

# E-Selectin and markers of HIV disease severity, inflammation and coagulation in HIV-infected treatment-naïve individuals

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## Abstract:

**Background:** E-selectin has been shown to play a role in atherosclerosis and to be increased in HIV-infected individuals due to chronic immune activation. There is a paucity of studies on E-selectin in HIV-infected treatment-naïve individuals.

**Objectives:** This study aimed to determine whether E-selectin levels were increased in HIV-infected treatment-naïve individuals and whether these correlated with markers of disease severity, inflammation and coagulation to determine if this population is at risk for cardiovascular disease (CVD).

**Methods:** E-selectin levels were determined in 114 HIV-infected treatment-naïve and 66 HIV-negative individuals, compared between groups and correlated with markers of disease severity, inflammation and coagulation.

**Results:** There were statistically significant differences ( $p < 0.01$ ) in levels of WCC, CD4<sup>+</sup> count, %CD38/8, albumin, IgG, hsCRP and D-dimer between groups and no statistically significant differences in E-selectin ( $p = 0.84$ ) and fibrinogen ( $p = 0.65$ ) levels. E-selectin correlated with age ( $p = 0.02$ ) and gender ( $p = 0.01$ ).

**Conclusion:** E-selectin was a poor marker in this setting. There was no correlation with any of the markers of disease severity, inflammation and coagulation. E-selectin is most likely raised in an acute inflammatory setting, rather than chronic stage of HIV-infection. We recommend that other markers be utilized to identify patients at increased risk of CVD; as these were significantly increased untreated in individuals.

**Keywords:** E-selectin, inflammation and coagulation in HIV-infected treatment-naïve individuals.

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## Introduction

More than 30 years of intensive research has resulted in infection with Human Immunodeficiency virus (HIV) changing from a death sentence to a manageable disease.<sup>1</sup> HIV infection is characterised by ongoing immune activation and exhaustion of the T-cell pool which results in immunosenescence and progression to Acquired Immunodeficiency Syndrome (AIDS).<sup>2</sup> Chronic immune activation leads to the accelerated development of age-related disorders such as atherosclerosis and cardiovascular disease (CVD).<sup>3-6</sup> Atherosclerosis is a chronic process involv-

ing atherosclerotic plaque formation in the arterial wall.<sup>4</sup> Endothelial cell dysfunction is regarded as an early step in atherosclerotic plaque formation, where the defective endothelium interacts with pro-inflammatory stimuli such as oxidised low density lipoprotein and results in endothelial activation with expression of adhesion molecules.<sup>4</sup> The persistent immune activation associated with HIV-infection has been associated with an increased risk of CVD and this persists even after initiation of treatment.<sup>3,7</sup> HIV elite controllers who maintain viral suppression without treatment, have increased immune activation and associated CVD.<sup>8</sup> The drivers of this immune activation and subsequent atherosclerosis are thought to be mainly due to HIV-specific proteins, co-infections and gut translocation.<sup>9</sup> HIV-specific proteins may activate plaque macrophages and promote platelet adhesion, thereby accelerating disease and plaque rupture. An increased prevalence of plaques has been found in asymptomatic HIV-infection and in fact CVD is currently the third leading cause of death in HIV-infection.<sup>10</sup>

E-selectin is an adhesion molecule found on the surface of activated endothelial cells during periods of stress or inflammation.<sup>3,11,12</sup> E-selectin is expressed in response to pro-inflammatory cytokines such as tumour necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1.<sup>13,14</sup> It recruits leukocytes to the endothelium by binding ligands on their cell surfaces and facilitating their rolling on endothelial cells. This slows down the leukocytes and facilitates their eventual entry into the sub-endothelial space.<sup>11,12</sup>

Inflammation caused by HIV infection activates lymphoid B-cells to produce antibodies. The antibodies IgM and IgG are able to control viral replication, but not reduce it.<sup>15</sup> This causes continuous immune activation with ultimate B-cell dysfunction and polyclonal hypergammaglobulinaemia.<sup>16</sup> Studies have shown that increased IgG levels are associated with aggravated atherosclerosis and HIV disease progression.<sup>17,18</sup>

HIV disease severity is determined by the viral load and the CD4<sup>+</sup> count.<sup>2</sup> CD38 is a glycoprotein found on the surface of immune cells such as CD4<sup>+</sup> and CD8<sup>+</sup>-T lym-

phocytes, and is considered a marker of immune cell activation.<sup>2</sup> Studies have found that higher levels of activated T-cells expressed by levels of CD38 on CD8<sup>+</sup>-T-cells predict an adverse prognosis<sup>19</sup> and disease progression, regardless of the CD4<sup>+</sup> count in HIV infection.<sup>20</sup> Furthermore, the expression of CD38 correlates strongly with the viral load, suggesting that CD38/8 could be used as a marker of viral replication.<sup>20</sup>

Studies have shown that non-specific markers of inflammation such as albumin and high sensitivity C-reactive protein (hsCRP) are altered in HIV infection.<sup>21</sup> Serum albumin is a negative acute phase reactant and has been found to be a strong predictor of mortality<sup>22</sup> and progression to AIDS.<sup>22,23</sup> Increased catabolism of albumin due to inflammation, worsening of nutritional status and degradation in the liver due to chronic infection have been reported as causes for reduced levels.<sup>21,23,24</sup> HsCRP, an acute phase reactant protein produced by the liver in response to IL-6 indicates low grade inflammation and is thus useful in the assessment of cardiovascular risk.<sup>25</sup>

A hypercoagulable state also seems to exist in HIV infection.<sup>6,26</sup> Fibrinogen, an acute phase reactant, interacts with platelets resulting in their activation and fibrin formation. An increased fibrinogen level is associated with a 7-fold increase in all-cause mortality.<sup>27</sup> The degradation of fibrin generates soluble fibrin fragments such as D-dimer. Elevated levels of D-dimer are seen in all haematological disorders that cause activation of coagulation such as acute venous thromboembolism and CVD.<sup>28</sup> Elevated D-dimer levels have been shown to be a strong independent predictor of mortality and cardiovascular risk in HIV infection.<sup>28-30</sup>

Previous studies examining levels of E-selectin in HIV infection have been controversial.<sup>31-37</sup> The aim of this study was to determine whether E-selectin levels would be increased in a young HIV-infected treatment-naïve study population and whether these would correlate with markers of disease severity, inflammation and coagulation to determine if this study population is at increased risk for CVD.

## Methods

### Study setting and population

This was a cross-sectional study of 114 HIV-infected antiretroviral therapy (ART)-naïve and 66 HIV-negative individuals. All participants were recruited from an HIV counselling and testing (HCT) clinic in Emavundleni Crossroads, Cape Town. This clinic forms part of the Institute for Infectious Disease, Desmond Tutu HIV centre, University of Cape Town (UCT) and employs the national HCT algorithm with accredited rapid HIV tests.

Clinical information was recorded on a standard form by the investigator during the interview. Data collected included: (i) demographic details: age, gender and ethnic group; (ii) medical history: HIV status, date of HIV diagnosis and most recent CD4<sup>+</sup> count if available; (iii) medication use including vitamins and herbal preparations.

All participants older than 21 years of age, clinically healthy/asymptomatic, and ART-naïve were included. Participants with concurrent infections, tuberculosis (TB), on anti-TB or other antibiotic therapy and who were pregnant were excluded from the study.

### Laboratory measurements

Participants were initially screened for HIV using the HIV-1/2 Ag/Ab combo by Alere™. The confirmatory test was the Uni-gold™ Recombigen® HIV.

E-selectin was determined in duplicate on serum samples stored at -70°C using the E-selectin human ELISA kit (abcam®, Cambridge, UK). According to the manufacturer's specifications, samples are stable when stored at this temperature. The limit of detection is < 0.5 ng/mL and the average E-selectin concentration for this assay as determined by the manufacturers on 80 normal subjects was found to be 51.99 ± 26.65 ng/mL (range 11.78 – 160.72 ng/mL). A lyophilised internal quality control of known concentration was used to determine the coefficient of variation (CV).

The intra-assay CV was 8.9% and the inter-assay CV was 10.5%.

The white cell count (WCC) was analysed using fresh EDTA whole blood on the Siemens ADVIA 2120 (Berlin and Munich, Germany) haematology analyser.

The CD4<sup>+</sup> count and %CD 38/8 were determined by means of flow cytometry using fresh EDTA whole blood on the Becton Dickinson (BD) MultiTest CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC and CD8-FITC/CD38-PE/CD3-PerCP for CD4<sup>+</sup> and CD38<sup>+</sup> count respectively together with TruCOUNT tubes (BD Biosciences, San Jose, California) and analysed by BD FACS-Calibur.

The ACL TOP (Beckman Coulter Inc., Fullerton, CA) instrument together with the fibrinogen-C and D-dimer HS 500 reagent kit by HemosIL® (Bombay, United States) was used to determine fibrinogen and D-dimer concentrations respectively.

The IMMAGE analyser (Beckman Coulter Inc., Fullerton, CA) was used to determine hsCRP concentrations.

Serum IgG and albumin levels were determined using the Siemens ADVIA®1800 (Berlin and Munich, Germany) chemistry analyser.

All laboratory tests were performed at enrolment and serum was stored at -70°C for E-selectin determination. The E-selectin assay was performed by the same technologist.

### Statistical analysis

Data was analysed using STATISTICA v10.0 (Statsoft Inc.) and Microsoft® Excel® (Microsoft, Seattle, WA, USA). Descriptive statistics were used to analyse each parameter in terms of distribution: minimum, maximum, median, mean, standard deviation (SD) and statistical significance. Pearson correlation coefficient was performed for normally distributed data. The Spearman Rank correlation was performed for non-parametric data. Analysis of variance (ANOVA) was used to compare two or more groups of data that was normally distributed. A p-value ≤0.05 was considered statistically significant.

## Results

The two groups were well-matched for age with a mean age of  $32 \pm 7.7$  years for the HIV-infected group and  $29 \pm 8.3$  years for the control group. Furthermore, all the participants were recruited from the same HCT clinic and thus were of the same ethnicity and from the same socio-economic background.

Table 1 shows the mean  $\pm$  SD for the various analytes tested. We found that the albumin, WCC and CD4<sup>+</sup> count in the HIV-infected group were significantly lower compared to the control group. The % CD38/8, hsCRP and D-dimer levels were significantly higher in the HIV-infected group compared to the control group. Fibrinogen and E-selectin levels however exhibited no significant differences between the two groups.

**Table 1: Analyte levels (means  $\pm$  SD) in the HIV-infected and HIV-negative participants**

| Analytes                             | Reference Interval | HIV-infected (mean $\pm$ SD) | HIV negative (mean $\pm$ SD) | P- value |
|--------------------------------------|--------------------|------------------------------|------------------------------|----------|
| E-selectin (ng/mL)                   | 11.78-160.72       | 135.9 $\pm$ 60.7             | 137.0 $\pm$ 63               | 0.84     |
| WCC ( $\times 10^9$ cells/L)         | 4.0-10.0           | 4.92 $\pm$ 1.67              | 6.08 $\pm$ 1.96              | <0.01*   |
| CD4+count (cells/mm <sup>3</sup> )   | 500-1200           | 394.8 $\pm$ 216.4            | 823.9 $\pm$ 255.8            | <0.01*   |
| %CD38/8                              | 0.93-7.03          | 30.9 $\pm$ 19.6              | 13.2 $\pm$ 11.8              | <0.01*   |
| Albumin (g/L)                        | 35-52              | 40 $\pm$ 3.7                 | 44 $\pm$ 3.4                 | <0.01*   |
| IgG (g/L)                            | 7-16               | 28.4 $\pm$ 11.1              | 16.9 $\pm$ 4.3               | <0.01*   |
| Fibrinogen (g/L)                     | 2-4                | 2.81 $\pm$ 0.61              | 2.85 $\pm$ 0.66              | 0.65     |
| D-dimer (mg/L)                       | 0.0-0.25           | 0.32 $\pm$ 0.21              | 0.22 $\pm$ 0.09              | <0.01*   |
| Log <sub>10</sub> hsCRP (mg/L)       | -                  | 0.56 $\pm$ 0.58              | 0.29 $\pm$ 0.56              | <0.01*   |
| hsCRP (mg/L)                         | 0.0-7.5            | 8.15 $\pm$ 11.32             | 4.08 $\pm$ 5.33              | <0.01*   |
| Log <sub>10</sub> E-selectin (ng/mL) | -                  | 2.09 $\pm$ 0.2               | 2.1 $\pm$ 0.18               | 0.84     |

