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EPIVINF

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EPIVINF

Epigenetic regulation of host factors in viral infections

# D5.1 Result summary on Sirtuins abundancy and functionality in HIV and SARS-CoV-2 infected patients

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# 1. INTRODUCTION

The **EPIVINF** project aims to expand our knowledge of the role that epigenetics plays in viral infections and how epigenetic signatures predispose the host's individual response to the infection in terms of disease evolution, prognosis, risks factors, co-infections, and treatment. The objective of WP5 is to validate epigenetic signatures observed in HIV and SARS-CoV-2 infection using in vitro experimentation and animal models in order to advance identified lead candidates towards therapeutic use in personalized treatment strategies. The WP will test host factors identified in WP2-4 and which are involved in the epigenetic regulation of the antiviral immunity and implicated in HIV and SARS-CoV-2 related neurodysfunction. The work in WP5 will use in vitro and in vivo models to explore Epidrug-based strategies for the development of new immunotherapies and antiviral drugs to reduce disease burden of these infections.

# 2. DELIVERABLE. EVALUATION OF SIRTUINS

## 2.1 Purpose and scope of the document

This deliverable outlines the evaluation of Sirtuins as key modifiers of dysregulated epigenetic control of antiviral host factors, as part of Task 5.1 of the EPIVINF project. Sirtuins, a family of NAD+-dependent histone deacetylases, have been increasingly recognized for their role in regulating immune responses and antiviral defence mechanisms through epigenetic modulation. By focusing on these molecules, the deliverable serves as an essential step in establishing the technical groundwork for the broader validation studies envisioned in WP5.

The purpose of this document is to:

a) Detail the methodology and experimental setup employed to investigate Sirtuins abundancy and enzymatic activity in HIV and SARS-CoV-2 infections.
b) Provide initial findings that serve as a proof-of-concept for utilizing Sirtuins as therapeutic targets, supporting the broader aim of advancing epigenetic-based strategies for personalized treatment approaches.
c) Facilitate the subsequent validation and exploration of other lead candidates emerging from WP2-4, ensuring their seamless integration into the in vitro and in vivo experimental pipelines.

The scope of this deliverable encompasses:

a) The quantification of Sirtuins and its enzymatic effects in HIV and SARS-CoV-2 infections.
b) The development of quantitative assays, tools, and animal models required to test Sirtuins as therapeutic targets, ensuring reproducibility and scalability for future studies.
c) A framework for evaluating Epidrug-based strategies, new immunotherapies, and antiviral drugs that leverage Sirtuin-modulated pathways to reduce the disease burden of these infections.

By presenting the findings and methodologies related to Sirtuins quantification as an initial lead candidate, this document provides a foundational reference for future studies aimed at expanding the repertoire of epigenetic targets investigated in WP5. It also underscores the importance of refining and validating experimental approaches to maximize the translational potential of epigenetic discoveries made in earlier work packages.

# 3. SIRTUINS

Epigenetic regulation encompasses a wide range of mechanisms, from direct DNA and histone modifications that control transcription levels to interactions with messenger RNAs that regulate translation. Among these, histone deacetylases (HDACs) play a central role in modulating chromatin structure and transcription. HDACs are targets of various drugs with applications across multiple disease contexts, including cancers and HIV infection.

In a recent clinical trial conducted by partner IRSI, HDAC inhibitors such as romidepsin demonstrated potential not only to modulate viral gene expression but also to alter host transcriptional programs in immune cells, impacting antiviral host gene expression. These findings highlight a broader impact of HDACis on the host epigenome, including the potential regulation of members of the HDAC class III family—commonly known as Sirtuins. Sirtuins are NAD+-dependent deacetylases that serve as epigenetic regulators, and emerging evidence suggests that their expression may itself be modulated by viral infections through epigenetic mechanisms, not only providing an interesting field of study but also offering opportunities to therapeutically target such infections by epidrug based interventions.

