ASSSESSMENT OF IMMUNOPATHOGENIC RISK MARKERS FOR HIV ASSOCIATED NEPHROPATHY (HIVAN) IN ETHIOPIA

BY

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A THESIS SUBMITTED TO SCHOOL OF GRADUATE STUDIES OF ADDIS ABABA UNIVERSITY, DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND PARASITOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL MICROBIOLOGY

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By

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<tr>
<td>AAU</td>
<td>Addis Ababa University</td>
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<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
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<td>ACR</td>
<td>Albumin to Creatinine Ratio</td>
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<td>AIDS</td>
<td>Acquired Immuno Deficiency Syndrome</td>
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<td>APANs</td>
<td>APOL1 Associated Nephropathies</td>
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<td>APOL1</td>
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<td>Antiretroviral Therapy</td>
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<td>BUN</td>
<td>Blood Urea Nitrogen</td>
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<td>CBC</td>
<td>Complete Blood Count</td>
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<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
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<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
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<td>DMIP</td>
<td>Department of Microbiology Immunology and Parasitology</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>ESRD</td>
<td>End Stage Renal Disease</td>
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<td>FACScan</td>
<td>Fluorescent Activated Cell Sorter Scan</td>
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<td>FSGS</td>
<td>Focal Segmental Glomerulosclerosis</td>
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<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
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<tr>
<td>HAART</td>
<td>Highly Active Antiretroviral Therapy</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>HIVAN</td>
<td>Human Immunodeficiency Virus Associated Nephropathy</td>
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<td>Abbreviation</td>
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<td>IRB</td>
<td>Institutional Review Board</td>
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<td>KASPar</td>
<td>KBioscience Allele Specific PCR SNP assay</td>
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<tr>
<td>LDL</td>
<td>Lower Detection Limit</td>
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<tr>
<td>MALD</td>
<td>Mapping Admixture Linkage Disequilibrium</td>
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<td>MBL</td>
<td>Medical Biotech Laboratories</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>MYH9</td>
<td>Non Muscle Myosin Heavy chain 9</td>
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<tr>
<td>NASBA</td>
<td>Nucleic Acid Sequence Based Amplification</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>SNPs</td>
<td>Single Nucleotide Polymorphisms</td>
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<td>US</td>
<td>United State</td>
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<td>USRDS</td>
<td>United States Renal Data System</td>
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<td>VDRL</td>
<td>Venereal Disease Reference Laboratory</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Abstract

Background: HIV-1 infected patients are at risk of developing several types of chronic kidney disease, of which HIV-associated nephropathy (HIVAN) is the most prevalent. HIVAN is typically a complication of late stage HIV infection, associated with low CD4 cell counts and elevated serum HIV RNA levels. HIVAN is characterized: clinically by severe proteinuria and renal failure, and pathologically by a collapsing form of focal segmental glomerulosclerosis (FSGS), which rapidly progresses to end-stage renal disease (ESRD) in at risk individuals. Susceptibility to ESRD among HIV-infected individual, has been attributed to MYH9 E-1 and APOL1 G1 and G2 genetic variation. We determined the frequency of MYH9 E-1 and APOL1 G1 and G2 risk variants together with the prevalence of HIVAN among HIV infected individuals of Ethiopian population to determine whether the kidney disease genetic risk is all African or restricted to West Africa, and can explain the previously reported low risk of HIVAN among Ethiopians.

Objective: The objective of the study was to assess immunopathogenic risk markers for HIV-Associated Nephropathy in Ethiopia

Methods: We studied a cohort of 200 HIV-infected individuals (120 patients already on ART and 80 ART naïve patients) who were treated in ART clinic of Tikur Anbessa Teaching Hospital. We sought clinical evidence for HIVAN (serum creatinine > 1.4 mg/dl or proteinuria > 30 mg/dl at a spot urine sample). Genetic analyses include the genotyping of the MYH9 E-1 risk haplotype and APOL1 G1 and G2 risk markers (variants). Statistical analysis compared clinical and genetic indices for HIV-infected individuals of Ethiopian population and overall Ethiopians, in comparison to those reported for HIV-infected African Americans, overall African Americans, West Africans and non-Africans.

Results: In our study, none of the HIV-infected patients of Ethiopian populations showed clinical criteria for HIVAN. This absence of clinically apparent HIVAN was statistically significant difference from that reported for African Americans. The genetic indices showed that, 56% of HIV infected individuals of Ethiopians carried the MYH9 E-1 risk haplotype. This frequency of MYH9 E-1 risk haplotype is almost consistent with the frequency reported for African Americans and West Africans. The frequency of APOL1 G1 and G2 risk variants
were zero percent in all of the 200 HIV-infected individuals of Ethiopian population. Global ancestry and the frequencies of the G1 and G2 APOL1 risk variants are not statistically different from their frequencies in the general Ethiopian population, but are significantly and dramatically lower than those observed among HIV-infected African Americans, overall African Americans and West Africans.

**Conclusion:** Although 56% of HIV infected individuals of Ethiopian populations have MYH9 E-1 risk haplotype and that seems consistent with the previously reported African Americans and West African populations risk for HIVAN, the coinciding absence of HIVAN and the APOL1 risk variants (G1 and G2) among HIV-infected individuals of Ethiopians support a western and west central Africa, rather than all African ancestry risk for end stage renal disease, and can readily explain the lack of HIVAN among individuals of Ethiopian populations.

**Keywords/phrases:** HIVAN, ESRD, MYH9 E-1 risk haplotype, APOL1 G1 and G2 risk Markers, Serum creatinine, Proteinuria
1. Introduction

1.1. Background

Human immunodeficiency virus (HIV)-infected patients are at risk for developing several types of chronic kidney disease (CKD), of which HIV-associated nephropathy (HIVAN) is the most prevalent (Kopp et al., 2003). HIVAN is a kidney syndrome in HIV-1 seropositive patients, characterized clinically by severe or heavy proteinuria, kidney dysfunction and rapid progression to kidney failure, and pathologically by a collapsing form of focal segmental glomerulosclerosis (FSGS) (Kopp et al., 2003; Lu and Ross, 2005). It was initially described in 1984 by Rao et al., (1984) who reported a pattern of sclerosing glomerulopathy in HIV-1 seropositive patients in New York City. HIV-Associated Nephropathy became well recognized and soon was found to be the most common cause of chronic kidney failure and end-stage renal disease (ESRD) in HIV-infected Patients (Klotman et al., 1999; Kopp et al., 2003). It is now the third leading cause of end stage renal disease (ESRD) in African Americans between the ages of 20 and 64 years (Lu and Ross, 2005).

Most patients affected with HIVAN are of African descent. Blacks are 12 times more likely to develop HIVAN than non-black patients (Abbott et al., 2001). HIV-associated nephropathy (HIVAN), the classic kidney disease of HIV infection, occurs almost exclusively in patients of African descent, creating the potential for an epidemic of HIV-related kidney disease among the estimated 25 million people living with HIV in sub-Saharan Africa (UNAIDS, 2006). The association of HIVAN with African Americans (AAs) was recognized in multiple early studies (Lucas et al., 2004; Szczech et al., 2004). Among HIV infected patients, AAs had a 31- fold higher risk of ESRD compared with European Americans (EAs) (Lucas et al., 2007). The association with African ancestry has also been reported in Europe and Africa (Cohen and Kimmel, 2007). The exclusiveness of HIVAN to individuals of African descent implied the existence of genetic variation. A strong genetic predisposition exists for the development of HIVAN, with the overwhelming majority of cases occurring in individuals of African descent (Kiryluk et al., 2007).

Recently, scanning of kidney podocytes by mapping admixture linkage disequilibrium (MALD) identified DNA sequence variants (risk markers) within a region of chromosome 22 that are highly
associated with the increased prevalence of HIVAN and other common forms of chronic kidney disease in African heritage population (Kao et al., 2008; Kopp et al., 2008; Tzur et al., 2010). Almost two and half years ago, two published studies demonstrated a strong association of genetic variants in the MYH9 gene, which is located on chromosome 22, encoding the molecular motor protein non-muscle myosin heavy chain IIA, with HIVAN in African Americans (Kao et al., 2008; Kopp et al., 2008). These two studies used mapping by admixture linkage disequilibrium (MALD) in African Americans to identify the MYH9 gene, as a single major disease susceptibility locus for HIVAN. The association in these studies was strongest with a set of four single nucleotide polymorphisms (SNPs) Comprising the rs4821480, rs2032487, rs4821481, and rs3752462 which defined a risk haplotype, termed the E-1 haplotype (Kopp et al., 2008). These two studies showed that the MYH9 E-1 risk haplotype has a frequency of 60% in African Americans but only 4% in European Americans. But thus far causal mutations in MYH9 gene have not been identified (Nelson et al., 2010). However, recent availability of population based whole sequence information, enabled two other research teams (Genovese et al., 2010; Tzur et al., 2010) to independently and simultaneously discover the likely causative mutations in the neighboring APOL1 gene, which is located near to MYH9 gene on the same chromosome number 22. These two research teams indicated that, the trypanolytic APOL1 G1 (rs73885319 and rs60910145) and G2 (rs71785313) variants, common in people of African ancestry, were suggested to grant adaptive advantage in the face of current or past Trypanosoma brucei rhodesiense epidemics, whilst conferring risk for the group of Chronic kidney diseases in general and HIVAN in particular. The APOL1 risk markers (APOL1 G1 and G2) for renal disease occur in more than 30% of African-American chromosomes (Genovese et al., 2010).
1.2. Statement of the problem

In the United States (US), HIVAN became the third leading cause of ESRD (the near total loss of kidney function), in African American men between the ages of 20–64 years (USRDS, 1999). Also in the US, a study in the primary care setting found a prevalence of 3.5% among people living with HIV (Ahuja et al., 1999), while an autopsy series found it in 12% of renal biopsies (Shaninian, 2000). Given the regional burden of HIV disease, if these statistics were to be extrapolated to sub-Saharan Africa, between 770,000 and 2.6 million HIVAN cases would be predicted (Naicker, 2010; Fabian et al., 2009). One would expect such large numbers would be noticed, however, there are few data to suggest a similar prevalence of HIVAN in Africa. There is a paucity of data on the prevalence of HIVAN among population in sub-Saharan Africa. Han and colleagues found that 53% to 79% of kidney biopsies of HIV-positive black patients in South Africa demonstrated HIVAN (Han et al., 2006).

Initial clinical studies in large East Coast urban centers in the United States indicated that 10% of HIV-1–infected patients appear to develop renal disease, of which 90% showed clinical and/or pathological features consistent with HIVAN (Langs et al., 1990). Subsequent studies from France, Brazil, and Thailand confirmed this remarkable susceptibility to HIVAN in HIV-1–infected patients of African descent (Praditpornsilpa et al., 1999). A more recent autopsy-based survey reported that 12% of African-American patients dying of HIV-1 infection have histologically confirmed HIVAN (Shahinian et al., 2000). Since 1991, USRDS reports have included information regarding the prevalence of HIVAN in patients with end-stage renal disease (ESRD) and indicated that the incidence of acquired immunodeficiency syndrome (AIDS) in patients with ESRD increased steadily from 0.1% during 1987 to 1991 to 1.05% in 1999 (a renal biopsy was not mandatory for the diagnosis).

