

Effects of micronutrient status on oxidative stress and exocrine pancreatic function in patients with chronic pancreatitis

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Micronutrient deficiency and oxidative stress in relation to pancreatic exocrine insufficiency among chronic pancreatitis (CP) patients needs closer scrutiny. In this study, we examined the role of micronutrients (Zn and Cu) on oxidative stress related parameters and stool elastase-1 in tropical chronic pancreatitis (TCP) and alcoholic chronic pancreatitis (ACP) patients. We also compared oxidative stress parameters in CP patients with low and normal pancreatic stool elastase-1, estimation of which is the best available test for detecting pancreatic exocrine insufficiency. Ninety-one (56 male and 35 female) TCP cases, 84 ACP cases and 113 (60 male and 24 female) healthy controls were studied. Levels of reduced glutathione (GSH), ascorbic acid and zinc and activities of glutathione peroxidase (GPx), superoxide dismutase (SOD) reduced significantly, while thiobarbituric acid reactive substance (TBARS) and copper level increased significantly in erythrocytes of both ACP and TCP patients in comparison to healthy controls. However, we did not find differences in these parameters between diabetic and non-diabetic TCP patients or between diabetic and non-diabetic ACP patients. The study suggested an association between pancreatic exocrine insufficiency and oxidative parameters, while zinc deficiency was found to be correlated with SOD and pancreatic exocrine insufficiency in CP, irrespective of its etiology.

Keywords: Copper, Oxidative stress, Pancreatitis, Pancreatic exocrine insufficiency, Trace elements, Zinc, Ascorbic acid, Elastase-1, Antioxidant enzymes

Chronic pancreatitis (CP) is an inflammatory disease associated with irreversible exocrine insufficiency, which in due course may cause fibrosis and diabetes mellitus and with significantly increased risk of developing pancreatic cancer. This disease is common in India, particularly in South India. Kerala state in India has the highest incidence of this disease. Excessive alcohol consumption has been identified as the primary etiologic factor in numerous studies of CP, although fewer than 5% of heavy drinkers develop CP¹.

Tropical chronic pancreatitis (TCP) is a form of CP characterized by recurrent abdominal pain, pancreatic calculi and diabetes with young age of onset, malnutrition and severe pancreatic damage¹. Despite many decades of intensive research, the pathogenesis of TCP is not fully understood. The majority of

available data indicates that CP begins within the pancreatic acinar cell². Clinical and experimental studies have indicated that antioxidant deficiency may play a role in the pathogenesis of CP³⁻⁶. Dietary deficiency or painful exacerbations may influence antioxidant levels. In one study, deficiency in the intake of selenium, vitamins A, C, E and riboflavin has been reported in dietary patients with CP, as compared to a healthy control group⁷. Another study has demonstrated depleted antioxidant levels in patients with CP, being lowest during painful exacerbations of the process⁸. Evidence from a double-blind, randomized, controlled trial has suggested that antioxidant therapy consisting of a cocktail of common antioxidants may be beneficial to ameliorate pain associated with CP⁹. However, another group has shown that antioxidant therapy does not reduce pain in patients with painful CP of predominantly alcoholic origin¹⁰.

In this study, the role of copper and zinc on oxidative stress related parameters and stool elastase-1 in TCP and ACP (alcoholic CP) patients has been studied and compared with healthy controls. We have also compared the oxidative stress parameters in CP

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Abbreviations: ACP, alcoholic chronic pancreatitis; AP, acute pancreatitis; CP, chronic pancreatitis; GPx, glutathione peroxidase; GSH, reduced glutathione; NO, nitric oxide; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance; TCP, tropical chronic pancreatitis.

patients with low and normal pancreatic stool elastase-1. Estimation of fecal elastase-1 is one of the best available tests for determining pancreatic exocrine insufficiency¹¹.

Materials and Methods

Glutathione (Sigma–Aldrich, Steinheim, Germany) and other fine chemicals of analytical grade (Qualigens Fine Chemicals, Mumbai, India) were used.

