ZONULIN-1 AS A POTENTIAL BIOMARKER FOR DETECTION OF IMMUNOLOGICAL NONRESPONDERS AMONG HIV INFECTED PATIENTS

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In cooperation with the Immunity and Infection research group from the “Institut d’Investigació Sanitària Pere Virgili” (IISPV)

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Index

Summary **4**
Key words **4**
1. Introduction **5**
1.1. HIV: aetiology and epidemiology 5
1.2. HIV structure 6
1.3. Replication cycle 7
1.4. Pathogenesis of the infection 9
1.5. HIV detection systems 11
1.6. Treatment 12
1.6.1. Immunological nonresponders (INRs) 12
1.7. HIV and the gastrointestinal tract 13
1.7.1. Zonulin-1 as a biomarker of intestinal mucosal barrier function 14
1.7.2. IFABP and EndoCAb 15
2. Hypothesis and Objectives **17**
3. Materials and methods **18**
3.1. Study design 18
3.2. Measurement of Zonulin-1 levels 19
3.3. Measurement of EndoCAb and IFABP levels 20
3.4. Statistical analysis 20
4. Results **21**
4.1. Patients' characteristics 21
4.2. Zonulin levels related to baseline low $CD4^+$ T-cell counts (controls vs cases) 22
4.3. Zonulin levels as a potential marker of immunodiscordants' response (IRs vs INRs) 22
4.4. Biomarkers' correlations 23
4.4.1. Zonulin-EndoCAb 23
4.4.2. Zonulin-IFABP 24
4.5. Logistic regression 25
5. Discussion **26**
6. Conclusions **28**
7. Bibliography **29**

Summary

Human Immunodeficiency Virus (HIV) infection is still a major global health issue. Immunological nonresponders (INRs), which take up about 30% of HIV patients, do not fully recover $CD4^+$ T-cell levels to optimal values upon administration of antiretroviral therapy. Since the gastrointestinal tract is largely affected due to HIV infection, potential biomarkers of gut damage have been studied. Zonulin, the only known modulator of tight junctions, was used in this project to discriminate between INRs, immunological responders and controls in subjects starting first antiretroviral therapy. Zonulin levels of INRs turned out to be significantly lower than controls ($p < 0.01$), indicating an important dysregulation of intestinal mucosal barrier function in INRs, and also leaving zonulin as a potential biomarker in the detection of INRs.

Keywords

Biomarkers, EndoCAb, gastrointestinal tract, Human Immunodeficiency Virus (HIV), IFABP, Immunological nonresponders (INRs), zonulin

1. Introduction

1.1. HIV: aetiology and epidemiology

Human Immunodeficiency Virus (HIV) is a retrovirus of the *Lentivirus* genus of the *Retroviridae* family, subfamily *Orthoretrovirinae* (1,2). HIV is the cause of Acquired Immunodeficiency Syndrome (AIDS), which causes a weakening of the immune system making the patient prone to getting life-threatening infections and various types of cancers. The virus can be transmitted via body fluids such as blood, vaginal fluids, rectal fluids, semen and pre-seminal fluid, and breast milk (1). HIV mainly infects T cells, whose primary function is the regulation of the cellular and humoral immune responses. However, HIV can also infect other cell types such as macrophages, immature dendritic cells and resting T-cell subsets (3).

When the infection happens, some patients may experience no symptoms whatsoever. If symptoms arise, they are similar to those present in influenza infection: headaches, fever, sore throat, amongst many others (1). Patients who display severe or durable symptoms during the acute phase tend to progress more rapidly to AIDS (3). This acute infection, which can last for a few weeks or months, is the phase in which the virus is most infectious (4).

The acute infection becomes an asymptomatic infection, lasting ten years or longer. Although symptoms may not arise during this phase, the virus can also be transmitted. If not treated, most infected patients eventually develop AIDS. These patients have severely compromised immune systems, having a CD4 cell blood count below 200 cell/mm^3, compared to an average CD4 cell count of 500-1500 cell/mm^3 (1,4). Therefore, they are at high risk of contracting uncommon infections than the general population (opportunistic infections). There is also a higher risk for certain cancers, especially lymphomas and Kaposi sarcoma (skin cancer). After being diagnosed with HIV, it is common to have regular blood tests to check the CD4 cell count (1).

It is estimated that HIV was introduced to the human population between 1920 and 1940 (1). HIV continues to be a significant global health issue, having claimed 34.7 million lives so far. An estimated 37.6 million people were living with HIV at the end of 2020, over two-thirds of whom are in the WHO African Region. In 2020, 690 000 people died from HIV-related causes and 1.5 million people were newly infected (4).

The new proposed global target is 95-95-95, which consists of three main objectives: [1] detecting 95% of infected patients, [2] having 95% of diagnosed patients under treatment and [3] having 95% of patients under treatment with an undetectable viral load (4).

1.2. HIV structure

There are two groups of HIV: HIV-type 1 or HIV-1 and HIV-type 2 or HIV-2, being the former the leading agent of AIDS worldwide. The latter is mainly found in some regions of Africa. Despite both groups having the same basic structure, differences can be found when the genome organisation is considered (3). It has been proven that HIV-1 evolved from a non-human primate immunodeficiency virus, and HIV-2 evolved from West African sooty mangabeys (2).

The retrovirus genome consists of two copies of single-stranded RNA molecules of positive polarity, which are identical (3,5). Its main components are the three structural genes [1] *gag*, which encodes for proteins of the outer core membrane, capsid protein, nucleocapsid and for a nucleic-acid stabilising protein, such as p24, p7 and p6 (core) and p17 (matrix); [2] *env*, which encodes for the viral envelope glycoproteins, gp120 and gp41, whose primary function is the recognition of cell surface receptors; [3] and *pol*, which encodes for the three essential enzymes for viral replication: reverse transcriptase, integrase, and protease as well as for the RNase H (2,3). However, six regulator genes (*tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*) can also be found (2,5,6). Other genes coding for accessory proteins exist as well, not required for replication, despite their function being less understood (3,6).

In its provirus form, the HIV genome is flanked by two repeated sequences, known as Long Terminal Repeats (LTRs), which allow integration into the target genome (5).

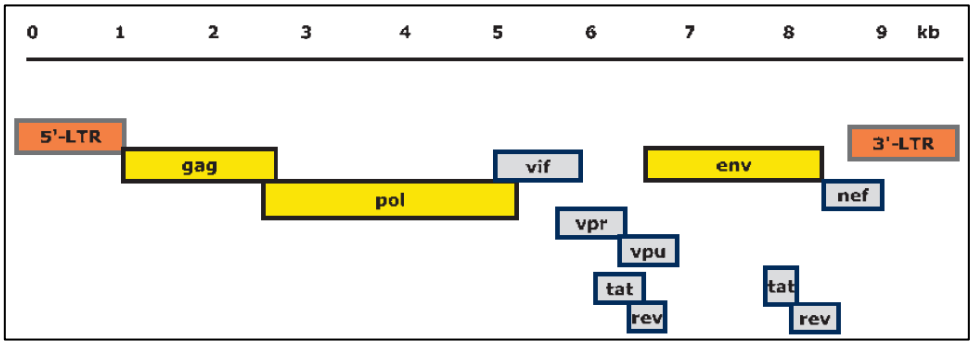

Figure 1. Structure of HIV-1 genome. The figure describes the genes that code for both structural and regulatory proteins. Neither 5-LTR nor 3'-LTR are translated into proteins but allow integration into the target genome. In the case of HIV-1, the genome consists of 9.2-9.6kb. LTR: long terminal repeat; gag: group-specific antigen; pol: polymerase; env: envelope. Extracted from Seitz R., Human Immunodeficiency Virus (HIV) (2).

