Study protocol

Accurate staging of immuno-virological dynamics during acute HIV infection

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1. OBJECTIVE OF THE STUDY

1.1 GOAL OF THE STUDY

This study will evaluate how immunological and virological profiles of the different anatomical compartments evolve in patients before and after treatment initiation in individuals starting treatment during acute HIV seroconversion. Monitoring of immune parameters will allow us to assess the presence of chronic inflammation and immune exhaustion in subjects that start treatment in early stage of infection as compared to those who start treatment in the chronic phase of infection. Additionally, we aim to evaluate how early treatment affects composition of gut microbiota and how that is linked to immune homeostasis in gut-associated lymphoid tissue (GALT). By this longitudinal study design, we will be able to assess if and how the viral reservoir, established during early infection stages, evolves during the course of infection, while patients remain on combination anti-retroviral therapy (cART). Comparing blood and tissue compartments at different stages of infection will assess compartmentalisation of the reservoir.

1.2 HYPOTHESIS

We hypothesize that the magnitude of innate immune responses at different stages of acute HIV infection is directly linked to the strength of antigenic stimulation and impacts the development of adaptive immune responses. We further hypothesize that the initial viral load burden (linked to the degree of antigenic stimulation) impacts the reservoir size and therefore the immunological and virological profile of the patients is directly linked to the different stage of infection. Given that HIV has strong tropism to the GALT and does severe damage to the intestinal mucosal system early in infection, we hypothesize that early treatment initiation will allow for the maintenance of gut epithelial integrity and therefore GALT and gut microbiome homeostasis. Furthermore, we believe that the size and anatomical localization of the reservoir is directly correlated to the stage at treatment initiation. If this is the case, accurate staging will provide an invaluable tool to compose specific patient cohorts for HIV cure studies in the future. We will use Fiebig staging or more accurate staging if this becomes available.

1.3 RATIONALE

Human immunodeficiency virus (HIV) is the causative agent of the acquired immunodeficiency syndrome (AIDS). The world health organisation (WHO) has estimated that 78 million people have been infected since the beginning of the HIV pandemic, and 35 million people have died from AIDS[1]. Today, approximately 37 million people are still infected with HIV-1, out of which 17 million are currently on combined antiretroviral therapy (cART)[1]. Since its development, cART has saved and increased the quality of millions of peoples' lives. Although, the treatment efficiently supresses viral replication and prevents progression to AIDS, **current cART regiments are not able to eliminate the latent viral reservoir** that is established within the first days of infection[2,3]. This reservoir is responsible for **viral rebound** at treatment interruption[4].

One of the major disadvantages of cART is chronic treatment necessity. The burden of chronic treatment, along with treatment costs, difficulties to access treatment in some parts of the world and ongoing chronic inflammation despite viral suppression, create an **urgent need for a cure**. For the vast majority of

patients, a sterilising cure (complete elimination of the viral reservoir) is not feasible. Therefore, the focus is on a **functional cure** or the so-called "HIV remission" by achieving a substantially **small viral reservoir** that can be spontaneously controlled by immune system without the need of cART. Although this has been achieved in a small group of patients, immunological processes behind it are not well understood[5].

So far, only **early cART initiation** has proven a positive effect on limiting the size of the viral reservoir[6–8]. **Recent HIV-cure trials** have been performed on **chronically infected patients that started therapy late in a chronic phase of infection** and have not been able to show an effect on the size of the reservoir. Instead of using patients with such heterogeneous viral reservoirs, **we believe that these trials should first be performed on a cohort of patients with a well-characterised, small HIV reservoir**, who are close to a functional cure. We believe that **Fiebig staging** (Figure 1, below) forms a **suitable tool** to define such cohort, but in order to validate the value of these stages, we need to investigate whether or not they **are well correlated with the immune, virological and microbiome parameters** in early cART initiated patients.

Fiebig stages have been characterised by Fiebig et al.[9], using markers that allow for precise "serum based" staging of HIV infection. As shown in the research methodology, the first stage is characterised by the presence of viral RNA (stage I), subsequent stages are characterised by the presence of p24 antigens (stage II); p24 antibodies (stage III); and increasingly specific Western blot patterns (stage IV – VI)[9,10]. It is currently not known how **immune responses and viral reservoir development** in the different anatomical compartments are linked to these **Fiebig stages**.
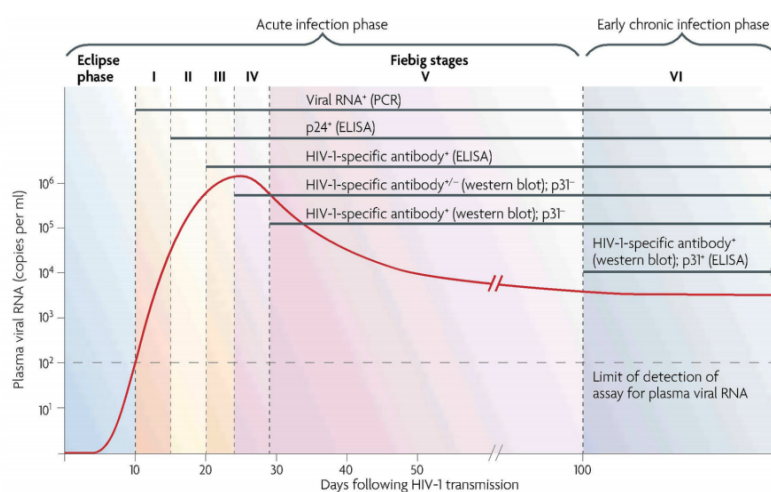
Figure 1: Laboratory staging of HIV infection. The first four Fiebig stages are characterised by the subsequent detection of viral RNA, p24 antigen, antibodies and a semi-positive western blot (some, but not all bands are present). (McMichael et al., 2010[29]).

First, we aim to perform **in-depth profiling of immune responses initiated in acute infection at different Fiebig stages** to understand which specific immune responses are the most efficient in restricting the establishment of viral reservoir in different anatomical tissues.

Primary HIV infection is characterized by an increase in the levels of acute-phase proteins, as well as a cytokine storm, activation of the complement system and a rapid rise in plasma viremia[11]. The earliest innate immune responses include: formation of neutrophil extracellular traps, activation and decline in numbers of dendritic cells[12,13] and activation of natural killer and natural killer T cells[14–16]. Chronic HIV infection is characterised by **chronic immune activation**, which leads to persistent **inflammation**[17] and **immune exhaustion**[18,19]. Mainly virus-specific CD8 T cells undergo functional exhaustion, lose effector functions and fail to control viral infection[20].

Most of the **acute HIV infection studies** have been conducted during the **decline of viral load**. Recently, magnitude and kinetics of CD8 T cell activation have been evaluated during hyperacute HIV infection[21], however, **precise in-depth characterization of innate immune responses at different Fiebig stages** and how these are linked to the **establishment of adaptive immune responses** have not been performed.

Although cART-mediated viral suppression causes significant reductions, many **immune activation pathways remain abnormal during suppressive cART**, especially in patients who initiated cART during later infection stages. We believe that **chronic inflammation and immune exhaustion will be prevented by early treatment initiation**.

Integration of viral DNA into the host resting CD4 T cell genome has been shown to occur during early infection and leads to the formation of a very stable latent viral reservoir[22–24]. **In-depth analysis of viral reservoir dynamics** will allow us to determine if **infection of different cell subsets** occurs in a **similar manner in blood and anatomical compartments** and if the **Fiebig stages** can be used as a **tool** for the prediction of infected CD4 T cell subsets. In addition, we aim to map **how seeding of the viral reservoir occurs in different anatomical compartments** *in vivo* and how this reservoir evolves under treatment. Recent data have shown **residual levels of continuous virus production** in lymphatic tissues[25,26], which may be responsible for constant replenishment of the viral reservoir[26].

Lastly, analysis of microbiome composition will allow us to determine impact of HIV infection and early treatment on gut microbiome composition that is inherently linked to GALT integrity and immune activation.

References:
1. UNAIDS. Global Aids Update. (2016).
2. Chun, T.-W., Moir, S. & Fauci, A. S. HIV reservoirs as obstacles and opportunities for an HIV cure. *Nat. Immunol.* **16,** 584–589 (2015).
3. Whitney, J. B. *et al.* Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. *Nature* **512,** 74–77 (2014).
4. Chun, T. W., Davey, R. T., Engel, D., Lane, H. C. & Fauci, A. S. Re-emergence of HIV after stopping therapy. *Nature* **401,** 874–5 (1999).
5. Sáez-Cirión, A. *et al.* Post-Treatment HIV-1 Controllers with a Long-Term Virological Remission after the Interruption of Early Initiated Antiretroviral Therapy ANRS VISCONTI Study. *PLoS Pathog.* **9,** (2013).
6. Ananworanich, J., Dubé, K. & Chomont, N. How does the timing of antiretroviral therapy initiation in acute infection affect HIV reservoirs? *Curr. Opin. HIV AIDS* **10,** 18–28 (2015).
7. Malatinkova, E. *et al.* Impact of a decade of successful antiretroviral therapy initiated at HIV-1 seroconversion on blood and rectal reservoirs. *Elife* **4,** 1–17 (2015).
8. Laanani, M. *et al.* Impact of the timing of initiation of antiretroviral therapy during primary HIV-1 infection on the decay of cell-associated HIV-DNA. *Clin. Infect. Dis.* **60,** 1715–1721 (2015).
9. Fiebig, E. W. *et al.* Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *AIDS* **17,** 1871–9 (2003).
10. Cohen, M. S., Gay, C. L., Busch, M. P. & Hecht, F. M. The detection of acute HIV infection. *J Infect Dis* **202,** S270-277 (2010).
11. Stacey, A. R. *et al.* Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections. *J. Virol.* **83,** 3719–33 (2009).
12. Killian, M. S., Fujimura, S. H., Hecht, F. M. & Levy, J. A. Similar changes in plasmacytoid dendritic cell and CD4 T-cell counts during primary HIV-1 infection and treatment. *AIDS* **20,** 1247–1252 (2006).
13. Almeida, M., Cordero, M., Almeida, J. & Orfao, A. Different subsets of peripheral blood dendritic cells show distinct phenotypic and functional abnormalities in HIV-1 infection. *AIDS* **19,** 261–271

14. Alter, G. *et al.* Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *J. Infect. Dis.* **195,** 1452–1460 (2007).
15. Borrow, P. & Bhardwaj, N. Innate immune responses in primary HIV-1 infection. *Curr. Opin. HIV AIDS* **3,** 36–44 (2008).
16. Clay, C. C. *et al.* Neuroinvasion of fluorescein-positive monocytes in acute simian immunodeficiency virus infection. *J. Virol.* **81,** 12040–12048 (2007).
17. Keating, S. M., Javobs, E. S. & Norris, P. J. Soluble mediators of inflammation in HIV and their implications for therapeutics and vaccine development Sheila. *Cytokine Growth Factor Rev.* **23,** 193–206 (2012).
18. Porichis, F. & Kaufmann, D. E. Role of PD-1 in HIV pathogenesis and as target for therapy. *Curr. HIV/AIDS Rep.* **9,** 81–90 (2012).
19. Sciaranghella, G., Tong, N., Mahan, A. E., Suscovich, T. J. & Alter, G. Decoupling activation and exhaustion of B cells in spontaneous controllers of HIV infection. *AIDS* **27,** 175–180 (2013).
20. Velu, V., Shetty, R. D., Larsson, M. & Shankar, E. M. Role of PD-1 co-inhibitory pathway in HIV infection and potential therapeutic options. *Retrovirology* **12,** 14 (2015).
21. Ndhlovu, Z. M. *et al.* Magnitude and Kinetics of CD8^{+} T Cell Activation during Hyperacute HIV Infection Impact Viral Set Point. *Immunity* **43,** 591–604 (2015).
22. De Rijck, J., Vandekerckhove, L., Christ, F. & Debyser, Z. Lentiviral nuclear import: A complex interplay between virus and host. *BioEssays* **29,** 441–451 (2007).
23. Siliciano, R. F. & Greene, W. C. HIV latency. *Cold Spring Harb. Perspect. Med.* **1,** 1–20 (2011).
24. Siliciano, J. D. *et al.* Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat. Med.* **9,** 727–728 (2003).
25. Fletcher, C. V *et al.* Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues. *Proc. Natl. Acad. Sci. U. S. A.* **111,** 2307–2312 (2014).
26. Lorenzo-Redondo, R. *et al.* Persistent HIV-1 replication maintains the tissue reservoir during therapy. *Nature* **530,** 51–56 (2016).

