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MIR-34 EXPRESSION LEVELS IN RELATION TO NLRP3 INFLAMMASOME ACTIVATION IN THP1-ASC-GFP CELLS

Pilot Study

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Abstract

Inflammation is the body's innate immune system responding to harmful stimuli. The body reacts to this stimuli by forming and activating inflammasomes. Inflammasome protein activation plays major roles seen in metabolic and autoimmune disorders, showing the importance of comprehending the processes involved. The NLR protein family is involved in regulating innate immune responses. These types of proteins can sense pathogen-associated molecular patterns as well as damage-associated molecular patterns. Several miRNA families have been known to be regulators of the NLRP3 inflammasome, indicating that an improved understanding of how miRNAs work together to balance the inflammatory response is an area to be focused on. As well as increasing understanding of the miRNA networks and how those can be used to optimize the response of the inflammasome. The aim of this study was to determine the expression levels of the miR-34 family in relation to the NLRP3 inflammasome activation. Using THP-1 cells, mirRNA was isolated from cells taken at different time points after stimulation of the cells with LPS and ATP, followed by performing a two-step RT-qPCR. The Livak method with the RNU48 reference gene was used and indicated potential downregulation of the miR-34 family during NLRP3 inflammasome activation. Further studies should be carried out to confirm these findings.

List of abbreviations

ALR	AIM2-like Receptor
ASC	Apoptosis-associated Speck-like protein containing C-terminal
ATP	Adenosine Triphosphate
BP	Base Pair
CARD	Caspase Activation and Recruitment Domain
CCM	Cell Culture Media
CLR	C-type Lectin Receptor
DAMP	Damage-associated molecular pattern
gDNA	Genomic DNA
IL-1β	Interleukin-1β
LPS	Lipopolysaccharides
NLR	NOD-like Receptor
NTC	Non-Template Control
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PMA	Phorbol Myristate Acetate
PRR	Pattern Recognition Receptor
PYD	Pyrin Domain
RLR	RIG-I-like Receptor
RT	Reverse Transcription
RT qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
TLR	Toll-like Receptor

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Introduction

NLRP3 Inflammasome

Inflammation is the body's immune system responding to harmful stimulus, tissue damage, and infections. Formation and activation of inflammasomes is the way the body reacts to this harmful stimuli. Inflammasomes are large multiprotein complexes that are seen in the cytosol of immune cells that are involved with the inflammatory caspases activation (Broz & Dixit, 2016). Caspases are known to be a family of cysteine proteases which serve an important role in apoptosis, a form of cell death, or the activation of cytokines. Caspases can further be sectioned into either an initiator or an effector caspase (Martinon & Tschopp, 2004). The initiator caspase activates the effector caspase, however, the initiator caspase is automatically activated during apoptotic conditions (Shi, 2004).

Inflammasome protein activation plays major roles seen in metabolic and autoimmune disorders, showing the importance of comprehending the processes involved. Inflammasome pattern recognition receptors (PRRs) can be organized into five groups depending on the structural forms and features. These groups include the RIG-I-like receptors (RLRs), toll-like receptors (TLRs), C-type lectin receptors (CLRs), AIM2-like receptors (ALRs), and NOD-like receptors (NLRs). Based on the PRRs location, they can be divided into classes; membrane-bound PRRs including the TLRs and CLRs, or into the cytoplasmic PRRs which include the NLRs and RLRs (Kumar, Kawai & Akira, 2011).

The NLR protein family is involved in regulating innate immune responses. These types of proteins can sense pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs) (Schroder & Tschopp, 2010). PAMPs such as fungal zymosan and bacterial lipopolysaccharide (LPS) have been known to activate NLRP3 and can cause the release of interleukin-1β (IL-1β) when there is a presence of adenosine triphosphate (ATP) (Lamkanfi, Malireddi & Kanneganti, 2009). DAMPs such as particles of substances and crystals are also known to activate the NLRP3 inflammasome. The NLR subclass mainly detects DAMPs, while the TLR subclass mainly detects PAMPs. (Martinon, Petrilli, Mayor, Tardivel & Tschopp, 2006).

The NLR family can be divided into five subfamilies; the NLRA, which can be distinguished by the acidic transactivation domain; the NLRB, which contains a baculovirus inhibitor of apoptosis protein repeat; NLRC, which contains a caspase activation and recruitment domain (CARD); NLRP, which contains a Pyrin domain (PYD); and NLRPX, containing an undefined domain (Ting et al., 2008). The NLRP3 inflammasome consists of NLRP3, apoptosis-associated speck-like protein containing C-terminal CARD (ASC) and procaspase-1. Stimulation of the cells begins oligomerisation of the NLRP3, promoting the clustering of ASC with NLRP3 using a PYD interaction. The CARD of both the procaspase-1 and ASC then react to form catalysis of the procaspase-1 forming caspase-1 (Kumar et al., 2011). These interactions can be seen in Figure 1.

Caspase-1 is known to trigger pyroptosis (Hornung & Latz, 2010). Pyroptosis can be used as a way to lyse macrophages, causing cell death. The event of pyroptosis and the resulting processes that occur are suspected to be a cause of inflammation as well as coinciding with the secretion of IL-1β and IL-18. IL-1β and IL-18 are known as proinflammatory cytokines (Kumar et al., 2011). The NLRP3 inflammasome plays a large role in the production of the inflammatory cytokines interleukin-1β and IL-18. These cytokines cause a variety of biological issues seen with infection, autoimmune processes and inflammation. (Guo, Callaway & Ting, 2015).