Consecutive CP patients attending the Pancreas Clinic, Amrita Institute of Medical Sciences were recruited in the study. The study was approved by the Institutional Ethics Committee. Written informed consent was obtained from the subjects before enrolment. Chronic pancreatitis patients were diagnosed on the basis of pancreatic calcification (ultrasonography/computerized tomography) and/or parenchymal or ductal changes on imaging (CT/ERCP/MRCP/EUS). Tropical chronic pancreatitis (TCP) was defined as young age of onset (< 35 yrs), non-alcoholic CP patients who have also had no obvious etiological factors of CP on investigation. Alcoholic chronic pancreatitis (ACP) was defined as CP patients consuming alcohol ≥ 80 g/day for ≥ 5 yrs. Patients with pancreatic cancer, pancreatic surgery, pseudocyst or common bile duct obstruction and those consuming protein and vitamin supplements were excluded from the study.

Diabetes mellitus was diagnosed if the fasting plasma glucose value was equal to or greater than 126 mg/dL confirmed on two occasions and/or a plasma glucose value equal to or greater than 200 mg/dL after a 2 h glucose load confirmed on two occasions, and/or there were requirements for insulin or oral hypoglycemic drugs. History of illness including presenting complaints, duration of illness, pain and diabetes mellitus and risk factors, such as alcohol and smoking were recorded. Demographic parameters and anthropometric measurements were elicited and a detailed physical examination conducted. BMI was calculated by the formula: Weight in kg/height in m².

Venous blood was collected in EDTA tubes, centrifuged (1000 g, 10 min) at 2°C and washed three-times with cold normal saline. The precipitate was resuspended in 30 ml of chilled distilled water and mixed to obtain blood hemolysate for further analysis.

Stool samples of CP cases were collected and stored at -4°C for <1 week prior to testing. Serum

albumin¹² and plasma ascorbic acid¹³ (vitamin C) were measured. Reduced glutathione (GSH) content¹⁴, thiobarbituric acid reactive substance (TBARS)¹⁵ and activities of glutathione peroxidase (GPx)¹⁶ and superoxide dismutase (SOD)¹⁷ in erythrocyte were estimated. Erythrocyte zinc and copper were estimated using air-acetylene flame atomic absorption spectrophotometer (3110, Perkin-Elmer, Waltham, MA, USA) as described elsewhere^{18,19}.

Pancreatic elastase-1 in stool was measured by using commercially available ELISA kit (Bioserv Diagnostics, Rostock, Germany) according to manufacturer's instructions.

Statistical analysis

Statistical analysis was done using SPSS version 11 software (SPSS Inc, Chicago, USA). Differences in mean were calculated using One-way analysis of variance with Scheffe post-hoc test. Non-parametric Mann-Whitney U test and Kruskal-Wallis test, as appropriate were used to compare variables without a normal distribution. Linear correlation between the two groups was evaluated by calculating the Spearman rank correlation coefficient. Two-tailed P values less than 0.05 were considered statistically significant.

Results

The demographic characteristics of study population are given in Table 1. Of the 175 cases, there were 91 TCP cases (35 males and 56 females) and 84 alcoholics (all males). No significant change was observed in mean age and body mass index (BMI) among the tested groups. Number of diabetic patients among ACP and TCP was comparable. Majority of alcoholics were smokers.

Table 1—Demographic characteristics of study population

	Healthy controls (n = 113)	Tropical chronic cases (TCP) (n = 91)	Alcoholic chronic cases (ACP) (n = 84)
Age (Yrs) (Mean \pm SD)	36 \pm 11.54	35 \pm 13.7	40 \pm 11.8
BMI (Mean \pm SD)	20.52 \pm 3.17	19.44 \pm 4.02	19.64 \pm 3.21
Gender (Male: female)	60:53 (88.3%)	56:35 (62.5%)	84:0 (100%)
Diabetes	0	53 (58.24%)	44 (52.38%)
Smokers	0	12 (13.18%)	67 (79.8%)
Pain	0	71 (78%)	59 (70.2%)

Serum albumin level (Fig. 1) and plasma vitamin C (Fig. 2) were significantly lower in ACP and TCP cases, as compared to controls.

We observed significant reduction in antioxidant enzyme levels, such as erythrocyte GSH, GPx, SOD and increased erythrocyte TBARS in both TCP and ACP cases, as compared to healthy controls. While erythrocyte TBARS level was elevated in TCP cases as compared to ACP cases, erythrocyte GSH level was significantly reduced in ACP, as compared to TCP cases (Table 2)

Both diabetic and non-diabetic CP cases showed significant reduction in erythrocyte GSH, GPx, SOD and plasma vitamin C (Fig. 3), whereas erythrocyte TBARS was elevated as compared to healthy controls (Table 3). No significant difference in these parameters was observed between diabetic and non-diabetic CP cases (Table 3) and also between diabetic and non-diabetic TCP patients or between diabetic and non-diabetic ACP patients (Table 2). Erythrocyte zinc was significantly lower, whereas erythrocyte copper level was elevated in both ACP and TCP patients, as compared to healthy controls (Table 2).