Viral particles are round, have a diameter of around 100 nm and are surrounded by a lipoprotein-rich membrane derived from the host cell (2,3,6). On top of the membrane, heterodimer complexes are found. Such complexes are formed by gp120 bound with gp41. The matrix protein gp17 is bound to the inside of the viral lipoprotein membrane. The virus membrane and the matrix protein include the capsid, composed of polymers of p24, the core antigen. The capsid contains two copies of HIV RNA combined with a nucleoprotein, and the enzymes reverse transcriptase, integrase, and protease (3). Furthermore, several RT/RNase H and integrase molecules are bound to the nucleic acid (2).

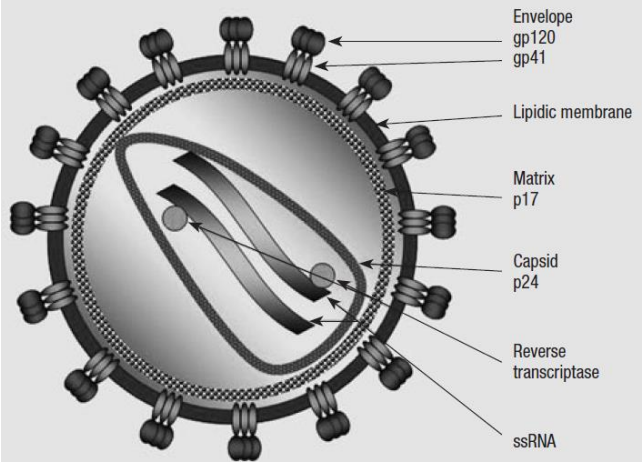

Figure 2. Structure of the HIV particle. ssRNA: single-stranded RNA. Extracted from Fanales-Belasio E et al., HIV virology and pathogenetic mechanisms of infection: a brief overview (3).

1.3. Replication cycle

The HIV replication cycle can be summarised in six steps: [1] binding and entry, [2] uncoating, [3] reverse transcription, [4] provirus integration, [5] virus protein synthesis and assembly, and [6] budding (3).

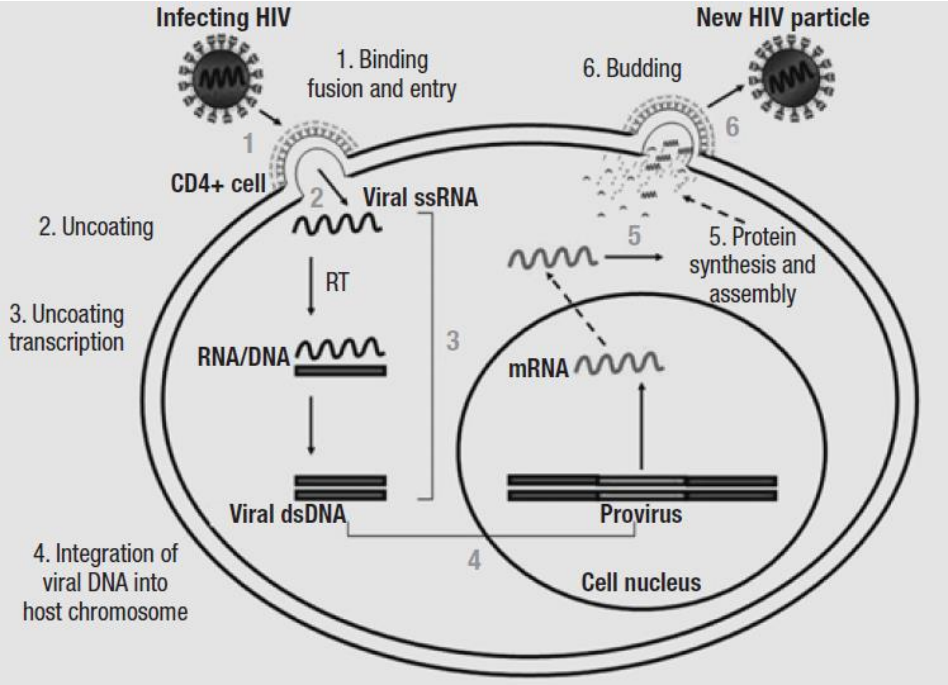

Figure 3. HIV replication cycle. RT: reverse transcriptase; dsDNA: double-stranded DNA. Extracted from Fanales-Belasio E et al., HIV virology and pathogenetic mechanisms of infection: a brief overview (3).

The entry pathway begins once gp120 has bound CD4, a monomeric glycoprotein found on the surface of immune system cells, such as T-lymphocytes or monocytes/macrophages (3). Therefore, all CD4 positive cells, such as T helper cells or macrophages, are susceptible to HIV infection (2). Upon binding, the virus envelope undergoes a conformational change, which allows a specific gp120 domain to bind chemokine receptors. The most common coreceptors are CXCR4 and CCR5. The differential expression of chemokine receptors on cell targets is a significant determinant of the HIV-1 tropism (3). This tropism is related to the gp120 aminoacidic sequence and its spatial conformation (5). Some strains of HIV-1 preferentially bind the β-chemokine receptor CCR5, which are known as macrophage-tropic (M-tropic) or R5 viruses. Other strains use preferentially CXCR4 for entry, known as T-lymphocyte-tropic (T-tropic) or X4 viruses (3). However, some variants have been described that use both coreceptors equally (5). This double binding allows a conformational change that affects gp41, forming a channel that inserts into the plasma membrane and allows membrane fusion (2,3).

Following membrane fusion, the virus uncoats into the cytoplasm of the target cell. The now free viral RNA is converted into proviral DNA due to the action of the reverse transcriptase (RT) and the integrase (5). The viral reverse transcriptase has a high mutation rate due to a lack of proofreading activity (1 error per 10 000 nucleotides), making not uniform the viral population on an infected individual (6). It is estimated that the viral RT makes one error, at least, per transcription round (2). After integration, caused by the integrase into a random place of the human host cell genome, the viral genome can stay latent, replicate in a controlled manner, or experience a massive replication which will have a cytopathic effect on the infected cells (2,5). Upon cell activation, transcription of proviral DNA into mRNA occurs, which migrates into the cytoplasm and allows structural proteins of new virions to be synthesised (3). NF-κB represents the main regulatory element of the transcription of the HIV genome after latency, and so does the Nuclear Factor of Activated T Cells (NFAT) (5). However, cleavage of the precursor molecules by the viral protease is necessary for generating infectious viral particles (2,3,5). The viral protease does the processing of Gag and Gag/Pol on five specific sites. The assembly of the HIV-1 particle is caused by the oligomerisation of Gag proteins (2,7).

In inactive CD4 lymphocytes, the viral genome can be partially converted into DNA. Upon cell activation, integration and conversion can be finalised. This non-integrated proviral form can become an essential reservoir during the first week after infection and may be used as a biomarker for recent infection (5).

