

Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Unravelling viral epitranscriptomics during SARS-CoV-2 infection

B.1.2 Abstract

The RNA modification N⁶-methyladenosine (m⁶A) plays an important role in processes related to gene regulation, such as embryonic development, metabolic diseases, and cancer. Recently, it has been shown that m⁶A modifications on the RNA transcripts of more than 15 viruses can influence viral replication. Although various aspects of SARS-CoV-2 have been thoroughly studied, little is known about the molecular mechanisms underlying the interaction between the host and viral RNAs. Two studies showed that m⁶A levels in COVID-19 patients are correlated with disease severity.

Previous methods to identify m⁶A modifications are biased and laborious. Fortunately, direct RNA sequencing (DRS) can be used to recognise m⁶A modifications more precisely and more efficiently. We propose to study the role of m⁶A RNA modifications in the host-virus interaction during SARS-CoV-2 infection.

First, the DRS method will be improved and validated for the measurement of m⁶A modifications in context of SARS-CoV-2. Second, we will identify m⁶A modifications with DRS on RNA transcripts in samples of a) human cell lines, b) human organoids, and c) patient-derived samples to find associations between m⁶A modifications and cell types, time after infection, the viral replication rate, and COVID-19 severity. Third, we will look closely at the influence of m⁶A modification levels on the SARS-CoV-2 replication rate, by administrating drugs that alter m⁶A levels. Additionally, antiviral drugs will be tested to determine whether they influence m⁶A levels.

Taken together, this research will provide insights in the role of epitranscriptomics during viral infection and could provide targets for antiviral therapeutics.

B.1.3 Layman's summary

RNA molecules fulfil essential roles in all living cells. Chemical modifications of RNA are important in the regulation of processes underlying embryonic development, metabolic diseases and cancer (Batista 2017). These modifications are generally referred to as "The Epitranscriptome". Recently, it has been shown that one of these RNA mutations, called m⁶A, is also widely present on the RNA transcripts of more than 15 viruses. This modification can either increase or decrease the viral replication rate, dependent on the virus type and the location of the modification (Kennedy et al. 2017). Viral epitranscriptomics is the study of RNA modifications during viral infection.

Identifying these m⁶A modifications was previously laborious, prone to bias, and results were of limited resolution (H. Liu et al. 2019). Fortunately, a new, elegant technique was proven to be efficient, quick and precise in the identification of m⁶A modifications, called direct RNA sequencing (DRS) on nanopore sequencers.

The COVID-19 pandemic, which has caused over 5.2 million deaths since 2019, has boosted an enormous research effort into SARS-CoV-2 (WHO 2021b). This research has mostly focused on the topics such as a) the identification of the virus, b) how the virus replicates and spreads, c) how COVID-19 can be treated, and d) vaccine development. Although the viral epitranscriptome of SARS-CoV-2 could be relevant in all these topics, it has only been investigated in a handful of studies. Since there is some evidence that certain m⁶A modifications are associated with more severe COVID-19 in patients (Meng et al. 2021), it is essential to further understand the role of m⁶A RNA modifications in the host-virus interaction during SARS-CoV-2 infection.

Therefore, we propose to study m⁶A modifications, with the cutting-edge technology of direct RNA sequencing.

First, we will optimise and validate the DRS method on SARS-CoV-2 samples and on human infected cells, so that this technique can be used in an efficient high throughput manner on many samples.

Secondly, we will identify m⁶A modifications in context of SARS-CoV-2 infection in a) human cell lines, b) human organoids, and c) patient-derived samples. In order to get a big picture of m⁶A during infection, we will identify these modifications on different time points after infection, on different cell types, and in samples of patients with different COVID-19 severity (asymptomatic, mild, severe, death, and long-COVID). This will create a large data set of m⁶A modifications, which will be used to identify associations between m⁶A and various aspects of SARS-CoV-2 infection.

Thirdly, we will be looking more closely at the influence of m⁶A modification levels on the SARS-CoV-2 replication. Therefore, we will either increase or decrease m⁶A levels in human cells, and see how the viral replication changes after this. Additionally, we will be testing some of the currently used antiviral drugs, that show some benefits in severely affected patients, to see whether the clinical benefit can be partially explained by changes in m⁶A levels.

This study will provide more insights in the SARS-CoV-2 epitranscriptome, which could benefit the development of antiviral therapies in the future.

B.1.4 Keywords

Epitranscriptomics, SARS-CoV-2, N⁶-methyladenosine (m⁶A), direct RNA sequencing

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic (What)

a) Background

Similarly to the epigenetic code found on DNA molecules, RNA molecules can also be subjected to internal base modifications. Epitranscriptomics is the study of these internal base modifications on RNA transcripts. A wide range of RNA modifications has been identified, with the addition of a methyl group to the N⁶-position of adenosine (m⁶A) being the most abundant mRNA modification (Batista 2017). The methyl group is placed on the adenosine molecule by so called writer proteins. In mammalian cells, this step is performed by a heterodimer of the enzymes METTL3 and METTL14 (J. Liu et al. 2014). Once this methyl group has been added to the adenosine, the m⁶A RNA modification can be recognised by reader proteins YTHDC1-2 in the nucleus (Xiao et al. 2016) and by reader proteins YTHDF1-3 in the cytoplasm (A. Li et al. 2017; Wang et al. 2014, 2015). The methyl group can be removed from the adenosine nucleotide by the eraser proteins ALKBH5 (Zheng et al. 2013) and FTO (Bartosovic et al. 2017; Jia et al. 2011).

m⁶A has been shown to play an important role in RNA transcript processing by influencing splicing (Kasowitz et al. 2018; Xiao et al. 2016), translation (Meyer et al. 2015; Zhao, Roundtree, and He 2017), and RNA stability (Spitale et al. 2015). Thus, it is no surprise that the m⁶A modification is relevant in various biological processes that are dependent on highly regulated gene expression, such as embryonic development (Kasowitz et al. 2018), metabolic diseases (Batista 2017; Wu et al. 2017), and cancer (Cui et al. 2017; He et al. 2019).

