Development of circRNA Detection Protocol for circHIPK3

and its Potential Application in Identifying Novel Biomarkers for Sepis

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Preface

The thesis 'Development of a circRNA Detection Protocol for circHIPK3 and its Potential Application in Identifying Novel Biomarkers for Sepsis' is written for the submission for the master's degree of *Biology of Disease* at the Universiteit Utrecht. This thesis is the final result of a six-month internship at the Epigenetics and Epigenomics Translational Research Team at INCLIVA, Valencia. During my internship here, I have been performing many laboratorial procedures and protocols for my first time. This steep learning curve has brought me a lot of experience, knowledge, sometimes frustration but mostly more curiosity to science. I want to express my gratitude to Dr. José Luis García Giménez for his guidance, brainstorm sessions and expertise during my project. But also, the freedom and responsibilities that were essential for my personal development.

Secondly, I want to thank Elena Nacher-Sendra, Irene Cánovas-Cervera, Marta Seco and Enric Dolz-Andrés for teaching me how to perform the protocols and analysis which were the basis of my research. Moreover, thanks to all the members of the Research Team, as everyone was always prepared to help me when I was lost. I also want to thank prof. dr. H.A.H. (Karin) Kaasjager for finding time to be my second examiner.

Finally, I would like to mention the support of my boyfriend and my Valencian friends through all the stages of my time in Valencia as living and working in another country always brings extra challenges. Of course, it has been a labour of love and I hope you enjoy reading it as much as I enjoyed writing it.

Jessica van der Mannen

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Abstract

Background – Sepsis is a life-threatening condition, characterised by a dysregulated host response to an infection, resulting in endothelial dysfunction, microvascular thrombosis and organ dysfunction. Surviving patients are often left with chronic illnesses and long-term complications. Early diagnosis with adequate biomarkers is essential in the diagnosis, prognosis, treatment and clinical outcome of septic patients. This thesis aims to discover novel biomarkers in the field of epigenetics, with a focus on circular RNAs (circRNAs) and specifically circHIPK3. CircRNAs are covalently closed loop molecules and are involved in several cellular processes, including the immune response.

Methods – A cell culture of human umbilical vein endothelial cells (HUVECs) were treated with extracellular histones to reproduce a severe sepsis pathological condition. After four hours of treatment, the HUVECs were lysed, and their RNA was extracted. The RNA samples underwent fifteen minutes of RNase R or mock treatment to eliminate all linear RNA. CircHIPK3 expression levels were determined using SYBR-Green RT-qPCR while the expression levels of its downstream miRNA targets were determined using TaqMan RT-qPCR. Primers specific for circHIPK3 were designed with Primer-BLAST.

Results – The designed primer-pair proved to be specific for the target circHIPK3. The widely used GAPDH reference gene primer-pair also bound to circGAPDH after RNase R treatment. RNase R treatment was most effective when performed after RNA extraction without extra clean-up, maximizing RNA concentration and purity. The SYBR-Green RT-qPCR assay exhibited high sensitivity for detecting GAPDH and HIPK3, down to 1 ng RNA concentration. Shorter RNase R treatment times (as short as 10 minutes) were sufficient to degrade linear RNA without significantly affecting detection of circGAPDH and circHIPK3. CircHIPK3 levels and its downstream targets in HUVECs remained relatively unchanged following histone treatment. In HEK-239 cells, downstream miRNA levels remained unchanged under different histone concentrations. CircHIPK3 levels in patient plasma were undetectable.

Conclusions – The *in vitro* experiments with HUVECs exposed to extracellular histones did not show significant alterations in circHIPK3 expression and its downstream targets. Furthermore, circHIPK3 levels were undetectable in patient plasma. The study developed a protocol for circHIPK3 detection and contributed to the understanding of circRNAs' potential as sepsis biomarkers, but further optimization and validation are needed for patient samples.

Layman summary

Sepsis is a severe condition that occurs when the body's response to infection becomes dysregulated, leading to organ dysfunction. Patients that survive are often left with chronical illnesses or long-term complications. Early detection of sepsis is crucial for effective treatment. However, there is a lack of useful biomarkers to improve early detection, prognosis and start of adequate treatment to prevent death and the development of chronical complications. This study focus on the field of epigenetics to find new biomarkers. Epigenetics is a field of biomedical sciences that concentrates on the changes in gene activity and gene expression levels in, among others, circular RNAs (circRNAs). CircRNAs are covalently closed loops that are derived from parts of their precursor mRNAs. They are able to regulate various cellular processes through several mechanisms and have been associated with disease development, including sepsis. This study develops a protocol to be able to detect circRNA, with a specific focus on circHIPK3, levels *in vitro* and eventually *in vivo*. Therefore, we treated human umbilical vein endothelial cells (HUVECs) with extracellular histones to mimic sepsis pathology *in vitro*, extracted their RNA and treated these extracts with RNase R to eliminate all linear RNA, so only the circRNAs would be left. Gene expression levels were determined with SYBR-Green RT-qPCR where household gene circGAPDH functioned as a reference. Downstream expression levels of miRNA targets of circHIPK3 were determined with TaqMan RT-qPCR. Our results show that the developed protocol is specific and sensitive for even small concentrations of circHIPK3 in HUVECs. Furthermore, the RNase R treatment application time was determined to be after RNA extraction to yield the highest RNA concentration and purity for analysis. CircHIPK3 and its downstream miRNA targets were not differentially expressed after HUVECs were treated with extracellular histones. It is possible that circHIPK3 levels are unaffected following histone-mediated cellular stress. Moreover, circHIPK3 levels were undetectable in patient plasma, probably due to the low levels of RNA in the samples. This thesis developed a protocol for the detection of circHIPK3 and potentially other circRNAs. However, it needs to be optimized for patient samples. Despite these challenges, it lays the groundwork for future research in this area. By understanding the genetic markers associated with sepsis, healthcare professionals may diagnose and treat patients more effectively, leading to improved clinical outcomes.

Table of Contents

1. Introduction

Sepsis is a life-threatening condition, characterized by organ dysfunction and tissue damage, which is caused by a dysregulated host response in reaction to an infection (1,2). It accounts for ~20% of all deaths worldwide and is responsible for approximately 11 million deaths annually (3). Sepsis is recognized as the final common process to death resulting from several severe infectious microorganisms. The dysregulated host response includes sustained elevated levels of pro-inflammatory as well as anti-inflammatory cytokines as a result of an altered immune and metabolic cell processes (4). This dysregulated homeostasis results in, among others, the excessive formation of neutrophil extracellular traps (NETs) which consists of chromatin fibre structures with antimicrobial peptides, enzymes and histones (4–7). The extracellular histones that are released during NETosis and the pro-inflammatory cytokines aggravate the process of adherence and migration to the endothelium, leaving it leaky and with loss of function (4,7). This activates the coagulation cascade and the release of pro-coagulant factors, resulting in microvascular thrombosis and impaired blood flow to potentially every organ in the body (7). The combination of pro-inflammatory cytokines and a disrupted blood flow leads to tissue damage, (multiple) organ dysfunction and, eventually, septic shock (SS) and death (7,8).

Even though patients are treated in hospital for the underlying initial infection and receive hemodynamic support to maintain blood pressure and organ perfusion, they are often left with chronic critical illness (7). Such as persistent inflammation, immunosuppression and catabolism syndrome (PICS), physical and cognitive impairments and cardiovascular disease (4). Early diagnosis and treatment of sepsis is important in improving patient outcomes (9). The identification and application of new biomarkers for sepsis would be useful for this early diagnosis, predicting disease severity and mortality, and even support decision-making processes related to treatment and therapies during patient hospitalization. However, there is a lack of complete understanding of the pathophysiology, diagnosis, and treatment of sepsis, making it difficult to develop novel biomarkers (10).

Therefore, the aim of this thesis is to discover novel biomarkers for the diagnosis of early sepsis and SS in hospitalized patients to enhance future research of septic pathophysiological processes and improve disease diagnosis and prognosis.

Currently, clinics are using several laboratory tests to diagnose septic patients, including levels of procalcitonin and of C-reactive protein, and many other biomarkers (7,9). However, most of these biomarkers lack sensitivity and specificity (9,11). Therefore, there is a need to have more accurate biomarkers available in order to diagnose a patient quickly and adequately in order to commence the most appropriate treatment strategy and enhance clinical outcome (9).

Epigenetics is a promising field of biomedical science that has the potential to decipher intricate molecular and transcriptional cellular pathways to help explain the pathological disease onset and progression. Epigenetic mechanisms are defined as changes in gene activity and expression that are not related to changes in the DNA sequence and are stable enough to alter cell transcription (12). Epigenetic mechanisms include mainly DNA methylation, histone post-translational modifications (PTMs) and noncoding RNAs (ncRNAs), such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) (13,14).

Importantly, epigenetic mechanisms contribute to the function of both innate and adaptive immunity, and profound changes in histone PTMs, DNA methylation and altered expression of miRNAs during and after sepsis have been described (15). Particularly, circRNAs have been closely related with the control of immune response by controlling the function of T-cells, macrophages and other immune cells (16). Therefore, this thesis will focus on the methodology of detection of circRNAs and its optimization.

CircRNAs are involved in gene regulation, splicing, and translation as well as cell differentiation, apoptosis, autophagy and proliferation (17). These non-coding RNAs derive their name from their structure as covalently closed loop molecules (18,19). CircRNAs are produced through a non-canonical splicing process where a downstream 5'-end is covalently joined to an upstream 3'-end, aided by the spliceosome (18,20). This process is referred to as back-splicing and the resulting circRNA may include exons and introns from its processor pre-mRNA (20). CircRNAs contribute to cellular processes through six different processes, see *figure 1* for a schematical overview of the biomolecular activities (21,22).

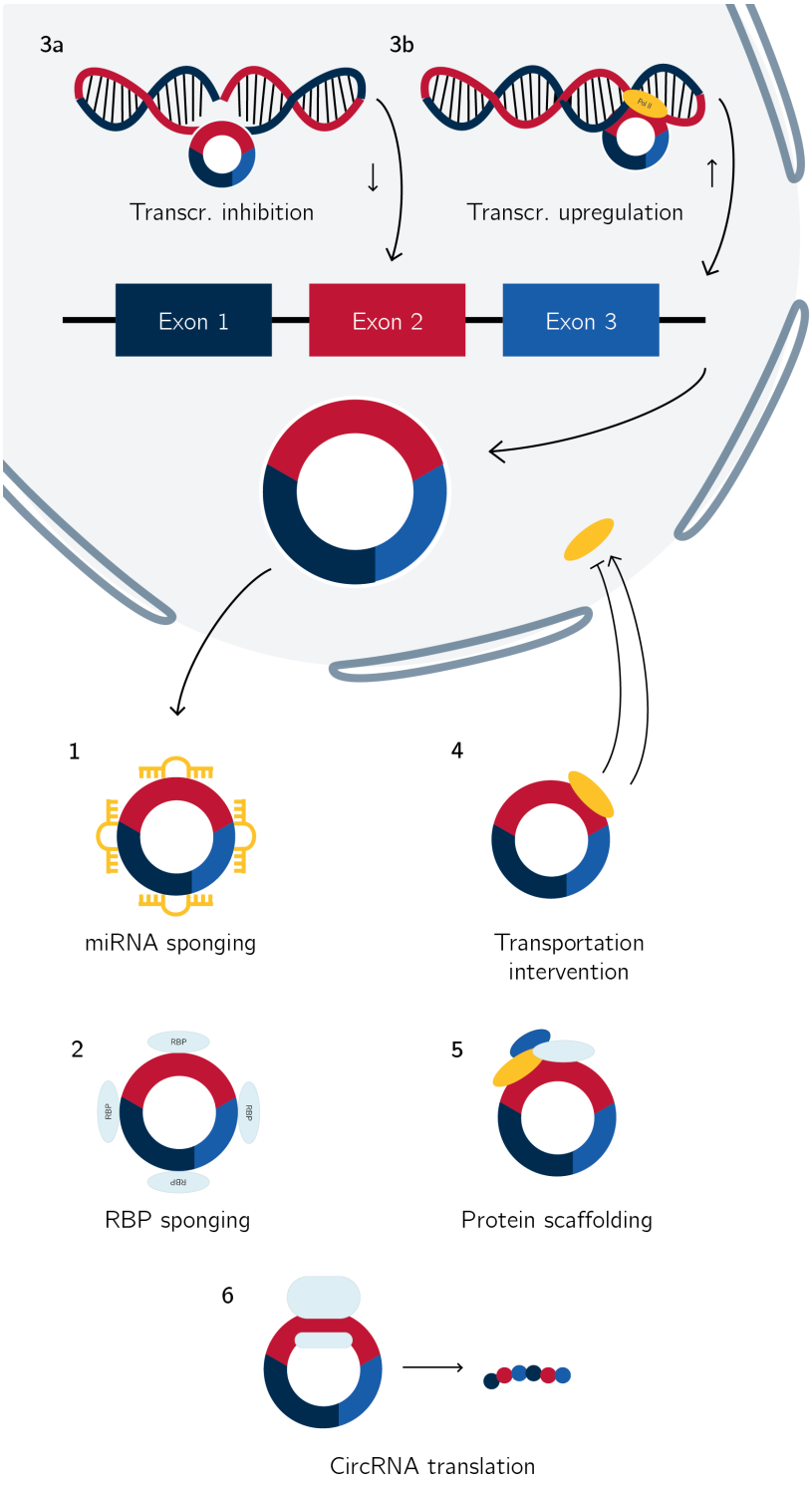

Figure 1 – Circular RNA mechanisms to alter cellular processes*. 1) microRNA sponging to prevent them from inhibiting their target messengerRNA; 2) RBP sponging to prevent them from blocking translation; 3) Respective inhibition and upregulation of transcription; 4) Either preventing or promoting the translocation of proteins; 5) Protein scaffolding to promote protein-interactions; and 6) The translation of circRNA into proteins.*

1. miRNA sponging

   One of the main activities of circRNAs to affect cellular processes is through the sponging or miRNA on their specific miRNA binding site(s). Because miRNAs normally interfere with the translation of messenger RNA (mRNA), sponging of miRNAs results in an upregulated translation of the mRNA target proteins.
2. RNA-binding protein (RBP) sponging

   Similarly, circRNAs can sponge RBPs in the cytosol to prevent them from blocking mRNA translation initiation.
3. Transcription intervention

   In the nucleus, transcription is stopped or paused when circRNA binds to its target DNA locus, forming a hybrid R-loop structure. On the other hand, circRNAs can interact with the RNA polymerase II complex to enhance transcription of its parent genes.
4. Protein transportation intervention

   A well lesser studies mechanism of circRNA's influence on cellular processes is the sequestering or translocating of proteins between subcellular compartments, inhibiting or enhancing downstream effects.
5. Protein scaffolding

   Furthermore, circRNAs are able to bind to proteins, acting as scaffolds to facilitate interactions between several proteins.
6. CircRNA translation

   Finally, even though circRNAs are known as non-coding RNAs, selected research has shown that some circRNAs can be translated into proteins through the recruitment of ribosomes via internal ribosome entry sites (22).