Our observations indicate that viral infections can trigger epigenetic changes that extend to host proteins, such as Sirtuins, which are directly involved in downstream regulation of other host factors critical for antiviral defence and disease progression. Notably, Sirtuin 2 (SIRT2), a member of the Sirtuin family, has demonstrated strong effects on HIV control in our studies. Beyond its role in viral regulation, SIRT2 has also been implicated in the progression of neurodegenerative diseases such as Alzheimer's Disease and aging, underscoring the broader biological significance of its epigenetic regulation and its direct effect on host gene expression.

Understanding the players and mechanisms involved in these epigenetic cascades will be essential to fully grasp the impact of epigenetic dysregulation in viral infections. Such insights will also help identify targetable factors within these cascades that can restore "healthy" epigenetic signatures of key antiviral host factors, particularly those involved in immune responses.

In WP5, we will focus on host factors identified in WP2-4, with a specific emphasis on effector molecules involved in epigenetic pathways, including those whose expression is under epigenetic control. Preliminary data from SARS-CoV-2 studies suggest that Sirtuins play a critical role in the immune response to infection and are associated with disease severity during acute stages of COVID-19. Furthermore, our findings suggest that Sirtuins, particularly SIRT1 and SIRT2, are centrally involved in the control of both HIV and SARS-CoV-2 infections, as well as in neurofunction associated with disease progression.

In Task 5.1, we aim to determine the abundance and activity of SIRT1 and SIRT2 across various stages of these infectious diseases, spanning from acute infection to chronic neurological dysfunction. This task will guide the design and validation of experimental approaches to evaluate Sirtuins as potential targets for epidrug interventions. While the Sirtuin family represents a promising candidate for such interventions, additional host factors identified in WP2-4 may demonstrate stronger associations with disease progression and could be prioritized for therapeutic development.

Given the growing body of evidence on the roles of SIRT1 and SIRT2 in HIV and SARS-CoV-2 infections, this deliverable will focus on exploring their functional relevance and therapeutic potential. These findings will contribute to the broader goal of Task 5.1 to validate epigenetic targets and develop strategies for restoring effective antiviral immunity.

## 3.1 SIRTUIN ABUNDANCY

Sirtuins, as key regulators of gene activity through their histone deacetylase function, have the potential to drive downstream epigenetic cascades that may lead to impaired pathogen control and disease progression. Investigating their abundance in infected individuals is therefore critical to understanding their role in host-pathogen interactions and their potential as therapeutic targets.

Plasma levels of sirtuins can serve as biomarkers for understanding systemic responses in HIV and SARS-COV-2 infections. This measurement will help to:

>Evaluate the extent of systemic immune activation and inflammation in individuals with HIV.

> Correlate circulating Sirtuin levels with disease progression, viral load, and immune dysfunction.

>Investigate their relationship with HIV-associated comorbidities, such as neurologic dysfunction.

To assess Sirtuin abundance, we focused on SIRT1 and SIRT2, two members of the Sirtuin family that have shown significant relevance in the context of HIV and SARS-CoV-2 infections.

We quantified their levels in plasma using biospecimens from individuals across different stages of HIV and SARS-CoV-2 infections. For absolute quantification of Sirtuins proteins, we have employed commercially available ELISA kits and Olink® proximity extension assays. Our analysis revealed distinct patterns of SIRT1 and SIRT2 abundance in HIV and SARS-CoV-2 infections, which were stratified by disease stages:

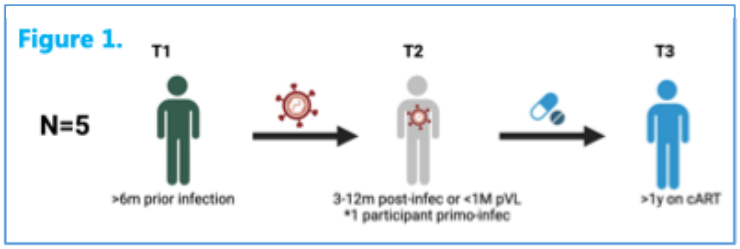
### 3.1.1 HIV INFECTION

In the context of HIV infection, Sirtuins have been implicated in several key processes:

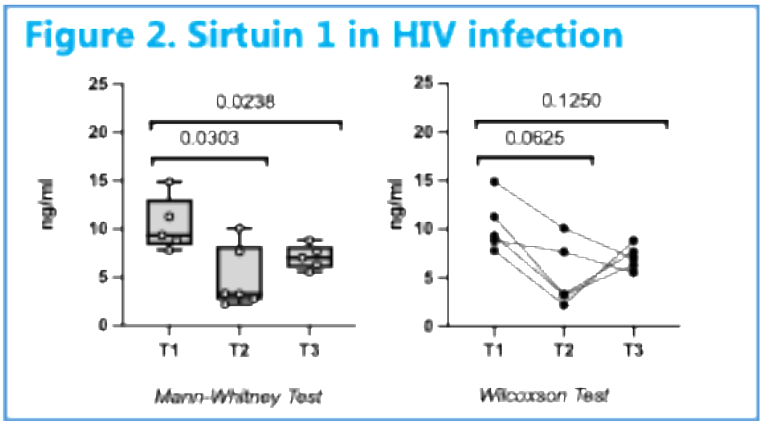
a) Viral Replication and Transcription: SIRT1 modulates the acetylation status of transcription factors, including NF-κB and p53, which are critical in HIV transcription and latency.
b) Immune Dysregulation: Both SIRT1 and SIRT2 are involved in modulating inflammation and immune responses. Dysregulation of these pathways is a hallmark of chronic HIV infection and contributes to immune activation and exhaustion.
c) Host-Pathogen Interactions: SIRT1 influences the HIV reservoir and latency reactivation, while SIRT2 may play a direct role in cell cycle regulation and infection dynamics in specific cell types, in particular macrophages and CD4+ T cells.

#### 3.1.1.1 HIV INFECTION COURSE

To evaluate the longitudinal expression and abundancy of Sirtuins, a pilot study was performed in longitudinally stored samples from 5 individuals (Figure 1). The samples from 5 seronegative individuals at high risk of infection (CHECKEAR Cohort) were used for pre-infection timepoints (>6 months prior infection). Then, a second timepoint sample was used to evaluate sirtuins levels at acute stages of infection and in the absence of treatment (<6months after infection). Finally, a to evaluate the impact of ART treatment onthe expression of sirtuins, a third timepoint was included (>1 year on ART treatment).

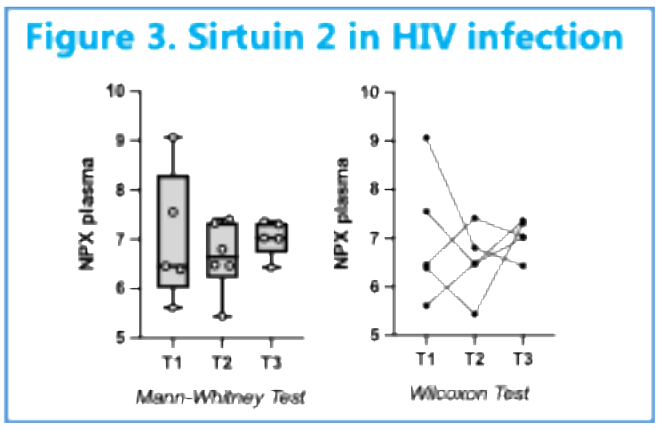



***SIRTUIN 1:*** The abundancy of Sirtuin 1 was evaluated using commercially available ELISA kit. Despite the low number of participants tested, a statistically significant reduction of Sirt1 abundancy in acute phase of HIV infection was detected (Figure 2). Moreover, once treatment was administered, the levels of the protein tend to be partially restored.