In recent years, m⁶A modifications on viral RNA molecules have been studied and it has been shown that m⁶A is involved in the viral replication cycle. HIV-1 has been used as a model virus in the field of viral epitranscriptomics, and multiple studies indicate the complex spatiotemporal effect of m⁶A modifications on HIV-1 replication and its viral particle production. Dependent on the position of the m⁶A modification on the transcript, the location in the cell, and the time after infection, m⁶A is either positively or negatively associated with viral replication (Kennedy et al. 2016; Lichinchi, Gao, et al. 2016; Lu et al. 2018; Tirumuru et al. 2016). m⁶A modifications have also been shown to increase or decrease viral replication and viral particle production in over 15 other viruses (Kennedy et al. 2017). For example, in Hepatitis C Virus (HCV) and in Zika Virus (ZIKV) m⁶A negatively regulates the viral infection by reducing viral particle production in the infected cells (Lichinchi, Zhao, et al. 2016; Mcintyre et al. 2016). On the contrary, in Influenza A Virus (IAV), m⁶A residues enhanced viral gene expression and pathogenicity in mice (Courtney et al. 2017). The situation is more complex in the Hepatitis B Virus (HBV), as the position of the m⁶A modification on the viral transcript influences the RNA stability and reverse transcription rate of the viral genome (Imam et al. 2018). Although increasingly more is being understood about the various effects of viral m⁶A modifications on viral infections, the epitranscriptome of many viruses remains unknown, as studying the epitranscriptome of viruses is not yet a standard practice in the field of virology.

COVID-19, the disease that is caused by the respiratory virus SARS-CoV-2, has caused over 5.2 million deaths world-wide since the beginning of the pandemic in late 2019 (WHO 2021b). The disease has spread to every country on the planet (WHO 2021b) and measurements against the virus are still having profound impact on daily life, society, the economy, and politics. Understandably, this has resulted in extensive research efforts into SARS-CoV-2. The research is mostly centred around topics of a) the identification of the virus, b) how the virus causes disease, c) how it replicates and spreads, d) how rapidly it mutates, and e) how COVID-19 can be treated (NIH 2021a). Nevertheless, the SARS-CoV-2 epitranscriptome remains mostly unstudied, with currently only few reports available on PubMed (NIH 2021b), although it could potentially be associated with each of these topics, as this has been proven to be the case for other RNA viruses (Kennedy et al. 2017). So far, m⁶A modifications have been identified in infected cell lines and extracted patient lymphocytes (Meng et al. 2021). This promising study also showed a correlation between human RNA transcripts in lymphocytes that are both hyper-methylated and upregulated after SARS-CoV-2 infection and more severe disease in COVID-19 patients (Meng et al. 2021). Unfortunately, due to the used technique in this study, the position of the m⁶A modifications on the RNA transcripts could not be identified, thus resulting in a limited research scope.

Conventional techniques to study m⁶A RNA modifications *in vivo* include PA-m⁶A-seq, miCLIP, and Me-RIP-seq, which all use antibodies to bind to the m⁶A modification regions. Each of these techniques has severe shortcomings: a) indirect measurement of m⁶A modifications, b) low sensitivity of antibodies for m⁶A modifications, c) cross-reactivity of antibodies, d) they require a specific protocol for each RNA modification, e) introduction of biases by the multi-step experimental protocols, f) high false-positive rate, g) limited quantitative information, and h) incomparability between studies because of the use of different antibodies (H. Liu et al. 2019; Xu and Seki 2020). Fortunately, direct RNA sequencing (DRS) on nanopore sequencers from Oxford Nanopore Technologies offers a new method to study RNA modifications directly on individual, full-length RNA molecules with single nucleotide resolution. This allows for the identification of transcript isoforms and provides both qualitative and quantitative data on m⁶A modifications (Garalde et al. 2018; Leger et al. 2019; H. Liu et al. 2019). The full length SARS-CoV-2 RNA genome and transcripts of SARS-CoV-2 RNA have successfully been sequenced with direct RNA sequencing in multiple studies (Kim et al. 2020; Taiaroa et al. 2020; Vacca et al. 2020; Viehweger et al. 2019).

a) Overall aim

To circumvent the limitations of the currently conventionally used m⁶A identification techniques, and to expand the research scope of m⁶A studies, it is important to study the epitranscriptome of SARS-CoV-2 with direct RNA sequencing. m⁶A modifications on both the viral SARS-CoV-2 transcripts and human transcripts will be studied, as it has been shown that also the human epitranscriptome can change upon viral infection and is associated with COVID-19 disease outcome (Meng et al. 2021).

Therefore, the overall aim of this proposal is to understand the role of m⁶A RNA modifications in the host-virus interaction during SARS-CoV-2 infection.

b) Sub-objectives

We have defined three subobjectives to answer the overall aim of this proposal.