CircRNAs may have a substantial role in various human pathologies, according to the differential expression of circRNAs during disease progression (19,23), this includes cancers (19,24–31), diabetes (26,32,33), cardiovascular diseases (34,35), central nervous system (29,36,37) and sepsis (8,20,38–49). One abundant circRNA in human cells is the circular homeodomain-interacting protein kinase 3 (circHIPK3 or hsa_circ_0000284), derived from exon 2 of the *HIPK3* gene, spanning 1.099 base pairs (23). CircHIPK3 is abundant in the cytoplasm of different human cells and is able to sponge multiple miRNAs (23,50). These include miR-29b-3p (24–26,32,34,38), miR-124-3p (24,27–

31,36,37,39,51), and miR-148b-3p (35,39). All exerting a regulatory role in the translation of their target mRNAs and proteins and ultimately the development of sepsis and the aforementioned diseases. Since circHIPK3 is three times more stable than its parent HIPK3 mRNA (52), and abundantly present in various human cell types, it could be a suitable novel biomarker for the early detection of sepsis and SS (53).

Various research already linked under- or overexpression of circHIPK3 to the progression of sepsis. Xiao *et al.* suggested that in a healthy individual, intestinal mucosa circHIPK3 levels increase following acute injury, enhancing epithelial repair and ongoing epithelium renewal. However, their mouse CLP-model and patient mucosal intestine tissue exhibited decreased circHIPK3 levels, contributing to sepsis pathology (38). On the other hand, Han *et al.* demonstrated that serum of severe acute kidney injury (SAKI) patients, their SAKI-mice model and LPS-treated kidney cells presented higher levels of circHIPK3 in their blood and that the downregulation of circHIPK3 alleviated disease (39). Similar results were found by Lu *et al.* where higher serum levels of circHIPK3 were found in septic patients as well as *in vitro* septic cells (52). The knock-down of circHIPK3 alleviated cell damage. Therefore, changes in circHIPK3 levels are likely to contribute to the sepsis pathology. However, additional research is needed to confirm the clinical relevance between circHIPK3 levels and the development of sepsis and SS.

In this thesis an *in vitro* septic model is used to propose a protocol to detect circHIPK3 as a potential biomarker. This protocol could then be applied to study the circulating levels of various circRNAs in serum retrieved from septic and SS patients to demonstrate the use as a validation and diagnostic tool for sepsis disease progression. The main research questions that will be discussed in this thesis are as follows:

1. *How can circular RNAs be used as a biomarker for the diagnosis and prognosis of sepsis in hospitalized patients?*

With the following sub-questions:

   1.1. How can circHIPK3 be used as a biomarker for the diagnosis of sepsis in an in vitro *model of sepsis?*

   1.2. Which of the circHIPK3 downstream targets are altered in both the in vitro *model of sepsis and in septic and SS patients?*

Before the use patient samples, an *in vitro* cellular model was used where human umbilical vein endothelial cells (HUVECs) were treated with extracellular histones to reproduce a pathological condition which occurs in severe cases of sepsis (5). This previously described model, optimized by the Epigenetics and Epigenomics Translational Research Team at INCLIVA was used to obtain RNA to develop an optimized protocol for the detection of our circHIPK3 and its targets. After optimization, the protocol was applied to patient serum retrieved from hospitalized septic and SS patients of intensive care unit of the *Hospital Clinico Universitario de Valencia* to validate the developed protocol. Subsequently, downstream targets of circHIPK3 were checked in two different *in vitro* cellular models to help understand the sepsis pathologic pathway. The results help to enhance the development of biomarkers for the diagnosis and prognosis of sepsis and improve clinical outcome.

Previous research has shown that patients with sepsis or septic shock have altered levels of circulating circHIPK3 (38,39,52). It is therefore expected that this biomarker will show a positive result as being used as a biomarker.

2. Materials and Methods

2.1 Cell culture

Human umbilical vein cells (HUVECs) (REF. C2519A, Lonza, Basel, Switzerland) were cultured in completed EGM-2 Endothelial Medium Bulletkit (REF. H3CC-3162, Lonza, Basel, Switzerland) supplemented with 1% penicillin-streptomycin. Flasks were coated in 0,5% gelatine (REF. G9391-500G, Sigma-Aldrich, Burlington, Massachusetts, USA) for 5 minutes to ensure attachment of cells. HUVEC cultures were incubated in a Heracell 150i CO_2 incubator (Thermo Scientific, New York, NY, USA) in a humidified atmosphere with 5% CO_2 and 37°C. See appendix 1 for the complete protocol of the HUVEC culture.

2.2 Histone purification, quantification and treatment

As high levels of circulating histones are important mediators in sepsis and SS (5), HUVECs were treated with extracellular histones to reproduce the release of extracellular histones after NETosis in early stages during sepsis. See appendices 2 and 3 for the complete protocol for histone purification, quantification and treatment.

Purification

Histones were collected from HeLa cells (ATCC, Manassas, Virginia) which were cultured in Iscove's Dulbecco's Modified Eagle Medium (DMEM) High Glucose (REF. 11965092 Invitrogen, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Subsequently, histones were purified following a previously optimized protocol (5,54). HeLa cells were washed with cold PBS and centrifuged for 5 minutes at 2.000 rpm. The dry pellet was resuspended with 1 mL of Hypotonic Lysis Buffer (10 mM Tris-HCl pH=8, 1 mM KCl, 1,5 mM MgCl2) with 10 µL/mL of 95°C orthovanadate and 2 µL/mL of proteases inhibitor (Fisher Scientific, Hampton, USA). Cells were left rotating in a 4°C room for 30 minutes and then centrifuged for 10 minutes at 10.000 rpm. Finally, the dry pellet was resuspended thoroughly with 400 uL of 0.4 M H_2SO_4 and kept in rotation overnight at 4°C. On the following day, the cell suspension was centrifuged for 10 minutes at 13.000 rpm in 4°C. The supernatant, which contained the histones, was supplemented with 132 uL of 100% tricarboxylic acid to precipitate the histones. The mixture was incubated on ice for 30 minutes and centrifuged for 10 minutes at 13.000 rpm in 4°C. The supernatant was discarded, and the pellet was

washed with 500 uL of 4°C acetone. The cell suspension was centrifuged for 10 min at 13.000 rpm in 4°C and washed and centrifuged again. Finally, the pellet was resuspended in 50 uL of ddH_2O and stored in the freezer.

Quantification

In order to add the desired concentration of histones to the HUVEC cultures, the histone concentration of the previously prepared histone samples was measured following the Bradford Protein Assay. Firstly, 195 mL of 20% Bradford Reagent (REF. 500-0006, Bio-Rad Laboratories, Life Sciences Group, Hercules, California, USA) was added to the to a 96-well plate. Then, 5 uL of five different samples with known histone concentration (0 ug/mL; 0,25 ug/mL; 0,5 ug/mL; 1 ug/mL; and 2 ug/mL) and 5 uL of the histone sample were added to the wells and adequately mixed. The plate was incubated for 5 minutes at room temperature before being put in the spectrophotometer SpectraMax Plus 384 (Molecular Devices, Canada) to measure the shift in absorption in 595 nm. From the absorption rate of the known histone concentrations, the concentration of the desired histone sample was calculated.

Treatment

12-well plates were prepared with gelatine and completed EGM-2 growth medium before adding the cells. The HUVEC culture flasks were washed three times with 5 mL PBS before adding 1,5 mL of trypsin and incubated for 3 minutes in an incubator with 5% CO_2 and 37°C. Subsequently, cells were collected from the flask and centrifuged for 5 minutes at 10.000 rpm. The supernatant was discarded, and the pellet was resuspended in 1 mL of EGM-2. 10 uL of this cell suspension was mixed with 90 uL of PBS and 10 uL of this mixture was used to count cells using a hemocytometer. From the calculated number of cells in the cell suspension, the required volume was calculated in order to add ±100.000 cells/well. The cells were left in the incubator overnight. The next day, the wells were washed twice with 500 uL PBS. The calculated volume of the desired histone concentration was added to the wells and supplemented with EGM-2, hence every well had a final volume of 500 uL. Treatment was accomplished in the incubator for 4 hours. See *Table 1* for an overview of the desired histone concentration and respective volumes of histones and EGM-2 that were added. Each histone treatment condition was measured in quadruplets.

Table 1 – Once the main text is complete, I will add the table description.

Histone concentration [ug/mL]	Histone volume [uL]	EGM-2 volume [uL]
0	0	500
100	74,4	425,6
150	111,4	388,6
200	148,8	351,2
250	185,9	314,1

2.3 Flow cytometry

The cytotoxicity of the histone treatment on the HUVECs was evaluated with a flow cytometer (Backman EPICS XL-MCL) in the Flow Cytometry Unit of the Central Core Research Facilities in the Medicine and Dentistry School (University of Valencia). With this approach, we studied cell viability, apoptosis and necrosis, using the Annexin-V kit (REF. ANXVKF-100T, Immunostep S.L., Salamanca, Spain). Four replicates of the five histone treatment conditions of the HUVECs were prepared as previously described. After 4 hours of histone treatment in the incubator, the medium was collected from the wells and put in respective Eppendorfs. 500 uL of trypsin was added and incubated for 3 minutes at 37°C to collect all the remaining attached cells. The trypsin and cells were collected and added to the respective Eppendorfs. Eppendorfs were centrifuged for 5 minutes at 1.500 rpm. The pellets were resuspended in 100 uL of Annexin Binding Buffer mixture (1 mL Annexin Binding Buffer and 9 mL PBS), 5 uL of Annexin V and 5 uL of Propidium Iodide. The reaction was incubated for 15 minutes in a dark place. Lastly, ±250 uL of Annexin Binding Buffer mixture was added and transferred to a flow cytometry tube for analysis in the flow cytometer.

2.4 RNA extraction

HUVECs were treated with different histone concentrations as previously described. After 4 hours of histone treatment, the medium was discarded. The wells were washed twice with 500 uL room temperature PBS and 500 uL of Lysis Binding Solution (REF. 8540G21, Invitrogen, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) was added to every well. The lysis reagent was incubated for 5-10 minutes at room temperature. Subsequently, the wells were completely scratched with a scratcher to ensure all cells

were de-attached from the well walls. The scratcher was cleaned in between wells with alcohol and water to prevent exchange between different histone treatment conditions. The total volume was taken up and put in Eppendorf. The cells were stored at -20 °C overnight. The following day, mRNA was extracted using the *mir*Vana kit (REF. AM1560, Invitrogen, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) following manufacture's manuals. In short, 100 uL of miRNA Homogenate Additive was added, mixed well and left to incubate on ice for 10 minutes. Next, 1 mL of Acid-Phenol:Chloroform was added, vortexed and centrifuged for 5 minutes at 10.000 rpm at room temperature. The aqueous phase was transferred to a fresh tube where 1,25 mL of 100% ethanol was added. This mixture was pipetted onto a Filter Cartridge on top of the Collection tube and centrifuged for 15 seconds at 10.000 rpm whereafter the flow-through was discarded. This step was repeated until all mixture was passed through into the Collection tube. Three Filter Cartridge washing steps followed: once with room temperature 700 uL miRNA Was Solution 1 and twice room temperature 500 uL Wash Solution 2/3, finished with one final additional minute of centrifuging. All flow-through was discarded. The Filter Cartridge was transferred into a fresh Collection Tube and 100 uL of 95 °C Elution Solution was applied to the centre of the filter. The tube was centrifuged for 25 seconds at maximum rpm to recover the RNA. The tube with the flow-through and thus the RNA was stored at -20 °C. See appendix 4 for the complete protocol of the cell culture RNA extraction.

2.5 RNase R treatment

CircRNA is more stable than its mRNA precursor as it does not get degraded by exonucleases because of the lack of 5'- and 3'-end (18). Theretofore, all samples were treated with RNase R to degrade all linear RNAs and leave only circRNAs. First, a working solution of 1 U/uL of RNAse R enzyme (REF. RNR07250, Epicentre, Middleton, WI, USA) was created by diluting 20 U/uL RNase R enzyme with RNAse-free water (55). Two different master mixes were prepared: mock-treatment and treatment. The former master mix consisted of 1,5 uL RNase R Reaction Buffer (0,2 M Tris-HCl pH=8, 1 mM KCl, 1 mM MgCl2) (REF. RNR07250, Epicentre, Middleton, WI, USA) and 3,5 uL RNAse-free water per sample. The treatment master mix consisted of 1 uL RNase R enzyme working solution, 1,5 uL RNase R Reaction Buffer and 2,5 uL RNAse-free water per sample. Whereafter 10 uL of the RNA extract was treated with 5 uL of either the control or

treatment master mix. This reaction was incubated for 15 minutes at 37 °C. See appendix 5 for the step-by-step protocol.