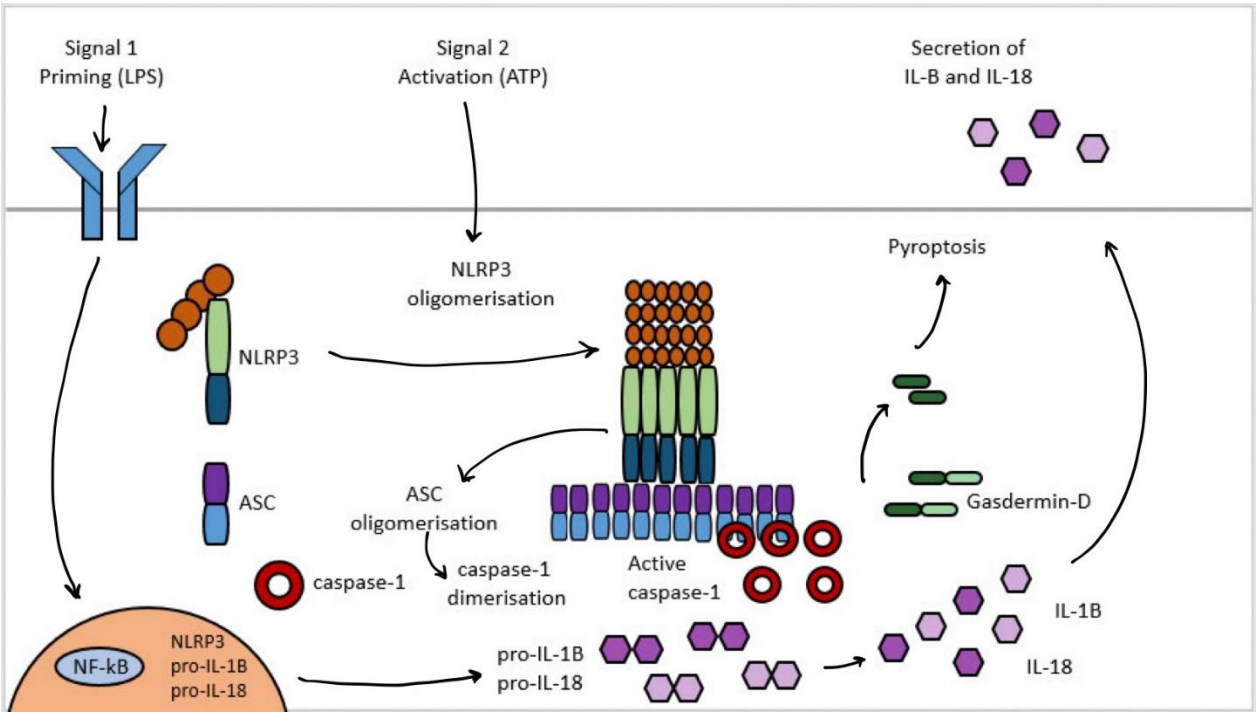

Figure 1. NLRP3 inflammasome activation diagram. The NLRP3 inflammasome requires two different signals to activate, a priming Signal 1 which activates NF-kB and an activating Signal 2 which begins oligomerisation. The priming signal then begins transcription of pro-IL-1B and NLRP3 as well as resulting in post-translational modifications. The ASC is recruited by PYD interactions with the NLRP3, forming large oligomers. ASC interacts with caspase-1 generating active caspase-1. The active caspase-1 converts pro-IL-1B and pro-IL-18 to active forms, which are then secreted. Cleavage of Gasdermin-D by caspase-1 occurs, resulting in pyroptosis, which is an inflammatory cellular death (Coll, O'Neill & Schroder, 2016).

MicroRNA

MicroRNAs, also known as miRNAs, are a group of small-single stranded RNAs that can silence the expression of genes by their interaction with mRNA. miRNAs are typically known to have a length between 20-23 base pairs (BP) and appear to play a role in gene expression seen in plants and animals as negative regulators (Ambros, 2001). miRNAs over the years have been classified into different categories based on the sequence of pre-miRNAs, the structure of pre-miRNAs and the mature miRNA. These categories are known as miRNA families. In relation to inflammatory responses, miRNA families have been seen to target specific proteins that are involved during regulation of inflammation, as well as being expressed in immune cells. With the constant down or upregulation of the immune response genes, the main step is to coordinate the inflammation response. Similarly seen with protein coding genes, miRNA transcripts change expression during the response process. Several miRNA families have been seen to be misexpressed in many different human autoimmune diseases. The effects of the downregulation or upregulation of these miRNA families show the capacity that miRNAs have to regulate functionalities within mature cells (O'Connel, Rao & Baltimore, 2012). Activation of the NLRP3 protein has been commonly seen in relation with the suppression of different miRNA families (Wang et al., 2015; Zhou et al., 2018; Liu, Zhao, Shan, Gao & Wang, 2019).

The miR-34 family of miRNA consists of 3 subfamilies; a,b and c. These subfamilies all contain the same seed sequence of GGCAGUG (Engkvist et al., 2017). Within humans, the miR-34 family makes up three miRNAs which are encoded by two separate genes. MiR-34a has its own transcript, while

miR-34b and miR-34c share a transcript (Hermeking, 2009). The miR-34 family has been very outstanding in cancer research, as it is seen as a major regulator of tumor suppression. When miR-34 is overexpressed it represses many oncogenes, this results in the increase of cancer cell death as well as metastasis inhibition (Li et al., 2009). The miR-34 family was seen to be deregulated within human cancers. These discoveries led to the miR-34 family becoming the first miRNA family seen to reach phase 1 clinical trials (Agostini & Knight, 2014). In association with tumors, chronic inflammation is a common symptom seen in cancer patients (Hussain & Harris, 2007). In addition to the discovery that miR-34a was a regulator of tumor suppression, it was also seen having an anti-inflammatory effect with the downregulation of TNF-a and IL-6. It was also discovered that miR-34a is affected by inflammatory stimuli, being found that miR-34a was downregulated once PAMP stimulus with LPS was performed on murine macrophages (Jiang et al, 2012). Several miRNA families have been known to be regulators of the NLRP3 inflammasome, for example miR-33 is known to be a regulator of the NLRP3 inflammasome signaling pathway within macrophages (Xie et al., 2017), as well as miR-9 inhibiting the NLRP3 activation (Wang et al., 2017). These findings indicate that certain miRNA families can be used to regulate the activation of the NLRP3 inflammasome.

