Indoleamine 2,3 Dioxygenase, Age, and Immune Activation in People Living with HIV

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ABSTRACT:

Immune activation complicates HIV despite antiretroviral therapy (ART). Indoleamine 2,3 dioxygenase (IDO) catabolizes tryptophan (T) to kynurenine (K), regulating immune activity, and IDO activity increases with age. This study examines the relationship of IDO activity, bacterial translocation, and aging in people living with HIV (PLWH) persons on ART. Samples and data from PLWH on ART from Centers for AIDS Research Network of Integrated Clinical Systems and from matched HIV-uninfected patients (Controls) from Multicenter AIDS Cohort Study and Women's Interagency HIV Study were analyzed. The ratio of K to T (K/T) and neopterin were indicators of inflammation; 16S ribosomal DNA (16S rDNA) and lipopolysaccharide (LPS) were markers of bacterial translocation. Samples and data from 205 PLWH and 99 Controls were analyzed. PLWH had higher K/T values across all ages with a significant relationship between age and K/T for both groups. CD4 count or CD4 nadir had no association with K/T. There was no positive association between level of 16S rDNA or LPS detection and K/T. K/T and neopterin were associated. PLWH had elevated IDO activity, at younger ages, despite ART. This study suggests K/T ratio increases with age in both groups, and is elevated for PLWH at all ages compared to age matched Controls.

Significance of this study

What is already known about this subject?

- People living with HIV (PLWH) often experience the complications of aging earlier.
- PLWH, despite virologic control, have measurable increased levels of chronic inflammation.
- The evidence of immune regulation by indoleamine 2,3 dioxygenase (IDO) is more active in PLWH.

What are new findings?

- With aging, PLWH have elevated activity of IDO when compared with age matched control patients without HIV.
- Even younger PLWH have higher activity of IDO than all control patients without HIV.
- The IDO activity, thought to be driven by microbial translocation from the gastrointestinal tract, may be driven by multiple factors in PLWH.

How might these results change the focus of research or clinical practice?

- The chronic inflammation in PLWH could be from stimulation from the microbiome, but further studies need to be done to demonstrate the mechanism.
- In clinical practice, premature aging of PLWH should encourage vigilance for complications of aging in this population.

INTRODUCTION

Human Immunodeficiency virus (HIV) causes lifelong infection typified by immune activation and dysfunction that worsens as the infection progresses. Despite modern antiretroviral therapy (ART), older people living with HIV (PLWH) are at increased risk of cardiovascular disease or non-AIDS associated malignancies in part due to chronic inflammation and declining immune system function.[1 2] Several studies have suggested PLWH be considered to be of advanced age beginning at age 50.[3 4] The immune mechanisms of chronic inflammation and the impact on the cardiovascular system and risk for malignancy are still poorly understood. Measures of chronic inflammation have been shown to be associated with overall mortality and cardiovascular events in PLWH despite ART, even with CD4 cell recovery to >500 cells/mm^3.[1 5 6]

Reduction in plasma tryptophan in HIV patients was described in 1988; this was later learned to be due to increased degradation of tryptophan, not from low intake. L-tryptophan (2,3)-dioxygenase (TDO) and indoleamine 2,3 dioxygenase (IDO) both catabolize tryptophan to kynurenine in the first step of the

kynurenine biosynthetic pathway which is essential to the production of the neurotransmitter serotonin (5-hydroxytryptamine) and nicotinamide adenine dinucleotide (NAD/NADH). TDO is primarily active in the liver for dietary tryptophan metabolism.[7] IDO is produced by dendritic cells and regulatory T-cells and influences T-cell differentiation toward T-regulatory (T-reg) and away from helper cells (Th-17) by creating a kynurenine rich and tryptophan environment. The ratio of K/T also directly impairs T cell function.[8 9]IDO has been shown to be induced by INF-γ, TNF-α, TGF-β, and lipopolysaccharide (LPS).[7 8] Its activity can be measured by quantification of the ratio of kynurenine to tryptophan (K/T) in plasma or other tissues.[5] IDO plays a critical role in orchestrating immune tolerance in malignancy, tolerance of the gut microbiota, fetal tolerance during pregnancy, and various chronic infections. Chronic indoleamine 2,3 dioxygenase (IDO) activation has been implicated in multiple infectious diseases such as tuberculosis, influenza, leishmaniasis, and listeriosis.[10] IDO has also been shown to be the primary driver of peripheral tryptophan levels in PLWH. [11-14] Increased IDO activity has been associated with detectable levels of microbial translocation from the gastrointestinal tract, gut microbiota changes, as well as HIV disease progression and mortality rates in PLWH.[15]

IDO activity level increased with age in a general population cohort without known infections.[16] Additionally, higher levels of IDO activity have been associated with increased mortality in PLWH.[17] IDO promotes HIV-associated immune pathogenesis in humanized mice and macaques and is directly induced by HIV infection.[18] IDO activity along with neopterin, an indicator of inflammation, have been shown to decline in PLWH treated with ART.[19] Elevated IDO activity is associated with advancing HIV disease in humans.[8] Continuous bacterial translocation from the gastrointestinal tract of PLWH has been theorized to be a major driver of the chronic inflammation and progression of comorbidities that persists despite ART.[8 20 21]

This study examines the effect of age on IDO activity in PLWH who are stable on ART. Immune activation and bacterial translocation are also examined as possible associated factors with IDO activity level in

PLWH. The goal of the study was to explore associations between these parameters and their impact on the hypothesis that IDO activity associated with age or bacterial translocation is a key factor in chronic inflammation in PLWH.

METHODS

In this study, samples of plasma frozen and kept at -80 degrees C collected between 2000-2012 and de-identified data from PLWH who were virally suppressed, defined as viral load <50 copies/ml for at least 1 year, were obtained from the Centers for AIDS Research Network of Integrated Clinical Systems (CNICS). Samples of plasma frozen and kept at -80 degrees C, collected between 2003-2015 and data from age and sex matched HIV-uninfected (Control) patients were obtained from the Multicenter AIDS Cohort study (MACS) and the Women's Interagency HIV Study (WIHS).

These cohorts were used to ensure an adequate number of samples were obtainable for both PLWH and HIV-uninfected individuals at the extremes of age. In order to ensure adequate representation of ages, 40 PLWH and 20 Control patients from each age strata: 30-39, 40-49, 50-59, 60-69 and 70-79 years were requested. Information about age, sex, race, nadir CD4 count, concurrent viral load, concurrent CD4 count and complete blood cell count were obtained. Exclusion criteria included renal disease (characterized as eGFR < 60 cc/min or a serum creatinine >1.5 mg/dl), nephrotic syndrome, chronic hepatitis C virus (HCV), active hepatitis B (HBV), autoimmune disease, documented concurrent infection and receipt of immunosuppressive medications such as mycophenolate, cyclosporine, tacrolimus, chemotherapy, immunotherapy, intravenous immunoglobulin, or prednisone. This project was determined to be non-human subjects research by the Augusta University Institutional Review Board due to the de-identification of data and samples. Both repositories are approved by their respective institutional review boards and have consent for samples and data to be used for subsequent studies.

IDO-activity (HPLC): IDO-activity was assessed by de-proteinizing serum for HPLC analysis to detect kynurenine and tryptophan (K&T) as described.[22]

Markers of chronic inflammation, immune status and bacterial translocation: Samples were stored at -80 degrees C until thawing for analysis to minimize changes in neopterin levels prior to measurement.[23] Plasma samples were tested for neopterin using commercially available ELISA (IBL International, Mannedorf, Switzerland). Bacterial translocation was evaluated by detection of bacterial 16S ribosomal DNA (16S rDNA) by RT-PCR directly from plasma samples. The quantification of bacteria was based on a standard curve constructed from samples prepared with known quantities of *Campylobacter*. Bacterial translocation was evaluated by detection of LPS by commercial kit (Lonza QCL-1000).

Statistical Analysis: Descriptive statistics were performed for all subjects. Log transformations were used when needed if continuous data had a skewed distribution. Comparisons between Controls and PLWH were made using chi-square tests for categorical data and two sample t-tests with a Satterthwaite adjustment for continuous data. ANOVA was used to determine the differences between gender and race for K/T ratio. The association between K/T and covariates (age, CD4, CD4 nadir, levels of bacterial DNA, LPS, and neopterin) for Control and PLWH subjects was determined using ANCOVA homogeneity of slopes model separately for each covariate. A significant interaction between a covariate and group membership would indicate that the relationships were different. A sensitivity analysis was performed to determine the effect of removing PLWH subjects with undetectable viral loads. SAS© 9.4 (SAS Institute, Inc., Cary, NC) was used for all analyses. Significance was determined at a Type I error rate of 5%.

RESULTS

Samples and data from 205 PLWH on ART and 99 HIV-uninfected (Control) patients were analyzed. The groups did not differ by gender, race, or age. The groups were predominantly male (81% for both); 45%of PLWH and 58% of Controls self-identified as white, respectively. The pre-specified age ranges for assessment were represented; the mean age was 52 in both groups (range 35-83 and 35-87) (Table 1).

Table 1. Demographics for Controls and People Living with HIV (PLWH)

	PLWH	**Control**	
	n=205	**n=99**	**p-value**
Gender, n (column %)			
Male	166 (81)	80 (81)	0.97
Female	39 (19)	19 (19)	
Race, n (column %) *			
White	92 (45)	46 (58)	0.17
Black/African American	79 (39)	21 (26)	
Hispanic/Latino	25 (12)	11 (14)	
Other	9 (4)	2 (3)	
Age, median (min, max)	52 (35, 87)	52 (35, 83)	0.39

* 19 Control Females were missing Race

Renal function in PLWH was comparable to Control patients. As expected, the CD4 and CD4 nadir were significantly higher for Control subjects (Table 2).

Table 2: Laboratory comparison of Controls and PLWH

	PLWH **n=205**	**Control** **n=99**	**p -value**
CD4+ cells, median (min, max) Control n=77	512 (30, 2135)	859 (360, 1717)	<0.0001
CD4+ nadir, median (min, max) Control n=80	143 (0, 668)	581 (242, 1198)	<0.0001
K/T, median (min, max) *	0.065 (0.025, 0.361)	0.047 (0.016, 0.124)	<0.0001
Neopterin, median (min, max) *	6.4 (0.1, 79.5)	3.9 (0.5, 58.7)	<0.0001
LPS, median (min, max) *	0.17 (0, 1.02)	0.27 (0, 2.0)	<0.0001
16S rDNA PCR Ct, median (min, max)	n=172 26.9 (21.5, 28.5)	n=96 27.5 (24.8, 31.3)	<0.0001

* $\log_{10}$ transformation prior to analysis

K= kynurenine, T=tryptophan, PCR Ct= Polymerase chain reaction cycle threshold for 16S ribosomal DNA

Of note, 12% of the PLWH for whom we received samples had viral loads above the undetectable range at the designated time point. Removing their data in a sensitivity analysis did not change the final results thus these subjects were retained in order to maximize power. The distribution of K/T for both Control and PLWH subjects was no longer skewed after the log transformation. Outliers were not considered to be influential due to the small number present in relation to the total sample size for each group. (Figure 1). PLWH had higher K/T ratio values across all ages (Figure 2, p<0.0001) reflecting higher IDO activity.

Even younger (age < 50 y) PLWH had median K/T ratio values greater than older (age ≥ 50 y) Control patients (median (min, max): 0.047 (0.016, 0.124) vs. 0.065 (0.025, 0.361), respectively). Age was associated with significant increase in K/T as was hypothesized (Figure 2, $p<0.0001$) and this increase was not different depending on status of HIV infection ($p=0.92$, r^2 0.18 for the interaction). K/T ratios did not differ by gender or by race or ethnicity.

Current CD4+ cell count or CD4+ nadir cell count did not appear to have a relationship with K/T ratio (Supplemental Digital Content Figure 1). For PLWH as LPS increased, K/T decreased while there was no association between K/T and LPS for Controls (Figure 3, $p=0.0071$ for the interaction). For both PLWH and Controls there was no association between bacterial 16S rDNA PCR cycle threshold detection and K/T ratio (Figure 4). PLWH did have a lower cycle threshold for 16S rDNA PCR indicating more detection of 16S rDNA compared to controls (PLWH mean=26.6 (95% confidence interval 26.5-26.8), control=27.5 (95% confidence interval 27.3-27.6). As expected, both groups had positive association between K/T ratio and neopterin (Supplemental Digital Content Figure 2, $p<0.0001$). Neopterin levels were significantly higher for PLWH subjects compared to Controls (Table 1).

