Levels of Platelet Activating Factor and its metabolic enzymes in HIV-infected, naïve male patients

PAF and HIV-infected naïve patients

V.D. Papakonstantinou Dr¹, M. Chini MD², G.M. Stamatakis Dr¹, N. Mangafas MD², N. Tsogas MD², E. Fragopoulou Dr³, P. Gargalianos MD⁴, S. Antonopoulou Dr³, C.A Demopoulos Dr¹, M-C Lazanas MD²

¹Faculty of Chemistry, National & Kapodistrian University of Athens, Panepistimioupolis Zografou, 15771 Athens, Greece.
²3rd Internal Medicine Department-Infectious Diseases Unit, Red Cross General Hospital, Athens, Greece.
³Department of Nutrition-Dietetics, Harokopio University, Athens, Greece.
⁴1st Internal Medicine Department-Infectious Diseases Unit, “G. Gennimatas” Hospital, Athens, Greece.

* Correspondence author: Papakonstantinou D. Vasiliki. Faculty of Chemistry, National & Kapodistrian University of Athens, Panepistimioupolis Zografou, 15771 Athens, Greece.

papakonstantinou.v@gmail.com / 210.727.4470.
Abstract

HIV infection is characterized by immune activation and inflammation and HIV patients have a great risk to develop several morbidities such as cardiovascular disease.

Eight naïve, male, asymptomatic HIV-infected patients that did not meet the criteria for antiretroviral therapy initiation were assigned to the study for 12 months and Platelet Activating Factor (PAF) levels and its key metabolic enzymes were determined.

The results display stable PAF levels and enzymes’ activity during the study. These data indicate a stable inflammatory profile and come in accordance with the guidelines regarding the lack of need for antiretroviral therapy to these patients.

Monitoring PAF levels and metabolic enzymes, in parallel with the use of PAF inhibitors, could probably improve the quality of life in HIV-infected naïve patients by preventing the inflammatory manifestations of HIV infection.

Key-Words: PAF, PAF-CPT, lyso-PAF-AT, PAF-AH, Lp-PLA2, Inflammation, Non-AIDS morbidities, naïve, HIV infection
Περίληψη

Η HIV λοίμωξη χαρακτηρίζεται από ανοσολογική ενεργοποίηση και φλεγμονή. Οι ασθενείς έχουν μεγαλύτερες πιθανότητες από το μέσο πληθυσμό να αναπτύξουν μια πληθώρα παθολογικών καταστάσεων, αναφερόμενες ως μη συσχετιζόμενες με το AIDS εκδηλώσεις της HIV λοίμωξης, όπως η καρδιαγγειακή νόσος.

Οκτώ μη προθεραπευμένοι (naïve), άρρενες, ασυμπτωματικοί HIV+ ασθενείς που δεν ικανοποιούσαν τα κριτήρια για την έναρξη αντιρετροϊκής αγωγής, συμμετείχαν στη μελέτη και τα επίπεδα του Παράγοντα Ενεργοποίησης Αιμοπεταλίων (PAF) όπως και τα βασικά μεταβολικά του ένζυμα προσδιορίστηκαν για διάστημα 12 μηνών.

Τα αποτελέσματα δείχνουν ότι τόσο τα επίπεδα του PAF όσο και η δραστικότητα των μεταβολικών του ενζύμων δεν διαφοροποιούνται στο διάστημα των 12 μηνών. Τα δεδομένα αυτά υποδεικνύουν ένα σταθερό φλεγμονώδες προφίλ και υποστηρίζουν τις διεθνείς οδηγίες για τη μη χορήγηση αντιρετροϊκής αγωγής στους ασθενείς αυτούς.

Η παρακολούθηση των επιπέδων του PAF και των μεταβολικών του ενζύμων, σε συνδυασμό με την χρήση αναστολέων του PAF θα μπορούσε να βελτιώσει την ποιότητα της ζωής των naïve ασθενών, εμποδίζοντας τις μακροπρόθεσμες φλεγμονώδεις εκδηλώσεις της HIV λοίμωξης.