Finally, the viral particle buds from the plasma membrane of the infected cells (7). During the budding process, the virus lipid membrane may incorporate various host cell proteins and become enriched with phospholipids and cholesterol (3). Afterwards, progeny virions are released from infected cells (6).

The cell is not a friendly environment for the virus. It encodes specific factors that can inhibit HIV at various steps of its life cycle. However, HIV-1 has evolved to evade or counteract these antiviral host factors (6). For example, HIV-1 is stable over several hours against gamma irradiation, ultraviolet light or ultrasonic waves (2).

1.4. Pathogenesis of the infection

The pathogenesis of the infection and its progression depend on both the infecting virus and the host's immune system response. Their balance will determine the outcome of the infection (3).

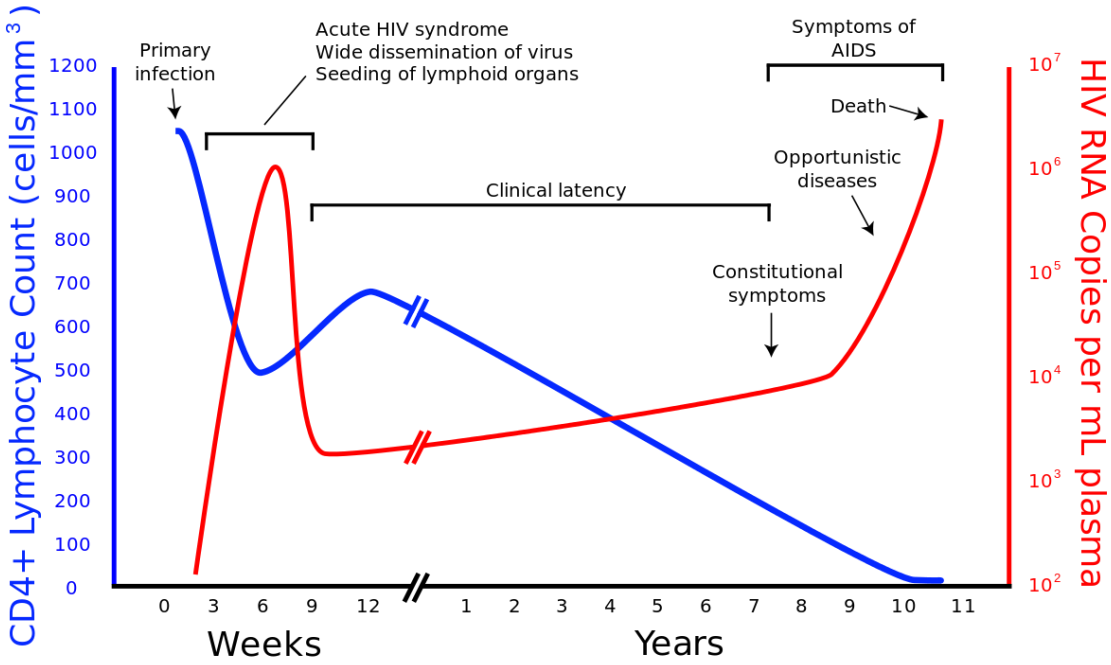

Figure 4. Temporal course of the HIV infection. Image extracted from Wikimedia Commons. Retrieved February 14, 2022, from https://commons.wikimedia.org/wiki/File:Hiv-timecourse_copy.svg.

During primary infection, also known as the acute phase, HIV-1 levels are incredibly high but short-lived since the immune system can generate an adequate response to control viral replication. Viremia declines over the first weeks until a steady level is reached (which may even be under detection level), but so does the CD4 cell count, a consequence of the high viremia levels. Seroconversion, defined as the time of appearance of the first HIV-specific antibodies, can last from 3 to up to 5 weeks (3).

A few weeks after acute infection, once seroconversion is achieved, most patients enter an asymptomatic or latency period, in which HIV levels drop, characteristic of lentiviruses (3,4). This state is usually accompanied by the absence of symptoms (3). Once infected, the cell will last in this state for the remainder of its life span (6). However, HIV continues to replicate in the so-called HIV reservoirs, body compartments where there is a low expression of viral antigens (3). Thus, the infection can be spread either by infection of new cells or by replicating the already existing ones. Resting T-cell subsets are vital since they can keep the virus in an integrated proviral form. Furthermore, cells infected but in a latency state will not show viral antigens and, therefore, will not be eliminated (6). Moreover, there is a progressive loss of CD4 cells and a weakening of the immune system (3).

Infection does not progress the same in all patients, and infected individuals can be classified based on their infection course. The classification includes progressors, rapid progressors, non-progressors, long-term non-progressors, and elite controllers.

Some patients may expose an efficient control of the infection by themselves. When talking about elite controllers or long-term non-progressors, viral replication control and non-progressor phenotypes are attained thanks to several host, immune and viral factors. Host factors include specific HLA alleles. Immune response factors are related to innate immunity, cellular immune response, and humoral immune response, including neutralising antibodies. Virological factors include low levels of virus, nucleic acids, and proteins as well as defective or attenuated viruses (low replication capacity or defective in some proteins). Since many combinations are plausible to obtain such a phenotype, the group of elite controllers and long-term non-progressors is not homogeneous (8). Concretely, elite controllers are defined as patients who have, at least, three HIV determinations below the level of detection in the absence of antiretroviral treatment for, at least, a year; as well as a lack of evolution, stable levels of CD4 cells (greater than 400-500 cells/mm^3), strong immune responses and no signs of clinical progression (3,8,9).

The mechanism by which they control HIV has been studied because they could become a great model to find a functional cure for HIV.

If the patient cannot contain the virus, the immune system continues to weaken, and the CD4 count drops to < 200 cell/μL. In this state, the patient is prone to opportunistic infections, which can provoke life-threatening diseases. This stage is called AIDS (3).

1.5. HIV detection systems

HIV testing is a two-step process. First, a screening test is done, which gives results in around 30 minutes and can be performed at home. A laboratory or follow-up test is required to confirm the diagnosis (1). Early identification greatly improves treatment options and reduces the risk of transmission (4).

Approximately 24 hours after infection, the first progeny virus is released from the infected cells. On day 11 post-infection, HIV RNA can be detected in blood and, after two weeks, HIV DNA can be detected in blood lymphocytes in an integrated form. Antibodies can be detected after 3-5 weeks post-infection. When it comes to HIV detection systems, two types can be used: antibody detection or virus detection (2).

HIV antibody screening tests, such as ELISA, are used in primary diagnosis but require a confirmatory test if a positive result is obtained. Such tests can only be performed 3-5 weeks post-infection, once seroconversion is achieved (2,10).

Confirmatory tests are used to prevent false-positive results, which are very common in cases of individuals with autoimmune diseases or pregnant women. An example would be the Western blot assay. Only if the criteria for the confirmatory tests are fulfilled can the HIV infection be confirmed. In case of a positive result, and due to the consequences of the diagnostic, it is recommended to perform a second test with an independently taken blood sample (2,10).

Viral isolation is often unsuccessful and costly when it comes to virus detection. Using a combination of monoclonal and polyclonal antibodies, and using the ELISA sandwich technique, the p24 protein can be detected. However, a positive p24 antigen test must also be confirmed. Furthermore, genome detection can also be performed via direct amplification of the target sequence or signal amplification. If needed, virus particles can be concentrated, e.g., by ultracentrifugation (2).

