Interaction of Antiretroviral Drugs with the Microbiome & Implications on the development of cardiovascular diseases in HIV+ persons: A Study Protocol

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Study protocol

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Abstract

Background

The longevity of people living with HIV has been enhanced by the introduction of antiretroviral (ARV) drugs in the mid-90s. Antiretroviral therapy (ART) is now mandatory for all persons who test HIV positive in South Africa and the government policy is to start treatment upon diagnosis. The ART is based on protease inhibitors (PIs) and nucleoside reverse transcriptase inhibitors (NRTIs) and both groups of drugs have been reported to have deleterious side effects manifesting as HIV-associated lipodystrophy syndrome, metabolic syndrome and cardiovascular diseases. HIV infection also causes dislocation of the gut microbiota which also contribute towards the observed systemic inflammation even before patients are on ART. The aim of this study is to find the relationship between HIV infection, ART, endothelial function, and gut microbial dynamics and how they influence development of cardiovascular disease (CVD) and metabolic syndrome. Presented here is the detailed study protocol.

Methods

This is a longitudinal study to be conducted over a period of two years. Newly diagnosed patients of both genders enrolled for the ART programme will be recruited and blood, stool and anthropometric measurements will be collected every 6 months. Endothelial function, measured by non-invasive methods i.e. flow-mediated dilation and retinal microvasculature assessment. Lipid profile, viral load and other relevant blood parameters will be measured using routine methods employed by the National Health Laboratory Services in Mthatha, South Africa. Stool samples will be used for metagenomic analysis to characterise changes in the gut microbial richness and abundance. The 16S rRNA gene will be PCR amplified and amplicons will be sequenced using a next-generation sequencing platform. Multivariate analysis will be used to determine the nature of the relationship between blood chemistry parameters, gut microbial profile, endothelial function and anthropometric data.

Impact of project

The study aims to determine changes in vascular function and gut flora in the persons living with HIV on the ART programme, subsequently providing a platform for development of corrective and therapeutic nutraceuticals and probiotics for the same population.

Background

Human Immunodeficiency Virus (HIV) patients are now living longer as a result of use of antiretroviral (ARV) drugs in addition to improved lifestyle choices. The ARV-enhanced longevity is accompanied by increased incidences of lipodystrophy, metabolic syndrome (MetS) and endothelial dysfunction, thus increasing the risk of cardiovascular diseases among HIV patients (1). The ARV therapy (ART) cause
dyslipidaemia and promote biosynthesis of atherogenic cholesterol, thus reducing endothelial function. Accordingly, the patient’s vasculature is strained, resulting in increased risk to cardiovascular diseases. Furthermore, changes in the lipid profile and development of metabolic syndrome is also a function of gut microbial dynamics which is also affected HIV infection. A number of researchers have already reported on the impact of HIV on the general richness and abundance of gut microbiome but there are no studies showing how the resultant changes affect the global lipid profile of HIV positive individuals, as well as the accompanying vascular function. Taken together, HIV and ART directly influence endothelial function, lipid metabolism and the gut microbiome, however not enough knowledge exist to explain the exact mechanisms involved. As such, there is need to simultaneously study the changes in viral load, gut microbial dynamics, endothelial function and metabolic syndrome in HIV patients taking ARV.

Preliminary data (2) show that HIV patients in South Africa exhibit dyslipidaemia, and there appears to be differences between the sexes. Current literature also suggests that metabolic syndrome can be a function of dysbiosis of the gut microbiome. Turnbaugh et al. (3) provided evidence of variation in gut microbial communities at phylum level when comparing lean and obese twins, with obesity being associated with reduced microbial diversity. Obesity, together with other metabolic diseases such as type 2 diabetes mellitus, rheumatoid arthritis, and systemic lupus erythematosus, is associated with chronic inflammation. There is now data suggesting that changes in gut microbial composition is responsible for the inflammation (4) as well as the obesity.

The success of antiretroviral therapy (ART) has been monumental in improving life expectancy of human immunodeficiency virus (HIV) positive individuals, but the long term effects of controlled viral presence and ART are becoming more common. A unique variant of metabolic syndrome, characterised by lipid imbalances, body fat redistribution, and insulin resistance, is increasingly being observed in HIV patients on ARVs (5), making ART an interface of non-communicable and infectious diseases. Several studies (5–9) have reported that both HIV and the accompanying ART are cardiovascular disease risk factors because of their direct impact on lipid and glucose metabolism and accumulation. In a meta-analysis, the risk of developing cardiovascular disease in the HIV population has been calculated at 61% higher in comparison to the general population, dependent on the duration of anti-retroviral treatment (10). Protease inhibitors and nucleoside reverse transcriptase inhibitors used in HIV treatment are known to change the dynamics of gut microflora and are linked to changes in lipid profile leading to development of obesity and cardiovascular diseases. In other published studies, the role of HIV in altering the composition of gut microbiota has been documented.

Viral infection has both direct and indirect impacts on the progression of dyslipidaemia since the virus infects macrophages and triggers inflammatory reaction as exhibited by elevated levels of TNF-α (11). Indirectly, HIV infection results in the translocation of gut microbiota, which subsequently upsets enteric health leading to enteropathy. Monaco and co-workers (12) concluded that bacterial microbiome is directly influenced by the changing viral load and CD4 cell count, thus connecting HIV to microbiome induced systemic inflammation that would result in dyslipidaemia. Nucleoside reverse transcriptase inhibitors are implicated in the development of lipodystrophy syndrome (13) while protease inhibitors
result in dyslipidaemia (9), resulting in co-existence of both lipid disorders in HIV patients undergoing treatment.

Although the link between HIV infection, ART, metabolic diseases, and the enteric microbiome has been suggested, there is still need for comprehensive investigations to confirm this relationship. Thus, there is a need to simultaneously investigate and characterize the changes in gut microbial dynamics, endothelial function and in their relation to changes in viral load of HIV positive individuals on ART. This is what this study aims to do. In addition, use of metagenomic methods will enable detection of a wide range of microbial and viral diversity that is affected by shifts in the host physiology and produce data that will further clarify the HIV-microbiome-lipid complex. The results of this project are important when recommending individualised medication and diets for people living with HIV, a disease with a particularly high prevalence in South Africa. Unfortunately, there is very little data available from Sub-Saharan Africa (SSA) although it is the most affected area in the world with 70% of people living HIV (14). Despite a great decline in HIV-associated morbidity and mortality due to the highly effective special government funded roll-out programs which greatly improved the anti-retroviral therapy coverage by up to 75% in some regions (15), HIV-infection is still the top cause of death in SSA followed by non-communicable diseases connected to vascular disease (e.g. stroke, diabetes, ischemic heart disease and hypertensive disease (2, 16–18)).

Aims And Objectives

The primary goal of the study is to determine the association between HIV infection, gut microbial dynamics, and cardiovascular disease in person living with HIV who are taking of ART (Figure 1). Such data will enable inference and quantification of the relationship between ART, gut microbiome, and overall lipid profile in HIV patients. Since the enteric microbiome influences lipid deposition and consequently endothelial function, the study also aims to determine how changes in the microbiomes of HIV patients on ART affect endothelial function. The following specific objectives will be pursued to achieve these goals:

1. Characterise the gut microbiome of newly diagnosed HIV ARV-naïve patients and HIV on ART patients using 16S rRNA amplification

2. Quantify changes in cholesterol accumulation in HIV patients that are HIV ARV-naïve and HIV patients on ART.

3. Determine vascular function in both HIV ARV-naïve and HIV on ART patients using flow mediated dilation using ultrasound and retinal vascular imaging.

4. Assess the prevalence of metabolic syndrome in HIV ARV-naïve and on ART patients in Mthatha, South Africa.

Methodology
Study Population

Mthatha is the capital of the O.R. Tambo district, a region with an estimated population of over 1,300,000 inhabitants, 54% and 46% female and male respectively. Unlike most towns in South Africa there is no economic activity sustaining the town economy and was created as the capital of the former Transkei Bantustan (homeland). Higher education, public services and health are the dominant industries. Prevalence of HIV varies between 12.38% and 52%, making it one of the HIV hotspots in the country.

Study design

This protocol describes a longitudinal study designed to determine the effect of living with HIV and ARVs on the gut microbial dynamics over a two-year period. Gut dysbiosis will be considered the outcome of study and frequency of occurrence will be compared between HIV positive and HIV negative participants. Convenience sampling will be employed until the numbers required for each demographic is reached in Participants living with HIV presenting at gateway clinic for routine ARV collection will be recruited cases and persons presenting at the same clinic and are HIV negative individuals will be used as controls. The sample size will be calculated using the following formula:

\[ n = \frac{p(1-p)z^2}{d^2} \]

Where \( n = \text{sample size} \), \( p = \text{the proportion of people who are HIV positive in the region} \), \( z = \text{the critical z value for 95% confidence level} \), and \( d = \text{the margin of error (5%)} \).

For the pilot study, a sample size of 300 participants will be used for gathering preliminary data and to optimise the procedures and protocols. Participants comprising both rural and urban residents of the Eastern Cape of South Africa, will be recruited from the Gateway Clinic in Mthatha. Participants will be 50% male and 50% female. Half of the participants of each sex will be HIV positive. Of all the HIV patients, half will be ARV-naïve and the other half will be on ART (Figure 2).

Ethical approval

Ethical consent and permission to carry out the research project was obtained from the Faculty of Health Sciences Research and Ethics Committee (Protocol Number: 119/2018) at Walter Sisulu University (WSU). The study has been registered with the Pan African Clinical Trial Registry (PACTR202108587192356). Only patients who volunteer to participate will be considered for the study. All participants will be requested to read and sign consent forms prior to taking part in the study. Anthropometric data will be recorded and a food frequency questionnaire will be administered and
participants will be categorised according to their major food group types. The data will be anonymized and stored in a safe place. Data storage and usage will follow the National Data Protection Guidelines.

Selection of participants

Participants will be selected on the basis of the following inclusion and exclusion criteria:

Inclusion criteria

1. 18-65 years of age at the time of signing consent form
2. Signed consent form
3. Resident of Mthatha and surrounding rural areas for the past 12 months
4. Have received counselling pre – and post-testing for HIV
5. Reports overnight fast

Exclusion criteria

1. Currently using antibiotics
2. Currently using antidiabetic medication
3. Recent history of gastrointestinal diseases
4. Tested COVID19 positive in the three months prior to the data collection

Participant recruitment

Prior to sampling, trained health workers will explain the goal of the project, anticipated output of the study and why it is important for people to volunteer. This session will serve as a knowledge transfer activity to attract volunteers and to educate the community on HIV/AIDS biomedical issues.

Anthropometric measurements

The National Health and Nutrition Examination Survey (19) recommendations will be used for all anthropometric measurements. Body mass, height, waist and hip circumference will be assessed to determine weight categories of participants. Body mass be measured using an electronic balance and a stadiometer will be used to measure height and the two parameters will be used to compute body mass index (BMI). The waist circumference will be measures at the middle of the iliac crest and the tenth rib and the hip circumference will be measure at the widest point of the buttocks. A flexible tape will be used for both waist and hip measure and the parameters will be used to calculate the waist to hip ratio also used to characterise fat distribution.
Determination of lipid profile and blood chemistry

Blood for laboratory analysis will be collected by a trained professional after an overnight fast. A venous catheter will be placed in the morning, and the first blood samples will be collected from 08h00 for the analysis of glucose, lipid profile (total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol and triglycerides), C-reactive protein.

Assessment of vascular function

Flow mediated dilatation

Endothelial function will be measured using flow mediated dilation (FMD) as described by Strijdom and coworkers (2). The FMD technique involves ultrasonic assessment of the percentage increase in brachial artery diameter from baseline conditions to maximum vessel diameter during hyperaemia induced by inflation and deflation of a sphygmomanometer cuff to suprasystolic levels for 5 minutes. Flow mediated dilation of the brachial artery will be assessed using an ultrasound machine that measures baseline and maximum arterial diameter during hyperaemia following the guidelines proposed by Thijssen et al. (20). The percentage change will be taken to represent endothelial function and thus functional status of the vasculature.

Retinal vessel analyses

Another non-invasive technique involving retinal microvasculature assessment will also be used to assess vascular function (Figure 2). Other recent studies have reported that wider retinal venules are linked to cerebral hypoxia, endothelial dysfunction, and inflammation, in contrast to narrower retinal arterioles, which are linked to elevated blood pressure and endothelial dysfunction. A handheld digital retinal camera will be used to take images of both left and right retinal vessels, and the images will be stored in tagged image file (TIF) format. Vasculature dimensions will be analysed using semi-automated MONA REVA software (Vito Belgium), independent of participant characteristics to reduce bias. Retinal venules and arterioles within the 0.5 – 2.0 optic nerve head diameter from the margin of the optic nerve will be assessed as described by Everson (20), Marincowitz (21), and Hosák (22).

Characterisation of gut microbiome diversity

Stool sample collection
Faecal samples will be collected using EasySampler® Stool Collection Kit (GP Medical Devices ApS, Holstebro, Denmark). Participants will be supplied with the gloves and collecting material and will be given easy-to-follow instructions on how to collect the stool. Samples will be kept on ice during transportation from the clinic to the laboratory and total nucleic acids will be extracted using a commercial kit at the end of each sampling day. The DNA will be stored at -20°C until further processing. Backup samples will be stored at -80°C until needed for further analysis.

16S rRNA sequencing

DNA integrity will be validated by A polymerase chain reaction (PCR) as earlier reported by (23). The 16S rRNA gene will be PCR amplified and amplicons will be sequenced using a next-generation sequencing platform. Multiple software will be used for data analysis but the initial 16S rRNA amplicons will be analysed using QIIME2 (24).

Data analysis

Comparisons of statistical means will be done using factorial ANOVA with ARV therapy, gender and HIV status as the categorical factors for all quantitative variables. Multivariate analysis will be conducted to determine the interrelationships between gut microbiome, HIV status, ARV therapy, endothelial function and the lipid profile. All analysis will be done using R, a language and environment program for statistical computing will be used to assess microvascular changes across the groups. An $\alpha$ of 0.05 will be use as the significance level.

COVID-19 Related Protection measures

In addition to wearing gloves, researchers and participants will be required to wear surgical face masks and an alcohol-based sanitiser will be used to clean surfaces and instruments before and after use. Disposal surgical gowns will be worn by researchers taking anthropometric measurements and blood samples and disposed after every patient. COVID-19 exposure history of participants will be noted in the questionnaire (25)

Data Management and Utilisation
Participant data will only be used for research purposes and their identities will remain anonymous. A participant will be allowed access to his/her data only if interest is indicated at the onset. During analysis names and participant IDs will not be used unless their use is pertinent for the analysis. Sequence data from the next-generation sequencing will be deposited in the NCBI database.

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Figures
Figure 1

Summary of study aim, objectives and anticipated outcomes.
Figure 2

Experimental design and protocol