/dl) | 0.2 (0.1-1.5) | 0.1 (0.1-0.4) | n.s. |

Data are expressed as median (range) or number (percent); *UB*, unbound bilirubin concentration; *DB*, direct bilirubin concentration; *K*, binding constant; *n.s.*, not significant

Fig.1

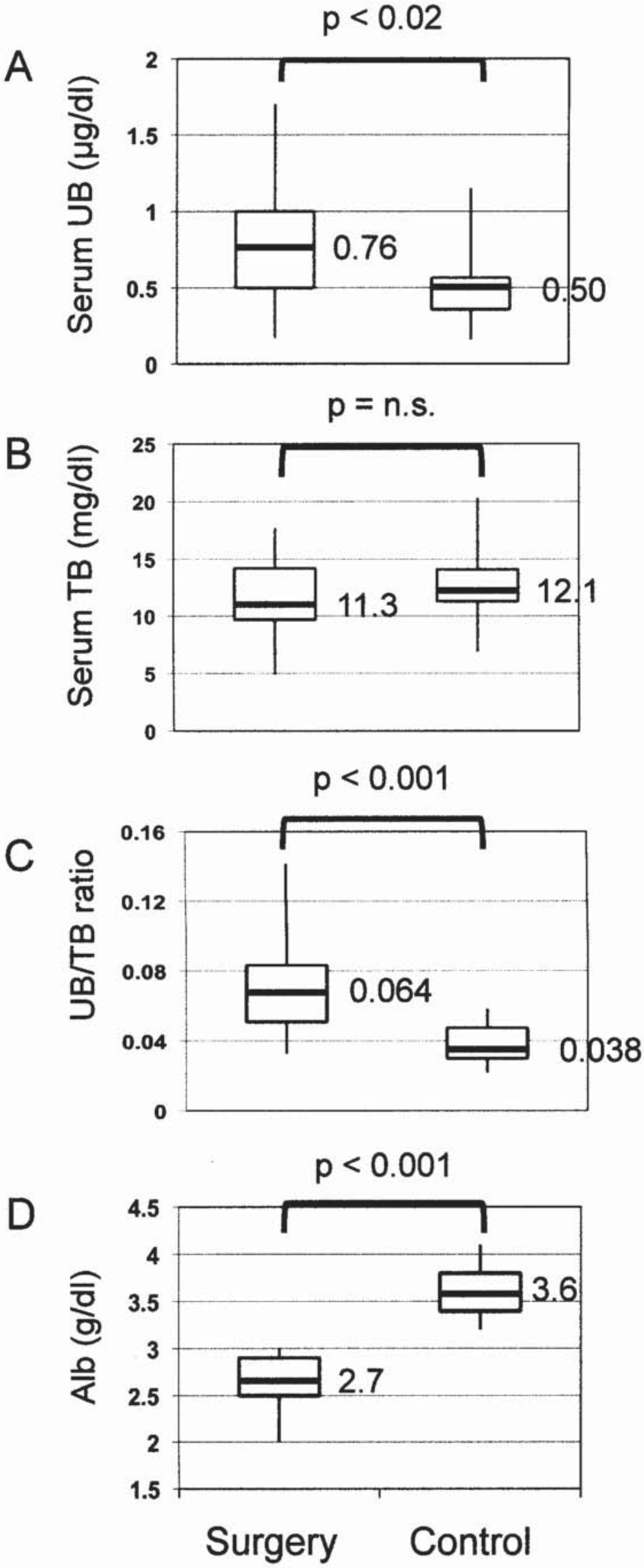
p < 0.02
A
Serum UB (μg/dl)
2
1.5
1
0.5
0
0.76
0.50
p = n.s.
B
Serum TB (mg/dl)
25
20
15
10
5
0
11.3
12.1
p < 0.001
C
UB/TB ratio
0.16
0.12
0.08
0.04
0
0.064
0.038
p < 0.001
D
Alb (g/dl)
4.5
4
3.5
3
2.5
2
1.5
3.6
2.7
Surgery
Control

Fig.2