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Title:

HIV Sequencing After Treatment Interruption to Identify the Clinically Relevant Anatomical Reservoir

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Study protocol

In depth sampling and subsequent treatment interruption to identify the HIV-1 anatomical reservoir.

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Department of thoracic and vascular surgery, Ghent University Hospital

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1. Objective of the study:

Goal of the study

The main goal of this study is to identify and characterise the anatomical component of the replication competent HIV-1 reservoir.

Hypothesis

We hypothesize that the clinically relevant HIV-1 reservoir is hiding in various but specific anatomic compartments and is able to rebound when therapy is stopped.
This reservoir is probably smaller than the HIV-1 reservoir hiding in the blood but could be more transcriptional active because of its specific environment, possibly influenced by lower concentrations of the antiretroviral therapy.

Rationale

With combined antiretroviral therapy (cART), HIV-1 infection can be suppressed, but not cured. A reservoir of HIV-1 infected cells remains impervious to treatment and reinitiates viral infection once treatment is stopped. The cellular spectrum of these HIV-1 cellular reservoirs is highly diversified, and its role varies according to the environment of the anatomical sites in which the virus hides (e.g. cell type, accessibility of cART). Hence, a key question in HIV-1 cure research is: Where in the body of patients receiving cART is the virus hiding? Current studies have mainly looked at the CD4 T cell compartment of the peripheral blood, which forms the easiest compartment to study, but may not be representing the actual replication competent HIV-1 reservoir. Although technologies like droplet digital PCR (ddPCR) (1-3) single HIV-1 genome sequencing (4,5), Linear Amplification and target captured based HIV-1 integration site sequencing (6) have been introduced by us and others to investigate the viral reservoir, a comprehensive analysis that combines these tools to identify the source of the viral rebound in a relevant clinical setting is lacking. Previous studies that tried to answer this question were limited in scope as they only performed phylogenetic analysis on plasma (7) pre- and post-cART, or they only evaluated integration sites and performed phylogenetic analysis in the blood pre- and on cART (8).

The current proposal will, for the first time, identify the source of the viral reservoir by phylogenetically backtracking the viral genome of the rebounding virus to the sequences of viral DNA in different anatomical compartments. The subsequent characterization of the viral reservoir markers (size, integration sites, methylation profile, stimulation and inhibition assays) will enable us to understand how this viral rebound occurred.

This study proposal is unique as it combines new emerging technologies assessing highly relevant patient derived samples in a unique clinical and research setting.

References:

1. Kiselinova M et al. Plos One (2014); 9: e85999

2. Malatinkova E et al. J Clin Microbiol (2014): doi:10.1128/JCM.03087-14

3. De Spiegelaere W et al. Analytical biochemistry (2013); 439: 201

4. Josefsson L et al. Proc Natl Acad Sci U S A (2011); 108: 11199

5. Josefsson L et al. Plos Pathog (2013); 9: e1003432

6. Schmidt M et al. Nature methods (2007); 4: 1051

7. Chalmet K et al. BMC infectious diseases (2010); 10: 262

8. Maldarelli F et al. Science (2014); 345: 179

2. The present study

2.1 Study population

2.1.1: Number of subjects

10 patients with undetectable viral load under stable antiretroviral therapy with no potential contraindications for a temporary treatment interruption will be informed and further guided if interested.

2.1.2: Inclusion and exclusion criteria:

In order to be eligible, study participants **must meet all the following criteria:**

- Documented HIV-1 infection, subtype B
- Able and willing to provide written informed consent
- Age = or >18 years < 65 years
- Nadir CD4 count >=300/µl. CD4 count at screening > 500/µl
- Patient should take ART for at least 2 years with no changes in the ART for at least 90 days prior to study entry. Patients should be on an integrase inhibitor + 2 nucleoside analogs based regimen.
- Patient should have a viral load < 20 copies/ml determined by CobasTaqMan HIV-1 test v2.0 assay for at least 2 years. (Occasional "blips" will be permitted) (A blip is defined as an intermittent viremic episode with a viral load above detection level but below 200 copies/ml and a return to an undetectable level in a next control, if more than six months prior to the study entry)
- Last viral load undetectable
- Ability to attend the complete schedule of assessments and patient visits. Ability and willingness to have blood and tissue samples collected and stored indefinitely and used for various research purposes. Women of childbearing potential or their partner should use double barrier contraception during the study. Females of reproductive potential (women who have not been post-menopausal for at least 24 consecutive months, i.e., who have had menses within the preceding 24 months, or women who

have not undergone surgical sterilization, specifically hysterectomy, or bilateral oophorectomy and/or bilateral salpingectomy), will need a negative serum or urine pregnancy test within 48 hours prior to study entry.
NOTE: Acceptable documentation of hysterectomy and bilateral oophorectomy, bilateral salpingectomy, tubal micro-inserts, partner who has undergone vasectomy, and menopause is participant-reported history. All participants must agree not to participate in the conception process (e.g., active attempt to become pregnant or to impregnate, sperm donation, or in vitro fertilization). Participants must agree to use barrier protection for all sexual activity and if participating in sexual activity that could lead to pregnancy, the participant/partner must use at least two reliable forms of contraceptives (condoms, with or without a spermicidal agent; a diaphragm or cervical cap with spermicide; an IUD; or hormone-based contraception) during the study.