As stated above, it has been observed that human cancers cause a vast amount of miRNA families to have low levels of expression, indicating that miRNAs actively participate in tumor suppression. The miR-34 family has been known to be expressed at reduced levels or lost in varying types of cancers. The miR-34 family is a direct transcriptional target of p53, a tumor protein (He et al., 2007). p53 is the most commonly mutated gene in human cancers, indicating the large role it plays in preventing cancer (Surget, Khoury & Bourdon, (2013). It has been proposed that the NLRP3 inflammasome and the p53 pathway may intersect at the inflammasome adaptor molecule ASC (Haasken & Sutterwala, 2013).

Several important questions and hypotheses remain in the study field of miRNAs and how they influence inflammasome function. With the many findings that different miRNA families affect activation of different inflammasomes, the studies seem endless yet promising. An improved understanding of how miRNAs work together to balance the inflammatory response is an area to be focused on, as well as understanding the miRNA networks and how those can be used to optimize the response of the inflammasome.

Aim and objective

The aim with this thesis project is to measure the miRNA expression levels of the miR-34 family to evaluate the effect of different durations of PAMP and DAMP stimuli. This data will also be used to evaluate if the expression levels increase or decrease with the activation of the NLRP3 inflammasome, determining if the miR-34 family is a potential regulator of the NLRP3 inflammasome.

The main objectives in this thesis project involve isolating miRNA from THP1 cells to measure the expression levels of the miR-34 family during stimulation of the NLRP3 inflammasome. With the use of the two-step reverse transcription quantitative polymerase chain reaction (RT qPCR) mechanism to quantify the data for evaluation.

The information provided by this work will allow further studies to be carried out, whether that be to further investigate the miR-34 family or to focus on other miRNA families that may be involved with NLRP3 regulation and activation. Very few studies have been done on the miR-34 family specifically for the NLRP3 inflammasome regulation and activation.

Materials and methods

Ethical aspects

The cells used were THP-1 cells from a human, there are no ethical issues as the use is *ex vivo/in vitro*, meaning experimentation will occur outside of the natural environment of the cells. The project follows the All European Academics the European Code of Conduct for Research Integrity as well as the Swedish Research Counsels guidelines. THP-1 cells will be used as they are a known human cell line that is commonly used to study immune responses. The University of Skövde has permission to use THP-1 cells at a Biosafety level 2 containment.

Cell culturing

THP1-ASC-GFP cells (Invivogen) at a passage level of five were used. The cells were cultured with RPMI 1640 (w. L-glutamine), 10% heat inactivated FBS, 1 mM sodium pyruvate, 0.45% glucose solution, 10 mM HEPES and pen-strep 100X (Sigma), with the addition of zeocin (Invivogen) to a concentration of 200 µg/mL. Incubation was performed at 37 °C with 5% CO_2. During media change, the cells were spun down at 1500 g in the Hettich Universal 32 centrifuge. The cells were cultured to a final cell count of approximately 50 million at a passage level of seven in 50 mL. Differentiation from monocyte to macrophage was performed with the use of Phorbol Myristate Acetate (PMA) (Invivogen) at a concentration of 0.5 µg/mL. PMA exposure lasted for four hours, a cell culture media (CCM) change was performed to remove the PMA, and then incubation without PMA was carried out for 18 hours. Before the addition LPS, a control sample was taken. Stimulation with LPS-B5 ultrapure (Invivogen) was done at a final concentration of 100 ng/mL, and the cells were incubated at 37 °C for three hours. Another control sample was taken. Stimulation with ATP (Sigma) was done at a final concentration of 5 mM. Samples were then taken at varying time points as seen in Table 1. After sample 1.1 was taken, the remaining sample plates had LPS added immediately. After sample 1.2 was taken, the remaining sample plates had ATP added immediately. Stimulation of the cells were performed in chronological order. Each sample plate was scraped and washed with cold phosphate buffered saline (PBS) to remove the cells. The samples were at a volume of 1000 µL, they were centrifuged at 4 °C in a refrigerated centrifuge (Biofuge) at 400 g, the supernatant was removed, and the sample was stored in the form of a cell pellet at -80 °C. Three biological replicates were completed.

Table 1. Samples taken during cell stimulation with LPS and ATP.

Sample	What was performed and the corresponding time points
1.1	0 min - control, before LPS was added.
1.2	0 min - control, after LPS but before ATP was added.
1.3	20 min - after ATP was added.
1.4	40 min - after ATP was added.
1.5	60 min - after ATP was added.
1.6	90 min - after ATP was added.
1.7	120 min - after ATP was added.
1.8	180 min - after ATP was added.
1.9	20 hours - after ATP was added.
1.10	24 hours - after ATP was added.

MicroRNA isolation

Total RNA was isolated using the mirVana miRNA Isolation Kit (ThermoFisher). During isolation of replicate three, samples one through five followed the protocol for smaller concentrations while samples six through ten followed the normal protocol (ThermoFisher). For almost all samples, 300 µL of the aqueous phase was recovered in step E. 3, however for samples 3.1-3.5, 600 µL of the aqueous phase was recovered accidentally. During isolation of replicate two, centrifugation times and speeds were increased. The samples were spun down for ten minutes at 200 g in miniSpin centrifuge (Eppendorf) and then five minutes at 300 g. Samples nine and ten had an additional centrifugation for 5 minutes at 300 g. Once isolation was completed for all biological replicates, concentrations and purities of all samples were measured using the Nanodrop spectrophotometer (ThermoFisher).

RT-qPCR

All samples were diluted based on the concentration readings seen in Table 3 in the appendix, to a final concentration of 2 ng/µL. The RT reactions were performed according to the TaqMan Small RNA Assays protocol (ThermoFisher), the kit used was the TaqMan MicroRNA Reverse Transcription Kit (ThermoFisher) and the primers were the TaqMan MicroRNA Assays (ThermoFisher). Three primers were used for each sample in all three biological replicates; miR-34a, miR-34b, and miR-34c. The primer for the endogenous reference gene RNU48 was also used for each sample in replicate one. The primer sequences are shown in Table 2.