2. THE PRESENT STUDY

2.1 STUDY POPULATION

2.1.1 Number of subjects

We will enroll a minimum of 30 patients and a maximum of 50 participants (Fiebig stages I – VI) in the study to **establish a cohort** of early treated patients. We aim at including at least 15 people with an incomplete Western Blot (Fiebig I->IV) and as a control we will also include patients with complete Western Blot and history of a semi-recent infection (Fiebig V-VI).

Before sampling, patients willing to participate will be enrolled following **informed consent**. Study participants will be precisely **staged** at the moment of diagnosis, using the criteria defined by **Fiebig** et al.[9] or more up to date serological staging if such becomes available.

2.1.2 Inclusion and exclusion criteria

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

- Documented recent HIV-1 infection, early diagnosis.
  - clinical symptoms of acute seroconversion and incomplete Western Blot or
  - negative screening test within the past 6 months and incomplete Western Blot or
  - risk contact within the <3 months and presumable primo-infection with or without clinical symptoms and incomplete Western Blot.
- Able and willing to provide written informed consent

- Age = or >18 years < 65 years
- Ability to attend the complete schedule of assessments and patient visits for patients participating in option A schedule (described below), or ability to attend a partial schedule of assessments and patient visits for patients participating in option B (described below).
- Ability and willingness to have blood and tissue samples collected and stored indefinitely and used for various research purposes.

Potential participants meeting any of the following criteria will NOT be enrolled in the study (=**exclusion criteria**):

- Previous or current history of opportunistic infection (AIDS defining events as defined in category C of the CDC clinical classification), consisting of chronic HIV-1 infection.
- Evidence of active HBV infection (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).
- Evidence of active HCV infection: HCV antibody positive result within 60 days prior to study entry with positive HCV viral load or, if the HCV antibody result is negative, a positive HCV RNA result within 60 days prior to study entry.
- Current or known history of cardiomyopathy or significant ischemic or cerebrovascular disease.
- Current history of cancer.
- 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 > 3xULN
- 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.

Patients will be staged according to Fiebig et al. (2003)[9]. Figure 1 (above) gives a schematic view of the Fiebig stages. Standard laboratory tests will be performed to assure accurate staging. Accurate staging is part of standard of care in the situation of aute seroconversion.

2.1.3 Participation Options

We foresee 2 options to participate in the study.
Option A:
Prospective, when the diagnosis was made very recently, the patient will be eligible for option A**.**
Option B:
The patient prefers not to participate in the first in depths sampling, but is willing to participate in a selected number of sample collections during follow up as specified in the IC **(option B1)** or the patient decides to participate during a later phase, because the patient already started treatment during acute seroconversion in the past (**option B2**)

TIMEPOINT	OPTION A *= patient agrees to the complete deep sampling program*	OPTION B 1/ 2 *= patient agrees to a subset of sampling*
Day 0	- Peripheral venous puncture 12 x 9 mL - Urine sampling - Stool sampling - Lumbar puncture - Inguinal LN resection - Left colonoscopy + biopsy	Patient can prospectively (B1) or retrospectively (B2) decide to participate in a part of the proposed sampling in option A.
At undetectable VL (UVL)	- Leukapheresis - Lumbar puncture - Urine sampling - Stool sampling - Sperm/cervico-vaginal sampling	Patient can prospectively (B1) or retrospectively (B2) decide to participate in a part of the proposed sampling in option A.
At 1 year after UVL	- Leukapheresis - Lumbar puncture - Urine sampling - Stool sampling - Sperm/cervico-vaginal sampling - Inguinal LN resection - Left colonoscopy + biopsy	Patient can prospectively (B1) or retrospectively (B2) decide to participate in a part of the proposed sampling in option A.
Month 1 – 24 after start of treatment at M1, M3, M6, M12, M18, M24	Additional 3 x 9mL blood collection as part of a routine blood draw.	A minimum of 2 additional blood draws/ year.

2.2 STUDY DESIGN AND INTERVENTION

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

Note: a consortium agreement is being prepared to extend the project to a multicentre study.

2.2.1 Primary objectives and outcomes

Immunological parameters:
- Immunoassays to measure human pro-inflammatory mediators.
- Immunoassays to measure mediators of immune resolution.
- Assays to measure markers of microbial translocation.
- Flow cytometric assays to assess phenotype (activation and exhaustion) of innate immune cells: monocytes, dendritic cells (DC), natural killer (NK) cells, granulocytes and adaptive immune cells: T cells and B cells.
- Flow cytometric assays to assess function of immune cells, such as antigen uptake and phagocytosis, chemotaxis, oxidative burst, cytotoxicity and cytokine secretion, production of pro-inflammatory and stimulatory/inhibitory factors, viral inhibition capacity.
- Analysis of infiltrating immune cells in tissues.
- Biomarker evaluation on a cellular (multiparameter flow cytometry) and plasma (HIV antigen, antibody affinity for antigen, antibody affinity to Fc receptors, antibody subclassing and antibody glycosylation) level.

Gene expression analysis/transcriptomics

- Full transcriptome analysis (RNA seqencing) of human genes associated with immunity from blood and tissue material.
- RNA sequencing (RNAseq) analysis of various immune cells and cells expressing cell-associated viral RNA (CA RNA).
- Transcriptome analysis to map micro RNA (miRNA) and long non coding RNA (lncRNAs)

Therapeutic drug monitoring
- When on stabile cART, TDM (therapeutic drug monitoring) studies to look at cART penetration in peripheral tissues.

Microbiome
- Compositional analysis of gut microbiota in the lower intestinal tract.

Virological parameters
- Quantification of viral markers as total HIV DNA, integrated HIV DNA, episomal DNA (2LTR circles) and cell-associated RNA in different anatomical reservoirs and different cell subsets.
- Integrations sites sequencing of the region of the human genome where the virus integrates 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
- Single genome sequencing/ full-length sequencing of proviral HIV
- Exosomes analysis to determine whether HIV co-factors are present in exosomes and play a role in infection

2.2.2 Procedures

2.2.2.1 Eligibility screening and inclusion in the study

Study participants will be selected from patients already in follow up (for **option B**) or patients starting follow-up at UZ Gent (**option A or B**). External laboratories will be informed if a positive screening result with incomplete Western Blot by the Aids reference lab. They will get information about the possibilities for the patient to participate in the study. If samples, send from external labs, hospitals or through the general practitioner from high risk groups give a positive screening result with an incomplete confirmation, the applicant will be notified immediately to refer the patient as soon as possible to an Aids reference center. 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. Patients that are already in a follow up and that fulfil the inclusion criteria will be proposed to participate starting from a later time point.

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 possible timing of primary HIV infection
- 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.
- Hematology- leukocytes, WBC differentiation.
- Lymphocyte typing- B and T lymphocytes, T helper/suppressor cells.
- Plasma/serum levels of D-dimer, CRP, complement C3, complement C4.
- Hepatitis HCV & HBV serology if necessary.
- Pregnancy test among women.
- ECG and lung X-ray if required for pre-operative assessment related to the study procedure.
- Assessment of good peripheral venous access for leukapheresis.

2.2.2.2 In depth sampling OPTION A

Day 0: first visit to the clinic after positive screening test for HIV-1: study protocol is explained; eligible subjects are invited to participate. If patients are willing to participate, they are invited to start the study protocol as described below (option A). If not, standard routine clinical care is continued (option B is still possible).

An in-depth sampling will be planned before therapy initiation. 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 1 day. This will be planned as soon as possible after the first contact and HIV-1 diagnosis. This will allow us to start treatment immediately after without any delay. The lymph node resection will take place under short general anaesthesia, for the comfort of the patient.

- Within 24 to maximum 84 hours patients that are included in the study and willing to participate are admitted to the hospital for following procedures: peripheral blood sampling (approx. 100 ml or 12 x 9 ml), CSF puncture, left colonoscopy, inguinal lymph node excision and stool collection. These investigations are part of the **study protocol** and will be only done in patients that are willing to participate and sign the informed consent form.
- After these investigations, treatment is started immediately based on a quadruple regimen with an INSTI, boosted PI and 2 NNRTI's as the guidelines recommend. The start of cART is part of **routine clinical care** as guidelines recommend early treatment initiation.
- Follow-up visits are arranged at our aids reference clinic at 1 week post-intervention, 1 month (M1), 3 months (M3) and 6 months (M6). **The 1 week post-intervention visit is an additional study visit. The other visits are part of routine clinical care.**
- At viral load <20 copies/ml, a leukapheresis, lumbar puncture and stool collection is planned. These investigations are part of the **study protocol**.
- One year after the first visit, a second in depth sampling is planned, as part of the **study protocol**: repeating colonoscopy, lymph node excision, lumbar puncture, semen, urine and stool sampling and leukapheresis. The lymph node resection at this stage will again take place under short general anaesthesia. The colonoscopy can take place under sedation or under the same general anaesthesia, depending on the patient request for general anaesthesia.
- At every standard blood drawing for **routine follow-up** an extra 3 tubes of blood of approx. 30 ml will go the lab for analyses following the **study protocol**.

PART of routine clinical care:

- Intake visit Day 0 at positive screening in the aids reference center.
- General lab analysis with confirmation test, viral load, blood count and CD4 count, co-infection screening and biochemistry.
- Start of cART early after diagnosis.
- Follow-up at M1, M3 and M6: clinical and biochemical follow-up.

PART of the study protocol:
- Explanation of the study protocol at the intake visit D0 and if willing to participate signing of ICF and planning of additional investigations.
- Sampling before treatment initiation: blood sampling, CSF puncture, left colonoscopy and inguinal lymph node excision.
- 1 week post-intervention: clinical control as part of the study visit.
- Extra blood drawing at routine blood analysis at M1, M3, M6, M12, M18 and M24.
- Sampling at reaching undetectable viral load: stool, urine and genital sampling, leucapheresis and CSF puncture.
- Sampling one year after reaching undetectable viral load: stool, urine, genital and blood sampling, CSF puncture, left colonoscopy and inguinal lymph node excision.

If one of these invasive procedures would reveal any abnormalities, unknown to the patients and his physician, diagnostics and therapeutics will be effectuated as to be expected in these cases (ex biopsy, exeresis of a suspect lesion). Follow-up of these results will be foreseen by the treating physician of the patient.

Note: the sampling can be adapted based on individual characteristics of the patients. If patients are not willing to have one of the procedures or there is a contra-indication, this part will be left out. We aim to include at least 30 of our patients for the entire study protocol and all the procedures it involves.

2.2.2.3 In depth sampling OPTION B

Patients that decide not to start the study protocol can still decide to participate in the later phase. This option B group can be divided in 2 subgroups:
- B1: The group of patients that decide prospectively not to participate in the first in depths sampling, but is willing to participate in a selected number of sample collections during follow up as specified in the IC.
- B2: The group of patients that participated as part of the early seroconverter cohort in the study (Reference number: EC 2013/468) or that started treatment early during acute seroconversion over the last 4 years and are interested to participate starting from a later time point and in a selected number of sample collections during follow up as specified in the IC.

2.2.2.4 Sampling will consist of:

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

(PBMCs). 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 one year interval following the initial collection. If contra-indicated this procedure will be replaced by a peripheral blood draw of 12x9ml. The first leucapheresis will be performed once undetectable viral load is reached, the second leucapheresis will be performed one year after the first leacapheresis time point (max interval 16 months).

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 twice, before cART is initiated and after one year of chronic treatment after undetectable viral load.

Colonoscopy
We will perform collection of gut-associated lymphoid tissue (GALT) via biopsy of rectal mucosa. Rectal mucosal sampling will be obtained by sigmoidoscopy, collecting up to 10 mucosal biopsies. If patients agree the second phase of the sampling will consist of a full length colonoscopy with biopsies taken at the terminal ileum and colon.

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 sigmoidoscopy and rectal mucosal biopsy will perform rectal biopsies. This procedure will be performed twice for each participant. The sigmoidoscopy will be performed before cART is initiated, the (second) full length colonoscopy will be performed one year after reaching an undetectable viral load during chronic treatment.

Lumbar puncture
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; lumbar puncture will not be performed if evidence of intracranial pressure is identified. This procedure will be performed three times for each participant: before cART is initiated, at the time of reaching undetectable viral load and one year after reaching an undetectable viral load during chronic treatment.

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: at the time of reaching undetectable viral load and one year after reaching an undetectable viral load during chronic treatment.g. Urine sample

A mid-stream urine sample will be collected. This means that you don't collect the first or last part of urine that comes out. This reduces the risk of the sample being contaminated with bacteria. Urine will be collected in a sterile container and stored in the fridge immediately (room: 120 040). We will collect urine at two time points at the time of reaching undetectable viral load and and one year after reaching an undetectable viral load during chronic treatment.

References:
1.   Molina A et al. Blood reviews (2003); 17: 249
2.   Price RW et al. Aids (2001); 15: 125
3.   Zara F et al. Sexually transmitted infections (2004); 80: 108
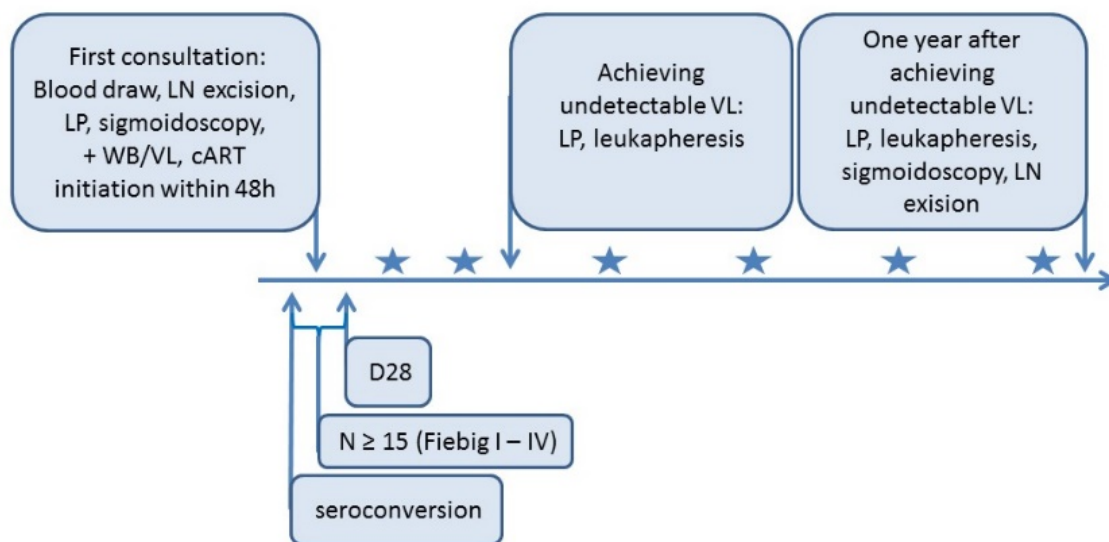
2.2.3 Flowchart

Figure 2: Sampling schedule. LN = lymph node. LP = lumbar puncture. WB = western blot. VL = viral load. Stars represent blood draws at 6, 12, 24, 36, 48 and 60 weeks post infection

2.3 DESCRIPTION OF THE LABORATORY RESEARCH TECHNIQUES

2.3.1 Immunological analysis

- Immunoassays to measure human pro-inflammatory mediators and markers of microbial translocation using ELISA or Luminex:
  IL-1α, IL-1β, IL-6, IL-7, IL-17a, TNF-α, IFN- γ, myeloperoxidase, neutrophil elastase, cathepsin G, neutrophil defensins, nitric oxide, reactive oxygen species, I-FABP, sST2, zonulin-1, EndoCab IgM, LBP, sCD14, KT ratio and others

- Immunoassay to measure the inflammation marker CRP by immunoturbidimetric assay

- Immunoassays to measure mediators of immune resolution using mass spectrometry:
  lipoxins, resolvins, maresins, protectins

- Flow cytometric assays to assess phenotype of innate immune cells: monocytes, dendritic cells (DC), natural killer (NK) cells, granulocytes and adaptive immune cells: T cells and B cells

- o Lineage markers: CD45 (leukocyte common antigen), CD3, CD4, CD8, CD45RA, CD45RO (T cell lineages markers), CD19 (B cell), CD56, NKG2A/NKG2C, 2-domain and 3-domain KIR (NK cell), CD66b (neutrophil), CD68 and CD33 (macrophage), CD14 (monocyte), CD11c (mDC), CD123 (pDC)
  - o Activation markers: CD69, CD38, HLA-DR, CD62L, CD16, CD25
  - o Cell proliferation marker: Ki67
  - o Terminal differentiation and proliferative history marker: CD57
  - o DC maturation markers: CD80, CD83, CD86
  - o Immune exhaustion (Plasma markers: D-dimer, soluble CD163, soluble CD14, IP-10, IL-6 ; HIV-specific T cell: PD-1, LAG-3, Tim3 expression)

- Flow cytometric assays to assess function of immune cells
  - o Phagocytosis (neutrophils, monocytes, dendritic cells)
  - o Chemotaxis (neutrophils, monocytes)
  - o Oxidative burst (neutrophils, monocytes)
  - o Cytotoxicity and cytokine secretion (NK and T cells)
  - o Immune signalling (secretion of cytokines, chemokines, immune-stimulatory or immune-inhibitory factors upon *in vitro* cell stimulation)
  - o Viral inhibition assay (VIA) will be performed to measure the ability of NK and CD8 T cells to inhibit HIV-1 replication. In brief, sorted NK cells or CD8 T cells are incubated with HIV-1-infected autologous CD4 T cells. At predetermined time points, supernatant will be collected and the amount of HIV-1 will be quantified using p24 ELISA.

- Infiltrating innate immune cells and lymphoid tissue fibrosis on formalin fixed tissue samples
  To be able to **quantify** the number of **infiltrating innate immune cells**, fixed tissue sections will be stained. We will evaluate the infiltration of neutrophils (anti-human (αh) neutrophil defensins), macrophages (αhCD68, αhCD33), dendritic cells (DC) [αhDC11c (BDCA-1), αhCD123 (BDCA-2)], natural killer (NK) cells (αhCD56, αhNKp46) and T cells (αhCD8 and αhCD4).

- Biomarker evaluation on a cellular (multiparameter flow cytometry) and plasma level (HIV antigen, antibody affinity for antigen, antibody affinity to Fc receptors, antibody subclasses and antibody glycosylation).
  - o PBMCs will be stained for cell associated RNA, isolated by fluorescent activated cell sorting (FACS) and subjected to RNA seq analysis to identify markers of latency. The identified markers will be validated by multiparameter flow cytometry and examined in the relation to previously identified cellular markers of latency.
  - o Plasma samples will be used to assess the affinity of antibodies to different viral antigens. Next, affinity of antibody Fc fragment to Fc receptors (FcRs) will be assessed by Biacore (GE Healthcare) or Evalution platform (MyCartis). Additionally, antigen-specific antibody subclasses (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM) will be determined by Luminex platform or Evalution (MyCartis) technology. Antibody glycosylation profiles will be assessed by labelling glycans with 8-aminopyrene-1,3,6-trisulfonic acid and analysing by capillary electrophoresis.

2.3.2. Inflammatory parameters

We would like to look for biomarkers of axonal injury and neurodegeneration in the cerebrospinal fluid. We will closely collaborate with Prof Magnus Gisslen, University of Gothenburg and expert 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.

2.3.3. Gene expression analysis/transcriptomics

- Transcriptome analyses with the use of quantitative PCR (qPCR) or RNA sequencing (RNAseq) technology will be used to explore expression profiles of genes associated with immunity. Sorted immune cells or cells excised by laser capture microdissection from fixed/frozen tissue specimens will be subjected to RNAseq technology. Single RNAseq data analysis pipelines will be used (i.e. SINCERA) and ready to be implemented for determining cell type specific gene signatures. This includes quality control (i.e. FASTQC), read mapping (i.e. Tophat), cell type identification and signature & driving force analysis (i.e. SINCERA). Total RNAseq will be performed on Illumina Hiseq 2500 with 10-100 ng input of ribodepleted RNA per patient. Data analysis will involve quality control, mapping of the reads (Tophat) and gene quantification together with differential expression analysis (edgeR). Next, Ingenuity Pathway analysis (IPA) will allow performing gene set enrichment analysis and gene ontology.
- The expression profile of specific RNA factors related to immunity or potentially playing a role in HIV replication will be determined by qPCR (restriction factor analysis). Technology as described above.
- RNAseq analysis of cells expressing cell-associated RNA (CA RNA) will be used to explore the changes in gene expression of infected cells. Technology as described above.
- Transcriptome analysis to map micro RNA (miRNA) and long non coding RNA (lncRNAs). Technology as described above. The non-coding (nc)RNAs are known to control and direct transcriptional and posttranscriptional processes in, offering unique possibilities for pathogens to hijack the cellular machinery and reshape gene expression in their favor. Therefore, studying transcriptome changes upon viral infection with a focus on ncRNAs will be crucial to elucidate the pathways of viral persistence. These expression datasets will be overlapped to investigate broad viral response mechanisms as well as virus- specific changes to the host transcriptome. Transcription factor analysis will be included to investigate how the transcriptome changes can be explained. Functional validation will focus on ncRNAs as these molecules are shown to be crucial for viral persistence.

2.3.4. Therapeutic drug monitoring (TDM)

To estimate the viral reservoir in the different anatomical compartments, the determination of the local concentrations of the administered drugs is of great importance. Sufficient knowledge on drug penetration in peripheral tissues of the current therapies in use is missing. Some studies show that due to lower concentration of certain antiretrovirals in lymph nodes, persistent viral replication occurs[7]. This source of continuous viral replication could be the *in vivo* 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.

2.3.5. Microbiome monitoring

Stool microbiome will be analysed using next-generation sequencing (NGS) of 16S rRNA gene amplicons to identify bacteria at genus/species level and assess the corresponding gut enterotype.

2.3.6. Virological analysis

- Cell sorting of the different cell populations.

  To investigate the **viral reservoir in different cell subsets**, we will isolate immune cells from the collected biopsies and sort different cellular subsets (methods for cell isolation and sorting have been optimised in our lab for the HIV STAR study), if the amount of immune cells recovered allows for further differentiation.

  PBMCs will be isolated from peripheral blood samples by density gradient centrifugation. CD4 isolation will be performed by magnetic beads (negative selection). Cell subsets will be isolated by flow cytometry cell sorting, using antibody labelling based on cell surface markers. From the blood compartment we will isolate CD8 T cells (CD45+CD3+CD8+), monocytes (CD45+CD14+), NK cells (CD45+CD56+CD16+) and B cells (CD45+CD19+). From the pre-sorted CD4 T cell fraction, we will further differentiate Tcm-Tem, Ttm and naïve T cells based on the expression of CD45RO, CD45RA, CD27 and CCR7.

  A fraction of the lymph node biopsy and 2 colon biopsies will be stored for further immunohistochemical analysis. Colon samples will be treated using an optimised protocol for cell isolation, after which CD45+ immune cells will be isolated using fluorescence activated cell sorting (FACS). Cells extracted from the lymph node will be pre-sorted by CD4 negative selection and sorted based on the Tcm-Tem phenotype (cfr above markers).

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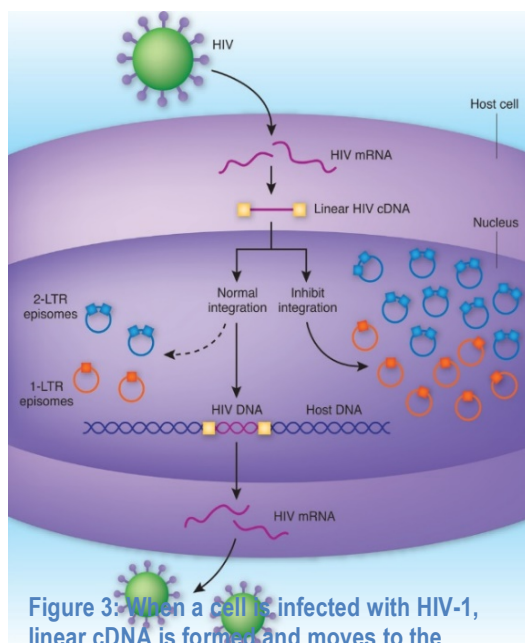

Figure 3: When a cell is infected with HIV-1, linear cDNA is formed and moves to the nucleus, where it is incorporated into the host genome as a provirus. When integration was not successful, unintegrated sequences recombine at their long terminal repeat (LTR), to form 1- or 2-LTR circles. Figure: Schacker[34]

Our data have shown that HIV-1 is present in different anatomical compartments throughout the body[1]. Sorted cell populations from the different tissues will be evaluated using a set of viral reservoir assays based on HIV-1 RNA/DNA intermediates. These assays have been translated by our group from *in vitro* research towards the clinic, using state of the art droplet digital (dd) PCR technology, which allows absolute ultrasensitive quantification of viral DNA and RNA. The cellular fractions from blood and the anatomical compartments will be separated and subjected to **quantification of total HIV-1 DNA, 2LTR** (long terminal repeat) **circular HIV-1 DNA, integrated HIV-1 DNA and cell-associated RNA**.

We will perform **quantification of episomal HIV-2LTR circles** in different anatomical sites to detect ongoing viral replication. These 2LTR

circles have been recognised as a marker of **ongoing replication**. 2LTR quantification assays have been translated from *in vitro* to *in vivo* settings, mainly in PBMCs[2]. Our current data show high levels of 2LTRs in PBMCs of recent seroconverters.

Integrated HIV-1 DNA is a marker for the amount of infected cells and will be quantified using the *Alu*-HIV PCR method, as described by De Spiegelaere et al. This technique relies on the use of a nested PCR, where a specific human repeat element, the *Alu* element, is recognised by the first primer. The second primer of the first PCR recognises an HIV sequence.[3]

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 have adapted these assays towards a clinical user-friendly ddPCR platform[5].

- 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.

- 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.

- Exosomes:
  As exosomes are excreted from typical reservoir cells such as CD4+ T cells, one can hypothesize that viral DNA or other viral markers are present in these exosomes even under full suppression of the virus and therefore can serve as future markers for HIV-1 latency in patients. The presence of co-factors in exosomes and/or differential compositions will be verified in exosomes obtained from blood samples of HIV-1 infected patients. For exosome proteomics, we will use a Stable Isotope Labeling by Amino acids in Cell culture (SILAC) approach. The principle of SILAC labeling is based on metabolic incorporation of given non-radioactive isotopic forms of an amino acid in the culture media into cellular proteins. Typically, SILAC experiments start with two cell

populations: one is labeled in the culture medium composed of heavy isotopic $^{13}C_6$ L-lysine and/or $^{13}C_6^{15}N_4$ L-arginine for 6 doublings, while the other is maintained in the same medium but with normal L-lysine and/or L-arginine for the same period. Proteins are extracted from the two cellular populations respectively, 1:1 mixed and subjected to mass spectrometry. The relative intensities of mass spectrometric peak(s) generated from a protein reflect its relative abundance. A complete analysis of bioactive molecules packaged in the exosomes will be performed including:

- o Identification of specific cellular markers of latently infected cells by proteome analysis
- o Identification of viral proteins packaged in exosomes through mass spectrometry
- o identification of viral RNA species by standardized RT-PCR techniques
- o identification of cellular RNA (specific miRNA)
- o identification of viral DNA through ligation-mediated PCR and subsequent Illumina sequencing
- o comparison of exosomes from latently infected cells before and after reactivation with Latency Reversing Agents (LRAs)

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

  - o 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 harbouring 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).

  - o 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 were 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).

- Single genome sequencing/ full-length sequencing of proviral HIV
  Full-length genome sequencing of proviral HIV will be performed by nested PCR with Illumina MiSeq. This strategy enables to quantify the relative proportion of sequence-intact viruses in the sorted cell populations. DNA amplicon sequences will be aligned with an HXB2 reference and jModeltest v0.1.1 will be used to infer the best phylogenetic model to explain the alignment sequence evolution. Identification of hypermutated sequences will be performed using hypermut 2.0. Stop codons, sequence inversions and deletions in viral open reading frames will be identified using automated sequence analysis approaches, using standard software.
  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. Ananworanich, J., Dubé, K. & Chomont, N. How does the timing of antiretroviral therapy initiation in acute
2. infection affect HIV reservoirs? *Curr. Opin. HIV AIDS* **10,** 18–28 (2015).
3. Malatinkova, E. *et al.* Accurate quantification of episomal HIV-1 two-long terminal repeat circles by use of optimized DNA isolation and droplet digital PCR. *J. Clin. Microbiol.* **53,** 699–701 (2015).
4. De Spiegelaere, W. *et al.* Quantification of integrated HIV DNA by repetitive-sampling Alu-HIV PCR on the basis of poisson statistics. *Clin. Chem.* **60,** 886–895 (2014).
5. Rasmussen, T. A., Tolstrup, M., Winckelmann, A., Østergaard, L. & Søgaard, O. S. Eliminating the latent HIV reservoir by reactivation strategies: Advancing to clinical trials. *Hum. Vaccines Immunother.* **9,** 790–799 (2013).
6. Kiselinova, M. *et al.* Comparison of droplet digital PCR and seminested real-time PCR for quantification of cell-associated HIV-1 RNA. *PLoS One* **9,** 1–8 (2014).
7. Schmidt M et al. Nature methods (2007); 4: 1051
8. Fletcher CV et al. *Proceedings of the National Academy of Sciences of the United States of America* 2014,**111**:2307-2312.
9. 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:

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.

Lumbar Puncture (LP)

Possible complications 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.

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

This 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, vaginal lavage and urine sampling

These are non-invasive procedures and not associated with serious adverse effects.

Anaesthesia

Lymph node excision is done under short general anaesthesia in most cases, because of better tolerability and comfort for the patient. 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 the psychological effect of recent HIV diagnosis

Recent diagnosis makes patients more vulnerable to participate in a study without having balanced very well their engagement. In recent studies performed at the HCRC we included both treatment experienced but also treatment naïve patients (HIV STAR: 2015/0894, Amfar Study EC 2013 468). In the study that included patients with a recent diagnosis, blood sampling and gut biopsies were taken. Patients have a strong interest in the scientific results and we evaluated the study design by standardised questionnaires and most participants did have a very positive experience. We are continuously optimizing the procedures based on this received feedback. In addition, patients will be questioned about their engagement multiple times after the first sampling phase. The protocol will be rediscussed with them and they have the possibility to end their engagement at any time during the course of the study. Patients that present with acute seroconversion are included in the multidisciplinary care path at our AIDS reference centre, where they have the possibility to discuss their recent diagnosis and the psycho-social consequences with a team of doctors, social nurses and psychologists. We have from previous sampling studies created an intern peer reviewing system where we discuss potential issues and cases with Prof. Dr. Dirk Vogelaers.

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) 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. The clinical studies that we set up are presented at the yearly BREACH (organisation unifying all AIDS reference centres in Belgium) congress and the advice of the positive counsel is asked. In order to optimise patient inclusion recruitment will be organised in cooperation with the partner institutions Institute of Tropical Medicine Antwerp and the University Hospital Brussels.

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 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=50) 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 differences between different patient cohorts and compartments within this specific patient cohort while still adhering to the budget constraints.

4.2 DATA ANALYSIS

Samples and results will be anonymised by Prof. Dr. Linos Vandekerckhove and Karen Vervisch and analysed under supervision of postdoctoral students Dr. Eva Maltinkova and Dr. Magdalena Sips at HIV Cure Research Center.

4.3 STATISTICAL ANALYSIS

Patients will be divided into groups depending of Fiebieg stage at the diagnosis. Statistical comparisons of various parameters will be performed with the Kruskal-Wallis test. *P*-values of pairwise comparisons will be adjusted using Dunn's method. Reported *P*-values will be two-sided and values <0.05 will be considered significant. Spearman's rank correlation coefficient will be used to examine the relationship between immunological and virological parameters, such as activation status of innate immune cells and the size of HIV DNA viral reservoir seeded early in infection. When appropriate, logarithmic transformation of the data will be performed to facilitate normalization of the data distribution and to perform parametric tests. Adjustment for multiple testing will be done using Bonferroni correction. Statistical analyses will be performed using SPSS or GraphPad Prism programs.

4.4 STORAGE AND SHIPMENT OF THE SAMPLES

4.4.1 Sample processing

PBMCs will be stored in liquid nitrogen at Bimetra biobank, UZ Gent at -196°C in FCS/DMSO for further analysis. PBMC's will be used for immunological analysis as described on heading 2.3.1 and 2.3.2 of the protocol.
Plasma samples will be stored at -80°C in HIV cure research center lab, room number 120 040; MRBII building, second floor and used both for biomarker evaluation (protocol:2.3.1) and virological assays (protocol: 2.3.6).

Also DNA will be stored at -20°C, RNA isolates will be stored at -80°C in HIV cure research center lab room 120 040. DNA/ RNA will be used for gene expression analysis as described in the protocol 2.3.3. and for virological analysis (protocol: 2.3.6).

Stool samples will be stored at -80°C in HIV cure research center lab room 120 040 and used for micobiome evaluation (protocol:2.3.5).

Formalin fixed paraffin embedded (FFPE) samples will be stored in HIV cure research center labs room 120 040 and used both for biomarker evaluation (protocol: 2.3.1) and virological assays (protocol: 2.3.6). Analysis of HIV integration sites and HIV methylome (protocol: 2.3.6) are done in collaboration with Lisa Frenkel, Seattle Children's Hospital and anonymized samples will be send over to the Frenkel lab for analysis. Single HIV genome sequencing and full length HIV sequencing (protocol: 2.3.6) will be performed on RNA/DNA in collaboration with the Mathias Lichterfield lab Lichterfeld at MIT and Harvard, Boston, USA and anonymized samples will be send over to the Frenkel lab for analysis.

Cerebrospinal fluid will be used for biomarkers of axonal injury and neurodegeneration in the cerebrospinal fluid. We will closely collaborate with Prof Magnus Gisslen, University of Gothenburg and expert in the field, doing additional testing of CSF measuring inflammatory parameters like neurofilament in his lab. Therefore cerebal fluid samples will be stored in HIV cure research center labs room 120 040 and send over to Gotenburg for analysis. Samples that are not used at the Frenkel, Lichterfield and Gisslen lab will return to the Bimetra biobank or will be destroyed. Samples are analysed by PhD students of the HIV Cure Research Group of Prof Vandekerckhove that perform a research stay in a foreign lab.

As this are very precious samples, samples will be stored at the Bimetra biobank, UZ Gent or at the HIV cure research center lab, roomnumber 120 040; MRBII building, second floor for 20 years.

5. DURATION AND TERMINATION OF STUDY

The clinical study (including sampling before therapy initiation, during follow-up) 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 September 2017 until the 1st of January 2023. 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.

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. Patients do not receive compensations but transport will be reimbursed for visits that are not part of routine clinical practice.

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. The end of the study is defined as the sampling at the third time point.

11. DISSEMINATION

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

12. CONTACT PERSON

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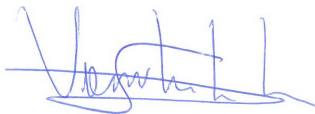- Dr. VAN DER MOEREN NATHALIE

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