In healthy individuals, SIRT1 plays a homeostatic role in regulating immune responses, inflammation, and cellular metabolism. Plasma SIRT1 levels are stable and associated with a balanced immune system. SIRT1 maintains low levels of inflammation by deacetylating NF-κB and other pro-inflammatory mediators and also supports efficient mitochondrial function, redox balance, and autophagy. Acute HIV infection is characterized by a massive peak in viremia, immune activation, and cytokine production. This leads to significant immune and metabolic dysregulation. The decreased SIRT1 plasma levels (or "activity") observed can be associated with HIV-induced inflammation and oxidative stress that may suppress SIRT1 expression. This is thought to be due to NAD+ depletion, which is required for SIRT1 activity, as metabolic dysregulation and inflammation reduce NAD+ availability. Another explanation could be that HIV proteins (e.g., Tat) may directly interfere with SIRT1 activity, enhancing viral transcription by preventing SIRT1-mediated repression of the HIV LTR. However, in some immune cell subsets (e.g., macrophages or CD4+ T cells), SIRT1 may be upregulated as a protective response to oxidative stress or inflammation. Finally, ART administration effectively suppresses viral replication, reducing systemic inflammation and immune activation, but does not fully restore immune homeostasis or eliminate latent reservoirs. In line with this, our data show only partial restoration of SIRT1 levels after 1 year on ART. Although by then, ART has reduced inflammation and immune activation, plasma SIRT1 levels not be back to normal as chronic immune dysregulation and persistent underlying inflammation may prevent full restoration. Additionally, residual reservoirs and low-level viral transcription might continue to modulate SIRT1 expression in certain immune cells.

***SIRTUIN 2:*** Our earlier studies in chronically HIV infected individuals on and off ART suggested that SIRT2 levels may be considerably lower than SIRT1 in the plasma. Thus, the differential protein levels in the course of HIV disease were determined by Olink® (proximity extension assay) technique. No differences were observed between timepoints, mainly due to the high variability of relative protein abundancies between participants and across longitudinal timepoints (Figure 3).



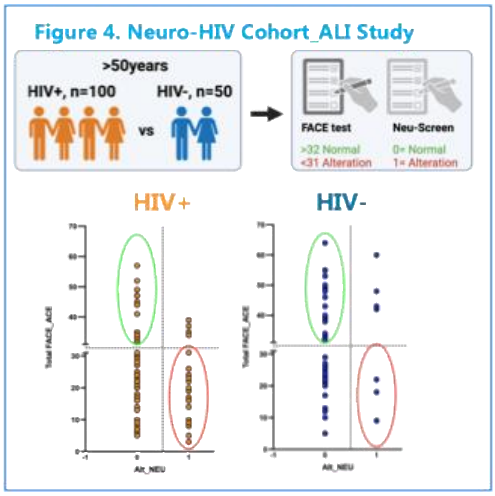

SIRT2, a cytoplasmic deacetylase, plays critical roles in cellular metabolism, oxidative stress response, and cell cycle regulation. Its functions differ from SIRT1, but it also intersects with pathways relevant to HIV infection, including immune activation, viral replication, and latency. SIRT2 is expressed in a wide range of tissues, particularly in metabolically active cells and regulates cytoplasmic and mitochondrial homeostasis, cell cycle progression, and inflammation. Given that SIRT2 suppresses inflammatory pathways, such as TNF-α and IL-1β production, by deacetylating transcription factors like NF-κB and maintains redox balance, mitochondrial function, and glucose metabolism, a decreased SIRT2 level or activity was expected during acute infection. The inflammatory milieu of acute HIV infection, characterized by a cytokine storm, likely suppresses SIRT2 activity. This could result from oxidative stress or immune activation disrupting SIRT2's regulatory functions. Also, HIV-mediated NAD+ depletion may impair SIRT2's deacetylase activity, similar to SIRT1. Finally, near-normal plasma levels would be expected, however, chronic low-grade inflammation and ART-related effects may prevent complete normalization, particularly in tissues affected by HIV

reservoirs or ART toxicity. The inflammatory milieu of acute HIV infection, characterized by a cytokine storm, likely suppresses SIRT2 activity. This could result from oxidative stress or immune activation disrupting SIRT2's regulatory functions.