\*indicate statistical significance (p < 0.05)

E-selectin levels were correlated with all the other analytes tested in both groups. There was a significant correlation between E-selectin and albumin in the HIV-neg-

ative group and a near significant correlation with the WCC in the HIV-positive group. Furthermore, E-selectin did not correlate significantly with any of the other analytes in both groups (Table 2)

**Table 2: Correlation between E-selectin and other analytes in both groups (Spearman r and p values)**

| <b>Analytes compared to E-selectin</b> | <b>r- value for HIV-infected</b> | <b>p-value for HIV-infected</b> | <b>r-value for HIV-negative</b> | <b>p-value for HIV -negative</b> |
|----------------------------------------|----------------------------------|---------------------------------|---------------------------------|----------------------------------|
| CD4+count                              | -0.14                            | 0.13                            | 0.03                            | 0.83                             |
| %CD38/8                                | -0.02                            | 0.88                            | -0.04                           | 0.81                             |
| Albumin                                | 0.04                             | 0.66                            | 0.25                            | 0.05*                            |
| IgG                                    | -0.04                            | 0.67                            | -0.15                           | 0.22                             |
| hsCRP                                  | 0.07                             | 0.44                            | 0.07                            | 0.60                             |
| D-dimer                                | -0.00                            | 0.99                            | 0.06                            | 0.62                             |
| Fibrinogen                             | 0.15                             | 0.16                            | 0.03                            | 0.79                             |
| WCC                                    | 0.18                             | 0.07                            | -0.05                           | 0.69                             |

\*indicate statistical significance (p < 0.05)

After finding a near significance between E-selectin and the WCC in the HIV-infected group, further correlations were performed with E-selectin and the differential

count. We found that all the parameters in the differential count contributed to the near significance of the WCC. However, we concluded that the neutrophils contributed the largest portion, due to the significant positive correlation (r = 0.83) observed (Table 3).

**Table 3: Correlation between E-selectin and differential count parameters in the HIV-infected group**

| <b>Differential count parameters.</b> | <b>HIV-infected</b> |                | <b>HIV-negative</b> |                |
|---------------------------------------|---------------------|----------------|---------------------|----------------|
|                                       | <b>r-value</b>      | <b>p-value</b> | <b>r-value</b>      | <b>p-value</b> |
| Neutrophils                           | 0.83*               | <0.01          | 0.90*               | <0.01          |
| Lymphocytes                           | 0.44                | <0.01          | 0.51                | <0.01          |
| Monocytes                             | 0.59                | <0.01          | 0.67                | <0.01          |
| Eosinophils                           | 0.29                | <0.01          | 0.09                | 0.47           |
| Basophils                             | 0.48                | <0.01          | 0.37                | <0.01          |

## Discussion

Chronic inflammation in HIV infection is an important cause of disease progression and is associated with an increased risk of inflammation-associated conditions such as CVD and cancer.<sup>3,29</sup> The purpose of this study was to determine E-selectin levels in ART-naïve HIV-infected ART-naïve individuals, compare these to E-selectin levels in controls and correlate them with markers of HIV disease severity, inflammation and coagulation. We hypothesized that E-selectin and other markers of CVD would be increased in our untreated HIV-infected group due to heightened levels of inflammation.

Various parameters of inflammation and immune activation, such as D-dimer and hsCRP have been studied previously and shown to be independent risk factors for the development of heart disease.<sup>38,39</sup>

Previous studies on local HIV populations both treatment-naïve and on treatment have found abnormal serum protein electrophoresis patterns suggestive of polyclonal hypergammaglobulinaemia and increased IgG.<sup>40,41</sup>

Previous studies have found that ART is unable to repair the immune damage caused by HIV infection, thus recommendations for earlier initiation of ART have resulted.<sup>42</sup> In resource-limited settings the roll-out of ART remains a challenge<sup>43</sup> and therefore, it becomes important to identify individuals who may be at increased risk of these conditions.

The underlying pathophysiology of CVD is atherosclerosis, which leads to endothelial dysfunction.<sup>44</sup> E-selectin, preferentially found on endothelial cells is a marker of endothelial dysfunction.<sup>45</sup> Furthermore, cytokines released during the inflammatory process activate endothelial cells, resulting in the up-regulated expression of E-selectin.<sup>11</sup>

Previous studies examining the effects of HIV infection on E-selectin levels have been controversial.<sup>31-37</sup> Some studies have shown that HIV TAT-protein directly upregulates E-selectin.<sup>31</sup> Kristoffersen et al found no difference in the E-selectin level before and after ART initiation; however they found that ART significantly increased cholesterol, triglyceride and LDL-cholesterol levels, which are major contributors to atherosclerotic plaque formation.<sup>33</sup>

Rönsholt et al found that E-selectin levels were decreased by ART but never normalised. Furthermore, none of the parameters tested, including E-selectin, correlated with the CD4<sup>+</sup> count or viral load.<sup>34</sup> Two studies by Graham et al found E-selectin levels to be increased during the acute phase of HIV infection but not during the chronic phase,<sup>35,36</sup> suggesting that E-selectin is a positive acute phase reactant. This was an important finding and could explain the lack of increased E-selectin in our study, as our cohort were all in the chronic stage of the infection.

Calza et al found that E-selectin levels were significantly increased in the HIV-infected ART-naïve group compared to the HIV infected group on ART; these were also higher than the HIV negative control group. Furthermore, they found that E-selectin levels correlated with an elevated viral load and decreased CD4<sup>+</sup> count. However, the HIV positive treatment-naïve group were older (median age 40 years) than our study group and consisted of 5/50 females compared to the HIV negative control group with a median of 32 years consisting of 13/51 females. Our study had a predominance of younger females in both groups and we showed that age and gender did impact on the results. The Calza study gave no indication that statistical analysis was corrected for age and gender.<sup>37</sup>

Our HIV-infected group, although clinically asymptomatic, all presented in the chronic stage of infection which was evident in the decreased CD4<sup>+</sup> count; with a mean of  $394.8 \pm 216.4$  cells/mm<sup>3</sup> compared to  $832.9 \pm 255.8$  cells/mm<sup>3</sup> in the control group ( $p < 0.01$ ). In our study we found both the ART-naïve HIV infected group ( $135.9 \pm 60.7$  ng/mL) and the control ( $137.6 \pm 63.0$  ng/mL) group's E-selectin to be in the upper limit of normal reference range, suggesting that our cohort may have underlying chronic inflammation regardless of HIV infection. A review on the immune system of African children found that malnutrition and micronutrient deficiency from infancy may cause irreversible damage to the immune system leading to compromised immune surveillance from early childhood.<sup>43</sup> This predisposes individuals to opportunistic infections which promote an altered immunity, early senescence of the immune system and a depleted naïve CD4<sup>+</sup> T-cell pool. We hypothesize that since our study population is from a low socio-economic setting where children often do not receive the adequate nutrition needed for

sustained growth and strong immunity, this may be the source of underlying chronic inflammation.

Analysis of markers tested for inflammation found that hsCRP was significantly higher ( $p=0.01$ ) in the HIV-infected group compared to the control group, confirming the presence of increased inflammation in our HIV-infected group. Albumin levels were significantly lower ( $p<0.01$ ) in the HIV-infected group compared to the controls further supporting the presence of ongoing inflammation. In addition, hsCRP showed a significant inverse correlation with albumin ( $r= -0.32$ ,  $p<0.01$ ) highlighting the value of these markers in this setting. IgG was found to be significantly increased ( $p<0.01$ ) in the HIV-infected group compared to the controls, suggesting the presence of marked B-cell activation and possibly indicating the presence of ongoing bacterial translocation due to a “leaky gut”.<sup>29</sup> The % CD38/8<sup>+</sup> T-cells was significantly higher ( $p<0.01$ ) in the HIV-infected group compared to the controls, confirming significant cellular activation is taking place due to the viral load and ongoing immune stimulation.

Markers of coagulation tested indicated no difference ( $p=0.65$ ) in the fibrinogen levels of the HIV-infected group. Fibrinogen is an acute phase reactant and therefore may not be raised in a more chronic stage of infection. Importantly, the D-dimer was significantly higher ( $p<0.01$ ) in the HIV-infected group. The SMART study found that D-dimer was an independent risk factor for all-cause mortality<sup>30</sup>, suggesting that our study group is at increased risk. Since the Strategies for Management of Antiretroviral Therapy (SMART) study, various other studies have examined the association between HIV infection, anti-retroviral therapy ART and D-dimer with all-cause mortality.<sup>27,46,47</sup>

In addition, D-dimer levels in our study showed inverse relations with the CD4<sup>+</sup> count and albumin ( $r= -0.35$  and  $r= -0.41$  respectively, both  $p<0.01$ ) and positive correlations with the viral load, hsCRP and IgG ( $r=0.34$ ,  $0.27$  and  $0.41$  respectively, all  $p=<0.01$ ), thus the D-dimer would be a valuable marker of immune activation and possible risk for thrombosis in this setting.

E-selectin levels showed significant positive correlation ( $r=0.18$ ,  $p=0.02$ ) with age, which supports the age-relat-

ed increase in E-selectin levels. This can possibly be explained by the oxidative stress theory of age: that older individuals experience increased oxidative stress on tissues, such as endothelial cells causing the up-regulation of adhesion molecules such as E-selectin.<sup>48,49</sup> E-selectin levels were found to be significantly higher in males ( $p=0.01$ ). However, after correcting E-selectin for gender, no difference was found for these levels in the HIV-infected group compared to the controls, and males still had higher E-selectin levels regardless of their HIV status. Both the significance of age and gender has been demonstrated in various other studies.<sup>50-52</sup> After the age of forty, the risk of CVD is increased by up to 49% in males and 32% in females, with males being 3.4 times more susceptible due to their naturally higher expression of E-selectin.<sup>50</sup> E-selectin levels were correlated with all the markers of disease severity, inflammation and coagulation in our entire cohort and we found no correlation with any of the parameters tested. We also correlated E-selectin with all the parameters in the HIV-infected group and controls respectively and found that E-selectin correlated significantly ( $p=0.05$ ) with albumin in the control group and neared significance with the WCC( $p=0.07$ ) in the HIV infected group.

The WCC was significantly decreased in the HIV-infected group which may reflect both a decreased CD4<sup>+</sup> count and decreased neutrophil count. We therefore further correlated the WCC with the differential count subsets to determine which leukocyte contributed to the correlation. Interestingly, the WCC correlated most strongly to the neutrophil count. This may reflect the increased trafficking and consumption of neutrophils at sites of infection or inflammation, effectively removing them from the circulation.

## Conclusion

Our study had a few limitations. We had a small study population and did not have any participants who were on ART to compare to our study population. Also, we only determined E-selectin and did not determine other emerging cardiovascular risk markers. Although HIV-infection leads to increased CVD and we have previously determined that our cohort indeed has such a risk, we found no significant difference in E-selectin levels between HIV-infected and non-infected individuals. We concluded that E-selectin is not a valuable marker during

the chronic stage of HIV infection and recommend that tests such as D-dimer, hsCRP, albumin and IgG be utilized to identify individuals who may be at increased risk of disease progression or CVD in HIV infection. The cost-effectiveness of a combination of these markers will need to be determined in resource-limited settings.

Future studies that take into account the time of seroconversion may find E-selectin to be a better marker of early, acute inflammation and follow-up studies, monitoring the development of CVD or atherosclerosis plaque development in this cohort would be of value.

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### Conflict of interest

None.

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