1. The detection of m⁶A RNA modifications with direct RNA sequencing (DRS) will be improved and validated in context of SARS-CoV-2 infection
2. Associations between m⁶A modifications on human and SARS-CoV-2 RNA transcripts will be measured during SARS-CoV-2 infection. This will include m⁶A measurements:
 - a. at different time points of infection
 - b. in patient samples of different COVID-19 severity and disease outcomes
 - c. in different human cell lines
 - d. in different human organoid types
 - e. in combination with knocked-out m⁶A writer proteins
3. The effect of drug intervention on m⁶A levels and on SARS-CoV-2 reproduction will be determined.
 - a. inhibitors to m⁶A writers (3-deazaadenosine), readers (by knock-down), and erasers (meclofenamic acid) will be tested in order to identify potential new antiviral strategies to target COVID-19.
 - b. known antiviral SARS-CoV-2 drugs (remdesivir, favipiravir, and tocilizumab) will be tested in order to identify whether their effect on viral replication can be partially explained by epitranscriptomic mechanisms via altered m⁶A levels

Knowledge about the association between m⁶A modifications on both human and SARS-CoV-2 RNA transcripts and viral replication could teach us more about the complex replication process of SARS-CoV-2 and the relation between the viral and human epitranscriptome. This knowledge could potentially be harnessed to develop new therapeutic interventions for COVID-19 and other coronaviruses.

B.2.2 Approach (How)

This research project is subdivided into three parts, that each focus on one of the study subobjectives.

1. The detection of m⁶A RNA modifications with direct RNA sequencing (DRS) will be improved and validated in context of SARS-CoV-2 infection

Although m⁶A detection with DRS-ONT has been shown to be both sensitive and specific in yeast cells and human cell lines (Garalde et al. 2018; Leger et al. 2019; H. Liu et al. 2019), the technique will first be optimised on SARS-CoV-2 study materials and SARS-CoV-2 infected human cell lines, before the rest of the study will be conducted. Optimising will include some variations on the RNA extraction procedure, to determine which results in the highest yield of unfragmented RNA. In addition, sample preparation will be scaled-up and optimised, to allow for high-throughput sequencing with multiple samples being sequenced in parallel on the nanopores. This will ensure a more efficient and standardised sequencing approach. This direct RNA sequencing method will be validated via the principle of *orthogonal method validation* (Eurachem 2014), in which the sequencing results are compared with the results of three currently conventionally used techniques for m⁶A detection: PA-m⁶A-seq, miCLIP, and Me-RIP-seq.

Multiple sequencing metrics that are obtained from both SARS-CoV-2 study materials and SARS-CoV-2 infected human cell lines will be used to compare the methods; a) the number of m⁶A modifications identified in each sample, b) the false-positive rate, c) the false-negative rate, and d) the resolution of the m⁶A identification (how certain is it that m⁶A occurred on a specific position). In addition, as part of the sequencing method improvement, different computational tools that have been developed to obtain m⁶A modification information from the direct RNA sequencing data, are compared to determine which tool is most appropriate for the analysis of SARS-CoV-2 and human transcripts. The tools Methylartist, ELIGOS, EpiNano, and Tombo (Cheetham, Kindlova, and Ewing 2021; Jenjaroenpun et al. 2021; H. Liu et al. 2019; Stoiber et al. 2017) are validated with the same metrics as mentioned above. Additionally, it is determined whether these tools have a bias for identifying m⁶A at certain positions in the transcript (e.g. 5'UTR bias or a bias for a specific m⁶A motif sequence). This will result in a standardised and validated sequencing and analysis method, that will be used throughout the rest of the study.

2. Measurement of m⁶A modifications on viral and human transcripts during SARS-CoV-2 infection

After the DRS and computational analysis will have been optimised and validated, m⁶A modifications will be identified in a wide range of different samples, in order to find potential associations between m⁶A modifications and various aspects of the host-virus interaction during SARS-CoV-2 infection. m⁶A modifications on both viral and human RNA transcripts will be identified in:

- a. Human cell lines of kidney (HEK-293), liver (Huh-7), lung (A549), colon (NCM460), and heart (AC16) origin. This will include samples taken at different time intervals after SARS-CoV-2 infection, to determine whether there are time-dependent differences between stages of infection. The samples will be grown in our laboratory, with the help of experts that have previously used these exact cell lines for (direct RNA) sequencing in context of SARS-CoV-2.
- b. Human organoids of kidney, lung, colon, and heart origin. This will also include samples taken at different time intervals after infection. These organoids will be obtained from collaborations with leading experts labs that have currently used these organoid types in SARS-CoV-2 studies (Han et al. 2021; Mills et al. 2021; Xia et al. 2020). We will form a partnership with the Hubrecht Institute to grow and maintain most of these organoids, as this institute has the most expertise in this field within our region.
- c. Patient biopsy samples that have been taken as part of their COVID-19 treatment, and patient lymphocyte samples that have been donated for research purposes. The patient biopsies will include samples of more severe COVID-19 disease, whilst the lymphocyte samples will be obtained from patients of varying disease severity and outcome (asymptomatic infection, mild disease, severe disease, fully recovered, and long-COVID) that participate in studies from the University Medical Center Utrecht.

The combination of all these different samples will result in a large data set, that will be mined to generate a bigger picture of the involvement of m⁶A modifications in various aspects of infection.

In each sample, the amount of m⁶A will be identified, as well as the positions of the m⁶A modifications on the viral and human transcripts. When focussing on the SARS-CoV-2 transcripts, it will be interesting to see which m⁶A modifications are present, and whether this is different a) at various times after infection, b) in different cell types, and c) in patient of different COVID-19 severity. When focusing on the human transcripts, the same aspects are interesting, but, giving the size of the human transcriptome, the amount of transcripts is much larger, and more diverse than is the case for the SARS-CoV-2 transcripts. Therefore, we will focus on human transcripts that are a) shown to be relevant in viral infections, such as interferon-related transcripts, ACE-2 transcripts, and immune-related transcripts, b) highly up-regulated after infection c) highly down-regulated after infection, d) highly methylated after infection, or e) highly demethylated after infection. This will create multiple sets of genes that are relevant during SARS-CoV-2 infection and that have an altered epitranscriptome upon infection. These sets will then be used to compare patient samples of different COVID-19 severity and patient outcomes to see whether there are associations between certain m⁶A modifications and COVID-19 severity and disease outcome.

