School of Medicine
Department of Pathology & Microbiology

THE SPECTRUM OF HEPATIC PATHOLOGY IN HIV INFECTED ADULTS AT AUTOPSY AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA.

By CHIBAMBA N. MUMBA

A DISSERTATION SUBMITTED TO THE UNIVERSITY OF ZAMBIA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE OF MASTER OF MEDICINE IN PATHOLOGY.

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DECLARATION

I, **Chibamba Ngo'malala Mumba** declare that this dissertation represents my own work. This work has not been done in Zambia before and neither has it been published for any qualification at the University of Zambia or any other University. Various sources to which I am indebted are clearly indicated in the text and in the references.

CANDIDATE: DR. CHIBAMBA N. MUMBA.

Signed ……Date…….

SUPERVISOR: DR. VICTOR MUDENDA.

Signed……. Date…….

CERTIFICATION OF APPROVAL

DISSERTATION TITLE: THE SPECTRUM OF HEPATIC PATHOLOGY IN HIV INFECTED ADULTS AT AUTOPSY AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA.

This dissertation for Dr. **CHIBAMBA N. MUMBA** has been approved as partial fulfilment of the requirements for the award of the **Master of Medicine in Pathology** at the University of Zambia.

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ABSTRACT

Background:

With the increasing availability of highly active antiretroviral therapy (HAART), people are living longer with human immunodeficiency virus (HIV) infection. Effective antiretroviral therapy (ART) for HIV has resulted in a great reduction of acquired immunodeficiency syndrome (AIDS)-related deaths. In the ART-era, liver disease has become one of the most common non-AIDS related cause of death among HIV infected patients. In those co-infected with hepatitis C virus (HCV), end-stage liver disease has emerged as one of the leading causes of morbidity and mortality in patients living with HIV. The true extent and variety of liver pathology affecting HIV-infected adults in Zambia remains largely unknown. The objective of this study was to describe the spectrum of hepatic pathology in HIV infected adults at autopsy at a tertiary referral center.

Methods:

A total of 139 paraffin embedded postmortem liver biopsies from 139 HIV seropositive adults who died at the University Teaching Hospital (UTH) between 2006 and 2013 were evaluated. Serial sections from each liver biopsy where stained with hematoxylin and eosin, Masson's trichrome**,** Periodic acid Schiff and methenamine silver stains. A Ziehl Neelsen stain was done where tuberculous infection was suspected histologically. Histology was then reviewed without blinding to the clinical details. Data was then compiled and examined using SSPS version 20.0.

Results:

The median age of the patients was 36 years (IQR 11). 64% (89) were male. Liver pathology was found in 93.5% of the patients with only 6.5% demonstrating normal histology. The commonest histologic findings were portal tract fibrosis and non-specific portal chronic inflammation, 73% and 60.3% respectively. Caseating granulomatous hepatitis was seen in 41.6% of the patients and macro-vesicular steatosis 26.5%. Females were significantly more likely to have steatosis than males ($p<0.001$). Non-specific sinusoidal dilatation and congestion were seen in 21.9% of the patients, parenchyma necrosis 16.7% and chronic hepatitis 8.8%. Parenchymal necrosis was

associated with being on HAART (p=0.004). The only neoplasm seen was cavernous hemangioma in 1.5% of the patients.

Conclusion:

Liver pathology is common in HIV-infected adults at autopsy at the UTH. The spectrum of hepatic pathology in HIV positive patients at UTH is almost exclusively due to structural change and infection/inflammation. Of the infectious/inflammatory causes, tuberculous infection presenting as granulomatous hepatitis is the most common. Female gender and HAART are associated with steatosis and parenchymal necrosis respectively. AIDS defining neoplasms seem uncommon in the liver.

DEDICATION

To my parents, Richard and Bernadette Mumba, for always being my rock.

ACKNOWLEDGEMENTS

This Dissertation would not have been possible without the help of several people. I would like to thank my supervisor, Dr. Victor Mudenda, for guiding me through the various stages of my dissertation journey.

I am truly indebted and thankful to Dr. Jonathan Sitali, Dr. Kondwelani Mateyo, Dr. Temba Sikwila and Dr. Chisambo Mwaba; your insights shaped this document.

A special thanks to my colleagues Dr. Peter Julius, Dr. Luchenga Mucheleng'anga, Dr. Dennis Musonda and Dr. Sergio Muwowo for encouraging me throughout this research.

My gratitude to the staff in histology laboratory at University teaching hospital. Your tireless work in preparing the histology slides is the backbone of this research work.

Finally, but not the least, I am sincerely and heartily grateful to Ms. Precious Goma for supporting me throughout my dissertation writing.

LIST OF ACRONYMS / ABBREVIATIONS

AIDS- Acquired immunodeficiency syndrome

ALT- Alanine amino transferase

AST- Aspartate amino transferase

ART- Anti retroviral therapy

cART- combined antiretroviral therapy

CMV- Cytomegalovirus

CVD- Cardiovascular disease

HAART- Highly active antiretroviral therapy

HBV- Hepatitis B virus

HCC- Hepatocellular carcinoma

HCV- Hepatitis C virus

HIV- Human immunodeficiency virus

KS- Kaposi sarcoma

PEPFAR- President's Emergency Plan for AIDS Relief

UNZABREC- University of Zambia Biomedical Research Ethics Committee

UTH- University Teaching Hospital

DEFINITIONS

Portal tract fibrosis: Formation of new collagen fibers in the portal tract. The fibrosis may be confined to the portal tract or bridge between a portal tract and another portal tract or a terminal hepatic vein.

Nonspecific chronic portal inflammation: Chronic inflammatory cell infiltration of the portal tract comprising lymphocytes and some plasma cell, which is not diagnostic of any specific infection or inflammatory process.

Caseating granulomatous hepatitis: A focal accumulation of epithelioid cells, which are modified macrophages with abundant cytoplasm and often curved, elongated nuclei. The collections of macrophages have central areas of coagulative necrosis (caseous necrosis) and may be associated with multinucleate giant cells.

Macro-vesicular steatosis: The accumulation of excess lipid in the hepatocytes. It is characterized by a single large fat vacuole or several smaller ones occupying the greater part of a hepatocyte, pushing a nucleus to the periphery.

Micro-vesicular steatosis: The accumulation of excess fat in hepatocytes. It is characterized by finely divided fat and a central nucleus.

Sinusoidal dilatation: Regular dilatation of the hepatic sinusoidal network.

Parenchymal necrosis: Substantial areas of liver cell death. It may be massive with multi lobular necrosis involving a substantial part of the whole liver or multi lobular necrosis characterized by confluent necrosis involving the whole or several adjacent lobules.

Chronic hepatitis: A case was defined as having chronic hepatitis if it had any of the following or a combination of:

(a) lymphoid aggregates and lymphoid follicles with germinal centers in the portal tract;

(b) heavy lymphocytic or lymphoplasmacytic infiltrate in the portal tract with or without interface hepatitis (inflammatory infiltrate extends from portal tract into the adjacent parenchyma and there is destruction of hepatocytes);

(c) hepatocellular damage and inflammation in the lobule (lobular hepatitis)

Acute hepatitis: Presence of hepatocyte cell swelling and/or ballooning degeneration and lymphocytic infiltrate within the parenchyma and sometimes the portal tract. A few neutrophils and eosinophils may also be seen. These features can be seen in combination with some form of necrosis, either confluent necrosis or bridging necrosis.

Portal granuloma: Collection of activated macrophages in the portal tract without any associated caseation.

Cholestasis: Presence of visible bile in the tissue section.

Bile duct proliferation (ductular reaction): A reaction of ductular phenotype, seen as an increase in ductular structures.

Cavernous hemangioma: Benign vascular tumor composed of dilated vascular channels.

CMV infection (cytomegalovirus): Infection will be defined by the presence of markedly enlarged cells that contain characteristic “owl’s eye” inclusions that are visible on H&E stained tissue sections.

Cryptococcal infection: Presence of round to oval yeast with narrow based budding whose mucopolysaccharide capsule material stains with mucicarmine or periodic acid Schiff.

Elevated liver function tests: Elevation of Alanine aminotransferase (ALT) and/or Aspartate aminotransferase (AST) and/or alkaline phosphatase (ALP) by greater than three times the upper limit of normal.

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CHAPTER ONE

1.0 BACKGROUND

Human Immunodeficiency Virus (HIV) infection only came to prominence in the early 1980's and since then it has spread globally resulting in a worldwide pandemic. HIV infection causes progressive destruction of an individual's immune system resulting in the Acquired Immunodeficiency Syndrome (AIDS) and inevitably death[1]. However, with the increasing availability of effective highly active anti-retroviral therapy (HAART) more people are surviving longer with HIV and presenting with liver disease[2]. Liver disease has therefore emerged as a significant cause of morbidity and mortality in patients living with HIV[3].

The spectrum of liver disease that occurs in HIV-infected people includes both those seen in the immune-competent as well as those specific to the immune-compromised. Of note is the fact that the incidence and severity of liver disease in HIV-infected patients is significantly different from other patient-populations and has been further modified by the advent of cART[4]. Furthermore, the pattern of liver pathology tends to differ from region to region[5]. As at present, the true extent and variety of liver pathology affecting HIV-infected adults in Zambia, from either the pre-ART era or after the introduction of ART remains largely unknown.

This study therefore is meant to determine the different hepatic diseases and lesions in HIV-infected adults at autopsy at the University Teaching Hospital in Lusaka. The benefit will include the documentation of different liver pathology in HIV-infected adults which will provide baseline information for policy makers to develop means of preventing and treating liver disease in HIV infected patients.

1.1 STATEMENT OF THE PROBLEM

Liver disease is emerging as a significant cause of morbidity and mortality among HIV infected patients[3]. The types of liver diseases differ regionally. To our knowledge, no data on the spectrum of liver pathology in Zambia was available prior to this study. This study has described the different hepatic pathologies that affect HIV infected patients at autopsy.

1.2 STUDY JUSTIFICATION

In Zambia, state provision of antiretroviral therapy began in late 2002[6] and became free in June 2004 due to the commitments made by the Global Fund and PEPFAR. As at the end of 2010, 72 percent of the 480,000 people in Zambia needing ARV treatment were receiving it[7]. This number rose to 671,066 in 2014[8]. With the increase in the provision of ARV therapy, people with HIV infection are living longer. Annual AIDS-related mortality dropped approximately from 58,000 in 2000 to 19,000 in 2014[8]. Therefore, as has been found elsewhere, it is expected that more HIV-infected patients will present with liver disease and it is liver disease that is going to be the most common cause of mortality and morbidity[2]. This study gives us insights into the spectrum of liver pathologies and their causes in HIV infected Zambian adults at a tertiary hospital. The information may be used to come up with strategies aimed at reducing this burden of disease among HIV patients.

1.3 STUDY QUESTION

What is the histologic spectrum of Liver pathology in HIV infected adults at autopsy at the University Teaching Hospital?

1.4 NULL HYPOTHESIS

There is no hepatic pathology seen at autopsy in adult HIV infection.

1.5 STUDY OBJECTIVES

General Objective

To describe the spectrum of hepatic pathology in HIV-infected adults at Autopsy at the UTH, Lusaka.

Specific Objective

1. To determine the types of infections in the liver at autopsy in HIV-infected patients.
2. To determine the types of neoplasms in the liver at autopsy in HIV-infected patients.
3. To determine the types of non-specific and specific structural changes in the liver at autopsy in HIV-infected patients.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of HIV/AIDS

As of 2014, the number of people living with HIV globally was 36.9 million, which accounts for 0.5% of the world's population. Most of these (25.8 million, 70%) live in sub-Saharan Africa, which has an adult prevalence rate of 4.8%[9]. Most of the countries in sub-Saharan Africa have generalized HIV epidemics, with national prevalence rates greater than 9%. 6.8 million people are living with HIV in South Africa, making it the country with the highest number of people living with HIV. Swaziland, with a prevalence rate of 27.7% has the highest rate in the world[10].

In Zambia, according to the 2013-14 Zambia Demographic Health Survey, 13% of adults aged 15-49 years are infected with HIV. Among women aged 15 – 49 years, the HIV prevalence rate is 15%, while among men aged 15 – 49 years, the HIV prevalence rate is 11%. Provincial differentials indicate that Muchinga province has the lowest HIV prevalence (6%), while Copperbelt province has the highest prevalence (18 %) [11].

2.2 Epidemiology of liver disease in HIV/AIDS

Persons with HIV/AIDS are afflicted by different diseases which include secondary infections and tumors. The primary causes of death are AIDS, liver-related, cardiovascular disease (CVD) - related and non AIDS malignancy[2]. In the era of combination anti-retroviral therapy (cART) and improved survival[12], liver disease has become a leading cause of morbidity and mortality among HIV-infected persons[13-15]. Liver disease has emerged as the most common non-AIDS related cause of death among HIV infected patients, accounting for 14 – 18% of all deaths[2, 13]. In some series, nearly half of deaths among hospitalized HIV infected patients in the ART era have been attributed to liver disease[16, 17].

In the United States, death rates among patients living with HIV have been falling over time[12]. The decline in morbidity and mortality due to AIDS in attributed to the use of ART[12]. Deaths attributed to AIDS decreased from 3.79/100 person years in 1996 to 0.32/100person years in 2004, but there was proportionate increases in deaths involving liver disease, bacteria/sepsis, gastro intestinal disease, non AIDS malignancies, and renal disease, with hepatic disease being the only reported cause of death with an absolute rate increase over time. The percentage of deaths due exclusively to non AIDS defining illnesses rose from 13.1% in 1996 to 42.5% in 2004, the most frequent of which were cardiovascular, hepatic disease, pulmonary disease, and non AIDS malignancies[13].

A study done in France from 1998 to 1999 by Bonnet *et al* found that AIDS-related illnesses were no longer the major causes of death in HIV infected patients on cART[14]. Another study done in France 10 years after the introduction of cART, found that the proportion of liver-related deaths among HIV-infected persons increased from less than 2% in 1995 to 17% in 2011[18].

There is very little data on liver disease in HIV patients on the African continent. In a study done in Kenya in 2007 looking at liver biopsies in HIV patients who presented with febrile illnesses and inconclusive initial investigations, Shavadia J *et al* found that disseminated Mycobacterium tuberculosis was the most common cause of pyrexia in this population of patients[19]. In South Africa, Garcia-Jardon M *et al* did an autopsy study of 86 patients with HIV and found that 10% had liver disease[20]. Specific information on the types of liver disease were not provided in this study.