Finally, all RNA samples were quantified using Nanodrop 2000 (Thermo Scientific, New York, NY, USA) in order to add an equal concentration of RNA to every well-reaction in the RT-qPCR. Furthermore, Nanodrop was used to assess the purity of the treated samples.

2.6 Primer design and validation

Since the detection of circRNAs is a new in the field of epigenetics, there lacks a previously designed and tested primer-pair for the detection of circRNAs. Therefore, we designed and validated our own primers which we can use in the detection and quantification of our circRNA of interest: circHIPK3. As previously mentioned, circHIPK3 is derived from exon 2 of the *HIPK3* gene (23). The nucleotide sequence for circHIPK3 starts at the 510th nucleotide and ends at the 1609th nucleotide of the *HIPK3* transcript variant 1. From the CircInteractome database (56), we identified that the first 100 and the last 98 nucleotides are part of the backsplice junction, forming the circHIPK3 variant. See *figure 2* for a schematic representation of the circHIPK3 genetic format. Since we are interested in the detection of the circRNA and all linear RNA was degraded during RNsae R treatment, our

Figure 2 – The nucleotide sequence of circHIPK3*. (hsa_circ_0000284) As part of the HIPK3 transcript variant 1 (NM_005734). In red the backsplicing regions from which the circHIPK3 is covalently closed as a loop. In yellow and orange, the PCR-product, where orange depicts the overlapping part of PCR-product and one backsplicing region.*

primer and primer-product had to cover the nucleotide sequence which is represented on exon 2. For the design of the primer, we used Primer-BLAST (57) on the NM_005734 template with the following specific parameters: PCR Product Size: minimal 100 bp, maximal 300 bp; Max Melting Temperature Difference 1°C; Primer Size: 18-22 bp; GC Clamp: 1; Primer GC content (%): 40/60 and Maximum Self & Pair Complementarity: 4/2. From the results we picked the primer-pair that would only cover the circHIPK3 transcript. We used the following primer-pair: F: 5'-GCTTTCAGCACCGTAACCATAC-3' and R: 5'-GATACATGACTGGCCGACCC-3'.

The primer-pair was validated using gel electrophoreses. A 2% working solution of agarose powder in TAE buffer was made and microwaved until all agarose had dissolved. Subsequently, 0,5 uL of SERVA DNA Stain G (REF. 39803.01, Quimigen, Madrid, Spain) was added and mixed gently. This mixture was poured into the mal with slots and dried for 15 minutes. All samples were completed with 1 uL of loading buffer. The electrolyte bath was filled with TAE 1x buffer and 4 uL of DNA ladder (REF. 3422A, Takara Bio USA, Inc., CA, USA) and 4 uL of samples were added to every respective slot. The bath was running on 80 Volt for 40 minutes. Finally, the the agarose gel was taken from the bath and put in the AMERSHAM ImageQuant800 imaging system with 535 nm light filter to image the RNA bands. The band sizes were manually compared on the computer to the DNA ladder.

For the reference gene we aimed to use a known and widely used housekeeping gene. We therefore analysed the identified circGAPDH templates from CircInteractome and confirmed that the used GAPDH primer pair F: 5'-TGGCAAATTCCATGGCACCG-3' and R: 5'-GACTTGATTTTGGAGGGATCTCGC-3' attaches to the circGAPDH (hsa_circ_0025178) sequence and gives a primer-product of 102 bp. Additionally, we examined with SYBR-Green RT-qPCR if the total amount of primer-product would decrease but not be totally eliminated when treating the samples with RNase R.

2.7 RT-qPCR

SYBR-Green

The SYBR-Green method was used for the detection of circHIPK3 because it binds to all double-stranded DNA molecules and does not require the design of a probe for a nucleotide-specific sequence. Firstly, a reverse transcription (RT) step was performed with all the samples before continuing with the polymerase chain reaction (PCR). For the

reverse transcription, the RT-kit (REF. 4366596, Applied Biosystems, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) was used to create a master mix with 2 uL random primers (5x), 0,8 uL of dNTPs (100 mM), 1 uL Multiscribe Reverse Transcriptase (50 U/uL), 2 uL of Reverse Transcription Buffer (10x) and 1 uL of RNAse inhibitor (20 U/uL) per sample. In a 96-well plate, 6,8 uL of this mixture was added and complemented with 200 ng of the samples and RNase-free water to a final volume of 20 uL. Reverse transcription was performed in the ThermoCycler 9800 Fast Thermal Cycler (Applied BioSystems, Thermo Fisher Scientific, New York, NY, USA) under the following conditions: 25°C for 10 minutes, 25°C for 2 hours and then 85°C for 5 minutes.

Subsequently, the PCR was performed using the SYBR-Green kit (REF. K0222, Thermoscientific, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania). Every desired well in a 384-well plate was prepared with 5 uL the SYBR-Green, 3,4 uL RNAse-free water and 0,3 uL of both the sense and antisense strand for the specific primer for each target gene. Finally, 1 uL of the cDNA from the RT-reaction was added to each corresponding well, hence every well had a final volume of 10 uL. Quantification was performed using the QuantStudio5 Real-Time PCR System (Thermo Scientific, New York, NY, USA) under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C to activate the polymerase, followed by 40 cycles for amplification, in which first the temperature was held at 95°C for 15 seconds and then dropped to 60°C for another minute. It then increased again to 95°C for 15 seconds, then decreased to 65°C where it remained for 1 minute, and finally increased again to 95°C, at which point was held for 15 seconds. Appendix 6 contains the full SYBR-Green RT-qPCR protocol.

Taqman

The TaqMan technology was used to quantify the miRNA targets of circHIPK3. Because this technology uses a TaqMan probe with a fluorescent dye and a quencher molecule, it is more specific and preferred in the quantification of known nucleotide sequences. For the RT-reaction, a master mix was created from the RT-kit (REF. 4369016, , Applied Biosystems, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) with 2 uL Reverse Transcription Buffer (10x), 0,4 uL dNTPs (100 mM), 0,25 uL of RNase inhibitor (20 U/uL), 4 uL of Multiscribe Reverse Transcriptase (50 U/uL) and 8 uL of the RT-primer pool (5x) with all desired RT-primers for the target genes, complemented with RNase-free water. To every desired well in the 96-well plate, 14,66 uL of RT-master mix is added and 200 ng

of the samples complemented with RNase-free water to a final volume of 20 uL per well. Reverse transcription was performed in the ThermoCycler 9800 under the following conditions: 16°C for 30 minutes, 42°C for 30 minutes and then 85°C for 5 minutes.

Succeeding, the PCR was performed for every target gene for which different master mixed were created. Per sample 5 uL TaqMan Gene Expression Mastermix (2x) (REF. 4369016, Applied Biosystems, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania), 0,5 uL TM primer for the specific target gene and 3,5 uL of RNAse-free water was added to the well of a 384-well plate. Every well was complemented with 1 uL cDNA of the sample to achieve a final volume of 10 uL. Quantification was performed using the QuantStudio5 Real-Time PCR System under the following conditions: 10 minutes at 95°C, followed by 45 cycles in which first the temperature was held at 95°C for 15 seconds and then dropped to 60°C for another minute. See appendix 7 for the complete protocol.

2.8 Statistical analysis

All data were analysed and visualized using GraphPad Prism version 9.5.0 for macOS (GraphPad Software, San Diego, California, USA). Data were checked for normality with the Shapiro-Wilk test ($p < 0,05$).

To compare Ct value or 2^{-DDCt} between two groups, an unpaired t-test ($p < 0,05$) was used. For multiple-group comparisons, an ordinary one-way ANOVA ($p < 0,05$) was employed, followed by Dunnett's multiple comparisons test for post-hoc analysis ($p < 0,05$). Non-normalized datasets were analysed using the non-parametrical Kruskal-Wallis test ($p < 0,05$) was used.

Data were presented as the mean ± standard deviation (SD).

3. Results

This work was designed to optimize the method of detection of circRNAs and its potential to be used as biomarkers for sepsis and septic shock. The experimental design for a preliminary approach to detect circHIPK3 was to generate a bio-model of acute damage induced by extracellular histones, which are considered Damage Associated Molecular Patterns (DAMPs) and has been previously described by the research group (5). This experimental model consisted of the use of an *in vitro* cell culture of human umbilical endothelial vein cells (HUVECs) treated with increased amounts of extracellular histones obtained from HeLa cells.

3.1 Bradford assay

We assessed the histone concentration obtained from acid extraction procedure of HeLas cells using the Bradford assay so we could treat HUVECs with a similar histone concentration each time. *Figure 3* presents the results of the Bradford assay, illustrating the absorbance values at 595 nm for a series of 4 BSA stock solution of known concentrations and the samples of interest. The absorbance values are plotted against the protein concentrations. The protein standards had the following average absorptions: 0 ug/uL: 0,278 ± 0,004 (N=3), 0,25 ug/uL: 0,486 ± 0,007 (N=3), 0,50 ug/uL: 0,633 ± 0,007 (N=3) and 1 ug/uL: 0,811 ± 0,010 (N=3). The protein standard calibration graph in *figure 3* shows a linear relationship between absorbance and protein concentration, with

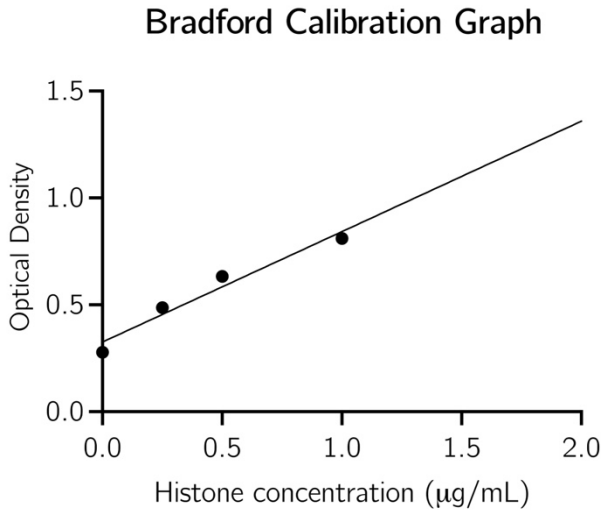

Figure 3 – Bradford Calibration Graph. *The histone concentrations from the HeLa cells were quantified using a Bradford Assay where four protein standards had the following average absorptions: 0 ug/uL: 0,278 ± 0,004 (N=3), 0,25 ug/uL: 0,486 ± 0,007 (N=3), 0,50 ug/uL: 0,633 ± 0,007 (N=3) and 1 ug/uL: 0,811 ± 0,010 (N=3). The linear regression line has the following formula: Optical Density=0,326*histone concentration+0,517.*

increasing absorbance at 595 nm as protein concentration increases. The linear regression line had the following formula:

$$Optical\ Density = 0{,}326 * histone\ concentration + 0{,}517$$

From this formula, the final volumes for histone treatment were interpolated, see *table 1* for an overview of the final volumes per histone concentration.

3.2 Extracellular histones induce apoptosis and necrosis in HUVECs

The previously described flow cytometry protocol evaluated the cytotoxic effect of extracellular histones on HUVECs. Four replicates of HUVECs were treated with 5 different extracellular histone concentrations: 0, 100, 150, 200 and 250 ug/mL. This fell in the range of previously described extracellular histone concentrations for HUVECs (5). The results are shown in *figure 4*. The results showed a decrease in cell viability corresponding with a higher concentration of extracellular histones, compared to the control condition (0 ug/mL), see *figure 4a.* Additionally, *figures 4b-f* showed an increase in Annexin V- and/or propidium iodide-positive cells with a higher concentration of extracellular histones. These increasing values correspond with the earlier findings

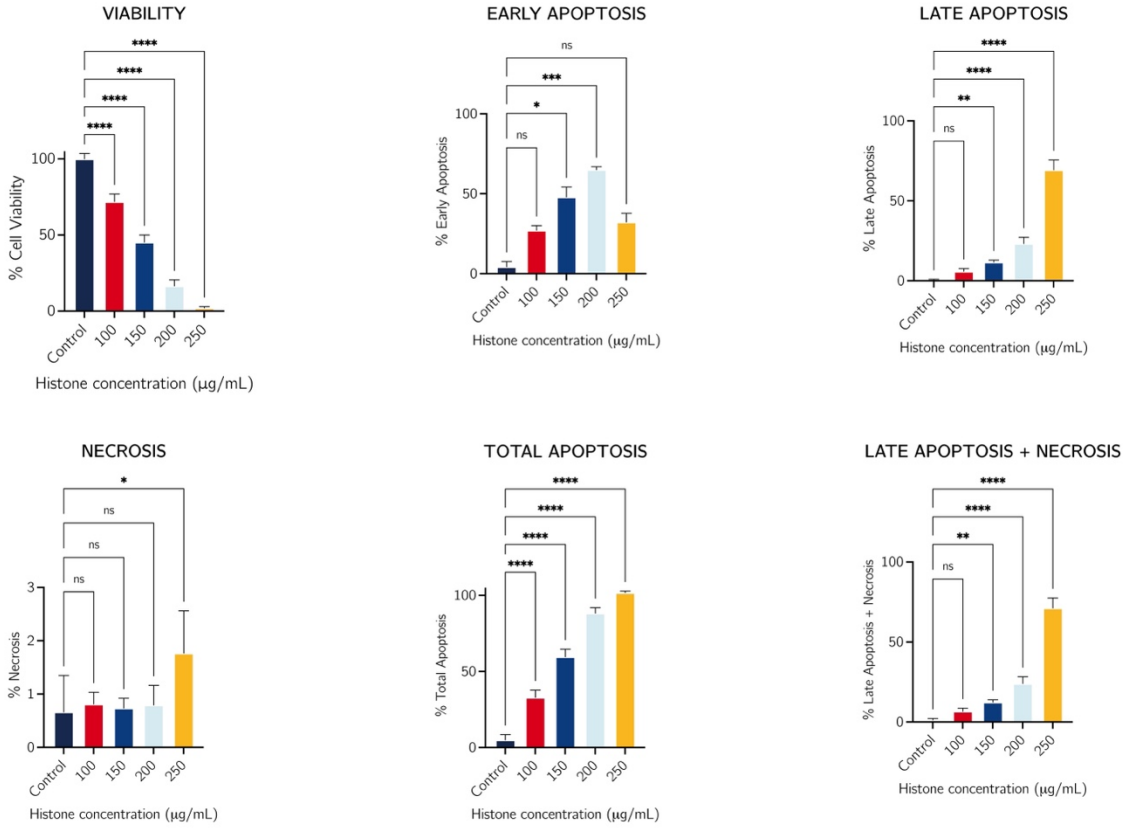

Figure 4 – Effect of extracellular histones on cellular viability, early apoptosis, late apoptosis and necrosis. *Increasing histone concentrations decrease cellular viability by activating the inflammasome and inducing increased apoptosis and necrosis.*

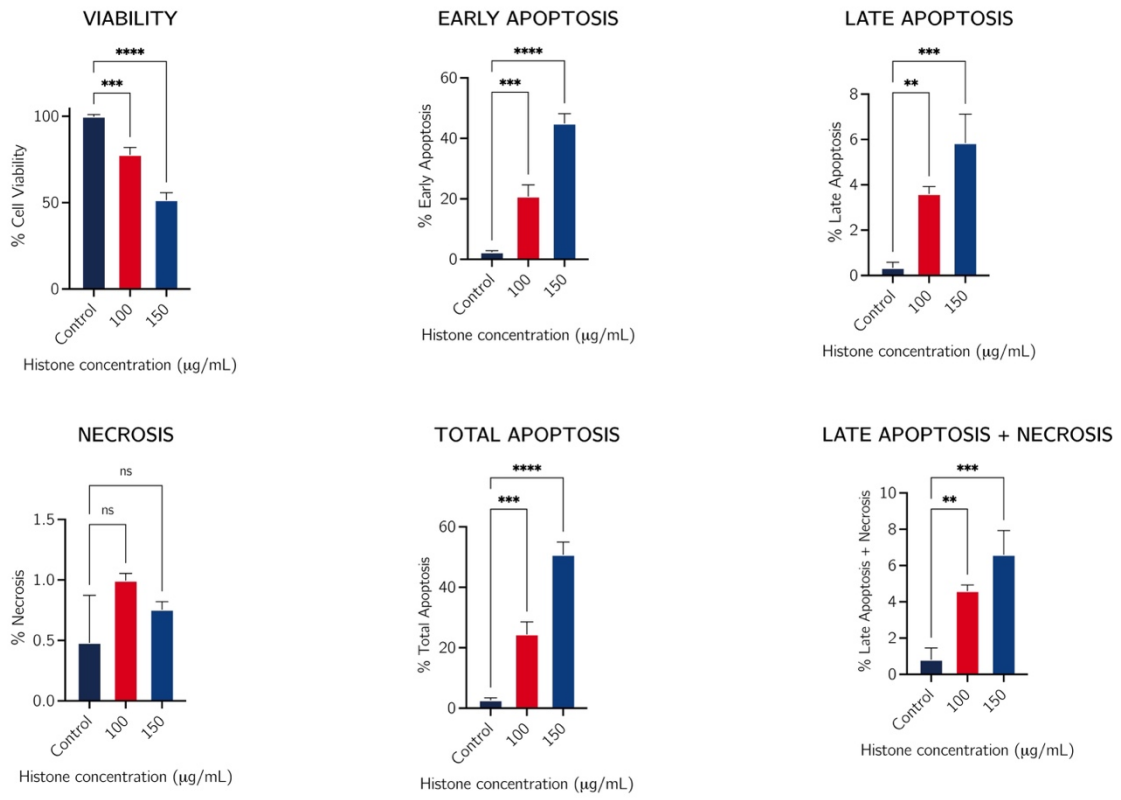

Figure 5 – Replicate of the first experiment. *Viability decreases while apoptosis and necrosis increase when HUVECs are exposed to increasing histone concentration.*

where extracellular histones were found to mediate the activation of the NLRP3 inflammasome to induce late apoptosis and necrosis (5). The experiment was replicated by quadruplets of 3 different extracellular histone concentrations: 0, 100 and 150 ug/mL, which yielded similar results, see *figure 5*. Both experiments showed ± 50% cell viability when 150 ug/mL of extracellular histones were added, which confirmed successful treatment without killing all cells for subsequent extraction of RNA.

3.3 Designed primers are specific for circHIPK3

After RNA extraction, RNase R treatment and SYBR-Green RT-qPCR of HUVECs, the amplified DNA products were visualized and analyzed using agarose gel electrophoresis. *Figure 6* displays the gel image, indicating the migration pattern of the amplified DNA fragments. The DNA ladder in lane 1 and 17 served as a size reference, allowing estimation of the fragment sizes in the experimental samples. The experimental lanes (lanes 2 to 14) exhibited bands at ~265 bp, indicating successful amplification of the target PCR-product using our own-designed primers. The size of the observed bands corresponds to the expected product size based on the primer design and target sequence in Primer-BLAST. These results indicate that the primer pairs were successful in specifically amplifying the

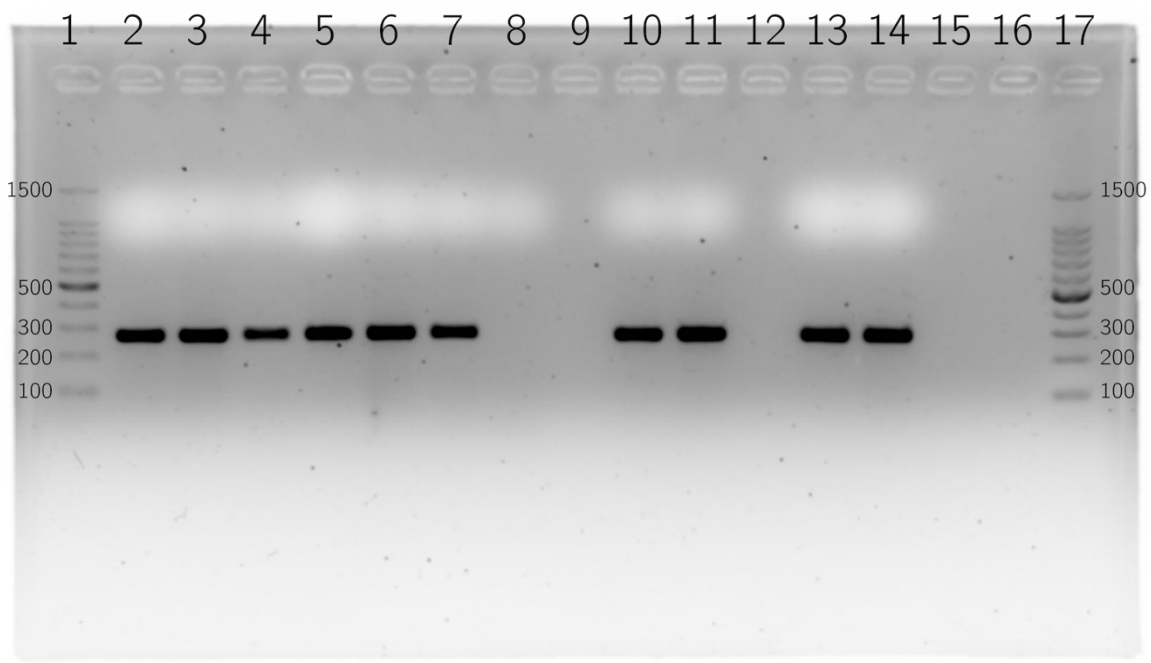

Figure 6 – Designed primers are specific for circHIPK3*. Lanes 1 and 17 contain the DNA Ladder with a maximum size of 1500 bps and minimum size of 100 bps as a size reference. The following lanes contained RNA samples that have been treated with RNase R: 2, 3, 4, 10 and 11. Contrarily, lanes 5, 6, 7, 13 and 14 contained RNA samples with mock-treatment. Lane 8 contains a blank and lanes 9, 12, 15 and 16 have been left open on purpose.*

intended target region. Importantly, no contamination or smearing was observed, indicating the absence of nonspecific amplification or degradation of the samples. The absence of additional bands or artifacts suggests that the designed primers exhibited high specificity and minimized unwanted amplification and the absence of a primer dimer by-product. The specificity of the SYBR-Green RT-qPCR assay was also again confirmed by the absence of non-specific amplification or primer-dimer artifacts. Only single peaks corresponding to the target genes were observed in the melt curve analysis. Furthermore, the primer-pair worked on both the RNase R treated samples (lanes 2, 3, 4, 10 and 11) and mock-treated samples (lanes 5, 6, 7, 13 and 14) as predicted.

3.4 GAPDH primer-pair binds to circGAPDH

To investigate if the widely used GAPDH reference mRNA and its primer-pair also binds to circGAPDH, we assessed the RNA levels in 12 RNase R treated and 12 untreated HUVEC samples. *Figure 7* shows the comparison of threshold cycle (Ct) values between treated and untreated samples. The mean Ct value in the mock samples was 15,79 ± 1,278 (N=12), indicating a relatively higher abundance of GAPDH. In contrast, the mean Ct value in the RNase R-treated samples was 20,14 ± 0,474 (N=12). Statistical analysis using an unpaired t-test demonstrated a significant difference in Ct values between the treated and mock

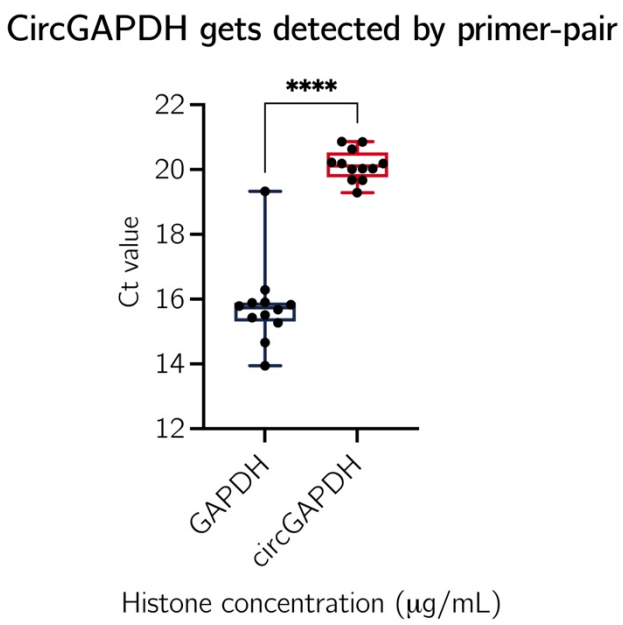

Figure 7 – GAPDH primer-pair also detects circGAPDH. *The Ct values increased significantly (p < 0,0001) after RNase R treatment from 15,79 (N=12) to 20,14 (N=12). The remaining circGAPDH after treatment was detected by the used GAPDH primer-pair.*

samples (p < 0,0001) and a corresponding decrease in the RNA levels of GAPDH. Even though we did not directly measure the relative linear GAPDH levels in both samples, the significant difference in RNA levels between treatment and mock treatment aligns with the expected function of RNase R, which selectively removes linear RNA, thereby reducing the abundance of GAPDH mRNA in the samples. Furthermore, since we detected Ct-values even after RNase R treatment, suggested that the used primer-pair also binds to the undegraded circRNA. Suggesting that this primer-pair holds as a reference gene even after RNase R treatment.

3.5 RNase R treatment should be done after RNA extraction, without extra clean-up

The aim of this experiment was to evaluate when RNase R treatment would be done in the protocol to yield the best results. For this we analysed RNA concentration and purity in HUVECs RNA-extracts. Three treatment conditions were tested: (1) RNase R treatment before the first clean-up step during the RNA-extraction protocol, (2) RNase R treatment after RNA-extraction, and (3) RNase R treatment after RNA-extraction with all clean-up steps repeated from the RNA-extraction protocol. All conditions were also performed with mock-treatment. The analysis aimed to determine the optimal timing for RNase R treatment to ensure high RNA concentration and purity.

Table 2 – Nanodrop results from HUVEC extracts during different RNase R treatment conditions. *Values in green have optimal RNA purity, values in black have average RNA purity and values in red indicate contaminated RNA samples by phenols..*

Sample	RNA [ng/uL]	260/280	A260/230
During extraction	7,4	1,92	0,82
During extraction-mock	41,3	1,56	0,46
After extraction	440,4	1,94	1,97
After extraction-mock	349,2	2,03	1,86
With extra clean-up	17298	1,10	1,00
With extra clean-up-mock	17237	1,08	1,00

Table 2 presents the results of the experiment, displaying the RNA concentration and purity values measured using a Nanodrop spectrophotometer. The RNA concentrations are expressed in ng/µL, while the purity is assessed by the 260/280 and A260/230 ratios. For the RNase R treatment during RNA-extraction condition, the RNA concentration was 7,4 ng/µL, with 260/280 and A260/230 ratios of 1,92 and 0,82, respectively; and mock-treatment yielded 41,3 ng/uL, with 260/280 and A260/230 ratios of 1,56 and 0,46. In the RNase R treatment after RNA-extraction condition, the RNA concentration was 440 ng/µL, with 260/280 and A260/230 ratios of 1,94 and 1,97, respectively; and mock-treatment yielded 349,2 ng/uL with 260/280 and A260/280 ratios of 2,03 and 1,86, respectively. Lastly, in the RNase R treatment after RNA-extraction with an additional clean-up step condition, the RNA concentration was 17298 ng/µL, with 260/280 and A260/230 ratios of 1,10 and 1,00, respectively; and mock-treatment yielded 17237 ng/uL with 260/280 and A260/230 ratios of 1,08 and 1,00, respectively. The 260/280 and A260/230 purity values were evaluated using *Table 3* (58).

Table 3 – Interpretation of Nandrop RNA purity (58).

Ratio	Value	Purity
260/280	2,0-2,2	Optimal RNA purity
	> 1,7	Acceptable RNA purity
	< 1,7	Contaminated RNA with aromatics
A260/230	> 2	Optimal RNA purity
	< 1,8	Contaminated RNA with salts, carbohydrates and phenols
	< 1,5	High contaminated RNA with salts, carbohydrates and phenols

The highest average RNA concentration was observed when RNase R treatment was performed after RNA-extraction. Additionally, these samples were overall purer. These findings suggest that performing RNase R treatment after RNA-extraction is the most effective approach to maximize RNA concentration in HUVECs.

3.6 Protocol shows high sensitivity for RNA detection

To assess the specificity and sensitivity of the SYBR-Green RT-qPCR assay, we performed a dilution curve analysis for the two target genes, GAPDH and HIPK3. The dilution curve allowed us to determine the lowest RNA concentrations at which reliable detection and quantification were achieved. For this, the RNA extracts obtained from HUVECs were diluted to four new concentrations: 1 ng, 25 ng, 50 ng and 100 ng and finally their respective number of copies. *Figure 8* shows the dilution curve profiles for GAPDH and HIPK3. Each data point is a measured in quadruplets and represents the relative Ct value obtained from qPCR analysis at different RNA input concentrations. The x-axis represents the number of copies of the target gene, while the y-axis represents the corresponding relative Ct values. The relative Ct value is calculated from the highest number of cycles needed for the lowest RNA volume (1 ng). For both GAPDH and HIPK3, the dilution curve profiles exhibited a consistent trend of decreasing Ct values with increasing RNA input concentrations. This indicates a reliable and consistent detection of the target genes

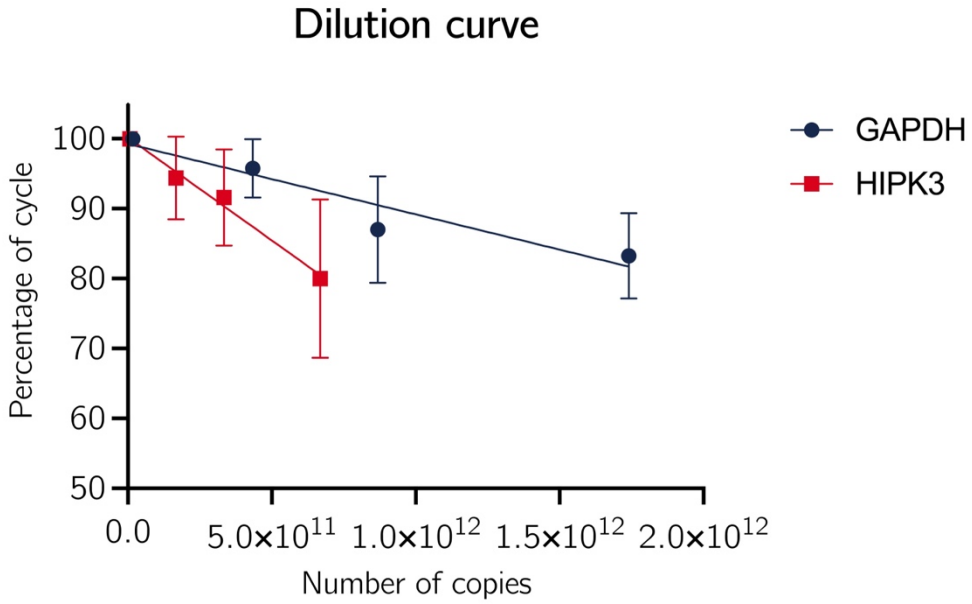

Figure 8 – Dilution curve. *Per different volume of RNA (1 ng, 25 ng, 50 ng and 100 ng) the relative difference in Ct value was calculated, with the lowest volume (1 ng) as reference. RNA volumes were converted into number of RNA copies to match the PCR-product. Both dilution curves depict a linear decreasing line. Even at the lowest volume (1 ng) there was a clear detection signal (GAPDH: 22.41 (N=5) and HIPK3: 28,09 (N=5), suggesting a high sensitivity of the assay.*

throughout the dilution series. The sensitivity of the assay was determined by the lowest RNA concentrations at which reliable detection was achieved. We observed consistent and reproducible Ct values for both GAPDH and circHIPK3 down to a concentration of 1 ng of input RNA. At this concentration, clear amplification signals were obtained: mean Ct value for GAPDH: 22,409 ± 3,236 (N=5) and for HIPK3: 28,090 ± 2,297 (N=5). This allows for accurate quantification of the target genes. Based on the dilution curve analysis, our findings demonstrate the high sensitivity of the SYBR-Green RT-qPCR assay for the detection of GAPDH and HIPK3. The absence of non-specific amplification and primer-dimer artifacts confirms the assay's specificity. Moreover, the reliable detection of both genes down to 1 ng RNA concentration indicates the assay's sensitivity.

3.7 Shorter RNAse R treatment time does not influence Ct value

The aim of this experiment was to investigate the influence of RNase R treatment time on the Ct values of two target genes, GAPDH and circHIPK3. We aimed to determine if a shorter treatment time had any effect on the Ct values, reflecting the RNA levels of the target genes. For this, 48 HUVEC RNA extracts were either treated with RNase R for 10 or 15 minutes and a final volume of 200 ng was. *Figure 9* illustrates the comparison of Ct values for circGAPDH and circHIPK3 under different RNase R treatment times. The x-axis represents the treatment time (10 and 15 minutes), while the y-axis represents the corresponding Ct values. To statistically analyze the data, we performed an unpaired t-

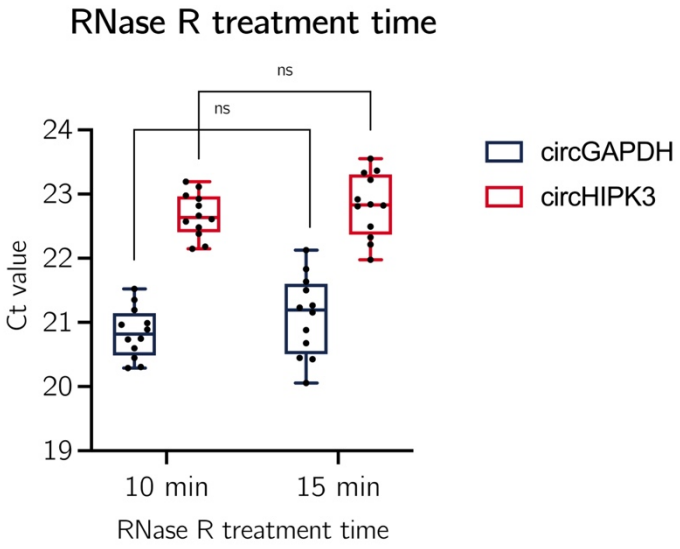

Figure 9 – Shorter RNase R treatment time does not influence circRNA concentration. *HUVEC samples were treated for either 10 or 15 minutes with RNase R. CircGAPDH Ct values increased insignificantly (p=0,2239) from 20,84 to 21,10 and circHIPK3 values increased insignificantly (p=0,3963) from 22,67 to 22,82.*

test to compare the Ct values between the 10-minute and 15-minute treatment groups for each target gene. The Ct values for circGAPDH were 20,840 ± 0,395 (N=12) and 21,100 ± 0,624 (N=12) respectively, while the Ct values for circHIPK3 were 22,670 ± 0,343 (N=12) and 22,82 ± 0,496 (N=12), respectively. The analysis revealed no significant differences in Ct values for either GAPDH (p = 0,2239) or circHIPK3 (p = 0,3963) when compared to the RNase R treatment time. These results indicate that a shorter RNase R treatment time, as short as 10 minutes, is sufficient to effectively degrade linear RNA molecules without significantly impacting the detection and quantification of GAPDH and circHIPK3. Furthermore, we observed that a 15-minute treatment time exhibited a greater SD (circGAPDH: 0,6240 and circHIPK3: 0,4956) compared to 10-minute treatment (0,3946 and 0,3434 respectively. The wide range and high variability of longer treatment indicate a greater dispersion of data points. This suggest that 15-minute treatment is influenced by various factors, leading to inconsistent and less reliable measurements. Additionally, the 10-minute treatment showed a higher level of precision and stability in RNA concentration measurements.

3.8 CircHIPK3 levels in HUVECs are unchanged following histone treatment

The aim of this experiment was to examine the effect of three different histone treatment concentrations (0 ug/mL, 100 ug/mL, and 150 ug/mL) on difference in circHIPK3 levels in HUVECs, calculated as 2^{-DDCt}. For this, each quadruplicate per different histone concentration received either RNase R or mock treatment. The 2^{-DDCt} value was used to quantify and compare gene expression levels between different experimental conditions, providing a relative measure of gene expression changes. The treated samples were analysed on changes in circHIPK3 levels, and the mock-treated samples were analysed on changes in HIPK3 levels. CircGAPDH and GAPDH were used as a reference gene for the respective analysis. *Figure 10* displays the results of the experiment, illustrating the 2^{-DDCt} value circHIPK3 (*figure 10a*) measured in RNA extracts from HUVECs treated with different histone concentrations. Likewise, in *figure 10b* the relative levels for HIPK3 obtained from HUVEC treated with different histone concentration are displayed. Furthermore, the 2^{-DDCt} value was calculated per different histone concentration for circHIPK3 relative to HIPK3 (*figure 11*).

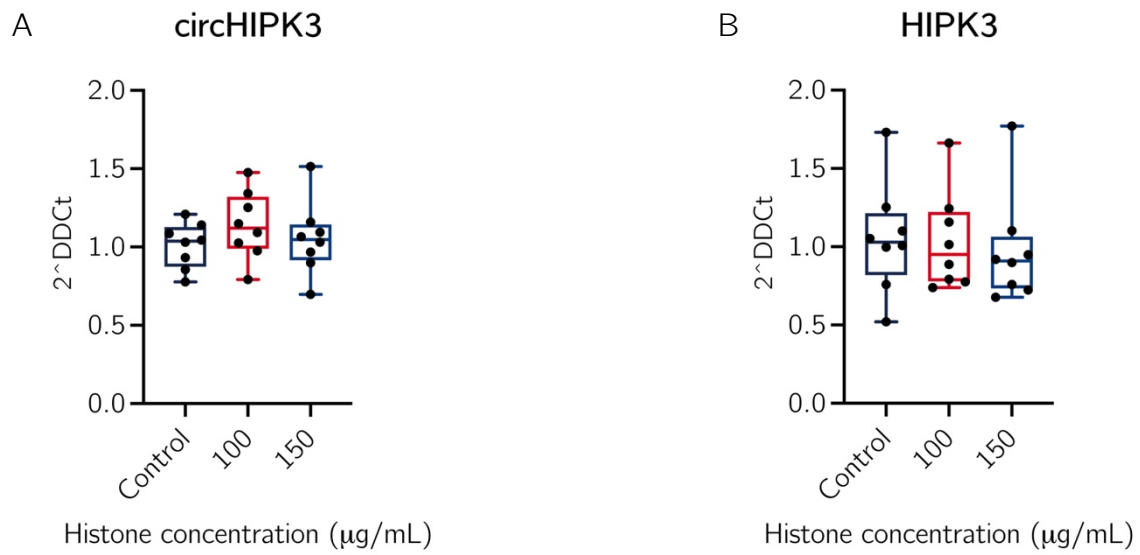

Figure 11 – The relative gene expression of circHIPK3 and HIPK3 are unchanged following histone treatment. *Relative gene expressions were calculated with the reference genes circGAPDH and GAPDH levels respectively. A) Treated samples: Control: 1,010 ± 0,146 (N=8), 100 ug/mL: 1,138 ± 0,217 (N=8) and 150 ug/mL: 1,054 ± 0,234 (N=8). Statistical analysis showed no significant difference between the three groups (p=0,4474). B) Mock-treated samples: Control: 1,053 ± 0,354 (N=8), 100 ug/mL: 1,034 ± 0,314 (N=8) and 150 ug/mL: 0,975 ± 0,350 (N=8). Likewise, statistical analysis did not show any significance between the three treatment groups (p=0,6565).*

For the RNase R treated samples, the following 2^{-DDCt} were found: Control: 1,010 ± 0,146 (N=8), 100 ug/mL: 1,138 ± 0,217 (N=8) and 150 ug/mL: 1,054 ± 0,234 (N=8). The 2^{-DDCt} values for the mock-treated samples were: Control: 1,053 ± 0,354 (N=8), 100 ug/mL: 1,034 ± 0,314 (N=8) and 150 ug/mL: 0,975 ± 0,350 (N=8). Finally, the relative gene expression levels between circHIPK3 and HIPK3 per different histone concentration were: Control: 1,127 ± 0,477 (N=8), 100 ug/mL: 1,309 ± 0,539 (N=8) and 150 ug/mL: 1,786 ± 1,129 (N=8). To evaluate the statistical significance of the observed differences, a

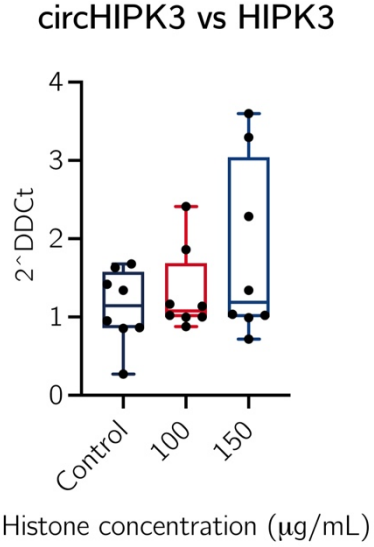

Figure 10 – The relative gene expression of circHIPK3 is unchanged compared to HIPK3 following histone treatment. *Relative gene expression of circHIPK3 was calculated with HIPK3 as a reference. The following 2^{-2DDCt} values were found: Control: 1,127 ± 0,477 (N=8), 100 ug/mL: 1,309 ± 0,539 (N=8) and 150 ug/mL: 1,786 ± 1,129 (N=8). No statistical significance was found between the different histone concentrations (p=0,5054).*

one-way ANOVA was conducted. The analysis demonstrated no significant difference among the histone treatment concentrations with p-values of 0,4474 (treated samples), 0,6565 (mock-treated samples) and 0,5054 (circHIPK3 vs HIPK3 levels). These results suggest that circHIPK3 and HIPK3 levels in HUVECs remain relatively unchanged following histone treatment. The small mean differences observed for the 2^{-DDCt} across the histone treatment concentrations indicate a consistent maintenance of circHIPK3 expression levels, comparable to the control condition.

3.9 miRNA levels of downstream target miR-124 in HUVECs are changed following histone treatment

In this experiment we aimed to investigate if some miRNAs, which are the downstream targets of circHIPK3 showed changes in their relative expression levels under different histone concentrations (0 ug/mL, 100 ug/mL and 150 ug/mL). Changes in gene expression levels of the three genes of interest miR-29b, miR-124 and miR-148b were measured with TaqMan RT-qPCR and expressed in 2^{-DDCt} values relative to the reference gene RNU48 in HUVECs. The analysis aimed to assess any changes in downstream miRNA levels following histone treatment. *Figure 12* illustrates the results of the experiment, presenting the mean gene expression levels (2^{-DDCt}) for each target gene and histone

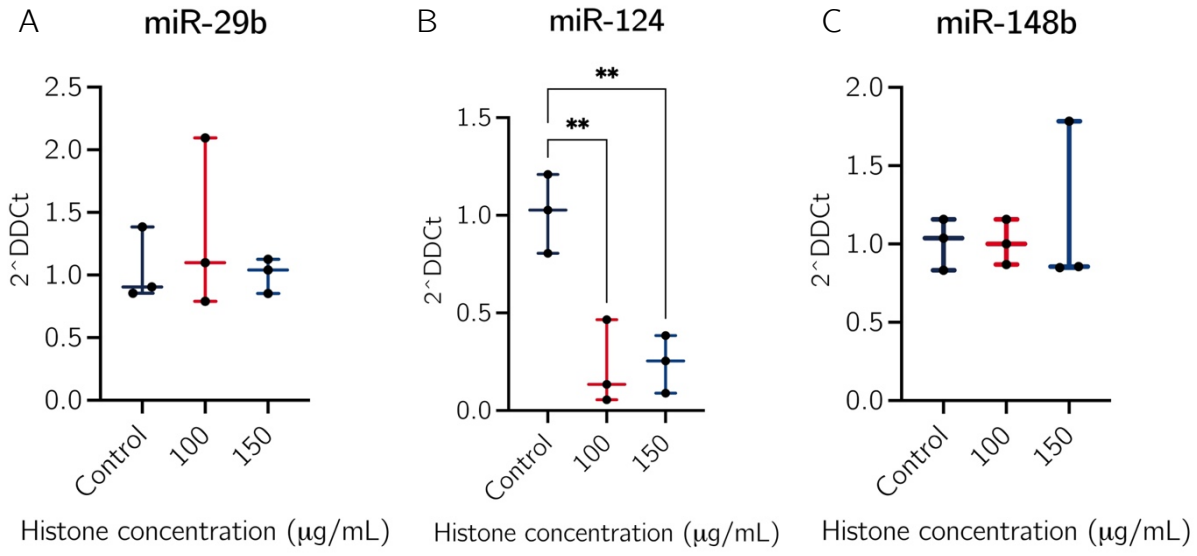

Figure 12 – Different histone concentrations do not have an effect on the downstream targets of circHIPK3. *A) Relative gene expression levels of miR-29b: Control: 1,048 ± 0,292 (N=3), 100 ug/mL: 1,327 ± 0,682 (N=3) and 150 ug/mL: 1,007 ± 0,140 (N=3). B) miR-124: Control:1,015 ± 0,202 (N=3), 100 ug/mL: 0,218 ± 0,217 (N=3) and 150 ug/mL: 0,242 ± 0,148 (N=3). C) miR-148b: Control: 1,009 ± 0,165 (N=3), 100 ug/mL: 1,009± 0,145 (N=3) and 150 ug/mL 1,164 ± 0,538 (N=3). All RNA expression levels were measured relatively to RNU48. Statistical analysis showed no significant change in gene expression levels with increasing histone levels for miR-29b (p=0,640) and miR-148b (p=0,950). However, miR-124 levels showed a significant decrease for increasing histone levels between the control (p=0,004 and p=0,005, respectively).*

treatment concentration. For the target gene miR-29b (*figure 12a*), the mean gene expression levels relative to RNU48 were 1,048 ± 0,292 (N=3), 1,327 ± 0,682 (N=3) and 1,007 ± 0,140 (N=3) for the control, 100, and 150 histone concentrations, respectively. Similarly, for the target gene miR-124 (*figure 12b*), the mean gene expression levels relative to RNU48 were 1,015 ± 0,202 (N=3), 0,218 ± 0,217 (N=3) and 0,242 ± 0,148 (N=3) for the control, 100, and 150 histone concentrations, respectively. In the case of target gene miR-148b (*figure 12c*), the mean gene expression levels relative to RNU48 were 1,009 ± 0,165 (N=3), 1,009 ± 0,145 (N=3) and 1,164 ± 0,538 (N=3) for the control, 100 ug/mL, and 150 ug/mL histone concentrations, respectively.

Statistical analysis was performed using a one-way ANOVA to determine any significant differences in the gene expression levels of the target genes among the histone treatment groups. The analysis revealed no significant effect of the histone treatment concentrations on the expression levels of genes miR-29b (p=0,640) and miR-148b (p=0,950) compared to the control group in HUVECs. These findings indicated that the downstream miRNA levels in HUVECs remained relatively unchanged following the histone treatment. However, further analysis revealed a significant effect of histone treatment concentrations on the expression levels of miR-124 (p=0,004). Post-hoc analysis showed a significant decrease in the gene expression levels of miR-124 between the control group and the 100 ug/mL histone concentration group (p=0,004) and between the control group and the 150 ug/mL histone concentration group (p=0,005). These results indicate that the expression levels of miR-124 were significantly altered following histone treatment with increasing histone concentrations.

3.10 Downstream miRNA levels in HEK-239 cells are unchanged following histone treatment

Since the downstream miRNA targets of circHIPK3 are also expressed in Human embryonic kidney 293 cells (HEKs), we were interested to check if the downstream targets would be differentially expressed in that cell-line under increasing histone concentrations. For this, we used the RNA-extracts from HEK-239 cells and histone concentrations of 0 ug/mL, 150 ug/mL and 200 ug/mL. These histone concentrations were determined during flow-cytometry where we aimed to find the histone

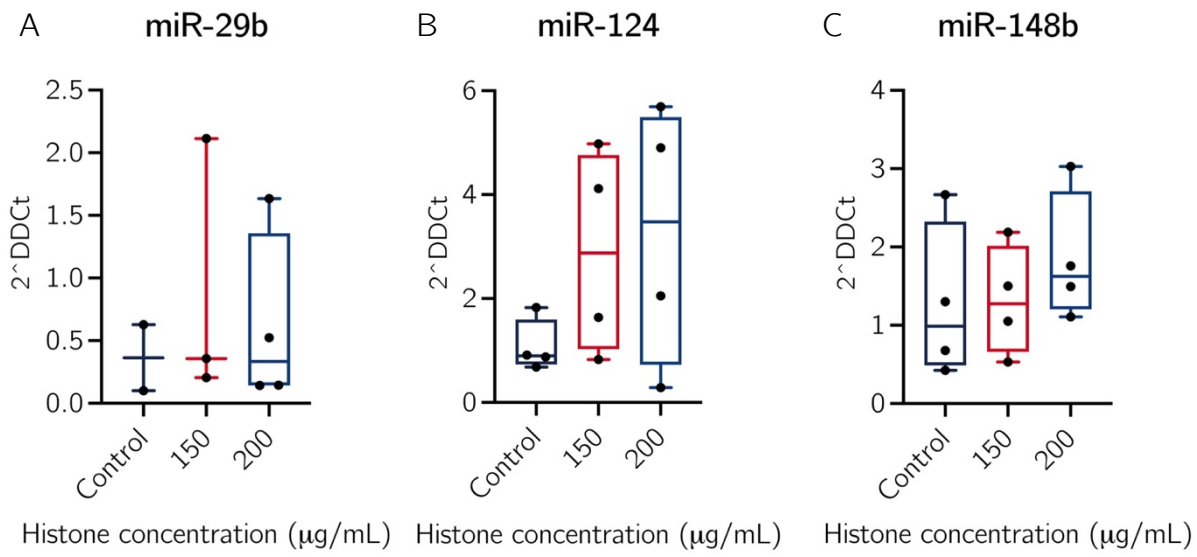

Figure 13 – Different histone concentrations do not have an effect on the downstream targets of circHIPK3 in HEK-239 cells. *A) Relative gene expression levels of miR-29b: Control: 0,365 ± 0,373 (N=2), 150 ug/mL: 0,892 ± 1,061 (N=3) and 200 ug/mL: 0,612 ± 0,706 (N=4). B) miR-124: Control: 1,075 ± 0,512 (N=4), 150 ug/mL: 2,890 ± 1,973 (N=4)) and 200 ug/mL: 3,234 ± 2,510 (N=4)). C) miR-148b: Control: 1,268 ± 1,003 (N=4), 150 ug/mL: 1,318 ± 0,702 (N=4) and 200 ug/mL: 1,847 ± 0,831 (N=4). All RNA expression levels were measured relatively to RNU48. Statistical analysis showed no significant change in gene expression levels with increasing histone levels for all the target genes miR-29b (p=0,775), miR-124 (p=0,265) and miR-148b (p=0,587).*

concentration that inflicts 50% viability. Changes in gene expression levels were measured with TaqMan RT-qPCR and expressed in 2^{-DDCt} values relative to the reference gene RNU48. *Figure 13* displays the results of the experiment, presenting the mean gene expression levels (2^{-DDCt}) for each target gene and histone treatment concentration. For the target gene miR-29b (*figure 13a*), the mean gene expression levels relative to RNU48 were 0,365 ± 0,373 (N=2), 0,892 ± 1,061 (N=3) and 0,612 ± 0,706 (N=4) for the control, 150, and 200 histone concentrations, respectively. Similarly, for the target gene miR-124 (*figure 13b*), the mean gene expression levels relative to RNU48 were 1,075 ± 0,512 (N=4), 2,890 ± 1,973 (N=4) and 3,234 ± 2,510 (N=4) for the control, 150, and 200 histone concentrations, respectively. In the case of target gene miR-148b (*figure 13c*), the mean gene expression levels relative to RNU48 were 1,268 ± 1,003 (N=4), 1,318 ± 0,702 (N=4) and 1,847 ± 0,831 (N=4) for the control, 150, and 200 histone concentrations, respectively.

Similar statistical analysis was performed to determine any significant differences in the gene expression levels of the target genes among the histone treatment groups. The analysis revealed no significant effect of the histone treatment concentrations on the expression levels of all the target genes miR-29b (p=0,775), miR-124 (p=0,265) and miR-148b (p=0,587) compared to the control group. These findings indicate that the

downstream miRNA levels in HUVECs remained relatively unchanged following the histone treatment.

3.11 CircHIPK3 levels are undetectable in patient's plasma

Finally, we aimed to assess the levels of circHIPK3 in plasma samples obtained from sepsis patients and control subjects. A total of six sepsis patient serum samples and six control serum samples were extracted for RNA, treated with RNase R, and measured following the SYBR-Green RT-qPCR protocol as described in the *Materials and Methods* section. However, due to the limited availability of plasma, a reduced amount of RNA (127,920 ± 36,702 ng, N=22) was used per sample instead of the intended 200 ng. The results of the qPCR analysis are displayed in Table 4. All samples were measured in triplicates, but it is worth noting that mock-treated samples control 3 and control 5 did not contain any RNA and therefore do not have available data (n.d.a.). Upon analysis, it was observed that most replicates of the patient samples did not yield any detectable signal for HIPK3 and circHIPK3. Moreover, the Ct-values for GAPDH were on average a lot higher (32,645 ± 2,966 (N=10)) than in the HUVEC experiments (15,79 ± 1,278 (N=12)), indicating lower RNA levels in the patient's serum. This result suggests that the amount of RNA in patient samples was below the detection limit of the applied protocol.

Table 4 – Average Ct values for GAPDH, circGAPDH, HIPK3 and circHIPK3. *All samples have been measured in triplets, however, not all replicates yielded a Ct value and is therefore not represented. Most HIPK3 and circHIPK3 values are undetermined which means that there is too little RNA to be detected within the SYBR-Green assay. For Control 3 and Control 5 there is no data available (n.d.a) under the mock-treated samples.*

Sample Name	Av. Ct-value GAPDH	Av. Ct-value circGAPDH	Av. Ct-value HIPK3	Av. Ct-value circHIPK3
Patient 1	27,916 ± 0,113 (N=3)	28,396 ± 0,297 (N=3)	31,827 ± 0,201 (N=3)	33,620 (N=1)
Patient 2	31,846 ± 0,245 (N=3)	31,573 ± 0,359 (N=3)	33,182 ± 0,580 (N=2)	33,811 (N=1)
Patient 3	33,009 ± 0,953 (N=3)	33,155 ± 0,181 (N=3)	Undetermined	Undetermined
Patient 4	30,766 ± 0,217 (N=3)	34,220 ± 2,208 (N=3)	33,826 (N=1)	Undetermined
Patient 5	28,712 ± 0,252 (N=3)	35,890 ± 1,916 (N=2)	32,713 ± 0,726 (N=2)	Undetermined
Patient 6	31,870 ± 0,382 (N=3)	31,640 ± 0,210 (N=3)	Undetermined	Undetermined
Control 1	34,038 ± 0,436 (N=3)	33,390 ± 0,146 (N=3)	Undetermined	Undetermined
Control 2	36,154	34,188 (N=1)	Undetermined	Undetermined
Control 3	n.d.a.	Undetermined	n.d.a	Undetermined
Control 4	34,138 ± 1,341 (N=3)	35,336 ± 1,167 (N=2)	Undetermined	Undetermined
Control 5	n.d.a.	34,331 ± 0,883 (N=2)	n.d.a.	Undetermined
Control 6	38,005 (N=1)	35,418 (N=1)	33,586 (N=1)	Undetermined

4. Discussion

The aim of this thesis was to optimize the detection method of circular RNAs and explore their potential use as biomarkers for sepsis and septic shock. Specifically, we aimed to investigate the use of circHIPK3 as a biomarker for the diagnosis and prognosis of sepsis in hospitalized patients, because of the previous literature proposing this circRNA as a potential biomarker (38,39,52). To address this objective, we designed a series of experiments and evaluated the expression patterns of circHIPK3 and its downstream targets in a cellular model and plasma samples from patients admitted in the intensive care unit of the *Hospital Clinico Universitario de Valencia*.

Our first sub-question aimed to assess the utility of circHIPK3 as a diagnostic biomarker for sepsis in an *in vitro* model. For this we treated HUVECs with extracellular histones to reproduce the release of extracellular histones after NETosis in early stages during sepsis. Our findings in HUVEC showed that circHIPK3 expression levels were not significantly altered with increasing concentrations of extracellular histones. This finding was not expected because of our previous literature research where circHIPK3 is altered both in an *in vitro* model of sepsis and *in vivo* septic mice and patient (38,39,52). These studies reported altered expression of circHIPK3 in LPC-induced cells and serum of patients. Even though these results were obtained from a different cellular model than we have used, other research has found significant changes in circHIPK3 levels in HUVECs (59,60). This suggests that circHIPK3 can indeed be detected in HUVEC but may not be directly influenced by histone-mediated cellular stress. Therefore, it could be interested to evaluate the change in gene expression of different circRNAs when HUVECs are treated with extracellular histones.

Alternatively, to confirm this result, we examined the expression levels of circHIPK3 downstream targets, including miR-124, miR-29b, and miR-148b in HUVECs exposed to increased histone concentrations. Only miR-124 showed a significant increase in expression, while miR-29b and miR-148b remained unaffected. However, it is worth noting that the Ct values of miR-124 were on average 39,132 ± 2,259, suggesting very low expression levels and therefore suggesting not very reliable results. Generally, Ct values higher than 30 are considered as 'undetectable' (61). Therefore, this outcome should be interpreted with high caution. Additionally, in HEK-293 cells, none of the downstream targets showed significant up- or down-regulation following increased histone

concentrations. However, the wide range of miR-124 expression in HEK-293 cells with increasing histone concentrations (Control: 1,145, 100 ug/mL: 4,150 and 150 ug/mL: 5,403) suggest that there is greater variation in the data, possible due to the effects of extracellular histone cytotoxicity.

In patient plasma samples, we encountered challenges in detecting circHIPK3 and HIPK3 levels. The Ct values of the reference gene GAPDH in patient plasma were considerably higher compared to the cellular HUVEC model whilst HIPK3 and circHIPK3 levels were undetectable, indicating low RNA abundance in the samples. Despite quantifying RNA concentrations using a nanodrop and adding 127,920 ± 36,702 ng per sample, it appears that there is a difference in detecting RNA between plasma and cell culture in terms. This could be the fact that RNA is generally present as fragmented molecules instead of intact transcripts in plasma (62). Furthermore, the patient samples showed on average a 260/280 ratio of 1,138 ± 0,268 and a A26/230 ratio of 0,230 ± 0,231. The first ratio suggests sample contamination with aromatics while the latter ratio suggest a very high sample contamination with salts, carbohydratesPla and phenols. This finding should be considered when continuing with the establish protocol for detecting circRNA in patient samples. Although recent studies have proposed that circRNAs are highly stable, there is still a need to completely characterize the stability and integrity of different circular RNAs in different types of biospecimens.

Furthermore, future directions should investigate the difference in expression levels of various circRNAs and their associated miRNAs in patient samples to contribute to creating a more whole epigenetic profile for sepsis and SS patients. Circulating levels of other circRNAs have been shown to be altered during sepsis disease progression (8,41,42,44,46,47,53) and could potentially be used as biomarkers. All circRNA biomarkers could improve our understanding of sepsis pathophysiology and could be used in the clinic to quickly diagnose patients and treat them accordingly to improve clinical outcome.

In conclusion, our study developed a protocol for the detection of circHIPK3 and explored the use of circHIPK3 as a biomarker for sepsis diagnosis and prognosis. While the expression levels of circHIPK3 and its downstream targets did not show significant alterations with increasing histone concentrations in HUVECs and HEK-293 cells, our findings contribute to the development of a new procedure to be implemented in the

laboratory which may set the basis for the future research of circRNAs and their potential role as biomarkers of sepsis and SS patients. However, the challenges encountered in detecting circRNAs in patient plasma samples emphasize the need for further optimization and validation of protocols for biomarker discovery. Ultimately, a more complete epigenetic profile of hospitalized patients can be developed to improve clinical decision-making, improve sepsis diagnosis and prognosis, and prevent chronic illness following sepsis.

4.1 Limitations

There were several limitations when conducting this research. First, we did not directly measure the presence of linear mRNA after RNase R treatment. This could have provided valuable insights into efficiency of the RNase R treatment and the possible presence of residual linear mRNA following treatment. Any residual linear mRNA would be picked up by the used primers and labeled as circRNA since the used primers are able to bind to the linear and circular form of our target and reference RNA. Therefore, it would be of additional value to perform an evaluation of the RNA Integrity Number (RIN) to assess RNA quality. Furthermore, the small volume used for patient samples (67,583 ± 33,264 µL) compared to the standard volume (200 µL) used for similar assays (63) may have resulted in too low concentrations of RNA which contributed to the difficulty in detecting (circ)RNA. The relative concentrations of RNA in the patient samples could be checked by measuring the mRNA levels in these samples and compare them to previously conducted mRNA RT-qPCR assays. This could help determine if the low RNA levels are part of patient samples or if there are technical issues with our suggested protocol.

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Appendix

Appendix 1: Cell culture protocol

Materials

- HUVECs
- Culture medium
- Penicillin (50 U/mL)
- Streptomycin (50 ug/mL)
- Gelatine
- Distilled water
- PBS tablet
- Trypsin

- T-25/T-75/Petri-dish
- 15 mL/30 mL tube
- Pipet (sizes: 1000 mL & 10 mL & 25 mL)
- Incubator
- Hot bath
- Centrifuge
- Cryotube

Day 1

Prepare the gelatine mix that is used to plate the flasks or Petri-dishes to ensure attachment of the HUVECs:

1. Mix 0,5 g of gelatine with 100 mL of distilled water in a bottle.
2. Let it mix with a magnetic stirrer until it is dissolved.
3. Autoclave to sterilize.

Prepare the growth medium:

1. Follow the instructions of the manufacturer and mix all supplements well.
2. Add 5 mL of s/p.

Day 2

Ensure that the materials that are going to be used at the same temperature as the cells, to avoid possible damage. Take the gelatine, PBS, culture medium, and trypsin out of the refrigerator and put them in a hot bath for 20-30 minutes to warm up and reach the temperature of the culture (about 30-36 °C).

1. Discard the medium of the old flask.
2. Wash three times with ± 5 mL PBS.
3. Add 1,5 mL of trypsin to separate the adhered cells.
   1 mL in the T-25, 1,5 mL in the T-75 and 2 mL in the Petri-dishes.
4. Incubate the cells at 37 °C for about 2-3 minutes. Check after a couple of minutes if the cells are detached.
5. Neutralize the trypsin with ± 5 mL growth medium. Discard the medium from the top of the bottom and make it run down over all the cells. Take up the cells and medium and repeat the taking up and discarding 1 or 2 times.
6. Pass the mixture of cells and medium into a 15 mL tube.
7. Repeat steps 5 and 6 for all the flask until you caught all the cells.
8. Centrifugate for 5 min at 1000 rpm.
9. Discard the medium, leaving the pellets with cells at the bottom of the tube.
10. Store the tube at -20°C for experiments or continue with cell passage or conservation.

Cell passage

After the collection of the cells, you can either use them for your experiments or pass them to a different flask to ensure continuous growth. For each day you want them to grow with enough medium.

1. Coat the flask or Petri-dish with the gelatine mixture: 3 mL for a T-75 flask or 5 mL for a Petri-dish.
2. Let it set for 5 minutes at room temperature and discard the spare.
3. Add culture medium: 5 mL for the T-25, 10 mL for the T-75 and 15 mL for the Petri-dish.
4. Resuspend the pellet from the 15 mL tube with X uL of medium. The amount of medium that needs to be added depends on the number of new flasks: add 500 uL of medium per flask. Ensure homogeneity by pipetting up and down.

Note: If you don't want to prepare a lot of different flasks, or you want to leave the cells for a longer period without having to repeat everything. You can resuspend the cells (step 4) in a greater amount of medium.

5. Take 500 uL of the resuspended mixture in the 15 mL tube and add to the new flask directly into the growth medium. Repeat for all the new flasks. Finally, swing north-south-east-west to ensure homogeneity.
6. Store it in the 37°C incubator.

Conservation

1. Write down the cell type, number of passes, date of conservation and name on the cryotube (pink lid).
2. Add 1 mL of freezer medium (10% DMSO and 90% FBS).
3. Resuspend the pellet of cells from the 15 mL tube. Pipet up and down to ensure homogeneity.
4. Pass the total mixture in the cryotube (on the side of the tube).
5. Store at -80°C.

Appendix 2: Histone purification, quantification and treatment protocol

Materials

- Buffer lysis
- Protease inhibitor
- Orthoborate
- PBS
- H_2SO_4
- 100% TCA
- Acetone
- ddH_2O

Day 1

Thaw all the tubes with the pellet for 10 minutes at room temperature before you start and put the centrifuge at 4°C.

1. Wash the cells with 500 uL cold 4°C PBS and resuspend.

   Note: If the pellet is big, use 1 mL and put in two different Eppendorfs.
2. Transfer the suspension to an Eppendorf.
3. Centrifuge for 5 min at 2.000 rpm in 4°C.

4. Discard the supernatant by suctioning.

Start with preparing the buffer mixture. Usually, you will have 10 new Eppendorf tubes with cells. And you need to add 1 mL of buffer mixture per tube. Therefore, you can prepare 10 mL of buffer. The buffer contains lysis to break the cells, orthoborate and protease inhibitor to protect the histones and prevent them from degrading and for maintaining them.

5. Create buffer mixture: add 10 mL of hyptonic lysis buffer
6. Add one protease inhibitor tablet (final concentration: 2 uL per 1 mL of buffer)
7. Add 100 uL of 95°C orthovanadate (final concentration: 10 uL per 1 mL of buffer)
8. Resuspend pellet in 1 mL of mixture.
9. Rotate suspension for 30 min in a cold room (4°C).
10. Centrifuge for 10 min at 4°C at 10.000 rpm.
11. Resuspend the pellet in 400 uL of H_2SO_4 (sulfuric acid) at 0.4 M.

    Note: the amount of H_2SO_4 is in relation to the desired concentration (e.g., if you want 0.4 M, you must add 400 uL). Important to resuspend very well (or vortex), with no clumps left in the solution as the nuclei have to be suspended.
12. Put the suspension in rotation overnight at 4°C.

At the end of the day, you will have the cells ruptured and free histones in the solution, ready to isolate them on day 2 and measure the concentration.

<u>Day 2</u>

On the second day you are going to isolate and count the histones.

1. Collect the Eppendorfs that were left in rotation in sulfuric acid overnight.
2. Centrifuge for 10 min at 13.000 rpm at 4°C.
3. Transfer the supernatant to a new Eppendorf tube. (You want to keep the supernatant since it contains the histones.)
4. Add 132 uL of 100% TCA drop-by-drop and invert the tube several times to mix.

   Note: TCA is used because it will bind to the histones and causes them to precipitate.
5. Incubate on ice for 30 min, inverting the tube every 10 min.
6. Centrifuge for 10 min at 13.000 rpm at 4°C. Discard the supernatant with a pipette.
7. Wash the pellet with 500 uL of 4°C acetone.

   Note: The histones will form a viscous mass, therefore it is important to resuspend the histones every time to ensure proper washing. OR: Carefully remove supernatant with pipette and wash histone pellet with ice-cold acetone without

disturbing it. Acetone is used to remove acid from the solution without dissolving the protein pellet.

8. Centrifuge for 10 min at 13.000 rpm at 4°C. Discard the supernatant. Perform two washes (steps 7 & 8).
9. Let the pellet dry and the remaining acetone to evaporate.
10. Resuspend pellets in 50 uL of ddH_2O and store in freezer.

    Note: When the pellet is big, use 100 uL of ddH_2O.

In this step you have isolated the histones and are ready to measure the concentration.

Measure histone concentration

The Bradford Assay is used for measuring histone concentration. When the Bradford reagent binds to histones, it transforms from a brown colour to a blue colour. This creates a shift in absorption maximum (~470 nm to ~595 nm).

1. Create a mixture of Bradford Reagent and sterile H20 (1:5).
2. Add 195 uL of Bradford Reagent to the wells where you want the reaction.
3. Add 5 uL of the following into the 96-well plate:
    a. Five different histone standards: 0 ug/uL; 0,25 ug/uL; 0,5 ug/uL; 1 ug/uL; and 2 ug/uL.
    b. All the desired samples.

    Note: For each reaction, have triplicates. Make sure you mix properly by pipetting up and down.
4. Incubate for 5 min at room temperature.
5. Put the 96-well plate in the spectrophotometer and measure the absorption of the histone standards and the samples (595 nm).
6. Calculate the histone concentration in each sample by using the equation of the histone standard wells and its standard line with the measured absorption:

$$concentration\ [ug/uL] = \frac{absorbtion - \ slope\ intercept}{slope\ coefficient}$$

7. Finally, calculate the volume of each sample you have to take for every desired histone concentration treatment:

$$volume_s[uL] = \frac{concentration_t[ug/mL]/_{1000} * \ volume_t[uL]}{concentration_s[ug/uL]}$$

Appendix 3: Histone treatment and flow cytometry

Materials

- Growth medium (completed EGM-2 with 10% FBS and 5 mL p/s)
- PBS
- Gelatine
- Annexin binding buffer
- Annexin V
- Propidium iodide (PI)
- Trypsin

- Eppendorf
- Counting plate
- 96 well plate

Day 1

It is important to count the cells to know how much you have and to take the same amount for every reaction. This protocol starts after the collection of the pellet in a 15 mL tube.

1. Coat the wells with 400 uL of gelatine. Let it set for 5 minutes and discard the extra.
2. Add 500 uL of complete growth medium to every well.
3. Resuspend the pellet with 1 mL of medium.
   Note: If the pellet is big, use 2 mL of medium. Do not forget to multiply with 2.000 uL in step 7.
4. Add 10 uL of the cell suspension and 90 uL of PBS in an Eppendorf. The final volume is 100 uL, and the dilution is 1:10.
5. Take 10 uL from the mixture and put on a plate for counting under the microscope.
6. Count the cells. And calculate the number of cells:

$$N_{cells} = \Sigma Q_{1-4} * 10.000 * dilution\ (e.g. 10)$$

7. Add approximately 120.000 cells per well. Calculate the required volume:

$$x\ [\mu L] = \frac{120.000 * 1.000\ [uL]}{N_{cells}}$$

8. Wait 24 hours for the cells to grow.

Day 2

First, treat the cells with histones.

1. Check the cells under the microscope to see if they're at 80% confluency.
2. Take up all the cell culture medium from the wells.
3. Wash all the wells twice with 500 uL PBS.
4. Add 500 uL of growth medium to all the wells.
5. Per different condition, take up the required amount of growth medium and add the same calculated number of histones. This is to have the same volume in every well, but with different concentrations of histones.

   Note: This calculation is done in the Excel file/step 6 of Measure histone concentration
6. Leave the wells in the incubator for 4 hours.

Annexin V protein is a commonly used approach for studying apoptotic cells. The protein binds to the exposed phosphatidylserine on the outside surface of the plasma membrane. Healthy cells normally do not express this on the outer surface and will therefore not be picked up by the assay. Propidium Iodide (PI) is a red-fluorescent nuclear and chromosome counterstain. PI cannot enter a healthy cell, only cells where the membrane is disrupted.

1. Mix 1 mL of Annexin Binding Buffer with 9 mL of PBS (1:10).
2. Take up the cell culture media from the wells and add to their respective Eppendorfs.
3. Add 500 uL trypsin to the wells.
4. Incubate for 2-3 minutes at 37°C.
5. Pipet the trypsin a couple of times in the wells (hard!).
6. Add all the trypsin to the respective Eppendorf.
7. Centrifuge at 1.500 rpm for 5 minutes
8. Discard the supernatant.
9. Resuspend the cells in 100 uL of Annexin Binding Buffer and add 5 uL of Annexin V and 5 uL of Propidium Iodide (PI).
10. Incubate for 15 minutes in a dark place because PI is light sensitive.
11. Add ~250 uL of Annexin Binding Buffer and resuspend the pellet.
12. Add the suspension to a round flow cytometry tube.

    Note: If you have to wait, put the tubes on ice to pause the reaction.
13. Analyse the cells with the flow cytometer.

Appendix 4: Cell culture RNA extraction

Materials

- mirVana kit (ref. AM1560, Invitrogen, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania)
- Sterile PBS
- Lysis Binding buffer (REF. 8540G21)
- MiRNA Homogenate Additive (REF. 8526G)
- Acid-Phenol:Chloroform
- Elution solution (REF. 9911G2)
- 100% ethanol
- miRNA Wash Solution 1 (REF. 8680G1)
- miRNA Was Solution 2/3 (REF. 8562G1)

- Eppendorfs
- Scratcher
- Centrifuge
- Filter Cartridges
- Collection tubes

Lysis

1. Remove medium from the wells.
2. Perform 2 washes with 500 uL PBS.
3. Add 500 uL of Lysis Binding buffer to every well.
4. Wait 5 minutes in room temperature.
5. Scratch the wells with a scratcher.

   Note: Make sure you wash the scratcher in-between wells with alcohol and water.
6. Take up the mixture and clean the wells thoroughly and put in Eppendorfs.
7. Put in freezer.

*Mir*Vana RNA isolation

1. Add 50 uL of miRNA Homogenate Additive to the Eppendorfs.

   Note: The desired added volume is 1/10, so if you have 500 uL cell suspension, add 50 uL.
2. Mix well by vortexing or inverting the tube.
3. Leave the mixture on ice for 10 minutes.

4. Add 500 uL of Acid-Phenol:Chloroform.

   Note: Also here, the added volume depends on the start volume (1:1).

   Note: Be sure to withdraw from the bottom phase in the bottle of Acid-Phenol:Chloroform, because the upper phase consists of an aque- ous buffer.
5. Vortex for 30-60 seconds to mix.
6. Centrifuge at 10.000 g at room temperature for 5 minutes.
7. Transfer the aqueous phase to a fresh tube and note the volume.

   Note: when the start volume is ± 500 uL, the aqueous phase is ± 300 uL
8. Preheat Elution Solution (or just nuclease-free water) to 95 °C.
9. Add 375 uL of room temperature 100% ethanol to the aqueous phase.

   Note: Also here, the added volume depends on the volume (1:1,25).
10. Place a Filter Cartridge into one of the Collection tubes and pipet the lysate/ethanol mixture onto the Filter Cartridge.

    Note: Only a maximum of 700 uL can be applied at a time, so for bigger volumes, apply the mixture in successive applications to the same filter.
11. Centrifuge for 15 seconds at 10.000 rpm.
12. Discard the flow-through and repeat until all of the mixture is through the filer.
13. Apply room temperature 700 uL miRNA Wash Solution 1 to the Filter Cartridge and centrifuge for 10 seconds. Discard the flow-through.
14. Apply room temperature 500 uL Wash Solution 2/3 and centrifuge similarly.
15. Repeat the washing step 14.
16. Discard the flow-through and spin for 1 additional minute to remove residual fluid from the filter.
17. Transfer the Filer Cartridge into a fresh Collection Tube and apply 100 uL of 95 °C Elution Solution or nuclease-free water to the centre of the filter and close the cap.
18. Spin for 25 seconds at maximum speed to recover the RNA.
19. Collect the eluate which contains the RNA and store it at -20 °C.

Appendix 5: RNase R treatment

Materials

- RNse R enzyme (ref. BioSearch technologies, E011-20D1)
- Nuclease-free water

- RNase R Reaction Buffer (ref.BioSearch Technologies, SS000769-D1)

- Eppendorfs
- Centrifuge
- Filter Cartridges
- Collection tubes

Method

1. Briefly centrifuge RNase R enzyme.
2. Dilute 20 U/uL RNAse R enzyme with RNase-free water to achieve a 1 U/uL working solution. Pipet up and down at least 10 times and briefly centrifuge. Keep enzyme on ice.
3. Prepare two master mixes:
    a. 1,5 uL RNase R Reaction Buffer (3,3 uL for 2 samples + excess) and 3,5 uL nuclease-free water (7,7 uL for 2 samples + excess).
    b. 1 uL of working solution (2,2 uL for 2 samples + excess), 1,5 uL RNase R Reaction Buffer (3,3 uL for 2 samples + excess) and 2,5 uL nuclease-free water (5,5 uL for 2 samples + excess).
4. Take 10 uL of the RNA extraction samples and put in new Eppendorfs.
5. Add 5 uL of either one of the master mixes to each corresponding RNA sample. Ensure good mixture and briefly centrifuge.
6. Incubate for 15 minutes at 37 °C and keep samples on ice.

Appendix 6: SYBR-Green RT-qPCR

SYBR Green binds specifically to double-stranded DNA molecules and becomes fluorescent upon binding, emitting a green fluorescence when exposed to specific wavelengths of light.

Materials

- RNA samples
- Nuclease-free water
- SYBR Green kit (ref. K0222) Thermoscientific, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania)
- Sense & antisense strand
- Nuclease-free water

- 96-well plate
- RT machine
- PCR machine

Reverse Transcription

1. Perform a quantification of the samples with Nanodrop.
2. Prepare the RT MasterMix: 2 uL of Pool RT primers (5x), 0,8 uL dNTPs 100 mM, 1 Multicribe Reverse Transciptase (50 U/ul), 2 uL of 10x Reverse Transcription Buffer, 1 uL of RNase inhibitor 10 U/uL and 3,2 uL of nuclease-free water.
   Note: These volumes are per sample. Create a Master Mix for all samples + 1,1% error.
3. Add 10 uL of the MasterMix to every well in the 96-well plate you want a reaction.
4. Add 200 ng of each RNA sample to the 96-well plate and complete with RNAse-free water to a volume of 10 uL. Including the blank.
5. Cover the plate with Parafilm and centrifuge at 1.000 rpm for 1 minute.
6. Perform the RT-reaction with the following cycle: 10 minutes at 25°C → 2 hours at 37°C → 5 minutes at 85°C.
7. Store at -20 °C.

PCR

1. Prepare the PCR MasterMix per desired target: 5 uL of SYBR Green, 0,3 uL of sense strand, 0,3 uL of antisense strand and 3,4 uL of nuclease free water.
   Note: These volumes are per sample and specific per target. Create multiple Master Mix for all samples + 1,1% error.
2. Add 9 uL of the respective MasterMix to every well in the 384-well plate that you want a reaction in for that target. Repeat for all Master Mixes.
3. Add 1 uL of the respective sample to all wells. Including three blanks.
4. Cover the plate with Parafilm and centrifuge at 1.000 rpm for 1 minute.

Perform the PCR-reaction with the following cycle: 2 minutes at 50°C → 10 minutes at 95°C → 40x cycle of: remain 15 seconds at 95°C & 1 minute at 60°C → 15 seconds at 95°C → 1 minute at 65 °C → 15 seconds at 95°C.

Appendix 7: TaqMan RT-qPCR

TaqMan is a probe-based technology used in real-time quantitative polymerase chain reaction (qPCR) to detect and quantify specific DNA or RNA sequences. TaqMan probes are labelled with a fluorescent dye and a quencher molecule.

Materials

- Nuclease-free water
- RT-primers
- Samples
- 10x Reverse Trasnciption Buffer
- dNTPs 100 mM
- RNAse inhibitor 20 U/uL
- Multicribe Reverse Transciptase, 50 U/ul
- Taq-Man Master Mix
- TM-probes

- Eppendorfs
- RT-plate
- Parafilm
- Centrifuge
- RT-machine
- PCR-plate
- PCR-machine
- USB-stick

Reverse Transcription

Work at the genomic section bench and clean before with ethanol and RNAse ZAP and work on top of a clean paper sheet.

1. Create the RT-primer pool by adding nuclease-free water with the RT-primers.
2. Create the master mix by mixing:
    I. 2 uL of 10x Reverse Trasnciption Buffer
    II. 0,4 uL of dNTPs 100 mM
    III. 0,25 uL of RNAse inhibitor 20 U/uL
    IV. 4 uL of Multicribe Reverse Transciptase, 50 U/ul

V. 8 uL of RT-primer pool (5x)

3. Add 14,66 uL of the master mix to every well of the RT-plate where you want a reaction.
4. Add 5,34 uL of the samples or nuclease-free water to every well for a final volume of 20 uL per well.
   Note: Pay attention to not hover above the wells you want to fill with anything else than the pipet point.
5. Cover the plate with Parafilm and centrifuge at 1.000 rpm for 1 minute.
6. Perform the RT-reaction with the following cycle: 30 minutes at 16°C → 30 minutes at 42°C → 5 minutes at 85°C.

PCR

1. Prepare the PCR MasterMix per desired target: 0,5 uL of TaqMan Small RNA assay, 5 uL of TaqMan MasterMix II (2x) and 3,5uL of nuclease free water.
   Note: These volumes are per sample and specific per target. Create multiple Master Mix for all samples + 1,1% error.
2. Add 9 uL of the respective MasterMix to every well in the 384-well plate that you want a reaction in for that target. Repeat for all Master Mixes.
3. Add 1 uL of the respective sample to all wells. Including three blanks.
4. Cover the plate with Parafilm and centrifuge at 1.000 rpm for 1 minute.
5. Perform the PCR-reaction with the following cycle: 10 minutes at 95°C → 45x cycle of: remain 15 seconds at 95°C & 1 minute at 60°C.