Λέξεις κλειδιά: PAF, PAF-CPT, lyso-PAF-AT, PAF-AH, Lp-PLA2, φλεγμονή, μη συσχετιζόμενες με το AIDS εκδηλώσεις, naïve, HIV λοίμωξη
Introduction

Thirty years after the recognition of HIV, scientists are optimistic enough to discuss about the “end of AIDS”\(^1\). Despite this fact, HIV infection continues to be a major health problem with many victims in Greece\(^2\) just as the rest of the world\(^3\). The introduction of combination Antiretroviral Therapy (cART) has managed to reduce dramatically the viral load and raise the CD4 cells\(^4\). Despite all the beneficial effects of the antiretroviral therapy, HIV patients have a lower lifespan than the average population and the majority of them express a panel of pathological conditions called all together non-AIDS morbidities, with inflammation being their cornerstone\(^5\). Existing data support an association of the development of the non-AIDS morbidities with many factors such as the residual HIV replication, the microbial translocation, any kind of co-infections and the antiretroviral therapy\(^6\).

Cardiovascular disease, and particularly atherosclerosis, is one of the most prevalent among all non-AIDS morbidities\(^7,8\). The exact mechanism may not be clear yet, but studies showed the possible implication of various inflammatory agents\(^9\). The combination of the administered antiretroviral drugs plays a major role as it has been shown that different ART regimens have a pronounced effect either beneficial or harmful, based on various markers of inflammation, coagulation and immune activation\(^10,11\)\(^{\text{ENREF 9}}\). Furthermore, HIV patients usually have serum lipid abnormalities, the long-term effects of which on CVD in combination
with ART, remain unknown. As a result, several studies tried the administration of statins, in order to prevent CVD, with promising results.

One of the most potent factors of inflammation, with major role in atherosclerosis is Platelet Activating Factor (PAF). PAF is a glycerylether phospholipid identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine and a significant signaling molecule of the immune system, considered as a primitive and universal cellular mediator, having both physiological and pathological role. Considering PAF biosynthesis, there are two distinctive pathways namely de novo and remodeling, with key-enzymes being dithiothreitol-insensitive PAF-cholinephosphotransferase (PAF-CPT, EC 2.7.8.16) and lyso-PAF:acetyl-CoA acetyltransferase (lyso-PAF-AT, EC 2.3.1.67), respectively. PAF catabolism is moderated by PAF-specific acetylhydrolase (PAF-AH, EC 3.1.1.47), and its plasma isoform, Lipoprotein-associated phospholipase A2 (Lp-PLA2). PAF has been implicated in HIV infection, as its biosynthesis is induced by the viral protein Tat. Its action has been related to many disorders of the central nervous system such as AIDS dementia. Moreover, in a recent study from our group, it was shown that each antiretroviral regimen effects by a different way PAF levels and its metabolic enzymes. The aim of the recent study was to monitor PAF levels and its metabolic enzymes for 12 months in HIV infected individuals prior to introduction of antiretroviral therapy.
Patients and Methods

**Study design**

Study enrollment began after obtaining approval from the scientific board of the Red Cross General Hospital of Athens in Greece and all volunteer patients have signed the informed consent according to the Declaration of Helsinki. The volunteers (n=10) were recruited from the 3rd Internal Medicine Department-Infectious Diseases Unit, Red Cross General Hospital, Athens, Greece. All participants were male, treatment naïve, and asymptomatic HIV-infected patients as determined by the presence of antibodies against HIV measured by enzyme-linked immunosorbent assay (ELISA) and confirmed by Western blot. All volunteers did not meet the criteria for antiretroviral therapy according to the for ART initiation according to the European and international guidelines. The average age of the group was 33 ± 12 years and 40% of them were smokers. Patients with any inflammatory conditions such as diabetes mellitus, hypertension, allergy, periodontitis or on any medication were excluded from the study (n=2). Blood samples were collected for a 12-month period by 6 fasting blood draws.

**Quantitation of enzyme assay-derived PAF**

The isolation and purification of PAF was according to the method of Demopoulos et al. Briefly, 10 mL of blood were collected from each patient and poured immediately into 40 mL of absolute ethanol. The
mixture was stirred and centrifuged at 300 xg for 10 min at room temperature. The supernatant and the pellet were extracted separately according to the Bligh and Dyer method and the chloroform phase in each case was stored at -20°C. The supernatant chloroform extract contains PAF that is loosely bounded to plasma proteins and lipoproteins, named free PAF, while the pellet extract contains PAF strongly bounded to cellular structures, named bound PAF. Total PAF levels occur from the sum of Bound and Free PAF. PAF is firstly purified by silicic acid column chromatography and secondly by HPLC (Hewlett-Packard series 1100) on a cation-exchange column. The final samples were dissolved in BSA (1.25% in saline) and PAF levels were measured by the aggregatory activity (by a 400 VS aggregometer Chrono-Log, USA) towards washed rabbit platelets. PAF levels are expressed as fmol/mL of blood.

**Isolation of plasma, platelets, leukocytes and erythrocytes**

An amount of 9mL blood was obtained from each volunteer in 1 mL of an anticoagulant solution of sodium citrate / citrate acid. The sample was centrifuged at 194xg for 10 min at 25 °C. Then four simultaneous procedures were carried out:

a) Isolation of plasma

The supernatant called PRP (Plasma Rich in Platelets), was centrifuged at 1,400xg for 20 min at 25 °C. The supernatant, called PPP (Plasma Poor in
Platelets), was further centrifuged at 20,000xg for 1h at 4 °C, in order to remove viral load as pellet, and then was aliquoted and stored at -80 °C.

b) Isolation of human platelets

The pellet of the PRP centrifugation (platelets) was re-suspended in 1 mL of a buffer containing 50 mM Tris- HCl (pH 7.4). Subsequently, platelets were sonicated in ice bath (4x15 s) and centrifuged at 500xg for 10 min at 4 °C. Platelets homogenate after protein determination by Bradford method was aliquoted and stored at -80 °C.

c) Isolation of human leukocytes

In the pellet of the first centrifugation (leukocytes and erythrocytes) saline was added until the volume of 10 mL and after mild mixing by inversion, the solution was separated in two parts of 5 mL each. In each part the isolation of the leukocytes from the contaminating erythrocytes was achieved by erythrocyte sedimentation. 1.7 ml of dextran solution (3% dextran in NaCl 0.15 M) was added in each part and the mixtures were kept for 1 h at room temperature. The leukocyte-rich supernatants of the two aliquots were pooled and centrifuged at 500xg for 10 min at room temperature. The supernatant was removed and contaminating erythrocytes of the sediment pellet were lysed with the addition of 5 mL of a lysis solution consisting of 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA and then removed by centrifugation at 300xg for 10 min at room temperature. The isolated leukocytes were re-suspended in 1 mL of a
buffer containing 50 mM Tris-HCl (pH 7.4), sonicated in ice bath (4x15 s) and centrifuged at 500xg for 10 min at 4 °C. Leukocytes homogenate after protein determination by Bradford method were aliquoted and stored at -80 °C.

d) Isolation of human erythrocytes

After the addition of dextran solution and the removal of the leukocyte-rich supernatants, 0.5 mL of the pellet (erythrocytes) were resuspended in 2.5 mL of saline and further centrifuged at 200xg for 10 min at 25 °C in order to remove contaminating plasma remains. The supernatant was removed; the pelleted erythrocytes were resuspended in 2.5 mL of a buffer containing 50 mM Tris-HCl (pH 7.4) and homogenized by potent stirring in vortex. The homogenized erythrocytes were further centrifuged at 20,000xg for 1h at 4 °C, in order to remove whole cells, debris and contaminating viral load in the pellet, while the supernatants, erythrocytes homogenate, after protein determination by Bradford method were aliquoted and stored at -80 °C.

Enzymatic Assays

**PAF-CPT activity assay**

The assay was performed on the homogenates of leukocytes and platelets as previously described. Briefly, the reaction was carried out at 37 °C for 20 min in a final volume of 200 μL containing 100 mM Tris-HCl (pH 8.0), 15 mM dithiothreitol (DTT), 0.5 mM EDTA, 20 mM MgCl2, 1mg/mL BSA,
100 μM CDP-Choline, 100 μM 1-O-alkyl-2-sn-acetyl-glycerol (AAG, added in the assay mixture in ethanol), and the sample (0.05 mg/mL final concentration of protein). The mixture of Tris, DTT, EDTA, MgCl₂ and BSA was incubated in 37°C for 5 min. Initially, the homogenized sample was added in the mixture. After 30s, AAG was added and 30s later the reaction was started by addition of CDP-Choline. The reaction was stopped by adding 0.5 mL of methanol after 20 min.

**lyso-PAF-AT activity assay**

The assay was performed on the homogenates of leukocytes and platelets as previously described.²⁵ Briefly, the reaction was carried out at 37 °C for 30 min in a final volume of 200 μL containing 50 mM Tris-HCl (pH 7.4), 0.25 mg/mL BSA, 20 μM lyso-PAF and 200 μM acetyl-CoA and the sample (0.125 mg/mL final concentration of protein). The reaction was started by the addition of the homogenized sample and was stopped after 30 min by adding 0.5 mL of methanol.

**Determination of PAF specific activity**

After the assay of PAF-CPT and lyso-PAF-AT, PAF was extracted according to the Bligh–Dyer method and was separated by thin-layer chromatography (TLC) on Silica Gel G coated plates with a development system consisted of chloroform:methanol:acetic acid:water (100:57:16:8, v/v/v/v). PAF band was scrapped off, extracted using Bligh-Dyer and finally quantitated by the washed rabbit platelet aggregation assay.¹⁵
**PAF-acetylhidrolase activity assay**

PAF-AH in HLs, HPs, ERCs as well as Lp-PLA2 in plasma were determined by the trichloroacetic acid precipitation method using \[^{3}H\] PAF as a substrate, as previously described \(^{25}\). Briefly, the reaction took place for 30 min at 37 °C in a final volume of 200 μL. Initially 50 mM of Tris/HCl buffer (pH 7.4) was incubated with 4 nmol of \[^{3}H\]-PAF (20 Bq per nmol) \[^{3}H\]-acetyl PAF/PAF solution in BSA (1% in saline) for 5 min. The reaction started by the addition of homogenized samples (0.25 mg/mL in the case of HLs, 0.5 mg/mL in the case of HPs, 2.5 mg/mL in the case of ERCs or 2 μL in the case of plasma) The reaction was terminated by the addition of BSA solution (0.75 mg/mL) followed by precipitation with trichloroacetic acid (TCA, 9.6% v/v). The samples were then placed in an ice bath for 30 min and subsequently centrifuged at 16,000xg for 5 min. The \[^{3}H\]-acetate released into the aqueous phase was measured on a liquid scintillation counter (1209 Rackbeta Pharmacia, Wallac, Finland).

**Biochemical markers and immunological analysis**

Clinical biochemical markers were measured by a Siemens Dimension RxL automatic analyzer. CD4 cell counts were defined using Tetra One System on the EPICS XL flow cytometer, while viral load was determined using the Versant HIV-1 RNA 3.0 assay.
**Statistical analysis**

Normal distribution was checked by P-P graphs using Shapiro-Wilk criterion. Differences within each group during the overall 12-month treatment were determined by non-parametric Friedman (displayed as $p_{\text{time}}$) analysis. Wilcoxon test was used to compare within each group the difference of specific time point with the baseline values (displayed as $p_{\text{specific-time-point}}$) criterion. Correlation analysis was performed using Spearman’s coefficient and viral load changes are reported in a logarithmic scale for convenience and not in order to normalize the value. Statistical significance was considered as $p<0.05$. The analysis was performed using IBM SPSS Statistics 20.
Results

*Anthropometric and biochemical characteristics of the volunteers*

Despite the increased viral load, all patients had CD4+ T cells within the minimum normal range. This fact is actually the reason they didn’t meet the criteria for antiretroviral administration. Among all the characteristics they were measured (table 1), only HDL was found to be quite below the normal range during the study. However, the statistical analysis revealed some significant differentiations during the 12-month study such as glucose ($p_{\text{time}}=0.008$), LDH ($p_{\text{time}}=0.033$), albumin ($p_{\text{time}}=0.012$) and platelet count ($p_{\text{time}}=0.034$). Individual alterations ($p<0.05$) at specific time points compared to baseline are also detected and specifically, CD4+ T cells are increased at the 6th month, blood urea nitrogen is decreased at the 12th month and white blood cells are increased at the 6th month.

**Table 1**: Anthropometric and biochemical characteristics of naïve HIV patients during 12 months.
All the results are expressed as median values and interquartile range (25th-75th). $p_{time}$ displays the difference within the group during the overall 12-month study. *: displays the significant difference of each time point with the baseline value. $p<0.05$

**PAF levels of HIV naïve patients**
PAF levels (Bound, Free and Total, Fig 1) are not differentiated either throughout the study period or at any specific timepoint.

*Figure 1: PAF levels of naïve patients i) Bound PAF, ii) Free PAF and iii) Total PAF is expressed in fmol/mL of blood.*
**Specific activity of PAF biosynthetic enzymes**

The specific activity of the two PAF biosynthetic enzymes, PAF-CPT and lyso-PAF-AT, is not differentiated either throughout the study period or at any specific timepoint in both leukocytes and platelets (Fig 2).
Figure 2: Specific Activity of PAF biosynthetic enzymes of naïve patients i) PAF-CPT in Leukocytes, ii) lyso-PAF-AT in Leukocytes, iii) PAF-CPT in Platelets and iv) lyso-PAF-AT in Platelets. The enzymatic activity is expressed in pmol/min/mg of protein.
Specific activity of PAF catabolic enzymes

The specific activity of PAF catabolic enzymes does not exhibit any differentiation either throughout the study period or at any specific timepoint in all blood cells and plasma (Fig 3).

Figure 3: Specific Activity of PAF catabolic enzymes of naïve patients i) PAF-AH in Leukocytes, ii) PAF-AH in Platelets, iii) PAF-AH in Erythrocytes and iv) Lp-PLA2 in plasma. The enzymatic activity is expressed in pmol/min/mg of protein or μL of plasma.
Correlation analysis between biochemical markers, PAF and its metabolic enzymes

In order to identify whether alterations of one biochemical marker may affect alterations of the others, the differences (Δ) of the biochemical marker values from 0 to 12 months were intercorrelated. The characteristics that were correlated were: PAF levels (Bound, Free and Total), PAF biosynthetic enzymes (PAF-CPT and lyso-PAF-AT in leukocytes and platelets), PAF catabolic enzymes (PAF-AH in leukocytes, platelets, erythrocytes and plasma) and all the characteristics of table 1.

The tables 2 and 3 present only the statistically significant correlations that were occurred during the analysis.

The results display that ΔFree PAF levels are positively correlated to ΔPAF-CPT in platelets and ΔLp-PLA2. ΔPAF-CPT in platelets is positively correlated to ΔLp-PLA2 while Δlyso-PAF-AT in platelets is negatively correlated to ΔPAF-AH in platelets.

Table 2. Spearman partial correlation coefficients between the difference of PAF levels and the difference of PAF metabolic enzymes activity (from 0 to 12 months)

<table>
<thead>
<tr>
<th></th>
<th>ΔFree PAF</th>
<th>ΔPAF-CPT (Platelets)</th>
<th>Δlyso-PAF-AT (Platelets)</th>
<th>ΔPAF-AH (Platelets)</th>
<th>ΔLp-PLA2</th>
</tr>
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<tbody>
<tr>
<td>ΔFree PAF</td>
<td>0.905</td>
<td>(0.002)</td>
<td>0.755 (0.031)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔPAF-CPT (Platelets)</td>
<td>0.905</td>
<td>(0.002)</td>
<td>0.790 (0.020)</td>
<td></td>
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</tr>
</tbody>
</table>
The values displayed are Spearman partial correlation coefficients and p (in parentheses).

ΔBound and ΔTotal PAF levels are negatively correlated to ΔBUN. ΔPAF-CPT in leukocytes is positively correlated to ΔALP. ΔLyso-PAF-AT in platelets is negatively correlated to ΔSGOT,AST, and ΔCK and positively to ΔHDL, ΔRBC and ΔHCT. ΔPAF-AH in platelets is positively correlated to ΔSGOT,AST and ΔCK while ΔPAF-AH in erythrocytes is negatively correlated to ΔHGB.