2.3 Causes of liver disease in HIV/AIDS

There are many causes of liver disease in HIV-infected patients. It may arise as a result of the HIV itself, cART used to treat the infection and infectious complications of HIV infection[21]. Opportunistic infections and cancers are seen more frequently in HIV-infected patients, and several ailments that are common in the community may be seen with increasing frequency in this patient population[21].

The burden of non-AIDS morbidity and mortality has changed in the ART era and so has the types of liver disease the clinician is likely to come across among HIV-infected patients[22]. In the western world, before the availability of ART, opportunistic infections, such as those caused by *Mycobacterium avium* complex or HIV-related cholangitis dominated the expression of liver disease and patient survival[23]. A retrospective study done in San Francisco, California from 1981-1985 by Schneiderman *et al* that reviewed hepatic histology in 26 biopsies and 59 autopsies found that macro-steatosis and non-specific portal inflammation were the most common histologic abnormalities, 42.4% and 35.3% respectively. Intra-hepatic AIDS-specific opportunistic infections and malignancies were detected in 42% of both biopsy and autopsy groups, with *Mycobacterium avium-intracellulare* being the most frequent pathogen seen at 16.5%. Kaposi Sarcoma, at 12.9%, was the most common post-mortem AIDS-related hepatic finding. Intra-hepatic lymphoma 4.7%, CMV hepatitis 9.4%, and hepatic mycoses were less frequently observed[24].

Wilkins M.J. et al examined 101 liver biopsies in HIV-positive patients in the United Kingdom from 1984-1989. Only nine showed no abnormalities. The commonest histological findings were either fatty change or changes related to co-existent chronic viral hepatitis. Granulomas were seen in 15 cases, four of which were positive for acid-fast bacilli. A range of organisms were recorded: CMV 4, *Histoplasma capsulatum* 1, *Pneumocystis carinii* 2 (now known as *pneumocystis jeroveci pneumonia*), *Cryptococcus neoformans* 1 and *Leishmania donovani* 1, two cases of non-Hodgkin lymphoma, but no cases of Kaposi sarcoma[25].

A prospective study by Teerha P. *et al* in Hat Yai, Thailand from 1995-1996, found that *Mycobacterium tuberculosis* was the most common histological finding (15 cases - 32.6%). Other findings included Histoplasmosis (6 cases - 13%), hepatitis B virus and HCV infection (2 case – 4.4%), fatty liver (2 cases - 4.4%), drug-induced hepatitis (1 case - 2.2%) and non-specific changes (5 cases - 10.9%), Cryptococcosis (6 cases - 13.0%), AFB-negative granulomatous hepatitis (8 cases - 17.4%), Penicillosis (4 cases - 8.7%)[26]. They did not state weather the patients where on ART or not.

An autopsy study done in Mumbai, India from 1999-2003, by Amarapurkar *et al* revealed that tuberculosis was the commonest liver pathology, presenting in 31.6% of the patients. Other findings included fatty change 10%, HCV infection 10%, HBV infection 1.6% and normal histology 10%[27]. Lanjewar *et al* in a retrospective and prospective study where they looked at 155 autopsies and 16 biopsies at a tertiary level hospital in Mumbai, India, from 1988-2002, also found similar trends, with tuberculosis being the most common liver pathology, 41%, followed by Cryptococcosis 5%, CMV infection 3% and Hepatitis B Infection at 3%. Normal livers were found in 26% of the cases[23]. Both studies were done around the time ART was being introduced, and these patients were not on ART.

Pereira *et al* in a more recent study done in Brazil at a tertiary care teaching hospital examined 52 percutaneous liver biopsies of 50 HIV-infected patients not on ART and found that 24% had tuberculosis, 10% Cryptococcus,10% Kaposi Sarcoma and 4% Hodgkin's Lymphoma[28].

In the ART era, the spectrum of liver disease among HIV-infected patients has shifted towards co-infection with HCV, HBV, drug-induced hepatotoxicity, alcohol-related and nonalcoholic fatty liver disease[22, 29, 30].

Hepatitis B and C Viral infection

In HIV-infected persons taking ART, liver disease caused by chronic viral hepatitis has emerged as a leading cause of morbidity and mortality, due in large part to HCV co-infection resulting from shared risk factors for the two viruses[31]. In the United States and Europe, approximately 30% of HIV infected persons are co-infected with Hepatitis C Virus (HCV) [31] and worldwide, 10% have Chronic Hepatitis B virus infection[32]. In sub-Saharan Africa, a systematic review and meta-analysis of sixty studies by Barth R. E. *et al* showed that among HIV infected individuals, the mean HBsAg and anti-HCV prevalence rates were 15% and 7% respectively. They concluded that many HIV infected patients in sub-Saharan Africa are HBV or HCV co-infected and "HIV is associated with a higher prevalence of both HBV and HCV in the region"[33]. However, this association was less evident than that observed in the West. A study done in Zambia by Kapembwa

et al found that 9.9% of HIV positive blood donors were HBsAg positive while 1.2% were HCV antibody positive[34]. From the above it seems that the prevalence of HBV among HIV positive patients is higher than that of HCV in Africa and Zambia in particular. The opposite is true for countries in the West.

A Swiss HIV Cohort Study found that HIV and HCV co-infection was associated with quicker progression to AIDS and a slower CD4 count recovery than in those with HIV mono-infection[35]. HIV and HCV co-infection is associated with a lower rate of spontaneous HCV RNA clearance, with chronic HCV infection developing in 80% of these patients. HIV in turn accelerates HCV related liver disease once chronic HCV infection is established, by increasing progression of fibrosis[36].

Worldwide, approximately 10% of HIV infected patients have chronic HBV infection[32]. Like in HCV and HIV co infection, HIV co infection reduces the ability to clear HBV infection after exposure, which in turn causes increased HBV DNA and 23% of the patients developing chronic HBV infection[37]. Chronic HIV and HBV co infection is associated with higher liver related mortality than in HIV or HBV mono infection because of increased rate of progression of fibrosis[38].

Alcohol

Rosenthal E, *et al* in the French MORTAVIC Study showed that there was a steady increase in the number of HIV-infected patients consuming excessive alcohol[18]. A study done in the United States showed that 8% of the HIV positive Cohort and 15% of current alcohol drinkers were heavy drinkers, which is about twice as prevalent as that in the general population[39]. In another study, excessive alcohol consumption was the most common co-morbidity and was twice as high in those who died from liver disease than in those dying from other causes[40]. Alcohol abuse is prevalent among HIV infected patients and can independently contribute to liver disease progression[41].

Drug induced hepatotoxicity

Hepatotoxicity in HIV infected patients can result from the variety of pharmacologic agents used to treat them. Different patterns of hepatotoxicity may be seen and these include hypersensitivity, idiosyncratic reaction, mitochondria injury, immune reconstitution inflammatory syndrome (IRIS) and steatosis[1].

Severe hepatotoxicity as a result of cART has been reported in 10% of patients, with life threatening events reported at a rate of 2.6 per 100 person years[42]. Most anti-retroviral drugs used in the treatment of HIV infection can result in liver injury[21]. Both Nucleoside and non- nucleoside reverse transcriptase inhibitors, and protease inhibitors can cause hepatotoxicity with elevated liver enzymes, with Nevirapine and Efavirenz being the most usually implicated drugs[1]. Hypersensitivity reactions are usually seen with Nevirapine, Abacavir, and Efavirenz and occurs a week after starting therapy. Mitochondrial toxicity results in impaired fatty acid oxidation and micro vesicular steatosis. This is associated with use of Didanosine or Stavudine.

When HBV or HCV infection is present, immune reconstitution with liver injury may occur[1].

Some of the other factors associated with ART related hepatic injury include preexisting advanced fibrosis, pretreatment elevated ALT or AST, alcohol abuse, older age, female gender, first exposure to ART, and concomitant tuberculosis medication[42, 43, 44].

Drugs used to treat fungal infections like ketoconazole and fluconazole can be associated with elevation in transaminases in 10 to 20% of cases[1]. They cause Hepatocellular injury[3].

Isoniazid and rifampicin, used in the treatment of mycobacterium tuberculosis can produce abnormalities in liver function tests[1]. They can also cause Hepatocellular injury. Ethambutol on the other hand is associated with cholestatic injury[31].

Hepatic steatosis

Hepatic steatosis is seen in one third of AIDS patients at autopsy or on biopsy, and is usually mild to moderate in degree and is peri portal in distribution[1]. Causes of hepatic steatosis may include chronic alcoholism and non-alcoholic fatty liver disease (NAFLD) [1]. NAFLD may progress to non-alcoholic Steatohepatitis (NASH) which may lead to fibrosis and eventual cirrhosis[45]. The prevalence of NAFLD is higher in patients with HIV (30-40%) than in the general population (14-31%) [46, 47]. The risk factors for NAFLD may be similar in those with or without HIV[48], but other factors like HIV or ART may contribute to NAFLD[49]. NAFLD is associated with accelerated liver fibrosis progression [50], as well as increased liver-related mortality[50, 51].

Hepatocellular carcinoma

The MORTAVIC study showed that HCC as a cause of death has been increasing progressively[18]. Hepatocellular carcinoma (HCC) was found to account for 25% of liver related deaths among HIV infected people in 2005, an increase from 15% in 2000[52]. HCV, HBV and alcoholic cirrhosis, are the leading causes of HCC worldwide[53].

AIDS RELATED LIVER DISEASE

Infection:

Several opportunistic infections can affect the liver in HIV/AIDS patients. *Mycobacterium avium* complex is the most common. Involvement of the liver by *Mycobacterium tuberculosis* has been reported in about 8% percent of patients with extra pulmonary tuberculosis and HIV infection[54, 55]. Other infections that may be found in the liver in AIDS include CMV, fungi like *Cryptococcus neoformans* and *Histoplasma capsulatum*, disseminated *Herpes simplex* virus, *Varicella-zoster* virus, *Candida albicans*, *Aspergillus fumigatus*, *Toxoplasma gondii, and Strongyloides stercoralis*[54, 56, 57].

Neoplasms:

NHL and KS, which are AIDS-defining malignancies are seen in the liver in 33% and 9% of cases respectively[56, 58]. NHL usually appears in the liver in association with widespread dissemination. Most often, the lymphomatous infiltrate appears in the portal zones, and if extensive will be found throughout the hepatic lobules[1]. On the other hand, KS, when present in the liver will usually present around large portal veins and near the capsule[1].

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design and site

This was a descriptive retrospective cross sectional study done in Lusaka, Zambia, at the University Teaching Hospital, department of pathology and microbiology.

3.2 Study population

The study population comprised HIV infected adult subjects aged 18 years and older who died at UTH and were recruited into the sub-type C Neuro-AIDS pathogenesis in Zambia study between 1st August 2008 and 30th November 2013.

3.3 Sampling frame

All the liver samples obtained from the study population.

i. Inclusion Criteria

Samples that were properly labeled as liver and had a coding number.

Samples fixed in formalin.

ii. Exclusion Criteria

Samples from patients aged less than 18 years.

Samples from patients with missing files.

iii. Sample Size

270 autopsies were done during the study period, and 270 liver samples were collected and stored. Having removed autolysed and damaged samples, missing samples, samples without a patient file, pediatric cases and those from HIV negative patients, the sample size was 139 liver specimens.

3.4 Sample selection

All archived sequentially collected liver samples from the subtype C Neuro-AIDS pathogenesis in Zambia study between 1st August 2008 and 30th November 2013.

3.5 Tissue processing

Liver specimens were sampled from the sub type C Neuro-AIDS pathogenesis in Zambia study. These HIV infected patients had full autopsies in the department of pathology and microbiology at the UTH and liver samples obtained were fixed in 10% formalin.

All samples were submitted for tissue processing (appendix A), paraffin embedding and 3 micrometer thick tissue sections were obtained for histological assessment. The following stains were performed using standard operating procedures (appendix B):

a) Hematoxylin and eosin, to demonstrate the cationic and anionic tissue components and give a standard reference overview in the study of the tissue sections.

b) Periodic acid-Schiff, to demonstrate mucin and other glycogen containing molecules. It will also be used to demonstrate fungi and to assess basement membrane thickness.

c) Masson’s trichrome, to demonstrate collagen.

d) Gomori's methenamine silver, to identify fungi and visualize architecture of liver.

Other stains done where need arose included,

e) Ziehl Neelsen, to demonstrate alcohol and acid fast bacilli belonging to the genus *Mycobacterium*.

Immunohistochemistry was not employed in this study.

These slides were examined using a binocular light microscope, Olympus^{R} CX 31.

The primary outcome was a description of the hepatic pathologies in Adult HIV infection at autopsy.

The secondary outcome was a description of the infections and neoplasms in the liver at autopsy in Adult HIV infection.

3.6 Data management and analysis

i. Data collection tools

Data was collected from patient files and after microscopic examination entered onto a data collection tool (appendix C). The variables included;

Independent variables: age, sex, alcohol use, medication (ART, anti Tb drugs), liver function tests, hepatitis B and C status, and CD 4 count.

Dependent variables: histological findings, table 1.

Table1. Dependent variables.

Location	**Histologic findings (variables)**
Portal tract:	Chronic cellular infiltrate resembling hepatitis C
	Acute cholangitis
	Cholestasis
	Fibrosis
	Bile duct proliferation
	Granuloma
	Chronic inflammation
Parenchyma:	Chronic hepatitis
	Sinusoidal infiltration
	Polymorph infiltrate
	Granulomas consistent with Tuberculosis
	Cytoplasmic swelling
	Ground glass hepatocytes
	Fatty change (steatosis) • micro vesicular steatosis

	• macro vesicular steatosis
	Steatohepatitis
	Acute hepatitis
	Necrosis
	Sinusoidal dilatation
	Infections • fungi • viruses • bacteria • parasites
	Neoplasms

ii. Data analysis

Data was examined using SPSS version 20.

Frequencies, modes and means were used to analyze the types of infections, neoplasms and structural and non-structural changes on histological evaluation of the liver samples.

Cross table analysis and Pearson Chi square test and Fisher's Exact test were done to analyze the distribution of the study variables.

3.7 Expected outcome

The histologic appearance of the liver would be those of Mycobacterium infection.

The neoplastic appearance would be of lymphoma.

3.8 Ethical approval

Samples used were stored liver specimens collected as part of the sub type C Neuro-AIDS pathogenesis in Zambia study. Consent to obtain samples was given by the deceased next of kin before the autopsies were conducted (appendix D).

Data collected was delinked from name and other identifier details of the participant and confidentiality with regards to anonymised samples was maintained.

Approval to carry out the sub study was given by ERES CONVERGE IRB, approval number 2015-June-005 (appendix E).

CHAPTER FOUR

4.0 RESULTS

4.1 Patient demographics and characteristics:

Table 2 shows the demographic characteristics of the patients enrolled in the study. 139 HIV positive adult patients with a median age of 36 years (IQR 11) who died at UTH and underwent autopsy between 2008 and 2013 were enrolled. 89 (64%) were males and 50 (36%) were female. 67 (48%) patients were between the ages of 31-40 years, with the males making up 69% of the patients in this age group, figure 1. All the patients were black Africans. CD 4 counts were available for 33 (23.7%) of the 139 patients. The mean CD 4 count for the 33 patients was 152.42 cells/mm^3 (range 1-996). 27 (82%) of these patients had CD 4 counts less than 200 cells/mm^3, signifying advanced disease. 11 (33%) had CD 4 counts of less than 50 cells/mm^3. Slightly over half of the patients, 71 (51.1%) were on ART, while 64 (46%) were not. Information was missing for 4 patients. The ART regimens were almost all dual nucleoside reverse transcriptase inhibitor plus non-nucleoside reverse transcriptase inhibitor based, with the most frequent ART regimen used being tenofovir/emitricitabine and efavirenz (TDF/FTC/EFV), 41(58%), see table 3 and 4. Efavirenz was the most commonly used non-nucleoside reverse transcriptase inhibitor, 44 (79%). 41 (57%) patients were on anti TB medication while 42.4% were not. There was no data for 23 (16.5%) patients. 41 (29.5%) patients took alcohol while 31 (22.3%) didn't. There was no data for 67 (48.2%) patients. Of the available data, 32 (78%) were male while only 9 (22%) were female. For our cohort, 64% of the males took alcohol while only 41% of the females took alcohol.

Only eight patients were tested for HBsAg, with one having a positive test result. Only one patient was tested for HCV antibody, and the result was negative. Of the 74 patients with available liver function tests, 20 (27%) had elevated function tests while 54 (73%) had results within normal range.

Table 2: Baseline characteristics of the 139 patients that underwent postmortem.

Parameter	Frequency- N (%)
AGE (Years) Age categories: 16-20 21-30 31-40 41-50 51-60 61-70 71-80 >80	Median 36 (IQR 11) 4(3) 37(26) 67(48) 29(21) 1(1) 1(1) 0 0
SEX Males Females	 89 (64) 50 (36)
CD 4 COUNT (cells/mm^3) <200 <50	Mean 152.42 (range 1-996) 27(82) 11(33)
MEDICATION (Drug use) ART ANTI-TB	 71 (51.1) 41 (29.5)
ALCOHOL CONSUMPTION Male Female	41 (29.5) 32 (78) 9 (22)
HBsAg- 8 tested Positive Negative	 1 7
HCV ANTIBODY TEST-1 tested Positive Negative	 0 1
ELEVATED LIVER FUNCTION TESTS- 74 available results Yes No	 20 (27) 54 (73)

Figure 1. Chart showing the age distribution and sex of the patients.

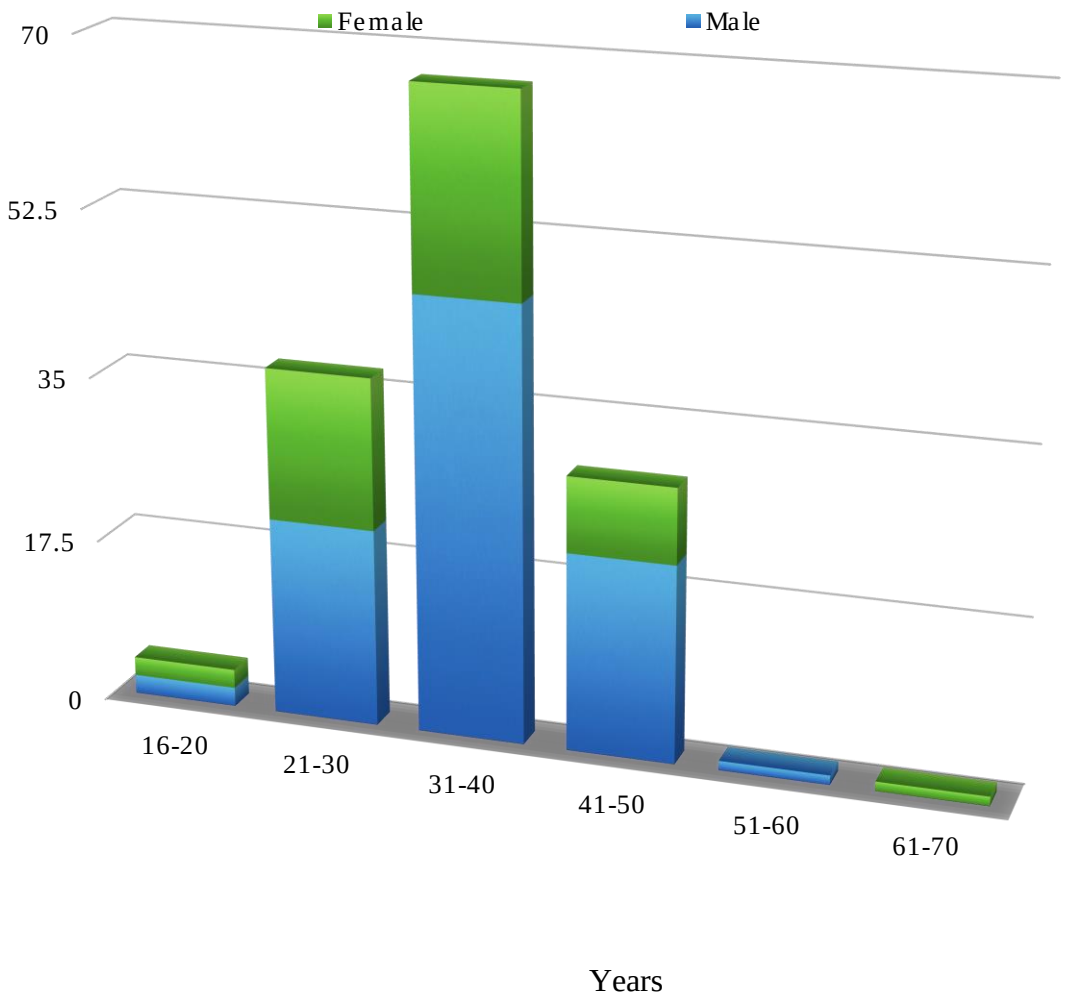


Table 3: ART regimens use and their frequencies.

	HAART regimen	**Frequency**
1	TDF/FTC/EFV	41
2	TDF/FTC/NVP	6
3	ABC/3TC/NVP	3
4	TDF/3TC/NVP	2
5	TDF/3TC/EFV	1
6	d4T/3TC/EFV	1
7	AZT/3TC/NVP	1
8	AZT/3TC/EFV	1
9	On HAART but regimen not known	15
Total		**71**

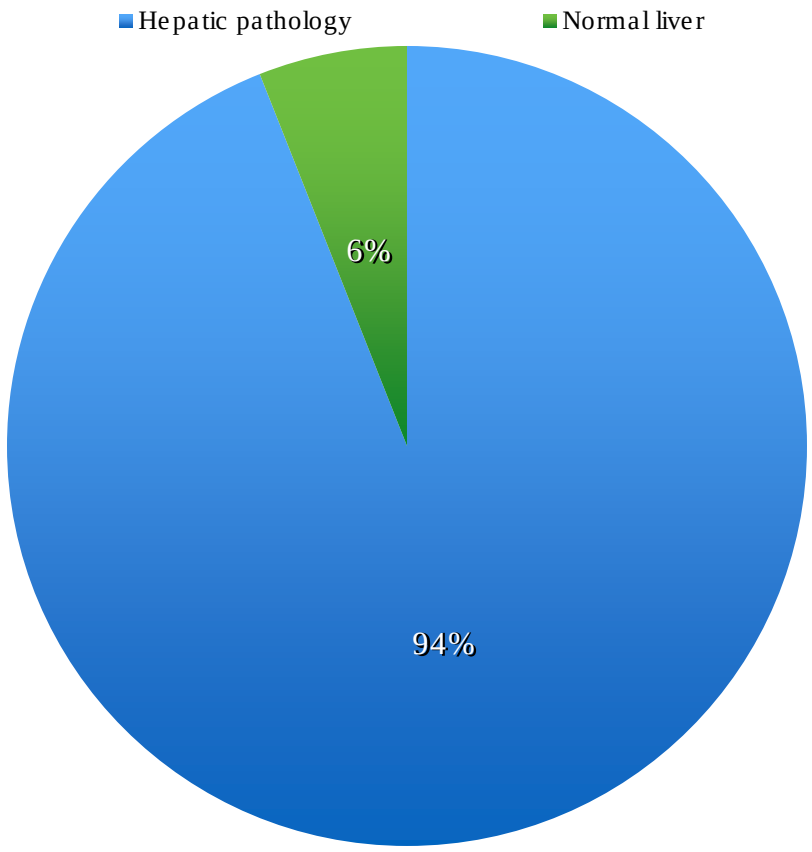
Table 4: Classes of anti-retroviral drugs patients were on.

	Nucleoside reverse transcriptase inhibitors	Frequency (%)
1	TDF/FTC	48(84)
2	TDF/3TC	3(5)
3	d4T/3TC	1(2)
4	ABC/3TC	3(5)
5	AZT/3TC	2(4)
	Non-nucleoside reverse transcriptase inhibitor	
1	Efavirenz	44(79)
2	Nevirapine	12(21)

4.2 Histopathological findings:

Significant hepatic pathology was found in 130 (94%) patients whose liver samples were evaluated histologically, figure 2.

Figure 2. Pie chart showing percentage of cases with liver pathology



Structural changes which included portal tract fibrosis, 101 patients (73%), with one case demonstrating cirrhosis, were the most common histopathological findings, figure 3, Table 5. This was followed in frequency by nonspecific portal chronic inflammation, 84 patients (60.3%) and caseating granulomatous inflammation (caseating granulomatous hepatitis), 57 patients (41.6%). The histologic features of caseating granulomatous inflammation were consistent with those of mycobacterial infection, figure 4. Of the 57 patients with caseating granulomatous hepatitis, 9 were positive for alcohol and acid fast bacilli, figure 5A and 5B. No other infections were found.

Figure 3. Masson trichrome stain demonstrating liver cirrhosis. Note the nodularity of this liver biopsy and the thick fibrous bands around these nodules, arrow (x 400 magnification).

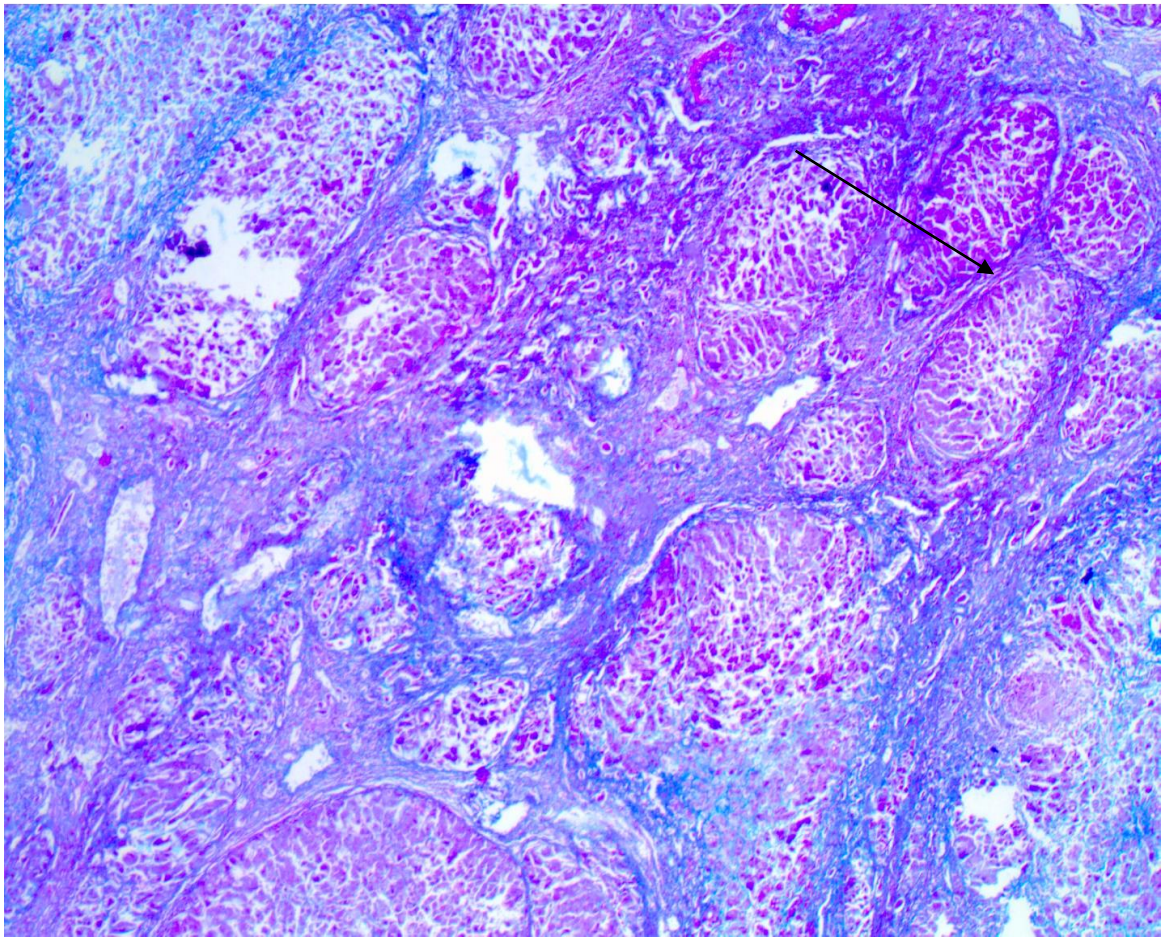


Table 5. Spectrum of hepatic pathology in HIV/AIDS (n=139)

Histology	Number of cases	Percentage (%)
Portal tract fibrosis	101	73
Nonspecific portal chronic inflammation	84	60.3
Caseating granulomatous hepatitis	57	41.0
Macro vesicular steatosis	36	26.5
Sinusoidal dilatation	30	21.9
Parenchymal necrosis	23	16.7
Chronic hepatitis	12	8.8
Micro vesicular steatosis	4	2.9
Acute hepatitis	3	2.2
Portal granuloma	3	2.2
Cavernous hemangioma	2	1.5
Cholestasis	1	0.7
Bile duct proliferation	1	0.7
Normal	9	6.5
Multiple (mixed) pathologies	112	81
1) Portal tract fibrosis and non-specific portal chronic inflammation	76	55
2) Steatosis and caseating granulomatous hepatitis	18	13
3) Caseating granulomatous hepatitis and parenchymal necrosis	9	6
4) Steatosis, granulomatous hepatitis, fibrosis/nonspecific chronic portal inflammation	10	7

Figure 4. Caseating granulomatous hepatitis. Also shown is a Langhans type multinucleated giant cell (arrow) x400 magnification, hematoxylin and eosin stain.