3. The effect of drug intervention on m⁶A levels and on SARS-CoV-2 reproduction will be determined. Once associations between m⁶A modifications and SARS-CoV-2 infection and COVID-19 severity have been identified, it will be important to determine whether these are just associations, or whether an epitranscriptomic mechanism could be the underlying driver. Therefore, the SARS-CoV-2 infected human cell lines will be subjected to various drugs that influence the m⁶A landscape. The m⁶A writer inhibitor 3-deazaadenosine (DAA), that has been shown to induce a slight reduction in HIV-1 viral replication (Mayers et al. 1995), and a knock-down of m⁶A readers will be used to decrease global m⁶A levels. Melofenamic acid, an inhibitor of the m⁶A eraser FTO that is currently used as a mild pain killer, will be administered to increase m⁶A levels in the infected cells (Cui et al. 2017). m⁶A modification detection will be performed to see if the modifications indeed changed upon these interventions. Both virus replication rate and virus particle production assays are used as metrics at different time intervals, to see whether the changed m⁶A landscape positively or negatively influences the SARS-CoV-2 infection of the cells.

Additionally, the effect of known antiviral drugs on m⁶A levels will be determined, to check whether their antiviral effect could be partially explained by changes in the epitranscriptome. The drugs remdesivir, favipiravir, and tocilizumab, which all have shown to have at least some clinical benefits in severely affected COVID-19 patients (Beigel et al. 2020; Lan et al. 2020; Shrestha et al. 2020) will be administered to the infected human cell line samples. m⁶A modifications on all the SARS-CoV-2 transcripts and on the human transcripts, which have been marked as transcripts of interest in part 2 of this study, will be measured to see whether the drugs had any effect on these specific modifications.

This study will make use of a cutting-edge technology to generate a unique dataset that can be mined to determine the association between m⁶A modifications and various relevant aspects of SARS-CoV-2 infection. Thereby, the knowledge of SARS-CoV-2 epitranscriptome and replication is increased, which has the potential of creating new innovative targets for therapeutic interventions against COVID-19.

Timeline of the research project

The figure below (fig.1) provides a schematic overview of the proposed research timeline.

The first year will be used for the approval of the patient sample collection, the patient sample collection itself, and the growth of the human cell lines and organoids. In parallel, the improvement and validation of m⁶A detection with direct RNA sequencing by comparison with the three conventionally used techniques will be performed. The computational tool comparison for the analysis of direct RNA sequencing m⁶A data will be included in the DRS method improvement and validation steps. The second year will be used to perform all the DRS-ONT sequencing experiments in the various beforementioned samples and settings. In the third year, the drug intervention experiments will be performed to determine the effect of various drugs on both m⁶A levels and SARS-CoV-2 replication.

Data analysis will be performed continuously over the course of the study, to ensure that appropriate interventions can be made if this would become necessary. In the fourth year, a thorough meta-analysis with other available methylome and transcriptome data sources will be conducted. There is also time reserved for repetition of experiments and additional experiments in this year, should this be needed. This research project will be concluded with the reporting all the gathered information to the scientific community and the general society. Finally, in line with open science guidelines, the (non-patient derived) sequencing data will be made available on publicly accessible platforms for future research purposes.

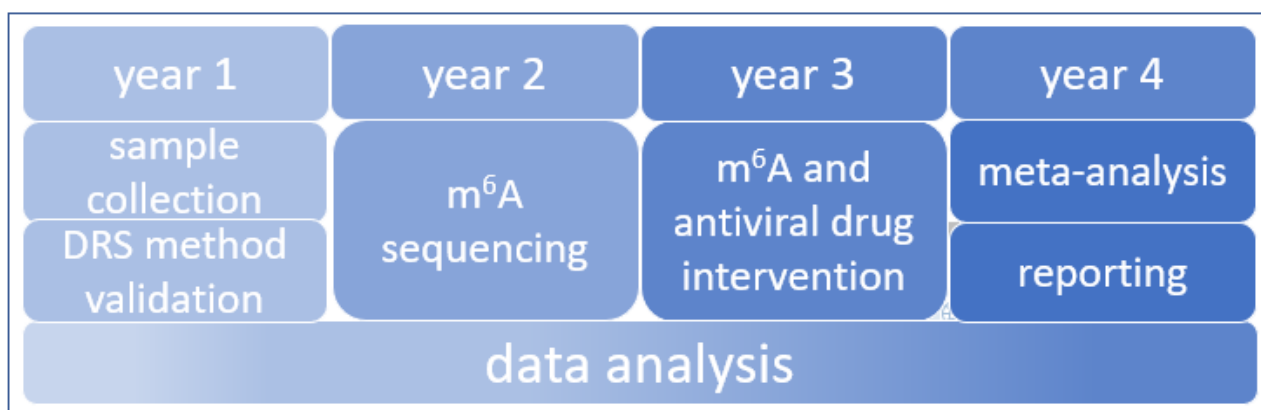


Figure 1: Schematic timeline of the epitranscriptomics study

DRS: direct RNA sequencing, m⁶A: nucleotide modification with a methyl group on adenosine N⁶-position

B.2.3 Feasibility / Risk assessment

Should DRS-ONT m⁶A detection turn out to be suboptimal for SARS-CoV-2 infected samples, analysis of the obtained samples will be performed with PA-m⁶A-seq, which is currently often used. Although the localisation of m⁶A modifications would become less precise, the data could still identify potential relationships between general m⁶A levels and a) the SARS-CoV-2 replication rate, b) differences between cell types, and c) COVID-19 patient outcomes, thereby providing enough insides to still help the field of viral epitranscriptomics and SARS-CoV-2 research forward.

As vaccinations campaigns are being rolled out and the treatment of COVID-19 improves, the number of severely affected patients will hopefully drop significantly in the near future. This might make patient enrolment and sample collection more challenging in the coming years. Would this be the case, we will collaborate with researchers that have previously collected and stored COVID-19 patient samples to continue our research efforts.

Since the growth and maintenance of organoids requires great expertise and specialist equipment, we will collaborate with various research groups that are specialised in kidney, lung, colon, and heart organoids, respectively. If our organoids would fail to grow as required, we will cooperate with groups that have previously stored SARS-CoV-2 infected human organoids to extract small amounts of RNA of these stored samples. As currently more and more human organoids for SARS-CoV-2 studies are being created (Clevers 2020), this seems to be a highly feasible alternative.

If the organoids RNA extraction would fail completely, we will still have the infected human cell lines to gather m⁶A data with regard to different cell types and time intervals after infection, since these are more easily maintained in the laboratory and have a shorter RNA extraction process.

RNA samples require very low temperature storage and delicate handling to limit the risk of RNA degradation. Should RNA degradation be frequent, another version of the direct RNA sequencing protocol, called sequence-specific DRS (ONT 2019) will be used to obtain sequencing data for chosen key regions of the fragmented transcriptome. Although this would require an additional design step, m⁶A modifications would still be identifiable in the data set, thus allowing for continuation of the study.

In the drug testing phase, it is important to test whether the drugs influence m⁶A levels and/or the SARS-CoV-2 replication rate in dose concentrations that would be reasonable to administer to human patients, as we want to test their potential as SARS-CoV-2 antivirals. However, should the m⁶A levels not change within the assigned dose range, a higher level of the drugs will also be tested, to determine the theoretical potential of the drug to alter m⁶A levels.

SARS-CoV-2 patient sample collection and processing of infected cells could impose serious health risks on the researcher that will need to be mitigated. These risks will be limited by vaccinating the research staff, wearing proper personal protective equipment, and by working in high level security laboratories.

Embedding of this project at Utrecht University

This research project will be embedded in the currently ongoing research lines in our research group at Utrecht University. Our Genome Biology and Epigenetics group specialises in research on chemical modifications on DNA molecules and histone proteins. As we aim to create a bigger picture of the complex interactions between different DNA, RNA and histone modifications we are capable of abstaining from tunnel vision on one specific mutation and instead incorporate the sequencing data of modifications in the wider context of the epigenome and epitranscriptome. As m⁶A is also present on DNA molecules, albeit in a lower abundance (Spingardi and Kriaucionis 2020), we already have some experience with m⁶A modifications in datasets. Although we have not yet used direct RNA sequencing for the identification of epitranscriptomic mutations ourselves, other research groups at the Utrecht University, that are part of the Utrecht Bioinformatics Center, have great expertise in the use of Oxford Nanopore sequencers and in the comparison of different sequencing techniques and computational tools. Furthermore, the Utrecht Bioinformatics Center will support us with high computational capacity, data storage, and general bioinformatics support.

Since the start of the COVID-19 pandemic, numerous research groups within the Utrecht University Medical Center have conducted studies on various aspects of SARS-CoV-2. The immunology and virology groups specialise in study of viral infections and viral replication in cell lines. We will collaborate with these groups to conduct the viral replication and viral particle production assays during the testing of the antiviral drugs and the m⁶A altering drugs. The Hubrecht Institute is world-wide renowned for its work on organoids from various cell types and tissues and has recently created intestinal organoids specifically for the study of SARS-CoV-2 infections, which are stored in an organoid biobank. We will collaborate with the institute to grow and maintain the human organoids. Since the Academic Medical Center has treated thousands of COVID-19 patients and has set up clinical studies on patient data and patient samples, there is already experience with the initiation of SARS-CoV-2 patient studies and patient sample collection, preparation, storage, and analysis. This will aid our proposed research project with the study set-up and supply of reliable COVID-19 patient samples.

For all these reasons, the Genome Biology and Epigenetics group and the wider Utrecht University research community are fully equipped to successfully conduct this ambitious research project.

B.2.4 Scientific and societal impact

By achieving the aims that have been defined for this research project, we will also try to create a positive impact both on the scientific community and on the general society.

a) Scientific impact

This study will aim to give the scientific community an improved DRS-ONT protocol for the detection of m⁶A modifications, that will have been evaluated and cross-validated with three commonly used m⁶A detection methods. This will result in a more streamlined approach, that could be adopted for the identification of various RNA modifications, since the DRS-ONT data itself also contains information on other RNA modifications. In addition to the benefits to the field of viral epitranscriptomics, this improved DRS-ONT protocol could also be used to study the human epitranscriptome in a wider context. This will allow for more precise, more efficient, and faster studies in the fields of e.g. cancer gene regulation, embryonic development, and metabolic diseases, which all show a deep interest in the epitranscriptome, but are currently reliant on more laborious techniques for their studies.

This research project is one of the first projects that studies viral epitranscriptomics with DRS-ONT. If successful, this could become a standard approach in viral epitranscriptomics, as it requires less experimental steps, has a shorter turnaround time, and can provide information on multiple RNA modifications, in all kinds of viruses. Additionally, this is one of the first viral epitranscriptomics studies that uses human organoids and patient-derived materials, as previous studies were mostly conducted on immortalized animal cell lines. An enormous data set of m⁶A information on various cell types, organoid types, time points after infection, and in patients with different disease outcomes will be the result of this study. The combination of this dataset and the validation of DRS-ONT for m⁶A identification will set a new standard of conducting viral epitranscriptomic studies and will hopefully result in the integration of this relatively new field in both virology and immunology.

The identification of m⁶A modifications in both viral and human transcripts in context of SARS-CoV-2 infection will provide more knowledge about the regulatory mechanisms of SARS-CoV-2 replication and its viral particle production that can be used in different subfields of virology. This knowledge could be used to develop therapies against SARS-CoV-2 or other (future) coronaviruses. Accordingly, the drug intervention studies could indicate whether changing m⁶A levels could influence SARS-CoV-2 infection and replication, thereby again providing new targets for therapeutic interventions. In the long term, more knowledge about SARS-CoV-2 replication in relation to m⁶A levels, could result in the development of an attenuated virus vaccine with manipulated m⁶A marks that could generate a more efficient immune response after vaccination, thus resulting in better acquired immunity and protection after vaccination. Higher levels of immunity would of course also greatly benefit the general society.

b) Societal impact

This study will improve and validate a method that can also be used to study other viruses. The m⁶A modification has already been identified in over 15 viruses that cause disease in humans, such as HIV-1, Zika virus, Influenza A virus, and Hepatitis viruses (Kennedy et al. 2017). Therefore, improvement of the direct RNA sequencing method for m⁶A identification could result in more research into these viruses that are relevant from a global health perspective. Furthermore, future emerging viruses could be subjected to this technique. As this will provide more information on the regulation of mechanism of viral replication, this may ultimately lead to improvements in prevention, treatment, and control of viral outbreaks.

This research could also have implications for the fight against the current COVID-19 pandemic, that greatly affects society. The m⁶A sites that will be indicated in this study, could potentially be targeted with specific, currently available drugs to alter the m⁶A levels and subsequently the viral replication of SARS-CoV-2. This would be a completely new approach to reduce SARS-CoV-2 replication and infection that could be used in addition to the limited arsenal of antiviral drugs that currently exists.

Furthermore, the data that will have been gathered from the samples from patients with different COVID-19 severity and outcomes, may result in the identification of more risk factors associated with severe disease, which in turn could result in better prevention and treatment.

At the time of writing, the fourth SARS-CoV-2 variant-of-concern, B.1.1.529, known as Omicron, is spreading across the globe (WHO 2021a). To determine the potential threat of such a variant, research is conducted quickly on the genome of the virus. The DRS-ONT method that we will fine tune for SARS-CoV-2 studies can be used to quickly and efficiently establish whether a new variant of the virus carries an altered epitranscriptome. This could help the scientific community to better understand whether changes in the viral replication are likely to occur, thus resulting in a more complete risk assessment for the effect on the general society.

Although this study focusses mostly on m⁶A modifications in context of viral infections, the improved DRS-ONT method can also be used to study m⁶A modifications on human RNA transcripts in a much wider context. This more precise and more efficient method could for example be used to study the human epitranscriptome in cancer cells. It is already known that certain m⁶A modifications are associated with poor cancer prognosis in e.g. gastric cancer (Zhang et al. 2019). A wider study in the epitranscriptome could result in the identification of more cancer associated modifications and biomarkers for prognosis prediction. In addition, this method could be used to quickly determine whether methylome altering potential anti-cancer drugs have the intended effect on m⁶A levels. Such a broader implementation in cancer research would hopefully benefit in better cancer treatment of numerous cancers in the future.

Given the number of people that could become patients of viral diseases or cancers, even if studies into m⁶A modifications would result in improvement of treatment in only a fraction of these disease cases, the potential benefit for patients would be enormous.

B.2.5 Ethical considerations

While the use of our transcriptome and epitranscriptome data could be of benefit in meta analyses of large datasets, it must be carefully considered which data is appropriate to make publicly available.

All patient-derived data will be securely anonymised, randomised and stored by following personal data protection guidelines that are the standard of practice in similar studies. Patient-derived human transcript data will not be made publicly available, as there is a risk of uncovering the patient's identity from either the transcriptome or epitranscriptome data, which would undermine the patient's right to privacy and anonymity. Contrary, data of the viral epitranscriptome, and of the human epitranscriptome from the human cell lines and organoids will be made publicly available, as this is an important part of open science and could contribute to future research.

Epitranscriptomic analysis of the patient samples could result in the identification of genetic mutations that are linked to certain diseases. Although it is not the aim of this study to find these genetic mutations, an ethical decision must be made if such mutations are identified serendipitously. Therefore, we will follow the standard of practice of genetic studies and ask in the informed consent procedure whether patients wish to be informed if certain mutations or risk factors are identified during the study. The patients personal decisions will be respected.

As this study will develop a streamlined direct RNA sequencing approach that can be used to study the viral epitranscriptome in multiple viruses, it is important to think about the appropriate model virus to conduct this method development on. Although it has been predicted that SARS-CoV-2 will be circulating worldwide for at least the coming decade (Peacock 2021), the impact of this virus is predicted to become less severe on individuals, because of vaccination, acquired immunity through infection, and improved patient care. Therefore, it might be more ethical to focus on viruses that will more severely affect patients in the future. However, SARS-CoV-2 currently affects millions of people from a range of socio-economic backgrounds and ethnicities from all of the world, which has a profound effect on society, the economy, and politics in many countries (WHO 2021b). Furthermore, it is expected that other coronaviruses could cause new pandemics in the future (Fan et al. 2019; B. Li et al. 2020; Wong, Lau, and Woo 2021), which increases the need for more knowledge about replication regulation in coronaviruses. Therefore, we consider SARS-CoV-2 to be a good model virus in the field of viral epitranscriptomics for the years to come.

B.2.6 Literature/references

- Bartosovic, Marek et al. 2017. "N6-Methyladenosine Demethylase FTO Targets Pre-mRNAs and Regulates Alternative Splicing and 3'-End Processing." *Nucleic acids research* 45(19): 11356–70.
- Batista, Pedro J. 2017. "The RNA Modification N6-Methyladenosine and Its Implications in Human Disease." *Genomics, Proteomics & Bioinformatics* 15(3): 154–63. <http://dx.doi.org/10.1016/j.gpb.2017.03.002>.
- Beigel, John H et al. 2020. "Remdesivir for the Treatment of Covid-19 - Final Report." *The New England journal of medicine* 383(19): 1813–26.
- Cheetham, Seth, Michaela Kindlova, and Adam Ewing. 2021. "Methylartist: Tools for Visualising Modified Bases from Nanopore Sequence Data." *bioRxiv*. <https://doi.org/10.1101/2021.07.22.453313>.
- Clevers, Hans. 2020. "COVID-19 : Organoids Go Viral." *Nature Reviews Molecular Cell Biology* 19: 5–6. <http://dx.doi.org/10.1038/s41580-020-0258-4>.
- Courtney, David G et al. 2017. "Epitranscriptomic Enhancement of Influenza A Virus Gene Expression and Replication." *Cell host & microbe* 22(3): 377-386.e5.
- Cui, Qi et al. 2017. "M(6)A RNA Methylation Regulates the Self-Renewal and Tumorigenesis of Glioblastoma Stem Cells." *Cell reports* 18(11): 2622–34.
- Eurachem. 2014. *Eurachem Guide: The Fitness for Purpose of Analytical Methods - A Laboratory Guide to Method Validation and Related Topics*. second.
- Fan, Yi, Kai Zhao, Zheng-Li Shi, and Peng Zhou. 2019. "Bat Coronaviruses in China." *Viruses* 11(3).
- Garalde, Daniel R et al. 2018. "Highly Parallel Direct RNA Sequencing on an Array of Nanopores." *Nature Methods* 15(3).
- Han, Yuling et al. 2021. "Identification of SARS-CoV-2 Inhibitors Using Lung and Colonic Organoids." *Nature* 589(7841): 270–75.
- He, Luer et al. 2019. "Functions of N6-Methyladenosine and Its Role in Cancer." *Molecular cancer* 18(1): 176.
- Imam, Hasan et al. 2018. "N6-Methyladenosine Modification of Hepatitis B Virus RNA Differentially Regulates the Viral Life Cycle." *Proceedings of the National Academy of Sciences of the United States of America* 115(35): 8829–34.
- Jenjaroenpun, Piroon et al. 2021. "Decoding the Epitranscriptional Landscape from Native RNA Sequences." *Nucleic acids research* 49(2): e7.
- Jia, Guifang et al. 2011. "N6-Methyladenosine in Nuclear RNA Is a Major Substrate of the Obesity-Associated FTO." *Nature chemical biology* 7(12): 885–87.
- Kasowitz, Seth D et al. 2018. "Nuclear M6A Reader YTHDC1 Regulates Alternative Polyadenylation and Splicing during Mouse Oocyte Development." *PLoS genetics* 14(5): e1007412.
- Kennedy, Edward M et al. 2016. "Posttranscriptional m(6)A Editing of HIV-1 MRNAs Enhances Viral Gene Expression." *Cell host & microbe* 19(5): 675–85.
- Kennedy, Edward M, David G Courtney, Kevin Tsai, and Bryan R Cullen. 2017. "Viral Epitranscriptomics." *Journal of Virology* 91(9): 1–10.
- Kim, Dongwan et al. 2020. "The Architecture of SARS-CoV-2 Transcriptome." *Cell* 181(4): 914-921.e10.
- Lan, Shao-Huan et al. 2020. "Tocilizumab for Severe COVID-19: A Systematic Review and Meta-Analysis." *International journal of antimicrobial agents* 56(3): 106103.
- Leger, Adrien et al. 2019. "RNA Modifications Detection by Comparative Nanopore Direct RNA Sequencing." *bioRxiv*: 1–29. <https://doi.org/10.1101/843136>.
- Li, Ang et al. 2017. "Cytoplasmic m(6)A Reader YTHDF3 Promotes mRNA Translation." *Cell research* 27(3): 444–47.
- Li, Bei et al. 2020. "Discovery of Bat Coronaviruses through Surveillance and Probe Capture-Based Next-Generation Sequencing." *mSphere* 5(1).
- Lichinchi, Gianluigi, Boxuan Simen Zhao, et al. 2016. "Dynamics of Human and Viral RNA Methylation during Zika Virus Infection." *Cell host & microbe* 20(5): 666–73.
- Lichinchi, Gianluigi, Shang Gao, et al. 2016. "Dynamics of the Human and Viral m(6)A RNA Methylomes during HIV-1 Infection of T Cells." *Nature microbiology* 1: 16011.
- Liu, Huanle et al. 2019. "Accurate Detection of M6A RNA Modifications in Native RNA Sequences." *Nature Communications* 10(4079): 1–9.
- Liu, Jianzhao et al. 2014. "A METTL3-METTL14 Complex Mediates Mammalian Nuclear RNA N6-Adenosine Methylation." *Nature chemical biology* 10(2): 93–95.
- Lu, Wuxun et al. 2018. "N(6)-Methyladenosine-Binding Proteins Suppress HIV-1 Infectivity and Viral Production." *The Journal of biological chemistry* 293(34): 12992–5.
- Mayers, D L et al. 1995. "Anti-Human Immunodeficiency Virus 1 (HIV-1) Activities of 3-Deazaadenosine Analogs: Increased Potency against 3'-Azido-3'-Deoxythymidine-Resistant HIV-1 Strains." *Proceedings of the National Academy of Sciences of the United States of America* 92(1): 215–19.

- Mcintyre, Alexa B R et al. 2016. "N6 -Methyladenosine in Flaviviridae Viral RNA Genomes Regulates Infection." *Cell Host and Microbe* 20(5): 654–65. <http://dx.doi.org/10.1016/j.chom.2016.09.015>.
- Meng, Yuting et al. 2021. "RBM15-Mediated N6-Methyladenosine Modification Affects COVID-19 Severity by Regulating the Expression of Multitarget Genes." *Cell Death & Disease* 12(732): 1–10.
- Meyer, Kate D et al. 2015. "5' UTR m(6)A Promotes Cap-Independent Translation." *Cell* 163(4): 999–1010.
- Mills, Richard J et al. 2021. "BET Inhibition Blocks Inflammation-Induced Cardiac Dysfunction and SARS-CoV-2 Infection." *Cell* 184(8): 2167-2182.e22.
- NIH. 2021a. "PubMed Database COVID-19." <https://pubmed.ncbi.nlm.nih.gov/?term=COVID-19> (November 25, 2021).
- . 2021b. "PubMed SARS-CoV-2 AND M6A OR Epitranscriptomics." <https://pubmed.ncbi.nlm.nih.gov/?term=SARS-CoV-2+AND+m6A> (November 25, 2021).
- ONT. 2019. "Sequence-Specific Direct RNA Sequencing (SQK-RNA002)." https://doi.org/https://community.nanoporetech.com/protocols/ss-direct-rna-sequencing-sqk-rna002/v/DSS_9081_v2_revK_14Aug2019 (November 27, 2021).
- Peacock, Sharon. 2021. "Newscast: Genomics for Dummies." *BBC*. <https://www.bbc.co.uk/programmes/p096mc5k>.
- Shrestha, Dhan Bahadur et al. 2020. "Favipiravir versus Other Antiviral or Standard of Care for COVID-19 Treatment: A Rapid Systematic Review and Meta-Analysis." *Virology journal* 17(1): 141.
- Spingardi, Paolo, and Skirmantas Kriaucionis. 2020. "How m(6)A Sneaks into DNA." *Nature chemical biology* 16(6): 604–5.
- Spitale, Robert C et al. 2015. "Structural Imprints in Vivo Decode RNA Regulatory Mechanisms." *Nature* 519(7544): 486–90.
- Stoiber, Marcus et al. 2017. "De Novo Identification of DNA Modifications Enabled by Genome-Guided Nanopore Signal Processing." *bioRxiv*. <https://doi.org/10.1101/094672>.
- Taiaroa, G. et al. 2020. "Direct RNA Sequencing and Early Evolution of SARS-CoV-2." *bioRxiv*. <https://doi.org/10.1101/2020.03.05.976167>.
- Tirumuru, Nagaraja et al. 2016. "N(6)-Methyladenosine of HIV-1 RNA Regulates Viral Infection and HIV-1 Gag Protein Expression." *eLife* 5.
- Vacca, D. et al. 2020. "Direct RNA Nanopore Sequencing of SARS-CoV-2 Extracted from Critical Material from Swabs." *MedRxiv*. <https://doi.org/10.1101/2020.12.21.20191346>.
- Viehweger, Adrian et al. 2019. "Direct RNA Nanopore Sequencing of Full-Length Coronavirus Genomes Provides Novel Insights into Structural Variants and Enables Modification Analysis." *Genome research* 29(9): 1545–54.
- Wang, Xiao et al. 2014. "N6-Methyladenosine-Dependent Regulation of Messenger RNA Stability." *Nature* 505(7481): 117–20.
- . 2015. "N(6)-Methyladenosine Modulates Messenger RNA Translation Efficiency." *Cell* 161(6): 1388–99.
- WHO. 2021a. "Update on Omicron." <https://www.who.int/news/item/28-11-2021-update-on-omicron> (December 2, 2021).
- . 2021b. "WHO Coronavirus (COVID-19) Dashboard." <https://covid19.who.int/> (November 25, 2021).
- Wong, Antonio C P, Susanna K P Lau, and Patrick C Y Woo. 2021. "Interspecies Jumping of Bat Coronaviruses." *Viruses* 13(11).
- Wu, Weiche et al. 2017. "AMPK Regulates Lipid Accumulation in Skeletal Muscle Cells through FTO-Dependent Demethylation of N(6)-Methyladenosine." *Scientific reports* 7: 41606.
- Xia, Siyu et al. 2020. "Long Term Culture of Human Kidney Proximal Tubule Epithelial Cells Maintains Lineage Functions and Serves as an Ex Vivo Model for Coronavirus Associated Kidney Injury." *Virologica Sinica* 35(3): 311–20.
- Xiao, Wen et al. 2016. "Nuclear m(6)A Reader YTHDC1 Regulates mRNA Splicing." *Molecular cell* 61(4): 507–19.
- Xu, Liu, and Masahide Seki. 2020. "Recent Advances in the Detection of Base Modified Cations Using the Nanopore Sequencer." *Journal of Human Genetics* (65): 25–33. <http://dx.doi.org/10.1038/s10038-019-0679-0>.
- Zhang, Cheng et al. 2019. "Reduced M6A Modification Predicts Malignant Phenotypes and Augmented Wnt/PI3K-Akt Signaling in Gastric Cancer." *Cancer medicine* 8(10): 4766–81.
- Zhao, Boxuan Simen, Ian A Roundtree, and Chuan He. 2017. "Post-Transcriptional Gene Regulation by mRNA Modifications." *Nature reviews. Molecular cell biology* 18(1): 31–42.
- Zheng, Guanqun et al. 2013. "ALKBH5 Is a Mammalian RNA Demethylase That Impacts RNA Metabolism and Mouse Fertility." *Molecular cell* 49(1): 18–29.