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Trace Element Levels, Cytokine Profile and Immune Activation Status in Plasma among Repeat Blood Donors with Asymptomatic HIV-1, HBV and HCV Infection

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Abstract

Imbalance of essential trace elements viz. Zinc, Selenium, Iron, Copper and Magnesium has been reported to influence disease course in HIV-1, HBV and HCV infections by altering immune status. A study was taken up to examine plasma levels of Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-10) categories of cytokines and immune activation markers (TNF-α, TNFR I and TNFR II) in an asymptomatic group of HIV-1, HBV and HCV infected blood donors in relation to trace elements. Plasma levels of Zn, Se and Mg were depressed in all the three groups of blood donors (P < 0.001 for all). Levels of Cu and Fe were depressed in HIV-1 infection (P < 0.001 for both), but elevated in HBV and HCV infections (P < 0.015 and < 0.001 for Cu and Fe in HBV and HCV infections respectively). IL-2 and IFN-γ were depressed in all the three groups of blood donors (P < 0.001). IL-4 and IL-10 levels were elevated in HBV and HCV infections (P < 0.001 for both). Immune activation markers were elevated in all the three groups of blood donors (P < 0.001 for all). HIV-1 infection showed positive correlations between Cu and IL-2, Zn and IFN-γ, and in HBV infection while positive correlations were found between Mg and TNFR I and TNFR II and Se with TNFR II. HCV infection showed a positive correlation between Se and IFN-γ (P < 0.001), Mg and IL-4 (P = 0.02), Fe and IL-10 (P < 0.01). The present study reveals possible relationship between trace element level alterations and alterations in cytokine and immune activation levels in HIV-1, HBV and HCV infection.

Keywords

HIV-1, HBV, HCV, Trace Elements, Cytokines, Immune Activation

1. Introduction

High prevalence of Human immune deficiency virus type 1 (HIV-1), Hepatitis B virus (HBV) and Hepatitis C virus (HCV) among blood donors has been recorded globally [1]. In the city of Delhi, the overall prevalence in blood donors has been reported to range from 0.28% to 0.30% for HIV-1, 1.15% to 1.33% for HBV and 0.57% to 0.67% for HCV respectively [2]. Studies indicate that an imbalance in T helper type 1 (Th1) and T helper type 2 (Th2) cytokine profile is associated with disease progression in infections due to HIV-1 [3], HBV [4] and HCV [5]. Further, studies have shown that changes in the intracellular environment induced by alterations in trace element status can cause immune dysfunction [6]. There are separate reports on cytokine alterations in HIV-1, HBV and HCV infections and that on trace element alterations in these infections [7] [8] [9]. However, there is hardly any report on relationship between trace element status and cytokine profile in HIV-1, HBV and HCV infections in same group of individuals. The present work aimed to study the impact of changes in a wide range of trace elements *i.e.* Zinc, Selenium, Iron, Copper and Magnesium (Zn, Se, Fe, Cu, Mg) on the status of immune activation and cytokine profile in an asymptomatic group of blood donors infected with HIV-1, HBV and HCV.

2. Materials and Methods

The project was approved by institutional review board including ethical clearance. The donors were explained the purpose of the study. Written consent for the study was obtained from the donors using a predesigned consent form in local language. During the period from July to December 2016, all the major blood banks including the private ones in the national capital territory of Delhi were requested to refer the blood donors found seropositive for HIV, HBV and HCV at their centres to the Centre for AIDS and Related Diseases, National Centre for Disease Control (NCDC). A total of 120 blood donors (all males), 40 each confirmed to be seropositive for HIV-1, HBV and HCV at NCDC were selected for the study. The period between detection of seropositivity for HIV-1, HBV and HCV in the blood banks referring the donors and confirmation of the same at Centre for AIDS and Related Diseases department, NCDC was considered duration of infection since diagnosis. In addition, since the donors were repeat donors donating blood at intervals of 4 - 8 months, whenever a seropositive blood donor was referred by a blood bank to our centre, test results for HIV, HBV and HCV at preceding blood bank attended by the seropositive donors were checked from the records available with blood banks to confirm seronegativity at the time of preceding donation. Forty, age and socioeconomically matched male blood donors seronegative for HIV-1, HBV and HCV infections and donating at the same blood banks were included as controls.

2.1. Epidemiological Information

A confidential interview was taken from the donors regarding age, marital status,

frequency of blood donation (for preceding one year), HIV related risk behaviours in terms of frequency (per week) of unprotected sex with heterosexual and/or homosexual partners as well as number of sex partners (for preceding one year). History of other risk factors viz. parenteral drug abuse as well as receipt of blood/ blood products were also obtained from the donors.

2.2. History of Dietary Intake, Alcohol Consumption and HBV Vaccination Coverage

A quantitative assessment of dietary intake was conducted by recall procedure for immediate past three typical days employing a food frequency questionnaire. Average daily intakes of total energy and protein were calculated as per Indian Council of Medical Research (ICMR) guidelines based on the quantities of various categories of raw food materials consumed as described earlier [10]. History regarding consumption of alcoholic drinks was elicited in terms of sessions/week (during preceding one year) while no consideration was given to brand or quantity of alcoholic drink consumed. The donors were enquired about the vaccination status against HBV from the records available with them.

2.3. Anthropometric Assessment and Clinical Assessment

Height (±0.1 cm) and weight (±0.1 Kg) were measured using standard techniques and body mass index (BMI) calculated by the formula weight (kg)/height (m^2) [11]. The seropositive cases were examined clinically by clinician at reference centre to determine the stage of illness. The cases were ruled out for any metabolic disorder, malignancy or immunosuppressive therapy.

2.4. Laboratory Studies

Fasting blood was collected and distributed as about 8 ml in plain sterile tube to yield serum for estimation of enzyme markers, serological assays for HIV-1, HBV and HCV infections, while about 4 ml of blood was distributed ethylene-diamine-tetra-acetic acid (EDTA) vial for routine haemoglobin, peripheral CD4 T lymphocyte count, cytokine, immune markers and trace element analysis.

2.5. Diagnosis of HIV-1, HBV and HCV Infection

Diagnosis of HIV-1 infection was based on commercial Enzyme- Linked Immunosorbent Assay (ELISA) test carried out at various blood banks in the city of Delhi and confirmatory western blot test carried out at AIDS Reference Centre, National Centre for Disease control (formerly National Institute of Communicable Diseases), Delhi [12]. HBV infection was diagnosed by detection of any of the three markers viz. hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc) employing commercial ELISA kits while diagnosis of HCV infection was carried out by detection of anti-HCV antibodies employing the ELISA and Recombinant Immunoblot Assay (RIBA) (Abott laboratories, North Chicago, IL, USA) [10]. All the donors and seronegative controls were screened for syphilis using com-

mercial Treponema Pallidum Haemagglutination (TPHA) kit (Fujirebio, Japan) to eliminate co-infection with syphilis [13].

2.6. Estimation of Enzyme Markers

Estimation of three enzyme markers as indices of liver function including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin in serum were carried out using auto-analyzer (Hitachi 912, Hitachi Ltd., Japan). Estimation of serum levels of albumin were carried out by techniques as described earlier [10]. Upper limit of normal values for ALT, AST, ALP and bilirubin was accepted as 55 U/L, 48 U/L, 115 U/L and 1.2 mg/dL respectively [14].

2.7. Enumeration of CD4 + T Lymphocytes

Enumeration of peripheral CD4 + T lymphocytes was done by flow cytometry. In brief, a standard flow cytometry method with lysed whole blood and a panel of two color combinations of fluorescein isothyocyanate and phycoerythrinconjuagated monoclonal antibody regents obtained from a single manufacturer (Becton-Dickinson, San Jose, California) were used to determine the expression of each antigen or antigen combination as described earlier [15].

2.8. Estimation of Trace Elements

Estimation of trace elements viz., Zn, Se, Fe, Cu and Mg in plasma was carried out using Flame Atomic Absorption Spectrophotometer (FAAS) (Thermo Electron Corporation, UK M6 Spectro with integrated software SOLAAR AA) as described earlier [16]. Commercially available standard solutions of trace elements from commercial source (Merck Private limited, Germany, concentrations 1000 ppm) were used to prepare suitable calibration curve for calculating trace element concentrations in plasma. All the working standard solutions were prepared in 10 mmol/L nitric acid. Plasma samples were diluted 100 fold for magnesium, 10 fold for copper, iron, selenium and zinc in 10 mmol/L nitric acid and then aspirated directly in FAAS. A blank was used for setting of zero absorbance of spectrophotometer.

2.9. Detection of Th1, Th2 Cytokines and Immune Activation Markers

Levels of T helper type1 (Th1) cytokines viz. interleukin-2 (IL-2) and gamma interferon (IFN-γ) and T helper type 2 (Th2) cytokines viz., Interleukin-4 (IL-4) and Interleukin-10 (IL-10) were measured in plasma using commercial ELISA kits (R&D Systems, Minneapolis, MI, USA) with sensitivity limits of 0.06 pg/ml, 8 pg/ml, 0.22 pg/ml and 0.17 pg/ml for IL-2, IFN-γ, IL-4 and IL-10 respectively. Plasma levels of immune activation markers viz., tumour necrosis factor alpha (TNF-α), tumour necrosis factor alpha receptor type l (TNFR p 55) and type ll (TNFR p 75) were measured using commercial ELISA kits from source as above with sensitivity levels of 0.49 pg/ml, 1.2 pg/ml and 2.3 pg/ml respectively. Coef-

ficient variation for all of these assays was found to be less than 10%. Readings below detection range were assigned zero values.

2.10. Statistical Analysis

Statistical analysis was carried out employing SPSS version 20. Characteristics of blood donors as assessed at enrolment were compared using χ^2 test for categorical variables and one way ANOVA for continuous variables with normal distribution while continuous variables not following normal distribution were compared by Kruskal Wallis test followed by multiple comparison using Mann Whitney test. P value < 0.05 was considered as statistically significant and it was adjusted for multiple comparisons. Fisher's exact test was used in which any of the expected cell frequency was less than five. Correlation of cytokine levels with trace element levels was evaluated by Spearman rank correlation test (due to lack of normal distribution in certain parameters).

3. Results

3.1. Demographic Profile, Risk Behaviour, Dietary Intake, CD4 Counts and Liver Enzyme Profile of the HIV-1, HBV and HCV Infected Donors

All the seropositive donors were males and unmarried belonging to the age group 24 - 33 years without any significant difference compared to seronegative blood donor control (mean ± SD for HIV-1, HBV, HCV and seronegative control as 29.2 ± 3.3, 30.5 ± 2.6, 29.2 ± 2.9, 30.0 ± 2.5 respectively). The mean duration between the preceding seronegative donation and detection of seropositivity for HIV-1, HBV and HCV in infected blood donors and seronegative donors ranged between 4 - 6 months with mean ± SD for HIV-1, HBV and HCV as 5.1 ± 0.8, 4.57 ± 0.5, 5.0 ± 0.8 and 4.8 ± 0.6 respectively (data not shown in table). Magnitude of exposure to unprotected sex with female CSWs could be elicited most in HIV-1 infected donors followed by HBV and HCV (Table 1). However none of the donors in any category gave history of other HIV related risk behaviour like parenteral drug abuse or receipt of blood transfusion. The proportion of individuals consuming alcohol was comparable between HIV-1, HBV and HCV infected donors as well as seronegative controls. Moreover the frequency of alcohol consumption was comparable between HIV-1, HBV, HCV and seronegative control groups (Table 1).

There was no alteration in CD4 T + lymphocyte count in all the subgroups of seropositive donors. On the other hand liver enzymes showed marginal elevation in all the subgroups of seropositive donors although the levels were comparable to seronegative controls. Serum bilirubin levels were unaltered in all the subgroups of seropositive donors. Energy intake and protein intake in all the three subgroups of seropositive donors were comparable to seronegative blood donor controls. There was no difference in the status of BMI and serum levels of albumin among the various groups of donors and controls (Supplementary Table 1). None of the donors received full or partial HBV vaccination.

Table 1. HIV related risk behaviour and alcohol intake in HIV-1, HBV and HCV infected blood donors.

			HIV-1 (n = 40)	HBV (n = 40)	HCV (n = 40)	Control (n = 40)	Statistical Comparisons
			Group I	Group II	Group III	Group IV	
H/O unprotected sex with female CSW*	Positive history	No (%)	31 (77.5%)	16 (40%)	9 (22.5%)	NA	χ^2 = 26.86 P < 0.000
	Frequency of Exposure/week	Range**	1 - 3	0 - 1	0 - 1	NA	f = 23.94 P < 0.000
		Mean ± SD**	2.1 ± 0.8	1.0 ± 0	1.0 ± 0	NA	
	No. of partners	Range**	4 - 6	2 - 3	2 - 3	NA	f = 77.56 P < 0.000
		Mean ± SD**	4.5 ± 0.8	2.4 ± 0.5	2.1 ± 0.3	NA	
Consumption of alcoholic drink*	Positive history	No (%)	15 (37.5)	14 (35)	12 (30)	10 (25)	
	Sessions/week	Range**	2 - 4	2 - 3	2 - 3	1 - 3	f = 0.63 P = NS (0.55)
		Mean ± SD**	2.1 ± 0.38	2.2 ± 0.46	2.2 ± 0.45	2.1 ± 0.8	

*During one year of pre-enrolment period; **Calculated for the donors with positive history only; None of the donors gave history of parenteral drug abuse or receipt of blood transfusion.

3.2. Plasma Trace Element Levels in HIV-1, HBV and HCV Infected Blood Donors

There was significant depression in plasma levels of Zn, Se, Fe, Cu and Mg in the group of blood donors infected with HIV-1 (P < 0.001 for all comparisons). In case of HBV and in HCV infected blood donors, plasma levels of Cu and Fe were significantly elevated as compared to controls. However, Zn, Se and Mg, levels in plasma were significantly depressed in both HBV and HCV infected blood donors compared to seronegative controls (P < 0.001 for all comparisons) (Table 2).

3.3. Plasma Cytokine Levels and Immune Activation Markers in HIV-1, HBV and HCV Infected Blood Donors

The plasma concentrations of Th1 type of cytokines *i.e.*, IL-2 and IFN-γ, were significantly decreased in all the three asymptomatic infected groups *i.e.*, HIV-1, HBV and HCV (P < 0.001). On the other hand, levels of Th2 type cytokines viz. IL-4 and IL-10 remained unaltered compared to control groups in case of HIV-1 (P = 0.39, P = 0.42 respectively). But in case of HBV and HCV, Th2 type of cytokines *i.e.*, IL-4 and IL-10 were found to be significantly elevated (P < 0.001 for all comparisons). Levels of three immune activation markers studied viz. TNF α, TNFR l and TNFR II were significantly increased in all the three groups of donors compared to seronegative controls (P < 0.001) (Table 3).

3.4. Correlation of Trace Element with Cytokines and Immune Activation Markers in HIV-1, HBV and HCV Infected Blood Donors

Correlation of trace element status with plasma cytokine levels in HIV-1, HBV and HCV infected blood donors showed that in case of HIV-1 infection a

Table 2. Plasma levels (mean ± SD) of trace elements in blood donors with HIV-1, HBV and HCV infections.

| Trace element level	Blood donor groups			Control
	HIV-1 (n = 40) Group I	HBV (n = 40) Group II	HCV (n = 40) Group III	(n = 40) Group IV
Zn(μg /dl)	50.78 ± 4.53*	54.28 ± 12.25*	54.40 ± 3.12*	83.08 ± 12.48
Se(μg /dl)	49.75 ± 14.60*	63.28 ± 12.70*	51.43 ± 7.94*	93.55 ± 9.08
Fe(μg /dl)	63.52 ± 8.94*	181.76 ± 16.92**	183.63 ± 13.55**	105.58 ± 25.50
Cu (μg /dl)	67.23 ± 12.98*	95.25 ± 10.60**	128.85 ± 16.89**	89.63 ± 12.46
Mg (mg/dl)	1.16 ± 0.27*	1.57 ± 0.50*	1.35 ± 0.21*	2.03 ± 0.62

*Significant depression ($P < 0.05$) compared to controls; **Significant elevation ($P < 0.05$) compared to controls; Multiple comparisons between the groups(by ANOVA): Cu: Overall $P < 0.001$; II vs. IV P < 0.03; rest all comparisons <0.001; Fe: Overall $P < 0.001$: II vs. III P = NS (0.63); rest all comparisons <0.001; Mg: Overall <0.001; I vs. III P = NS (0.12); rest all comparisons <0.001; Se: Overall $P < 0.001$; I vs. III P = NS (0.53): rest all comparisons <0.001; Zn: Overall $P < 0.001$; I vs. II P = NS (0.10); II vs. III < 0.95; rest all comparisons <0.001.

Table 3. Plasma levels (mean ± SD) of cytokines and immune activation markers in blood donors with HIV-1, HBV and HCV infection.

Immunological markers and their plasma levels (pg/ml)	Blood donor groups			Control
	HIV-1 (n = 40) Group I	HBV (n = 40) Group II	HCV (n = 40) Group III	(n = 40) Group IV
IL-2	4.19 ± 1.89*	5.53 ± 1.69*	0.30 ± 0.24*	16.37 ± 4.08
IFN-γ	23.1 ± 2.22*	23.93 ± 3.68*	27.53 ± 2.41*	50.53 ± 3.87
IL-4	2.5± 1.18	3.83 ± 1.97**	27.15 ± 6.37**	2.60 ± 1.27
IL-10	1.2 ± 0.27	25.03 ± 4.87**	29.98 ± 8.12**	1.23 ± 0.72
TNF-α	12.3 ± 5.21**	11.03 ± 2.37**	61.86 ± 21.67**	3.11 ± 0.48
TNFR I	1.6 ± 0.36**	1.31 ± 0.76**	1.02 ± 0.65**	0.31 ± 0.13
TNFR II	5.8 ± 1.38**	7.36 ± 2.76**	4.15 ± 2.01**	1.23 ± 0.36

*Significant depression ($P < 0.05$) compared to controls; **Significant elevation ($P < 0.05$) compared to controls; Multiple comparisons between the groups(by ANOVA): IL2: Overall $P < 0.001$; all comparisons <0.001; IFN-γ: Overall $P < 0.001$; I vs. II P = NS (0.23); rest all comparisons $P < 0.001$; IL-4: Overall $P < 0.001$; I vs. IV P = NS (0.80); rest all comparisons $P < 0.001$; IL-10: Overall $P < 0.001$; I vs. IV P = NS (0.85); rest all comparisons $P < 0.001$; TNF α: Overall $P < 0.001$; I vs. II P = NS (0.14); rest all comparisons $P < 0.001$; TNFR I: Overall $P < 0.001$; I vs. II P = NS (0.12); rest all comparisons <0.001; TNFR II: Overall $P < 0.001$; all comparisons $P < 0.001$.

marginal but significant positive correlation was found between Cu level and IL-2, Zn and IFN-γ (r = 0.33, P = 0.03 for both Cu and Zn) (Figure 1(a) and Figure 1(b)). However, no correlation could be demonstrated between the rest of the trace elements studied viz. Fe, Mg and Se with any of the Th1 or Th2 category of cytokines (Supplementary Table 2(a)). Correlation of trace element levels with the immune activation markers in HIV-1 infection showed a significant negative correlation between Zn and TNFR l (r = −0.31, P = 0.04) (Figure 1(c)).
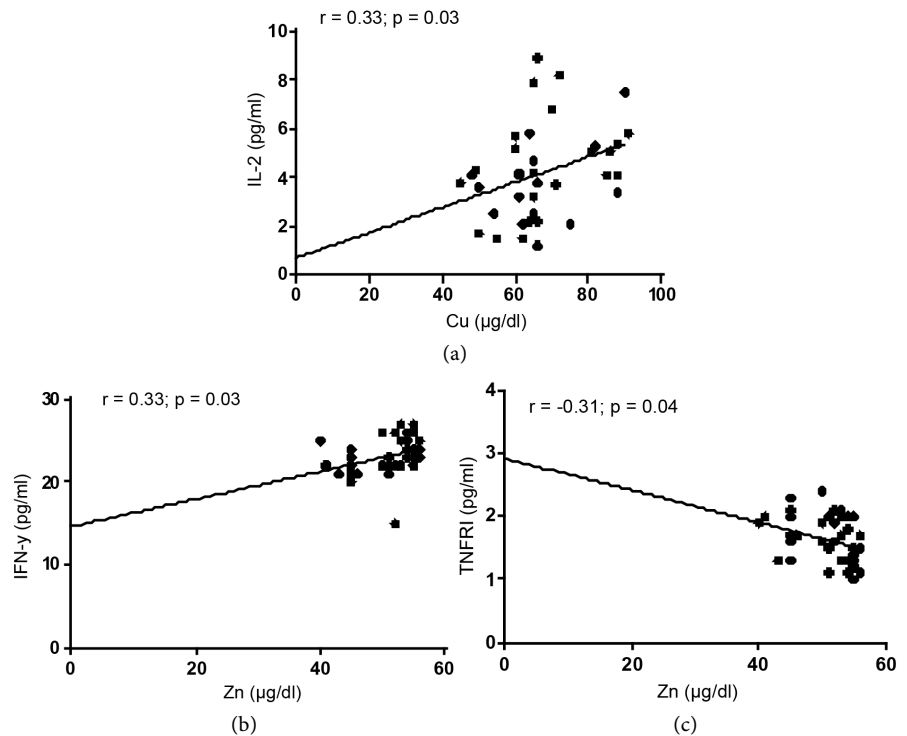
Figure 1. (a): Spearman rank correlation between plasma levels of copper (Cu) and interleukin 2(IL-2) in HIV-1 infected blood donors (n = 40; r = 0.33; P = 0.03); (b): Spearman rank correlation between plasma levels of Zinc (Zn) and interferon gamma (IFN-γ) in HIV-1 Infected blood donors (n = 40; r = 0.33, P = 0.03); (c): Spearman rank correlation between plasma levels of Zinc (Zn) tumour necrosis factor type-1 (TNFR I) in HIV-1 infected blood donors (n = 40; r = −0.31, P = 0.04).

In case of HBV infected blood donors significant positive correlations were found between Mg and TNFR l (r = 0.33, P = 0.04) and both Mg and Se with immune marker TNFR II (r = 0.33, P = 0.04; r = 0.42, P = 0.006 respectively) (Figures 2(a)-(c)). No correlation could be demonstrated in HBV infected blood donors between the levels of various trace elements and any of the Th1 and Th2 types of cytokines (Supplementary Table 2(b)).

In HCV infected blood donors samples, there was a positive correlation between Se andIFN-γ (r = 0.60, P < 0.001), Mg with IL-4 (r = 0.36, P = 0.02), Fe with IL-10 (r = 0.37, P = 0.01) (Figures 3(a)-(c)). However, a negative correlation was demonstrated between Cu level with Th2 type of cytokines IL-4 and IL-10 (r = −0.39, P = 0.01, r = −0.32, P = 0.03 respectively) (Figure 3(d); Figure 3(e)) and between Se and IL4 (r = −0.31, P = 0.04) (Figure 3(f)). No other correlation could be demonstrated between trace elements with cytokines and immune activation markers studied in case of HCV infection (Supplementary Table 2(c)).

4. Discussion

The HIV-1 seropositive donors in the present study showed higher magnitude of exposure to unsafe sex compared to HBV and HCV positive donors. However,
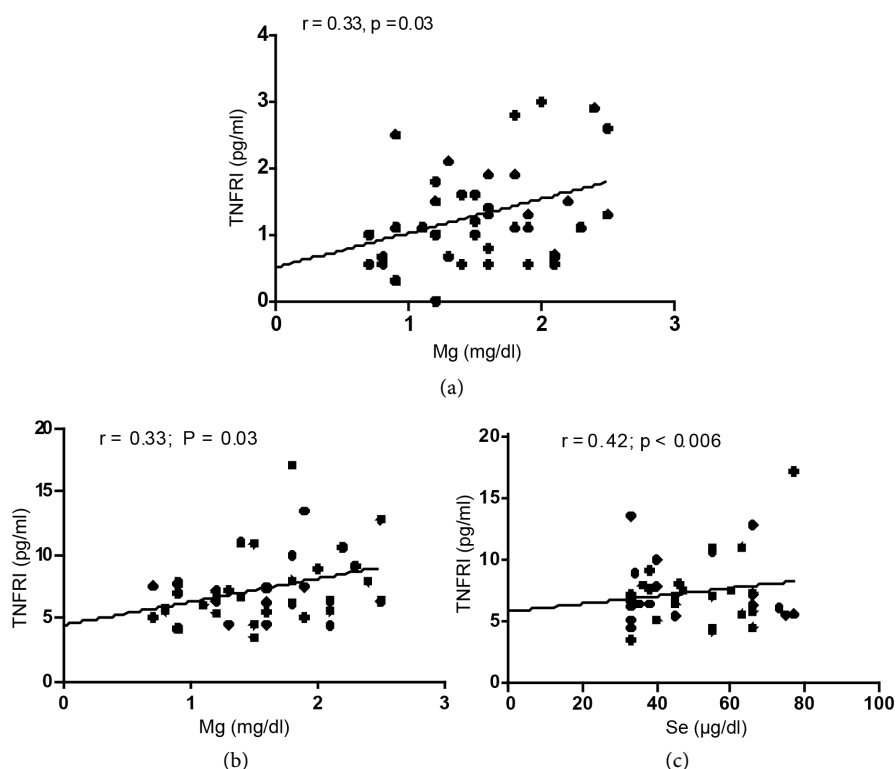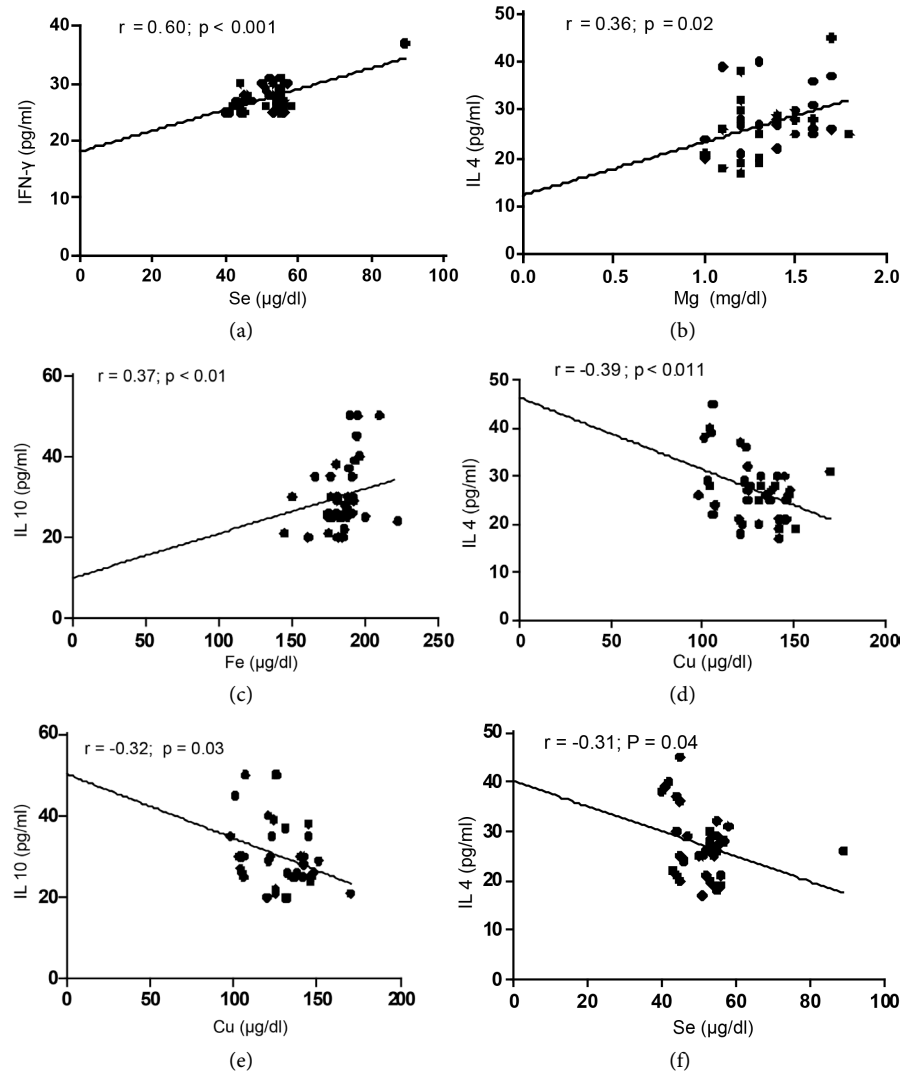
Figure 2. (a): 4 Spearman rank correlation between plasma levels of Magnesium (Mg) tumour necrosis factor type- 1 (TNFR I) in HBV infected blood donors (n = 40; r = 0.33, P = 0.03); (b): Spearman rank correlation between plasma levels of Magnesium and tumour necrosis factor type -2 (TNFR II) in HBV infected blood donors (n = 40; r = 0.33, P = 0.03); Spearman rank correlation between plasma levels of Selenium (Se) and tumour necrosis factor type-2 (TNFR II) in HBV infected blood donors(n = 40; r = 0.42, P = 0.006).

absence of such risk factor in perceptible proportion (22.5%) of the HIV-1 positive donors suggests acquisition of HIV-1 by other means like blood donation in unhygienic setup as pointed out in our earlier study [10]. Degree of consumption of alcoholic drinks was similar in all the subgroups of seropositive donors. A comparable proportion of seronegative control donors also revealed history of alcohol consumption which could be due to similarity in socioeconomic status between the seropositive subgroups and controls. The donors in the present study were repeat donors donating at multiple donation centres which provided us the opportunity of enrolling them at very early stages of HIV-1 or HBV or HCV infections based on recording of the interval between the point of detection of seropositivity in blood bank and preceding seronegative donation. This was further substantiated by the asymptomatic status, unaltered CD4 count, serum albumin and bilirubin levels as well as marginal alteration of liver enzyme profile comparable to control seronegative donors. Therefore, it may be presumed that the donors in the present study had pre-existing alterations in the trace element status prior to enrolment.

Trace elements play important roles in various components of both innate and acquired immune function [17]. Zinc promotes the growth, maturation and

Figure 3. (a): Spearman rank correlation between plasma levels of Selenium (Se) and IFN gamma (IFN-γ) in HCV infected blood donors (n = 40; r = 0.60, P < 0.001); (b): Spearman rank correlation between plasma levels of Magnesium (Mg) and interleukin 4 (IL-4) in HCV infected blood donors (n = 40; r = 0.36, P = 0. 02); (c): Spearman rank correlation between plasma levels of Iron (Fe) and interleukin 10 (IL-10) in HCV infected blood donors (n = 40; r = 0.37, P = 0.01); (d): Spearman rank correlation between plasma levels of Copper (Cu) and interleukin 4 (IL-4) in HCV infected blood donors (n = 40; r = −0.39, P = 0.01); (e): Spearman rank correlation between plasma levels of Copper (Cu) and interleukin 10 (IL-10) in HCV infected blood donors (n = 40; r = −0.32, P = 0.03); (f): Spearman rank correlation between plasma levels of Selenium (Se) and interleukin 4 (IL-4) in HCV infected blood donors (n = 40; r = −0.31, P = 0.04).

activity of numerous immune cells, such as B and T lymphocytes and natural killer cells [18] and functions as a signalling molecule for defence cells [19]]. Zinc deficiency may also be accompanied by an imbalance of Th1 and Th2 cell function resulting in dysregulated resistance to infection [20]. Selenium (Se) is an essential micronutrient that affects optimal immune responses. Through its incorporation into selenoproteins, Se is involved in regulating oxidative stress,

redox, and other crucial cellular processes in nearly all tissues and cell types, including those involved in innate and adaptive immune responses [21]. The measurement of trace elements especially viz, Se and Zn has been considered to predict disease progression in many viral infections e.g. HIV-1, HBV and HCV [9] [22] [23]. Iron plays important roles in anti-microbial host responses, firstly by synergistic effects towards anti-microbial radical formation [24] and secondly by directly altering immune cell proliferation and anti-microbial immune effecter pathways [25]. Conversely, excess iron promotes the formation of intracellular free radicals, which can cause oxidative damage [26]. Requirement of copper for optimal innate immune response has been demonstrated in animal models [27] and cell culture models using mouse macrophages [28], where the effects could be reversed by administration of Cu. Nitric oxide (NO), an important mediator of intracellular killing of microbes, is degraded during copper deficiency [29]. Some of the recent research showed that interleukin-2 induced T cell proliferation is reduced even in marginal copper deficiency [30]. Magnesium acts as a cofactor for immunoglobulin synthesis, immune cell adherence, antibody-dependent cytolysis and macrophage response to lymphokines.

Present study showed significantly depressed plasma levels of Zn, Se, Fe, Mg and Cu in blood donors infected with HIV-1. Zinc is a component of HIV-1 nucleocapsid proteins and is used for gene expression, multimerization and integration in HIV-1 and this may explain the low plasma zinc levels observed in our study [31]. Low level of selenium in our study is in agreement with the view that HIV-1 requires large amount of selenium for its replication in the cell, which could deplete host level of selenium [7] and such decline in Se status has been reported to occur even in early stage of disease when malnutrition or mal-absorption cannot be a factor [22]. The Se deficient HIV-1 infected patients are nearly 20 times more likely to die from HIV related causes than those with adequate selenium levels [32]. Under low selenium conditions, increased oxidative stress and apoptosis activate the virus, which replicate at higher rates to escape from a dying cell [33]. Experimental evidence had shown that serum iron may decline in early asymptomatic HIV infection probably because of impaired absorption although they may however increase with progression of the disease as iron accumulates in the macrophages and other cells [34]. Depleted serum Mg concentration in early HIV-1 infection has been linked with common complaints of lethargy, weakness fatigue and decreased mentation in HIV infection [35]. Reduced level of Cu observed in our study could be due to reduction in the synthesis of ceruloplasmin by liver, possibly associated with HIV-1 infection [36].

The plasma trace element status in both HBV and HCV infected blood donors in the present study showed significantly low level of Zn, Se and Mg concentrations and elevated level of Cu and Fe as compared to seronegative controls which is in agreement with the study by Hatano *et al.*, [23] as well as by Kalkan *et al.*, [37]. Studies have shown that lower concentration of selenium in the serum and erythrocytes in hepatitis B and C patients [38] [39] responds poorly to

bacterial and viral infections leading to faster progression of both HBV and HCV infections [40] [41]. Reduced levels of selenium can be explained by the fact that selenium is used as a co-factor for glutathione peroxidise (GPX) enzyme. Se-dependent GPX and anti-oxidative ability are linked with reduced level of selenium micronutrient levels, although HCV infection has been reported to be potent in inducing oxidative stress suggesting that this mechanism is unique to this virus [42]. Elevated plasma copper levels in HBV and HCV as observed in the present study is considered to be due to redox process and oxidative stress [43]. Elevated level of Fe observed in the present study could reflect interruption of Fe metabolism during acute stage resulting into increased accumulation in liver [44]. It has been shown that hepatitis B surface antigen (HbsAg) positive cases have decreased magnesium levels in the serum suggesting the reflection of defence strategies by the organism [45].

The present study demonstrated a significantly low level of Th1 type of cytokines (IL-2 and IFN-γ) in blood among HIV-1 infected group when compared to seronegative controls, while the Th2 type of cytokines *i.e.* (IL-4 and IL-10) remained unaltered. Many reports describe a shift from Th1 to Th2 cytokine profile during the progression of HIV-1 disease [46] [47]. There are numerous reports documenting association of imbalance of Th1 and Th2 categories of cytokines leading to disease progression in HIV, HBV and HCV infection [48] [49] [50]. It is generally accepted that qualitative defect in T cell function in terms of change in plasma cytokine profile from Th1 dominant to Th2 dominant can be evident before quantitative decline in CD4 + T lymphocyte count at the asymptomatic stage of HIV infection [48]. An ongoing progression of HIV-1 infection in our group of asymptomatic HIV-1 infected blood donors was further evident from raised levels of immune activation markers *i.e.* TNF-α and sTNFR levels as reported in many studies [51] [52].

Both HBV and HCV infections in the present study showed dominance of Th2 category of cytokines and elevation of immune activation markers. It could be hypothesized that these donors were likely to be future potential persistent carriers for both these infections [53] [54], since balance between immunostimulatory and inhibitory cytokines has been shown to prolong inflammation leading to chronicity of liver disease in both these infections [54]. Patients with HBV and HCV infection have been reported to be characterterized by elevated plasma levels of TNF-α, TNFR I and TNFR II as well as TNF-α production by in vitro [55] [56].

The present study showed a significant positive correlation between depression of Zn and Cu levels and Th1 type of cytokines in HIV-1 infection which is in agreement with studies [57] [58] showing that intracellular deficiency of Zn and Cu can lead to decrease in the synthesis and secretion of Th1 type of cytokines *i.e.* IL-2. Further, transcriptional activity of the IL-2 gene is suppressed in Zinc and Cu deficiency when T cells are treated with phytohemaglutinin or phorbolmyristate acetate [59].

It has been shown that in Zn deficiency generation of IL-2 and IFN-γ are de-

creased where as the production of Th2 type of cytokines IL-4 and IL-10 are not affected [60]. A positive correlation between low level of Cu and depression in Th1 cytokine IL-2 level observed in our study is in agreement with the findings reported by Prasad [61], who demonstrated impaired secretion of IL-2 receptor (a marker of early T cell activation) by peripheral blood mononuclear cells from healthy subjects receiving a low Cu diet. Negative correlation of Zn with TNFR l in HIV-1 infected plasma samples of blood is consistent with the study of Prasad [60], who reported decreased plasma Zn to be associated with increased plasma oxidative stress markers and increased generation of inflammatory cytokines.

Positive correlation between Se with IFN-γ in HCV infection observed in the present study has been demonstrated in mouse model experiments [6], where mice fed with deficient selenium diet resulted in decrease in interferon gamma levels in serum while positive correlation between Fe and IL-10 corroborate with the study of Baum *et al.* [8] who have shown that increased iron transferritin saturation (TS) is significantly associated with elevated Th2 cytokine profile in HCV positive patients. The positive correlations between Mg and IL-4 in our study substantiate the possibility that magnesium deficiency may affect antibody synthesis due to impaired signal to B cells by activated T helper cells [62].

Information on the inter-relationship between trace elements, cytokines and immune activation is limited to HIV-1 infection only [35], while there is hardly any such information available in HBV and HCV infections. There are numerous reports on alteration of trace elements in advanced stages of HIV, HBV and HCV infections, when interplay of multiple factors like decreased intake, impaired absorption and altered metabolism come into play [31] [37] [44]. However, data of such alterations in asymptomatic stage of these infections are lacking. Admittedly, however, our study to examine alteration of cytokines and activation markers was based on quantitation of plasma levels of cytokines and activation markers. More in depth studies like quantitation of mRNA expression of the cytokines and activation markers by in vitro cultured lymphocytes in presence of trace elements would have provided better insights into the alteration of these markers which we could not undertake due to resource constraints.

Nevertheless, the present report provides information on the association between trace element alteration and cytokine imbalance at early asymptomatic stage of HIV, HBV and HCV infections in absence of obvious protein calorie malnutrition that tends to point out that trace element alterations can exist at early asymptomatic stage since the donors in our study had unaltered parameters like BMI, protein intake, calorie intake, serum albumin levels comparable to control population.

Conflict of Interest

We declare that we have no conflict of interest.

Acknowledgements

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Appendix

Supplementary Table 1. CD4 Count, liver enzyme profile, albumin, energy intake, protein intake and BMI in various subgroups of seropositive donors.

	HIV-1 (n = 40)	HBV (n = 40)	HCV (n = 40)	Control (n = 40)
CD4 cells/μl	828± 175	844 ± 149	886 ± 133	907 ± 159
ALT(U/L)	32.6 ± 30.1	30.1 ± 22.1	27.7 ± 21.9	26.4 ± 10.7
AST(U/L)	28.7 ± 18	24.2 ± 8.7	22.8 ± 9.2	24.9 ± 9.1
ALP(U/L)	92.6 ± 34.4	88.9 ± 33.9	85.9 ± 29.3	79.8 ± 20.6
Bilirubin (mg/dL)	1.1 ± 0.5	1.2 ± 0.7	1.3 ± 0.7	1.0 ± 0.20
Albumin(g/dL)	4.0 ± 0.5	4.1 ± 0.5	4.3 ± 0.5	4.2 ± 0.50
Energy (K cal/kg body weight)	37.1 ± 3.4	36.6 ± 2.9	35.1 ± 2.8	38.3 ± 0.42
Protein intake (gm/day)	60.8 ±4.6	59.4 ± 5.3	59.8 ± 7.2	61.5 ± 4.7
BMI	19.9 ± 1.2	20.9 ± 2.8	20.4 ± 1.4	21.1 ± 4.2

Note: There was no statistical difference in the above parameters between HIV-1, HBV, HCV; infected donors and that of seronegative controls in multiple comparisons as well as in individual comparisons between the above subgroups (by ANOVA).

Supplementary Table 2. Spearman rank correlations between plasma levels of trace elements and cytokines in HIV-1, HBV and HCV infected donors. (a) HIV-1; (b) HBV; (c) HCV.

(a)

		IL2	IFN-y	IL-4	IL-10	TNF-α	TNFRI	TNFRII
Se	r	0.053	0.075	0.132	0.096	−0.602	0.093	0.035
	p	0.742	0.643	0.416	0.553	0.712	0.566	0.829
Zn	r	−0.142	0.339	−0.092	0.290	0.145	−0.318	0.228
	p	0.379	0.033*	0.568	0.061	0.300	0.045*	0.155
Fe	r	−0.067	0.269	−0.200	−0.167	0.169	−0.026	0.133
	p	0.690	0.093	0.215	0.301	0.296	0.870	0.411
Cu	r	0.335	−0.118	0.060	0.089	−0.130	0.090	−0.070
	p	0.034*	0.468	0.700	0.582	0.017	0.597	0.666
Mg	r	−0.008	−0.090	0.152	−0.159	−0.059	0.052	0.090
	p	0.959	0.578	0.349	0.320	0.713	0.748	0.550

(b)

		IL2	IFN-y	IL-4	IL-10	TNF-α	TNFRI	TNFRII
Se	r	0.115	−0.160	0.059	0.080	0.056	0.109	0.425
	p	0.477	0.314	0.714	0.957	0.730	0.502	0.006*
Zn	r	0.080	0.010	−0.144	0.233	−0.210	−0.174	−0.184
	p	0.623	0.949	0.372	0.146	0.193	0.281	0.254
Fe	r	−0.096	0.001	−0.013	−0.055	0.080	−0.025	−0.009
	p	0.557	0.993	0.933	0.732	0.623	0.874	0.954
Cu	r	0.105	−0.128	0.297	0.025	0.015	0.082	−0.130
	p	0.515	0.428	0.062	0.877	0.923	0.613	0.402
Mg	r	−0.153	−0.110	−0.049	−0.270	−0.060	0.330	0.331
	p	0.344	0.498	0.702	0.091	0.710	0.030*	0.036*

*indicates significant correlation.

(c)

Se	r	−0.123	0.600	−0.317	−0.110	0.218	0.275	0.275
	p	0.440	0.001*	0.046*	0.490	0.170	0.063	0.081
Zn	r	0.024	0.221	−0.171	−0.140	−0.016	0.267	0.267
	p	0.878	0.163	0.283	0.380	0.918	0.090	0.090
Fe	r	−0.052	0.094	0.153	0.373	0.171	−0.136	−0.136
	P	0.745	0.555	0.338	0.017*	0.283	0.393	0.393
Cu	r	0.105	0.201	−0.395	−0.326	0.004	0.310	0.310
	p	0.510	0.205	0.011*	0.039*	0.978	0.520	0.047
Mg	r	0.050	0.186	0.366	−0.190	−0.029	0.292	0.292
	P	0.752	0.242	0.020*	0.233	0.854	0.063	0.063

*indicates significant correlation.