In a recent USRDS-based report of 3,653 patients with ESRD secondary to HIVAN during 1992 to 1997, HIVAN was the cause of ESRD more strongly associated than all others with African-American population affiliation (Abbott et al., 2003). This well-documented enhanced susceptibility of African Americans to HIVAN in HIV-1 infection is even greater than the predilection of the same group to hypertensive and diabetic renal damage (Fogo, 2003). A recent study reviewing
autopsies of HIV-1 seropositive patients found that among black patients, 12% had HIVAN at the time of death. The higher prevalence of HIVAN in this series may be due to advanced AIDS in many of these patients (Shahinian et al., 2000). With the continued increase of HIV-1 infection around the world; HIVAN is likely to become an increasingly prominent problem. In 2003 alone, more than 3 million people in sub-Saharan Africa were estimated to have been infected with the virus. Worldwide, only 7% of people who need anti-retroviral therapy have the means of obtaining it. This could translate into a large number of patients with end-stage renal disease due to HIVAN (UNAIDS, 2002). ESRD is a major and growing public health challenge. In the United States treatments such as, dialysis or transplantation are used to extend life of the patient while in most countries such modalities of renal replacement treatment are often not available, and the disease is fatal (USRDS, 2007).

In 2005, a US research team proposed that as the number of African-Americans living with HIV increased, such a scenario was likely to develop in the United States. Since HIVAN itself is not systematically monitored on a national level, the researchers used proxy data on end-stage renal disease (ESRD) among HIV-positive African-Americans to predict how ART use was likely to affect HIVAN prevalence between 2002 and 2020. Their mathematical model suggested that even with ART considerably slowing the rate at which people progressed to ESRD, there would still be a steady increase in the absolute number of HIV-positive African-Americans with ESRD over time (Schwartz et al., 2005). According to the most recent report from the United States Renal Data System (USRDS), which was the source of data for the 2005 study, overall HIV-associated ESRD incidence decreased 2% from 1996-1998 to 2006-2008, while prevalence more than doubled (USRDS, 2010). In addition, for unclear reasons, ART does not always prevent HIVAN from emerging or progressing, possibly because infected kidney cells may be a protected reservoir for the virus in individuals with HIVAN, or due to poor adherence to treatment. Finally, another factor to consider in resource-constrained settings where ART failure is more likely to be determined on clinical or immunological grounds, is that a much larger proportion of people on ART may have detectable viral loads for longer periods of time than in industrialized settings, which could increase the risk of HIVAN developing or progressing (USRDS, 2010).
Many studies have proposed that, the exclusiveness of HIVAN to individuals of African descent implied the existence of genetic variation. A strong genetic predisposition exists for the development of HIVAN, with the overwhelming majority of cases occurring in individuals of African descent (Kiryluk et al., 2007). Recently, two research groups (Kopp et al., 2008; Kao et al., 2008) have identified genetic risk markers (alleles) on chromosome 22 of kidney podocytes at MYH9 gene. These researchers indicated that among the many MYH9 risk variants, the MYH9 E-1 haplotype (comprises 4 SNPs: rs 4821480, rs 4821481, rs 2032487, and rs 3752462), which is very powerfully associated with the increased risk for HIVAN in African Americans that leads to ESRD. The population attributable risk for HIVAN in African Americans related to MYH9 E-1 haplotype is 70% with an odds ratio of 5.9 (Kopp et al., 2008). Population attributable risk reflects the percentage of disease that would disappear if the risk alleles were replaced by neutral alleles. In this case, 79% of HIVAN in African Americans (AAs) could be prevented if European derived or non African derived MYH9 variants were inherited. 60% of African Americans in the general population possessed 1 MYH9 E-1 risk allele compared with 4% of European Americans (EAs), and 35% AAs are homozygous for MYH9 E-1 risk alleles compared with only 1% of EAs. Ethnic differences in MYH9 genotype distributions seem to account for the excess risk of ESRD due to HIVAN (Kopp et al., 2008; Kao et al., 2008). The frequency of MYH9 E-1 risk haplotype in the general population of sub-Saharan Africa is in the range of 50 to 80%, where the highest value (80%) is found in Yoruba (West African population) (Oleksyk et al., 2010).

Very recently, the availability of population based whole sequence information, enabled two other research teams (Tzur et al., 2010; Genovese et al., 2010) to independently and simultaneously discover another genetic risk markers on the same chromosome 22 of kidney podocytes at APOL1 gene. The genetic risk markers of this gene were found near to MYH9 gene. And it was indicated that convergent human evolution under the powerful selection pressure of an infectious pathogen called Trypanosoma brucei rhodesiense, which is the etiologic agent of sleeping sickness (African trypanosomiasis), favored the spread in western Africa of two sets of APOL1 coding sequence variants (APOL1 G1 and APOL1 G2) which are highly associated with the kidney disease risk phenotypes previously attributed to MYH9, and biologically shown to confer protection from a lethal form of African sleeping sickness. The APOL1 G1 and G2 risk markers (alleles) for kidney disease occur in more than 30% of African American chromosomes (Giulio et al., 2010). Higher
frequency of APOL1 risk alleles also found in the Yoruba population of West Africa country (Tzur, et al., 2010).

Although HIVAN risk is an African problem, but to date the frequency of MYH9 E-1 risk haplotype and APOL1 G1 and G2 risk haplotypes in HIV-1 infected Ethiopian population have not been tested in association with HIVAN. Therefore, in this study we assembled a dataset of HIV-infected individuals from Ethiopia and we included 200 HIV infected individuals from ART clinic of Tikur Anbesa Teaching Hospital, Addis Ababa, Ethiopia. Following a careful clinical evaluation for HIVAN, the frequencies of the major MYH9 E-1 risk haplotype including their 4 SNPs (rs 4821480, rs 4821481, rs 2032487, and rs 3752462) and the frequencies of the APOL1 risk haplotypes (the G1 and G2 risk variants) were determined and the results were compared with allele frequencies in different countries.

2. Review of the literature

Epidemiologic data from the USA consistently demonstrate a significantly greater risk for end-stage Renal disease (ESRD) among African Americans (incidence rate of 1010 per million populations) compared with European Americans (incident rate 279 per million populations) (USRDS, 2008). This discrepancy is evident for many major etiologies of ESRD, including sporadic cases of focal segmental glomerulosclerosis (FSGS), non-diabetic ESRD associated with hypertension and HIV-1 associated nephropathies, and of these it was found that HIVAN has ≥18-fold increase risk in African Americans (AAs) as compared with European Americans and other non-African descent populations (USRDS, 2008; Kitiyakara et al., 2003). It has long been noted that African Ancestry population (e.g, African Americans) are more likely to develop kidney disease and have a poorer prognosis than their European descent counterparts. Family clustering of disparate etiologies of kidney diseases has also been reported in African American families (Freedman et al., 1995). In the United States (US), African Americans have approximately 3–4-fold higher rates of ESRD compared to European Americans (Coresh et al., 2007). Importantly, these discrepancies were shown to persist even after socioeconomic differences were considered, leading many to hypothesize that a population-based genetic predisposition, likely related to African ancestry, is responsible for the observed elevated risk for ESRD among African Americans. A similar discrepancy in ESRD incidence rates of lesser magnitude is also well documented for Hispanic
Americans (incidence rate of 520 per million populations) (USRDS, 2008). The reason behind the increased predilection among black persons for the development of HIV-associated nephropathy is not clear. In general, black persons have a higher incidence of other renal diseases (e.g., diabetic nephropathy, Hypertensive nephropathy); therefore, they may have an underlying genetic predisposition to severe renal disease, regardless of the etiology. The type of host response to the HIV infection itself may be what determines whether or not nephropathy develops in a specific individual (USRDS, 2008).

**Genetic factors in HIVAN**

Concordant with the above epidemiological data and the suggested hypothesis, two recently published studies demonstrated a strong association of genetic variants (risk markers or factors) in the MYH9 gene, which is located on chromosome 22, encoding the molecular motor protein non-muscle myosin heavy chain IIA, with HIVAN in African Americans (Kao et al., 2008; Kopp et al., 2008). These two studies used mapping by admixture linkage disequilibrium (MALD) in African Americans to identify the MYH9 gene, as a single major disease susceptibility locus for HIVAN. The association in these studies were strongest with a set of four single nucleotide polymorphisms (SNPs) comprising the rs 4821480, rs 2032487, rs 4821481, and rs 3752462 which defined a risk haplotype, termed the E-1 haplotype (Kopp et al., 2008).

Although many MYH9 single nucleotide polymorphisms (SNPs) were found to significantly associate with HIVAN, any of the three highly correlated SNPs: rs4821480, rs2032487, and rs4821481 in intron 23 plus rs3752462 in intron 13, defined an extended -1 (E-1) haplotype that was more informative than any SNP for association with kidney disease (Kopp et al., 2008; Nelson et al., 2010). The MYH9 E-1 risk haplotype was associated with HIVAN, Focal Segmental Glomerulosclerosis (FSGS), and non-diabetic ESRD (OR = 5.9, 2.8, 7, p<10^-8) (Kopp et al., 2008). The extended haplotype spans 14.9 kb, extending across two haplotype blocks that encompass introns 12–23. All of the MYH9 single nucleotide polymorphisms (SNPs) most strongly associated with kidney disease falls within this extended block (Kopp et al., 2008). The MYH9 E-1 haplotype explains nearly all of the excess burden of major forms of kidney disease in African Americans; for example, the attributable risks are 100% and 70% for HIVAN and FSGS, respectively. The association of MYH9 E-1 risk markers (alleles) with HIVAN is particularly worrisome for sub-
Sahara Africa where risk alleles are predicted to be at high frequency and more than 22 million adults are infected with HIV-1 (Oleksyk et al., 2010).

Kopp et al., (2008) and Kao et al., (2008) identified MYH9 genetic variants associated with HIVAN in independent African–American samples. Kopp et al., (2008) reported the MYH9 E1 haplotype conferred an odds ratio 5.9 for HIVAN and the E1 haplotype is present in 60% of African–Americans as compared with 4% of European Americans. Kao et al., (2008) estimated that the prevalence of HIVAN would be reduced by 70% if African–Americans had inherited European ancestry at the MYH9 locus.

The epidemiological association between individuals of African ancestry and higher incidence of ESRD due to HIVAN and others has also been reported outside of the USA (Anochie et al., 2008; Han et al., 2006) and in the case of HIVAN was further suggested to be specifically related to West African Ancestry. A subsequent analysis of the Human Genome Diversity Panel found the frequency of MYH9 E-1 risk haplotype followed a gradient, or cline, being most frequent within sub-Saharan African populations (range 50–80%), less frequent in populations from the Middle East (9–27%) and Europe (0–9%), and rare or absent in Asia, the Americas, and Oceania (Oleksyk et al., 2010). The frequency (distribution) of MYH9 E-1 risk haplotype including its 4 SNPs (rs4821480, rs2032487, rs4821481, and rs3752462) in different countries and populations is shown on (Table-1).
### Table - 1: The frequency of MYH9 E-1 haplotype and its pathogenic risk markers (alleles) in HIV infected individuals and general populations of different countries

<table>
<thead>
<tr>
<th>Group</th>
<th>Population</th>
<th>No</th>
<th>rs4821480 allele (%)</th>
<th>rs4821481 allele (%)</th>
<th>rs3752462 allele (%)</th>
<th>rs2032487 allele (%)</th>
<th>E1-haplotype Allele (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV infected individuals</td>
<td>African Americans</td>
<td>298</td>
<td>67</td>
<td>62</td>
<td>72</td>
<td>64</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>European America</td>
<td>125</td>
<td>4</td>
<td>4</td>
<td>31</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Africans</td>
<td>Bantu Kenya</td>
<td>12</td>
<td>67</td>
<td>67</td>
<td>17</td>
<td>NA</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Yoruba west Africa</td>
<td>25</td>
<td>88</td>
<td>80</td>
<td>12</td>
<td>NA</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>San</td>
<td>7</td>
<td>64</td>
<td>64</td>
<td>14</td>
<td>NA</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Mbuti</td>
<td>15</td>
<td>53</td>
<td>50</td>
<td>20</td>
<td>NA</td>
<td>50</td>
</tr>
<tr>
<td>East-Asia</td>
<td>Chinese</td>
<td>45</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Japanese</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>26</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Europe</td>
<td>French</td>
<td>29</td>
<td>5</td>
<td>NA</td>
<td>66</td>
<td>NA</td>
<td>5</td>
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<td></td>
<td>Italian</td>
<td>14</td>
<td>8</td>
<td>NA</td>
<td>50</td>
<td>NA</td>
<td>8</td>
</tr>
<tr>
<td>Middle east</td>
<td>Mozabite</td>
<td>30</td>
<td>19</td>
<td>20</td>
<td>52</td>
<td>NA</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Palestinian</td>
<td>51</td>
<td>14</td>
<td>21</td>
<td>55</td>
<td>NA</td>
<td>12</td>
</tr>
</tbody>
</table>

Note: NA – no data available; a, and b, represents source of references. a- stands for Kopp et al, 2008 and b-stands for Oleksyk et al, 2010
Recently, Pattaro et al., (2009) replicated the genetic association of serum creatinine levels with the MYH9 gene polymorphisms in European HIVAN individuals, indicating that this gene may influence kidney function in non- Africans as well. Interestingly, two of the SNPs reported by Kopp et al., (2008) to comprise part of the E-1 risk haplotype observed among HIVAN in African Americans, were also tested by Pattaro et al., (2009) but were not associated with elevated creatinine levels of ESRD due to HIVAN among Europeans. Cumulatively, these lines of evidence suggest a marked effect of MYH9 polymorphisms on kidney function or disease susceptibility. However, the question as to whether the same protective or risk polymorphisms similarly affect populations of various distinct geographic origins remains open and many subsequent studies did not address completely, the global distribution of MYH9 E-1 risk alleles in HIV infected individuals, and the historical reasons for this health disparity remained elusive.

By implication, the "MYH9-associated nephropathy" true causative variant should also be a prerequisite for HIVAN risk. In African Americans, typical estimate of HIVAN frequency among HIV positive individuals is about 3.5% (Ahuja et al., 1999). In contrast, previous research (Behar et al. 2006) has reported the absence of HIVAN in 126 HIV positive Israelis of Ethiopian origin, and have since confirmed this finding recently in a much larger cohort of Ethiopian HIV patients (unpublished data). It also appears that HIVAN risk, and by implication "MYH9-associated nephropathy" risk, is not an Africa-wide phenomenon, but rather restricted to certain regions of Africa, including parts of western, central and southern Africa, and virtually absent in parts of northeast Africa, such as Ethiopia (Tzur et al., 2010).

Nunez et al., (2010) recently identified that not all HIV infected patients who are at risk based on their MYH9 E1 alleles develop HIVAN although polymorphisms in the MYH9 gene are strongly associated with HIVAN in African Americans. These researchers then indicated that; because the majority of HIV infected MYH9 risk homozygotes do not develop nephropathy, so additional environmental and/inherited factors are clearly required.
Almost two and half years ago, mapping by admixture linkage disequilibrium (MALD) identified DNA sequence variants particularly the E-1 haplotype variant within a region of chromosome 22 that are highly associated with the increased prevalence of HIVAN (a common non diabetic forms of chronic kidney disease) in African heritage populations (Kopp et al., 2008; Kao et al., 2008 ). These researches first concentrated on the MYH9 gene as the most likely culprit gene to harbor a causative mutation, despite the causal mutations in MYH9 gene has not been identified so far (Nelson et al., 2010). However, they (Kopp et al., 2008; Kao et al., 2008) noted that the MYH9 gene was sitting almost immediately next to the APOL1 gene that encodes apolipoprotein L-I (APOL1), which is involved in the resistance to infection by Trypanosoma brucei, the cause of African trypanosomiasis or sleeping sickness in sub-Saharan Africa. And they suggested that the MYH9 might have been dragged along by its neighboring gene, rather than conferring a population benefit in itself.

As soon as that paper was published, just by considering the suggested hypothesis and by using the recent availability of population based whole sequence information, two papers came out showing that there are in fact two mutations in the APOL1 gene that are even more significantly associated with ESRD than all previously reported genetic variations in MYH9 that are common in African chromosomes but absent from European, Chinese, or Japanese chromosomes (Tzur et al., 2010; Genovese et al., 2010). Then the researchers examined the frequencies of these mutations in a sample set of 676 individuals from 12 African populations, including 304 individuals from four Ethiopian populations (Tzur et al., 2010). This was coupled with the corresponding distributions for the African ancestry MYH9 risk mutations. They found a pattern of reduced frequency of the APOL1 mutations in north eastern Africa, in contrast to most of the central, western, and southern African populations examined. “Especially striking was the complete absence of the APOL1 missense mutations in Ethiopia”. But the frequency of MYH9 risk variants in north eastern Africa was relatively high and was consistent to most of the central, western, and southern African populations. The frequency of APOL1 G1 and G2 risk variants in different countries and populations are shown on (Table-2).
Table-2: The frequency of APOL1 G1 and G2 pathogenic risk markers (alleles) in HIV infected individuals and general populations of different countries

<table>
<thead>
<tr>
<th>Group</th>
<th>Population</th>
<th>N</th>
<th>G1rs73885319 allele (%)</th>
<th>G1 rs60910145 allele (%)</th>
<th>G2 rs71785313 allele (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV infected individuals</td>
<td>African Americans</td>
<td>38</td>
<td>44.7</td>
<td>42.1</td>
<td>27.6</td>
</tr>
<tr>
<td></td>
<td>Hispanic Americans</td>
<td>10</td>
<td>55</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Americans</td>
<td>African Americans</td>
<td>148</td>
<td>20.9</td>
<td>20.4</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>Hispanic Americans</td>
<td>378</td>
<td>5.7</td>
<td>5.6</td>
<td>4.4</td>
</tr>
<tr>
<td>West-Central Africans</td>
<td>Cameron, Far North/ Chad</td>
<td>64</td>
<td>0.8</td>
<td>0.0</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Cameron (Somie)</td>
<td>65</td>
<td>16.4</td>
<td>15.3</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>Congo</td>
<td>55</td>
<td>10.9</td>
<td>9.3</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>Ghana (Asante)</td>
<td>35</td>
<td>40.9</td>
<td>41.2</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>Ghana (Bulsa)</td>
<td>22</td>
<td>11.4</td>
<td>11.4</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td>Nigeria (Yoruba)</td>
<td>59</td>
<td>46</td>
<td>45</td>
<td>7</td>
</tr>
<tr>
<td>Ethiopians</td>
<td>Afar</td>
<td>76</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Amhara</td>
<td>76</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Maale</td>
<td>76</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Oromo</td>
<td>76</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Other Africans</td>
<td>Malawi</td>
<td>50</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>Mozambique</td>
<td>51</td>
<td>12.0</td>
<td>12.0</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>Sudan</td>
<td>30</td>
<td>0.0</td>
<td>1.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Europe</td>
<td>Caucasian</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chinese and Japanese</td>
<td>Many ethnic groups</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: population shown in brackets describes ethnic groups. a, b, and c represents source of references. a-stands for Tzur et al, 2010. b-stands for Rosset et al. c-stands for Consortium TGP (2010).
Tzur et al., (2010) indicated that Convergent human evolution under the powerful selection pressure of an infectious pathogen, favored the spread in western Africa of two sets of APOL1 coding sequence variants which are highly associated with the kidney disease risk phenotypes previously attributed to MYH9, and biologically shown to confer protection from a lethal form of African sleeping sickness. According to this report the two West Africa sets of APOL1 G1 risk variants (rs73885319 and rs60910145) are missense mutations in the last exon of the APOL1 gene (S342G and I384M) respectively is a neighboring gene, located 14 Kbp 3’ down stream from MYH9, and demonstrate that these are more strongly associated with ESRD due to HIVAN than previously reported MYH9 variants (the E-1 risk haplotype).

The genetic basis for the well documented increased susceptibility to Focal Segmental Glomerulosclerosis, HIVAN, and hypertension-related ESRD among African and Hispanic Americans was recently attributed to their shared African ancestry (USRDS, 2009; Kopp et al., 2008; Shlush et al., 2010). Specifically, the trypanolytic APOL1 G1 (rs73885319 and rs60910145) and G2 (rs71785313) variants, common in peoples of African ancestry, were suggested to grant adaptive advantage in the face of current or past Trypanosoma brucei rhodesiense epidemics, whilst conferring risk for the group of kidney diseases noted above, which have been designated as APOL1-associated nephropathies (APANs) (Genovese et al., 2010; Tzur et al., 2010). While the association between the G1 and G2 APOL1 variants and APAN susceptibility was confirmed in two independent surveys of African Americans (Genovese et al., 2010; Tzur et al., 2010) and an additional set of Hispanic Americans (Tzur et al., 2010), their phylogeographic distribution in a global set of healthy individuals was pivotal in understanding their evolution in the context of African population genetics. The G1 and G2 APOL1 variant frequencies obtained from the 1000 Genomes Project sequences (Consortium TGP, 2010) demonstrated that the APOL1 G1 and G2 risk alleles (rs73885319, rs60910145, and rs71785313) are frequent in West African Yoruba while absent among European, Japanese, or Chinese individuals. Moreover, an independent dataset comprised from West-Central and East African Ethiopian population showed a within African genetic structure with the G1 risk alleles restricted to west African population and absent in the Ethiopian population (Tzur et al., 2010). The frequencies of the G1 and G2 risk alleles among African Americans with APANs were similar to those observed in their presumed ancestral West
African populations (Tzur et al., 2010; Rosset et al., 2010). A similar comparison for HIV-infected individuals of Ethiopian ancestry is still missing in the literature.