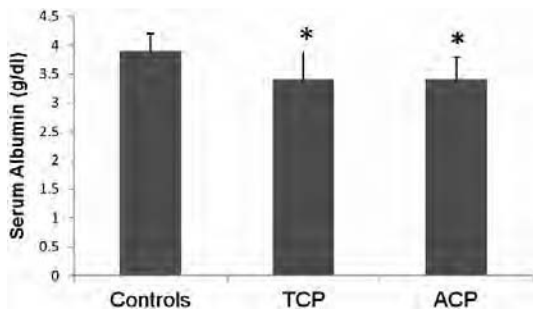


Fig. 1—Serum albumin level in TCP and ACP patients and healthy controls [Values represent mean ± SE]

Pancreatic elastase-1 in stool was measured in 101 CP cases (34 ACP and 67 TCP). It was found to be lower ($\leq 200 \mu\text{g/g}$) in 67 (66.3%) chronic pancreatitis cases while 34 (33.7%) had normal elastase-1. We compared blood antioxidant levels between CP patients with lower and normal elastase-1

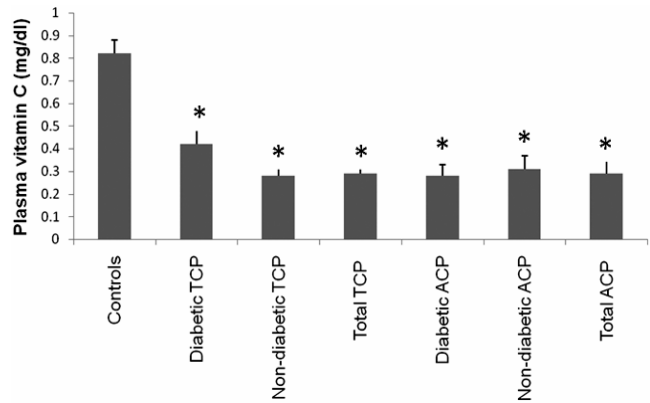


Fig. 2—Plasma vitamin C level in diabetic and non-diabetic TCP and ACP patients and healthy controls [Values represent mean ± SE]

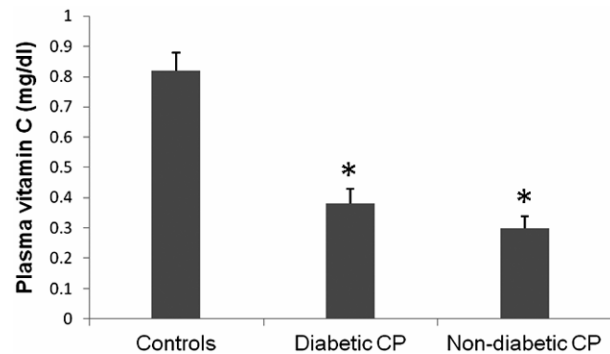


Fig. 3—Plasma vitamin C level in diabetic and non-diabetic CP patients and healthy controls [Values represent mean ± SE]

Table 2—Reduced glutathione (GSH) content, thiobarbituric acid reactive substances (TBARS) level, activities of glutathione peroxidase (GPx) and superoxide dismutase (SOD), zinc (Zn) and copper (Cu) content in erythrocytes of diabetic and non-diabetic TCP and ACP patients and controls [Values represent mean ± SE]

	Controls (n = 113)	TCP patients (n = 91)			ACP patients (n = 84)		
		Diabetic (n = 53)	Non-diabetic patients (n = 38)	Total TCP patients	Diabetic (n = 44)	Non-diabetic (n = 40)	Total ACP patients
GSH ^a	8.59 ± 0.21	4.61 ± 0.15 ¹	5.39 ± 0.22 ¹	6.21 ± 0.25 ¹	6.02 ± 0.32 ¹	6.51 ± 0.41 ¹	5.07 ± 0.25 ^{1,3}
TBARS ^b	5.62 ± 0.13	8.29 ± 0.34 ¹	8.55 ± 0.37 ³	10.1 ± 0.51 ^{1,3}	11.05 ± 0.6 ¹	9.52 ± 0.6 ¹	7.44 ± 0.33 ¹
GPx ^c	19.06 ± 0.33	14.6 ± 0.45 ¹	13.95 ± 0.55 ³	15.41 ± 0.54 ¹	14.11 ± 0.57 ¹	15.99 ± 0.82 ²	15.83 ± 0.48 ¹
SOD ^d	2984.87 ± 49.96	2267.54 ± 108.08 ¹	2373.62 ± 72.76 ³	2179.82 ± 74.36 ¹	2265.72 ± 78.31 ¹	2185.01 ± 92.26 ²	2304.77 ± 86.66 ¹
Zn ^e	37.14 ± 0.51	23.87 ± 0.64 ¹	25.84 ± 0.89 [*]	24.85 ± 0.76 ^{1,3}	26.64 ± 0.98 ¹	29.13 ± 1.25 ¹	27.88 ± 0.61 ¹
Cu ^f	3.2 ± 0.06	3.73 ± 0.13 ²	3.79 ± 0.14 ²	3.76 ± 0.14 ¹	3.47 ± 0.16	3.82 ± 0.16 ²	3.64 ± 0.16 ¹

¹P<0.001 compared to controls; ²P<0.05 compared to controls; ³P<0.001 compared between ACP and TCP patients
a, μmol/g Hb, b, nmol/g Hb, c, nmol of NADPH oxidized/ min/g Hb, d, IU/g Hb, e, μg/g Hb, f, pg/g Hb

Table 3—GSH content, TBARS level, activities of GPx and SOD, zinc and copper content in erythrocytes of diabetic and non-diabetic chronic pancreatitis (CP) patients and controls

	[Values represent mean \pm SE]		
	Controls (n = 113)	Diabetic CP cases (n = 97)	Non-diabetic CP cases (n = 78)
GSH (μ mol/g Hb)	8.59 \pm 0.21	5.38 \pm 0.2*	5.93 \pm 0.24*
GPx (nmol of NADPH oxidized/ min/g Hb)	19.06 \pm 0.33	14.33 \pm 0.37*	14.95 \pm 0.5*
SOD (IU/g Hb)	2984.87 \pm 49.96	2266.55 \pm 64.72*	2281.73 \pm 59.02*
TBARS (nmol/g Hb)	5.62 \pm 0.13	9.8 \pm 0.39*	9.02 \pm 0.35*
Zn (μ g/g Hb)	37.14 \pm 0.51	26.5 \pm 0.94*	26.24 \pm 0.93*
Cu (pg/g Hb)	3.2 \pm 0.06	3.775 \pm 6.58*	3.63 \pm 0.15*

*P<0.001 compared to control

levels. Plasma vitamin C was significantly lower in CP cases with low elastase-1 (Table 4), as compared to CP cases with normal elastase-1 (P<0.001). Among trace elements, erythrocyte zinc was significantly lower in CP cases with low elastase-1, as compared to normal elastase-1 cases (P<0.001). Erythrocyte zinc was positively correlated with pancreatic elastase-1 ($r = 0.565$, P<0.001) and erythrocyte SOD ($r = 0.45$, P<0.001), indicating that CP patients with lower elastase-1 tend to have lower zinc and SOD levels.

Discussion

Oxidative stress caused by an imbalance between the production of free radicals and antioxidants plays an important role in various inflammatory diseases. Oxidative stress and free radical-mediated pancreatic injury have been postulated to be an important etiopathogenic mechanism of CP²⁰. Clinical complications of CP, such as diabetes⁴, smoking⁴ and painful exacerbations⁸ may also influence antioxidant levels.

Significant reduction in antioxidant enzymes and elevated lipid peroxidation products in both TCP and ACP cases as compared to controls (Table 3) indicated severe oxidative stress in CP. The relationship between zinc and SOD in our results supported the hypothesis of free radical mediated injury in pancreatic inflammation²¹.

GSH has roles in acinar stimulus-secretion coupling, maintenance of the cytoskeleton and

Table 4—GSH content, TBARS level, activities of GPx and SOD, zinc and copper content in erythrocytes and plasma vitamin C levels in chronic pancreatitis (CP) patients with and without normal stool elastase-1

	[Values represent mean \pm SE]	
	CP cases with low stool elastase-1 (n = 67)	CP cases with normal stool elastase-1 (n = 34)
GSH (μ mol/g Hb)	5.93 \pm 0.32	5.49 \pm 0.29
GPx (nmol of NADPH oxidized/min/g Hb)	14.55 \pm 0.58	15.78 \pm 0.62
SOD (IU/g Hb)	2284.15 \pm 81.02	2067 \pm 103.98
TBARS (nmol/g Hb)	10.01 \pm 3.92	9.11 \pm 3.8
Vitamin C (mg/dl)	0.18 \pm 0.02	0.76 \pm 0.11*
Zn (μ g/g Hb)	23.8 \pm 8.6	32.1 \pm 9.0*
Cu (pg/g Hb)	3.61 \pm 0.9	3.8 \pm 0.7

* P<0.001 compared to CP cases with low stool elastase-1

appropriate protein folding in the endoplasmic reticulum²¹. GSH depletion may contribute to impaired zymogen granule transport (secretory block) and premature activation of pancreatic proenzymes. In a randomized clinical trial, GSH supplementation has shown beneficial effects by protecting visceral organ function in patients with acute pancreatitis²². A compromised pancreas as a source of ROS could release xanthine oxidase into the circulation²³, leading to increased lipid peroxidation and decreased protein sulfhydryl in extra-pancreatic organs/tissues²⁴. Therefore, ROS may function as an interorgan signal substance responsible for the development of pancreatitis-associated multi-organ dysfunction syndrome. When NO, lipoxidative damage and glutathione levels in pancreas, lung and circulation have been studied in rats with alcohol-induced AP, it has been observed that NO increases in both pancreas and lungs in AP. Thus, NO contributes to the pathogenesis of AP under oxidative stress²⁵.

Significant increase in copper levels in CP cases as compared to controls in this study was in agreement with previous reports^{26,27}. This was related to elevated ceruloplasmin levels, probably secondary to chronic inflammatory state of the disease because inflammatory cytokines are known to induce expression of ceruloplasmin²⁶. A previous

study has indicated that metallothionein may be upregulated in CP patients²⁸ and this partly might explain increased copper levels in CP cases. Copper is an integral part of many important enzymes involved in a number of vital biological processes. Although normally bound to proteins, copper may be released and becomes free to catalyze the formation of highly reactive hydroxyl radicals. Data obtained from *in vitro* and cell culture studies have largely been supportive of copper's capacity to initiate oxidative damage and interfere with important cellular events²⁹. It has been observed that copper ions bind to sulfhydryl groups in cells and inactivate the action of certain enzymes, such as glucose-6-phosphate dehydrogenase and glutathione reductase³⁰, both of which are necessary for the reduction of oxidized glutathione to reduced form of glutathione. Each of these effects would tend to decrease the GSH content of erythrocytes and make them more vulnerable to oxidative effects of copper. It has been also demonstrated that cellular damage and apoptosis could result from copper accumulation in the organisms³¹.

Zinc deficiency may be the effect of reduced absorption and can be a contributory factor in disease progression via the reduction of free radical scavengers, increased oxidative stress and increased collagen deposition³². Other possible effects could be an alteration in immune function³³.

Further evaluation of oxidative stress parameters in CP cases with and without pancreatic exocrine insufficiency showed that plasma vitamin C was significantly lower in CP cases with low pancreatic elastase-1. However, we did not notice any significant differences in GSH, GPx, SOD and TBARS. This indicated that deficiency of antioxidant might influence pancreatic exocrine insufficiency at least when vitamin C level was considered. However, we did not find significant differences in these parameters between diabetic and non-diabetic CP patients.

In conclusion, we observed elevated oxidative stress, reduced zinc and elevated copper levels in CP cases. We also noted an association between pancreatic exocrine insufficiency and oxidative parameters in CP, irrespective of its etiology. However, pathophysiology of CP is complex; environmental as well as genetic mechanisms are believed to play a role. Further work is needed to characterize the mechanistic effects of some of our

findings which could help in explaining the variable clinical phenotype of this disease.