<u>Potential participants meeting any of the following criteria will not be enrolled in the study:</u>

- Previous or current history of opportunistic infection. (AIDS defining events as defined in category C of the CDC clinical classification).
- History of resistance to antiretroviral drugs, documented by genotyping.
- Hepatitis B surface antigen positive or HBV viral load positive in the past and no evidence of subsequent seroconversion (=HBV antigen or viral load negative and positive HBV surface antibody).
- HCV antibody positive result within 60 days prior to study entry or, if the HCV antibody result is negative, a positive HCV RNA result within 60 days prior to study entry
- Significant risk of HIV superinfection during treatment interruption.
- Current or known history of cardiomyopathy or significant ischemic or cerebrovascular disease.
- History of HIV-related thrombocytopenia.
- Active renal disease (defined as a glomerular filtration rate (calculated by Cockroft Gault equation) below 50ml/min or the presence of HIVAN in the past medical history.
- Current or known history of cancer (with the exception of in situ cervix carcinoma or squamous cell carcinoma of the skin) within five years prior to screening.
- Pregnancy or breastfeeding.
- Any conditions, including psychiatric and psychological disorders, which will in the opinion of the investigator interfere with the trial conduct or safety of the participant.
- Previous participation in a trial evaluating an immune modulating agent
- Abnormal laboratory tests results at screening:
  1. Confirmed Hemoglobin <11g/dl for women and <12 g:dl for men
  2. Confirmed platelet count < 100000/l
  3. Confirmed neutrophil count <1000/µl
  4. Confirmed AST and/or ALT > 2xULN

The following treatment will be prohibited three months before screening and during the course of the study:

1. immunosuppressive drugs (inclusive corticosteroids) with the exception of drugs used for topical use.
2. Immunomodulatory drugs including but not limited to Granulocyte colony stimulating factors, Granulocyte-monocyte stimulating factor, interleukin 2, 7 & 15.

- Active drug or alcohol use or dependence that, in the opinion of the site investigator, would interfere with adherence to study requirements.
- Acute or serious illness, in the opinion of the site investigator, requiring systemic treatment and/or hospitalization within 60 days prior to entry.

Criteria for ATI Discontinuation (ART resumption)
Subjects will be advised to reinitiate ART after ATI for any of the following

- HIV-1 RNA above 200 copies/ml on 2 consecutive determinations at least 3 days apart.
- A single HIV-1 RNA above 1000 copies/ml
- CD4+ T cell counts below 350 cells/mm^3 on 2 consecutive determinations at least 2 weeks apart
- CD4+ T-cell count decline of > 50% from baseline prior to ATI
- Clinical progression to CDC Category B or C disease
- Diagnosis of Acute Retroviral Syndrome.
- Pregnancy
- Subject requests re-initiation of ART

VL is done 2-3/week up to week 4 of ATI, then every week until treatment reinitiation.

2.1.3 Prior and concomitant therapy: patients before starting the study will, if they are currently on another regimen, be switched to an integrase inhibitor based regimen, at the latest 90 days prior to study initiation (Current treatment options in infectious diseases, (2014)6:144-158).

2.2 Study design and Intervention:

This is a single arm mono-centric non-randomized (prospective) study.

2.2.1 Primary objectives and outcomes:

- Quantification of viral markers as total HIV DNA, episomal DNA (2LTR circles) and cell-associated RNA in different anatomical reservoirs.
- Genetically link the viral reservoir in different anatomical reservoirs to the rebounding virus found in the plasma after therapy-stop by doing phylogenetic analysis.
- Integrations sites sequencing to look for clonal expansion.
- Methylation profiles in the human genome where HIV-1 integrates.
- Stimulation and inhibition assays to characterise the replication competent viral reservoir

- TDM (therapeutic drug monitoring) studies to look at cART penetration in peripheral tissues.
- Human markers to screen for inflammatory parameters related to HIV-1 infection especially in CSF.

2.2.2 Procedures

2.2.2.1: Eligibility screening and inclusion in the study:

Study participants will be selected from patients in regularly follow-up at UZ Gent. Screening evaluation will be performed prior to study entry. The patient will be invited to participate in the trial and provided information about the aims and risks of the project. After obtaining written informed consent, clinical eligibility will be assessed. Patients will be eligible for enrolment if they fulfil all the inclusion criteria. Patients will not be eligible for the study if they meet any of the above described exclusion criteria. If patients are not on an integrase inhibitor based regimen, they should be switched 3 months prior to study initiation.

The following will be assessed at this visit:
- Assessment of subject eligibility according to the inclusion and exclusion criteria
- Demographic and epidemiological data
- Medical history (past and current), including HIV-associated conditions
- Full antiretroviral history (including result of resistance assays if available)
- Review of non-antiretroviral medication within the last 30 days with special attention for immunomodulatory and immunosuppressive drugs and anticoagulation therapy.
- Physical examination including vital parameters, height and weight
- HIV viral load and CD4 T cell count.
- Laboratory test (non-fasting): hemoglobin, absolute neutrophil count (ANC), platelets, creatinine, electrolytes, liver enzymes AST & ALT, coagulation. Hepatitis HCV & HBV serology if necessary.
- Pregnancy test among women.
- ECG and lung X-ray
- assessment of good peripheral venous access