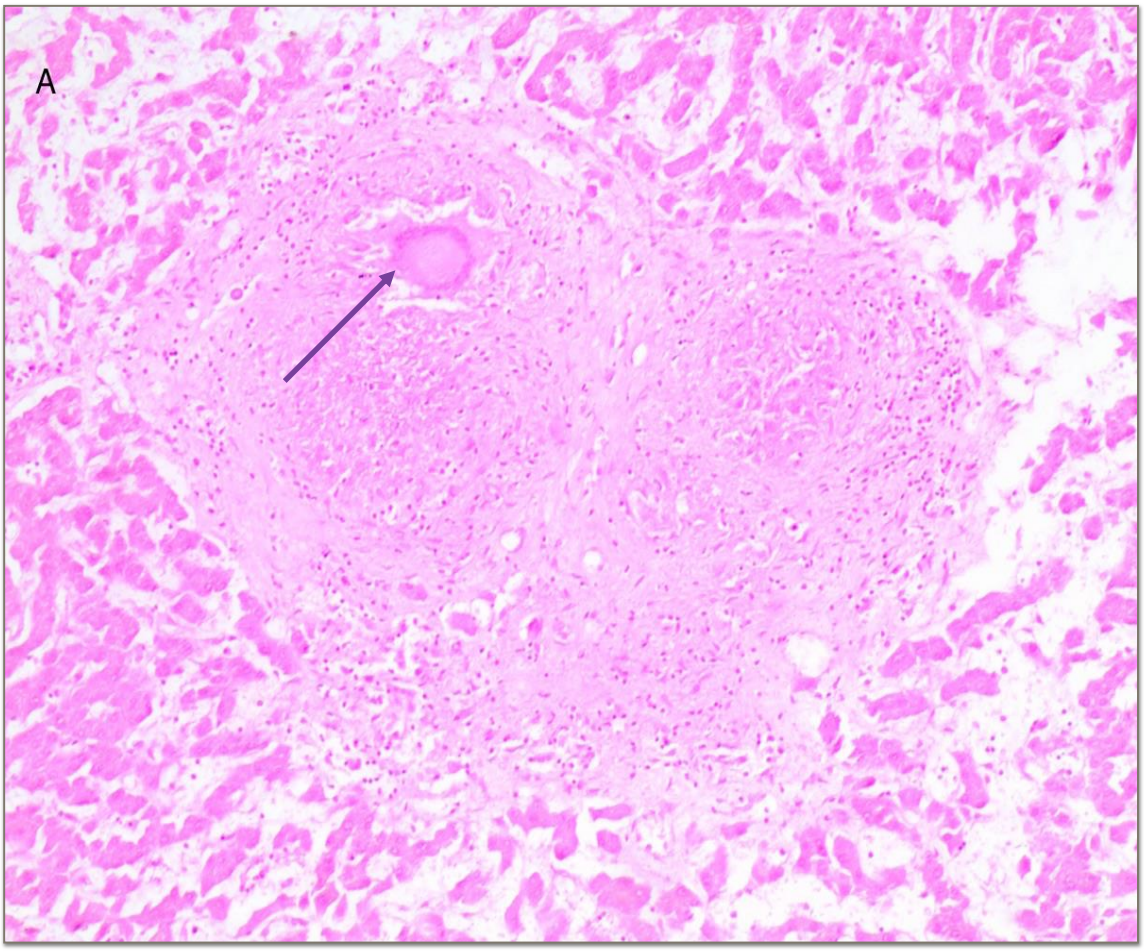


Figure 5A. Ziehl Neelsen stain highlighting the alcohol and acid fast bacilli, arrow. (X 400 magnification).

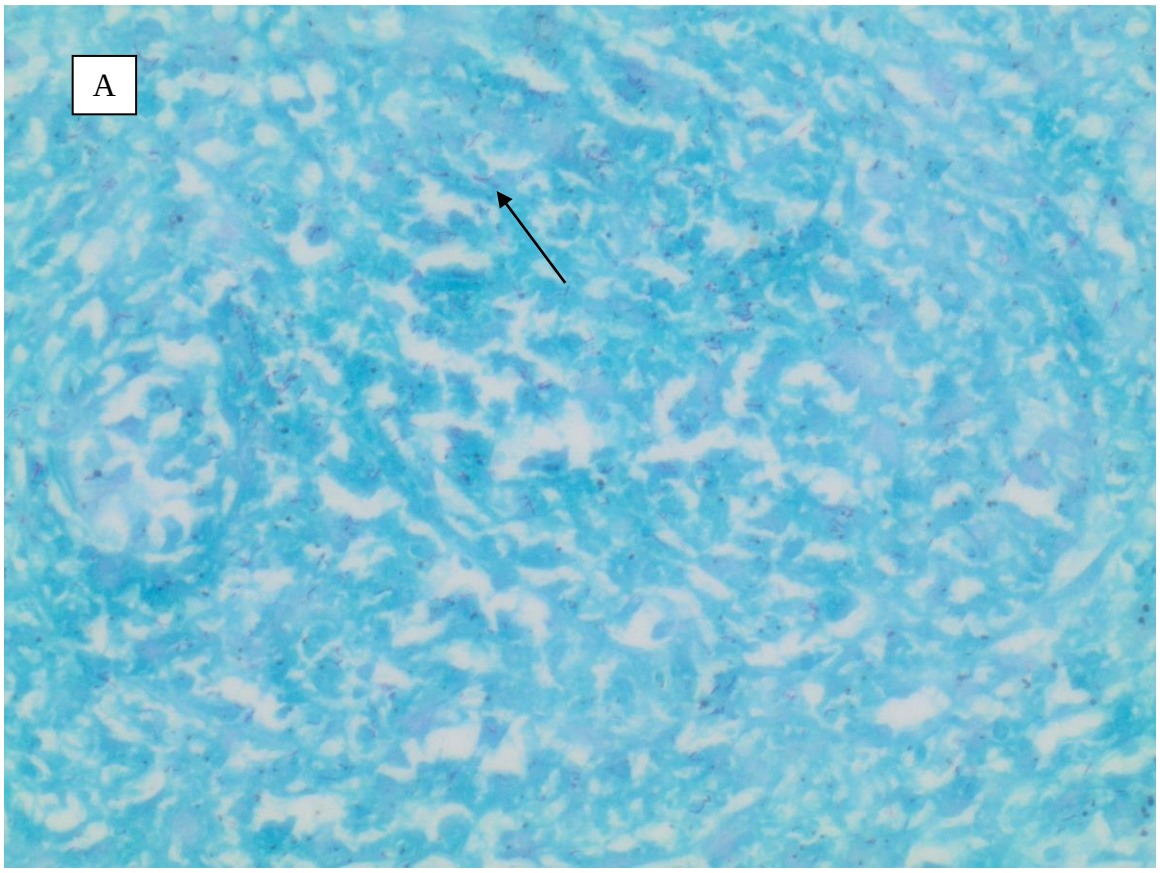
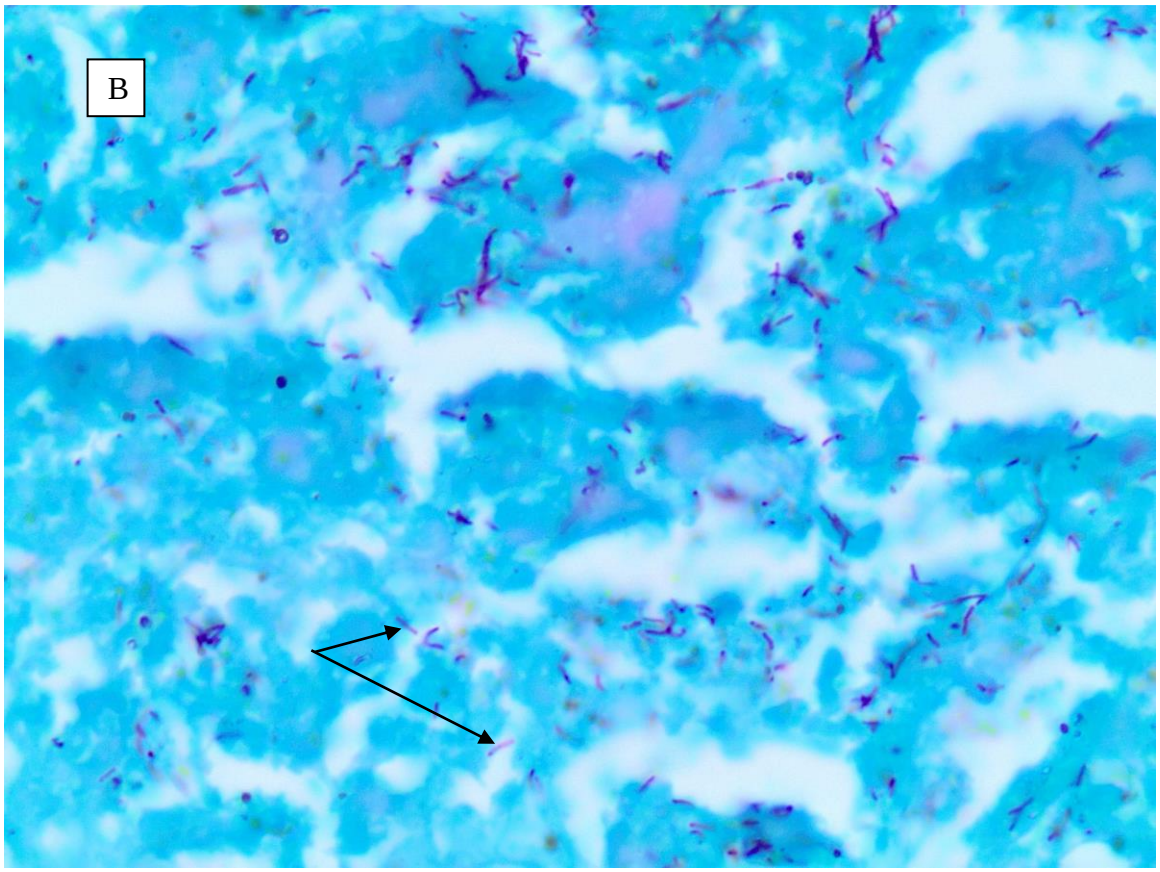


Figure 5B. Ziehl Neelsen stain (X 1000 magnification, under oil), shows the alcohol and acid fast bacilli (arrows).



Other findings included macro vesicular steatosis in 37 patients (26.5%), figure 6. This finding was not association with being on ART or anti TB drugs (p= 0.116, 0.989 respectively). Females were more likely to have steatosis than males (p=0.000).

Sinusoidal dilatation was found in 30 patients (21.9%) and parenchymal necrosis 23 patients (16.7%), figure 7. Patients with parenchymal necrosis were significantly more likely to be on ART (p=0.004).

12 patients (8.8%) had chronic hepatitis, 4 (2.9%) micro vesicular steatosis, 3 (2.2%) acute hepatitis, 3 (2.2%) portal granuloma, 1 (0.7%) cholestasis and 1 patient (0.7%) bile duct proliferation.

Multiple pathologies were found in 112 patients (81%), with the most common combination being portal tract fibrosis and nonspecific portal chronic inflammation, 76 patients (55%). Other combinations included; 18 patients (13%) with steatosis and caseating granulomatous hepatitis, 9 patients (6%) caseating granulomatous hepatitis and parenchymal necrosis, and 10 patients (7%) with steatosis, caseating granulomatous hepatitis and portal tract fibrosis/nonspecific portal chronic inflammation, table 5.

The sex distribution of the major histologic findings is highlighted in figure 9. For all the histologic lesions except steatosis, the distribution was skewed towards males.

Figure 6. Macro vesicular steatosis with granulomas, arrow. Insert shows Langhans type giant cell in a granuloma. Hematoxylin and eosin stain (X 400 magnification).

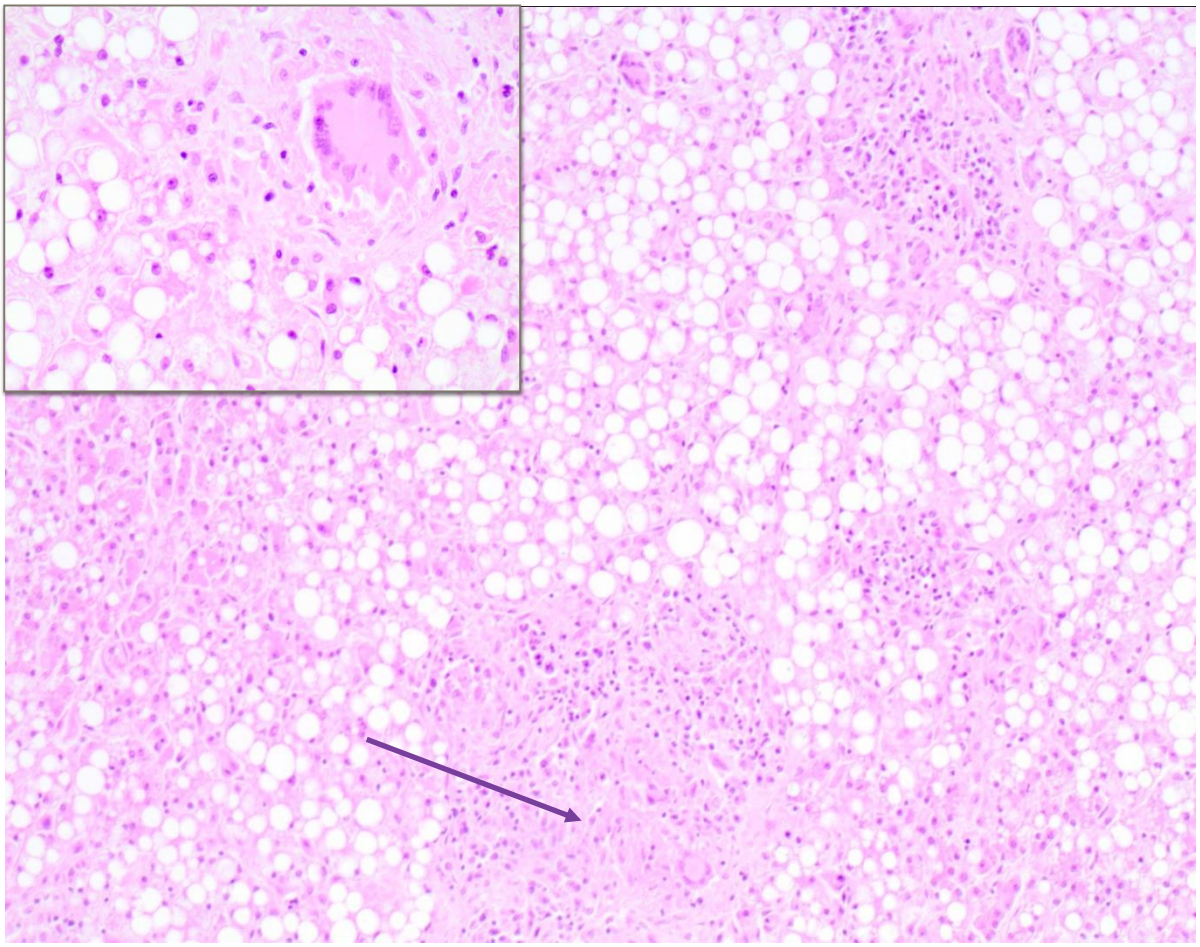
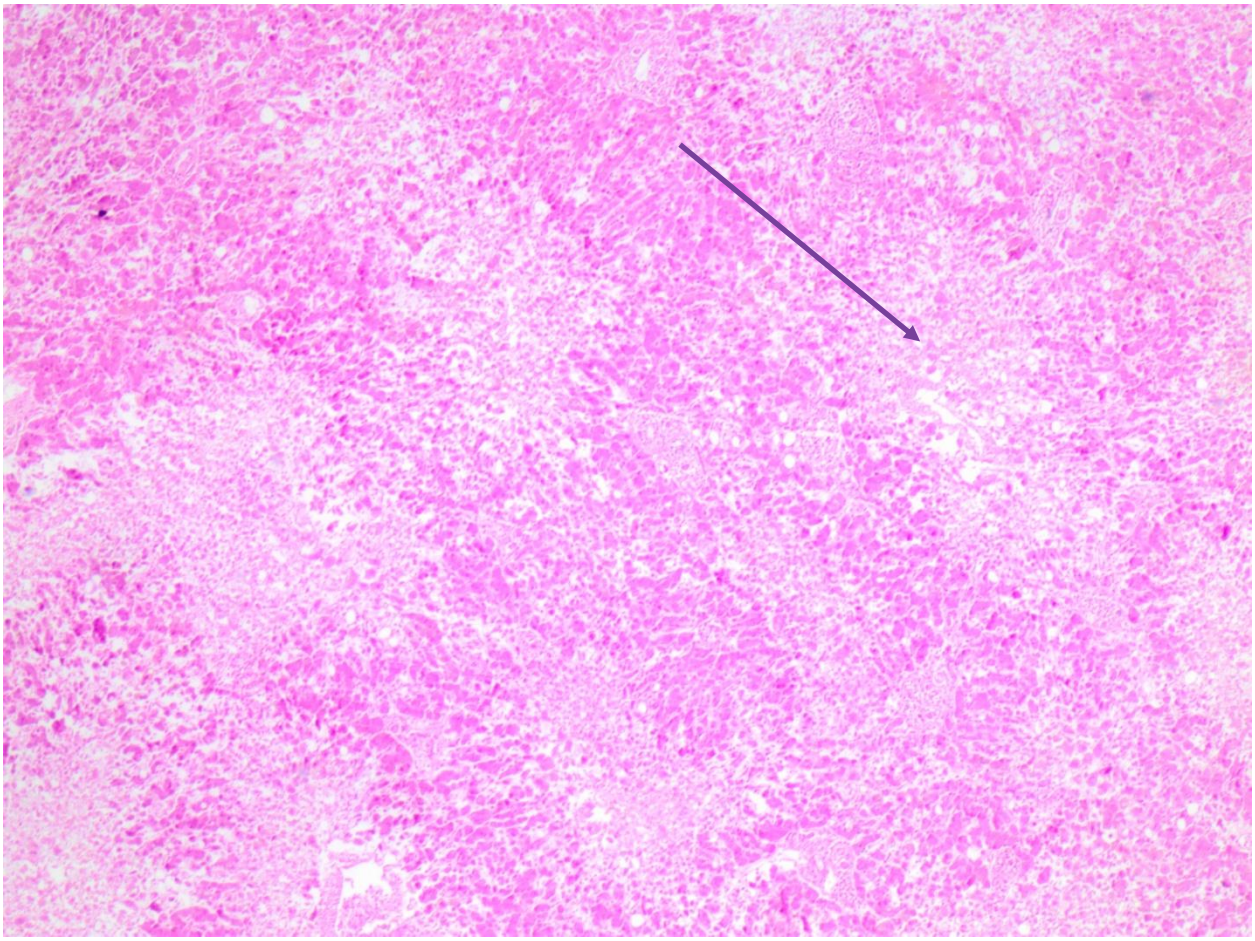


Table 6: Association of various categorical variable with histopathologic findings.

HISTOLOGY	Number of Cases-N (%)	**P values**		
		HAART	**Anti TB drugs**	**Sex** (male/female)
Portal tract fibrosis	101(73)	0.993	_	0.906
Nonspecific portal chronic inflammation	84(60.3)	_	_	0.416
Caseating granulomatous hepatitis	57(41.0)	0.366	0.185	0.388
Macro vesicular steatosis	36(26.5)	0.116	0.989	<0.001
Parenchymal necrosis	23(16.7)	0.004	0.405	_

Figure 7. Sub massive necrosis with a centrilobar (peri venular) distribution, arrow. X 400 magnification.



Only 2 patients (1.5%) had neoplasms, both of which were cavernous hemangiomas, figure 8. 9 (6.5%) patients had histologically unremarkable liver samples.

Figure 8. Hemangioma demonstrating dilated engorged vascular channels. Hematoxylin and eosin stain (X 400 magnification).

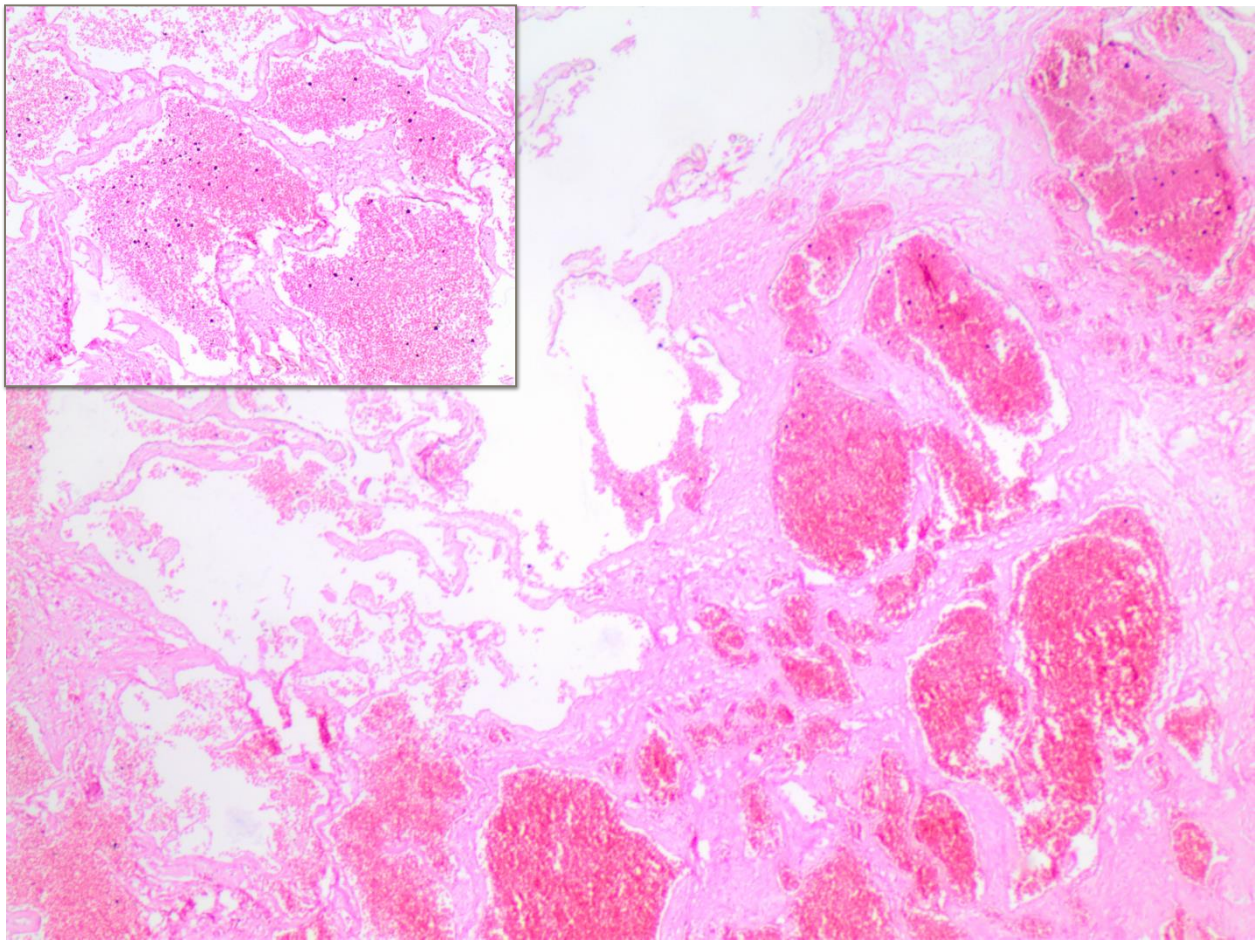
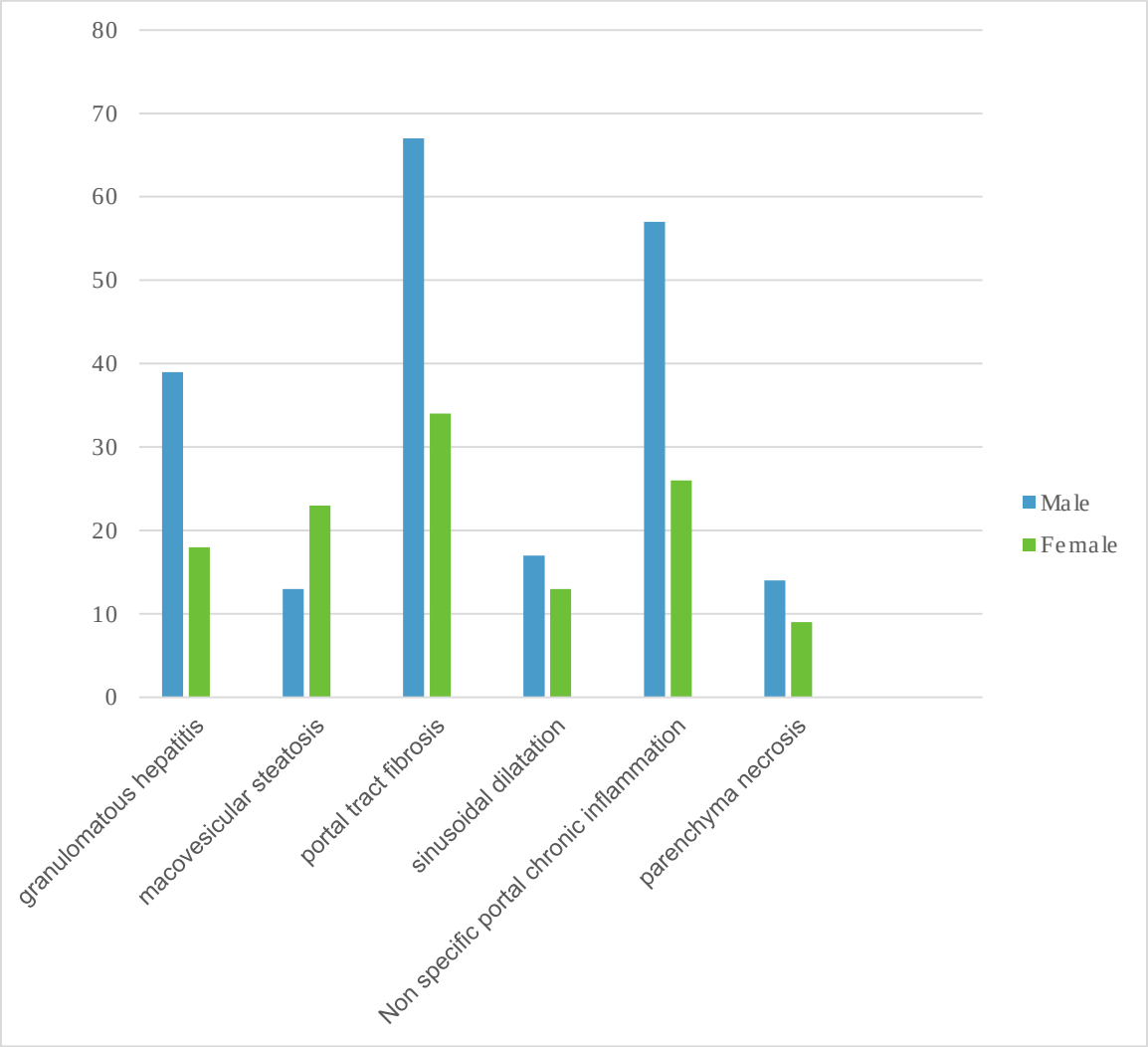


Figure 9. Chart showing the sex distribution of the different histologic findings.



CHAPTER FIVE

5.0 DISCUSSION

Autopsy studies have been used from time in memorial to study disease in humans and as a window into the different pathologies that can affect different population groups. Several studies have been done in HIV-infected adult patients, but this is the first study in Zambia that has looked at liver disease in this patient population. Many studies have shown that in the era of ART, liver disease among HIV infected patients has become one of the leading causes of morbidity and mortality [2, 13-17].