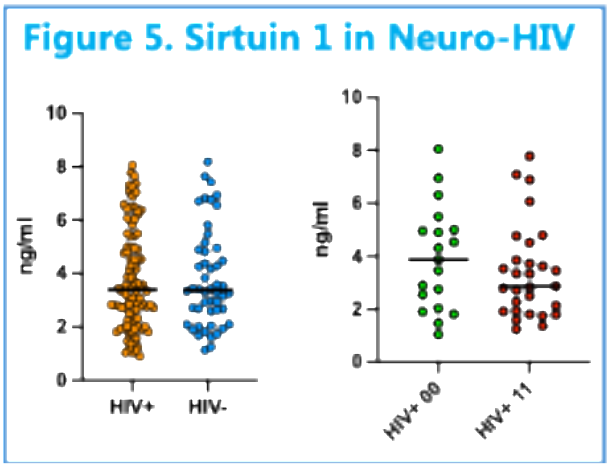
Overall, to confirm these hypotheses and determine SIRT1 and SIRT2 profiles in plasma, larger number of participants will need to be evaluated. Given the unexpectedly high inter-patient variability, especially for SIRT2, and to reach the statistical power of such analyses, we have established collaborations with an acute infection cohort in Lima, Peru (SABES cohort), where more than 200 new infections were diagnosed in individuals who were also sampled prior to infection. Sample availability is limited but we are conducting feasibility analyses on both, plasma and cell pellets to conduct scientifically sound analyses.

#### 3.1.1.2 NEUROLOGICAL CONSEQUENCES

HIV infection significantly impacts the central nervous system (CNS), leading to neurological complications such as HIV-associated neurocognitive disorders (HAND), neuroinflammation, oxidative stress, and neurodegeneration. Sirtuins (SIRT1–SIRT7) are involved in neuroprotection, metabolism, and inflammation, making them critical in understanding how HIV infection and antiretroviral therapy (ART) affect neurological outcomes. Here, we have explored the levels of sirtuins in samples from the ALI Study (Figure 4). The ALI Study explored pre-clinical symptomatology suggestive of Alzheimer Disease and neurocognitive dysfunction through a comprehensive test evaluations and biological markers testing (pTau, Nfl, BDNf) in ART treated HIV infected individuals and age- and sex-matched HIV negative individuals of more than 50 years of age.

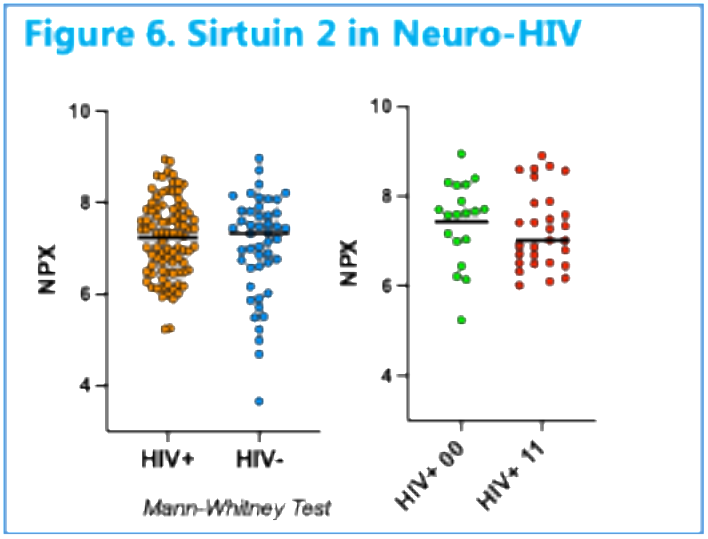



***SIRTUIN 1:*** SIRT1 plays a pivotal role in counteracting the neurodysfunction associated with HIV by regulating inflammation, oxidative stress, and neuronal survival. When quantifying the absolute plasma levels of Sirt1 in ALI Study participants, we did however not detecte differences between HIV infected participants and HIV uninfected, probably due to the partial homeostatic restoration of plasma viral load and inflammation due to ART treatment (Figure 5, 00=No alteration, 11=Alteration). However, Sirt1 tended to be reduced in HIV infected individuals with worse neurological status in ALI Study (Figure 5, right hand panel). Given this inside and based on the literature is tempting to speculate on potential mechanisms explaining a reduction of SIRT1 in individuals with neurological complains: In brief, HIV proteins such as Tat and gp120 suppress SIRT1 expression in neurons and glial cells, compromising its neuroprotective functions and exacerbating inflammation, while Tat directly inhibits SIRT1 activity, worsening neuronal damage. Similarly, it has been observed that persistent oxidative stress further depletes NAD+, a critical co-factor for SIRT1, suppressing its activity. Importantly, a reduction on SIRT1 levels facilitates the activation of microglia, leading to the release of neurotoxic cytokines like TNF-α and IL-1β, which damage neurons, while astrocyte dysfunction



impairs their neuroprotective role, amplifying neuroinflammation. Moreover, mitochondrial dysfunction also ensues, as decreased SIRT1 levels hinder mitochondrial biogenesis, exacerbate energy deficits, and increase susceptibility to oxidative damage, further promoting neurodegeneration. Additionally, SIRT1 dysfunction disrupts the expression of genes critical for synaptic plasticity, leading to cognitive and memory deficits, while HIV-associated neurotoxins worsen neuronal and synaptic damage. These combined effects highlight the central role of impaired SIRT1 activity in the progression of HAND.

***SIRTUIN2:*** SIRT2, also plays essential roles in neuroprotection, cytoskeletal regulation, inflammation, and oxidative stress and its dysregulation also has profound implications for the central nervous system (CNS) in the context of HIV infection. Similarly to Sirt1 expression levels, in ALI Study there was not statistical differences in relative SIRT2 levels measured by Olink ® between HIV infected and uninfected groups (Figure 6). However, there was a trend towards reduced levels of SIRT2 in HIV infected individuals who showed cognitive alterations compared with those that performed well in cognitive evaluations (Figure 6, 00=No alteration, 11=Alteration). As SIRT2 plays a significant role in maintaining neuronal health and regulating neuroinflammation, and its dysfunction may be implicated in the neurodysfunction associated with HIV. During HIV infection, chronic inflammation and oxidative stress, exacerbated by viral proteins such as Tat and gp120, disrupt SIRT2 activity. This disruption contributes to cytoskeletal instability, as SIRT2 is essential for deacetylating tubulin and maintaining axonal integrity. Additionally, SIRT2 dysfunction in microglia and astrocytes amplifies neuroinflammation by allowing the unchecked release of neurotoxic cytokines, such as TNF-α and IL-1β, which further damage neurons. The loss of SIRT2's regulatory role in oxidative stress exacerbates mitochondrial dysfunction and neuronal injury, while impaired cytoskeletal repair mechanisms contribute to synaptic and cognitive deficits observed in HIV-associated neurocognitive disorders (HAND).




As a conclusion, to confirm these suggestions and the SIRT1 and SIRT2 levels associated with neurological changes, higher number of participants will be evaluated for plasma levels. Also, CSF plasma levels will be explored with cohorts from OSR and USAAR partners.

### 3.1.2 COVID

In the context of COVID-19 infection, sirtuins have been implicated in several critical processes:

a) SIRT1 and SIRT2 regulate key host factors involved in viral replication and transcription. SIRT1 modulates the acetylation status of transcription factors like NF-κB and p53, which are crucial for the inflammatory response and potentially influence SARS-CoV-2 replication. SIRT2, through its role in deacetylating tubulin and regulating the cytoskeleton, may impact intracellular viral trafficking and assembly.

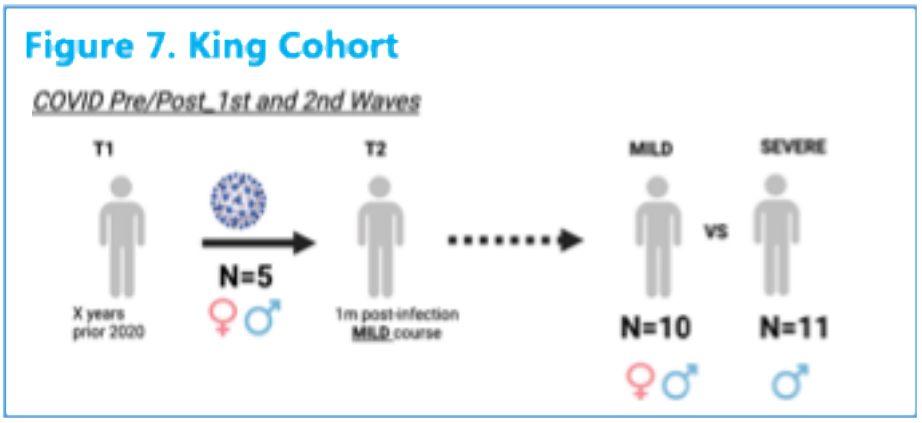
b) Both SIRT1 and SIRT2 are essential in modulating inflammation and immune responses, which are profoundly dysregulated in severe COVID-19. SIRT1's anti-inflammatory effects, mediated by the deacetylation of NF-κB, may be suppressed during the cytokine storm, allowing for hyperinflammation. Similarly, SIRT2's

involvement in regulating oxidative stress and microglial activation links it to the neuroinflammatory milieu observed in COVID-19-associated neurological dysfunction.

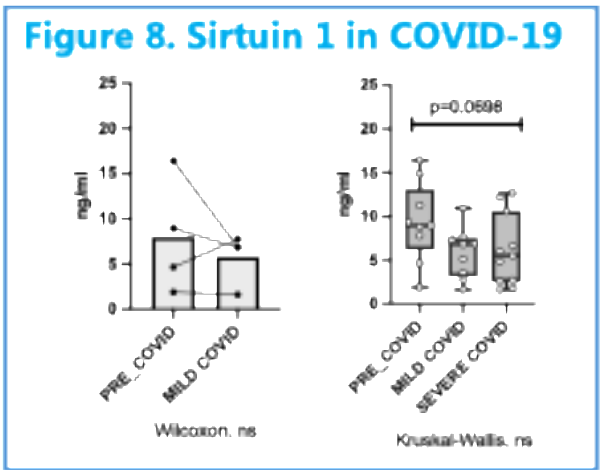
c) SIRT1 influences the cellular response to SARS-CoV-2 infection by modulating metabolic pathways and mitochondrial function, which are essential for viral replication and host cell survival. SIRT2, through its role in cell cycle regulation, may impact infection dynamics in specific cell types, such as macrophages and epithelial cells, which are targets of SARS-CoV-2.

#### 3.1.2.1 NATURAL COURSE: ACUTE PHASE MILD

To evaluate the longitudinal expression and abundance of Sirtuins and its relationship to COVID-19 disease, we first conducted a study on longitudinally stored samples from five individuals that included sampling time points before the emergence of the COVD19 pandemic (2020) and one month after infection with SARS-COV-2, which in all the 5 tested individuals showed a mild course of the disease (Figure 7). Additionally, a cross-sectional study was conducted, using stored samples from five individuals (PRE-COVID, n=10) and five more individuals with a mild course of the disease (MILD group, n=10), as well as samples from participants who experienced a severe course of the disease (SEVERE group, n=11) to ascertain sirtuin levels in relation to disease severity (Figure 7).




***SIRTUIN 1:*** As with the HIV infection study, the abundance of Sirtuin 1 was evaluated using a commercially available ELISA kit. In the longitudinal analysis, one of the five participants showed undetectable levels at the Pre-COVID timepoint, reducing the number of participants available for statistical analyses Figure 8).

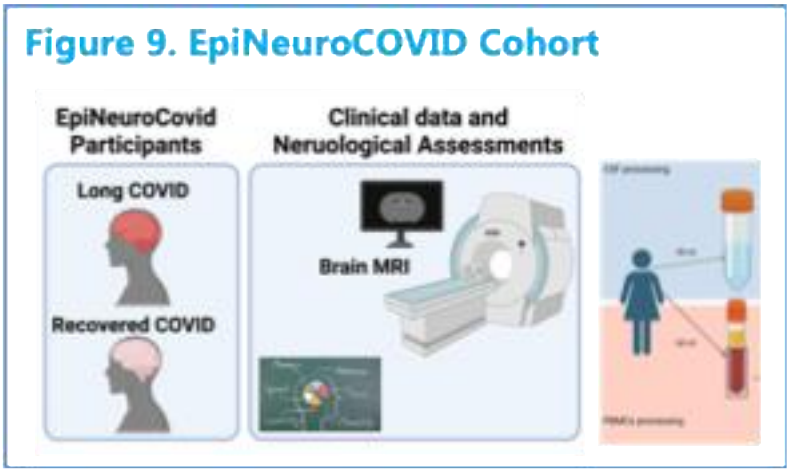



A number of studies have indicated that SIRT1 plasma levels may differ between non-COVID individuals, those with mild COVID-19, and those with severe COVID-19. In non-COVID individuals, SIRT1 levels are typically stable, reflecting a balanced state of cellular regulation and minimal systemic inflammation. In contrast, among patients with a mild course of COVID-19, it has been reported that SIRT1 levels are slightly elevated, which likely reflects the body's initial immune response to infection and the associated mild inflammation suggesting a role for SIRT1 in mitigating excessive inflammatory responses and promoting cellular homeostasis. On the toher hand, in patients with severe COVID-19 infection, an significant decrease in SIRT1 plasma levels has been reported. This reduction is thought to contribute to the dysregulated immune response and heightened inflammatory state observed in severe cases.

SIRT1 is known for its anti-inflammatory and antioxidative properties, and its deficiency can exacerbate cytokine storms and oxidative stress, leading to severe tissue damage and poor clinical outcomes. Our comparison shows indeed a reduced SIRT1 levels in more severe COVID-19, however without reaching statistical significance, possibly due to statistical power limitations.

#### 3.1.2.2 NEUROLOGICAL CONSEQUENCES

Recent studies indicate that both SIRT1 and SIRT2 are involved in LongCOVID conditions, which is characterised by the persistence of symptoms following a SARS-CoV-2 infection. As stated above, SIRT1 is recognised for its anti-inflammatory and antioxidative properties, and its levels are frequently diminished in severe cases of LongCOVID, potentially contributing to the chronic inflammation and immune dysregulation observed in this condition. SIRT2 is involved in cellular stress responses and mitochondrial function. Dysregulation of SIRT2 may also contribute to the ongoing symptoms and mitochondrial dysfunction observed in LongCOVID patients. To study the differential levels of both proteins in Long-COVID participants, we have access to samples from the EPINEURO study which is conducted in our labs and which has enrolled during 2024 more than 40 participants, including a group of individuals who have recovered from SARS-CoV-2 infection without longitudinal sequalae (ReCov) and Long-COVID patients with neurocognitive impairment (LCNI). All of these participants have been screened for different neurological assessments, including brain MRI and large volume (30ml) of CSF samples have been collected from the LCNI group (Figure 9). The study is ongoing and proteomics analyses will employ ELISA and Olink testing methods to measure the levels of SIRT1 and SIRT2 in Long-COVID disease, providing valuable insights into the role of these proteins in the disease process.




# 4. CONCLUSION

Due to a reduced number of participants and the sensitivity required for detecting low sirtuin levels, there has been a delay in the deliverable for measuring SIRT1 and SIRT2 levels in HIV and COVID-19 patients. The lower-than-expected participant numbers have impacted our ability to obtain statistically significant data. Additionally, the difficulty in assessing enzymatic activity measurements due to low levels of detection has further complicated our efforts. The advanced techniques needed to accurately measure sirtuin levels demand additional time to ensure reliability and precision. We are committed to delivering high-quality results and are working diligently to overcome these challenges by i) increasing the number of participants and including measurements in CSF samples (SABES and EPINEURO cohorts, respectively), ii) customizing high-sensitivity tests (MesoScale Discovery technique) for Sirtuin 2, iii) measuring enzymatic activity in samples with higher levels of proteins and including cell lysate measurements.

As stated in the original proposal for the project, Sirtuins have emerged from our earlier work as potential candidates for epigenetic therapeutic intervention but emerging data from the work in EPIVINF may prioritize other molecular targets. Thus, based on our experience with measuring sirtuin molecules, we will extend this

expertise to validate other lead candidates derived from WP2-4, while also working on increasing the number of participants involved in these measurements.