SERUM FIBRONECTIN LEVELS IN PEOPLE LIVING WITH HIV/AIDS

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THIS DISSERTATION IS SUBMITTED TO THE DEPARTMENT OF MEDICAL LABORATORY SCIENCES

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CERTIFICATION

This is to certify that Eyisi Blessing Nwamaka PG/MSc/07/47282, an M.Sc. student of the Department of Medical Laboratory Sciences, Faculty of Health Sciences and Technology, College of Medicine, University of Nigeria, Enugu Campus, majoring in CLINICAL CHEMISTRY has satisfactorily completed the requirements for the research work.

The results embodied in the work have not been submitted in part or full to any Diploma or Degree of this or any other University.

Supervisor : **Chief**

Onyeanusi J. C.

Signature: ----------

Date: ----------

DEDICATION

This work is dedicated to the Almighty God who manifested Himself to me as 'Jehovah Osuofia.'

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I wish to thank the following people for their contributions towards making this dream a reality: my supervisors, Chief Onyeanusi J. C., Dr. Ogbu I.S.I. for their guidance, Okechukwu Adum for all his assistance in the analytical process, my brothers and sisters for their prayers and encouragement, Chidi of H.G. & W. consultants Ltd, Chijioke Mbah for assisting in the sample collection, Chinedu Okoroafor and Michael Emeka for assisting in the statistical analysis. Also not left out is Chioma Igwe who helped in the typesetting of the work. May God reward all of you.

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LIST OF ABBREVIATIONS

AIDS	-	Acquired Immune Deficiency Syndrome
HIV	-	Human Immunodeficiency Virus
CD4	-	Cluster of Differentiation Cells
HREC	-	Health Research Ethics Committee
UNTH	-	University of Nigeria Teaching Hospital
ECM	-	Extra Cellular Matrix
CIG	-	Cold Insoluble Globulin
CAD	-	Coronary Artery Disease
MTCT	-	Mother to Child Transmission
CCR5/CxCR4	-	Chemokine Receptors
RNA	-	Ribonucleic Acid
DNA	-	Deoxyribonucleic Acid
CDC	-	Centre for Disease Control

PGL	-	Persistent Generalized Lymphadenopathy
PID	-	Pelvic Inflammatory Disease
BSA	-	Bovine Serum Albumin
HOS	-	Human Osteosarcoma
TCR	-	T Cell Receptor
APC	-	Antigen Presenting Cell
MHC	-	Major Histocompatibility Complex
DM	-	Diabetes Mellitus
EDTA	-	Ethylene Diamine Tetraacetic Acid
OD	-	Optical Density
HAART	-	Highly Active Antiretroviral Therapy
PB	-	Polybrene
LCK	-	lymphocyte-specific Protein Tyrosine Kinase.

ABSTRACT

Serum fibronectin level was investigated in people living with HIV/AIDS. A total of 161 subjects were recruited into the study. Out of this, 98 (60.9%) constituted the patient population while 63 (39.1%) formed the controls. The age range considered was between 10 to 65 years. Ethical clearance was obtained from the Health Research Ethics Committee (HREC) of University of Nigeria Teaching Hospital, (UNTH) Ituku Ozalla. Informed consent was obtained from the participants and questionnaires were used to collect some vital data from them. Human fibronectin ELISA kit from WKEA Med Supplies Corp. was used to assay for fibronectin concentration while $CD4^+$ count was assayed using PARTEC $CD4^+$ easy count kit. The relationship between fibronectin level and $CD4^+$ count was investigated using Spearman's Correlation Coefficient. Test of significance for the following parameters was conducted using the Student's t-test and probability values less than 0.05 ($p<0.05$) was considered statistically significant: (i) fibronectin level in both test and control, (ii) $CD4^+$ count in both test and control, (iii) fibronectin level for $CD4^+$ count less than or equal to 200 ($CD4^+ \leq 200$) and $CD4^+$ count greater than 200 ($CD4^+ > 200$). The effect of duration of treatment with HAART on fibronectin level in the patient group was investigated using the Analysis of Variance Technique (ANOVA). From the result of the study, no correlation existed between fibronectin concentration and $CD4^+$ count in the patient population ($r=-0.084$, $p=0.1911$). The same holds for the control group ($r=-0.02$, $p=0.5931$). Comparison of fibronectin level between the test and control group showed a highly significant statistical difference ($p<0.001$). Equally, comparison of $CD4^+$ count between the test and control group showed a highly significant statistical difference ($p<0.001$). The study of the effect of treatment with HAART on the subjects showed a rise in fibronectin level between 1-5 years which is statistically significant ($p<0.01$). Fibronectin

level increased with increasing duration of treatment though there was a drop which is not statistically significant at 6-7 years of treatment.
From this study, we conclude that fibronectin concentration may not be used as a biological marker in HIV/AIDS patients.

CHAPTER ONE

INTRODUCTION

Fibronectin is a large molecular weight glycoprotein of about 440 kilodalton found as soluble dimer in plasma and an insoluble multimer in the basement membrane and intracellular matrix (Pankov and Yamada, 2002). It is composed of two similar but unidentical subunits. The characteristic features of fibronectin include its various molecular and biologic interactions which can be assigned to the different domains of the molecule. Fibronectin has been shown to bind Candida albicans, protozoan pathogens such as trypanosoma cruzi and leishmania, and to the envelope glycoprotein of viruses (Protor, 1987). Fibronectin is capable of binding to various complement components possibly making clearance of viral antigen-antibody-complexes, which are observed in the circulation during viral infection, more rapid. Thus virus-binding ability of fibronectin may play a role in modifying viral infections in the body.