Patient demographics and characteristics:

The study population was composed predominantly of patients between the ages 31-40 years, figure 1, and most of these patients were male. The Zambia Demographic Health Survey of 2013-14 gives an HIV prevalence rate of 15% amongst women and 11% amongst men in this age group. Therefore, it is expected that the majority of our patients should be female. The study population is skewed towards male predominance. One of the explanations could be that the mortality rate amongst women is lower than men even though the prevalence rate of HIV is higher in women. This could be explained in several ways. Firstly, women in the urban areas of Zambia have better health seeking behavior than men[59], hence they get treated early and therefore prevent the dire complications of HIV infection. Secondly, proportionally more women are on ART than men, "even when the higher HIV prevalence in females is accounted for" [60].

The CD 4 counts for our patient population were low, table 2, with 82% having CD 4 counts less than 200 cells/mm^3. Previously, this was the threshold for starting ART, but this was changed in 2013. The current Zambian and WHO guidelines put the threshold at less than or equal to 500 and 350 cells/mm^3 respectively [61, 62]. With the scaling up of availability and access to ART, more HIV patients will be on treatment. In our study, 51.1% of the cohort was on ART. This is figure is expected to proportionally increase.

Histologic findings:

In this study, we have shown that significant liver pathology is found in 94% of HIV patients at autopsy, with only 6% (9) cases having histologically normal livers. Similar findings were found in India in 2004 by Amarapurkar *et al*, where only 10% of the patients had histologically normal livers[24]. Bal *et al* also in India around the same time found a rate of 30% normal liver histology in this patient population[63]. Close to home, in South Africa, Garcia-Jarden M *et al* in an autopsy study found only 10% had liver disease[20]. This was the predominant noninfectious condition found.

The most common hepatic histologic findings in our study were structural changes, with portal tract fibrosis being the most common, 73%. One patient had cirrhosis. He was a 41 year old male patient whose serum was positive for HBsAg. These findings defer from studies done in India, Indonesia and the United Kingdom where steatosis and mycobacterial infection were the leading causes of hepatic pathology[19, 25,26,27,63]. The possible reasons for the differences could be the higher prevalence of infections like schistosomiasis in sub-Saharan Africa and the higher proportion of patients on anti tuberculous medication in the study as compared to those cited both of which may cause hepatic portal tract fibrosis.

The most common infection was mycobacterium tuberculosis presenting as caseating granulomatous hepatitis, 41.0% (57 patients). This finding is similar to studies done in Thailand, Brazil and India by Terrha *et al*, Pereira et al, Amarapurkar *et al*, and Lanjawar *et al*, who found mycobacterium in 32.6%, 24%, 31.6% and 41% of their cases respectively [23, 26, 27, 28]. Studies done in Zambia and other parts of sub Saharan Africa and West Africa have shown a link between extra pulmonary mycobacterial infection and co infection with HIV[64, 65, 66]. It is no wonder that the proportion of patients with hepatic mycobacterial infection was high in our cohort. Coupled with this is the fact that Africa carries the highest burden of mycobacterial infection[67].

Of the 57 patients with caseating granulomatous hepatitis, only 33% (19) were on anti TB drugs at the time of their death, while 46% (26) were not. Data was unavailable in 12 cases. Laboratory tests and imaging modalities are readily available at tertiary level

hospitals in Zambia for the diagnosis of pulmonary *Mycobacterium tuberculosis*. However, in the presence of severe immunosuppression, like in this study population with mean CD 4 count less than 200 cells/ml, a patient may not present with classic signs and symptoms. Secondly some of the signs and symptoms may be mistaken for other infectious diseases[66]. Extra pulmonary mycobacterium tuberculosis is usually a manifestation of disseminated disease. Bates *et al* found that all the cases of extra pulmonary tuberculosis they had, also had concurrent pulmonary tuberculosis. Finally lack of proper laboratory support especially in resource poor countries may result in missed and late diagnosis of mycobacterial infection. Non-caseating granulomas were seen in portal tracts in 2.2% (3) cases in our study. These samples were examined at multiple levels and a cause for these granulomas was not established. They may have represented cases of sarcoidosis or previous Schistosoma infection.

Steatosis (fatty change), mostly of the macro vesicular type was seen in 26.5% (36) of the cases. Only 2.9% (4 cases) had micro vesicular steatosis. These findings are comparatively similar to those of Bal *et al*, who in India found fatty change in 39% of their cases[63]. Other studies have a lower proportion of cases with fatty change. Amarapurkar in India found a proportion of 10%[27], while Teerha *et al* in Thailand found 4.4% of cases with fatty change[26]. Earlier studies done in the west showed higher proportions of fatty change with some studies demonstrating this change as being the most common hepatic abnormality in HIV patients [24, 25]. This study found no association between being on ART or anti TB drugs and steatosis (p=0.116, 0.989 respectively). Only 15% (13) of the males had macro vesicular steatosis. This figure was higher for the females with almost half of them, 48% (23) having steatosis. Steatosis was strongly associated with female sex. This study found that 64% (32) of the males took alcohol while a smaller proportion of females, 41% (9) took alcohol. Therefore, proportionally more males are expected to have alcohol-related steatosis than females. This then suggests that the large proportion of females seen with steatosis can be explained by them having a higher incidence of non-alcoholic fatty liver disease. According to the WHO report on obesity, women are more likely to be obese than men in Africa, and this may account for the high proportion of women having fatty change as opposed to the males. One of the other causes of hepatic steatosis is malnutrition, which was not assessed in our

cohort, but was observed by the postmortem performers. Steatohepatitis was not observed in any of the patients.

In different studies done in India around the same time, Amarapurkar *et al* and Bal *et al* found hepatic congestion in 23.3% and 9% of their cases respectively[27, 63]. 21.9% of the cases in our study showed sinusoidal dilatation with congestion. There are several causes of sinusoidal dilatation and congestion. The most common is venous outflow impairment. Other conditions in which patients can present with sinusoidal dilatation and congestion include tumors and granulomas, Crohn's disease, antiphospholipid syndrome and heroin addiction. It can also be seen in portal vein thrombosis, rheumatoid arthritis and Still disease. A few cases have been recorded in patients taking oral contraceptives [68, 69, 70]. We were unable to determine the cause of the sinusoidal dilatation and congestion in our patients, but they could be a result of terminal events, like heart failure.

This study found parenchymal necrosis in 16.7% (23) of the patients. Amarapurkar *et al* found 3.3%[27], while other studies have found in the range of 2-11%[24, 71]. The rate in this study was significantly higher than in these studies. Parenchymal necrosis was associated with ART and not with anti TB drugs. Special stains for fungi did not show any organisms. The commonest cause of necrosis is hepatitis, either viral or drug related. Other causes include hypo perfusion of the liver parenchyma and paracetamol poisoning[72]. More than half of the patients in the study cohort were on ART, while the numbers were low in the cited studies. This may explain the higher frequency of parenchymal necrosis in this study.

Histologic features resembling chronic hepatitis have been reported in several studies. In our study chronic hepatitis was observed histologically in 8.8% (12) of the study population. The histologic features were not classic for hepatitis B and C, and serology for the two viruses was not routinely done. These findings are similar to those of Wilkins et al, Amarapurkar *et al* and Lebovics *et al* and, who reported 10%, 5% and 4% respectively in the cases they studied [25, 27, 73]. In Amarapurkar's study, serology for hepatitis B and C was negative in all the cases[27]. Schneiderman *et al* in an earlier study found much higher occurrence at 35.3%[24].

Nonspecific acute hepatitis was seen in 2.2% (3) cases and had a lobular distribution. One of the possible causes includes viral hepatitis.

Other non-specific findings included cholestasis 0.7% (1) and bile duct proliferation 0.7% (1). Te HS reported that cholestasis is frequent in HIV infected patients and some of the causes include ART, antimicrobial agents and opportunistic infections of the liver[74].

The only tumor found was a cavernous hemangioma, which presented in 2 patients. One case was of a 47-year-old female who was on HAART and anti TB drugs while the other was of a 40 year old male who was on anti TB but not on HAART. Hemangioma is the most common benign tumor of the liver [68], irrespective of a patient's HIV status. We did not see any of the AIDS-defining neoplasms in the liver. These include non-Hodgkin lymphomas and Kaposi sarcoma.

Multiple hepatic pathologies were found in 81% of the patients. Portal tract fibrosis with nonspecific portal chronic inflammation was the most common combination, 55%. Portal changes typically predominate in mild chronic viral hepatitis and the presentation is usually with portal fibrosis and inflammation[68]. Steatosis with caseating granulomatous hepatitis, was seen in 13% of the patients. Only 6 of these patients had available CD 4 counts. 4 patients had CD 4 counts of less than 100 cells/mm^3, while the other two had counts of 117 and 304 cells/mm^3. Although the number of patients is small with available CD 4 counts, the trend suggests that low CD 4 counts may be a factor that determines a patient presenting with multiple hepatic pathologies. This is not unusual given that with advancing immunosupression HIV-infected patients are more likely to acquire multiple infections and inflammatory conditions.

CHAPTER 6

6.0 CONCLUSION

This study has demonstrated that the spectrum of hepatic pathology in HIV positive adult patients at autopsy at the UTH, which is a tertiary referral center for the entire country is almost exclusively due to structural changes and infection/inflammation. No AIDS defining neoplastic lesions were demonstrated. Of the infectious/inflammatory causes, *Mycobacterium tuberculosis* presenting as granulomatous hepatitis was the most common. Portal tract fibrosis and chronic inflammation are the most frequent nonspecific hepatic histologic changes while steatosis is the commonest specific change. Some of the determinants of hepatic pathology in our cohort included female gender, which was strongly associated with steatosis, while a patient being on HAART was more likely to have parenchymal necrosis.

6.1 STUDY LIMITATIONS

This being a retrospective study, a lot of clinical information was missing making it difficult to draw conclusions on associations.

More specialized tests like immunohistochemistry were not utilized. This meant we could not screen for particular viral infections like Hepatitis B, C and CMV when suspected. Culture was not done making typing of the mycobacterial infection difficult. So infection with features consistent with Mycobacterium had to be assumed to be due to *Mycobacterium tuberculosis* seeing that this is the commonest mycobacterial infection in our setting.

The study was on postmortem material from patients who died while admitted to hospital with very low CD 4 counts. Therefore, the information gained may not be translated to healthy HIV-infected patients with higher CD 4 counts.

6.2 RECOMMENDATIONS

In view of the aforementioned, it is recommended that HIV infected patients presenting at UTH should be classified as high risk and clinicians have a high index of suspicion for hepatic pathology when managing HIV positive patients as various clinical investigations are now available to ascertain a definitive diagnosis in most cases either via serology (HBV) or radiology imaging/ imaging guided biopsy for histopathology (structural changes and tuberculous granuloma).

Further follow-up areas of research would be to correlate the spectrum of hepatic histopathology findings with the CD 4 count and viral load so as to identify time points at which specific pathology can be targeted for investigation in monitoring hepatic disease progression during management. A prospective study to determine other factors associated with liver disease should be carried out.

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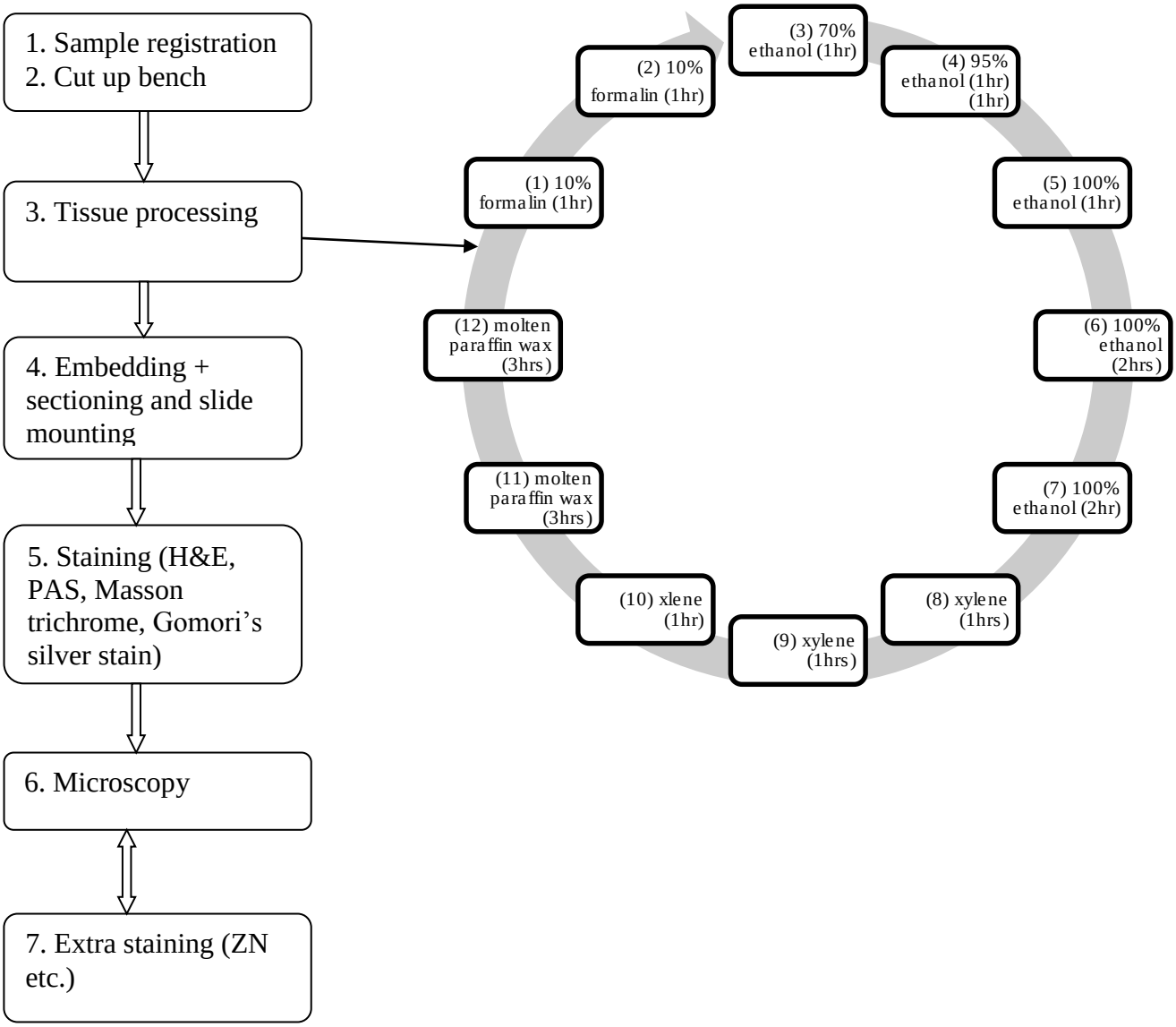
APPENDICES

A. TISSUE PROCESSING FLOW CHART



B. STANDARD OPERATING PROCEDURES **(SOP's)**

HEMATOXYLIN AND EOSIN STAINING PROTOCOL:

Principle

The oxidation product of haematoxylin is haematin, and is the active ingredient in the staining solution. Haematoxylin is not classified as a dye since the molecule possesses no chromophore. The *in situ* oxidation of haematoxylin is effected by the addition of a strong oxidant to the stain, in this case sodium iodate. Haematin exhibits indicator-like properties, being blue and less soluble in aqueous alkaline conditions and red and more soluble in alcoholic acidic conditions. In acidic conditions, haematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant, in this case aluminium. To ensure saturation of chemical binding sites, the stain is applied longer than necessary, resulting in the over staining of the tissues with much non-specific background colouration. This undesirable colouration is selectively removed by controlled leaching in an alcoholic acidic solution, (acid alcohol), the process being termed "differentiation". Differentiation is arrested by returning to an alkaline environment, whereupon the haematin takes on a blue hue, the process of "blueing-up". The haematin demonstrates cell nuclei.
Full cellular detail is obtained by counterstaining with the eosin mixture. Colour enhancement is achieved by fortifying the stain with phloxine, a chemical member of the same family as eosin (halogenated fluorosceins). The mechanism of their staining is not fully understood, but is believed to be of an electrostatic nature. Visualisations most acceptable to the histologist are obtained by applying the dyes in acidic conditions, whereby more intense specific colourations are obtained, the more acidic tissue components taking up the dye to a greater intensity, hence the addition of acetic acid.

Technical Points

1. (step 2) - The length of time necessary to over-stain the tissues will depend upon fixation and the type of alum haematoxylin employed. Lillie Mayer's alum haematoxylin-formalin fixed tissues should take 5 minutes.

Tissue Type	Haematoxylin	Acid alcohol 0.3%	Eosin	Comment
Routine tissues	4 minutes	See technical point 2	2 minutes	
Renal biopsies	10 minutes	1-2 seconds	2-4 minutes	Check staining

Decals	10 minutes	1-2 seconds	30 seconds	Check staining after blueing. Hx step may need to be repeated if prolonged decal.

2. (Step 4) - Differentiation with acid alcohol requires some practical experience to ascertain the correct end-point, since the acid solution alters the colour of the tissue to red. The correct end-point is when, after blueing up, the background is almost colourless. For renal biopsy sections, two quick dips in 0.3% acid alcohol are all that is required
3. (Step 6) - If Scott's tap water substitute is employed; blueing up is achieved in a much shorter time.
4. (Step 8) - Eosin is highly soluble in water. Over-staining is removed by washing in running water.
5. Fixation - Not critical. Acidic fixatives will give a more eosinophilic result. Picric acid containing fixatives give an overall enhanced result. Acidic decalcifying fluids give poor nuclear staining.
6. Renal biopsies - 10% buffered formalin. Sections cut at 2 micrometers

Method

1. Bring sections to distilled water
2. Stain nuclei with the alum haematoxylin (see note)
3. Rinse in running tap water
4. Differentiate with 0.3% acid alcohol (see note)
5. Rinse in running tap water
6. Rinse in Scott's tap water substitute (see note)
7. Rinse in tap water
8. Stain with eosin 2 mins
9. Dehydrate, clear and mount.

Results

Collagen.....pale pink
Muscle.....deep pink
Acidophilic cytoplasm.....red
Basophilic cytoplasm.....purple
Nuclei.....blue
Erythrocytes.....cherry red

Reagent Formulae

1. Lillie Mayer alum haematoxylin

aluminium ammonium sulphate ----- 200 g
haematoxylin (CI 75290) ---------- 20 g
ethanol ---------- 40 ml
sodium iodate ---------- 4 g
acetic acid ---------- 80 ml
glycerol ---------- 1200 ml
distilled water ---------- 2800 ml

In a 4L Ehrlenmeyer flask, to 1000 mls of the distilled water add the aluminium ammonium sulphate. Place the flask on a heater/stirrer, turn on the heater and allow to mix until the alum dissolves - this takes about 15 minutes. Remove the flask from the heater/mixer, allow cooling, and then adding the remaining 1800 mls distilled water - this will further cool the solution. Add the haematoxylin powder to the alcohol and dissolve as much of the powder as possible by shaking for a few minutes. Pour the strong alcoholic solution of haematoxylin into the cooled alum solution and stir to ensure all the powder is dissolved, preferably overnight. Add the sodium iodate, acetic acid, and finally the glycerol. Mix well, plug loosely and store.
It is appropriate to make up a batch of the required amount, dependent upon the usage rate.

2. Acid alcohol 0.3% Acid Alcohol

commercial grade ethanol ----- 2800 ml
distilled water ---------- 1200 ml
conc. hydrochloric acid --------- 12 ml

In a sufficiently large container, add the acid to the water, then add the alcohol and mix thoroughly. The generation of fine bubbles is an indication that mixing is thorough.

3. Scott's tap water substitute

sodium hydrogen carbonate --- 10 gm
magnesium sulphate ---------- 100 gm
distilled water ---------- 5 L

Dissolve the salts in the water. Store stock solutions at room temperature.

4. alc. acetified eosin/phloxine TQEH

1% eosin Y (CI 45380) ---------- 400 ml
1% aqphloxine (CI 45405) ----- 40 ml
95% alcohol ---------- 3100 ml
gl acetic acid ---------- 16 ml

Mix the above reagents together, and stir well. The solution keeps well.

References

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Lynch MJ, Raphael SS, Mellor LD, Spare PD and Inwood MJ, (1969), Medical Laboratory Technology and Clinical Pathology, 2nd edition, WB Saunders Co., Philadelphia London Toronto
LG Luna, Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, third edition, McGraw Hill.

PERIODIC ACID SCHIFF STAINING PROTOCOL:

1. Purpose

This stain is used for the demonstration of glycogen, mucin, and fungi; it is used for detection of glycogen in tissues such as liver, cardiac and skeletal muscle on formalin-fixed, paraffin-embedded tissue sections, and may be used for frozen sections as well. The glycogen, mucin, and fungi will be stained purple and the nuclei will be stained blue. Tissue sections are first oxidized by periodic acid. The oxidative process results in the formation of aldehyde groupings through carbon-to-carbon bond cleavage. Free hydroxyl groups should be present for oxidation to take place. Oxidation is completed when it reaches the aldehyde stage. The aldehyde groups are detected by the Schiff reagent. A colorless, unstable di-aldehyde compound is formed and then transformed to the colored final product by restoration of the quinoid chromophoric grouping.

2. Equipment, Reagents, Supplies

Equipment	Reagents	Supplies	PPE
Staining rack	Schiff's reagent	Positive control slide	Lab Coat
	1% Periodic Acid	Slides of interest	Gloves
	Distilled Water, Mayers Haematoxylin	Filter paper	Safety Goggles
	Xylene, Ethyl Alcohol, running tap water		

REAGENT PREPARATION

1. Periodic acid solution

Periodic acid	1 g
Distilled water	100 ml

2. Preparation of Schiff reagent

Dissolve 1 g of basic fuchsin and 1.9 g of sodium metabisulfite ($Na_2S_2O_5$) in 100 ml of 0.15 M hydrochloric acid (HCl). Shake the solution at intervals or on a mechanical shaker for 2 hours. The solution should be clear and yellow to light brown in color.
Add 500 mg of activated charcoal and shake for 1 to 2 minutes. Filter the solution through a No. 1 Whatman filter into a bottle. The filtered solution should be clear and colorless. If the solution is yellow, repeat the charcoal decolorization using a fresh lot of activated charcoal. Store at 4°C. Solution is stable for several months.

3. Specimen

Formalin fixed paraffin sections

4. Special safety precautions

General safety precautions as described in the UTH Safety Manual including Universal Precautions must be adhered to. Special safety procedures must be followed when

performing any procedure with corrosive reagents according to the Histology Safety Manual (HIS-SFT-v1).

5. Procedure Step-by step

Step #	Instruction
1.	Deparaffinize and hydrate to water.
2.	Rinse in distilled water
3.	Oxidize in 1% periodic acid solution for 5 minutes.
4.	Rinse in several changes of distilled water.
5.	Place in Schiff reagent for 15 - 20 minutes (Sections become light pink colour during this step).
6.	Rinse in running tap water for 5 minutes (Immediately sections turn dark pink colour).
7.	Stain the nuclei with Mayer's Haematoxylin for 1 minute. Differentiate and blue the sections.
8.	Wash in tap water for 5 minutes.
9.	Dehydrate in graded alcohol, clear in xylene (2 minutes each) and coverslip using a synthetic mounting medium DPX.
10.	Examine the control slide for the positive features and quality of stain.

6. Quality Control

The PAS stain with diastase or -amylase digestion has histochemical specificity for Glycogen. Skeletal muscle normally contains glycogen and is often recommended as a Positive control tissue. Kidney tissue can be used as a control demonstrating the features of the basement membrane (Tissue should be sectioned at 2µm).

<u>Schiff Reagent QC:</u>

- Test for Schiff reagent:

Pour 10 ml of 37% formalin into a watch glass. To this add a few drops of the Schiff reagent to be tested. A good Schiff reagent will rapidly turn a red-purple color. A deteriorating Schiff reagent will give a delayed reaction and the color produced will be a deep blue-purple.

- All solutions must be made in distilled water
- Use of any counter stain other than water weak Haematoxylin may mask results.

7. Reference range/Test Interpretation

Glycogen, neutral/sialomucins and some basement membranes ------red/purple
Fungi/various glycoprotein's---------- red/purple
Background ---------- blue
Nuclei ---------- blue

8. Notes, Limitations and Anything Else

a. The intensity of stain is dependent to some extent on the length of treatment with the periodic acid and Schiff reagent. For basement membranes, a longer time in periodic acid (10 minutes) and Schiff reagent (20 minutes) may give better results.
b. Earlier descriptions of the PAS procedure frequently recommended post-Schiff bisulfite rinses for the reduction of background. This is not necessary provided the slides are adequately rinsed in tap water.
c. Fixatives containing glutaraldehyde should be avoided if tissues are to be stained with the PAS technique. This is because glutaraldehyde contains two aldehyde groups; tissues fixed in glutaraldehyde contain free aldehyde groups capable of undergoing the Schiff reaction. This results in non-specific background staining.
d. Staining of glycolipids may be detected when frozen sections are used. In addition, staining of unsaturated lipids may occur in some cases due to the oxidation of carbon-to-carbon double bonds to produce Schiff-reactive aldehyde groups. However, glycolipids and unsaturated lipids rarely interfere with interpretation of results obtained from paraffin-embedded tissues as a significant loss of these molecules likely occurs during tissue processing.

9. Reference

1. http://www.ihcworld.com/_protocols/special_stains/pas.htm
2. John D. Bancroft (2007) Theory and Practice of Histological Techniques 6th Edition.

MASSONS TRICHROME STAINIG PROTOCOL:

1. **Purpose**

To differentiate between collagen and smooth muscle fibres or to demonstrate a change in the amount of collagen present. Other connective tissue elements can also be selectively demonstrated.

2. **Principle**

Masson trichrome uses two or more acid dyes of different molecular weights and contrasting colours to selectively color different basic tissue components on the tissue density. The general rule in trichrome staining is that a smaller dye will penetrate a tissue element but whenever a larger dye molecule can penetrate the same element, the smaller molecule will be replaced by it. The means by which selective sequential staining with a number of anionic dyes is achieved is almost certainly bound up with the physical nature of the dye and tissue and how they interact.

3. Equipment, Reagents, Supplies and PPEs (Personal Protective Equipment)

Equipment	Supplies	PPE
		Gloves. Lab coat

4. **Specimen**

5. **Special safety precautions**

General safety precautions as described in the UTH Safety Manual including Universal Precautions must be adhered to.

6. **Procedure**

.Take sections to water
.Wash in tap water
.Stain nuclei with Celestine blue –Heamatoxylin method
.Differentiate with 1% acid alcohol
.Wash well in tap water
.Stain in acid fuchsin solution for 5 minutes
.Rinse in distilled water
.Treat with Phosphomolybidic acid solution for 5 minutes
.Drain
.Stain with methylene blue OR in alanine blue solution for 2-5 minutes
.Rinse in distilled water
. Treat with 1% acetic acid for 2 minutes
.Dehydrate through alcohols
.Clear in xylene and mount in DPX

7. Quality Control

.Always include control sections of kidney or liver

.Optimize staining at each step

8. Reference range/Test Interpretation

Nuclei……..Grey/black

Muscle red blood cells, fibrin, cytoplasmic granules…….Blue

9. Notes and Limitations

Light green may be substituted for methyl blue or aniline blue

10. References

1. Ministry of Health Standard operating Procedure for level lll Hospitals (2008 Revision), Lusaka, Zambia.
2. Bancroft J.D. Stevens, A. Theory and Practice of Histological Techniques;
3. Churchill, Livingstone, London, 1982.
4. Bancroft, J.D.; Cook, H.C. Manual of Histological Techniques,
5. Churchill, Livingstone, London, 1984.
6. Bancroft JD, Gamble M, Theory and Practice of Histological Techniques;
7. Churchill, Livingstone, London, 2008.
8. Cook H.C.; Manual Histological Demonstration Techniques; Butterworth's, 1974.

9. Ministry of Health Standard operating Procedure for level lll Hospitals (2008 Revision), Lusaka, Zambia.

GROCOTT-GOMORI'S METHENAMINE SILVER STAINING PROTOCOL:

1. Principle

Chromic acid oxidation forms aldehydes from fungal cell wall polysaccharide components, which are subsequently demonstrated by reduction of an alkaline hexamine-silver complex. The reaction may be compared to that of the periodic acid Schiff reaction, (see PAS).

Grocott's alkaline hexamine-silver solution represents a vehicle which, upon reduction, precipitates nascent silver ions, thus blackening the site. This is known as an "argentaffin reaction".
Argentaffin reaction - *the ability of a silver complex solution to blacken a tissue element without the need of a reducing bath.* The term is adjectival and is applied to many methods, (eg von Kossa). The term "argentaffin reaction" should therefore not be used as a proper name.

2. Technical Points

1. A known positive control section must be used to ensure correct differentiation has been achieved.
2. Reagents should be prepared in a fume hood.

3. Method

1. Bring sections to distilled water.
2. Oxidise with 4% aq chromic acid at room temperature 1 hr
3. Wash in water for a few seconds.
4. Treat sections with 1% sodium metabisulphitee 1 min
5. Wash in running tap water 3 mins
6. Rinse thoroughly in distilled water.
7. Place in pre-heated working silver solution in a water bath at 60°C for 15 to 20 mins until section turns yellowish-brown (Check microscopically after washing in distilled water – fungi should be dark brown).
8. Rinse well in distilled water
9. Tone sections with 0.2% gold chloride 2 mins
10. Rinse in distilled water
11. Treat sections with 2% sodium thiosulphate 2 mins
12. Wash with running tap water 5 mins
13. Counterstain in working light green 15 sec
14. Rinse excess light green off slide with alcohol
15. Dehydrate, clear and mount.

4. Results

Fungi, Pneumocystis carnii, histoplasma spp ----------black
Inner parts of mycelia and hyphae ----------old rose
Leishmaniaspp, toxoplasma spp ----------negative
Mucin----------dark grey
Background ----------pale green

5. Reagent Formulae

Wear protective clothing, gloves and safety glasses when preparing reagents.

1. 4% aq Chromic Acid
   Chromium trioxide (analytical) ---- 4 g
   Distilled water ---------- 100 ml

2. Silver solution
   3% methenamine (= hexamine) ---- 23 ml
   5% silver nitrate ---------- 1.25 ml
   5% borax (sodium tetraborate) ---- 3 ml
   Distilled water ---------- 25 ml

3. 0.2% aq Sodium chloroaurate (yellow gold chloride)
   Gold Chloride (analytical) ---------- 1.0 g
   Distilled water ---------- 500 ml

4. 2% aq Sodium thiosulphate (hypo)
   Sodium thiosulphate ---------- 2.0 g
   Distilled water ---------- 100 ml
5. Working light green
   1% light green (CI 42095) in 1% acetic acid --- 10 ml
   Distilled water ---------- 40 ml

6. References

Grocott, R.G. 1955, A stain for fungi in tissue sections and smears. American Journal of Clinical Pathology, V25, p975
Luna L.G. Histopathological Methods and colour atlas of special stains and tissue artefacts, American Histo Labs Inc, Publications Division 1992.

ZIEHL NEELSEN STAINING PROTOCOL:

1.0 Purpose

To demonstrate acid fast bacteria belonging to the genus mycobacterium.

1.2 Principle

Mycobacterium possess a capsule containing a long chain fatty acid (mycolic acid) that makes them hydrophobic. The fatty capsule influences the penetration and resistance to removal of the stain by acid alcohol (acid fastness).The fatty capsule takes up the carbol fuchsin (primary stain) and resists decolorization (differentiation with acid alcohol) by acid alcohol. The speed with which the primary stain is removed by differentiation is proportional to the extent of the fatty coat. Either methylene blue or malachite green can be used as a counter stain. The principle is based on the primary stain that binds cell wall mycolic acids and the intense decolorization by acid alcohol does not release primary stain from the cell wall of AFB. The counter stain provides the contrasting background.

2. Equipment, Reagents, Supplies, Personal Protective Equipment (PPE)

Equipment	Reagents	Supplies	PPE
Staining Rack Staining trough	1. Filtered Carbol Fuchsin 2. Methylene blue or Malachite green 3. 1% Acid Alcohol 4. Alcohol 5. Xylene 6. Tap water 7. Distilled water	1. F i l t e r P a p e r	Gloves Proper PPE

PREPARATION OF REAGENTS

1. CARBOL FUCHSIN

Basic fuchsin	*1g*
Absolute alcohol	*10ml*
5% aqueous phenol solution	*100mls*

Dissolve carbol fuchsin in absolute alcohol then add 5% phenol solution. Mix well and filter before use.

2. DECOLOURIZING SOLUTION

70% Ethanol	*99mls*
Hydrochloric acid	*1ml*
Mix well.	

3. 0.2% METHYLENE BLUE

Methylene blue	*2mg*
Distilled water	*100mls*

Mix well

3. Specimen

Formalin fixed paraffin wax sections on frosted slides preferred. Sections must be appropriately cut, adequately dried.

4. Safety precautions

General safety precautions as described in the UTH Safety Manual including Universal Precautions must be adhered to.

5. Calibration procedures

NA

6. Procedure Step-by step

Describe the procedure step-by-step either in free text or table format:

Step #	Action
11.	Take test and control sections to distilled water
12.	Flood sections with filtered carbol fuchsin and stain for 30 minutes
13.	Wash well in tap water
14.	Differentiate well in 1% acid alcohol until sections are pale pink and red colour stops running (this usually only takes 2-5 dips).
15.	Wash well in tap water for tap water for 5 minutes then dip in distilled water
16.	Counter stain in 0.2% working methylene blue solution or malachite green until sections is pale blue or green (30 seconds -1 minute)
17.	Rinse in tap water then dip in distilled water
18.	Blot dry the slides after washing in water after the methylene blue counterstain. This will reduce the dehydration time and hence less leaching of the methylene blue.
19.	Dehydrate in alcohol then clear in Xylene and mount in DPX.

7. Quality Control

a) Always stain with a positive slide known to demonstrate the expected tubercle bacilli.
b) Avoid over – counterstaining as scanty organisms can easily be obscured
c) Before differentiation with acid alcohol, slides should be washed with 70% alcohol for about a minute to remove majority of the stain and this will reduce the differentiation time.
d) For easy and quick deparaffinization, sections should be place in Xylene right from the hot plate/oven while they are still warm

8. Calculation of results

Results are indicated as either positive or negative.

9. Reference range/Test Interpretation

Features	Result
Mycobacteria, hair shafts, Russell bodies, Splendore-Hoeppli immunoglobulins around actinomyces, fungal organisms	Red/Magenta
Background (Counterstain dependant)	Pale blue/Green

10. Alert/critical values, where appropriate

NA

11. Notes, Limitations and Anything Else

a) The blue counterstain may be patchy if extensive caseation is present. Care should be taken to avoid over-counterstaining as scant organisms can easily be obscured.
b) Decalcification using strong acids can destroy acid-fastness; formic acid is recommended.
c) Victoria blue can be substituted for carbol fuchsin and picric acid for the counterstain if color blindness causes a recognition problem.

12. Reference

1) Bancroft J.D. Stevens, A. Theory and Practice of Histological Techniques; Churchill, Livingstone, London, 1982.
2) Bancroft, J.D.; Cook, H.C. Manual of Histological Techniques, Churchill, Livingstone, London, 1984.
3) Bancroft JD, Gamble M, Theory and Practice of Histological Techniques; Churchill, Livingstone, London, 2008.
4) Cook H.C.; Manual Histological Demonstration Techniques; Butterworths, 1974.
5) Ministry of Health Standard operating Procedure for level lll Hospitals (2008 Revision), Lusaka, Zambia.

C. DATA COLLECTION TOOL

1. Serial number:
2. Age:
3. Sex:
4. Alcohol use: 1 yes
   2 no
   3 no data

5. On HAART; 1 yes
   2 no
   3 no data

6. Anti TB drug use: 1 yes
   2 no
   3 no data

7. Hepatitis BsAg: 1 positive
   2 negative
   3 not known

8. Hepatitis CV Ab: 1 positive
   2 negative
   3 not known

9. CD4 Count:

10. LFT's: 1 normal
    2 elevated
    3 no data

11. Portal tracts

    a) Chronic inflammation resembling hepatitis C; 1 present
    2 absent

    c) Fibrosis 1 absent
    2 confined to portal tracts
    3 portal –portal septa
    4 with distorted structure
    5 cirrhosis

    d) Bile duct proliferation 1 present
    2 absent

    e) Acute cholangitis 1 present

2 absent

f) Cholestasis 1 present
2 absent

g) Granuloma 1 present
2 absent

12. Parenchyma

a) Chronic hepatitis 1 present
2 absent

b) ground glass hepatocytes(hep B) 1 present
2 absent

c) Steatosis Micro vesicular 1 present
2 absent

Macro vesicular 1 present
2 absent

Both 1 present
2 absent

d) Steatohepatitis 1 present
2 absent

e) Acute hepatitis 1 present
2 absent

f) Caseating granuloma consistent with TB 1 present
2 absent

g) Necrosis 1 present
2 absent

h) Sinusoidal dilatation 1 present
2 absent

i) other 1 present
2 absent

13. Infections

a) Fungi

i) Cryptosporidiosis — 1 present 2 absent

ii) Histoplasmosis — 1 present 2 absent

iii) Other — 1 present 2 absent

b) Viruses

i) CMV — 1 present 2 absent

ii) Herpes — 1 present 2 absent

iii) Other — 1 present 2 absent

c) Bacteria

i) TB — 1 present 2 absent

ii) Other — 1 present 2 absent

d) Parasites — 1 present 2 absent

14. Neoplasms

a) Nodular regenerative hyperplasia — 1 present 2 absent

b) Capillary hemangioma — 1 present 2 absent

c) Hepatocellular carcinoma — 1 present 2 absent

d) Kaposi sarcoma — 1 present 2 absent

e) Lymphoma — 1 present 2 absent

f) Other — 1 present 2 absent

D. CONSENT FORM FROM SUBTYPE C NEURO-AIDS PATHOGENESIS IN ZAMBIA STUDY

CONSENT FORM FOR AUTOPSY, FOR THE NEXT OF KIN.

I give consent to have the deceased have tissues and blood samples be taken at the time of autopsy for the purposes of taking part in this Zambia HIV neuro AIDS study. I give my consent indicating voluntary and informed consent as next of kin to the deceased. I may withdraw my consent at any time without penalty or loss of benefits or treatment to which i am entitled. Doctors may choose not proceed to collect specimens or use such specimens for such study without my consent. Additionally, my withdrawal will not in any way affect how doctors treat me in regard to the deceased / next of kin

Declaration:

I understand what the study is all about and what is expected of if I participate in this study.

Interviewees
name.....

Signature / Thumb
print.....

Date.....
.....

Witness'
name.....

Signature.....
.....

Date.....
.....

Should you have any questions, you can contact Dr Constantine Malama, Kalingalinga Health Centre or Dr Victor Mudenda, at the UTH, Department of Pathology. You can also contact the Research and Ethics committee (REC).

Dr Constantine Malama

Project study medical doctor and principal investigator.

Mobile 097-9-070477

Dr Victor Mudenda

University teaching hospital

Department of Pathology and Microbiology

0966-750646

Dr Adelina Holgun

Research co-investigator

Mobile 0977-931184

Office260-252661

Dr KOR Chiyenu

Project medical doctor

Department of medicine

Mobile :0955-999357

ID ___ ___ ___ -___ ___ ___ Postmortem Information

Zambia HIV Neuropathogenesis

PATHOLOGY

Ward: ___ ___ ___ ___

AGE: ___ ___ SEX: O Male O Female

Clinical Diagnosis:_____

DOD: __ __/__ __/__ __ __ __ Time of D: ___ ___:___ ___ hrs

Date of AUTOPSY: __ __/__ __/__ __ __ __

Specimen collected: Date: ___ ___ /___ ___/___ ___ ___ ___

O Blood Time: _____

Purple top: _____ ml, # of vials: ____

Red top: _____ ml, # of vials: ____

Analysis: O HIV

Results: HIV: O Positive O Negative

O Plasma stored in -80 degree freezer

O CSF Time: _____

_____ ml, # of vials: _____

Analysis: O HIV

Results: HIV: O Positive O Negative

O CSF stored in -80 degree freezer

Tissues collected for MOLECULAR ANALYSIS

BRAIN REGION: Hemisphere

□ Frontal Lobe □ Right □ Left

□ Parietal Lobe □ Right □ Left

□ Temporal Lobe □ Right □ Left

□ Occipital □ Right □ Left

□ Hippocampus □ Right □ Left

□ Cerebellum □ Right □ Left

□ Basal Ganglia (Caudate/Putamen) □ Right □ Left

□ Lymph node □ Choroid Plexus □ Gut tissue

□Stored in cryovials, -80 degree freezer

Tissues collected for HISTOLOGICAL ANALYSIS

BRAIN REGION

□ Frontal Lobe □ Right □ Left

□ Hippocampus □ Right □ Left

□ Parietal Lobe □ Right □ Left

□ Temporal Lobe □ Right □ Left

□ Occipital □ Right □ Left

□ Basal Ganglia □ Right □ Left

□ Spleen □ Choroid Plexus □ Gut tissue □ Liver □ Lungs

□ Lymph node □ Kidneys/adrenals □ Pancreas □ Thyroid

□Stored in normal buffered formalin

Pathologist (Print)

Technician

_____

Date and Time picked up

E. ERES CONVERSE APPROVAL

33 Joseph Mwilwa Road
Rhodes Park, Lusaka
Tel: +260 955 155 633
+260 955 155 634
Cell: +260 966 765 503
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

6th March, 2016

Ref. No. 2015-June-005

The Principal Investigator
Dr. Chibamba Ngomalala Mumba
The University of Zambia
School of Medicine
Dept. of Pathology
P.O. Box 50110,
LUSAKA.

Dear Dr. Mumba,

RE: SPECTRUM OF HEPATIC PATHOLOGY IN HIV INFECTED ADULTS AT AUTOPSY AT UNIVERSITY TEACHING HOSPITAL ,LUSAKA.A SUB-STUDY OF THE SUB TYPE C NEURO-AIDS PATHOGENESIS IN ZAMBIA STUDY.

Reference is made to your corrections dated 5th February, 2016. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2015-June-005**
Approval and Expiry Date	Approval Date: 6th March, 2016	Expiry Date: 5th March, 2017
Protocol Version and Date	Version - Nil.	5th March, 2017
Information Sheet, Consent Forms and Dates	• English (Next of Kin Consent Form)	5th March, 2017
Consent form ID and Date	Version-Nil	5th March, 2017
Recruitment Materials	Nil	5th March, 2017
Other Study Documents	Data Collection Tool, Postmortem Information Sheet.	5th March, 2017
Number of participants approved for study	200 liver samples.	5th March, 2017

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Dr. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON