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ORIGINAL RESEARCH

The relationship between zonulin and liver function test in patients with human immune deficiency virus infection

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Background: The gastrointestinal tract (GIT) plays an integral role in the immunopathogenesis of human immunodeficiency virus (HIV). Although, changes in the concentration of serum zonulin, which is a marker of intestinal integrity, has been reported among HIV-positive patients, there is little data available on serum zonulin concentrations in HIV-positive patients on anti-retroviral therapy (ART) in Ghana. The aim of this study was to examine the changes in zonulin concentration in HIV patients and to examine the relationship between zonulin and liver function test.

Methods: This case-control study involved 86 HIV-negative participants, 85 treatment-naïve HIV-positive patients and 136 chronic HIV patients on ART. A total of 62 males and 245 females between the ages of 10 to 83 years were included in the study. Approximately 5 ml venous blood sample was collected from each participant into serum separator tubes (SST), and was analysed to determine serum concentrations for zonulin and liver functions.

Results: Serum zonulin concentration was significantly higher in those with primary HIV infection compared to the uninfected controls ($p < 0.0001$), while those with chronic HIV infection on ART had significantly lower serum zonulin concentration values compared to the primary HIV-infected individuals ($p < 0.0001$). Furthermore a negative correlation between serum zonulin and gamma-glutamyl transferase concentration values (GGT) (R = -3.13, $p < 0.05$) as well aspartate aminotransferase (AST) (R= -2.10, $p < 0.05$) concentration values were seen in this study.

Conclusion: Serum zonulin concentration was significantly increased in primary HIV (PHIV) patients as compared to the negative controls and those with chronic HIV (CHIV) infection.

Keywords: zonulin, primary HIV, chronic HIV, liver function

Introduction

Over thirty-five million of the world's population are estimated to be living with HIV, with sub-Saharan Africa alone accounting for more than 71% of this infection.[1] In Ghana alone, the prevalence of HIV is about 1.6%, with regional variations ranging below 1% in the northern region to about 2.8% in the eastern region.[2] HIV can be grouped into primary and chronic disease. Primary HIV infection (PHIV) is the initial phase after HIV acquisition, characterised by high viral replication, massive inflammatory response and irreversible immune-damage, particularly at the gastrointestinal level, while chronic HIV infection refers to HIV infection extending beyond six months.[3] HIV has a significant effect on the liver, especially in those with comorbidities and those who take alcohol. Some anti-retroviral medicines also have a negative effect on the liver, hence administration of anti-retroviral therapy (ART) to this group of patients will further lead to a significant impact on the hepatocytes.[4]

HIV usually infects the GIT-associated CD4+ T-cells leading to an increased permeability to bacterial endotoxins such as lipopolysaccharide (LPS), which can stimulate hepatocytes, Kupffer cells (KCs) and hepatic stellate cells (HSCs) to produce pro-inflammatory cytokines and chemokines that attract activated lymphocytes and monocytes to the liver, thus further inducing fibrosis.[5] Furthermore, it is hypothesised that increased levels of LPS contribute to chronic immune activation in HIV-infected patients.[5] The GIT microbiota is involved in the development and modulation of the immune system and it has been reported that signals from gut bacteria may be effective in the development of T-cells, T-helper type 1 and 2 cells, and T-helper 17 cells.[6] Zonulin, discovered about two decades ago, is the only physiological mediator known to control GIT permeability of tight junctions (TJs); therefore, increase in zonulin concentration is linked to increase in intestinal permeability,[7] which plays an integral role in the immunopathogenesis of HIV.[6] The physiological role of zonulin is unknown; however, it is believed that it is engaged in a variety of processes, including TJ regulation, which controls the passage of fluid, macromolecules, and leukocytes between bodily compartments. Another physiological function of zonulin is to guard against microbial invasion.[8] The mammalian small intestine is practically sterile in the absence of enteric illnesses. A leaky intestine can result from enteric microbes colonising the proximal gut (even if there is no visible mucosal injury or

development of particular toxins).[9] However, the mechanism(s) by which proximal bacterial contamination disrupts the normal regulation of intestinal TJ permeability remains unknown. Both normal enteric bacterial flora isolates (well defined for not carrying any known pathogenic features) and pathogenic bacteria have been shown to affect TJ competency, as evidenced by changes in epithelial resistance and enhanced inulin transit.[8] When bacteria come into contact with the intestinal mucosa, they release zonulin, which can be viewed as a host-independent defensive mechanism that reacts to the aberrant presence of microorganisms on the small intestine's surface, regardless of their pathogenic features or survival. Water is secreted into the intestinal lumen after zonulin-induced TJ opening, and bacteria are pushed out of the small intestine by hydrostatic pressure gradients.[10] Changes in zonulin concentration have been associated with increase in mortality of HIV patients,[11] and HIV medications,[12] hence the zonulin pathway in its innate immunity function could be a defence mechanism for the protection against HIV infection.[13] Research has shown that most chronic inflammatory diseases (CIDs) are associated with elevated zonulin concentration[14] and so it is expected that HIV infection could lead to dysregulation of zonulin metabolism since it has an effect on the immune system.

Effective treatment of HIV-infected patients decreases inflammation and immune activation.[15,16] Even though virological suppression is achieved by ART, the level of inflammatory markers such as zonulin could still remain elevated.[15] One study revealed that patients living with HIV on ART still have elevated markers of inflammation, coagulation and immune activation compared to uninfected individuals, despite virological control.[17] Furthermore, higher levels of inflammation have been reported in children with perinatally acquired HIV compared to uninfected controls[18,19] and this higher level of inflammation may persist despite viral suppression due to ART.[20]

The extent to which HIV infection and resultant immune compromise alter the gut microbiome remains controversial.[21] No well-controlled studies to date have evaluated the gut microbiota of the same individuals before and after infection with HIV.[21] Data on the relationship between zonulin and HIV infection in Ghana is very scanty; therefore, this study was aimed at elucidating the relationship between zonulin in treatment-naïve HIV-positive patients and HIV patients on ART and to examine the relationship between zonulin and liver function tests in HIV patients.

Methods

Study design/site

This case-control study was conducted at the Ho Teaching Hospital, Ho in the Volta Region of Ghana from January to May 2020.

Study population/eligibility criteria

Three hundred and seven (307) participants were involved in the study which consisted of 62 males and 245 females between the ages of 10 to 83 years. Eighty-six (86) of them were non-exposed and designated as negative, 85 were newly diagnosed HIV patients referred to as primary HIV (PHIV)/treatment-naïve HIV-positive patients who were not on ART and designated as HIV controls. Furthermore, 136 of the patients were chronic HIV patients (CHIV) on ART designated as HIV-ART.

Patients were included in the study if they met the following criteria: diagnosed and confirmed HIV-positive, followed up with frequent visits, and on ART for at least three (3) months in the case of the CHIV patients. Patients with active or chronic viral hepatitis, pregnancy, and those on concurrent hepatotoxic medication or on treatment with liver-supportive medicines were excluded.

Data collection and laboratory analysis

All persons gave their informed consent prior to inclusion in the study and written informed consent was also obtained from parents/guardians of those persons younger than 18 years.

Demographic characteristics and clinical history were obtained from patient records in their folders. Approximately 5 ml venous blood sample was collected from each participant into serum separator tubes (SST). Serum samples were stored in small aliquots at -21 °C until analysis. All samples were analysed within one week after collection. Serum biochemical assays (alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase [ALP] gamma-glutamyl transferase [GGT]), total protein, albumin, globulin and total bilirubin were determined using the Selectra Pro S (Vital Scientific B.V. Van Rensselaerwweg 4, NL 6956 AV Spankeren, The Netherlands) automated chemistry analyser using the procedure outlined for the equipment, while serum zonulin concentrations were determined in duplicate using human zonulin ELISA Kit (Immundiagnostik AG, Bensheim, Germany). The lowest limit of detection of the assay was 0.094 ng/ml. The intra-assay and

Table I: Grading of LFT abnormalities

Feature	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
ALT (U/L)	< 1.25	1.25–2.5	> 2.5–5.0	> 5.0–10	> 10
AST (U/L)	< 1.25	1.25–2.5	> 2.5–5.0	> 5.0–10	> 10
ALP (U/L)	< 1.25	1.25–2.5	> 2.5–5.0	> 5.0–10	> 10
GGT (U/L)	< 1.25	1.25–2.5	> 2.5–5.0	> 5.0–10	> 10
Bilirubin (µmol/L)	Normal	> 1.0–1.5	> 1.5–2.5	> 2.5–5	> 5

ALT – alanine transaminase, AST – aspartate transaminase, ALP – alkaline phosphatase, GGT – gamma-glutamyl transferase

Table II: Comparison of zonulin concentration (ng/ml) between HIV-infected and normal controls

Variable	Frequency	Minimum	Maximum	Mean	SD	*p*-value
Normal	86	27.000	65.000	39.407	8.084	**< 0.0001****
PHIV	85	41.000	67.000	57.618	7.157	
CHIV	136	15.000	60.000	34.066	10.582	**< 0.0001****
PHIV	85	41.000	67.000	57.618	7.157	

PHIV – primary HIV infection, CHIV – chronic HIV infection
*Significant at 5%, **Significant at 1%

inter-assay coefficients of variation ranged from 2.5% to 6.0% and from 7.7% to 8.3%, respectively.

Grading of liver function test (LFT) abnormalities was done according to the Acquired Immune Deficiency Syndrome (AIDS) Clinical Trials Group (CTG) criteria (Table I). Grade 1 indicates mild, Grade 2 moderate, Grade 3 severe and Grade 4 life-threatening values.

Statistical analysis

Unique codes were allocated to each participant in order to ensure data security and confidentiality. The normality test for variables under study was performed by Shapiro Wilk test followed by comparison between those with HIV and the negative controls by Mann–Whitney U-test. Continuous parametric variables were expressed as their mean ± standard deviation; continuous non-parametric variables were expressed as median (minimum and maximum), while categorical variables were expressed as frequencies and percentages. Comparisons of parameters were performed using the unpaired t-tests, Mann–Whitney U test, Kruskal–Wallis test, Pearson's r correlation test where appropriate. A *p*-value < 0.05 was considered statistically significant for all analyses. Data were analysed using the XLSTAT statistical software 2020 version and GraphPad Prism statistical software version 6.

Results

In all, 307 participants took part in the study consisting of 62 males and 245 females. The age range was between 10 to 83 years with mean age of the negative, PHIV and CHIV being 44.3, 45.1 and 46 years respectively. The mean body mass index (BMI) of the participants was 25.3, 25.0 and 25.3 kgm^{-2} for the negative, PHIV, and CHIV respectively. All those on ART took the same combination therapy consisting of AZT (Zidovudine), 3TC (Lamivudine) and EFV (Efavirenz), hence the effect of the ART on the liver was deemed to be same.

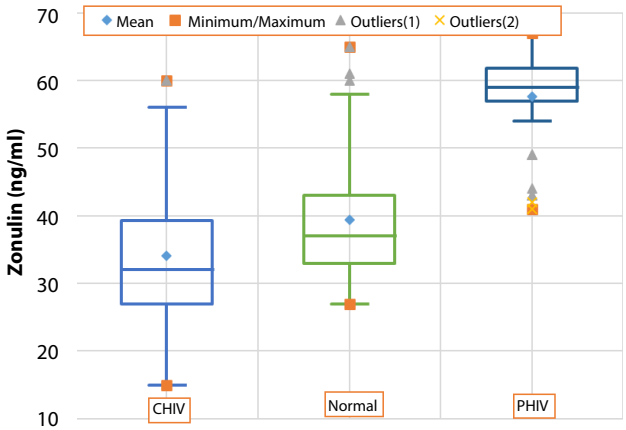

Figure 1: Box plots for zonulin according to the HIV infection category (PHIV, CHIV, negative control)

Table III: Correlation between zonulin and the biochemical markers of liver function test among those with CHIV

Variables	Age	BMI	Duration on ARTs (yrs)	Total protein	Albumin	Globulin	ALP	ALT	AST	GGT	Total Bili	Direct Bili	Indirect Bili	Zonulin
Age (Years)	1													
BMI (kg/m²)	0.079	**1**												
Duration on ARTs (yrs)	0.117	0.111	**1**											
Total protein (g/l)	**-0.319***	-0.073	-0.150	**1**										
Albumin (g/l)	0.128	0.159	0.128	-0.105	**1**									
Globulin (g/l)	-0.115	0.013	-0.083	**0.192***	-0.071	1								
ALP (U/L)	**-0.279***	-0.013	-0.118	0.076	-0.035	-0.069	**1**							
ALT (U/L)	-0.101	-0.040	-0.112	0.095	0.031	-0.097	**0.257***	**1**						
AST (U/L)	-0.142	-0.095	-0.073	**0.266***	0.007	-0.065	**0.226***	**0.667***	**1**					
GGT (U/L)	0.012	-0.021	0.050	0.033	0.132	-0.047	-0.005	0.156	**0.423***	**1**				
Total Bili (µmol/L)	0.034	-0.104	0.075	0.134	0.113	0.028	-0.052	0.161	**0.196***	**0.265***	**1**			
Direct Bili (µmol/L)	0.104	-0.095	-0.036	-0.026	0.010	-0.063	**-0.257***	0.059	0.064	0.097	**0.453***	**1**		
Indirect Bili (µmol/L)	0.000	-0.081	0.096	0.159	0.122	0.054	0.036	0.159	**0.195***	**0.260***	**0.945***	0.138	**1**	
Zonulin (ng/ml)	-0.009	0.117	0.062	0.014	-0.007	-0.025	0.054	-0.141	**-0.210***	**-0.317***	**-0.201***	-0.062	**-0.200***	1

BMI – body mass index, ALP – alkaline phosphatase, ALT – alanine transaminase, AST – aspartate transaminase, GGT – gamma-glutamyl transferase; *Significant at 5%, **Significant at 1%

Table IV: Comparison of categories of liver disease into their respective grades among those with CHIV infection to establish if significant differences exist in average zonulin concentration

Feature	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	*p*-value
ALT (U/L)	33.622	34.761	35.000	33.500		0.247
AST (U/L)	35.040	24.455				**0.001***
ALP (U/L)	24.172	27.000	19.00			0.972
GGT (U/L)	35.858	29.650	25.800	23.500	16.000	**0.001***
Bilirubin (µmol/L)	34.185	18.000				0.114

ALT – alanine transaminase, AST – aspartate transaminase, ALP – alkaline phosphatase, GGT – gamma-glutamyl transferase; *Significant at 5%

We used Mann–Whitney U test to compare zonulin concentrations between uninfected participants and those infected with HIV and observed significant statistical difference between the uninfected and infected participants ($p < 0.0001$). A further significant difference was observed between the PHIV and the CHIV ($p < 0.0001$) as shown in Table II and Figure 1. The results show that during the initial stages of HIV infection, plasma zonulin concentration increased, and with the progression of the disease, it may fall to concentrations lower than the uninfected group.

We examined the correlation between the liver function test and zonulin concentrations as shown in Table III. There was negative correlation between zonulin concentrations and GGT ($R = -3.13$, $p < 0.05$) as well as between zonulin concentrations and AST ($R= -2.10$, $p < 0.05$). Furthermore, positive correlations were observed between AST and GGT ($R = 0.423$, $p < 0.05$), AST and ALP ($R = 0.226$, $p < 0.05$), as well as AST and ALT ($R = 0.667$, $p < 0.05$), while negative correlation was shown between zonulin and total bilirubin ($R = -0.201$ $p < 0.05$). The rest of the biomarkers of liver function tests did not show any significant correlation with zonulin. Our results did not indicate any particular relationship between zonulin and BMI and age in the patients with CHIV infection.

We examined the average zonulin concentrations according to the grades of liver disease. We used Mann–Whitney U test to compare groups of two (AST, Bilirubin) and Kruskal–Wallis test to compare groups of more than two (ALT, ALP, GGT). The results are presented in Table IV. The results indicated that Grade 4 liver disease was associated with reduction in zonulin concentrations. There was significant rise in AST ($p < 0.001$) and GGT ($p < 0.001$) among those with CHIV but ALT, ALP and total bilirubin did not change significantly.