Among the spectrum of APOL1 associated nephropathies (APANs), the increased risk among African Americans is greatest for HIV-associated nephropathy (18 to 50 fold) (Eggers et al., 2004; Kopp et al., 2003). While comparable epidemiologic data for continental Africa is incomplete (Katz et al., 2010; Naiker et al., 2010), a glimpse into the epidemiology of HIVAN in East Africa is available from the reported absence of HIVAN among HIV-infected Israeli Ethiopians (Behar et al., 2006). And in this report the researchers considered that host genetic variation could contribute for HIVAN. Demonstration that the G1 and G2 alleles of APOL1 confer risk also for HIVAN among African Americans (Kopp et al., 2008; Tzur et al., 2010; Rosset et al., 2010), together with the reported zero frequency of these alleles in a sample set of 304 healthy Ethiopians (Tzur et al., 2010), strengthens the host genetic variation supposition. However, a formal genetic survey of the frequency of G1 and G2 risk alleles, together with assessment of kidney function in the same cohort of HIV-infected individuals of Ethiopian origin is needed to support this formulation. So, if significant frequencies of G1 or G2 risk alleles among Ethiopian HIV-infected individuals, not demonstrating clinical signs of HIVAN, would undermine our current understanding of the role of these risk alleles in the pathogenesis of HIVAN and by extrapolation the other APO Lipoprotein-1 Associated Nephropathies (APANs).
2.1. Pathogenesis of HIVAN

The pathogenesis of kidney disease associated with HIV infection is not completely understood. Researchers have been working to find whether HIVAN is caused by a direct effect of HIV-1 infection of renal parenchymal cells or an indirect effect of HIV-1–mediated immune dysregulation. The HIV genome has now been recovered from kidney tissue of HIV-infected patients with both HIVAN and healthy kidneys (Rappaport et al., 1994). Although HIV-1 viral messenger RNA and DNA have been detected in renal glomerular and tubular epithelial cells, the mode of HIV-1 viral entry into kidney cells has not been determined (Bruggeman et al., 2000). Renal epithelial cells lack both CD4 and CD4 co receptors (CXCR4 and CCR5) used by HIV-1 for cellular entry (Eitner et al., 2000). However, the expression of CXCR5, an HIV-2 co receptor, has been shown on podocytes (Huber et al., 2002). A “renal reservoir” of HIV-1 has been described during primary viral infection (Winston et al., 2001). Renal biopsies have revealed restoration of the architecture of the renal tubules and resolution of podocyte hypertrophy and glomerular collapse three months after the initiation of antiretroviral therapy. The number of podocytes expressing viral messenger RNA was unchanged. Similarly, Bruggeman et al., (2000) observed replication of HIV in renal cells of individuals with HIVAN despite treatment with antiretroviral agents and undetectable serum viral loads.

Local replication of HIV-1 in renal tubular cells has been confirmed (Marras et al., 2002). DNA extracted from renal tubular cells in 2 patients with HIVAN was used to amplify HIV-1 V3-loop or gp120 envelope sequences. Sequences were compared with the corresponding sequences in monocytes obtained from these same individuals. Sequences obtained from kidney tissue formed tissue-specific subclusters, suggesting local (renal) replication of HIV-1 (Marras et al., 2002). Normally, podocytes are postmitotic and do not proliferate. In HIVAN, podocytes appear to dedifferentiate and proliferate. Proliferation of dedifferentiated podocytes has been proposed as a common mechanism in the development of FSGS and HIVAN (D’Agati, 2008).

Nef is the major candidate of the many HIV-1 gene products that might be responsible for the collapsing glomerulopathy of HIVAN. A large body of evidence suggests that an abnormal response of podocytes to HIV-1 infection and/or HIV-1 proteins is the key event in HIVAN Pathogenesis (Sunamoto et al., 2003; Ross et al., 2003).
Recent results showed that HIV can directly infect the podocyte, and a series of interrelated pathways leads to loss of the differentiated phenotype with extensive proliferation and resultant glomerular collapse (Papeta et al., 2009). Others suggested that HIV-1 specific gene products may be involved in this process and interact with MYH9 gene. It also remains possible that other environmental and genetic co-factors are second hits necessary for the development of podocyte proliferation which results in HIVAN (Meredith et al., 2010).

2.2. Epidemiology of HIVAN

HIVAN, the classic kidney disease of HIV infection, occurs almost exclusively in patients of African descent, creating the potential for an epidemic of HIV-related kidney disease among the estimated 25 million people living with HIV in sub-Saharan Africa (UNAIDS 2006). Blacks are 12.2 times more likely to develop HIVAN than non black patients (Shahinian et al., 2000). HIVAN is the third leading cause of renal failure in African Americans aged between 20 and 64 years (Winston and Klotman, 1996). The disease usually presents late in the course of HIV infection (Winston et al., 1999). In untreated patients, HIVAN causes a rapidly progressive renal function deterioration often resulting in End Stage Renal Disease (ESRD) within 2 to 4 months of diagnosis (Carbone et al., 1989).

The prevalence of CKD in the various stages of HIV infection is difficult to assess. Proteinuria and elevated creatinine level have been found in 7% to 32% of HIV-seropositive patients and were associated with an increased rate of death in a study of 2038 female HIV infected patients (Szczech et al., 2004). The estimated prevalence of HIVAN has ranged from 3.5% in clinical studies to 12% in autopsy studies (Ahuja et al., 1999). There is a paucity of data on the prevalence of HIVAN among population in sub-Saharan Africa. Han and colleagues found that 53% to 79% of kidney biopsies of HIV-positive black patients in South Africa demonstrated HIVAN (Han et al., 2006). The incidence of HIVAN peaked in the United States during the mid-1990s (Ross and Klotman, 2002) and declined by 50% in the 1998 to 2001 time period, relative to 1995 to 1997, in association with the widespread use of HAART (Lucas et al., 2004). Although the incidence of HIVAN decreased during the HAART era, its prevalence is now increasing because of the aging of patients as a result of improved survival among those with HIV infection. The true prevalence of HIVAN
remained unknown because the diagnosis requires viral testing and renal histological analysis; more cases likely exist than are reported (Lucas et al., 2007).

2.3. Clinical presentation and Diagnosis of HIVAN

HIVAN is most often associated with low CD4 cell counts and elevated serum HIV RNA levels (Lucas et al., 2008; Berliner et al., 2008). However, it can also develop in patients with undetectable viral loads and high CD4 cell counts (Szczech et al., 2004; Izzedine et al., 2005). Patients are usually diagnosed when they have advanced kidney failure with severe proteinuria (Carbone et al., 1989). Despite the presence of heavy proteinuria, peripheral edema and hematuria are uncommon. Hypertension is also surprisingly rare in most patients with HIVAN, leading some to speculate that HIVAN is a salt-wasting disease (Abbott et al., 2001). There are no specific serologic or urinary markers for HIVAN. Co-infection with hepatitis B and C is common and should be considered in all patients suspected to have HIVAN, because they are associated with membranous nephropathy and membrano proliferative glomerulonephritis, respectively.

Kidney biopsy is the only test that can reliably diagnose the presence of HIVAN. But it is important to note that the definition of HIVAN does not mandate a formal renal biopsy, which is preferable, but usually not feasible (Bahar et al., 2006). So, the clinical definition used for HIVAN by Bahar et al., (2006) was: renal damage (i.e. plasma creatinine > 1.4 mg/dl, microalbuminuria > 30 mg/dl on a spot urine sample and GFR < 90 ml/min/1.73 m$^2$) and glomerular proteinuria with protein greater than 2 g/day in HIV-seropositive patient, without other predisposing factors for kidney disease, such as diabetes or hypertension, and with negative serological test results for hepatitis B and C. Histologically, proliferating glomerular epithelial cells are the prominent feature of the disease. The biopsy diagnosis in only 55-60% of patients is confirmed to be HIVAN by renal biopsy.

Renal ultrasound generally shows echogenic kidneys that are normal-to-large in size (Abbott et al., 2001). HIV-associated nephropathy (HIVAN) is commonly presents with the following sonographic findings: Enlarged, globular kidneys with marked echogenicity in a heterogeneous pattern, loss of corticomedullary definition, and obliteration of renal sinus fat. These findings may be more prominent in setting of clinical AIDS. HIV-positive patients may have HIVAN according to diagnostic criteria but still have few if any of the classic sonographic signs. According to the study by Di Fiori et al., only 20% of HIVAN patients present with enlarged kidneys and only 38% with
decrease of corticomedullary definition. Increased echogenicity (greater than liver and spleen) and a heterogeneous echopattern were the most common findings; these are seen in ~75% of cases. Globular morphology and loss of renal sinus fat were found in about half of cases. These classic findings are typically more apparent with more severe disease. The increased echogenicity associated with HIVAN often presents in characteristic patterns. Diffuse echogenicity is seen, but bands or patchy areas are more common. The latter pattern may be striking - bands of increased echogenicity may extend from the central kidney to the cortex.

2.4. Treatment and Prevention of HIVAN

Pharmacologic agents (drugs) that have been used in the treatment of HIVAN include: Angiotensin-Converting Enzyme (ACE) inhibitors, Corticosteroids, Cyclosporine, and Highly Active Antiretroviral Therapy (HAART). With any of these pharmacologic strategies, the goal of treatment is directed toward reducing HIV-1 replication and/or slowing the progression of kidney disease (Salman et al., 2006). Despite significant advances in the treatment of HIV infection, currently available therapy for HIV-Associated Nephropathy appears to be only partially protective. The widespread introduction of antiretroviral therapy (ART) was associated with an initial decrease in the incidence of HIV related end-stage renal disease, but there are still more than 900 new cases of end-stage renal disease attributed to HIV each year in the US (USRDS, 2000). Over the short-term, studies have also suggested that ART regimens that successfully suppress viral load, can temporarily at least improve renal function and may interrupt the course of severe conditions such as HIVAN. At the same time, however, it should be noted that ART may not, on its own, be adequate to interrupt the continuum of gradually worsening kidney disease once it has become established. For instance, a review of death certificates found that the percentage of deaths in HIV-infected people attributed to kidney disease actually increased from 6.3% to 9.1% in the US between the years 1995 and 2000 (after the ART era had begun) (Selik et al., 2002).

Treatment options (renal replacement therapies) for patients who have reached ESRD include hemodialysis, peritoneal dialysis, and renal transplantation (Naicker et al., 2006). Patients with HIVAN who progress to ESRD remain a clinical challenge. Physicians must anticipate progressive renal disease in patients with HIVAN and have as a goal the placement of an arteriovenous fistula in a timely manner for future use in hemodialysis. In current practice, hemodialysis is the accepted
modality of ESRD therapy in these patients (Salifu et al., 2010). Before the introduction of HAART, an analysis of the USRDS showed that HIV-infected recipients were at increased risk of death and graft loss compared with uninfected recipients of cadaver kidneys. As the survival of HIV infected patients has improved remarkably in the HAART era, renal transplantation as a form of renal replacement therapy is now being offered to HIV-infected patients with ESRD in some centers (Naicker et al., 2006). Analysis of USRDS transplant populations indicates that kidney transplantation in HIV-infected patients is plausible and ongoing. However, the appropriate use of immunosuppression and HAART still awaits the results of properly designed prospective clinical trials (Naicker et al., 2006).

As HIV infection is a risk factor for the development of Chronic Kidney Disease (CKD) such as HIVAN, the recommendations of the HIV Medicine Association of the Infectious Diseases Society of America suggesting screening for CKD in HIV-infected patients should be implemented (Gupta et al., 2005); screening tests should be similar to those for patients with diabetes mellitus to detect early renal involvement. Early detection and treatment of HIVAN to prevent or delay progression to ESRD is crucial. Preventive strategies need to be determined; prospective studies including ART, ACE inhibitor, and other therapeutic agents are required as a matter of urgency for developing countries in Africa, where the high burden of HIV disease coexists in stark contrast with limited healthcare resources (Naicker et al., 2006).
3. Significance of the Study

HIVAN is the third leading cause of end-stage kidney disease in the African-American population in United States. The incidence of renal involvement in Ethiopia and other African countries in individuals infected with HIV is not well documented. Furthermore, there is little information with respect to the efficacy of HAART treatment in diminishing renal involvement, either with respect to the timing of introduction of such therapy and prevention of HIVAN. The recent discovery of an important risk markers (alleles) for renal involvement in HIV infected individuals, whose frequency is greatly elevated in individuals of African ancestry, will allow more rational therapeutic approaches, and increase our understanding of the pathogenesis and suggest potential preventive and therapeutic approaches to the renal complications of HIV infection. This is of particular importance in those countries where renal replacement therapy by dialysis and transplantation for individuals who progress to end-stage kidney disease is not widely available.
4. **Hypothesis and Objectives of the study**

4.1. **Hypothesis**

- By considering the reported absence of HIVAN among HIV infected Israeli Ethiopians, we hypothesized that the Ethiopian HIV infected population is not at risk of developing kidney disease despite carrying the genetic MYH9 E-1 risk variants and this could be explained by the absence of identified risk markers in the APOL1 gene.

4.2. **Objectives of the study**

4.2.1. **General Objective**

- To identify the genes as immunopathogenic risk markers for HIV-Associated Nephropathy in Ethiopia.

4.2.2. **Specific Objectives**

1. To determine the prevalence of HIV Associated Nephropathy in Ethiopia.

2. To identify the genes of genetic risk markers for HIVAN, and to study its frequency.

3. To assess the correlation of HIVAN with the risk markers, the clinical treatment history and disease studies as determined by CD4 counts and viral load, and demographic parameters.
5. Materials and methods

5.1. Study Design and Study Period

The study design was a cross-sectional study in patients attending the ART clinic at Tikur Anbessa Teaching Hospital, Faculty of Medicine, Addis Ababa, Ethiopia. The study period was from January 30/2009 to December 1/2010.

5.2. Study Area and Study population

Patients were identified from the ART/HIV Clinic at Tikur Anbessa Teaching Hospital, Addis Ababa, Ethiopia. The hospital is located in the major metropolitan area and has many functional clinics, among this the HIV clinic has a schedule of six days per week for follow up patients and during this period 1000 patients are expected to visit the clinic. A total of 200 HIV-infected individuals seen for routine clinic visits were enrolled in the current study. Records from the ART clinic were reviewed to identify patients who meet clinical and laboratory criteria for entry and these patients were sequentially offered entry into this study. The objectives of the study were explained to all and consent obtained prior to any study procedures. Specimens were obtained from ART naïve patients and from those who are already on ART treatment. In addition, non-HIV-infected individuals (control groups) were enrolled. Here, control groups were identified as apparently healthy HIV-negative adults selected from the VCT-center of Tikur Anbessa Teaching Hospital and were matched with sex, age, as well as, other demographic parameters to the study participants. i.e. during the enrolment period the mean age of controls were 36.2 ± 8.1, the mean body mass index (BMI) were 18 ± 6.5, males and females were 44% and 56% respectively. Control groups with kidney diseases were not excluded.

5.3. Sample size

The sampling technique used is convenient sampling method. Considering tolerable type I and type II errors and by assuming a mean enrolment rate of >90% and an alpha error level of 5% and beta level of 80%, the needed sample size will be minimum of 60 in each patient group. Thus we enrolled a total of 200 HIV positive patients (120 patients already on ART and 80 ART naïve patients). Moreover we included 50 HIV negative healthy individuals that were controls.
5.4. The inclusion criteria

- Individuals with known HIV infection of greater than 3 months duration
- Follow-up at the ART clinic using the clinical data
- Signed informed consent
- Age ≥ 18 years

5.5. The exclusion criteria

- Known other causes of kidney disease or established kidney disease unrelated to HIV status
- Known hepatitis positive state
- Known VDRL positive state
- Pregnant women

5.6. Clinical and Socio-demographic data collection

Clinical and Socio-demographic data regarding each patient’s sex, age, temperature, pulse, blood pressure, body mass index (BMI), ethnicity, and parameters of special interest such as; known duration of HIV infection (time since diagnosis of HIV), Known duration of AIDS (time since diagnosis of AIDS), medical records of kidney disease and factors known to contribute to kidney disease, known systemic complications, AIDS status, as well as, treatment regimen status were collected using standardized questionnaire or data sheet format (see appendix-1). Subjects with a prior diagnosis of diabetes mellitus, hypertension, and known non-HIVAN kidney disease were excluded from the study. For each patient encounter, a numbered code was assigned, and only the coded number was available and thus all testing and information were kept confidential.
5.7. Laboratory methods

Routine preliminary laboratory studies were utilized: Complete Blood Count (CBC), CD4 and CD8 count, urinalysis dipstick test, kidney function indices (BUN & Creatinine tests), viral load test, hepatitis B and C tests, VDRL test, DNA extraction and genotyping.

5.7.1. Sample collection

5.7.1.1. Urine sample collection

Urine specimen was collected in a clean, sterile, leak-proof, and wide mouthed plastic container. 10 ml of urine sample was collected for the determination of urine microalbuminuria (this test was performed in the ART clinic at Tikur Anbessa Teaching Hospital).

5.7.1.2. Blood sample collection

Blood (whole blood) specimen was collected in EDTA containing vacutainer tube. Taking all aseptic precautions, 10 ml of blood sample was drawn then transported to Medical Biotech Laboratories (MBL) and the following tests were conducted for each enrolled subject: CBC, CD4 count, viral load count, plasma creatinine and BUN serology, hepatitis B and C serology, VDRL serology, DNA extraction and genotyping of the genetic MYH9 E-1 and APOL1 G1 and G2 risk markers status.

5.7.2. Sample preparation

Whole blood specimen was processed as follows (see figure-1): First it was centrifuged at 3500 rpm for 5 minutes. After centrifugation, the blood was separated into three components (Plasma on top, Buffy coat at the middle, and Packed RBC at the bottom of the tube).

Plasma was collected from the tube using plastic pasteur pipette and transferred to another clean test tube then the following tests were performed: BUN and Creatinine test, hepatitis B and C test, VDRL test, and viral load test.

The Buffy coat layer was separated from the tube by using plastic pasteur pipette. About 750 micro litter of Buffy coat was aspirated from the middle of the tube then transferred into a 1.5 ml micro
centrifuge tube (plastic vial) to be preserved at -20 °C. The stored buffy coat samples were then shipped in ice crystal to Molecular Medicine Laboratory in Rambam Health Care Campus and Rappaport Faculty of Medicine and Research Institute at Haifa, Israel, for DNA extraction and genotype determination.

5.7.3. Test procedure

5.7.3.1. Urinalysis test

Urinalysis was performed by using standardized chemstrip reagent strips (Roche Diagnostics, Mannheim, Germany). For those urinalysis test results of 1+ and 2+, urine albumin: creatinine ratio (ACR) was measured from the spot urine sample collected.

5.7.3.2. Complete Blood Count (CBC) test

CBC tests were determined using an automated hematology analyzer (Human 6700 version culter counter). Using this analyzer CBC tests such as: White blood cell (WBC) test, Differential count test, and hemoglobin (Hgb) tests were determined.

5.7.3.3. BUN and creatinine test

Plasma Creatinine and BUN tests were measured by spectrophotometer using Roche 5010 automated chemistry analyzer and Roche reagent kits (Roche Germany).

5.7.3.4. Hepatitis B, C and VDRL serology test

Hepatitis B, C and VDRL serology tests were done using methods based on Enzyme Immuno assay (EIA) kits supplied by manufacturers.

5.7.3.5. CD4+ T-cell count test

CD4+ T-cell counts were determined by FACScan (Becton Dickinson). MBL participates in External Quality Assurance Scheme with QASI, Health Sante, Canada, for CD4 count enumeration.
5.7.3.6. Viral load (HIV-1 RNA quantitation) test

As soon as plasma was extracted from whole blood 2-ml aliquots of plasma stored at -20 °C for viral load testing. HIV-1 viral load testing was done in batch and was not available immediately for real time monitoring of Antiretroviral (ARV) treatment, but results were communicated to the ART Clinic there after. Plasma viral load tests were done using the Nucleic Acid Sequence Based Amplification (NASBA) technology. NASBA assay was carried out according to manufacturers’ instructions, summarized below: HIV-1 RNA was extracted from 1.0 ml of plasma using a lysis buffer containing Guanidine Thiocyanate. Three synthetic RNA calibrators (Qa, Qb, Qc) of known high (10^6), medium (10^5), and low (10^4) concentrations (in copies/ml), respectively, were added. Each calibrator contained a short unique sequence differing from the HIV-1 wild type. RNA was extracted by using acidified silica, according to the procedure described by Boom et al., (1990). HIV-1 and calibrator RNA sequences were co-amplified by incubation of the extraction product with the primers and the enzyme mixture (Reverse Transcriptase, RNase H and T7 RNA Polymerase) at 41°C for 90 min. The RNA concentration of HIV-1, Qa, Qb and Qc amplicons were measured in four separated tubes. In each tube, the amplified RNA was captured with a biotin-labeled oligonucleotide bound to streptavidin-coated magnetic beads acting as the solid phase, and then hybridized with a specific ruthenium-labeled probe complementary to the HIV-1, Qa, Qb or Qc specific sequence. The magnetic beads carrying the hybridized amplicon-probe complex were captured on the surface of an electrode by means of a magnet. Voltage applied to the electrode triggered the electroluminescence reaction, in the presence of a buffer containing Tripropylamine. The light emitted by the ruthenium-labeled complex, measured by a photo - multiplier valve, was proportional to the amount of amplicon. Calculation of the relative amount of the four amplicons revealed the amount of HIV-1 RNA in the sample.

5.7.3.7. DNA extraction and genotype test procedure

These analyses were conducted in Israeli collaborating center in Haifa starting from July 2010 to September 2010. To do these procedures, the M Sc candidate first traveled to Israel caring a total of 250 buffy coat samples keeping them in ice crystal. After the samples reached to Rambam medical center laboratory of Haifa, Israel, DNA was first extracted from each sample then the MYH9 E-1, APOL1 G1 and G2 risk alleles were determined using KASPar (Kbioscience Allele Specific PCR
SNP genotyping assay) methodology by the principal investigator under supervision of the trained experts.

5.7.3.7.1. DNA extraction procedure

DNA from the buffy coat was extracted for PCR by the following procedure (Roche applied science kit, 2005): First the buffy coat samples were kept at room temperature to defreeze. DNA was then extracted using the Mag NA Pure LC 1.0 extraction instrument (Roche.cat.no.12 236 931 001) and its reagents. The instrument is a robotic work station which is used for automated isolation of nucleic acids (DNA, total RNA, mRNA) from different kinds of crude sample materials (whole blood, buffy coat, culture cells, tissue, bacteria or fungi). It uses a specially designed Mag NA Lc reagent kits that contain, buffers that are important for removal of PCR inhibitors, salts and proteins. It also contains lysis and binding buffers used for cell lysis and binding of total nucleic acids. A proteinase K containing buffer was used for digestion of proteins. A magnetic particle suspension was used for binding of total nucleic acids. An elution buffer was used which is important for elution of total nucleic acids, dilution of elutes and for reconstitution of Proteinase K. A 200µl of the thawed buffy coat specimen was pipetted into the sample cartilage which can hold 32 samples and put into the instrument after all the extraction reagents are ready in the instrument. The total time for automated purification of 32 samples including positive and negative controls was ~90 minutes. After genomic DNA was obtained from the buffy coat samples using the standard method mentioned above, the KBioscience Competitive Allele-Specific PCR SNP genotyping system (KasPar) was used to genotype all mutations reported herein. The 7 SNPs ( allelic mutations) evaluated by this method were the: rs4821480, rs2032487, rs4821481, rs3752462, rs73885319, rs60910145 and rs71785313.

5.7.3.7.2. DNA genotyping procedure

For MYH9 E-1 and APOL1 G1 and G2 risk marker status, the competitive allele-specific PCR SNP assay (KASPar) in routine use by the Israeli team was used. This assay utilizes FRET quencher cassette oligonucleotides, with sequences corresponding to detection of SNP addresses: rs4821480, rs2032487, rs4821482, and rs3752462 comprising the MYH9 E-1 haplotype, rs73885319 and rs60910145 comprises the APOL1 G1 haplotype, and rs71785313 comprising the APOL1 G2
haplotype. The KASPar methodology (Petkov et al., 2004) is a novel homogeneous fluorescent genotyping system. It has been developed at KBiosciences and is currently in use on a daily basis. KASPar offers the simplest, most cost-effective and flexible way to determine SNP genotypes. Analysis can be carried out in a variety of formats and the chemistry has been shown to function well in 96-, 384- and 1536-well plate formats. The system is comprised of two components, the Assay Mix (consists of three unlabelled primers; this is the SNP-specific component of the system) and the Reaction Mix (all other components required including the universal fluorescent reporting system). The KASPar assay system relies on the discrimination power of a novel form of competitive allele specific PCR to determine the alleles at a specific locus within genomic DNA for SNP typing. We genotyped the SNPs as follows: First primer design was made using the primer picker software. Then 2 micro litter DNA sample was added in a 384 micro titer plate. Then the assay mix that comprises the 3 unlabeled primers was combined to the reaction mix. Both of the combined assay mix and reaction mix were dispensed over DNA samples. After that the micro titer plates were sealed with optically clear seal. Then thermal cycled in a hydrocycler (PCR thermal cycler). After PCR product was obtained, the KASPar data was read by Fluorescence Resonance Energy Transfer (FRET) capable plate reader with the relevant filters (KASPar uses the fluors FAM and VIC for distinguishing between genotypes and ROX as a passive reference). Finally the FAM and VIC data were plotted on the X and Y axis, respectively. Genotypes can then be determined according to sample cluster.
Fig - 1 Flow chart of laboratory analysis scheme

Sample

Urine

whole blood

(For microalbuminuria)

Strip test

plasma

Buffy- coat (stored at -20 °C)

for CD4/CD8/CD3

and CBC testing

frozen at -20 °C for

HIV-1 viral load

BUN and Creatinine

Hepatitis B and C serology

VDRL test for syphilis

For E-1, G1, and G2 haplotype determination
5.8. Data management and Statistical analysis

All laboratory and clinical data were recorded on predetermined questionnaires during the study period. Each completed questionnaires were properly coded and key was prepared for each code. All data were first entered into an excel spreadsheet, cleaned, verified and then transferred for statistical calculation in SPSS 16.0 database. Throughout the study, double data entry were undertaken in order to assure quality of the data.

Our analysis of prevalence was based on viewing the number of HIVAN cases in a group of HIV carriers as a Poisson distributed random variable, with rate $\lambda=p*n$, where $n$ is the number of HIV carriers and $p$ is the HIVAN risk for a carrier from this group. We were interested in performing inference on $p$ and comparing it between populations. In particular, since we assume $n$ is given, we can calculate confidence intervals for $p$ based on confidence intervals for $\lambda$ generated with standard approaches (Johnson et al., 1969). This gives us a range of prevalence’s that are consistent with our observed prevalence.

Statistical analysis compared clinical and genetic indices for HIV-infected individuals of Ethiopians, in comparison to those reported for HIV-infected African Americans, overall African Americans, West Africans and non-Africans.

5.9. Ethical Considerations

The study was reviewed and approved by the Research and ethics committee of the Department of Microbiology, Immunology and Parasitology (DMIP), Department of Internal Medicine and the IRB of the Medical Faculty, Addis Ababa University and the IRB of the Rappaport Faculty of Medicine and Research Institute. Written informed consent was obtained from all the study participants (appendix-1), thumb prints were taken from those study participants who can not write. The consent form was prepared in Amharic (the local language) which was attached on the participant’s medical records. The privacy and confidentiality were maintained for each procedure.
6. Results

6.1. Clinical and Socio-demographic characteristics of the study population

The clinical and Socio-demographic characteristics of HIV infected population of Ethiopians examined in Tikur Anbesa Teaching Hospital is listed in Table-6.1. Of 200 patients screened for renal disease, the mean patient age was 38.1±9.3 years. Men and women accounted for 43.2% and 56.8% of the study populations, respectively. The mean patient body mass index (BMI) was 18.5 ± 7.1. Information regarding mode of HIV transmission was not available. The average time since diagnosis of HIV (known duration of HIV) was 3.7±2.4 years. A total of 77.2% were diagnosed as having AIDS. The average time since diagnosis of AIDS (Known duration of AIDS) was 3.5±2 years. Viral loads and CD4 cell counts were evaluated separately for treated and untreated patients. Most patients (60%) were treated with HAART at the time of kidney disease screening. The mean CD4+ T-cell count for treated patients and untreated patients were 362±178 cells/ml and 263±164 cells/ml, respectively. 70.1% of HIV-1 infected patients who were treated with ART had HIV-1 RNA (Viral load counts) below the lower limit of detection (LDL). Mean viral load count in copies/ml for treated and untreated patients were 8438±985 and 186,103±143,581 respectively. In this population, the effect of HAART was also clearly visible in significantly reduced viral loads both in terms of mean count and percentage of patients below detection level (p<0.0001). And of 200 HIV infected patients evaluated, none fulfilled clinical criteria set for HIVAN.
Table 6.1: Clinical and Socio-demographic characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number (N=200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>38.1±9.3</td>
</tr>
<tr>
<td>Sex Males (%)</td>
<td>43.2</td>
</tr>
<tr>
<td>Sex Females (%)</td>
<td>56.8</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>18.5±7.1</td>
</tr>
<tr>
<td>Known duration of HIV (years)</td>
<td>3.7±2.4</td>
</tr>
<tr>
<td>AIDS status (%)</td>
<td>77.2</td>
</tr>
<tr>
<td>Known duration of AIDS (years)</td>
<td>3.5±2</td>
</tr>
<tr>
<td>Renal failure (%)</td>
<td>0</td>
</tr>
<tr>
<td>HIVAN (%)</td>
<td>0</td>
</tr>
<tr>
<td>Treated by HAART (%)</td>
<td>60</td>
</tr>
<tr>
<td>CD4 counts (cell/ml)</td>
<td>336±179</td>
</tr>
<tr>
<td>CD4 counts for treated (Cell/ml)</td>
<td>362±178</td>
</tr>
<tr>
<td>CD4 counts for Untreated (cell/ml)</td>
<td>263±164</td>
</tr>
<tr>
<td>Viral load counts (copies/ml)</td>
<td>123,036±47,768</td>
</tr>
<tr>
<td>Viral load counts for treated (copies/ml)</td>
<td>8,438±985</td>
</tr>
<tr>
<td>Viral load counts for untreated (copies/ml)</td>
<td>186,103±143,581</td>
</tr>
<tr>
<td>Viral load counts (%&lt;LDL)</td>
<td>50.5</td>
</tr>
<tr>
<td>Viral load counts (%&lt;LDL) for treated</td>
<td>70.1</td>
</tr>
<tr>
<td>Viral load counts (%&lt;LDL) for untreated</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Values are expressed as mean±SD. LDL=Lower Detection Limit, BMI=Body Mass Index
6.2. Laboratory results linked with renal pathology based on urinalysis and Creatinine test

The laboratory results linked with renal pathology based on urinalysis and creatinine test is listed in table-6.2. The urinalysis and plasma creatinine test results were evaluated separately for treated and untreated patients. A total of 200 HIV infected patients were evaluated for kidney disease of these 120 (60%) were treated with HAART and 80 (40%) were not treated with HAART. Of 200 HIV infected patients, 2 patients (i.e. 1 patient who are on ART and 1 ART naïve patient) had proteinuria of 65mg/dl (+1) and 2 untreated (ART naïve) patients had proteinuria of 100mg/dl (+2) on a spot urine check up. For the results of (1+) and (2+) a test called albumin to creatinine ratio (ACR) was performed on the spot urine for determining kidney disease, but with great surprise all the results were under normal range. The mean creatinine level in treated and untreated patients was 0.84+0.24 and 0.87+0.17 respectively. This result was with in the normal range and in this regard none of the two groups (the treated and untreated HIV positive patients) had kidney failure. Comparative analysis was made between treated and untreated HIV-1 infected individuals but no statistical significant difference seen on abnormal urinalysis and creatinine values between the two groups (P>0.05). Besides analyzing the urinalysis and creatinine test result for HIV-1 patients listed above, the result of 50 healthy control groups were also analyzed. Accordingly, all the 50 HIV negative controls had proteinuria <30mg/dl and the mean creatinine level was 0.84+0.27 which is normal. So, in this study, none of the HIV positive patients (both treated and untreated with HAART) and HIV negative healthy controls fully met the prior criteria (the clinical definition) set for HIVAN.
Table 6.2: Laboratory results linked with renal pathology based on urinalysis and creatinine test

<table>
<thead>
<tr>
<th>Category (Controls)</th>
<th>Number</th>
<th>Urinalysis result</th>
<th>Creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV- (Controls)</td>
<td>50</td>
<td>Proteinuria (+1)</td>
<td>0.84±0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteinuria (+2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(&gt; 30mg/dl)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proteinuria (+2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100 mg/dl)</td>
<td></td>
</tr>
<tr>
<td>HIV+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>120</td>
<td>1 (0.83)</td>
<td>0.84±0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 (0.12)</td>
<td>0.87±0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (2.5)</td>
<td></td>
</tr>
<tr>
<td>Un treated</td>
<td>80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Values are expressed as mean ± SD or number (percent)

6.3. The frequencies of MYH9 E-1 haplotype risk alleles and its 4 SNPs

The frequencies of MYH9 E-1 haplotype genetic risk allele and its 4 SNPs (rs4821480, rs4821481, rs3752462, and rs2032487) were assessed and these frequencies were compared between the Ethiopian HIV infected subjects and HIV negative healthy controls. As shown in table-6.3 below, the frequency of the SNP rs4821480 was 57% for HIV infected individuals and 39% for healthy controls. The frequency of the SNP rs4821481 in HIV infected and healthy controls were 54% and 39% respectively. The frequency of the other SNP rs3752462 was also assessed for the two groups and it was found that higher (60%) in HIV infected individuals as compared to the relatively lower frequency (38%) in HIV negative healthy controls. Still the other SNP rs2032487 was assessed between the two groups and it was found that high frequency (52%) for HIV infected and a relatively lower frequency (31%) in HIV negative healthy controls. Finally the frequency of the E-1 haplotype that comprises the 4 SNPs stated above was assessed and the figure showed that still higher value (56%) in HIV infected individuals as compared to the relative lower frequency (35%) in healthy controls. With all of these results the statistical analysis between the two groups (HIV infected vs healthy controls) for these risk markers (alleles) evaluated here showed statistical significance difference (P<0.05).
Table 6.3: The Frequencies of MYH9 E-1 haplotype risk alleles and its 4 SNPs

<table>
<thead>
<tr>
<th>Group</th>
<th>Population</th>
<th>No</th>
<th>rs4821480 allele (%)</th>
<th>rs4821481 allele (%)</th>
<th>rs3752462 allele (%)</th>
<th>rs2032487 allele (%)</th>
<th>E1-haplotype allele (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-infected individuals</td>
<td>All ethnic groups</td>
<td>200</td>
<td>57</td>
<td>54</td>
<td>60</td>
<td>52</td>
<td>56</td>
</tr>
<tr>
<td>HIV-negative individuals (controls)</td>
<td>All ethnic groups</td>
<td>50</td>
<td>39</td>
<td>36</td>
<td>38</td>
<td>31</td>
<td>35</td>
</tr>
</tbody>
</table>

6.4. The frequencies of APOL1 G1 and G2 genetic risk markers (alleles)

Beside analyzing the frequencies of the MYH9 E-1 haplotype risk alleles and its 4 SNPs, as explained above, the frequencies of G1 rs73885319, G1 rs60910145, and G2 rs71785313 pathogenic risk markers (alleles) were also assessed in the Ethiopian HIV infected subjects and HIV negative healthy control groups. But with great surprise all these risk markers (alleles) have not found at the APOL1 gene in all of the ethnic groups of Ethiopian populations (both HIV infected and healthy controls) who are involved in the current study. i.e. The frequency of these two risk alleles (G1 and G2) were zero percent in both groups.
7. Discussion

This study is one of the first studies conducted to determine the prevalence of genetic risk factors or alleles (MYH9 E1 and APOL1 G1 and G2 risk markers) in relation to HIVAN in Ethiopia. The study was conducted on HIV infected patients who are referred from almost all regions of Ethiopia at Tikur Anbessa Teaching Hospital ART clinic, which was thought to concentrate the majority of HIVAN cases circulating in the general population. The prevalence of HIVAN among African Americans was estimated at between 3.5% and 10% in a primary care setting (Ahuja et al., 1999; D’Agati, 1997) and at 12% in an autopsy based study from patients treated with highly active antiretroviral treatment (Shahinian et al., 2000). All of these estimates are significantly higher (p<10^{-10} with a standard Poisson hypothesis test) than the previously maximal conservative estimate of 0.15% HIVAN cases among Israeli HIV patients of Ethiopian ancestry, and indicates a lack of the factors underlying HIVAN risk in African Americans among Israeli Ethiopians HIV carriers (Behar et al.; 2006). Repeating this analysis with the 200 Ethiopian HIV infected patients at the Tikur Anbessa Teaching Hospital, of whom none had HIVAN, yields CIs of [0%, 2.3%]. These data sets are consistent with each other.

Our clinical data from Ethiopians at Tikur Anbessa Teaching Hospital in Addis Ababa suggests the absence of HIVAN among HIV-1 infected individuals of Ethiopian patients. This result is consistent and confirms the previous reported absence of HIVAN in Israeli Ethiopians (Behar et al., 2006). The lack of HIVAN cases in our study is also consistent with the well documented low prevalence of renal disease in general and of HIVAN in particular in European, Hispanic, and Asian population. Conversely, there is consensus regarding the greater prevalence and an approximately 14 fold risk for HIVAN in patients of African ancestry. Therefore, the lack of HIVAN in our survey, is in striking contrast with the known high prevalence of HIVAN among populations at risk such as African Americans (Eggers and Kimmel, 2004; Kopp and Winkler, 2003). Our finding of zero prevalence of HIVAN in Ethiopia also contradicts the general impression that all individuals of African ancestry share a greater frequency of chronic renal disease and HIVAN (Naiker et al., 2003). Although HIVAN is a problem of African people as stated above, but in our study we found no single case of HIVAN. So, a few factors that may be potentially accounted for this difference was considered and further explained as follows:
The first point considered was a possible difference in viral subtype between Ethiopians and African Americans (AAs). A large body of evidence suggests that an abnormal response of podocytes to HIV-1 infection and/or HIV-1 proteins is the key event in HIVAN Pathogenesis (Sunamoto et al., 2003; Ross et al., 2003). Furthermore, recent results showed that HIV can directly infect the podocyte, and a series of interrelated pathways leads to loss of the differentiated phenotype with extensive proliferation and resultant glomerular collapse (Papeta et al., 2009). Bruke et al., (1997) indicated that HIV-1 has been stratified into 3 major phylogenetic groups; namely, M (major), N (new), and O (outlier). These groups can be sub classified further into subtypes that are endemic in different regions of the world. Accordingly, one can postulate different modes of susceptibility for acquiring HIVAN from different HIV-1 subtypes. For example; Subtype B of group M is predominant in North America and Europe, whereas subtype C of the same group is widely distributed in Africa and central Asia. Infection with subtype C in Israel previously was restricted to Ethiopians, whereas subtype B infects non-Ethiopian Israelis (Gehring et al., 1997). Therefore, Ethiopians and African Americans differ in the predominant subtype of HIV-1 infection. Soto et al., (1996) described greater susceptibility to HIV-1 subtype B infection because of increased affinity of this subtype to dendritic cells. Several studies suggested that the susceptibility, course, and progression of HIV infection could be related to the infecting viral subtype (Kanki et al., 1999). But other study compared the immune activation profile of patients infected with different HIV-1 subtypes and did not support the notion that HIV-1 subtype is a major determinant in the progression of HIV infection (Weisman et al., 1999). And so far no single study indicated the association between HIV-1 subtype and HIVAN. So, considering all these points, the explained differences in HIV-1 subtypes infecting African Americans and Ethiopians are not a sufficient explanation for our observation, although future studies certainly are needed to fully resolve this issue.

The second point we considered for the existed difference between Ethiopians (our study subjects) and African Americans in HIVAN status was the possible beneficial effect of HAART. Several studies indicated that Highly Active Antiretroviral Therapy (HAART) provides only partial protection and not effective drug for treating HIVAN cases completely. Although HAART protects HIVAN partially, it was indicated that it can reduce the incidence and improvement in the prognosis
of HIVAN (Atta et al., 2006; Lucas et al., 2004). Lucas et al., (2004) indicated that HAART prevents the development of HIVAN in at risk groups and it was associated with a 60% reduction in risk for developing HIVAN. Accordingly, one possible hypothesis to explain the virtual absence of HIVAN in Ethiopians in our study could be related to a beneficial effect of the HAART regimen administered to 60% of patients in the current study. HIVAN is considered a late manifestation of HIV-1 infection and previously was reported to occur in patients with CD4 counts less than 200 cells/mm$^3$ (Winston, et al., 1999). Although the mean CD4 count among Ethiopian patients in our study slightly exceeded this value, almost 30% of patients had a CD4 count less than 200 cells /mm$^3$ and some of them had greater viral load counts, suggesting less, rather than more effective HIV infection control. The proportion of persons under HIV care in the United States who had ever received HAART increased to more than 70% in 1998, (Cunningham, et al., 2000) which is almost similar to treatment coverage for Ethiopians in our study (60%). Thus, if Ethiopians share enhanced susceptibility to HIVAN with other groups of African origin, then few of our patients might have been expected to show clinical manifestations of HIVAN, whereas none did. Therefore, we thought that the observed absence of HIVAN in our study populations from Ethiopia cannot be attributed solely to better treatment modalities.

The other point we considered was the possible genetic differences or variations between Ethiopians and African Americans. Host genetic variation at one or several genetic loci seems to be a more plausible explanation for the observed low prevalence of HIVAN in our Ethiopian HIV-1 infected patients but greater susceptibility to HIVAN in African Americans for at least three reasons: First, it was indicated that African Americans have a well-documented enhanced susceptibility to other forms of renal injury, such as hypertensive, diabetic, and idiopathic focal segmental glomerulosclerosis forms of renal disease (Freedman et al., 2000; Fogo, 2003). Second, the markedly increased predilection is shared among individuals of African ancestry in the United States, Brazil, and Paris, making environmental factors less likely as a sole mechanism (Praditpornsilpa et al., 1999). And third, very recently higher frequencies of genetic variants (risk alleles) on MYH9 gene and APOL1 gene of kidney podocytes have been identified in African ancestry with strong association to HIVAN (Kopp et al., 2008; Tzur et al., 2010, Genovese et al., 2010). The predilection is so striking that many investigators ascribed this enhanced susceptibility to populations of African ancestry in general and not to African Americans per se, suggesting a
specific genomic effect common to all Africans. We consider this inference to be premature b/c the predominant geographic origin of individuals of African ancestry in the United States, Brazil, and Paris who were the scope of HIVAN studies reported to date is in west central Africa, extending from Senegal to Angola, in contrast to the distinct geographic origin of Ethiopians in east Africa. In addition to that, comparative data from various populations currently living in Africa (African samples who are affected with HIV) are not available, with the exception of a report from Zaire (Pakasa et al., 1993). And recently, Reich et al., (2001) showed that African populations have much greater levels of DNA sequence variation (genomic diversity) compared with populations of recent European or Asian ancestry. For any given DNA sequence haplotype block, contemporary African populations show a greater diversity of haplotypes and shorter distances of linkage disequilibrium compared with non-African populations. So, this suggests that even with in African population there is genetic variation and that could be the possible and plausible explanation for the differences in HIVAN status between our study population in Ethiopia and that of African Americans.

Besides analyzing the clinical data, the frequency or prevalence of MYH9 E-1 risk allele status was assessed and our results were compared with the risk frequencies obtained from the general diversity panel of HIV infected African Americans, general African populations and with populations other than Africans. Our result showed that HIV infected Ethiopians have high frequency of MYH9 E-1 risk alleles and this result is consistent with the reported high frequency of these alleles in HIV infected African Americans (Table-2). We also found consistent result when we compare our findings with the general African population data (Table-2). Conversely when we compare our results with non African populations (East Asia, Europe, and Middle East), our finding showed a significantly higher prevalence of E-1 risk allele. Although our study subjects have high frequency of E-1 risk allele, none of them develop HIVAN. But when we evaluate the other risk allele status (APOL1 G1 and G2) we found that a very low frequency (zero prevalence) in Ethiopians as contrasting to high prevalence in African ancestry reported in different studies (Tzur et al., 2010; Rosset et al., 2010; Genovese et al., 2010). To make it clearer, we also compared our results with the G1 and G2 frequencies obtained from the general diversity panel of African populations (table-2). On table-2 we have got the following information: Out of the four Ethiopian populations (Afar, Amhara, Maale, and Oromo) considered, all of them contained no risk alleles in
either G1 or G2. The West and West-Central African samples display a completely different picture, with allele frequencies as high as 41% for G1 and 20% for G2 in Ghana populations, and the allele frequencies as high as 45% for G1 in Yoruba (Nigeria) populations. When Comparing our 200 Ethiopian samples (Ethiopian HIV infected samples), and the four Ethiopian populations obtained from the general diversity panel of African populations (table-2) we observe that all pair wise comparisons show no significant difference in the total number of G1+G2 alleles (p>0.05, Fisher exact test for all pairs). Similar results are also obtained when G1 and G2 are considered separately. The Ethiopian population are significantly different from the combined West-Central African populations containing higher prevalence of G1 and G2 (p<0.0001, Fisher exact test for comparing any Ethiopian population to the pooled populations from Ghana, Cameroon and Congo on either G1, G2 or their combined prevalence). Thus, the complete absence of APOL1 risk mutations (absence of G1 and G2 risk markers) associated with APOL1 associated nephropathies (APANs) in our Ethiopian populations among HIV positive and healthy control groups serves as a potential explanation for the observed absence of HIVAN in Ethiopians.

In view of recent progress in our understanding of the genetic risk factors for HIVAN and the related spectrum of APANs, the coinciding absence of HIVAN and of the G1 and G2 risk states in the HIV-infected cohorts studied herein is not surprising and was previously shown in patients of non-African ancestry (Tzur et al., 2010; Genovese et al., 2010). However, in contrast to these reports, what surprised us is the HIV-infected cohorts in the current study belong to African continental population groups which is believed of having HIVAN and carrying high frequency of G1 and G2 risk alleles but none did. Examination of global genome wide genetic variation has consistently shown that the largest genetic distances are found between African and non-African populations (Behar et al., 2010; Li Tz, et al., 2008; Bryc et al., 2010). The same studies showed that the non-African human genetic variation is overwhelmed by constituent sub-Saharan African variation. A clear sub-Saharan continental genetic structure separating eastern and western African populations has been noted (Behar et al., 2010; Tishkoff et al., 2009). Specifically, a genome wide study of Ethiopian population clearly showed shared genetic components with African and Middle Eastern population (Behar et al., 2010). The genetic variation noted among Israeli Ethiopians overlapped with that observed in other Ethiopian population (Behar et al., 2010). Concordant with these previously noted results other study showed no significant genetic distances between HIV-
infected Israeli Ethiopians, HIV-infected Ethiopian and the general Ethiopian populations (unpublished data). Clear differences were noted with non-Ethiopian Israeli HIV-infected individuals, West African and non-African general populations and HIV-infected African Americans. Accordingly, the genetic structure difference observed for the G1 and G2 risk alleles is not between African and non-African populations but rather differences also observed among populations living in African continent. Combined with our clinical data, this African genetic structure seems to delineate the distribution of HIVAN, thus, lending strong support to our current understanding of the necessary genetic background for the development of HIVAN, and underscores the importance of APOL1 genetic risk in this pathogenesis.
7.1. Limitations of the study

- The first limitation of this study was: The study design, which should have been a more prolonged follow up study since HIVAN has a late manifestation.
- The second limitation of this study was: All ethnic groups all over Ethiopia were not found to be included in the study.
- The other limitation of this study was: The diagnostic method used for HIVAN was clinical which was known to be less sensitive to detect the disease specifically. i.e we didn’t apply the definitive diagnosis (biopsy) in this study, since biopsy requires a very invasive diagnostic work-up.
8. Conclusions and Recommendations

8.1. Conclusions

The prevalence of HIVAN in Ethiopia was determined. Our clinical data from Ethiopians at Tikur Anbessa Teaching Hospital in Addis Ababa suggests the absence of HIVAN among HIV-1 infected individuals of Ethiopian patients. This result is consistent and confirms the previous reported absence of HIVAN in Israeli Ethiopians. The lack of HIVAN cases in our study is also consistent with the well documented low prevalence of renal disease in general and of HIVAN in particular in European, Hispanic, and Asian population.

The frequency of the genetic risk alleles for HIVAN was also determined. Although the E-1 risk haplotype polymorphisms in the MYH9 gene are strongly associated with HIVAN in African Americans and West Africans, not all HIV infected patients who are at risk based on their MYH9 alleles develop HIVAN. This supportive evidence is also consistent with our results that most (56%) of the HIV infected patients carried the E-1 risk haplotype but none of them had HIVAN. So, our clinical and genetic results observed in HIV-infected individuals of Ethiopian HIV infected patients not showing the clinical stigmata of HIVAN explain the geographic demarcation of HIVAN risk as coinciding with the ancestry susceptibility based on G1 and G2 risk alleles of the APOL1 gene. It is this distribution which limits susceptibility to western Africa rather than to Africa as a whole. Epidemiological data concerning the spectrum of kidney disease associated with West African risk alleles, collected directly in western and southern Africa are still missing in the literature, as well as our understanding of the potential impact of various HIV types, gene-gene and gene-environmental effects on the pathogenesis of chronic kidney disease.

While our results are concordant and support the current understanding of the potential causative role of the G1 and G2 risk variants in the pathogenesis of HIVAN and the other forms of CKD in the spectrum of APOL1 risk, the exact molecular mechanism disrupting normal renal function remains a focus for future discovery.
8.2. Recommendations

Based on the findings of this study, we recommend that:

- Researchers to focus specifically on APOL1 gene, in order to know the exact mechanism of chronic kidney disease (e.g. HIVAN and ESRD) risk. This enables for the discovery of more appropriate drugs to combat the problem.
- Other researchers to extend the study to involve more/other ethnic groups within the country
References


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Appendices

Appendix 1: Patient Questionnaire Form (Data Collection Sheet)

HIVAN Study in Ethiopia

ART Card No __________ Sampling Date: __/__/___

I. Patient Identification

1. Code No __________

2. Gender: Male ______ Female ______

3. Date of birth ____/____/____

4. Age __________

5. Place of birth: Ethiopia, nearest city___________

Other country ____________________________

6. Patient ethnic group: Oromo ______ Amhara _______ Gurage ______

Somali ______ Tigray ______ Other ________

7. Parents ethnic group: Father ___________ Mother _________________

8. Religious affiliation: Orthodox ______ Protestant ________________

Muslim _______ Other ____________________

II. Patient Medical Record Information

9. HIV infection (date of diagnosis or notification) ____/____/____

10. AIDS diagnosis: Yes ______ No ______ Year of diagnosis ___/___/___

11. AIDS status: ______________________________________________________

12. Currently receiving ART treatment: Yes ____ No ___ Initiated date ___/___/___

13. Type of ART medication given: ________________________________________

14. Kidney disease: No _____ ESRD _____ CRF _____ Year of diagnosis __________

15. Kidney disease risk other conditions:

Diabetes I _____ Diabetes II ______ Hypertension _____ Glomerulonephritis _____
Cystic disease ______ Nephrotoxic drugs ___________ Others ____________

16. Other medications given ____________________________________________

17. Present acute infectious diagnosis:

   Urinary infection ______ Respiratory infection ______ Tuberculosis _________
   Soft tissue infection ___  Other infection of febrile illness ______  None ______

### III. Clinical and Laboratory data

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<tr>
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<td>CD4 (Cells/ml)</td>
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<td>Viral Load (Copies/ml)</td>
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Appendix 2: Information Sheet and Informed Consent form (English version)

Assessment of Immunopathogenic risk markers for HIV Associated Nephropathy in Ethiopia

Introduction and Purpose

You are being invited to participate in a research study entitled “Assessment of Immunopathogenic risk markers for HIV-Associated Nephropathy in Ethiopia”. Your doctor will explain the details of the study to you. You should feel free to ask any questions about the study, your participation in the study, or procedures required for participating. You must be at least 18 years old to participate. HIV including sexually transmitted infections (STIs) are major public health problems in Ethiopia. HIV infection can lead to a kidney disease. Very little is known, however, about the association of HIV infection and kidney diseases in our country. This study is designed to find out how many HIV-infected patients are also affected by kidney diseases. Moreover, this study will find out on the interaction among the different risk factors. Identifying risk markers that may lead to kidney diseases may be detected early by screening. There is a laboratory test available to determine whether or not HIV infected patients or other individuals will be at risk of developing kidney disease.

Study Design

We will offer and perform urine and blood testing for up to 200 eligible HIV patients and 50 controls presenting for follow-up at the Tikur Anbessa Teaching Hospital, Addis Ababa, Ethiopia. The study will run for a period of 1 year in Addis Ababa starting March 2009.

Study Procedure

If you decide to participate in this study, you will be asked to provide a medical history and give about 10 millilitres of urine sample and also blood for certain laboratory tests. If you are a known HIV-positive individual, we will also do an additional test for CD4 cell viral load to determine your immune status.
Possible Risks

The biggest risk identified in this study is your identification of HIV status. If you have a partner, we encourage you to undertake the counseling session along with your partner. Our counselors will educate you and your partner with regards to HIV transmission in the enrollment process and reinforced throughout the counseling. There are no risks, except for the usual risks associated with providing urine sample or blood drawing such as bruising, bleeding, and minor discomfort. In most situations the samples will be obtained during a routine follow-up visit as part of your normal diagnostic work-up.

Benefits

If you participate in this study, you will know your viral load to help you adjust your medications by your doctor. In addition, your participation will contribute to the understanding of the associations between HIV and kidney disease in our country. It is hoped that what we learn from this study will help to prevent the development of kidney disease that causes fatal consequences if not treated in the future. However, you may not directly benefit from participating in this study. There is no payment for your participation in this study. All extra laboratory tests performed for the study are for free. However, you will be re-imbursed modestly to cover your transport costs as well as compensation for the time lost in the clinic.

Confidentiality

Your name and other information that can be used to identify you will be kept private and confidential. The blood and urine samples will be sent to a laboratory for analysis. The researcher conducting this study will review your records and follow the progress of the research, but nothing that can be used to identify you will be used in reports of this study. Authorized representatives from the sponsor, any relevant governmental agency and the Institutional Review Board of your clinic (provided that such inspectors are legally obligated to protect any identifiable information from public disclosure, except where disclosure is otherwise required) may inspect your records. This proposal has been reviewed and approved by appropriate Ethics Review Committee whose task is to make sure that research participants are protected from harm. If you wish to find more about the Review Committee, contact us with a telephone no (011) 553 4945 or at AAU, Faculty
of Medicine, IRB office Tel: (011) 553 87 34, E-mail: aaumfibr@yahoo.com, and if you have any question about the study, contact the principal investigator, at Tel: (0911)-018826 or send E-mail at: yonahailbaht@yahoo.com

Consent

Participation in this research is voluntary. You have the right not to participate. Your choice will not affect your relationship with your doctor or your access to medical care. By signing this document, you do not give up any of your legal rights. A copy of this form will be kept in your medical records.

I have read this Patient Information and Consent Form, or this form has been read to me. I have had the opportunity to ask, and I have received answers to any questions I had regarding the study. I understand that if I have any additional questions, I may contact my treating physician. I agree to participate in this study and I have received a copy of this Patient Information and Consent Form.

Name of participant:

.......................................................... Date: ......................

Signature: ................................ Date: ..........................

Name of the principal investigator:

.......................................................... Date: ..........................

Signature: ................................ Date: ..........................

Name of physician:

..........................................................

Signature: ................................ Date: ..........................

(For those who can not Read the consent form)

Name of witness:

..........................................................

Signature: ................................ Date: ..........................

Appendix 3: Information sheet and Informed Consent Form (Amharic Version)
، willingness in the context of the current situation is essential, and it is important to prioritize the needs of the community. The situation requires immediate action, and the urgent need for resources and support should be acknowledged. It is crucial to ensure that the needs of the affected individuals are met in a timely manner. This can be achieved through effective communication and collaboration among various stakeholders. The situation demands a collective effort, and each individual has a role to play in addressing the challenges faced by the community. It is important to foster a sense of solidarity and to work together to overcome the difficulties. The situation requires a multi-faceted approach, and various strategies should be implemented to address the root causes of the issues. It is essential to prioritize prevention and to take proactive measures to mitigate the impact of the situation. The situation demands a sustained commitment, and it is crucial to ensure that the efforts are sustained over the long term. It is important to monitor the progress and to adjust the strategies accordingly. The situation requires a continuous assessment of the needs of the community, and it is important to ensure that the resources are effectively utilized. It is essential to promote transparency and to ensure that the decisions are made in the best interest of the community. The situation demands a strong sense of responsibility, and it is crucial to ensure that the efforts are directed towards achieving the desired outcomes.
Appendix 4: Declaration

I, the undersigned declare that this thesis is my original work. It has not been submitted for a degree in this or any other university and all the source materials used or quoted for this thesis have been duly acknowledged.

Name of the candidate: Yonas Haileselassie

Signature -------------------------------------------

Date -------/-------/---------- Place: Addis Ababa

This thesis has been submitted for examination with my approval as university advisors.

Name of the advisors:
Dr. Solomon Gebre-Selassie (MD, MSc)

Signature ---------------------------------------------

Date ------/-------/-------- Place: Addis Ababa

Dr. Dawit Wolday (MD, PhD)

Signature ---------------------------------------------

Date ------/-------/-------- Place: Addis Ababa