Fibronectin is seen in the immune complexes found in patients with some bacterial and rheumatic diseases. This protein may also increase the size of the immune complexes thereby facilitating their clearance (Torre et al, 1990). Viruses can interact with or modify the expression of fibronectin. The binding of fibronectin to viruses may play a role in the pathogenesis of viral infections, as is known to be the case in some bacterial infections, and thus may potentially modify viral infections in the body.

Fibronectin is a major component of the Extracellular matrix (ECM) in lymph node tissue, the main site of Human Immunodeficiency Virus (HIV) deposition and replication during the chronic phase of the infection (Marinka et al, 2002). Several monocyte functional abnormalities have been reported in AIDs including defective chemotaxis and phagocytosis (Courtney et al, 1987). The ability of viruses and bacteria to interact with the ECM) plays an important role in their infectivity and pathogencity (Marinka et al, 2002).

Fibronectin increases the phagocytic capacity of macrophages and neutrophils by increasing chemotaxis, phagocyte adherence and phagocytosis (Protor, 1987).

The activity of fibronectin in patients with HIV infection is of interest because fibronectin has been found to interact with phagocytes at several stages of the phagocytic process.

Some researchers have tried to define the interaction of fibronectin in patients with HIV/AIDs (Raffi et all, 1992; Torre et al, 1994; Marinka et al, 2002). However, there has not been resolution of the topic.

Marinka et al, (2002) investigated whether ECM fibronectin could affect the ability of HIV to infect lymphocytes. They came up with the conclusion that HIV infectivity of primary CD4 lymphocytes is enhanced by greater than one order of magnitude in the presence of super fibronectin, a multimeric form of fibronectin that closely resembles the in vivo matrix fribronectin. Giampaolo et al, (2002) did similar work as Marinka et al and came up with the same conclusion that the infectivity of primary CD4 cells is enhanced by super fibronectin.

This work is set to estimate the serum fibronectin levels in people living with HIV/AIDS infection with a view to finding whether it can be of any diagnostic importance in those individuals.

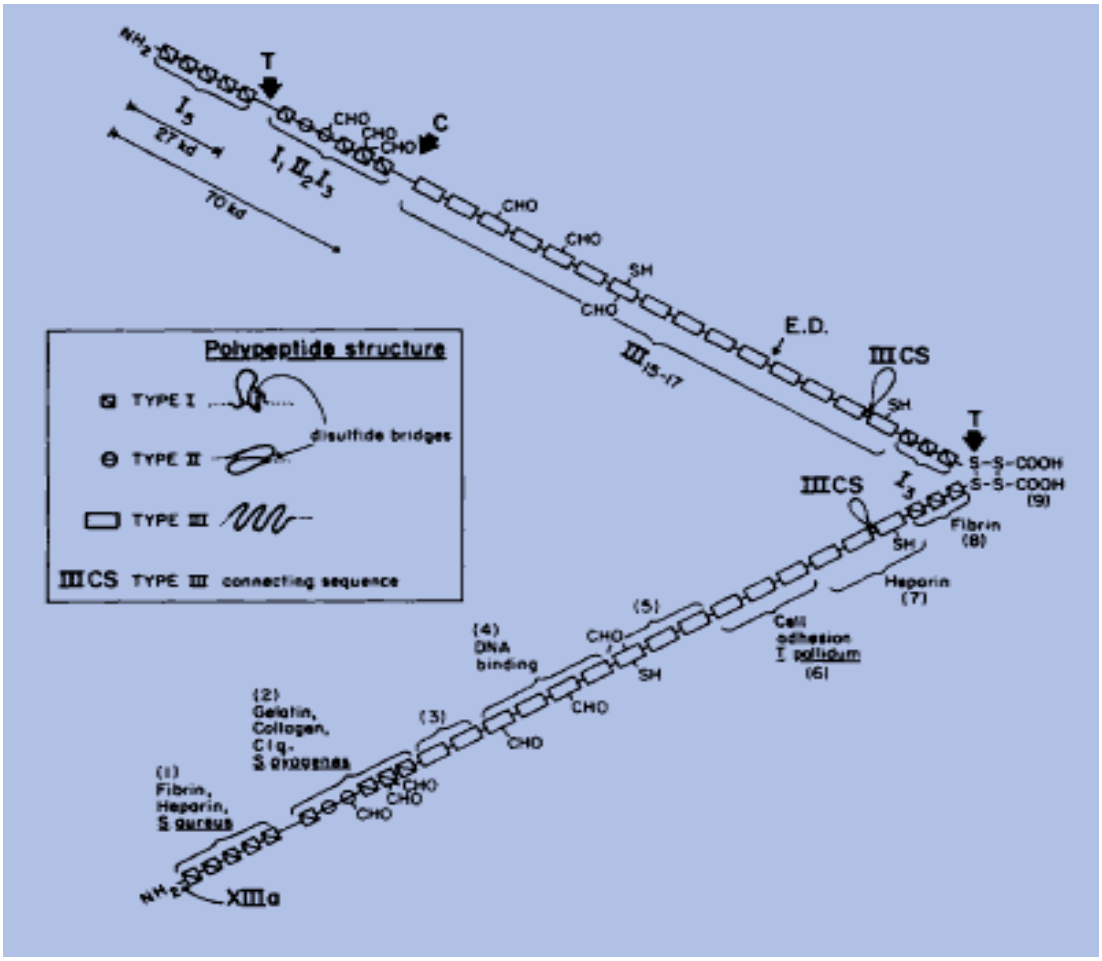
AIMS AND OBJECTIVES

1. To estimate the serum fibronectin levels in people living with HIV/AIDS infection
2. To determine the CD4 counts of these patients.

CHAPTER TWO

LITERATURE REVIEW

FIBRONECTIN STRUCTURE



2.1

Fibronectin (FN) is composed of two similar but unidentical subunits. It has a domain structure and the different domains account for the various molecular and biologic interactions of the molecule (Protor 1987). See figure 1.

It exists as protein dimer consisting of two nearly identical monomers linked by a pair of disulphide bonds (Pankov and Yamada, 2002).

Fibronectin exists in two different forms:-

Soluble plasma fibronectin (formerly called "cold insoluble globulin" (Cig): This is a major protein component of blood plasma (300µg/ ml) and is produced in the liver by hepatocytes.

Insoluble cellular fibronectin: This is a major component of the Extracellular matrix. It is secreted by various cells, primarily fibroblasts, as a soluble dimer and is then assembled into an insoluble matrix in a complex cell-mediated process. Other cells that secrete the type 2 fibronectin include chondrocytes, endothelial cells, macrophages as well as certain epithelial cells.

Fibronectin is rod-like and is composed of three different types of homologous, repeating modules namely type 1 (f_1), type II (f_2), and type III (f_3) modules.

F_1 module consists of 12 type 1 modules and is involved mainly in fibrin and collagen binding.

F_2 module consists of 2 type II modules and is involved mainly in collagen binding.

F_3 module consists of 4 type III modules. In addition to binding various forms of integrins, they also participate in heparin binding (Pankov and Yamada, 2002; Wikipedia, the free encyclopaedia).

2.1.1 PROPERTIES OF FIBRONECTIN

Fibronectin is ubiquitous in nature. It binds to membrane spanning receptors called integrins. It is secreted by mesenchymal cells and assembled into insoluble matrice (Bozzini et al, 1998).

It is located in cell surfaces, plasma, amniotic fluid, saliva, and virtually all body fluids and tissues (Torre et al, 1993). It cross-link collagen to bind cells together (Burtis et al, 2001).

In addition to integrins, fibronectin also bind ECM, components such as collagen, fibrin, laminin, heparin-sulphate proteoglycans (e.g. syndecans).

It may be considered as stable molecule since freezing or thawing do not alter their concentration either in serum or cerebrospinal fluid (CSF) from healthy volunteers (Torre et al, 1993).

The fibronectin protein is produced from a single gene, but alternative splicing of its pre -mRNA leads to the creation of several isoforms. Decreased fibronectin is seen in patients after trauma, burns, sepsis (Boughton et al, 1983). It is also seen in sickle cell anaemia (Emeribe et al, 2000).

Fibronectin is increased in morbidly obese individuals as a result of increased synthesis by lipocytes.

Raised plasma glucose has been found to increase fibronectin synthesis in trabecular meshwork cells (Sato and Roy, 2002). It is also increased in coronary artery disease (CAD) (Ozcelik et al, 2009). Altered fibronectin activities (i.e. decreased expression, increased degradation, and decreased organisation) has been associated with a number of pathologies including cancer and fibrosis (Williams et al, 2008).

2.1.2 FUNCTIONAL ROLES OF FIBRONECTIN

Fibronectin has numerous functions that ensure the normal functioning of vertebrate organisms (Pankov and Yamada, 2002). It is involved in many cellular processes including tissue repair, cell adhesion, growth, migration, differentiation, wound healing and embryogenesis. It can serve to organise cellular interactions by binding to different components of the ECM or membrane bound fibronectin receptors on cell surfaces (Ward and Marcey, 2001). It acts as a general adhesion molecule by anchoring cells to collagen or proteoglycan substrates. It is involved in phagocytosis as a non-specific opsonin (Raffi et al 1992; Torre et al, 1993).

Fibronectin plays a crucial role in wound healing (Grinnel,1984). The five phases of wound healing include:

i) Homeostasis

ii) Inflammation

iii) Cellular migration and proliferation

iv) Protein synthesis and wound contraction

v) Remodelling

The ECM facilitates wound healing by providing hemostasis, and in addition, an environment that intrinsically recruits new tissue cells to the wound site (Richard et al, 1999).

Along with fibrin, plasma fibronectin (the soluble form) is deposited at the site of injury, form a blood clot that stops bleeding and protects the underlying tissue. As repair of the injured tissue continues, fibroblasts and macrophages begin to remodel the area, degrading the proteins that form the provisional blood clot matrix and replacing them with a matrix that resembles the normal surrounding tissue. Firbroblasts secrete proteoses, including matrix metallo-proteins that digest the plasma fibronectin and then the fibroblasts secrete cellular fibronectin and assemble it into an insoluble matrix.

Fragmentation of fibronectin by proteoses has been suggested to promote wound contraction – a critical step in wound healing.

Fragmenting fibronectin further exposes its v-region, which contains the site of alpha 4-beta 1 ($\alpha_4\beta_1$) integrin – binding.

These fragments are believed to enhance $\alpha_4\beta_1$ integrins expressing cell binding, allowing them to adhere to and forcefully contract the surrounding matrix (Grinnel, 1984).

The ability of fibronectin to bind to several pathogens including viruses, and the interactions of fibronectin with several serum proteins such as complements and C-reactive protein suggest an important role in limiting viral infection and promoting healing process (Keski – Oja et al, 1987).

Fibronectin are necessary in embryogenesis, and inactivating the gene for fibronectin results in early embryonic lethality. Fibronectin are important for guiding cell attachment and migration during embryonic development. In mammalian development, the absence of fibronectin leads to defects in mesodermal, neural tube, and vascular development (George et al, 1993). Similarly, the absence of a normal fibronectin matrix in developing amphibians cause defects in mesodermal patterning and inhibits gastrulation (Daribere and Schwarzbauer, 2000).

Fibronectin is also found in normal human saliva, which helps prevent colonization of the oral cavity and pharynx by potentially pathogenic bacteria (Hasty and Simpson, 1987).

2.1.4 DIAGNOSTIC IMPORTANCE OF FIBRONECTIN

Fibronectin is an excellent marker of pre-term delivery (a delivery of about 37 weeks of gestation) since it leaks into the vagina if pre-term delivery is likely to occur. A systematic review of medical literature disclosed that fetal fibronectin is a good predictor of spontaneous preterm birth before cervical dilation (Honest et al, 2002). The test may be run on patients between 22-35 weeks gestation (Burtis et al, 2001) by collecting cervical and vaginal secretions with a swab (x3) and the concentration determined by immunoassay. Values greater than 50ng/ml indicate likely pre-term birth.

FIBRONECTIN IN OSTEOARTHRITIS

Fibronectin fragments are potentially earlier damage mediators in osteoarthritis than catabolic cytokines.

The fibronectin fragments upregulate matrix metalloproteins (MMP) expression, significantly enhance degradation and loss of proteoglycan (PG) from cartilage and temporarily suppress proteoglycan synthesis, all events observed in osteoarthritis. Fibronectin fragments may be involved in normal cartilage homeostasis as well. For example low concentration of fibronectin fragments enhance anabolic activities like protein

synthesis and could play a role in normal homeostasis. While some proteins decrease in osteoarthritis, the precursor of fibronectin fragments, native fibronectin, is elevated in the cartilage matrix in human osteoarthritis cartilage with the greatest increase near the articular surface or close to eburnated areas.

2.1.5 ROLE OF FIBRONECTIN IN CANCER

The adhesive glycoprotein, fibronectin and integrin receptors appear to play important roles in the progression of metastatic disease (Han et al, 2006). Fibronectin-integrin interactions are important in tumour cell migration, invasion and metastasis. In addition to promoting cell adhesion to the ECM, these proteins may also function in chemotaxis and control of proliferation. Peptide and antibody inhibitors of fibronectin and integrin functions have been shown to be effective inhibitors of metastasis, and are potentially important reagents for the study and control of cancer (Akiyama et al, 1995).

In lung carcinoma, fibronectin expression is increased, especially in non-small cell lung carcinoma. The adhesion of lung carcinoma cells to fibronectin enhances tumorigenicity and

confers resistance to apoptosis, including chemotherapeutic agents (Wikipedia, the free encyclopedia).

Fibronectin has been shown to stimulate the gonadal steroids that interact with vertebrate androgen receptors, which are capable of controlling the expression of cyclin D and related genes involved in cell cycle control. These observations suggest that fibronectin may promote lung tumour survival and resistance to therapy and it could represent a novel target for the development of new anti-cancer drugs. (Wikipedia, the free encyclopaedia)

2.2.1 **HIV INFECTION**

HIV is a lentivirus (a member of the family of retroviridae) that causes AIDS, a condition in which the progressive failure of the immune system allows life threatening opportunistic infections to thrive. HIV was discovered in 1981.

Location: HIV can be found in blood, semen, vaginal fluid, pre-ejaculate and breast milk.

Route of transmission: HIV can be transmitted through unsafe sex, contaminated needles, breast milk, and mother-to-child transmission (MTCT) i.e. peri-natal transmission.

HIV infects vital cells in the human immune system such as helper T cells (specifically $CD4^+$ T cells), macrophages, dendritic cells. When $CD4^+$ T cells number decline below a critical level (<200µl/ml), cell-mediated immunity is lost and the body becomes progressively more susceptive to opportunistic infections.

Two types of HIV have been characterized, viz: HIV–1 and HIV-2. HIV–1 is more virulent and more infective and is the cause of the majority of HIV infections globally (Wikipedia, the free encyclopedia).

2.2.2 HIV PATHOGENESIS

The worldwide spread of HIV–1 indicates that the virus effectively counteracts innate, adapted and intrinsic immunity. Despite its modest genome size (<10kb) and its few genes (HIV encodes three structural genes (gag, pol and env), HIV–1 excels in taking advantage of cellular pathway while neutralizing and hiding from the different components of the immune system. The HIV is made up of genetic material (RNA), enzymes and coating (cell membrane). These help the virus to enter and use other cells to make copies of itself.

HIV mainly infect white blood cells called T lymphocyte cells (T cells). HIV uses $CD4^+$ to gain entry into the host T cells and achieves this by binding of the viral envelope protein known as glycoprotein 120 (gp 120) to $CD4^+$ (Torre et al, 1994).

The $CD4^+$ T lymphocyte is the primary target for HIV infection because of the affinity of the virus for the $CD4^+$ surface receptor marker. The binding of $CD4^+$ creates a shift in the conformation of the gp 120 allowing HIV-1 to bind to a co-receptor expressed on the host cell. The co-receptors are chemokine receptors – CCR5 or CXCR4. Which of these co-receptors is used during infection is dependent on whether the virus is infecting a macrophage or T helper cell. (Wikipedia, the free encyclopaedia). Following a structural change in another viral trans-membrane protein called glycoprotein 41, HIV inserts a fusion peptide into the host cell that allows the outer membrane of the virus to fuse with the cell membrane.

HIV is one of the few retroviruses, meaning that it can convert its two strands of RNA into DNA by use of the enzyme – reverse transcriptase. After binding of the $CD4^+$ cell, the virus enters the cell, and using the reverse transcriptase enzyme, merges its RNA with the hosts genetic material. This causes the DNA in the

$CD4^+$ cell to make copies of the virus, a process called replication. Another enzyme called protease helps the new virus to form. The new virus then "binds off" the infected cells into the body where they infect more $CD4^+$ cells.

The presence of the virus causes a person's immune system to react by attacking the virus itself and any HIV-infected cells. This process results in the formation of antibodies. A person is said to be HIV positive if antibodies to the virus are detected by tests, indicating infection. As HIV-infected $CD4^+$ cells are destroyed or impaired, the person's immune system becomes less and less effective at fighting infection and disease. The person is said to be immune-compromised" or 'immuno-deficient'. Such people are more likely to develop unusual diseases called opportunistic infections that they would not get if their immune systems are healthy. As the number of $CD4^+$ cells decrease, the person is more likely to get sick and have more serious illnesses. When this is the case, the person is usually diagnosed with AIDS (M C Cune 2001)

2.2.2 **HIV CLASSIFICATION**

The center for Disease Control (CDC) classification system (revised in 1993) classified HIV infection on the basis of both the $CD4^+$ cell count and by the presence of HIV-related conditions while the World Health Organisation (WHO) system of classification (revised in 2007) is based only on the clinical manifestations.

While the CDC classification system grouped the HIV-related conditions into three categories (Asymptomatic, symptomatic, and AIDS), the WHO classification system grouped them into four classes (Primary, Asymptomatic, Symptomatic, and Progression from HIV to AIDS).

The CDC classification system: The revised CDC classification system for HIV-infected adolescents and adults categorizes persons on the basis of clinical conditions associated with HIV infection and $CD4^+$ T lymphocyte counts.

This system replaces the classification system published in 1986, which included only clinical disease criteria and which was developed before the wide spread use of $CD4^+$ T cell testing

$CD4^+$ T lymphocyte categories:

The three $CD4^+$ T lymphocyte categories are defined as follows:-

Category 1 : ≥500 cells/µl

Category 2 : 200-499 cells/µl

Category 3 : <200 cells/µl

These categories correspond to $CD4^+$ T lymphocyte counts per microlitre of blood and guide clinical therapeutic actions in the management of HIV-infected adolescents and adults. The lowest accurate, but not necessarily the most recent $CD4^+$ cell count should be used for classification purposes.

Clinical Categories: These are defined as follows:-

Category A: (Asymptomatic seropositive). This consists of one or more of conditions listed below in an adolescent or adult (≥ 13 years) with documented HIV infection. Conditions listed in category B must not have occurred.

Asymptomatic HIV infection, persistent generalized lympho-adenopathy (PGL), acute primary HIV infection with accompanying illnesses or history of acute HIV infection.

Category B: (Symptomatic). This consists of symptomatic conditions in an HIV-infected adolescent or adult that are not

included among conditions listed in clinical category C and that meet at least one of the following criteria:

a) The conditions are attributed to HIV infection or are indicative of a defect in cell mediated immunity.
b) The conditions are considered by physicians to have a clinical course or to require management that is complicated by HIV infection.

Examples of conditions in category B include but are not limited to: Bacillary angiomatosis, oropharyngeal (thrush) candidiasis, vulvo-vaginal: -Persistent, frequent or poorly responsive to therapy, cervical dysplasia (moderate or severe cervical carcinoma in situ; constitutional symptoms, such as fever (38.5^{o}c) or diarrhoea lasting greater than one month, PID especially if complicated by tubo-ovarian abscess, peripheral neuropathy, respiratory tract infection (tonsillitis, sinusitis, otitis media, pharyngitis).

Category C: (AIDS). Includes the clinical conditions listed in the AIDS surveillance case definitions e.g. HIV wasting syndrome, phenuocystis pneumonia, chronic herpes simplex, septicaemia, Kaposi sarcoma. (CDC 1993; CDC 1999, WHO 2007).

2.2.4 HIV/AIDS DIAGNOSIS

A person is diagnosed with HIV under the following conditions for persons aged 13 years or above.

a) Repeatedly reactive screening tests for HIV antibody (e.g. enzyme immunoassay) with specific antibody identified by the use of supplemental tests (e.g. Western blot, immunofluorescent assay).
b) Direct identification of the virus in host tissues by virus isolation.
c) HIV antigen detection or
d) A positive result on any other highly specific licensed test for HIV.

Serological tests are generally used for screening. The definition of AIDS include all HIV-infected individuals with $CD4^+$ cell count of <200 cells/µl (or percentage $CD4^+$ cell < 14%) as well as those that met the clinical conditions enumerated in stage 4 of the classification system (CDC 1993).

Flow Cytometry is the standard method for $CD4^+$ cells quantification.

2.2.5 HIV INTERACTION WITH FIBRONECTIN

Fibronectin can bind several viruses, fungi, and protozoa. Retroviruses bind to the heparin-binding domain of fibronectin. HIV-1 has been shown to bind to fibronectin, heparin, heparan sulphate, and antibody against fibronectin blocked binding. Bronchoaveolar lavage fluid from children with HIV-1 infection contained significantly higher concentrations of fibronectin than fluids from healthy children, suggesting that the ECM may play an important role in retroviral infection by binding and harbouring viral particles. $CD4^+$ and chemokine receptors are implicated in virus adsorption but alternative receptors such as fibronectn and membrane glycolipids can contribute to binding (pugliese et al, 2005). Binding of retroviruses to fibronectin is mediated by virus-associated heparan sulphate and inhibited by polybrene, heparinase and soluble heparan sulphate. The positively charged polybrene (PB) may interact with the negatively charged heparan sulphate on the viral particle and prevent virus binding to fibronectin. Heparinase inhibits binding to fibronectin by degrading virus-associated heparan sulphate. Likewise, free heparan sulphate competes with the virus for

binding to the heparin binding domain of fibronectin (Pedro et al, 2002).

Glycoprotein 120 envelope protein of HIV binds to the III-c region of fibronectin and results in increased viral adhesion to lymphocytes (Roger et al, 2009).

The ability of retroviruses to bind fibronectin has been utilized to transfer genes by co-localization of virus and target cells. This method, termed fibronectin-assisted gene transfer, improved retroviral transduction onto T-lymphocytes and hemopoietic stem cells (Martin et al, 2002).

Fibronectin is a major component of the ECM in lymph node tissue, the main site of HIV deposition and replication during the chronic phase of infection. Super fibronectin, an in vitro model for matrix fibronectin significantly enhances HIV infection of lymphocytes (Tellier et al 2000).

Giampaolo et al (2002), showed in their work that matrix proteins and their conformational status may play a role in the pathogenesis of HIV. They were able to show that superfibronectin (sFN) enhanced infection of primary $CD4^+$ T cells by both R_5 and X_4 strains of HIV-1. They used HIV pseudotyped with different envelope glycoproteins 120 and

HOS cells transfected with various chemokine receptors alone or in combination with the $CD4^+$ molecule. They showed that sFN-mediated enhancement required the $CD4^+$ receptor and does not alter the specificity of gp 120 for different chemokine receptors. Because the 111-C fragment of fibronectin also resulted in enhancement, they asked whether proteolysis of fibronectin-generated fragments are capable of enhancing HIV infection. They found out that progressive proteolysis of FN fragment by chymotrypsin correlates with an enhancement of HIV infection in both $CD4^+$ T cell line. Furthermore, incubation of HIV with sFN significantly prolonged infectivity at 37^oC compared with dimeric FN or Bovine Serum Albumin (BSA). They concluded from their results that polymerized (matrix) or degraded (inflammation-associated, but not dimeric (plasma) fibronectin are capable of enhancing infection by HIV-I independent of the co-receptor specificity of the strains. Moreover, virions bound to matrix FN maintain infectivity for a longer period of time than do virions in suspension.

Also Marinka et al (2000), investigated whether ECM could affect the ability of HIV to infect lymphocytes. They used sFN, a multimeric form of FN that closely resembles the in vivo matrix

FN. They showed that HIV-1_{111B} efficiently binds to multimeric FN and that HIV infection of primary $CD4^+$ T lymphocyte is enhanced by greater than one order of magnitude in the presence of sFN. This increase appears to be due to increased adhesion of viral particles to the cell surface in the presence of sFN followed by internalization of virus. Enzymatic removal of cell surface proteoglycans inhibited the adhesion of HIV-1_{IIIB}/sFN complexes to lymphocytes.

In contrast, antibodies to integrins had no effect on binding of HIV-1_{111B}/sFN complexes to lymphocytes. The III-C peptide alone also bound HIV- 1_{IIIB} efficiently and enhanced HIV infection, although not as effectively as sFN. HIV - 1_{IIIB} gp120 envelope proteins bind to the 111_1-C region within matrix fibronectin, and that this interaction may play a role in facilitating HIV infection in vivo, particularly in lymph node tissue.

2.3.1 FUNCTIONAL ROLES OF $CD4^+$ CELLS

The CD4 (Cluster of Differentiation 4) is a glycoprotein expressed on the surface of T helper cells, monocytes, mecropheges and dendritic cells. It was discovered in the late

1970s and was originally known as Len-3 and T4 (after the OK T4 monoclonal antibody that reacted with it) before being named CD4 in 1984. In human, the CD4 protein is encoded by the CD4 gene (Isobe et al, 1986).

The $CD4^+$ T lymphocytes co-ordinate a number of important immunologic functions and a loss of these functions result in progressive impairment of the immune response (Pedro et al, 2002).

CD4 is a co-receptor that assists the T cell receptor (TCR) with an Antigen presenting cell (APC). Using its portion that resides inside the T cell, CD4 amplifies the signal generated by the TCR by recruiting an enzyme known as the tyrosine kinase LCK, which is essential for activating many molecules involved in the signalling cascade of an activated T cell. CD4 also interact directly with Major Histo-compatibility complex (MHC) class II molecules on the surface of the APC using its extracellular domain. The extracellular domain adopts an antigen-like beta-sandwich with seven strands in 2-beta sheets, in a Greek key topology (Brady et al, 1993).

CD4 continues to be expressed in most neoplasm-derived T helper cells. It is therefore possible to use CD4 immuno-

histochemistry on tissue biopsy samples to identify most forms of peripheral T cell lymphoma and related malignant conditions (Kumerasen et al, 2003).

The antigen has also been associated with a number of auto immune diseases such as vitiligo and type 1 Diabetes mellitus (Zamani et al, 2010).

$CD4^+$ T cells play a crucial role in type 1 DM as a source of CD154. Without CD154, $CD4^+$ T cells were not capable of mediating help in disease development in non-obese mice. In fact, full expression of CD154 on the $CD4^+$ T cells seem to be essential in the normal spontaneous development of type 1 Diabetes Mellitus (Koji et al, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 STUDY POPULATION

A total of 161 subjects were recruited into this study. Out of these, 98 (60.9%) constituted the patients population while 63 (39.1%) represented the controls. The patients were known HIV seropositive individuals who are receiving treatment at the United State President's Emergency Plan for AIDS Relief/ AIDS Prevention Initiative in Nigeria (US PEPFAR/APIN) Clinic of the University of Nigeria Teaching Hospital, Ituku Ozalla, Enugu State. The controls were apparently healthy persons who were tested and found to be seronegative for HIV. They were drawn from the UNTH community (Staff and students) and also Garriki Area of Enugu Metropolis.

The consent of the participants were sought for and obtained after the whole procedure was explained to them. Those that objected were left out both in the patient and control population.

The following data were collected from the patients through the use of questionnaire: sex, age, duration of infection, symptoms and history of any disease, duration of treatment.

Ethical clearance was obtained from the Health Research Ethics Committee (HREC) of UNTH, Ituku Ozalla. Code N0. (NHREC/05/01/2008B).

Code No. NHREC/05/01/2008B

3.2 BLOOD SAMPLE COLLECTION AND STORAGE

1ml of venous blood were collected into EDTA anti-coagulated bottles and used to analyze for $CD4^+$ count within one hour of sample collection. 2mls of the same blood sample were collected into plain bottles and allowed to clot. After clotting and clot retraction, the samples were spun at 3000rpm for 10 minutes, separated into serum bottles and then stored at – 20^oC for onward use for the assay of fibronectin.

3.3 CD4 COUNT

The kit used for this assay is PARTEC CD4 easy count kit (code no: 05-8401) obtained from PARTEC GMbH AM Flug Platz 13. D-02828. Gorutz-Germany. The kit adopts the Flow Cytometric assay procedure.

Assay principle: Mouse monoclonal antibody MGM-241 recognizes the human $CD4^+$ antigens, a transmembrane glycoprotein (55KDa) of the immunoglobulin supergene family, present on a sub set of T-lymphocytes ("helper/inducer" T cells)

and also expressed at a lower level on monocytes, tissue macrophages and granulocytes. About 20-60% of human peripheral blood mononuclear cells as well as a sub population of monocytes but with a weaker signal are stained. $CD4^+$ is the primary receptor for the HIV.

Assay procedure: 20µl of EDTA anticoagulated whole blood were mixed gently with 20µl of $CD4^+$ monoclonal antibody (MAbPE) in a PARTEC test tube. These were incubated for 15minutes at warm temperature while being protected from light. Then 800µl of no lyse buffer were added, vortexed gently, and the blood sample analysed on a PARTEC Flow Cytometer for $CD4^+$ with the results expressed as cells per microlitre whole blood. Reference range: (500-1200) cells/µl

3.3.2 FIBRONECTIN

The kit employed here is Human Fibronectin ELISA kit obtained from WKEA MED SUPPLIED CORP, Chenguang Gardon, Qianjiu Street, Changelun 130012 China.

Assay principle: Microtitre plate wells coated with purified Human fibronectin antibody formed the solid phase. Fibronectin is then added, thereafter enzyme-labelled antihuman antibody to

form antibody antigen-enzyme-antibody complex. Addition of substrate yields a blue colour catalysed by horse radish peroxidase (HRP) enzyme. The reaction is stopped by adding Sulphuric acid and the colour change is measured Spectrophotometrically at 450nm wavelength. The concentration of fibronectin in the sample is then determined by comparing the OD of the samples to the standard curve.

Assay procedure:

PREPARATION OF STANDARD TUBES;

10 microtitre plate wells were used for the standard. The assay was done in duplicate. 100µl standard and 50µl standard diluent were added in plates 1 and 2 and mixed. 100µl was removed from each plate and added separately to plates 3 and 4 together with 50µl standard diluent. These were again mixed, 50µl discarded, and 50µl each transferred to plates 5 and 6-70 these were added 50µl standard diluents each, mixed and 50µl transferred to plates 7 and 8. 50µl standard diluents were added, mixed and 50µl removed and transferred to plates 9 and 10.

Finally, these were mixed, and 50µl discarded from each plate. The concentrations of the standards were respectively 1,500µg/l, 1000µg/l, 500µg/l, 250µg/l and 125 µg/l.

Preparation of Standard Curve

The average of the paired ODS of the standards were taken and the standard curve plotted using OD against concentration of the standard in µg/l.

Sample Assay:

10µl of the sample were added to the microtitre plates with 40µl sample diluents. These were well mixed, covered with the closure plate membrane, incubated for 30mins at 37^{o}C. After, they were washed five times using automatic washer and drained on an absorbent paper. The enzyme conjugate containing HRP and goat antihuman antibody were added, mixed, and incubated again for 30mins at 37^{o}C.

These were washed automatically for five times and drained on an absorbent paper. The substrates (A and B) were added, mixed well and incubated finally for 15mins at 37^{o}C. The reaction was stopped with the stop solution and the OD of each sample determined using micro plate reader (Mindray Analyser). The concentration of fibronectin was then read from the Standard Curve. Reference range: (70-1800)µg/l.

Statistical Analysis:

The statistical package used in this work is Graph Pad Prism version 10. The mean ± 2SD were determined both for $CD4^+$ and fibronectin for the tests and controls.

Analysis of variance (ANOVA) was used to compare the effect of duration of treatment with HAART on the patients.

Correlation study between fibronectin concentration and $CD4^+$ count was done using the Spearman's Correlation Coefficient. Test of significance was considered at $P < 0.05$ using the student's T-test.

CHAPTER FOUR

RESULTS

The result of this study showed that there was no correlation between $CD4^+$ count and fibronectin concentration in people living with HIV/AIDS. The correlation co-efficient for the test group is r=-0.084, P=0.1911. Similarly, in the control group, there was no correlation between $CD4^+$ count and fibronectin concentration (r=-0.02, P=0.5931).

The mean of both $CD4^+$ and fibronectin when compared between the test and control groups showed a significant statistical difference (P=0.001 for $CD4^+$ and P=0.003 for fibronectin).

Table 1: $CD4^+$ and FN for both test and controls

	$CD4^+$cells/µl	**FNµg/l**
Test (n = 98)	435.4 ± 261.5	491.3 ± 220.9
Control (n=63)	899.2 ± 258.1	675.2 ± 271.1
P–value	0.001	0.003

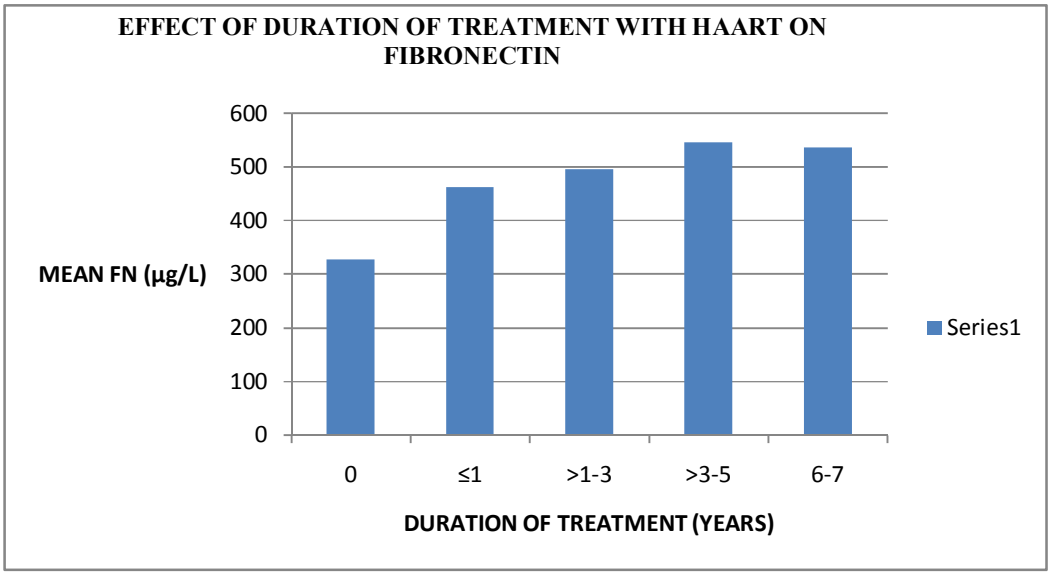
Table 2: Comparison of FN levels in patients with $CD4^+ \leq 200$ and $CD4^+ > 200$

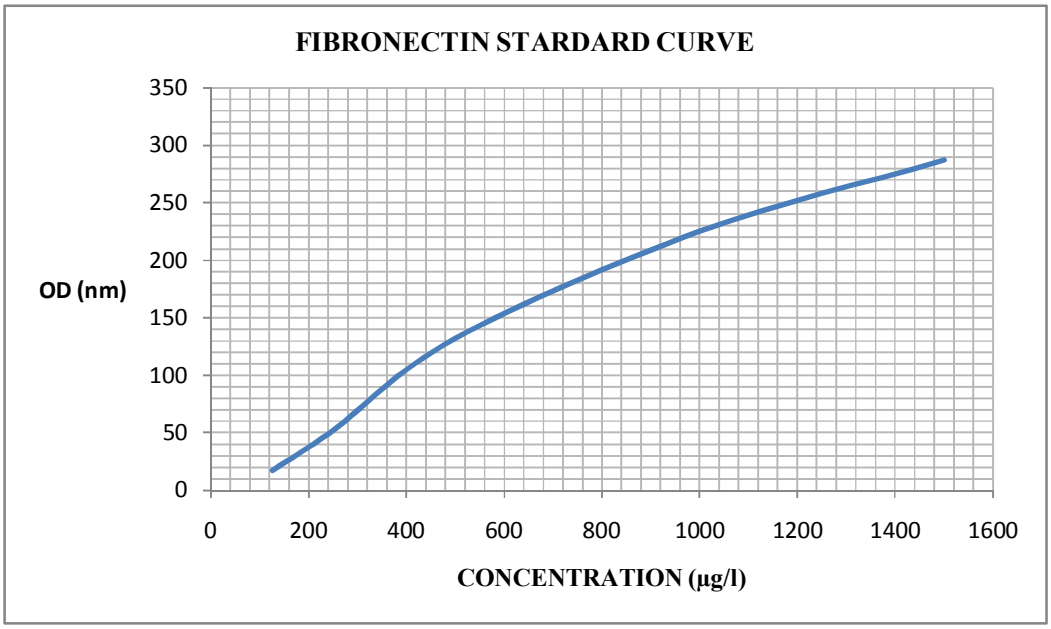
	$CD4^+ \leq 200$	**$CD4^+ > 200$**	**P – value**
FN	390.7±197.3	606.9 ± 235.8	< 0.05
N	23	72	

When fibronectin was compared between patients with $CD4^+$ count less than or equal to 200 ($CD4^+ \leq 200$) and those with $CD4^+$ greater than 200 ($CD4^+ > 200$), there was a significant difference in their mean ($P < 0.05$).

Table 3: Effect of duration of treatment with HAART on fibronectin

Duration of Treatment (Yrs)	Drug Naive Patients	≤ 1	<1-3	>3-5	6-7	P-value
FN	327.7	463.3	497.1	545.9	537.4	0.01
SD	166.1	201.1	273.3	161.3	244.1	
N	14	41	20	14	17	


EFFECT OF DURATION OF TREATMENT WITH HAART ON FIBRONECTIN
MEAN FN (µg/L)
600
500
400
300
200
100
0
0
≤1
>1-3
>3-5
6-7
DURATION OF TREATMENT (YEARS)
Series1


FIBRONECTIN STARDARD CURVE
OD (nm)
350
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
1400
1600
CONCENTRATION (µg/l)