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Research Paper

Levels of anti-myeloperoxidase antibodies in HIV positive patients at an HIV clinic in Harare, Zimbabwe

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ABSTRACT: *Coronary heart disease, preceded by atherosclerosis, is an emerging cause of non-AIDS deaths in HIV-positive patients. Inflammation and oxidative stress are implicated in atherosclerosis. Myeloperoxidase's location in leukocytes and its catalytic role in the formation of oxidative species link it to both. Presence of high titers of anti-myeloperoxidase antibodies in circulation is associated with elevated myeloperoxidase and its increased oxidative capacity and hence increased risk of arthrosclerosis and coronary heart disease. A cross-sectional study was carried out on 215 HIV-positive patients to determine the levels of anti-myeloperoxidase antibodies in sera and hence risk of coronary heart disease. Mean anti-myeloperoxidase antibody levels for the 215 participants were not elevated, though few patients (n=19, 8.8%) had elevated anti-myeloperoxidase levels. There was difference in the anti-myeloperoxidase levels when patients were stratified by different antiretroviral combinations. Interestingly, there was difference in the distribution of high density lipoprotein between those with normal and elevated anti-myeloperoxidase, respectively. There were no differences in mean levels of anti-myeloperoxidase antibody levels by body mass index, total cholesterol, hypertension, age, random blood glucose and history of smoking. Hence, measuring anti-myeloperoxidase antibodies could be useful, for predicting coronary heart disease risk, before changes in traditional risk factors become apparent.*

Keywords:– *ART, Coronary heart disease, HIV/AIDS, Inflammation, Myeloperoxidase*

I. INTRODUCTION

Myeloperoxidase (MPO) is a protein synthesized during myeloid differentiation that constitutes the major component of primary granules of macrophages, neutrophils and monocytes [1]. Phagocytes when stimulated respond with a burst of oxygen consumption which is converted initially to superoxide anion and then to hydrogen peroxide (H_2O_2) [1]. Myeloperoxidase, which is released from degranulation of neutrophil and monocyte granules, reacts with the H_2O_2 formed by the respiratory burst to form a complex that can oxidize a large variety of substances [1]. Among the latter is chloride, which is oxidized initially to hypochlorous acid, with the subsequent formation of chlorine and chloramines [1].Under normal circumstances, this forms the body's first line of defense against invading microbes [1].

Myeloperoxidase can impair high density lipoprotein (HDL) function [1]. In addition to playing a central role in cholesterol efflux and reverse cholesterol transport, HDL also possesses anti-inflammatory and anti-oxidative properties [2]. Dysfunctional HDL particles lack atheroprotective properties and promote pro-inflammatory effects. Myeloperoxidase can also increase protease activity at inflammatory sites by inactivating protease inhibitors and also acts as a detoxicant for H_2O_2, thereby preventing inactivation of proteolytic enzymes [3]. Myeloperoxidase can act as leukocyte-derived nitric oxide (NO) oxidase subsequently reducing the bioavailability of NO, contributing to endothelial dysfunction [1, 4]. Nitric oxide is a critical element in vascular homeostasis, and consequently insufficient production or increased scavenging of NO impairs vascular function and accelerates atherosclerosis [5]. Myeloperoxidase also oxidizes low density lipoprotein (LDL) particles; the oxidative modification of LDL is an early event of atherosclerosis [6], [7]. Oxidized LDL contributes to atherogenesis by promoting cholesterol deposition and transformation of macrophages into foam cells [6]. In addition to this myeloperoxidase is also involved in plaque destabilization and rupture [8]. Evidence for the role of myeloperoxidase in plaque destabilization was obtained from pathophysiological studies [9, 10].

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Circulating myeloperoxidase has been detected in higher quantities in patients with autoimmune disease accompanied by blood vessel necrosis characteristic of perinuclear anticytoplasmic autoantibody- (p-ANCA)-associated vasculitis, than in healthy controls [11]. These unusually high levels of myeloperoxidase in this group of patients are thought to predispose them to an increased risk of developing cardiovascular disease [6]. Vasculitis may occur as a primary auto-inflammatory process or may be secondary to another underlying disease such as inflammatory bowel disease, rheumatoid arthritis, neoplasia or viral infections [12]. We hypothesise that measuring levels of myeloperoxidase can be useful in predicting those at high risk of artherosclerosis and with it coronary heart disease due to HIV infection and/or antiretroviral treatment.

In a wide range of patients' populations, studies have reported an association of myeloperoxidase with cardiovascular disease and markers of atherosclerosis [13], [14], [15]. A study by Zhang and coworkers [13] in 2001 was the first epidemiological report accessing the association between myeloperoxidase and coronary artery disease and results showed patients having significantly higher concentrations of myeloperoxidase compared to controls [13], whilst a case-control study by Ndrepepa et al demonstrated an increase in myeloperoxidase levels in patients with coronary artery disease and correlated it to the extent and severity of atherosclerosis of coronary vessels [14]. Meuwese and co-workers studied association of myeloperoxidase with coronary artery disease, and demonstrated a positive correlation between myeloperoxidase and coronary artery disease [15].

Anti-myeloperoxidase antibodies are a group of auto-antibodies directed against myeloperoxidase found mainly in neutrophils and monocytes [16]. The antibodies of the immunoglobulin G type, are directed against antigens found in the cytoplasm of the neutrophils and monocytes [16]. Immunofluorescence on ethanol-fixed neutrophils shows two principal staining patterns, the cytoplasmic (c-ANCA) and perinuclear (p-ANCA). Anti-myeloperoxidase antibodies belong to the latter group [16]. There is evidence for a positive correlation between the titer of anti-myeloperoxidase p-ANCA antibodies and oxidative activity of myeloperoxidase, as well as the quantity of circulating myeloperoxidase [17]. As described earlier, myeloperoxidase is implicated in inflammation and oxidative stress, which causes atherosclerosis [18], [19], [20] and atherosclerosis, precedes coronary heart disease [21]. Therefore, by measuring anti-myeloperoxidase p-ANCA antibodies in HIV-positive patients we hope to predict the risk of coronary heart disease. To support our thinking, findings from a study by Hauer *et al*. in a cohort of 173 patients with ANCA associated renal disease showed that anti-myeloperoxidase p-ANCA positive patients had more atherosclerosis than a comparative group [22]. Hence the main objective of our exploratory study was to determine the levels of anti-myeloperoxidase p-ANCA antibodies in sera and hence determine coronary heart disease risk of HIV positive patients. The method designed to detect IgG class antibodies to myeloperoxidase in human sera is more commonly used to detect various autoimmune vasculitic disorders characterized by elevated levels of perinuclear-anticytoplasmic autoantibodies (p-ANCA) [23]. Our study is the first reported study in Zimbabwe that attempts to measure anti-myeloperoxidase p-ANCA antibodies in HIV patients.

II. MATERIALS AND METHODS

2.1 Ethical considerations

The study was approved by the Joint Research Ethics Committee of Parirenyatwa Group of Hospitals and University of Zimbabwe College of Health Sciences (JREC), Medical Research Council of Zimbabwe (MRCZ) and Research Ethics Committee (REK), Norway. Patients gave written consent after getting information regarding the study and data collected was used for research purposes only. Patients' specimens as well as records obtained during the study were assigned numerical codes to maintain confidentiality.

2.2 Study design and study site

A cross-sectional study of 215 out-patients attending an HIV Clinic in Harare, Zimbabwe

2.3 Study subjects and sample collection

Patients who gave written consent to take part in the study had demographic data collected in interviews and blood samples collected in plain tubes. Serum was separated within 12 hours and stored at -70^{o}C. The samples were transferred to a cold room with temperatures maintained in the 2-8^{o}C range 5 hours before analysis.

2.4 Sample size determination

The calculated minimum sample size was 138. It was calculated using the Dobson's formula as:
$N=Z^2p\ (1-p)/d^2$, where N = minimum number of samples, Z = test statistic at 95% confidence interval, P = proportion of the population with desired characteristic=10%, d=standard error=0.05. Hence calculated N= (1.96)2*0.1*0.9 / (0.052) =138.2976

2.5 Sample laboratory assays

The samples were brought to room temperature once and analyzed using the Diagnostic Automation Myeloperoxidase (MPO) ELISA test system (Diagnostic® Automation Incorporated, California, USA). The assay kit comes with a plate, conjugate, controls, calibrator, diluent, wash buffer, stop solution and the substrate (3, 3', 5, 5'-tetramethylbenzidine) [23]. Assay kit was brought to room temperature before the assay and calibrator was run in triplicate, for each assay run, reagent blank, negative and positive controls were also included in each assay as quality control measures. After a series of incubations and washings as prescribed by manufacturer, the plates were read using a micro well reader pre-set at 450nm. Optical density (OD) of each well was measured, and a calculation of the index value or OD ratio was made and the index values or OD ratios were converted to unit values for quantitative interpretations [23].

2.6 Principle of the method

The Diagnostic Automation Myeloperoxidase (MPO) p-ANCA ELISA test system is designed to detect IgG class antibodies to myeloperoxidase in human sera. Walls of plastic micro-well strips are sensitized by passive absorption with myeloperoxidase antigen [23]. The test procedure involves three incubation steps as described below.

2.6.1 Incubation step 1

Test sera (properly diluted) are incubated in antigen coated micro-wells. Any antigen specific antibody in the sample will bind to the immobilized antigen. The plate is washed to remove unbound antibody and other serum components.

2.6.1 Incubation step 2

Peroxidase conjugated goat anti-human IgG (γ chain specific) is added to the wells and the plate is incubated. The conjugate will react with IgG antibody immobilized on the solid phase in step 1. The wells are washed to remove unreacted conjugate.

2.6.3 Incubation step 3

The micro-wells containing immobilized peroxidase conjugate are incubated with peroxidase substrate solution. Hydrolysis of the substrate by peroxidase produces a color change. After a period of time the reaction is stopped and the color intensity of the solution is measured photometrically. The color intensity of the solution depends upon the antibody concentration in the original test sample.

2.7 Interpretation of results

2.7.1 Calculation of index values or Optical density (OD ratios) [23]

Index value/ OD ratio=OD of unknown sample/cut-off OD, where, cut-off OD= Correction factor*mean optical density of calibrator (Correction factor=1) [23]

2.7.2 Conversion of index values/OD ratios to unit values (AAU/l)

Anti-myeloperoxidase (AAU/L) = OD ratio*155, where 155 is the positive calibrator unit value [23].

2.7.3 Interpretation of index values/OD ratios

Table 1 Interpretation of Index values or OD ratios and AAU/L [23]

	Index Value or OD Ratio	**AAU/L**	**AAU/L**
Negative specimens	≤0.90	≤0.90 x 155	=139.5
Equivocal specimens	0.91 to 1.09	0.91x155 to 1.09x155	=141.05 to 168.95
Positive specimens	≥1.10	1.10x155	=170.5

We therefore adopted the cut off values for quantitative results as ≥170.5 AAU/L = elevated, and <170.5 AAU/L = normal.

III. RESULTS

A total of 215 adults on HIV monitoring and/or treatment were enrolled into the study, 50 (23.3%) were males, 185 (85.0%) were on different antiretroviral (ART) combinations as described earlier [24]. Table 1 shows the age and sex distribution of participants; more females were in the 30 to 39 year age group (n=64, 38.8%) compared to other groups. More males were aged 40 to 49 years (n=23, 46%). Of the 215 patients tested, 196 had normal and 19 (8.8%) had elevated anti-myeloperoxidase antibody levels. The patient results for anti-myeloperoxidase antibody levels were then categorized as normal or elevated and the two groups compared

for age, body mass index (BMI), total cholesterol (TC), total cholesterol/ high density lipoprotein (TC/HDL) ratio, high density lipoprotein (HDL) and random blood glucose (RBG) (Table 2).

Table 1: Age and sex distribution of HIV infected patients in the study

Age (years)	Males		Females	
	Number	(%)	Number	(%)
<30	6	12	25	15.2
30 – 39	10	20	64	38.8
40 – 49	23	46	49	29.7
50 – 59	8	16	20	12.1
60 – 69	3	6	7	4.2
Total	50	100	165	100

Table 2: Comparisons of categories of anti-myeloperoxidase antibody levels with age, BMI, TC, TC/HDL ratio, HDL and RBG

Categories of anti myeloperoxidase antibody levels		Age (years)	BMI (kg/m2)	TC (mmol/l)	TC/HDL ratio	HDL (mmol/l)	RBG (mmol/l)
Normal	Mean	40.2	25.5	4.6	3.9	1.3	5.3
	N	196	196	196	196	196	196
	SD	10.2	13.2	1.2	1.3	0.4	0.8
Elevated	Mean	41.2	23.6	4.3	4.7	1.0	5.1
	N	19	19	19	19	19	19
	SD	10.0	4.4	1.2	1.6	0.4	0.8
	P values	0.7040	0.742	0.201	0.003	0.047	0.471
Total	Mean	40.3	25.4	4.6	4.0	1.3	5.3
	N	215	215	215	215	215	215
	SD	10.1	12.7	1.2	1.3	0.4	0.8

There were no statistical differences in the distribution of age, body mass index, total cholesterol, random blood glucose and hypertension treatment ($p>0.05$) across both categories of anti-myeloperoxidase antibody levels (Table 2). However there were statistical differences in the distribution of high density lipoprotein and total cholesterol/high density lipoprotein ratio between those who had normal and elevated anti-myeloperoxidase antibody levels, respectively ($p<0.05$).

When stratified by antiretroviral drug combinations mean levels of anti-myeloperoxidase antibody were highest for patients on zidovudine/stavudine, nevirapine and lamivudine combinations and lowest for those on protease inhibitor-based second line drugs (Table 3). There was no difference in mean anti-myeloperoxidase antibody levels between groups of patients defined by their antiretroviral drug experience (naives, on different first line combinations and on PI-based second line, respectively (Table 3)

Table 3: Summary of anti-myeloperoxidase antibody levels by antiretroviral combinations

Antiretroviral drug combination	Mean	Standard deviation	Frequency/ %	p value
1. Antiretroviral drug-naïve	89.508125	46.617847	32 (14.9)	
2. TDF/NVP/3TC	107.018	89.090743	130 (60.5)	*γ=0.2843
3. TDF/EFV/3TC	106.40478	56.064327	23 (10.7)	*η=0.9743
4. ZDV or STV/NVP/3TC	117.59769	134.2021	13 (6.0)	*δ=0.7267
5. PI+	72.234118	40.166373	17 (7.9)	*φ=0.1957
Total	102.23563	81.724297	215 (100)	*ε=0.000

Key: TDF=Tenofovir, NVP=Nevirapine, 3TC=Lamivudine, EFV=Efavirenz, STV=Stavudine, ZDV=Zidovudine, PI+=PI Based Second Line, *ε=p value for Bartlett's chi2 (4) test for equal variances, *t tests for comparison between means of two groups i.e.*γ for groups 1 and 2, *η for groups 2 and 3, *δ for groups 3 and 4, *φ for groups 4 and 5

The t test comparison of means was also used to determine if there was significant difference in the mean anti-myeloperoxidase levels of antiretroviral-experienced and antiretroviral-naïve participants and in patient groups stratified by BP treatment, and smoking history; but there was no significant difference ($p>0.05$).

IV. DISCUSSION AND CONCLUSION

An earlier study done to investigate effects of the presence of autoantibodies to myeloperoxidase on the oxidative capacity of myeloperoxidase, showed positive correlation between myeloperoxidase activity and antibody titer, providing evidence that high titer of anti-myeloperoxidase antibodies depicts an increase in oxidative activity of myeloperoxidase [11]. This may be proof that presence of high titers of anti-myeloperoxidase, and hence myeloperoxidase, through various mechanisms has a net effect of increased inflammation, endothelial dysfunction and mural damage of blood vessels [25]. Based on the above assertions we conclude that there is a low prevalence of high coronary heart disease risk in the population of HIV positive patients that we studied, 19 subjects (8.8%) had elevated levels of the antibody and 196 (91.2%) had normal levels. Further, mean anti-myeloperoxidase antibody level (102.2 $\pm$ 81.7 AAU/L) was not elevated above the normal range.

As a way of assessing the relevancy of the test in predicting risk of developing cardiovascular diseases, a comparison was made between the anti-myeloperoxidase levels and traditional risk factors for cardiovascular disease. In this study, anti-myeloperoxidase levels were compared by body mass index (BMI).There was no statistical significant difference in the distribution of BMI across both categories of anti-myeloperoxidase levels ($p=0.742$). In the group with normal anti-myeloperoxidase levels mean BMI was 25.5kg/m^2, which falls within normal range. For participants with elevated levels of anti-myeloperoxidase, the mean BMI was 23.6kg/m^2.

In our study, of HIV patients, no statistical significance was demonstrated ($p=0.201$) between anti-myeloperoxidase levels and total cholesterol (TC) levels. However there was statistical significance ($p=0.047$) in the distribution of total cholesterol/high density lipoprotein ratio between the two anti-myeloperoxidase categories. The group with normal mean anti-myeloperoxidase had slightly elevated mean total cholesterol/high density lipoprotein ratio (4.6), whilst those with elevated anti-myeloperoxidase titers had normal mean total cholesterol/high density lipoprotein ratio (4.4). There was no statistical significance ($p=0.471$) in the distribution of random blood glucose across both categories of anti-myeloperoxidase levels and average random blood glucose level for both groups were within the normal ranges, as defined by World Health Organization (WHO) [26]. Hypertension and smoking are risk factors for cardiovascular disease [27], in our study, there was no difference in the mean levels of anti-myeloperoxidase antibody for those on hypertension treatment versus those who were not, $p=0.442$. A similar finding was observed in those who had a history of smoking versus non-smokers ($p=0.500$).

Antiretroviral therapy is thought to cause cardiometabolic side-effects, with a net effect of increasing the risk of cardiovascular disease [28]. However in this current study, there was no statistically significant difference in the mean anti-myeloperoxidase levels between antiretroviral therapy-experienced and antiretroviral therapy-naïve, $p=0.374$. There was, however, statistically significant difference in the levels of anti-myeloperoxidase by antiretroviral combinations ($p<0.001$), the greater proportion of those with elevated anti-myeloperoxidase being on triple combination of tenofovir/nevirapine/lamivudine (70.6%).

4.2 Conclusion

Mean anti-myeloperoxidase antibody titers of the 215 participants who attended HIV clinic were not elevated. Only 19 out of the 215 (8.8%) had elevated levels, showing a low prevalence of coronary heart disease risk, which is in agreement with results already reported, in which traditional methods, such as: lipids and Framingham risk scores were used [23], [29]. The results of our study, demonstrated no significant relationship between anti-myeloperoxidase levels and most traditional risk factors for cardiovascular disease, except HDL and TC/HDL ratio.

4.3 Limitations

The anti-myeloperoxidase p-ANCA antibody enzyme linked immunosorbent assay used here is said to be non-specific for disease and hence not diagnostic. Secondly, serum levels of anti-myeloperoxidase antibody and lipoproteins were determined in a single non-fasting sample that was obtained at a non-uniform time of the day [15]. Diurnal variation, variation over time such as: temporarily increased levels due to infection and differences in the time span from the last meal, could have affected our results [15], increasing error. In this respect, measurement of specific myeloperoxidase products, such as chlorotyrosine in HDL, may provide a better indication of myeloperoxidase activity and myeloperoxidase -induced damage. Furthermore, it has recently been suggested that myeloperoxidase levels after heparin administration are a better reflection of subendothelial myeloperoxidase. However, these analyses could not be performed in the present study [15]. Furthermore, our study attempted to use the anti-myeloperoxidase p-ANCA antibody test. Could this method be useful in our setting as a proxy for myeloperoxidase activity? The results from this study are promising and more carefully designed studies should be done in our setting, to help validate the test for anti-myeloperoxidase and set reference values for interpreting results. Longitudinal studies should also be carried out to ascertain changes in anti-myeloperoxidase antibody levels, and hence correlate the changes to any changes in traditional

risk factors over time. This may help explain the link and hence pathophysiology of coronary heart disease with respect to anti-myeloperoxidase pANCA antibodies and myeloperoxidase. Further studies may help answer whether anti-myeloperoxidase antibodies have clinical and diagnostic relevance in our setting.

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