Table 2. Primer Sequences used for each sample in all three biological replicates

miRNA	Primer Sequence	Accession Number
miR-34a	UGGCAGUGUCUUAGCUGGUUGU	MI0000268
miR-34b	UAGGCAGUGUCAUUAGCUGAUUG	MI0000742
miR-34c	AGGCAGUGUAGUUAGCUGAUUGC	MI0000743
RNU48	GATGACCCCAGGTAACTCTGAGTGTGTCGCTGATGCCATCACCGCAGCGCTCTGACC	NR_002745

The samples were run in a thermal cycler with the settings shown in Table 3.

Table 3. Thermal cycler settings

Step	Temperature (°C)	Time
Reverse Transcription	16	30 min
	42	30 min
Stop Reaction	85	5 min
Hold	4	Hold

qPCR was performed as stated in the TaqMan Small RNA Assays protocol (ThermoFisher). The PCR master mix that was used was the TaqMan Universal Master Mix II, no UNG (ThermoFisher), the microamp optical 96 well reaction plates (Applied Biosystems) were used as well as the 7300 real time PCR machine (Applied Biosystems). Samples for the miR-34a, miR-34b families, and the reference gene were plated onto the same PCR reaction plate, while miR-34c and the reference gene was plated on a separate plate. During qPCR, nuclease free water (VWR) was used as the non-template control (NTC). The Cq values obtained from qPCR were selected based on the rules seen in Table 4.

Table 4. Rules to determine usable Cq values obtained from qPCR.

Rule	Description
1.	Cq value must be at a 0.5 or lower range between the values.
2.	If three values were in the 0.5 range, the average of the three numbers would be the value used.
3.	If two of the three values were in the 0.5 range, the average of the closest two numbers would be the value used.
4.	If all three values were over the 0.5 threshold, the data would be removed from evaluation.
5.	If only two values were detected out of the three, and were not in the 0.5 or lower range, the data would be removed from evaluation.
6.	If only one value was detected out of the three, the data would be removed from evaluation.

Results

Cell culturing

During cell culturing and stimulation, it was observed that the pellets collected seemed to decrease in size over time after LPS and ATP addition. This was seen for both biological replicates one and two, but not for biological replicate three, as all samples in replicate three were invisible or nearly invisible.

MicroRNA isolation

For almost all samples, 300 µL of the aqueous phase was recovered in step E. 3, however for samples 3.1-3.5, 600 µL of the aqueous phase was removed and transferred to the new tube accidentally. This did not appear to affect the samples as the 260/280 and 260/230 ratios of purity and concentration readings seemed to follow the same trends as the other samples. After miRNA isolation was complete, the purity and concentration readings from the spectrophotometer for all three biological replicates were taken and can be seen in Table 5 in the appendix.

RT-qPCR

The Cq values determined by the rules in Table 4 were later evaluated using the Livak method to get the normalized expression values, fold change and log 2 fold change values which can be seen in Figures 2 and 3. The values for miR-34a and miR-34c seen in Figure 3A are all showing low expression. The values for miR-34a and miR-34b seen in Figure 3B are all showing low expression, while miR-34c has low expression values on all but two points, which show high expression.

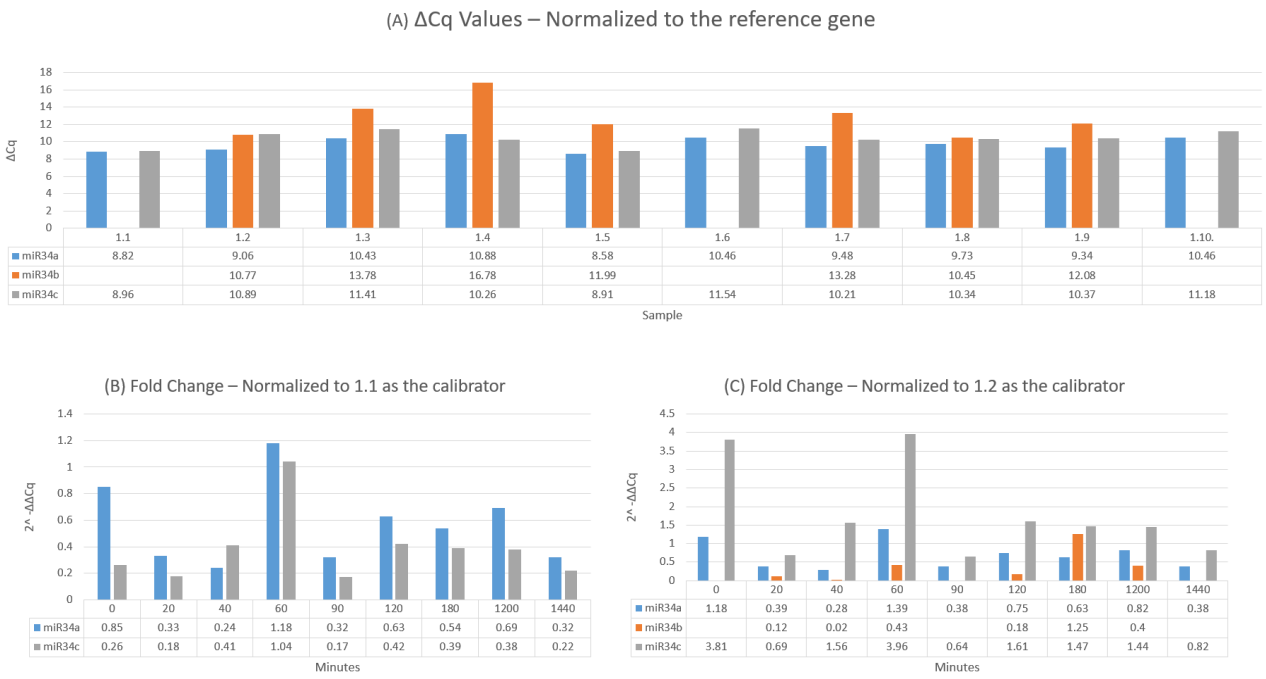

Figure 2. Representation of the processes from Cq values obtained from qPCR to normalized expression values. (A) Cq values are normalized to the Endogenous Control gene RNU48. Samples are displayed using 1.1 – 1.10, the corresponding time points are provided in table 1. (B) The differential expression of the miR-34 family calculated using the Livak method using the ΔCq value from sample 1.1 as the calibrator and represented in fold change. (C) The differential expression of the miR-34 family calculated using the Livak method using the ΔCq value from sample 1.2 as the calibrator and represented in fold change. A blank column indicates an unusable value due to the rules determined.

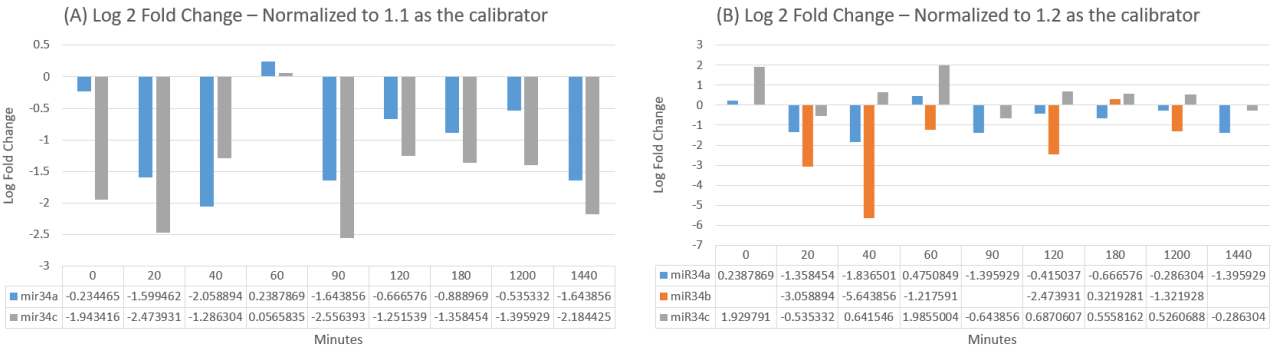

Figure 3. Visually accurate representation of the fold changes. (A) Log 2 fold change using the ΔCq value from sample 1.1 as the calibrator. (B) Log 2 fold change using the ΔCq value from sample 1.2 as the calibrator. A blank column indicates an unusable value due to the rules determined.

Discussion

Cell culturing

It has been hypothesized and discussed that 18-24 hour exposure to extracellular ATP can cause cellular apoptosis and therefore could be the cause of a decrease in sample size over time that was observed (Zheng, Zychlinsky, Liu, Ojcius & Young, 1991). The control samples were taken to be able to detect if anything occurs before and or during LPS stimulation.

MicroRNA isolation

The mirVana miRNA Isolation Kit was used to isolate the total RNA of the samples. All samples prior to resuspension had visible cell pellets. Prior to isolation, for biological replicate one, all samples had visible cell pellets after resuspension and centrifugation preparation steps. While in biological replicate two and three, most of the pellets were not visible after resuspension and centrifugation. To attempt to form a visible cell pellet, the centrifugation times and speeds were slightly increased, but this did not help much in forming visible cell pellets. This seemingly did not affect the results, as the concentration and purity readings of the isolated total RNA later on showed near normal and or expected values.

After miRNA isolation was complete, the 260/280 ratio and concentration readings from the Nanodrop for all three biological replicates can be seen in the appendix in Table 5. A Spectrophotometer uses photometry to measure the UV-Vis absorption levels of nucleic acids, which peak at the 260 nm point. The advantages of this technique is the ease of use, direct measurement feedback, and depending on the instrument used, it can detect and identify the contaminants it detects. The main disadvantage is that it cannot differentiate between RNA and DNA, and has limited sensitivity. The other instrument used in determining the concentration of samples is the Qubit which uses a fluorometric measurement with the use of dyes that can selectively bind to either RNA or DNA. The advantages of this technique is that it is specific, having measurement settings for different types of samples, it is highly sensitive, and is stated as being accurate even despite any contamination that may be present. However the disadvantages include a longer preparation and no purity information can be provided (Deben et al., 2013; ThermoFisher, 2020a). A Nanodrop spectrophotometer was used instead of the Qubit to measure the concentration and purity of the samples as S. Jurcevic (personal communication, March 16, 2020) stated previous success for miRNA studies with just the use of the spectrophotometer, as well as the large disadvantage with the Qubit not providing purity readings.

However, not using the Qubit in addition to the use of the Nanodrop spectrophotometer is seen as a large weakness during this study. As stated previously, the qubit is very specific and can measure different types of samples. The ability to measure for RNA in the samples would have confirmed the samples in fact had RNA and weren't just contaminated with DNA. It would've also been useful to use both the Qubit and Nanodrop to compare the differences in concentration readings, similarly done in the study by Deben et al. (2013), which states that Nanodrop gives higher readings in comparison to Qubit for RNA readings. However as stated by MIQE, it is advised to only select one method to use and report only those values (Bustin et al., 2009). A fragment analyzer could've also been used to confirm the samples contained miRNA by being able to measure the concentration of RNA fragments of certain sizes (Rodriguez & Vaneechoutte, 2019).

Another technique that could've been used to determine the contents of the samples include acrylamide and agarose gel electrophoresis. This would allow the RNA concentration to be qualitatively determined, as well as contaminates such as gDNA and degraded RNA to also be

determined. Further showing what is actually in the samples, and how much contamination is present (Wieczorek, Delauriere & Schagat, 2020). This was not performed due to lack of time.

Looking at some of the values for the spectrophotometer, contamination has occurred. For 260/280, a ratio of approximately 2.0 is seen as pure for RNA (Wilfinger, Mackey & Chomczynsky, 1997). A low 260/280 purity reading may be an indication of contamination from residual phenol which was used during extraction. As many of the samples have readings under 2.0 it could indicate again, excess phenol being left over in the samples during extraction (Matlock, 2015). The occasionally observed spectras portrayed by the spectrophotometer concluded that the occasional contaminant for some of the samples was indeed excess phenol as it followed the same pattern seen in other studies that involved phenol contamination during RNA extraction (Toni et al., 2018).

The low purity reading could also be due to a very low concentration reading, which can be seen for sample 2.4 in Table 5 in the appendix. The 260/230 ratio is less important as compared to the 260/280 readings as they are readings for contaminants, for example guanidine thiocyanate, which absorbs at that wavelength. An acceptable 260/230 ratio is approximately 1.7 or greater. A high purity ratio for 260/280 is not an immediate indication of an issue in the sample, and that the best indication of the purity and functionality of the sample is the performance during following applications (Matlock, 2015).

It is suggested to observe the spectral patterns to fully determine the overall quality of the sample (Matlock, 2015). However, this was not done in an extensive manner due to lack of knowledge, and the only results thoroughly observed and noted from the spectrophotometer were the purity ratios and concentration values. The samples used for RT-qPCR all came from replicate one which had better purity and concentration readings in comparison to replicates two and three.

RT-qPCR

Due to the abnormal concentration and purity readings seen in replicate two and three, it was decided to run qPCR on just biological replicate one, as the amount of reagents and time were running low hindering the ability to order more reagents as well as hindering the potential of creating additional biological replicates to supplement the study.

To prevent contamination during reverse transcription (RT), including a DNase step during the miRNA isolation, or using a no-reverse transcription (no-RT) control is advised. During reverse transcription in this study, a DNase step and a no-RT control were not used. The disadvantage to this is the potential of genomic DNA (gDNA) contamination (Promega, 2012), this could have also been detected early on if the Qubit had been used during miRNA isolation. Unwanted gDNA in samples can lead to affecting the accuracy of the RT-qPCR results by having unspecific amplification. The use of a no-RT control is costly and increases the work time, but is seen as necessary (Hashemipetroudi, Nematzadeh, Ahmadian, Yamchi & Kuhlmann, 2018). The RT control is used to detect gDNA contamination and an NTC is traditionally used to test primer-dimer formation (Laurell et al., 2012). Positively, the TaqMan MicroRNA Assay used during RT-qPCR in this study uses the stem-loop primer mechanism, which is not affected by gDNA contamination (Chen et al., 2005). Therefore, few samples as well as NTCs were successfully used to test RT-qPCR prior to performing it on the remaining samples. Indicating no contamination and usable samples.

To prevent the interpretation of the results from being subjective and skewed in favor of the values given, formulation of the rules to determine usable Cq values that were obtained from qPCR was performed prior to running qPCR. Cq also known as Ct values, are the amount of cycles it takes for the

fluorescent signal to surpass the background threshold level (Tellinghuisen & Spiess, 2014; ThermoFisher, 2016).

Two-step RT-qPCR is mainly used for detecting multiple targets within the same RNA sample in different reactions as well as having the ability to store the cDNA to use later on if needed. During this study, two-step RT-qPCR was performed due to its storage capabilities and affordability. However with two-step, there is a higher chance of contamination to occur in comparison to one-step, as two-step assays perform reverse transcription and qPCR in separate tubes, with different components and conditions. While one-step uses a single closed tube system (ThermoFisher, 2020b).

MiR-34a and miR-34b were plated onto the same PCR reaction plate, while miR-34c was plated on a separate PCR reaction plate. This was done because miR-34b and miR-34c share a transcript. Non-template controls were used to determine if contamination of DNA or RNA had occurred in the master mix. Looking at the raw data from the qPCR in Tables 6 through 10 in the appendix, all non-template controls came back as undetected, meaning there was no amplification and no contamination so the Cq values could be trusted and further evaluated.

A reference gene is used as it has constant expression levels for all samples and the levels are not affected by the treatments being performed during the study (Ram, Koramutla & Bhattacharya, 2017; Bio-Rad Laboratories, 2006). It should also have a similar threshold cycle to the gene being studied. Reference genes that meet these criteria are known as Housekeeping Genes (Kozera & Rapacz, 2013). RNU48 was used in this study as it is one of the two small nuclear RNAs used as a reference gene for human miRNA studies, the other being RNU44 (Wessels, Edwards, Zettler & Tayade, 2011). It is commonly seen as unacceptable to only use one reference gene when normalizing the data (Bustin et al., 2009), but with lack of time and funding, only one reference gene was used in this study. Several statistical programs such as geNorm, NormFinder, RefFinder and more can be used to help determine a good reference gene (Silveira et al., 2018).

Before normalizing the qPCR data, the reference gene expression values should be checked and confirmed to be constantly expressed in the study. This could have been done by measuring the selected reference gene as well as several other different reference genes with the samples and then making conclusions by using the geNorm algorithm. This would then provide validation of the expression levels and confirm the most stable reference gene to use (Cao et al., 2012). However, this was not done in this study and is seen as a weakness.

Prior to running qPCR on the samples, PCR amplification efficiency should be determined with the use of calibration curves, this however was not done, again due to lack of knowledge. This is seen as a weakness in this study, as it lowers the chances of being able to replicate this experiment accurately. Differences with PCR efficiency will result in differences in the calibration curves, resulting in differences between Cq values of the target sample and the reference gene, therefore giving misleading results (Bustin et al., 2009). The Livak method was used in this study, and the PCR amplification efficiency was assumed to be at 100%. This is seen as a weakness in this study, due to the fact that the efficiency was not determined to be 100% and the use of assumptions will lead to misleading results.

Looking at the qPCR data in Figure 2 and Figure 3, it can be predicted that the miR-34 family could be downregulated during stimulation with LPS and ATP. Without the ability to perform statistical testing on the data, the fold change values were evaluated individually and ranked using fold change is ≥2.0 for high expression and <2.0 for low expression which was also used in a study with miR-34a (Liu et al., 2015). Statistical analysis could not be performed due to the lack of biological replicates. It seems inefficient to make a significant fold change cut off without the proper use of statistical analysis. In several studies involving miR-34, 2.0 is commonly used as a significance fold change cut off, as well as the cut off value of $p<0.05$ or $p<0.01$ depending on the study, once statistical analysis has been performed (Xi, Zhang, Kong & Liang, 2018; Iannolo, Sciuto, Raffa, Pilato & Conaldi, 2018; Navarro & Lieberman, 2015).

Due to the lack of statistical analysis, comparison to other studies are based on the hypothesis that miR-34 is downregulated in this study. As previously stated, miR-34 is a direct translational target of p53 and that the NLRP3 inflammasome and the p53 may potentially intersect (Haasken & Sutterwala, 2013). In the study by Corney et al. (2010) it was indicated that miR-34a expression levels are decreased in 100% and miR-34b/c is decreased in 72% of human EOC samples with the p53 mutation. Similarly seen in the results of this study, the downregulation numbers from a log fold change were all within the range of zero to negative four. Similarly in the study by Xiong, Hu, Li, Zhou & Chen (2019), almost all samples in the study had the miR-34 family indicated as downregulated in gastric cancer tissues, with the downregulated samples having a fold change of less than zero. As stated by Hanazono et al. (2006), a p53 mutation can be seen in approximately 50% of gastric cancer patients. A large common factor is all seen within these studies, the involvement of p53 mutations.

Having seen that downregulation of the miR-34 family in many other cancers occurs in correlation to p53 mutations. The downregulation of miR-34 could indicate a future potential biomarker to use in the NLRP3 inflammasome, if it does indeed intersect at the adaptor molecule with the p53 tumor protein as predicted. The miR-34 family has already been indicated as a potential biomarker for many other cancers and diseases, due to its common dysregulation (Zhang, Liao & Tang, 2019; Li, Khanna, Li & Wang, 2011; Franchina et al., 2014; Misso et al., 2014). With the use of miRNA as biomarkers, the detection and diagnosis time is decreased leading to a faster treatment (Pogribny, 2018). However, the knowledge and information on different miRNA families are still lacking and there is a lot to discover, hence most of the miRNA families are shown as only a potential biomarker. This is mainly due to contradicting findings in independent studies, as well as not being disease specific (Pogribny, 2018). However, the knowledge on miRNAs has highly improved since the first discovery of miRNAs in 1993 (Lee, Feinbaum & Ambros, 1993). One of the very first uses of miRNA as a biomarker occurred in 2008 (Lawrie et al., 2008), which shows just how quickly the knowledge is expanding and the growing future use of miRNAs as biomarkers.

Conclusion

The aim of the study was to stimulate THP1-ASC-GFP cells to activate the NLRP3 inflammasome and measure the expression levels of the miR-34 family to observe what is occurring. This was done successfully, but without the ability to use statistical analysis due to the loss of the ability to run qPCR on all three biological replicates, the study became a pilot study.

The miR-34 family has been known to be lost or have low expression levels in many cancers (Misso et al., 2014). Using the findings provided by this study, it can be concluded that the miR-34 family expression levels during NLRP3 inflammasome activation tend to follow the trend of downregulation

as seen in many other studies involving the miR-34 family (Corney et al., 2010; Tanaka et al., 2013; Misso et al., 2014; Slabakova, Culig, Remsik & Soucek, 2017). Due to the lack of information in this area, this pilot study provided a good insight into the potential interactions occurring.

All in all, further studies should be carried out to confirm the findings provided by this pilot study. This study should be repeated as similarly as possible with the proper amount of biological replicates. A fragment analyzer should be used to check for miRNA. More reference genes should be used, as well as performing PCR efficiency tests. The reference gene expression levels should be evaluated and confirmed to be constant. Statistical analysis can then be performed on accurate data and give strong conclusive results.

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Appendix

Table 5. Purity and concentration readings for every RNA sample from the Nanodrop, 1.1 meaning all samples from replicate 1, 2.1 meaning all samples from replicate 2, and 3.1 meaning all samples from replicate 3.

Sample	Concentration (ng/uL)	260/280	260/230
1.1	20.1	2.00	0.56
1.2	21.7	2.00	1.48
1.3	6.2	2.17	1.66
1.4	26.5	2.16	1.86
1.5	22.8	2.00	1.72
1.6	25.5	2.00	0.55
1.7	15.4	1.95	0.39
1.8	13.1	1.95	0.14
1.9	7.9	2.04	0.43
1.10	20.1	1.99	0.57
2.1	16.7	1.85	0.81
2.2	21	2.02	1.07

2.3	10.5	1.92	0.75
2.4	4.3	1.38	0.90
2.5	12.6	1.78	0.90
2.6	23.2	2.08	1.83
2.7	14.6	2.01	0.76
2.8	16.9	2.04	0.89
2.9	4.4	2.14	0.84
2.10	9.5	2.01	0.41
3.1	5.6	2.26	0.62
3.2	5.5	3.57	0.65
3.3	10	1.95	0.20
3.4	7.5	2.03	0.56
3.5	5.8	2.13	0.18
3.6	23.2	1.7	0.14
3.7	11.1	1.87	0.25
3.8	16.4	1.8	0.55
3.9	30.8	1.94	1.06
3.10	36.8	1.96	1.04

Table 6. Raw qPCR data for miR-34a and non-template control for plate 1.

Sample	**Cq**	**StdDev Cq**	**Tm**
1.1	29.89	0.132	79.1
1.1	30.12	0.132	72.8
1.1	30.11	0.132	72.8
1.2	30.29	0.353	72
1.2	29.71	0.353	74.3
1.2	30.35	0.353	73.3
1.3	31.27	0.277	74
1.3	31.41	0.277	72.5
1.3	30.88	0.277	73
1.4	29.76	0.372	74.3

1.4	30.09	0.372	72.5
1.4	29.35	0.372	72.3
1.5	29.83	0.216	73.7
1.5	29.45	0.216	74
1.5	29.45	0.216	72.3
1.6	31.72	0.242	88.7
1.6	31.81	0.242	73.5
1.6	32.18	0.242	87.8
1.7	29.89	0.228	75.3
1.7	30.15	0.228	86.2
1.7	29.7	0.228	72.3
1.8	30.46	0.335	73.3
1.8	31.07	0.335	72.8
1.8	30.53	0.335	73
1.9	30.38	0.431	75.3
1.9	31.1	0.431	72
1.9	31.14	0.431	75.7
1.10	30.92	0.387	76
1.10	31.53	0.387	72
1.10	30.81	0.387	72
NTC	Undetermined		75.3
NTC	Undetermined		76
NTC	Undetermined		79.9

Table 7. Raw qPCR data for miR-34b and non-template control for plate 1.

Sample	**Cq**	**StdDev Cq**	**Tm**
1.1	32.28	0.772	74.3
1.1	33.14	0.772	73
1.1	31.6	0.772	74.3
1.2	34.07	1.097	73.3
1.2	32.03	1.097	75.3

1.2	32.36	1.097	72.3
1.3	34.87	0.364	77.8
1.3	35.24	0.364	72.3
1.3	34.51	0.364	74.3
1.4	36	0.796	73.3
1.4	35.66	0.796	77.8
1.4	34.48	0.796	72.3
1.5	33.65	0.409	72
1.5	29.45	0.216	72.3
1.5	33.15	0.409	76.5
1.6	32.84	0.745	72
1.6	34.33	0.745	74.3
1.6	33.67	0.745	78
1.7	33.79	0.108	73.3
1.7	33.59	0.108	81.4
1.7	33.76	0.108	72.8
1.8	31.21	0.193	73.3
1.8	31.03	0.193	76
1.8	31.42	0.193	72
1.9	38.34	2.59	73.5
1.9	33.91	2.59	72
1.9	33.81	2.59	73.3
1.10	35	0.648	78
1.10	20.45	0.648	72
1.10	33.71	0.648	72.8
NTC	Undetermined		76.3
NTC	Undetermined		77
NTC	Undetermined		74.5

Table 8. Raw qPCR data for miR-34c and non-template control for plate 2.

Sample	**Cq**	**StdDev Cq**	**Tm**

1.1	32.63	0.191	73.5
1.1	32.77	0.191	72.5
1.1	32.39	0.191	72.8
1.2	33.41	0.627	71.8
1.2	34.19	0.627	76
1.2	34.65	0.627	72.8
1.3	34.71	0.135	73.5
1.3	34.51	0.135	76.8
1.3	34.45	0.135	76.5
1.4	31.74	0.077	72.5
1.4	31.71	0.077	72.5
1.4	31.86	0.077	71.8
1.5	32.5	0.323	74
1.5	32.41	0.323	75.8
1.5	33.01	0.323	71.8
1.6	34.42	0.655	72.8
1.6	35.69	0.655	73.8
1.6	34.79	0.655	74.5
1.7	33.22	0.143	73.5
1.7	33.13	0.143	72.3
1.7	33.41	0.143	73
1.8	33.34	0.272	71.8
1.8	33.84	0.272	74
1.8	33.4	0.272	73.3
1.9	35.78	0.754	74.8
1.9	34.42	0.754	74
1.9	34.53	0.754	71.8
1.10	34.18	0.328	72.3
1.10	34.15	0.328	74
1.10	34.73	0.328	72.3
NTC	Undetermined		84

NTC	Undetermined	81.6

Table 9. Raw qPCR data for the RNU48 reference gene for plate 1.

Sample	**Cq**	**StdDev Cq**	**Tm**
1.1	21.18	0.062	76.5
1.1	21.29	0.062	74
1.1	21.18	0.062	72
1.2	21.34	0.072	72.8
1.2	21.2	0.072	89
1.2	21.25	0.072	83.3
1.3	20.95	0.037	80.7
1.3	20.87	0.037	81.4
1.3	20.9	0.037	72
1.4	19.06	0.016	72
1.4	19.05	0.016	72.5
1.4	19.03	0.016	73
1.5	21.01	0.017	72
1.5	21	0.017	72
1.5	20.98	0.017	73
1.6	21.38	0.108	73
1.6	21.39	0.108	72
1.6	21.57	0.108	73.5
1.7	20.37	0.085	72.5
1.7	20.4	0.085	74.3
1.7	20.53	0.085	72
1.8	20.6	0.275	72.3
1.8	21.09	0.275	72
1.8	20.62	0.275	72
1.9	21.79	0.119	73
1.9	21.89	0.119	72.5
1.9	21.66	0.119	72

1.10	20.4	0.039	73.5
1.10	20.45	0.039	73.3
1.10	20.37	0.039	76.3

Table 10. Raw qPCR data for the RNU48 reference gene and non-template control for plate 2.

Sample	Cq	StdDev Cq	Tm
1.1	23.59	0.037	71.8
1.1	23.66	0.037	72
1.1	23.65	0.037	74
1.2	23.58	0.055	71.8
1.2	23.47	0.055	72.5
1.2	23.53	0.055	89.1
1.3	23.17	0.059	81.3
1.3	23.08	0.059	73.3
1.3	23.19	0.059	71.8
1.4	22.43	0.546	71.8
1.4	21.39	0.546	72.8
1.4	21.64	0.546	71.8
1.5	23.51	0.081	74
1.5	23.64	0.081	72.3
1.5	23.49	0.081	72
1.6	24.25	0.472	73
1.6	24.05	0.472	72
1.6	24.95	0.472	73.3
1.7	24.23	0.69	73.5
1.7	22.97	0.69	74.8
1.7	23.12	0.69	82.7
1.8	23.32	0.12	73
1.8	23.09	0.12	71.8
1.8	23.15	0.12	71.8
1.9	24.1	0.087	71.8

1.9	24.2	0.087	72
1.9	24.03	0.087	74.5
1.10	22.89	0.118	73.5
1.10	23.12	0.118	74.3
1.10	22.96	0.118	73.5
NTC	Undetermined		88
NTC	Undetermined		79