DISCUSSION

This study analyzed samples and data from similar groups of PLWH subjects on ART and HIV-uninfected Controls drawn from two major national specimen and data repositories to examine the effect of aging on IDO activity, as measured by K/T ratio, according to HIV status. Large, national cohorts were used to maximize the age range able to be assessed, which is one of the strengths of this study. Ages were requested from specific age strata to ensure that extremes of age were represented, and the groups were well matched. It was found that PLWH on ART have elevated K/T compared to all Controls, even at younger ages. This supports previous studies' findings that PLWH do have a persistent chronic inflammatory state despite measured virologic suppression.[6 14 24 25] This study did support the main hypothesis that K/T would increase with advancing age in both groups in keeping with previously

208 published literature.[16 17] This study was the largest, multi-site cohort study with sufficient numbers for
209 statistical power in age strata to focus on both the effect of HIV and age on immune regulation. The
210 absolute differences between K/T ratio in the PLWH and Control groups was small, but statistically
211 significant. This difference may have clinical implications which are yet unknown as most studies that
212 showed increased mortality in PLWH or Controls with higher K/T ratios did not consider age. [4 5]
213 Similar to our finding of no relationship between IDO activity with microbial translocation, a recent
214 study by Chen and colleagues also found no relationship between IDO and markers of translocation, but
215 did find IDO correlated with total HIV DNA in peripheral blood, immune activation, and T cell
216 exhaustion.[25] These findings of no association of K/T and microbial translocation are unlike previously
217 published literature which proposed the link of translocation to the chronic inflammation in HIV.[8 20 21 26]
218 This may reflect advancements HIV care, specifically earlier implementation of ART which appears to
219 influence the residual HIV DNA in peripheral blood.[25] The CD4 nadir also did not associate with K/T ratio
220 in this cohort which might also be an effect of earlier ART initiation or more potent modern regimens
221 limiting the depopulation of the gut associated lymphatics or the innate immune system. In our study,
222 the K/T ratio was still indicative of ongoing inflammation in PLWH, as noted by the associated higher
223 levels of neopterin;[27] however, in this cohort of PLWH, inflammation does not appear to be driven by
224 microbial translocation from the GI tract as measured by plasma levels of bacterial 16S rDNA or LPS.
225 Limitations: the limitations of this study are numerous due to the inherent challenges of using specimen
226 and data repositories. 1. There may have been laboratory limitations due to the storage and
227 freezing/unfreezing of specimens. 2. Data was not available from all repositories regarding whether
228 subjects were fasting at the time of specimen collection and non-fasting state could lead to elevated
229 tryptophan levels. Whenever possible, fasting specimens were used. 3. There was an inadvertent
230 deviation from the study's protocol such that 12% of "well controlled" PLWH had detectable viral loads.
231 However, every attempt was made statistically to determine whether inclusion of those subjects would

impact the study results. The results were not significantly different regardless of their inclusion. 4. The timing of sample collection and transmitted lab data were requested to be the same, however it is unknown if that was always the case. 5. The samples from PLWH and the HIV-negative controls were collected from separate cohorts, thus may have been subject to differences in specimen collection, handling or storage which may have impacted results.[19] Co-existing infections were excluded where possible, but it is likely that there may have been subclinical or unreported infections at the time of specimen collection. We attempted to minimize the impact of unaccounted for coinfections by deriving Controls from cohorts identified as being at increased risk for HIV and thus having a heightened likelihood of having similar comorbidities and exposures as the PLWH cohort.[28] Medications have the potential to activate or suppress IDO function;[29] the attributable impact of specific ART regimens, duration on those regimens, and concomitant medications on IDO function was beyond the scope of this study. The LPS assay did show numerous zero values which may have led to too small a sample size when zero values were removed. Results should be interpreted with caution due to the limitations of the Lonza assay.

In conclusion, PLWH were observed to have K/T and neopterin levels indicative of increased inflammation at all age strata when compared age-matched to Controls. This cohort also demonstrated advancing age to be associated with an increase in the K/T ratio levels indicating increased IDO activity with age for the first time in PLWH. Also, markers of microbial translocation (16S rDNA and LPS) did not appear to correlate positively with K/T ratio for PLWH or Control subjects. This study suggests K/T ratio increases with age in both groups and is elevated for PLWH at all ages compared to age matched Controls.

Institutional Review: This study was deemed to not require ethics approval as it was judged to be not human subject research by the Augusta University Institutional Review Board as only de-identified data

and specimens were used and no prospective data or specimens were collected (Human subjects determination IRBHSD00000001). Informed consent was obtained prior to participation for all subjects by each of the respective cohort studies.

Conflicts of interest and sources of funding:

Potential conflicts of interest. ALM is a shareholder in NewLink Genetics Inc. and receives licensing income from this source. All other authors declare no conflicts.

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Footnotes:

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Figure captions:

Figure 1: People living with HIV (PLWH) had higher K/T ratios than HIV-uninfected Control (CNT) subjects. A $\log_{10}$ transformation (Log K/T) was used to minimize the influence of the outlying higher K/T values, especially in the PLWH. K= kynurenine, T=tryptophan

Figure 2. There was a significant positive association between Age and K/T ($p<0.0001$) for both PLWH (dotted line and open circles) and Control (CNT) (solid line and filled squares). PLWH had higher K/T across Age ($p<0.0001$). K= kynurenine, T=tryptophan

Figure 3: The relationship between LPS and K/T is significantly different for HIV-infected and HIV-uninfected (CNT) subjects ($p=0.0071$ for the interaction). There is no association for HIV-uninfected subjects (solid line and filled squares) and a significant negative relationship for HIV-infected subjects (dotted line and open circles). As LPS increases in HIV-infected subjects, K/T decreases.

Figure 4: There is not a significant association between 16S rDNA PCR cycle threshold gene expression and K/T levels ($p=0.28$) for both PLWH (dotted line and open circles) and Control (CNT) (solid line and filled squares). K= kynurenine, T=tryptophan, PCR= Polymerase chain reaction

Figure 1. Distribution of log K/T for Control and People living with HIV

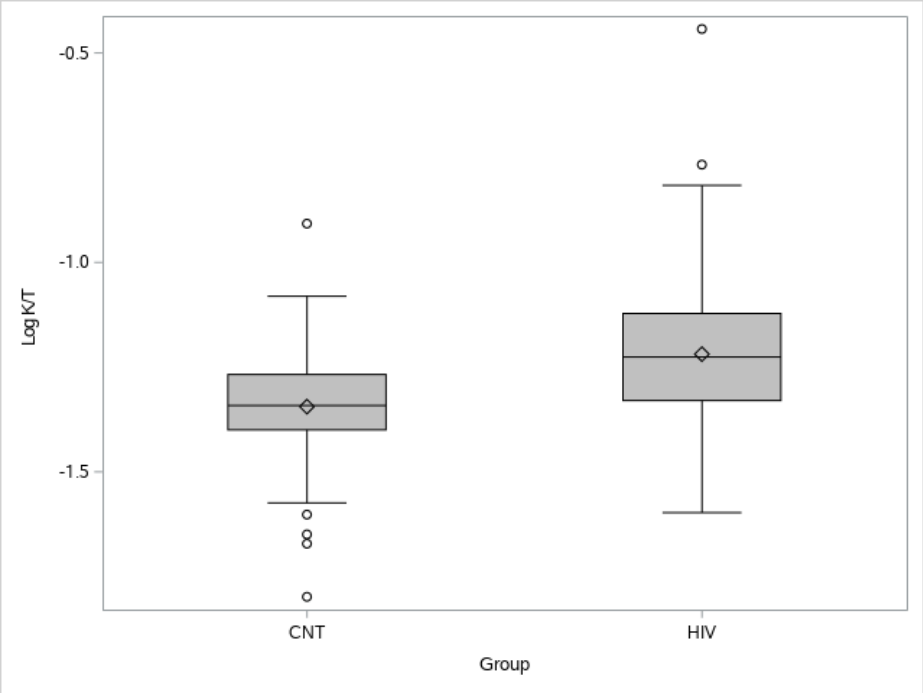

Figure 2. The Association between Age and K/T ratio

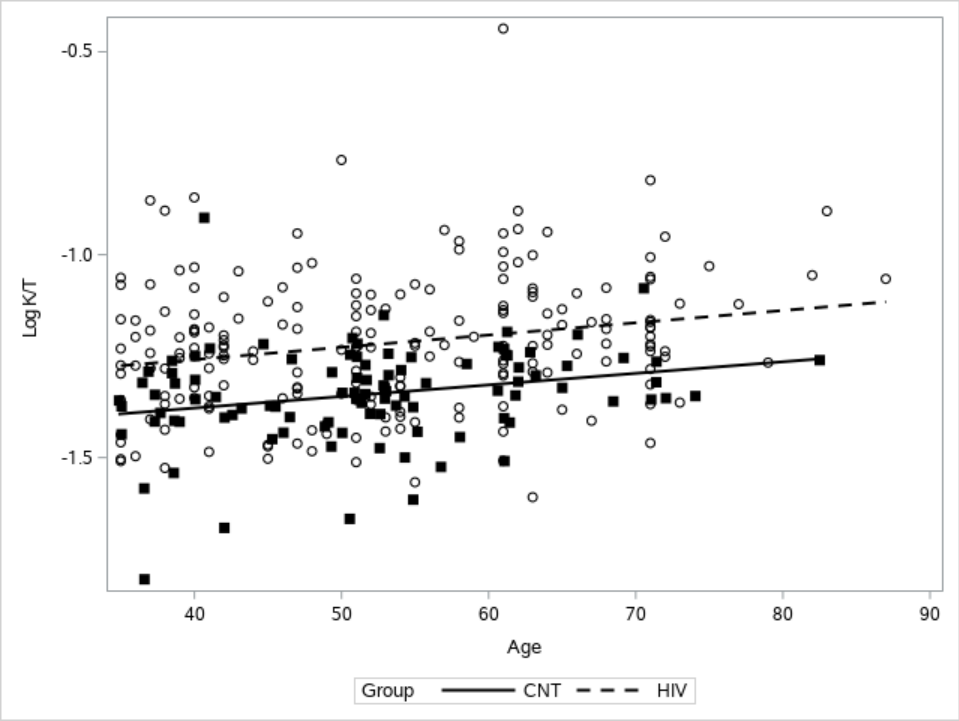

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Figure 3. Association between LPS and K/T

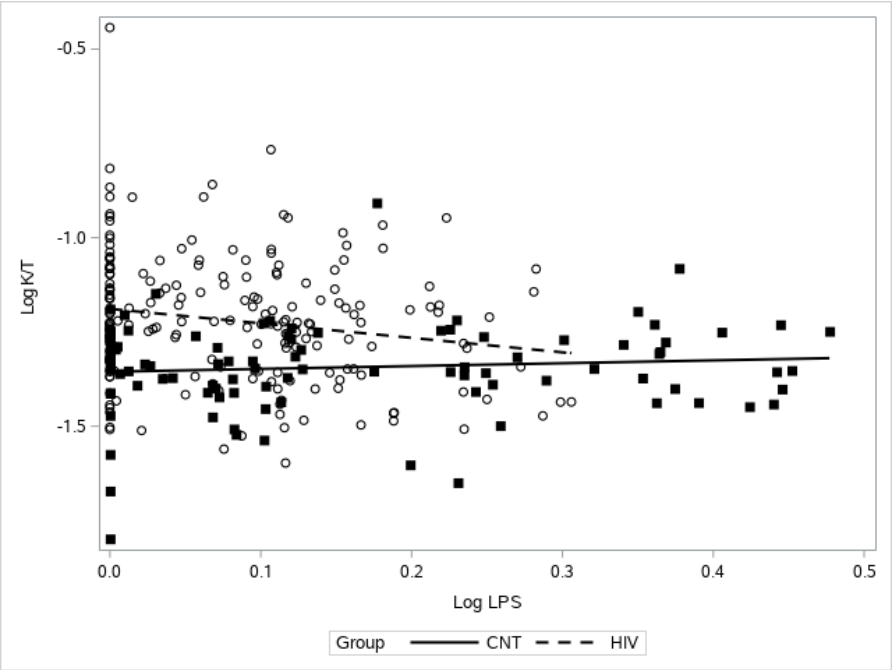

Figure 4. Association between 16S rDNA PCR and K/T