1.6. Treatment

HIV/AIDS is treated with antiretroviral therapy (ART), which stops the virus from multiplying. Despite ART being recommended only once the CD4 count has dropped or HIV complications have arisen, it is currently recommended for all people with HIV infections. The goal of ART is to maintain low virus levels in the blood, also known as viral load, so it cannot be detected and, therefore, it cannot be transmitted (1). However, its secondary goal is to allow restoration of CD4 T-cell count (10). ART has been described to reduce the risk of onward transmission by 96% (4). If the CD4 count drops before the treatment, it will usually go up with ART. This partial recuperation of the immune system causes the disappearance of HIV complications (1).

The main therapeutic options are nucleoside (NRTIs), nucleotide (NtRTIs) and non-nucleoside analogues (NNRTIs), reverse transcriptase inhibitors combined with protease inhibitors, and also integrase inhibitors. The most used combination consists of two NRTIs and one NNRTI. A genotypic test should be done before treatment to avoid resistance (2).

With treatment, most infected patients can live a healthy and everyday life. Since current treatments do not cure the infection, medication only works if taken every day. Stopping treatment may cause a viral rebound and CD4 count to drop. Also, if medicines are not taken regularly, the virus can become resistant, and treatment will stop working (1).

Nowadays, the main objective of the investigation is to find a functional cure, which consists of the suppression of HIV-1 viral replication permanently, even in the absence of antiretroviral therapy. However, the functional cure would not eradicate the virus, nor is it needed if levels are below detection (8).

1.6.1. Immunological nonresponders (INRs)

Approximately 30% of patients under treatment fail to recover their CD4 T-cell counts. Such patients are called immunodiscordants or immunological nonresponders (INRs) and tend to be associated with an increased risk of disease progression and death. It is believed that INRs tend to have both decreased or altered production of CD4 T-cells and excessive destruction of such. In a study by *Yeregui E. et al.*, INRs tended to be older, with lower baseline levels of CD4 cells and associated with intravenous drug use at enrolment (10).

1.7. HIV and the gastrointestinal tract

Increased intestinal permeability has been investigated as a critical element in the pathogenesis of many diseases, including autoimmune diseases such as HIV infection (11,12). If the gut barrier function has been impaired, it can become a key pathogenic component in such diseases. Therefore, an intimate cross-talk between epithelial cells and the immune system exists for the vigilance of the intestinal lumen (11,13,14).

HIV infection is characterized by systemic inflammation, an altered intestinal barrier, and gut microbiota dysbiosis (15–18). In adults, it is during the acute infection that the gastrointestinal tract is targeted, and it has been proven that HIV infection causes loss of $CD4^+$ T-cells as well as induces a shift in the composition of the gut microbiota (13,16,17,19,20). This is because the gastrointestinal tract is an essential site for HIV replication (15,16). In healthy patients, the gastrointestinal microbiome consists of an ecosystem of multiple microorganisms in a dynamic equilibrium. Such microorganisms shape many characteristics of the systemic immune system and the mucosal immune system (16). However, HIV-infected individuals have a very different gut microbiome than non-infected individuals, even when talking about patients who can control the infection thanks to ART. This change tends to enhance bacterial populations that are either pro-inflammatory or potentially pathogenic (15,16,19).

Furthermore, there has also been found a difference between patients undergoing treatment compared to untreated patients. Therefore, it is believed that the gut microbiota composition can contribute to chronic inflammation and disease progression (13,19). In a study by *Hunt P. et al.*, microbial translocation was predictive of mortality in patients under treatment (21).

Intestinal mucosa plays a role in immune functions as a barrier, and it also prevents bacterial translocation in addition to other roles in nutrient absorption (11,13,22,23). Once CD4 cells deplete in blood, they also do in the gastrointestinal tract. Therefore, there is an increase in the translocation of microbial products which leads to systemic immune activation (9,24).

Improved hygiene has reduced exposure to microorganisms, described as one of the possible causes of emerging immune-related diseases, particularly autoimmune diseases. In the pathogenesis of such diseases, two main factors have been described: genetic background and environmental exposure. However, a third element has been proposed:

increased intestinal permeability, probably influenced by gut microbiota composition. Intestinal permeability regulates molecular trafficking and leads to tolerance or immunity. Intracellular tight junctions (TJs) are essential in this process since they are highly dynamic. However, their mechanism is yet to be fully understood (25).

Some studies found that baseline translocation markers predicted the clinical outcome in elite controllers. Such results confirm the hypothesis that microbial translocation determines immune activation and clinical progression independently of viremia (9). On the other hand, immunological responders' gut microbiome is metabolically different from immunological nonresponders' (19). Many gut epithelial barrier function markers predicted mortality in INRs (21). Therefore, the immune system and the gut microbiome synergy could probably mediate solutions to inflammation and promote immune recovery (16,19).

1.7.1. Zonulin-1 as a biomarker of intestinal mucosal barrier function

Zonulin-1 is, so far, the only known physiologic modulator of tight junctions (TJs), which hold together epithelial cells (11,13,25). It can regulate intestinal permeability in a rapid, reversible, and reproducible way (11,21). Therefore, zonulin is involved in the pathogenesis of autoimmune diseases and can be used as a biomarker of gut barrier function (11,14,21,23,25). Zonulin-1 is also considered a marker of enterocyte function (14).

Tight junctions were thought to be an impermeable barrier blocking any paracellular passage. However, it is now known that tight junctions are dynamic structures. Zonulin can reversibly disassemble such structures, increasing intestinal permeability (11–13,23). It has been proposed that this mechanism works thanks to a PKC-dependent polymerization of actin microfilaments, leading to tight junctions' disassembly (11,23). The tight junction mechanism is yet to be fully understood (11).

Zonulin release happens mainly due to small intestinal exposure to bacteria and gluten. When exposed to enteric bacteria, small intestines release zonulin (11,25).

In a study made by *Nocella C. et al.*, differences in zonulin levels were found between healthy subjects and HIV treated patients. Furthermore, differences were also found between healthy subjects and naïve patients (12). It has been reported that decreased zonulin levels are correlated with increased mortality in such patients (13,14,21).

Therefore, it seems that the zonulin pathway protects against HIV infection (13). No information regarding its potential role in INRs has been previously described.

1.7.2. IFABP and EndoCAb

Many studies have hypothesised that microbial translocation markers could be associated with immune activation in HIV-infected patients, being independent predictors of the progression of the disease, but the clinical significance of such is yet to be entirely determined (26,27). Out of the many possible markers of microbial translocation, we decided to focus on two: IFABP and EndoCAb.

Intestinal fatty-acid binding protein (IFABP) is an intracellular protein whose primary expression is found in the epithelial cells of the small and large intestine mucosal layer. Such location allows the protein to leak into systemic circulation when intestinal mucosal damage occurs. Therefore, it works as a marker of enterocyte damage or death (14,22,28). Since there has been found a correlation between IFABP plasma concentration and small intestinal damage or diseases (e.g. sepsis, obesity, HIV…), IFABP has emerged as a possible non-invasive marker for evaluating gastrointestinal wall integrity loss and inflammation (22,28).

Circulating IFABP concentration has been positively associated with the innate activation of the immune system, .as well as with contribution to chronic and systemic immune activation in the HIV population (28). For example, in a study by *Prendergast AJ et al.*, levels of IFABP at six months were significantly higher ($P < 0.001$) than at six weeks in infants who had been infected with HIV, indicating progressive intestinal damage (20). Another study by *Sandler NG et al.* found that IFABP was slightly larger in patients who died. Furthermore, they also showed increased IFABP levels in HIV-infected subjects, associating higher levels with lower baseline $CD4^+$ T-cell counts (26). Finally, in another study by *Cheru LT et al.*, they found serum IFABP to be significantly higher in patients living with chronic HIV compared with elite controllers and HIV-negative controls. However, elite controllers still had higher levels of IFABP than HIV-negative controls, suggesting that there is intestinal damage as well in this type of patient (28).

EndoCAb, on the other hand, is an IgM antibody to the LPS core oligosaccharide. The levels of this molecule have been associated with the disease progression in HIV-1 infected individuals, having elevated levels compared to healthy subjects. Furthermore,

rapid progressors, which have a faster tendency to develop AIDS, had higher levels of EndoCAb in comparison to slow progressors or healthy controls (24). In the analysis of *Merlini E. et al.*, markers of microbial translocation did not predict disease progression, but this is in contrast with other data, provided by *Hunt et al.*, where they demonstrated an association between such markers and mortality (27). Finally, in a study by *Ancona G et al.*, they found that introduction of cART caused an increase in both IFABP and EndoCAb levels. This suggests that even if cART has immunological benefits, persistent gut damage still exists (18).

2. Hypothesis and Objectives

The antiretroviral therapy (ART) used to treat HIV/AIDS has two main objectives: maintaining a low viral load to avoid detection and transmission and restoring the CD4 T-cell count. However, ART is not infallible in the CD4 restoration. Approximately 30% of the patients under ART fail to recover their CD4 T-cell counts, and such patients are called immunological nonresponders (INRs).

Nowadays, there are no effective biomarkers that can allow us to differentiate between immunological responders (IRs) and immunological nonresponders (INRs) at the beginning of the infection. In the pathogenesis of autoimmune diseases, three factors have been described: genetic background, environmental exposure, and increased intestinal permeability. The latter is thought to be influenced by gut microbiota composition and intracellular tight junctions (TJs) which are vital due to their dynamism. Furthermore, *Serrano-Villar S et al.* stated that the gut microbiome of the immunological responders was metabolically different from the gut microbiome of immunological nonresponders.

Zonulin is the only known modulator of TJs, and its release happens due to exposure to enteric bacteria in the small intestines. Therefore, zonulin is involved in the pathogenesis of autoimmune diseases and can be used as a biomarker of gut barrier function. This is why we hypothesised that the levels of zonulin-1 measured at the beginning of ART could predict the patient's outcome in terms of being an immunological responder or an immunological nonresponder.

In conclusion, the objectives of this project were:

- To study zonulin-1 as a predictive biomarker of immunological response to ART.
- To establish whether there exists a correlation between zonulin-1, a TJs marker, and other dysbiosis markers, such as EndoCAb and IFABP.

3. Materials and methods

3.1. Study design

The study cohort consisted of 313 samples obtained from HIV patients who started the first ART therapy between 2011 and 2013 and aged over 18 years. Patients were monitored for 36 and the samples came from "Hospital Universitari Joan XXIII", Tarragona; from "Hospital de la Santa Creu i Sant Pau", Barcelona; and from "Hospital Virgen del Rocío", Sevilla. Storage of the samples at -80ºC was taken care of by the Biobank-IISPV (29).

The inclusion criteria were patients infected by HIV that had not started the ART before the study, which had a good response to the treatment (viral suppression after 36 months) and of which there was data on the CD4+ T-cell counts at the beginning of the study and after a period of monitoring of 36 months. Patients excluded from the study were: those who had started ART before the study; those taking drugs with known side effects, such as lipid-lowering agents; those suffering from opportunistic infections or inflammatory diseases; pregnant women; or patients that had received a vaccine during the year before the beginning of the study. The project was revised and approved by the ethics committee of each recruiting centre before the starting date. Furthermore, volunteers were informed of the implications of participating in the study, and they provided their written informed consent (29).

The study design ended up including 108 samples of which there were values of zonulin-1, EndoCAb and IFABP levels. Patients were initially divided into two groups: Controls (n = 53), which had an initial CD4+ T-cell count larger than 200 cells/µL and Cases (n = 55), which had an initial CD4+ T-cell count smaller than 200 cells/µL. Furthermore, Cases were divided into two groups based on their CD4+ T-cell count after 36 months (3 years). The first group were Immunological Responders (IRs) (n = 27), which had a CD4+ T-cell count larger than 250 cells/µL, meaning that ART helped to recover the T-cell count; and Immunological Nonresponders (INRs) (n = 28), with a CD4+ T-cell count smaller than 250 cells/µL, meaning that even if ART had made viral load indetectable, there had been no recuperation of the CD4+ T-cell count.

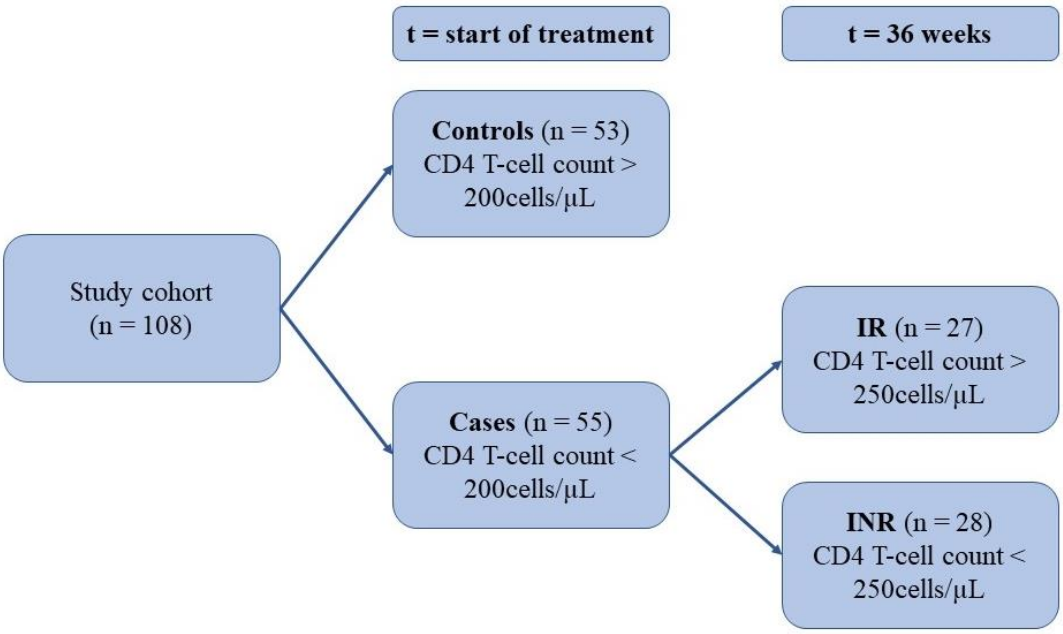

Figure 5. Description of the study cohort used for this study. The first classification (controls vs cases) was performed based on data available at the start of treatment. The second classification (IR vs INR) was performed based on data available at 36 weeks from the start of treatment. IR: immunological responders; INR: immunological nonresponders.

3.2. Measurement of Zonulin-1 levels

Zonulin-1 levels were measured with the Human Zonulin Elisa Kit from Elabscience®. In each ELISA plaque, there were duplicates of 20% of all the samples and a calibrator was used as a standardisation factor between different plaques to avoid the influence of temperature and other external factors.

The protocol of the kit went as follows and can be seen in Figure 6:

1. Addition of 100µL of either sample or standard to each of the wells with an incubation of 90 minutes at 37ºC. This would allow zonulin to bind to the antibodies present inside the well. Since some samples resulted in higher levels of zonulin-1 than the detection range of the kit, dilutions such as 1/2 and 1/5 were performed.
2. Discard of liquid, followed by the addition of 100µL of the Biotinylated Detection Antibody solution to each well, with an incubation of 60 minutes at 37ºC.
3. Aspiration and washing of the plate three times.
4. Addition of 100µL of the HRP conjugate working solution, with the following incubation of 30 minutes at 37ºC. Afterwards, aspiration and washing of the plate five times.
5. Addition of 90µL of the Substrate Reagent with an incubation of 15 minutes at 37ºC.
6. Addition of 50µL of the Stop Solution.
7. Immediate reading of the plate at 450nm with the following calculation of results.

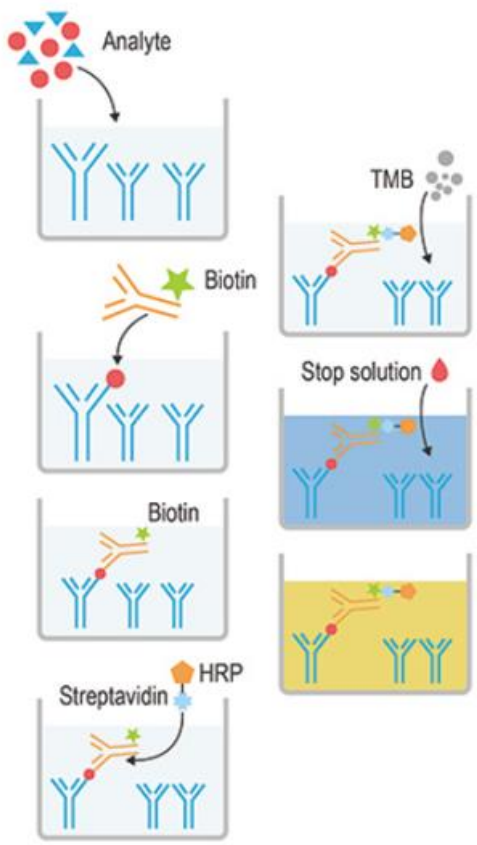

Figure 6. Followed protocol in the measurement of zonulin-1 levels. Image extracted from Elabscience®, retrieved March 29, 2022, from https://www.elabscience.com/p-human_zonulin_elisa_kit-41796.html.

3.3. Measurement of EndoCAb and IFABP levels

Measurement of both EndoCAb and IFABP levels were previously determined by ELISA assays using commercial kits of the HycultBiotech company (29). For the present work, circulating EndoCAb and IFABP concentrations from the 108 patients in the study were included in the database to evaluate their association with circulating zonulin-1 levels.

3.4. Statistical analysis

Statistical analyses were done using the SPSS software, and both Kruskall-Wallis and Mann-Whitney tests were performed to study the significance of the results. Bivariant correlations were also performed as well as a logistic regression. Results were considered to be significant if $p < 0.05$.

4. Results

4.1. Patients' characteristics

Table 1 summarises the characteristics of the patients included in this study. Despite not being statistically significant, it is easy to see a clear dominance of male patients of age around 50 years in all the study groups. In the case of INRs, their age is significantly higher when compared to both controls and IRs ($p = 0.024$ and $p = 0.046$, respectively). In the case of the CD4 cell-count, all values are statistically different from each other ($p < 0.001$ in all cases), but that is not the case in the CD8 cell-count, where only controls showed significantly higher values when compared to both IRs and INRs ($p = 0.006$ and $p < 0.001$, respectively). Finally, viral load was significantly higher in the case of INRs when compared to controls and IRs ($p = 0.010$ and 0.025, respectively). The suspected route of HIV transmission showed no statistical differences among groups.

*Table 1. Summary of patients' characteristics. Homo: homosexual; hetero: heterosexual; ADVP: drug addiction parenterally; hemoder: hemoderivates. * indicates statistical differences compared to control group. ° indicates statistical differences compared to IR group*

		Controls (n=53)	IRs (n=27)	INRs (n=28)
Gender, n (%)		48 (91%)	24 (89%)	24 (86%)
Age		48 [27-89]	48 [28-84]	53 [34-71]*°
CD4 (cells/µL)		348 [201-1159]	128 [6-199]*	59 [2-164]*°
CD8 (cells/µL)		1140 [315-2828]	761 [47-2736]*	488 [46-1347]*
Log Viral Load		5.56 [2.67-6.59]	5.96 [3.07-7.16]	5.55 [3.64-5.95]*°
Route of transmission (%)	Homo.	58	63	32
	Hetero.	40	41	46
	ADVP	8	4	11
	Hemoder.	-	-	4

4.2. Zonulin levels related to baseline low CD4+ T-cell counts (controls vs cases)

As can be seen in Figure 7, baseline zonulin levels were significantly higher ($p < 0.01$) in controls (baseline CD4+ T-cell count > 200cells/µL) compared to cases (baseline CD4+ T-cell count < 200cells/µL). Therefore, our results suggest that lower baseline levels of zonulin can be associated with a low $CD4^+$ T-cell count in naïve HIV-infected patients.

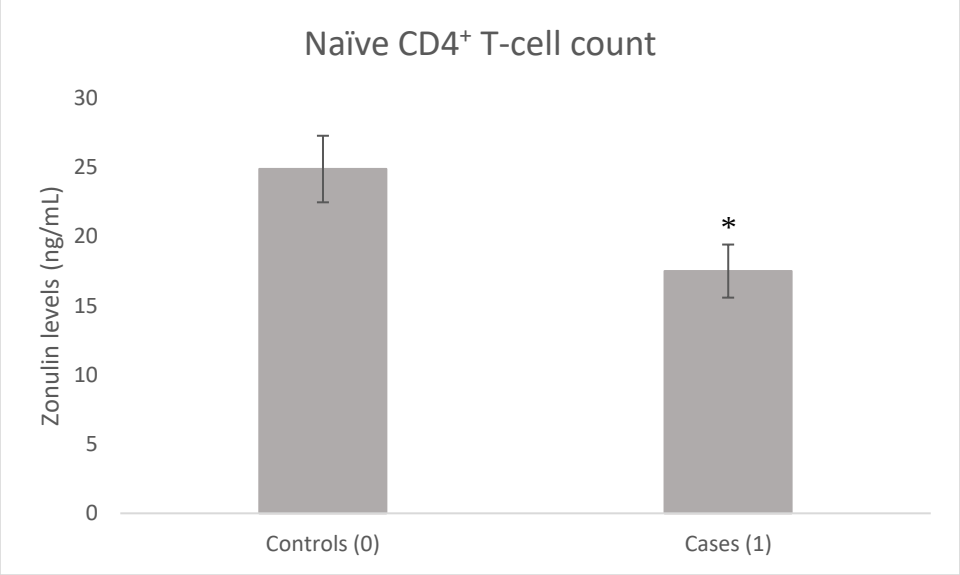

*Figure 7. Comparison of zonulin levels (ng/mL) between controls and cases at the beginning of treatment (baseline values). * refers to statistically significant values related to control values (p = 0.019).*

4.3. Zonulin levels as a potential marker of immunodiscordants' response (IRs vs INRs)

From what can be seen in Figure 8, zonulin levels were significantly higher ($p < 0.01$) in controls (baseline CD4+ T-cell count > 200cells/µL) than in INRs (CD4+ T-cell count < 250cells/µL after 36 weeks of treatment) from samples taken at the beginning of ART (baseline). Furthermore, zonulin levels also seem to be higher when comparing controls to IRs (CD4+ T-cell count > 250cells/µL after 36 weeks of treatment) and IRs to INRs, although statistical tests showed no actual significance ($p = 0.144$ and $p = 0.337$, respectively). These findings suggest a decrease in zonulin levels related to immune response; the worse the progression in the patient, the lower circulating zonulin concentrations.

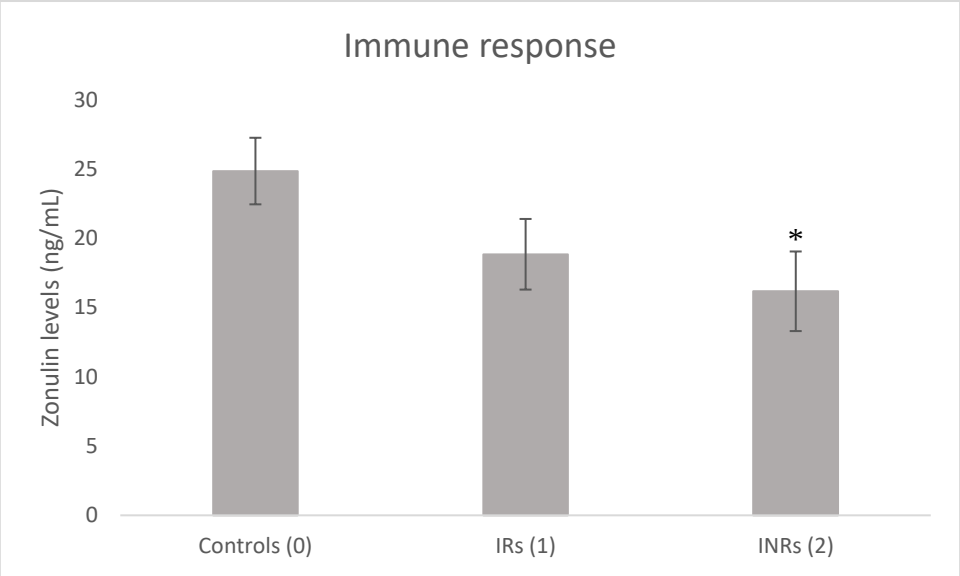

*Figure 8. Comparison of zonulin levels (ng/mL) between controls, IRs, and INRs, at the beginning of treatment. * refers to statistically significant values related to control values (p < 0.01).*

4.4. Biomarkers' correlations

In Table 2, circulating levels of both EndoCAb and IFABP are presented. As previously observed (29), circulating plasma EndoCAb concentrations were lower in INR patients whereas circulating IFABP concentrations were lower in control subjects. The only statistical difference can be found between EndoCAb and IFABP concentrations in the case of controls ($p < 0.001$).

*Table 2. Plasma levels of zonulin-1 (ng/mL), EndoCAb (ng/mL) and IFABP (ng/mL). Values from EndoCAb (ng/mL) and IFABP (ng/mL) were given by the "Immunity and Infection" research group of the "Institut d'Investigació Sanitària Pere Virgili"(IISPV). * indicates statistical differences compared to EndoCAb concentrations.*

	Controls	IRs	INRs
Zonulin (ng/mL)	24.87 ± 2.40	18.85 ± 2.55	16.18 ± 2.87
EndoCAb (ng/mL)	38.68 ± 2.42	38.99 ± 3.26	42.00 ± 6.20
IFABP (ng/mL)	799.86 ± 78.15*	856.30 ± 89.68	1099.38 ± 160.44

4.4.1. Zonulin-EndoCAb

In Figure 9, the correlation between zonulin levels (ng/mL) and EndoCAb levels (ng/mL) expressed as the logarithm of both values can be observed. This correlation is not significant since $p = 0.963$, results which were also previously shown by *Funderburg NT et al* (14).

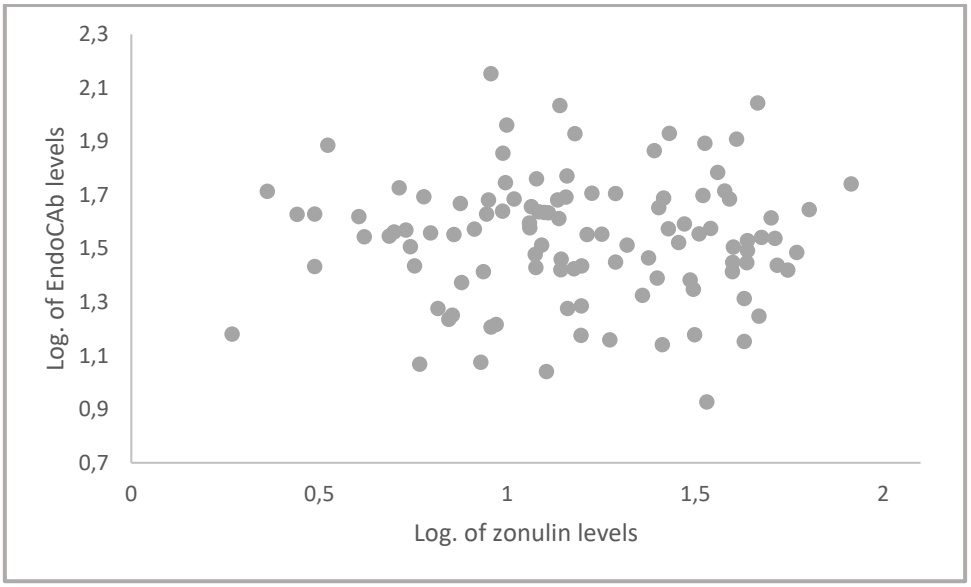

Figure 9. Correlation between the logarithms of zonulin levels (ng/mL) and EndoCAb levels (ng/mL). p = 0.963.

4.4.2. Zonulin-IFABP

In Figure 10, the correlation between zonulin levels (ng/mL) and IFABP levels (ng/mL) expressed as the logarithm of both values can be observed. This correlation is not significant since p = 0.626.

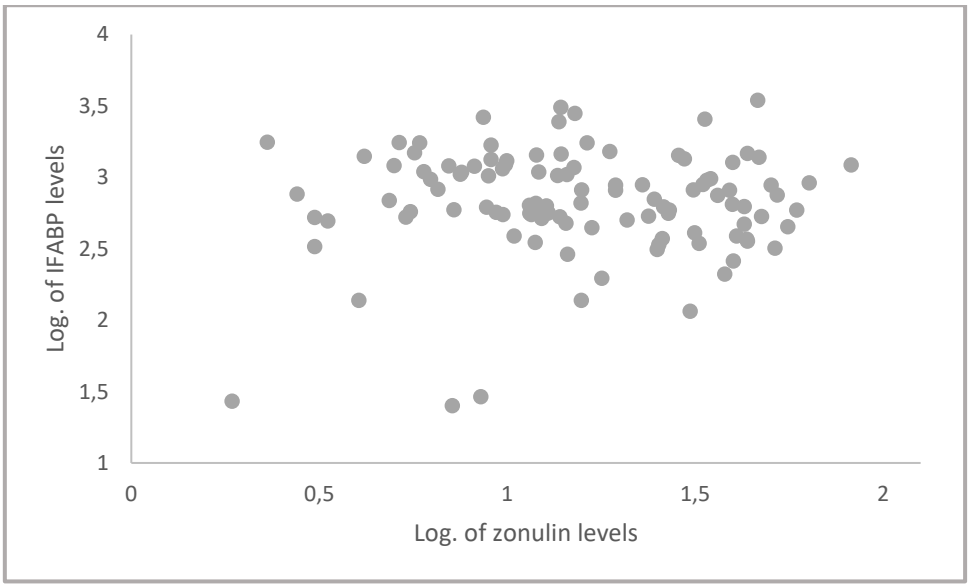

Figure 10. Correlation between the logarithms of zonulin levels (ng/mL) and IFABP levels (ng/mL). p = 0.626.

4.5. Logistic regression

Due to previous results, where there was a significant decrease in zonulin levels in INRs when compared to controls, logistic regression was performed to determine whether circulating zonulin-1 levels affect the probability of having poor immune restoration. In this statistical test, zonulin levels as well as EndoCAb and IFABP levels were included. Results of the logistic regression can be seen in Table 3. As stated by the significance, only zonulin values resulted significative in this case ($p = 0.039$); on the other hand, neither EndoCAb nor IFABP had an impact on the INR phenotype, due to their Odds Ratio being close to 1. Table 3 indicates that a decrease in one unit of zonulin concentration increases the probability of being INR by 1.036.

Table 3. Logistic regression between controls and INRs.

	B	Sig.	Exp(B)
Zonulin	-0.036	0.039	0.965
EndoCAb	0.000	0.979	1.000
IFABP	0.001	0.093	1.001
Constant	-0.491	0.412	0.612

5. Discussion

Patients affected by HIV have increased risks of being affected by non-AIDS related complications like metabolic disorders, among others, which cause significant morbidity and mortality in such patients (16,30). This has led to multiple studies where the relationship between the gastrointestinal tract and HIV-infection is studied. In many cases, a strong component in non-AIDS related complications is chronic inflammation, even in patients under effective ART (16,27,31). Such patients might have undetectable levels of viral molecules, but the virus is still present, which causes a chronic state of immune activation as well as persistent inflammation (32).

Concretely, our results show that zonulin levels were lower in those patients starting ART with worse immunological status (low $CD4^+$ T-cells). Furthermore, zonulin levels decrease the worse the immune response of the patient, being higher in controls than in immunological responders and immunological nonresponders to ART. As stated before, the logistic regression shows that a decrease in one unit of zonulin levels represents an increase of 1.036 in the probability of a patient being an immunological nonresponder. Finally, and even though both EndoCAb and IFABP had been already studied as biological markers of HIV due to their association with the gastrointestinal tract, zonulin showed no significant correlation with either of the two biomarkers.

In the acute phase of HIV infection, there is a massive target of the virus against the gut, causing $CD4^+$ T-cell depletion, which might not recover even after long-term ART, as it is the case of immunological nonresponders (31–33). This will end up affecting tight junctions, where zonulin is a regulator molecule, (31,32). Consequently, a decrease in the circulating zonulin-1 concentrations could be related to a shift in the microbiota composition towards pro-inflammatory and pathogenic species, as well as a release of bacterial compounds in the circulatory system, also known as microbial translocation (20,32,34). In fact, immunological nonresponders showed low EndoCAb concentrations but high IFABP levels compared to controls. Previously reports revealed high IFABP levels related to intestinal damage due to HIV whereas low EndoCAb levels as a consequence of immune cell depletion were associated with immune system activation and clinical progression (29).

Moreover, such processes end up activating chronic inflammation, entering a cyclic stage which only worsens over time (20,32,33). In our work, no significant correlation was found between zonulin-1 and EndoCAb or IFABP concentrations, suggesting that in this subset of patients, tight function damage was not directly related to decreased gut-mucosal integrity (IFABP) nor to LPS release to circulation (EndoCAb).

Zonulin is the only known modulator of tight junctions and is produced only by viable gut epithelial cells, serving as a marker of damage to the intestinal mucosa (14,31). These features make zonulin a potential biomarker of disease progression in HIV-infected patients. In a study by *Funderburg NT et al.*, zonulin, along with IFABP, was a predictive marker of mortality in HIV-infected patients, results which also concur with a study by *Hunt PW et al* (14,21,30). However, information about zonulin and its relationship to immunological nonresponders is still scarce, and only the study by *Wójcik-Cichy K et al.* described that mean levels of zonulin did not differ between HIV-infected patients and healthy controls or between patients with different degrees of CD4 recovery (31).

However, and with zonulin being a regulator of tight junctions, it is possible to hypothesise with other studies which relate both adherent junctions (AJ) and tight junctions (TJ) with immunological recuperation. In a study by *Tincati C. et al.*, INRs had a wider intercellular space in the colon tissue when compared to immunological responders, which was due to severe damage in both tight junctions (TJ) and adherent junctions (AJ). Furthermore, the extent of the epithelial barrier damage in the colon was also much larger in INRs when compared to both full responders and non-infected patients (35). In our results, zonulin levels were significantly lower in INRs when compared to controls. This could mean that lower zonulin levels would relate to dysregulation in tight junctions which might cause enlargement of the intercellular space, as previously related to immunological nonresponders (35). Consequently, these findings go along with results found in other publications, where it has been reported that zonulin can have a protective action against HIV infection (13).

Finally zonulin levels in IRs also showed lower values when compared to controls, despite not showing actual significance. Since IRs started ART with low $CD4^+$ T-cell count, the poor immune system could be related to gastrointestinal tract damage in HIV patients, even before starting ART therapy. Even though IRs have a less wide intercellular space in the colon tissue when compared to INRs (35), gastrointestinal tract damage is still present and might not be fully reversed by ART (33).

6. Conclusions

Not all patients infected with HIV have the same response to ART. Immunological nonresponders show less response to treatment and do not restore physiological $CD4^+$ T-cell levels. Therefore, it is essential to detect patients in this group to further monitor them, due to their higher tendency of developing non-AIDS complications.

Since one of the first targeted sites upon HIV infection is the gastrointestinal tract, many different studies have tried to find biomarkers that would corelate damage in the gut with the progression of the HIV or with how ART affects patients. In this project, zonulin has been proposed as a biomarker to distinguish INRs from IRs. Despite not having statistically significant correlations with other markers of gut epithelial damage such as IFABP or EndoCAb, zonulin levels are significantly lower in INRs when compared to controls, and lower yet not statistically significant when compared to IRs. It is expected, though, that a higher cohort of studies could allow further studies to obtain statistically significant results when comparing INRs and IRs.

In conclusion, in INR subjects there is an important dysregulation of intestinal mucosal barrier function, and these findings lead us to believe that zonulin can be a powerful biomarker of INRs, which might be further used in combination with other yet-to-be-known biomarkers of gut epithelial damage.

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