2.2.2.2: In depth sampling:

An in depth sampling will be planned before therapy interruption. Together with our collaborators we will put in place a standardised trajectory, aiming for the highest possible comfort for the participants. The patients will therefore be admitted in the hospital for 24h. Bronchoscopy, gastroscopy and coloscopy will be performed under anaesthesia. Before the anaesthesia, the anaesthesiologist will perform a lumbar puncture. During anaesthesia a bone morrow aspirate and an inguinal biopsy will also be effectuated. Leukapheresis can be organised on ambulatory basis within one month of the in depth sampling but not the day before or after due to increased bleeding risk. If leucapheresis is contra-indicated this will be replaced by a peripheral blood draw of 6x 9ml. Sperm collection will be done by standardised

protocol of the fertility clinic. Vaginal lavage will be done during hospitalisation or ambulatory at the gynaecology consultation.

2.2.2.3: Treatment interruption to allow viral rebound and subsequent sampling:

A second sampling will be foreseen after therapy-stop once viral rebound is detected and will consist on leukapheresis (or peripheral blood draw if contraindicated), lumbar puncture and genital sampling. As before there should be a timespan of minimum 24h between leukapheresis and puncture due to increased bleeding risk. For this sampling an overnight stay in the hospital will be arranged.

After the treatment interruption is initiated a follow-up of plasma viral load will be organized. Patients will be stopped at D1 (Friday) and a first control will be performed D4 (Monday) and D7 (Thursday). On an average we expect people to have rebounded before D12 and the second sampling will be planned like this. If the viral load control on D11 shows no signs of rebound, sampling will be postponed and further follow-up will be organized. If after 3 weeks the patient has not rebounded, we will discuss this result with the patient and we will propose weekly controls of viral loads and restart therapy after 2-3 months, after the second sampling. For patients without detectable viral load after 1 month, an additional sampling at that point (blood sampling, lumbar puncture and genital sampling) will be proposed.

Sampling will consist of

a: Leukapheresis

Participants will undergo leukapheresis at the Ghent University Hospital. Leukapheresis runs will last approximately three hours for the collection of > $1x10^8$ cells Peripheral Blood Mononucleair Cells. Leukapheresis is considered the gold-standard method for obtaining large volumes of peripheral cells from HIV-infected patients, and has been performed safely in individuals with and without hematologic malignancy and/or HSCT (1). Leukapheresis will be performed twice for each participant, with a minimum of three months interval following the initial collection. If contra-indicated this procedure will be replaced by a peripheral blood draw of 6x9ml.

b: Bronchial lavage and endobronchial ultrasounds guided sampling

Participants will undergo bronchial lavage and endobronchial ultrasound guided lymph node sampling at the pulmonary department of the University Hospital. The procedure is now routinely performed as part of clinical practice in patients with an unidentified lung disease (e.g. diagnosis for TB). This procedure will be performed only once for each participant.

c: Lymph node resection

The vascular surgery department will perform lymph node resection. Briefly, a small incision is made in the inguinal region (triangle of scarpa) allowing a lymph node resection. Lymph node resection will be performed once, before cART is stopped.

d: Gastroscopy and duodenal biopsy

Duodenal mucosal sampling will be obtained by gastroscopy with up to 10 mucosal biopsies obtained. This procedure will be performed only once for each participant.

e: Colonoscopy

We will take up to 10 mucosal biopsies, at the terminal ileum. We will also perform collection of gut-associated lymphoid tissue via biopsy of rectal mucosa. Rectal mucosal sampling will be obtained by anoscopy, collecting up to 10 mucosal biopsies.

Subjects will be counselled to avoid rectal trauma and the use of anticoagulants (e.g. aspirin, NSAIDS) before and after the procedure. Mucosal biopsies will not be taken if the participant has an increased risk for complications, including receptive anal intercourse within 3 days of the procedure, an active anal infection, or recent use of anticoagulants. Gastroenterologists who have training and clinical certification in anoscopy and rectal mucosal biopsy will perform rectal biopsies. This procedure will be performed only once for each participant.

f: Bone marrow biopsy

We will collect bone marrow tissue from aspirates during the study performed by the haematology department. This procedure will be performed only once for each participant before treatment stop.

g: Lumbar fluid

Cerebrospinal fluid (CSF) will be obtained for study purposes and processed in standardized fashion as previously described in the study of central nervous system HIV-1 infection (2). A maximum volume of 3-5 ml will be collected. Patients, currently on systemic anticoagulation (e.g. warfarin therapy), with a platelet count < 50,000 per uL or with clinical signs of systemic infection won't be included. Prior to lumbar puncture, a certified physician and study co-investigator will perform a cranial nerve exam and fundoscopic exam; lumbar puncture will not be performed if evidence of intracranial pressure is identified. This procedure will be performed two times for each participant.

h. Genital tract access

Sperm will be collected according to the standard operating procedures from our fertility department. Cervicovaginal secretions will be harvest as described by Zara et al. (3). This procedure will be performed twice for each participant.

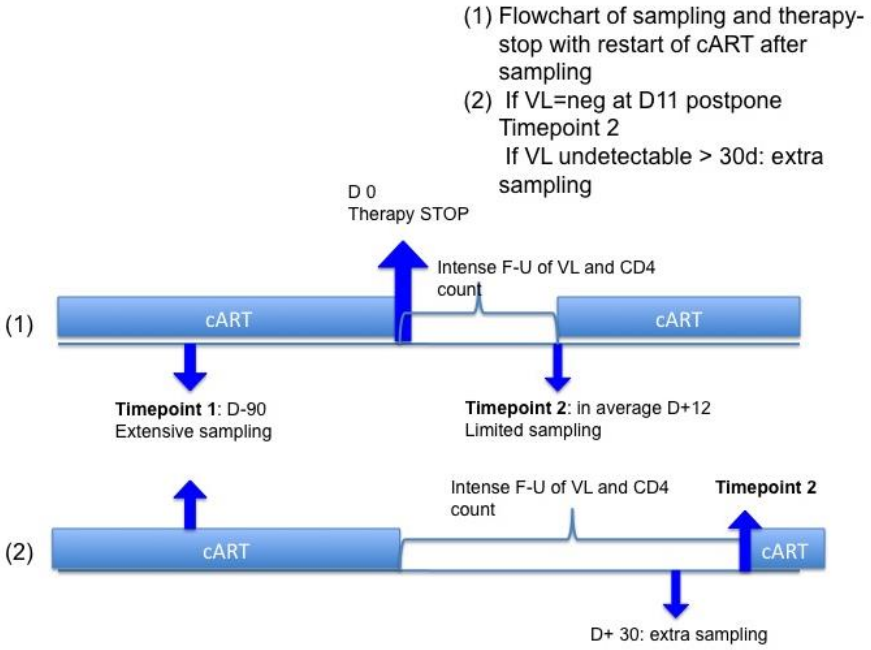
References:

1. Molina A et al. Blood reviews (2003); 17: 249

2. Price RW et al. Aids (2001); 15: 1251

3. Zara F et al. Sexually transmitted infections (2004); 80: 108

2.2.3 Flowchart



2.3 Description of the laboratory research techniques:

2.3.1: Cell sorting of the different cell populations: to investigate the viral reservoir in different cell subsets, biopsies from each site will be dissociated to a single cell suspension and mechanical disruption as described by Shacklett (1). After collagenase I treatment and density gradient centrifugation, cell suspensions will be divided into fractions using magnetic bead selection and flowcytometer sorting with the FACSJazz, with antibody labelling based on cell surface markers. The immune cells can be divided in different fractions based on the presence or absence of cell specific markers like CD3, CD4, CD45RA, CCR7, CD27, CD62L, CD95, CD122, CD14, CD123, CD71, and CD206. Sorting will be based on available literature describing different tissue related cell types that might be important for HIV replication and latency.

2.3.2 Phylogenetic analysis of the HIV-1 sequences present in the different cellular compartments:

In order to perform phylogenetic tree analysis, acquiring adequate sequencing input is of paramount importance. Conventional PCR and subsequent sequencing has important limitations when used to analyse mixtures of genetically divergent templates. The *taq polymerase* is prone to switch templates during the amplification process, thereby generating recombinants not reflecting the initial sequence. This can be avoided by using single genome amplification, initially described by the group of S. Palmer (2). Single genome-sequencing uses

Gag, Pol and Env sequences. Clonal sequences will be analysed using statistical phylogenetic techniques, including both maximum likelihood and Bayesian inference, in collaboration with Prof. Chris Verhofstede and Prof. Philippe Lemey (Rega Instituut Leuven, ERC 260864). This will allow to determine the compartmentalization structure of the viral population and to establish which reservoir sites contribute to the viral rebound upon therapy interruption.

If virus upon rebound can be linked to other anatomical compartments (while patients are on cART), then we will further support the data by identifying if these cellular populations were mainly clonal expanded by doing additional integration site sequencing cfr 2.3.4.

It might be important to approach the central nervous system differently compared to the other compartments for two reasons. First, due to the blood-brain barrier this reservoir might be more compartmentalized and secondly tissue sampling is not realistic. Most probably we will not be able to perform phylogenetic analysis on the lumbar fluid in patients on cART due to limited input material. Therefore, we will evaluate this reservoir mainly quantitatively in terms of viral rebound dynamics in the cerebrospinal fluid (CSF) compared to rebound in the peripheral blood (at day 12). An earlier or higher viral rebound in the CSF would then indicate that the replication competent reservoir is likely contained within the brain.

2.3.3 Quantitative assessment of the HIV-1 viral reservoir in the plasma and in different cellular fractions of the different anatomical sites.

To identify the nature of the replication competent HIV-1 reservoir (active cryptic replication versus dormant HIV-1 DNA) we will further characterize the viral reservoir in the body compartments containing viral DNA that is most closely related to the rebounding virus. The cell populations, sorted from the different tissues will be evaluated using a set of viral reservoir assays based on HIV-1 RNA/DNA intermediates, formed during each step of the replication cycle. The assays have been translated by my group from in vitro research towards the clinic, using state of the art droplet digital PCR technology, allowing absolute ultrasensitive quantification. The cellular blood fractions and cellular fractions from the anatomical compartments will be separated and subjected to quantification of cell-associated RNA, total HIV-1 DNA and 2 LTR circular HIV-1 DNA.

- Our data show that HIV-1 is present in different anatomical compartments throughout the body. We will perform total HIV-1 DNA quantification on the different cellular fractions to understand the size of the potential reservoir in the different compartments. Furthermore, we will evaluate a potential correlation of the size of total HIV-1 DNA in the cellular compartment with the identified source of rebound.

- Episomal HIV-2LTR circles are recognised as a marker of ongoing replication. 2LTR quantification assays have been transformed from in vitro to in vivo settings, mainly in PBMCs (3). Our current data show high levels of 2LTRs in PBMCs of recent seroconverters. For the first time we will perform quantification of 2LTR circles in different anatomical sites to detect ongoing replication.

- Full-length cell associated RNA forms a marker for HIV-1 DNA transcription and has been used in the context of clinical trials aiming at activating the latent reservoir (4). We adapted these assays towards a clinical user-friendly ddPCR platform (5). We will be the first group to map the HIV-1 transcriptional active sources in the body in patients on cART.

2.3.4: HIV-1 integration site analyses of the viral reservoir in different anatomical sites:

Integration site analysis will be performed to identify if the replication competent viral reservoir mainly clonally expanded (indicating homeostatic proliferation as mechanism for viral persistence. CD4+ T cell populations, sorted from the different tissues will be evaluated using LAM-PCR/nrLAM-PCR and deep sequencing (6). The LAM-PCR strategy uses a linear amplification by PCR with an outward-bound HIV-1 specific primer. These linear amplicons containing part of the human genome sequence of the integration site are subsequently analyzed by deep sequencing. Our strategy is based on a combination of target capture (TC) sample enrichment and next-generation sequencing. Our preliminary data shows integrated HIV-1 genome over all the chromosomes. We are currently evaluating the presence of unique integration sites. This project will constitute the first study to evaluate integration sites in anatomical compartment to map HIV-1 integration in vivo. We have recently optimized an assay to identify HIV-1 integration site in the patient genome. Our assay has been validated both on HIV-1 infected cells and patient-derived samples. We analysed HIV-1 infected patient-derived samples before treatment and analysed the integration site pattern.

2.3.5 Analysis of the HIV-1 methylome in different anatomical sites:

The HIV-1 methylome of proviral HIV-1 DNA will be assessed by bisulphite conversion and deep sequencing using an Illumina platform. Extracted DNA will be bisulphite treated to substitute all unmethylated Cytosines into Uracyls. Subsequently, a targeted PCR for the sense and antisense (as a control of the native sequence) strand will be performed to specifically isolate the HIV-1 CpG islands. Finally, methylation profiles are assessed by deep sequencing, enabling the analysis of separate HIV-1 DNA molecules within one sample.

2.3.6 Therapeutic drug monitoring (TDM): To estimate the viral reservoir in the different anatomical compartments, the local concentrations of the administered drugs is of great interest. There is not sufficient knowledge on penetration in peripheral tissues of the current therapies in use. Some studies show that due to lower concentration of certain antiretrovirals in lymphnodes, there is persistent viral replication (7). This source of continuous viral replication could be in vivo the origin of relapsing virus at treatment interruption. To determine treatment concentrations, we will work in collaboration with Prof. A. Verstraete and different techniques as tandem mass spectrometry and ultra performance liquid chromatography will be used.

2.3.7 Inflammatory parameters: we would like to look for biomarkers of axonal injury and neurodegeneration in the cerebrospinal fluid. We will collaborate with Prof Magnus Gisslen,

a specialist in the field, doing additional testing of CSF measuring inflammatory parameters like neurofilament protein light, neopterin and CSF/plasma albumin ratio (8). Other markers of immune activation in blood and/or CSF can also be assessed for example IL-6, TNF-alpha, HLADR+CD38+, CD14s, CRP and beta2microglubuline.

2.3.8 Stimulation and immunological inhibition assays: VOA (Viral Outgrowth Assay)/TILDA (Tat/rev Induced Limiting Dilution Assay).

Infectious units per million cells- IUPM

The principal approach for quantifying HIV-1 persistence during antiretroviral treatment (ART) is a viral outgrowth assay (VOA) performed on resting CD4 T cells. These cells do not produce virus without stimulation. Therefore, resting CD4 T cells are stimulated with phytohemagglutinin (PHA) in the presence of irradiated allogeneic peripheral blood mononuclear cells. These stimuli induce global T cell activation, which reverses latency at least in a fraction of cells carrying integrated HIV-1 genomes. The viruses released from these cells are expanded in MOLT4/CCR5 cell line and detected after 2 weeks in the supernatant. The frequency of latently infected cells, expressed in terms of infectious units per million (IUPM) resting CD4 T cells. In principle, this assay can also detect resting CD4+ T cells harboring labile unintegrated forms of HIV-1 DNA, although the frequency of cells containing unintegrated DNA during ART is low. Positive wells at each dilution were counted and the maximum likelihood method was used to calculate the frequency of cells with inducible replication competent HIV-1 (http://bioinf.wehi.edu.au/software/elda).

Tat/rev Induced Limiting Dilution Assay – TILDA

Enriched CD4+ T cells are re-suspended at 2 x~ 10^6 cells/ml in media and rested for 3–5 h at 37 °C, 5% CO2. CD4+ T cells are stimulated for 12 h with 100 ng/ml PMA and 1 µg/ml ionomycin (both from Sigma). The duration of 12 h was based on kinetic experiments demonstrating maximal production of tat/rev RNA and preserved cell viability at this time point. After stimulation, cells are serially diluted to 18 x~ 10^6 cells/ml, 9 x~ 10^6 cells/ml, 3 x~ 10^6 cells/ml and 1 x~ 10^6 cells/ml in culture medium.

The cell suspension from each serial dilution is distributed in 22 to 24 wells of a 96 well plate corresponding to 18,000, 9000, 3000 and 1000 cells per well. Pre-amplification was carried out. At the end of the pre-amplification, TE buffer was added to each well and the diluted PCR products was used as template for the tat/rev real-time PCR reaction (normal PCR). This reaction was performed using a real time PCR (Light Cycler 480, Roche Life Science). Positive wells at each dilution were counted and the maximum likelihood method was used to calculate the frequency of cells with inducible HIV msRNA.
(http://bioinf.wehi.edu.au/software/elda).

Biosafety: *All screening tests on patient material, isolation and sorting will be done in L2 facilities. Stimulation and culture of prelevated cells will be effectuated in an L3 facility.*

References

1. Shacklett BL et al. Methods in molecular biology (2009); 485: 359
2. Svicher V et al. Current HIV/AIDS reports (2014); 11: 186

3. Malatinkova E et al. J Clin Microbiol (2014): doi:10.1128/JCM.03087-14
4. Rasmussen TA et al. Human vaccines & immunotherapeutics (2013); 9: 790
5. Kiselinova M et al. Plos One (2014); 9: e85999
6. Schmidt M et al. Nature methods (2007); 4: 1051
7. Fletcher CV et al. *Proceedings of the National Academy of Sciences of the United States of America* 2014,**111**:2307-2312.
8. Jan Jessen Krut et al., Plos One (2014) e88591

3. Risks and advantages

3.1 Risks

In this project proposal, we have two important (risk related) ethical questions:

1) Is it safe to select "healthy volunteers (seropositive people with a normal life)" and

2) Can a treatment stop be carried out safely?

These two questions will be further addressed in this document. Informed consent will be obtained from each eligible subject prior to each procedure and study staff will transport all material for immediate processing at the HIV research virology laboratory. Patients may refuse a procedure, but remain enrolled in the study and undergo other tissue sampling, and/or analytical treatment interruption.

3.1.1 Risks related to patient sampling

For all individual procedures, the individual risk will be discussed with the patient by an open communication. For each procedure, the reported individual risks are low (typically 1/1000 for adverse events in all procedures), and are based on data from sick patients (e.g. lung disease for which bronchoscopy is needed). Also the patient's cumulative risk (all procedures taken together) will be discussed extensively. Risk estimation per procedure is shown below.

Endobronchial ultrasound guided lymph node biopsy (EBUS) is considered a safe procedure (Rintoul RC et al. Thorax, 2014). A retrospective analysis involving 16.181 patients, reported 23 serious adverse events (SAE) (0.14%) and 35 adverse events (AE) (by 0.22%) and no mortality. With EBUS, infectious SAE were most prevalent (down 0.07%), especially in patients with cystic lesions and sarcoidosis.

Limited data is available for bronchoalveolar lavage (BAL) in patients with no existing pulmonary disease, and this technique is considered to be safe in healthy subjects. In the case of extensive lavage (immuno-BAL), in patients with severe pulmonary disease, a self-limited fever and hypoxemia are the most frequent complications (American Thoracic Society, 2012).

Bone marrow aspirate: Large data series are published from the members of the British Society of Haematologists, reporting 54 890 bone marrow samples, between 1995 and 2001

(Bain BJ Clinical and laboratory haematology (2004). 26: 315). Total of 26 AE were reported directly linked to the procedures. The most frequently reported SAE was bleeding, in 14 patients, with emergency blood transfusion in 6 patients and leading to death in 1 patient (with underlying clotting problems). The most common risk factors associated with bleeding were myeloproliferative disease, aspirin use, or both; warfarin therapy, DIC and obesity. Other adverse effects are infection, long-lasting discomfort at the biopsy site, and penetration of the sternum, which can cause heart or lung problems.

Possible complications from a lumbar puncture (LP) comprise of: local discomfort with pain at the puncture site, during or after the puncture, radicular pain, bleeding, infection and post-puncture headache. A recent review (Wright et al J Neurol 2012; 259:. 1530-45) reported infections and bleeding, and each was seen in less than 0.01% of the cases. The incidence of post-puncture headache (PPH) is a fairly frequent complication and its incidence is highly dependent on the needle used. If an atraumatic needle is used, PPH is reported on average in 3-9% of the cases (Wright et al J Neurol 2012; 259:. 1530-45; Lavi et al Neurology 2006; 67; 1492-1494.). All the punctures within this study will be made with an atraumatic needle. Researchers investigated the prevention of PPH in an observational study with cross-sectional data analysis (Monteiro de Almeida et al Headache 2011; 51:. 1503-10). Total of 675 LPs were performed involving 477 volunteers (575 puncture in HIV-positive individuals and 100 in healthy volunteers). PPH was noted in only 0.9% of the subjects. Most cases of PPH (92%) recovered spontaneously or with medication. Three individuals were treated by an epidural blood patch. In UZ Gent an epidural blood patch is used with a protocol performed by an anaesthesiologist with specific expertise.

Biopsy of the gastrointestinal tract:

- A colonoscopy with biopsy carries a risk of bowel perforation in 1/1500. If this occurs, the perforation is recognised immediately and treated during the same endoscopy. As a result of the administration of analgesics and/or narcotic drugs other side effects can occur, such as low blood pressure, nausea, or twists. These are short-lived side effects and after a colonoscopy, the patient is kept in observation overnight, with monitoring of the pulse and blood pressure. Subjects will be counselled to avoid rectal trauma and the use of anticoagulants (e.g. aspirin, NSAIDS) before and after the procedure. Mucosal biopsies will not be taken if the participant has an increased risk for complications, including receptive anal intercourse within 3 days of the procedure, an active anal infection, or recent use of anticoagulants. Biopsies will be performed by gastroenterologists who have training and clinical certification in coloscopy/ anoscopy.
- The major complication of a gastroscopy with biopsy is aspiration pneumonia (<1/1000). The risk of perforation is very small (<1/1500).

Biopsy of the lymph node: Lymfocele is the main risk in removal of lymph nodes. This complication is infrequent (<1%) and can be surgically corrected. The infectious risk is <1% and easily treatable.

Leukapheresis: is considered a safe procedure. All studies available were done in patients with hematologic or solid tumours malignancies (ex. Reik, 1997). The most frequent side effect (39%) was paresthesia due to citrate-related hypocalcaemia (1 in 10). This could easily be managed with oral calcium supplements and/ or slower flow rates. Other reactions included hypotension (4%) and headache (1,4%). Patients under antihypertensive treatment, will be asked to interrupt this treatment the day of the procedure. The bleeding risk after the procedure is transitory increased. There is also risk of catheter problems during procedure. Leukapheresis will not take place if screening beforehand reveals poor peripheral venous access or electrolyte disturbances. In these cases, the procedure will be replaced by a peripheral blood puncture (6x9ml).

Semen sample and vaginal lavage: these are non-invasive procedures and not associated with serious adverse effects.

Anaesthesia: Most healthy people don't have any problems with general anaesthesia. Although many people may have mild, temporary symptoms, general anaesthesia itself is exceptionally safe, even for the sickest patients. The risk of long-term complications, much less death, is very small. In general, the risk of complications is more closely related to the type of procedure, the general physical health, than to the anaesthesia itself. Some of the factors that can increase the risk of complications include: smoking, obstructive sleep apnoea, obesity, high blood pressure, diabetes, other medical conditions involving your heart, lungs or kidneys, medications, such as aspirin, that can increase bleeding, history of heavy alcohol use, drug allergies and history of adverse reactions to anaesthesia. Rare complications, which may occur more frequently in older adults or in people with serious medical problems, include: temporary mental confusion, lung infections, stroke, heart attack, and death.

3.1.2 Risk due to intermittent stop of the medication

All patients undergoing treatment interruption will be counselled on safer-sex practices as the treatment interruption may increase risk of HIV-1 transmission. Only patients that can stop therapy safely (high CD4 nadir, no pre-existing resistance, low cardiovascular risk profile,..) will be selected for the study. Patients will be restarted on cART (the same regimen as prior to interruption) if they have a confirmed viral load >200 copies/mL (two positive measurement >200 – the DHHS definition of viral rebound for patients on ART) or a single value greater than 1000 copies/mL.

Several recent follow-up studies consider a controlled intermittent stop in the medication as safe (Chun TW et al AIDS (2010). 24: 2803, Allard SD et al Clin Immunol (2012) 142:. 252, Good

G et al AIDS (2013).; 27: 2679; Williams et al eLife JP (2014): e03821). Intermittent stop of the treatment was used extensively in the past in HIV research, for example, in the context of short-term treatment of acute (recent) HIV-1 infection in order to increase the therapy-free interval. In 2007, the SMART study was published in which an increased risk of cardiovascular mortality was demonstrated after stopping the medication. It was found that increased cardiovascular risk occurs in patients who suffer repetitive therapy stop with low CD4 cell counts (<100 CD4/ul) and with very high viral loads. In patients with high CD4 cell counts and low viral load before therapy, the stop in therapy is considered safe.

Therapy stop is the only way to achieve a viral rebound. In addition, there is the perception that therapy stop may be associated with viral drug resistance. Viral resistance occurs when only sub-therapeutic drug concentrations are present in the body. Viral resistance does not occur if therapy is not taken, because there is no selective pressure present, unless the antiviral cocktail that was stopped consisted of individual HIV-1 inhibitors with a very different half-life. This phenomenon is well known, therefore, I will work together with the virologist Prof Chris Verhofstede, and each therapy stop will be guided so that no HIV resistance occurs.

Patients are eligible **only if no drug resistance has been detected in the past**. With the current availability of different classes of antiretrovirals, an ART switch can help directly when a case of viral resistance occurs. Treatment history of the patients will be taken into account at inclusion, to be sure that a sufficient number of ART options remain, if necessary, to exchange the cART regime.

3.1.3 Risk of not reaching enough study participants:

This question rises whether this study will find enough motivated participants to participate. Therefore, an initial inquiry with several patients (N = 10) (on the advice of the Ethics Committee in UZ Gent) has already shown us that patients are indeed motivated to engage in such a study, provided that they are adequately informed about the scientific design and gain of the experiments. The burden of the intensive lifelong intake of ART medication causes HIV-infected patients, more than healthy volunteers, to actively participate in studies that contribute to the search for an HIV cure.

3.1.4 Risk of misperception of the study

An important ethical conundrum lay in the false perception patients may have with regard to this project. These “therapeutic misconception and misestimation” are well known concepts in bioethics. Patients that participate in this study’s might perceive the wrong expectations from the study. Although there will be no medical intervention (no admission of a study drug), the patient might perceive this study as having an impact on his ‘HIV reservoir’. They lead potential participants and/or practitioner to over-consider the very theoretical advantage (participating in a Cure diagnostic study) and under-consider the side effects of the study. It will be a matter of explaining the study correctly and designing a good balanced informed

consent that will be readily understandable to the potential participants.

4. Study analysis

4.1 Sample size calculation:

The number of patients (N=10) that we will investigate exceeds by far the numbers reported in previous studies on HIV-1 reservoir dynamics, and should provide ample power to reveal consistent phylogenetic patterns while still adhering to the budget constraints.

4.2 Data analysis:

Samples and results will be analyzed together with Prof Linos Vandekerckhove and Ward Despiegelaere at Ghent University.

For phylogenetic analysis we will collaborate with Prof. Chris Verhofstede and Prof. Philippe Lemey.

For TDM we will work in collaboration with Prof. A. Verstraete.

4.3 Statistical analysis:

Because of the small sample size of this cohort, we will only be able to look at individual results and perform non-parametric statistics on the cohort. Statistical significance (P values) of the results will be calculated by two tailed student t-test using SPSS. Correlations among variables will be tested by regression analysis. Proportions will be compared with Fisher's exact test using GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA, USA). The level of significance will be set at $P < 0.05$ and Bonferroni adjustment of P-values will be applied in case of multiple testing.

For phylogenetic analysis clonal sequences will be analysed using statistical phylogenetic techniques, including both maximum likelihood and Bayesian inference.

4.4 Storage and Shipment of the samples

All processed samples will be stored at a separate facility Biometra Biobank, UGent at -80 in FCS for further analysis.
DNA and RNA isolates will be stored at -20°C.
It is possible that samples will be shipped to internationally collaborating laboratories for assays described in this protocol.

5. Duration and termination of study:

The clinical study (including sampling and therapy-stop) will be performed over a timeframe of maximum 2 years. We expect to finish the analysis in 4 years. We hope to include patients starting from the 1st of January 2016 until the 1st of January 2018. Participation in this study is voluntary. Patients can refuse to participate or ask to end the participation before the final closure of the study, at any time. Patients themselves can be withdrawn from the study if they are not acting according to the procedures described or are not respecting the conditions.

We will start recruiting patients when approval of EC has been obtained and aim to include patients in the study before the beginning of March 2016. Together with the patients and with the collaborating disciplines a date for admission in the hospital will be arranged. Patients will stay overnight in the hospital during the sampling phase.
Approximately 3 months after initial sampling patients will be scheduled for a guided treatment interruption, followed by a smaller sampling after rebound, requiring again one night of observation in the hospital. After sampling the patient will restart therapy and will be followed-up closely until undetectable viral load.
During therapy stop a 2-3 times a week visit will be necessary.

6. Informed consent

The participant must personally sign and date the latest approved version of the informed consent form before any study specific procedures are performed. Written and verbal versions of the Participant Information Sheet and Informed Consent will be presented to the participants detailing no less than: the exact nature of the study; the implications and constraints of the protocol; the known side effects and any risks involved in taking part. It will be clearly stated that the participant is free to withdraw from the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal.

7. Costs and compensation for participating

Participation in this trial will not result in any additional cost to the patients and an additional compensation for the patients will be foreseen (400 euro/d of sampling).

8. Confidentiality

In accordance with the Belgian law concerning the private life protection (08 Dec 1992) and the patient's rights (22 Aug 2002) the information collected from participation in this study is protected. The participants have the right to request information of the existence of personal data held by the study coordinator and will have the right to rectify erroneous or inaccurate data. Representatives of the study coordinators, the Independent Ethical Committee and/or Regulatory Authorities will be granted direct access to the original medical records for verification of the clinical trial procedures and/or data, without violating the confidentiality according to the laws and regulations applicable in Belgium. By signing this informed consent form, participants are authorizing such access.
From patients agreeing to participate in this study, personal data and clinical information will be collected and coded. No reports containing the personal data will be publicly available.

9. ICH/GCP guidelines

This trial will be conducted in accordance with the protocol, current ICH-GCP guidelines and applicable law(s).Good Clinical Practice (GCP) is an international ethical and scientific quality

standard for designing, conducting, recording and reporting trials that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety and well being of trial subjects are protected, consistent with the principles that have their origin in the Declaration of Helsinki, and that the clinical trial data are credible.

10. Insurance

The coordinators of this study are liable, even without fault, for damage that the participants or legal successor has sustained and that has direct or indirect link with the trial. To that end the study has provided insurance coverage.
Patients in follow-up at the Ghent University Hospital are covered by the insurance of the Ghent University Hospital.

11. Dissemination

The study outcome and data will be published in peer-reviewed journals, aiming the highest impact factor in the field.

Contact person
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Signature Page

Investigator:

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Signature:

Date: