*Full Length Research Paper*

# DNA damage and plasma homocysteine levels are associated with serum metabolites and mineral constituents' profiles in children with persistent diarrhea

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This study describes the association between levels of DNA damage and homocysteine (Hcy) in persistent diarrheic (PD) patients and correlates them with serum biochemical metabolites and mineral components. PD patients (n = 36) age 4 - 6 years from Faisalabad hospitals were examined for anthropometric factors, plasma biochemical and mineral constituents. Compared to 36 normal controls, children with PD had significantly higher concentrations of LDL (p = 0.0001), ALT (p = 0.01), homocysteine (p = 0.001), TOS (p = 0.0001), TBARS (p = 0.001), K (p = 0.0001) and Mg (p = 0.0001) while serum triglyceride, total proteins, albumin, globulin, $T_3$, $T_4$, TAS, Na, Ca, Zn and Cu were significantly lower than those of healthy individuals. Both DNA damage and Hcy were positively linked with LDL-cholesterol, TBARS and K (all p values < 0.05). Both Hcy profile and percentage DNA damage in PD patients may impart role in the endothelium damage even in the normal range. PD patients have severe deficiency of macro- and micro-nutrients which may have resulted in enhancement of oxidative stress, DNA damage and Hcy levels in patients' plasma. Appropriate supplementation of macro- and micro-nutrients may decrease the DNA damage, Hcy levels and enhance the levels of health markers and decrease the mortality rate of PD patients.

**Key words:** Diarrhea, biochemistry, health marker, homocysteine, DNA damage.

## INTRODUCTION

Most diarrhea disorders form a continuum, with the majority of cases resolving within the first week of illness. However, a smaller proportion of diarrhea persists for longer than 2 weeks called persistent diarrhea (PD) and continues to be a major cause of morbidity and mortality in children. The highest incidence being in developing countries of the world and is a cause of 10 - 20% death of children. In Pakistan, the mortality rate for children less than five years of age is 139/1000 live births (Ahmed et al., 2001; Allen, 1998; Becker et al., 1991). Although global mortality rates may have been reduced, the overall incidence remains unchanged at about 3.2 episodes per child year (Bhutta, 2007; Black et al., 2003). In several large community-based studies, it has been shown that PD is directly responsible for 36 to 54% of all diarrhea related deaths (Bhutta, 2007; Cui et al., 1996).

Globally, the most important underlying trigger for PD is enteric infection and the consequences thereof. PD was seen with 23% of children's having bacterial and viral infections (Ahmed et al., 2001; Allen, 1998; Granot et al.,

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**Abbreviations: ALT,** Alanine amino transferase; **AST,** aspartate amino transferase; **Hcy,** homocysteine; **MDA,** melanodialdehyde; **ORS,** oral rehydration solution; **PD,** persistent diarrhea; **$T_3$,** triiodothyronine; **$T_4$,** thyroxine; **TBARS,** thiobarbituric reactive substances; **TOS,** total oxidant status; **TAS,** total anti-oxidant status.

2001; Kosek et al., 2003). PD causes loss of electrolytes and minerals and brings about alteration in serum metabolites, called health markers. Zinc deficiency is especially associated with longer episodes of diarrhea and higher intake of zinc may be required to make up enteric losses (Ahmed et al., 2001; Bhutta, 2007) to regulate its role in transcription function, antioxidant defense and DNA repair. It is being hypothesized that deficiency of zinc and other factors may increase some serum biochemical components, influence DNA integrity and protein content in serum (Ho, 2004). Quantification and correlations of these factors in children with diarrhea is important for determining the mechanisms by which disease occurs and spreads throughout a population (Kristen et al., 2005; Martin et al., 1999; Strand et al., 2004). Such studies may lead to practical approaches for prevention, diagnosis and treatment of PD patients.

## MATERIALS AND METHODS

### Patients and control subjects

This retrospective study was conducted on children with PD and admitted in Allied Hospital and Civil Hospital, Faisalabad, Pakistan after being approved by the University research ethical committee. When written consent of parents/guardians was accorded, age, gender, immunization status, history of fever or vomiting, any prior use of ORS or medication as well as nutritional status was recorded. A child was discontinued from the programme if she/he experienced any complications. Seventy two children were included in this study; 36 were PD patients and 36 were normal controls.

A complete physical examination of each child was performed by one of the physician attending to the patients. A total of seventy two venous blood samples (5 ml) were drawn into a test tube with anti-coagulant. Serum samples were distributed into small aliquots (1 ml) in Eppendorff tubes and preserved at 4°C for further analysis.

### Analytical

Serum glucose test was carried by commercially available kit (Fluitest Glu, Biocon Solutions Pte Ltd, Singapore). Cholesterol, triglycerides, HDL-and LDL-cholesterol were determined by using commercially available kits (Randox Labs Ltd, UK). Serum proteins, albumin and globulin were determined by using Bio Rays (Faisalabad, Pakistan) kits. Plasma triiodothyronine ($T_3$; ng/dl) and total thyroxine ($T_4$; ng/dl) concentrations were measured using ELISA (BioCheck Inc., USA) kits.

Total homocysteine (tHcy) was measured by an Hcy micro titer plate assay as described earlier (Erel, 2004). Plasma TOS and TAS were measured colorimetrically (Erel, 2005). TBARS levels were assayed at 532 nm (Erel, 2004) and expressed as MDA (µmol/l) level. DNA damage was measured by performing comet assay using the methods of Martin et al. (1999). The length of DNA migration in the comet tail was taken as an estimate of DNA damage and is presented as percentage of total DNA. All tests were performed in triplicate following strict external and internal quality control protocols. Reagents for calibration of instruments were supplied by manufacturers.

$Na^+$ and $K^+$ were analyzed using flame photometer, while atomic absorption spectrophotometer was used to measure Ca, Mg, Zn, Fe, Cu and Mn in serum samples, subjected to wet digestion prior to analysis as described earlier (Cui et al., 1996; Gruskin and

Sarnaik, 1997). Analysis of variance was applied to determine the difference between groups (Steel et al., 1997) and Duncan Multiple Range (DMR) test (Kristen et al., 2005) was applied using MStat C software to test the difference between means.

## RESULTS

The age of male and female healthy children (18 males and 18 females) ranged from 3 - 5.6 years while that of children with diarrhea (18 males and 18 females) ranged from 4-6 years. Body temperature of normal male and female was 97.89 and 98.01°F, respectively. While in children with diarrhea (both male and female) mean body temperature was 99.94 and 99.91°F, respectively. In addition to diarrhea, vomiting and stress symptoms were present in 95% of the children. Body mass index (BMI) was significantly (p = 0.0001) higher in normal children as compared to children with diarrhea (Table 1).

Mean serum biochemical constituents of normal as well as patients with diarrhea (both male and female children) are given in Table 1. Mean serum glucose (p = 0.0001) and cholesterol (p = 0.0215) concentrations were significantly higher in patients than in normal subjects. Overall mean triglyceride concentration was significantly (p = 0.0001) higher in females as compared to that of males and was significantly lower in children with diarrhea as compared to that in normal participants.

Overall mean serum HDL-cholesterol was significantly (p ≤ 0.0002) higher in males as compared to females. However, LDL-cholesterol concentration was significantly (p = 0.0001) higher in children with diarrhea as compared to normal individuals (Table 1) irrespective of their gender.

Mean serum proteins were significantly (p = 0.0007) lower in children with diarrhea than those in normal subjects (Table 1). Likewise, serum albumin was significantly higher (p = 0.0001) in healthy individuals. Overall mean serum globulin concentration in males was significantly (p = 0.0001) higher as compared to females and was more in normal children as compared to children with diarrhea.

Serum AST concentration was significantly (p = 0.0006) lower in children with diarrhea as compared to their normal counterparts. Serum ALT concentration was significantly (p = 0.0001) higher in patients than their normal peers (Table 1). Overall mean serum $T_3$ concentration decreased significantly (p = 0.001) in children with diarrhea as compared to normal ones. Serum $T_4$ concentration was significantly (p = 0.0001) lower in females and in children with diarrhea as compared to their male and female controls (Table 1).

Serum homocysteine concentration and TOS were significantly (p = 0.0001) higher in children with diarrhea (Table 1). Females had significantly lower overall TAS as compared to male and likewise children with diarrhea also showed significant lower amount of TAS. Similarly, DNA damage to lymphocytes was significantly higher (p = 0.0001) in individuals suffering from diarrhea than in

**Table 1.** Serum biochemical profiles of normal and diarrhea patients.

| Parameter | Male | | Female | | p |
|---|---|---|---|---|---|
| | Normal | Diarrhea | Normal | Diarrhea | |
| Body mass index (kg/m$^2$) | 15.64 ± 0.60[a] | 11.59 ± 0.37[c] | 13.47 ± 0.31[b] | 11.22 ± 0.19[c] | 0.0001 |
| Glucose (mg/dl) | 74.96 ± 2.08 [b] | 79.83 ± 1.83 [a] | 73.24 ± 2.42 [c] | 80.01 ± 2.69 [a] | 0.0001 |
| Cholesterol (mg/dl) | 105.85 ± 6.0 [b] | 115.13 ± 7.4 [a] | 99.49 ± 7.43 [c] | 104.9 ± 7.9[b] | 0.0215 |
| Triglyceride (mg/dl) | 155.51 ± 10.42[b] | 134.58 ± 4.10[d] | 173.74 ± 4.30[a] | 153.57 ± 4.4 [c] | 0.0001 |
| HDL-cholesterol  (mg/dl) | 32.95 ± 2.50[b] | 35.49 ± 3.19[a] | 26.95 ± 1.42[d] | 28.20 ± 0.83[c] | 0.0002 |
| LDL-cholesterol (mg/dl) | 112.28 ± 5.50 [c] | 128.54 ± 4.3[a] | 115.00 ± 4.22[b] | 128.87 ± 4.77[a] | 0.0001 |
| Total proteins (g/dl) | 7.82 ± 0.11[a] | 7.05 ± 0.10[b] | 7.78 ± 0.18[a] | 7.09 ± 0.18[b] | 0.0007 |
| Albumin (g/dl) | 5.74 ± 0.08[a] | 4.79 ± 0.11[b] | 4.73 ± 0.23[b] | 4.47 ± 0.17[c] | 0.0001 |
| Globulin (g/dl) | 2.08 ± 0.12[c] | 2.06 ± 0.10[c] | 3.05 ± 0.084[a] | 2.92 ± 0.10[b] | 0.0001 |
| AST (U/L) | 54.76 ± 2.71 | 50.81 ± 1.09 | 56.74 ± 2.29 | 54.14 ± 1.18 | 0.0006 |
| ALT (U/L) | 47.64 ± 0.64[b] | 47.11 ± 0.53[b] | 47.34 ± 0.57[b] | 57.80 ± 3.05[a] | 0.0001 |
| T$_3$ (ng/dl) | 2.57 ± 0.08[a] | 1.65 ± 0.05[c] | 1.89 ± 0.04[b] | 1.32 ± 0.06[d] | 0.001 |
| T$_4$ (µg/dl) | 12.26 ± 0.15[a] | 8.88 ± 0.21[c] | 11.44 ± 0.13[b] | 8.36 ± 0.08 [d] | 0.0001 |
| Hcy (µmol/L) | 29.83 ± 0.74[c] | 53.25 ± 0.88[a] | 36.08 ± 0.77[b] | 50.42 ± 1.64[a] | 0.0010 |
| TOS (µmol H$_2$O$_2$ equiv/L) | 14.11 ± 0.39[a] | 19.56 ± 0.40[b] | 12.67 ± 0.19[c] | 17.77 ± 0.29[d] | 0.0001 |
| TAS mmol trolox equiv/L | 1.39 ± 0.02[a] | 1.05 ± 0.03[b] | 1.34 ± 0.02[a] | 0.92 ± 0.02[c] | 0.0001 |
| TBARs (µmol MDA/L) | 0.25[b] | 0.32[a] | 0.23[b] | 0.34[a] | 0.001 |
| DNA damage (%) | 9[c] | 20[b] | 9.5[c] | 22 [a] | 0.0001 |

[a–d] Similar alphabets on mean values in a row do not differ significantly at p ≤ 0.05.

normal individuals.

Serum sodium (p = 0.0001), calcium (p = 0.0001), Mg (p = 0.0001), Zn (p = 0.0002) iron (p = 0.0001) and Cu (p = 0.001) concentrations decreased significantly in children with diarrhea (Table 2). Serum Mn levels did not change significantly (p = 0.589) in children with diarrhea.

DNA damage to lymphocytes was positively correlated with glucose (p = 0.020), LDL-cholesterol (p = 0.011), TBARS (p = 0.001) and potassium (p = 0.008) while negative relationship existed with total proteins (p = 0.008), thyroxine (p = 0.019), TAS (p = 0.018), zinc (p = 0.012), sodium (p = 0.034) and calcium (p = 0.018) (Table 3). Homocysteine was positively correlated with LDL-cholesterol (p = 0.012), TBARS (p = 0.004) and potassium (p = 0.015), while it was negatively associated with body mass index (p = 0.04), total proteins (p = 0.02) thyroxine (p = 0.024), sodium (p = 0.031), Zn (p = 0.012) and copper (p = 0.027) (Table 3). Both DNA damage and Hcy were non-significantly associated with iron.

## DISCUSSION

Diarrhea in young children is well recognized as a major cause of morbidity and mortality in many developing countries (Ahmed et al., 2001; Allen, 1998; Bhutta, 2007; Black et al., 2003; Kristen et al., 2005; Majumdar et al., 1997; Yusufzai and Bhutta, 2000). This is attributed to infectious and non-infectious causes of acute and chronic diarrhea and may last several days to few weeks. A decrease in BMI in children with diarrhea may be attributed to the persistency of diarrhea which results in the poor growth of children. In developing countries parti-cularly, children BMIs are significantly lower because of repeated episodes of illness, limited and poor quality of food and its availability (Bhutta, 2007; Black et al., 2003; Kosek et al., 2003; Strand et al., 2004). Malnutrition in young children contributes significantly towards the global burden of disease and the childhood-underweight is the leading cause of this disease (Bhutta, 2007; Wakwe and Okon, 1995; Weizman et al., 2002).

Serum glucose was high in children with diarrhea and was attributed to galactose's conversion to glucose by enhanced efficiency of galactose-1-phosphatase (Ahmed et al., 2001; Bhutta, 2007). Low concentration of chole-sterol in PD patients may be due to low intake of food or low fat in the diet which eventually led to deficiency of cholesterol, and HLD or malabsorption of fat from the intestine (Ahmed et al., 2001; Bhutta, 2007; Black et al., 2003) may have caused this change. In the present study, decrease in total proteins in children with diarrhea may be related to its decreased synthesis by the liver or its increase rate of catabolism due to diarrhea. Hypo-proteinemia in children with diarrhea may be due to the enteric proteins loss (Weizman et al., 2002). Fall in serum albumin level in children with diarrhea may be due to dilution by an excess of protein free fluids (ORS) given during treatment. Decrease in serum globulin content in children with diarrhea may be due to a decrease in specific immunoglobulin fraction due to immuno-

**Table 2.** Gender dependent serum macro and micro minerals in normal and patients with diarrhoea.

| Parameter | Male | | Female | | p |
|---|---|---|---|---|---|
| | Normal (n = 18) | Diarrhoea (n = 18) | Normal (n = 18) | Diarrhoea (n = 18) | |
| Sodium (mg/dl) | $27.15 \pm 1.17^a$ | $21.35 \pm 0.52^c$ | $25.27 \pm 0.70^b$ | $20.19 \pm 0.16^d$ | 0.0001 |
| Potassium (mg/dl) | $0.71 \pm 0.07^c$ | $1.14 \pm 0.04^a$ | $0.77 \pm 0.06^b$ | $1.15 \pm 0.06^a$ | 0.0001 |
| Calcium (mg/dl) | $17.25 \pm 0.78^b$ | $14.30 \pm 0.21^c$ | $17.89 \pm 0.99^a$ | $12.96 \pm 0.26^d$ | 0.0001 |
| Magnesium (mg/dl) | $5.17 \pm 0.07^c$ | $5.47 \pm 0.07^a$ | $5.10 \pm 0.07^d$ | $5.26 \pm 0.07^b$ | 0.0001 |
| Zinc (µg/dl) | $0.16 \pm 0.01^a$ | $0.10 \pm 0.01^c$ | $0.18 \pm 0.02^a$ | $0.13 \pm 0.02^b$ | 0.0002 |
| Manganese (µg/dl) | $0.09 \pm 0.00$ | $0.098 \pm 0.00$ | $0.11 \pm 0.00$ | $0.12 \pm 0.00$ | N.S. |
| Copper (µg/dl) | $0.12 \pm 0.01^a$ | $0.08 \pm 0.01^b$ | $0.10 \pm 0.01^{ab}$ | $0.08 \pm 0.01^b$ | 0.0010 |
| Iron (µg/dl) | $3.12 \pm 0.21^a$ | $2.94 \pm 0.20^b$ | $2.93 \pm 0.19^b$ | $2.80 \pm 0.21^c$ | 0.0001 |

[a–d] Similar alphabets on mean values in a row do not differ significantly at $p \leq 0.05$.

**Table 3.** Correlation coefficients of serum homocysteine and DNA damage of lymphocytes of patients with diarrhea and biochemical characteristics, hormonal, macro and micro minerals' levels.

| Parameters | Homocysteine (µmol/L) | DNA (%) |
|---|---|---|
| Body Mass Index (BMI; $kg/m^2$) | - 0.960 (0.040) | - |
| Glucose (mg/dl) | - | 0.980 (0.020) |
| LDL-Cholesterol (mg/dl) | 0.988 (0.012) | 0.989 (0.011) |
| Total Proteins (g/dl) | - 0.980 (0.020) | -0.992 (0.008) |
| $T_4$ (µg/dL | - 0.976 (0.024) | - 0.981 (0.019) |
| TAS (mmol trolox Equiv./l) | - | - 0.982 (0.018) |
| TBARS (MDA, µmol/l) | 0.985 (0.004) | 0.925 (0.001) |
| Sodium (mg/dl) | - 0.969 (0.031) | - 0.966 (0.034) |
| Potassium (mg/dl) | 0.985 (0.015) | 0.992 (0.008) |
| Calcium (mg/dl) | - | - 0.982 (0.018) |
| Zinc (µg/dl) | - 0.983 (0.012) | - 0.895 (0.012) |
| Copper (µg/dl) | - 0.973 (0.027) | - |
| Iron (µg/dl) | - | - |

Values in parenthesis are p-value for significance at $p \leq 0.05$.

compromization causing globulin to decrease.

AST and ALT determine hepatocellular injury and may help in monitoring the status of liver during PD. ALT was significantly higher in children with diarrhea indicating hepatocellular disease (Bhutta, 2007). On the other hand AST activity was related to damage of cells in kidney, pancreas and erythrocytes.

Serum $T_3$ concentration was significantly low in children with diarrhea. Although some $T_3$ is produced in the thyroid, approximately 80 - 85% is generated outside the thyroid primarily by conversion of $T_4$ by selenium-dependent 5'deiodinase in the liver and kidney. Also, $T_4$ was significantly low in children with diarrhea. This may be indicator of increased rate of conversion of $T_4$ to $T_3$ at the level of liver and/or kidney of the diarrheal children in order to keep up their normal base metabolic rate (Kristen et al., 2005).

Testing for tHcy in children with PD may be useful for investigating and assessing the nutritional status (Allen,

1998; Strand et al., 2004; Ueland et al., 1993) as it is influenced by blood folate, and vitamin supplementation (Granot et al., 2001; Victoria et al., 2000). As the TOS increased and TAS decreased significantly (p = 0.0001) in children with diarrhea, these may have contributed in hyper-homocysteinemia (Ahmed et al., 2001; Bhutta, 2007). As children with diarrhea are particularly vulne-rable to nutritional deficiencies, it is expected that Hcy will rise in PD (Bhutta, 2007; Ho, 2005). Diarrhea and other gastrointestinal disorders are associated with an elevated level of oxidative stress and lipid per oxidation with a lower level of antioxidant defences (Ho, 2004; Yusufzai and Bhutta, 2000).

In the present study hyponatremia may be due to sodium depletion during diarrhea. Normally, plasma sodium and osmolality within narrow limits is being taken care by thirst directed fluid intake and by renal water excretion by ADH (Kristen et al., 2005). Serum potassium concentration was significantly high in children with

diarrhea. Wakwe and Okon (1995) studied the plasma electrolytes pattern of children with PD and reported similar results. Serum zinc concentration was significantly lower in children with PD. These results were in line with the findings of earlier workers (Ahmed et al., 2001; Allen, 1998; Bhutta, 2007; Cui et al., 1996). As zinc is considered to be important in childhood growth and development, zinc deficiency has been shown to alter intestinal water/electrolyte transport (Bhutta, 2007; Majumdar et al., 1997). An association between diarrheal episodes and low plasma copper and zinc has been demonstrated (Wakwe and Okon, 1995; Yusufzai and Bhutta, 2000). Serum iron concentration was significantly increased in children with acute diarrhea. Its higher concentration may have caused gastrointestinal side effects such as nausea, vomiting, constipation, and abdominal distress as reported earlier.

DNA damage was positively correlated with glucose, TBARS, LDL-cholesterol and potassium and negatively correlated with total proteins, thyroxine, total antioxidant status, sodium, zinc and copper in patients with diarrhea. Granot et al. (2001) studied a population and found that there was increased concentration of reactive oxygen species and depleted antioxidant defences in PD patients. Body mass index, total proteins, thyroxine, sodium and Zn were negatively related while LDL-cholesterol, TBARS, and potassium were positively correlated with homocysteine (Table 3).

The present study indicated that subjects having PD had lower antioxidant status and caused a significant increase in the damage of DNA and reduced TAS as described earlier (Bhutta, 2007; Ueland et al, 1993). It is not known if these patients were taking any antioxidant in addition to their medicine or any other supplementation as well as intervention in their feeding habits. This study thus draws attention for increasing TAS, decreasing TOS, TBARS and DNA damage by supplementation of the macro and micronutrients in children with diarrhea for prevention, cure and control of PD.

## ACKNOWLEDGEMENTS

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