Discussion

We examined the relationship between zonulin concentration and HIV infection and assessed the changes in the zonulin concentration in PHIV-infected persons without ART and CHIV-infected individuals on ART. We also examined the relationship between zonulin and biomarkers of liver function across the various grades of liver diseases. Our study showed that significantly higher serum zonulin concentrations characterised the early stages of HIV infection as compared to negative controls. This result is consistent with earlier findings which showed increase in zonulin concentration following HIV infection.[22] Zonulin concentration has also been previously shown to have a good classification performance for distinguishing PHIV infection from CHIV.[22] This is in spite of the fact that the researchers could not ascertain the exact time of exposure to the HIV virus.[3] Our results also showed significantly higher plasma zonulin concentrations among those with PHIV who were not on ART as compared to those with CHIV who were on ART. This is in consonance with what was reported by Pastor et al., who stated that plasma zonulin and stool lactoferrin were significantly higher in primary HIV-infected individuals as compared to chronic HIV-infected individuals.[22] Those with CHIV also had significantly lower zonulin concentration as compared to the negative controls and PHIV participants. It is unclear why plasma zonulin concentrations were significantly lower in CHIV patients, but greater gut epithelial cell death or malfunction as the disease progresses may decrease its expression.[22]

Our results also showed a negative correlation between plasma zonulin concentrations and both GGT and AST with reduced zonulin concentration corresponding to increased levels of these liver enzymes. Zonulin is secreted mainly from the liver, but also from enterocytes, adipose tissue, brain, heart, immune cells, lungs, kidney, and skin.[23,24] Therefore, with the increased levels of AST and GGT which are suggestive of reduced liver function, it is not surprising that zonulin concentration in patients with elevated liver enzymes had decreased zonulin concentration since the liver will not be able to secrete enough zonulin when its function is drastically reduced. GGT is a microsomal enzyme present in hepatocytes and biliary epithelial cells, renal tubules, pancreas and intestine.[25] It can also be found in cell membrane acting as transporter of peptides into the cells across the cell membrane and takes part in glutathione metabolism. Serum GGT activity is largely associated with the hepatobiliary system though higher concentrations are found in renal tissue.[25] The results of this study showed that elevated liver enzymes in CHIV may be due to the effect of the HIV infection on the hepatocytes and not the ART and corroborates previous findings which suggested that hepatic injury may occur in HIV, even in the absence of ART.[26] The fact that our results indicated that the more severe form of liver disease, Grade 4 (life-threatening) was associated with significant reduction in zonulin, also points to the fact that as liver function decreases, secretion of zonulin decreases. This is in consonance with a previous finding which reported that decreased zonulin concentration correlated with increased mortality in HIV patients.[27] A strong correlation existed between AST and ALT among those with CHIV infection. AST elevations often predominate in patients with cirrhosis and even in liver diseases that typically have an increased ALT.

Consequently, persistent elevation of ALT for more than six months after an occurrence of acute hepatitis is used in the diagnosis of chronic hepatitis and liver injury.[28] Furthermore, elevation in ALT levels is greater in patients with nonalcoholic steatohepatitis than in those with uncomplicated hepatic steatosis.[29] Caution must, however, be exercised in the use of AST to evaluate hepatocellular damage since it occurs in other tissues in the body like the heart and red blood cells, however, an increase in AST in conjunction with elevation in ALT is indicative of hepatocellular injury.[30]

This study has several limitations, which include a disproportionately higher number of females as compared to males because we used secondary data which had more females than males and lack of microbiome data. We could also not ascertain the first weeks or days of infection since symptomatic phase can occur several weeks after initial viral exposure. Furthermore, we could not distinguish whether the increased or decreased zonulin concentration were as a result of the anti-retroviral medicines, leaky gut or duration of the infection. We could also not determine CD4 count and viral load. Nevertheless, this is the first study to evaluate zonulin as a biomarker of intestinal integrity among Ghanaians with primary and chronic HIV infections.

Conclusion

Our study showed that zonulin concentration was significantly increased in PHIV infection as compared to the HIV-negative participants. The highest concentration of zonulin was observed in PHIV followed by negative controls and the least concentration was observed in those with CHIV. This observation suggests that the zonulin pathway in its innate immunity function may be protective against HIV infection. This protective role may be eroded with prolonged HIV infection coupled with prolonged treatment with ART. There was significant decrease in zonulin concentration among those with Grade 4 liver function abnormalities with AST and GGT showing the greatest increase among those severe liver disease.

Conflict of interest

The authors declare no conflict of interest.

Funding source

No funding was required.

Ethical approval

The study was approved by the Research Ethics Committee at the University of Health and Allied Sciences and was conducted according to the Declaration of Helsinki. All persons gave their informed consent prior to inclusion in the study and written informed consent was also obtained from parents/guardians of those younger than 18 years.

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