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References

- Balakrishnan V, Nair P, Radhakrishnan L & Narayanan V A (2006) *Indian J Gastroenterol* 25, 74-81
- Stevens T, Conwell D L & Zuccaro G (2004) *Am J Gastroenterol* 99, 2256-2270
- Verlaan M, Roelofs H M, van-Schaik A, Wanten G J, Jansen J B, Peters W H & Drenth J P (2006) *World J Gastroenterol* 12, 5705-5710
- Braganza J M & Dormandy T L (2010) *JOP* 11, 99-112
- Leung P S & Chan Y C (2009) *Antioxid Redox Signal* 11, 135-165
- Chvanov M, Petersen O H & Tepikin A (2005) *Philos Trans R Soc Lond B Biol Sci* 360, 2273-2284
- Rose P, Fraire E, Hunt L P & Acheson D W (1986) *Hum Nutr Clin Nutr* 40, 151-164
- Braganza J M, Hewitt C D & Day J P (1993) *Pancreas* 2, 80-85
- Bhardwaj P, Garg P K, Maulik S K, Saraya A, Tandon R K & Acharya S K (2009) *Gastroenterology* 136, 149-159
- Siriwardena A K, Mason J M., Sheen, A J, Makin A & Shah N (2012) *Gastroenterology* 143, 655-663
- Lüth S, Teysen S, Forssmann K, Köbel C, Krummenauer F & Singer M V (2001) *Scand J Gastroenterol* 36, 1092-1099
- Okamura M (1980) *Clin Chim Acta* 103, 259-268
- Beutler E, Duron O & Kelly B M (1963) *J Lab Clin Med* 61, 882-888
- Paglia D E & Valentine W N (1967) *J Lab Clin Med* 70, 158-169
- Winterbourn C C, Hawkins R E, Brian M & Carrell R W (1975) *J Lab Clin Med* 85, 337-341
- Jain S K, McVie R, Duett J & Herbst J J (1989) *Diabetes* 38, 1539-1543
- Burtis C A, Ashwood E R & Tietz N W (1986) *Tietz Text Book of Clinical Chemistry*, pp 589, W.B. Saunders
- Kenney M A, Ritchey S J, Culley P, Sandoval W, Moak S & Schilling P (1984) *Am J Clin Nutr* 39, 446-451
- Blomfield J & Macmahon R A (1969) *J Clin Pathol* 22, 136-143
- Braganza J M, Lee S H, McCloy R F & McMahon M J (2011) *Lancet* 377, 1184-1197
- Girish B N, Rajesh G, Vaidyanathan K & Balakrishnan V (2011) *Indian J Gastroenterol* 30, 84-88
- Huang Z W, Tang J Z, Chen Y, Yuan D S, Shen Y B & Wang W (2005) *Zhongguo Wei Zhong Bing Ji Jiu Yi Xue*. 17, 673-674
- Gomez-Combronero L, Camps B, De La Asuncion JG, Cerda M, Pellin A, Pallardo F V, Calvete J, Sweiry J H, Mann G E, Vina J, Sastre J (2000) *J Pharmacol Exp Therapeu* 293, 670-676

- 24 Folch E, Gelpi E, Rossello-Catafau J & Closa D (1998) *Dig Dis Sci* 43, 2405-2410
- 25 Andican G, Gelisgen R, Unal E, Tortum O B, Dervisoglu S, Karahasanoglu T & Burçak G (2005) *World J Gastroenterol* 11, 2340-2345
- 26 Dabrowski A & Gabryelewicz A (1992) *Int J Pancreatol* 12, 193-199
- 27 Fabris C, Farini R, Del Favero G, Gurrieri G, Piccoli A, Sturniolo G C, Panucci A & Naccarato R (1985) *Clin Biochem* 18, 373-375
- 28 Segal I, Sharer N M, Kay P M, Gutteridge J M & Braganza J M (1996) *QJM* 89, 45-53
- 29 Milnerowicz H, Chmerek M, Rabczyński J, Milnerowicz S, Nabzdyk S & Knast W (2004) *Pancreas* 29, 28-32
- 30 Gaetke L M & Chow C K (2003) *Toxicology* 189, 147-163
- 31 Attri S, Sharma N, Jahagirdar S, Thapa B R & Prasad R (2006) *Pediatr Res* 59, 593-597
- 32 Saravu K, Jose J, Bhat M N, Jimmy B & Shastry B A (2007) *IJCCM* 11, 74-80
- 33 Keen C L & Gershwin M E (1990) *Annu Rev Nutr* 10, 415-431