Table 3. Spearman partial correlation coefficients between the difference of PAF metabolic values (levels and enzymes) and the difference of several clinical characteristics (from 0 to 12 months)
<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \Delta \text{PAF-CPT} ) (Leukocytes)</td>
<td>0.762</td>
<td>(0.028)</td>
</tr>
<tr>
<td>( \Delta \text{lyso-PAF-AT} ) (Platelets)</td>
<td>-0.707, -0.707, -0.810</td>
<td>(0.050, 0.050, 0.015)</td>
</tr>
<tr>
<td>( \Delta \text{PAF-AH} ) (Platelets)</td>
<td>0.755, 0.857</td>
<td>(0.031, 0.007)</td>
</tr>
<tr>
<td>( \Delta \text{PAF-AH} ) (Erythrocytes)</td>
<td>-0.735</td>
<td>(0.038)</td>
</tr>
<tr>
<td>( \Delta \text{Lp-PLA2} )</td>
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The values displayed are Spearman partial correlation coefficients and p (in parentheses).

**Discussion**

Thirty years after the advent of AIDS, the researchers have managed to turn HIV infection into a chronic disease while the prolongation of patients’ lifespan is accompanied by a variety of non-AIDS morbidities. These conditions are typically associated with systemic or chronic inflammation,
immune activation and immunosenescence, and they usually appear as cardiovascular or renal disease as well as several types of cancer. In the present study, PAF levels of HIV, naïve patients are measured for the first time along with PAF basic metabolic enzymes activity for 12 months. The results show a firm stability in PAF levels and enzymes which indicate a stable inflammatory profile of these patients and probably support the decision of not prescribing antiretroviral therapy in patients with CD4 count of 500 cells/μL or higher. Despite the patients do not show any significant deviations, there are some single cases that display trends of increase in PAF levels which can be attributed to the respective increase of the two biosynthetic enzymes as it is well known that the whole PAF system in the organism is being characterized by a dynamic relation and balance.

Concerning the anthropometric and biochemical characteristics of naïve HIV, there some significant alterations. The decrease at the glucose levels of the patients comes in accordance with the reported disturbances in glucose homeostasis in HIV, naïve patients. Moreover, the reported decreases in albumin levels and in platelet count are quite prevalent in HIV naïve patients and associated with the disease severity and several morbidities, respectively.

Regarding the correlation analysis between the difference of the baseline and the 12th month, a lot of outcomes have been detected. As referred in
the introduction, PAF levels in blood can occur from different sources including the *de novo* biosynthetic pathway of blood cells. Considering that, the positive correlation between \( \Delta \text{Free PAF levels} \) and \( \Delta \text{PAF-CPT in platelets} \) is reasonably expected. Besides, \( \Delta \text{Free PAF levels} \) are positively correlated to \( \Delta \text{Lp-PLA2} \), which can be ascribed as a compensatory mechanism to down-regulate raised Free PAF levels \(^{31}\). As a result, the detected positive correlation of \( \Delta \text{PAF-CPT in platelets} \) with \( \Delta \text{Lp-PLA2} \) is reasonable, considering Free PAF as the intermediate molecule of this correlation. It is not known why the increases of Free PAF levels correlate only with the increase of PAF-CPT in platelets and not with the respective one in leukocytes and this requires further investigation. \( \Delta \text{Bound and} \) \( \Delta \text{Total PAF levels} \) are negatively correlated to \( \Delta \text{Blood Urea Nitrogen} \) and this result may be due to a possible kidney dysfunction as PAF is also implicated in renal pathophysiology \(^{32}\).

Due to the shortage of similar studies and the absence of standardized methods for the determination of PAF levels and metabolic enzymes it is hard to find suitable controls to compare with. However, Lp-PLA2 has been studied thoroughly with a standardized methodology. Detopoulou et al \(^{33}\) and Tsoupras et al \(^{34}\) studied Lp-PLA2 in healthy volunteers and their comparison with the HIV patients of the present study reveals similar Lp-PLA2 specific activity.

In conclusion, the present study reveals a steady PAF-related inflammatory profile in naïve HIV patients. Monitoring PAF levels and
enzymes in these patients, along with the use of PAF inhibitors, could probably improve their quality of life by reducing the various non-AIDS morbidities. Further studies could possibly reveal the possible mechanisms behind the observed correlations and contribute to the debate of “when to start” the antiretroviral therapy.

Study limitations

The main limitation of this research study is the number of the patients. The reason is the chosen methods for PAF which are quite strenuous and time consuming, but more sensitive and trustful than other rapid methods. In order to eliminate this parameter, the writers have chosen quite carefully the statistical analysis. In addition, female patients have not been included in the study as the menstrual cycle affects the metabolism of PAF.

References