"Missing link between NLRP3 mediated neuroinflammation and micro RNA in Alzheimer's disease"

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE
OF

MASTER OF TECHNOLOGY
IN
BIOMEDICAL ENGINEERING

Submitted by:

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JULY, 2021

CANDIDATE'S DECLARATION

I, Ankita Das (Roll No.: 2K19/BME/05), student of M.Tech Biomedical Engineering, hereby declare that the project dissertation titled "**Missing link between NLRP3 mediated neuroinflammation and micro RNA in Alzheimer's disease**" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship, or other similar title or recognition.

Place: Delhi

Date: 05-07-2021

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CERTIFICATE

This is to certify that the M.Tech. Synopsis entitled "**Missing link between NLRP3 mediated neuroinflammation and micro RNA in Alzheimer's disease**" submitted by **Ms. Ankita Das (2K19/BME/05)** in partial fulfillment of the requirement for the award of the degree of Master of Technology from Delhi Technological University, is an authentic record of the candidate's own work carried out by her under my guidance. To the best of my knowledge this work has not been submitted in part and full for any Degree or Diploma to this University or elsewhere.

Place: Delhi

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ACKNOWLEDGEMENT

I would like to express my deepest gratitude to our research guide as well as head of the department **Prof. Pravir Kumar, Department of Biotechnology, Delhi Technological University** for his unstinted inspiration, invaluable guidance, encouragement, keen interest, good wishes and valuable suggestions throughout my entire research tenure.

I am thankful to Chitranjan Mukherjee for giving me moral boost and making my hopes alive with energy and enthusiasm to carry out the present work and helping hand at every step. I am also very much thankful to PhD scholars of our laboratory Sudhanshu Sharma, Rohan Gupta, Dia Advani, and Rahul Tripathi for helping me. My sincere thanks to my parents and family member for encouragement and moral support I received during the tenure of study.

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ABSTRACT

NOD-LRR and pyrin domain containing protein 3 (NLRP3) is abundantly expressed in macrophages and is involved in neuroinflammation in the brain. Together with an adaptor-protein called apoptosis associated speck like protein and pro-caspase-1, it forms an inflammasome complex. Inflammasome complex produces IL-1β and IL-18 and can trigger inflammation. NLRP3 inflammasome mediated chronic neuroinflammation was found to be a major factor in several neurodegenerative diseases (NDDs) like Alzheimer's disease (AD). Several reports elucidated the role of inflammasome in AD such as Aβ aggregation and Tau hyper-phosphorylation were found to be elevated by NLRP3 mediated neuroinflammation. Taking into account of the functional importance of NLRP3 inflammasome, it is needed to identify the regulatory mechanisms which control the expression of the NLRP3 gene. One such regulatory mechanism is MicroRNA (miRNA) mediated regulation where a miRNA can target the untranslated regions (UTRs) of the target gene thus reducing the expression of that gene. In this study, different step by step computational analysis were performed which included healthy brain tissue expression, presence of polymorphism at 3' UTR of NLRP3, ability to regulate the transcription factors of NLRP3, and microarray analysis of dysregulated miRNAs in AD patients. After that a thorough insilico validation was performed which involved pathway analysis, miRNA sponge analysis, and mRNA-miRNA binding site accessibility prediction by evaluating RNA secondary structure. Finally, ten novel miRNAs (hsa-17-5p, hsa-20b-5p, hsa-27a-3p, hsa-186-5p, hsa-30d-5p, hsa-30a-5p, hsa-30e-5p, hsa-338-3p, hsa-223-3p, and hsa-548a-3p) were reported having the most potentiality to regulate the expression of NLRP3 in AD.

Keywords: NLRP3, Inflammasome; Neuroinflammation; Innate immunity; Alzheimer's disease; Micro RNA; RNA secondary structure; Neurodegeneration.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
NDD	Neurodegenerative disorder
MiRNA	Micro RNA
UTR	Untranslated region
TF	Transcription factor;
SNP	Single nucleotide polymorphism
LncRNA	Long non coding RNA
CeRNA	competitive endogenous RNA

1. INTRODUCTION:

Alzheimer's disease (AD) has the most prevalent among the neurodegenerative diseases in the world preceding Parkinson's disease. AD is seen mostly in the elderly population who are aged over 65 years. According to the Taylor et al., by the year 2050, over 13.8 million people aged over 65 years are feared to be affected by the disease in the United States alone [1]. Two common forms of AD are there— "EOAD" (early onset of Alzheimer's disease) and "LOAD" (late onset of Alzheimer's disease). The latter is observed more frequently [2]. The basic symptoms involve dementia, cognitive impairment, decreased ability of judgment, difficulty in performing familiar tasks, having trouble with spatial activities etc. [3], [4]. The exact cause of the disease is not very clear which makes it an idiopathic disorder. According to the most accepted amyloid cascade hypothesis, transmembrane protein APP, which is involved in neuronal growth and survival, is sequentially cleaved by β-secretase and γ-secretase thus forming insoluble Aβ oligomers and aggregate in extracellular spaces [5]. Aβ aggregation is the prime molecular pathogenesis in AD patients. Tau is another important protein; phosphorylated form of which is responsible for stabilizing microtubules inside the neurons. In AD patients, hyper-phosphorylation of the protein results into destabilization of the microtubules which in turn creates aggregation of impaired helical filaments forming NFT (neuro fibrillary tangles) and ultimately deregulation of axonal transport occurs [6]. Researchers suggested several forms of neuronal insults such as oxidative stress, mitochondrial dysfunction, ubiquitin proteasome dysfunction, endoplasmic reticulum stress and neuroinflammation, could induce the AD pathogenesis [7], [8].

Microglia are the residing macrophage cells which are responsible for surveillance inside the central nervous system and protects it from infection and neuronal damage or protein aggregation and keeps the hemostasis maintained [9]. It gets activated and induce neuro-inflammation when damage signal is generated. Activated microglia could be both neuroprotective and neurotoxic. If the inflammation caused by activated microglia removes the infection or damage and after that inflammation gets stopped, it is neuroprotective but if the inflammation continues and starts to damage the area, it could be neurotoxic [10]. Inflammasome complex is the main source of neuro-inflammation in CNS [11], [12]. Inflammasomes are large multimeric protein complex assembly having ability to induce neuroinflammation. NOD-like receptor pyrin domain containing 3 (NLRP3) is one of the three major inflammasome complexes having potential involvement in AD pathogenesis [13]. When microglia is activated, inflammasome activates caspase-1 which then cleaves inactive pro-IL-

-1β and pro-IL-18 into active mature IL-1β and IL-18 molecules and produce inflammation [14]. Expression of NLRP3 was found to be significantly upregulated in the post mortem samples of the brain tissues of AD patients [15]. Recently Elijovich et al. showed significant level of IL-1β in AD patients [16]. Sutinen et al. reported that upregulated IL-18 triggers BACE1 and PSEN1 to form Aβ oligomers in AD [17]. It is also found that IL-18 induces production of GSK-3β and Cdk5 which are involved in tau hyper-phosphorylation in AD [18]. Inhibiting NLRP3 was reported to reduce Aβ burden and improve cognition in transgenic APP/PS1 mice [19]. So it is very clear that neuro-inflammatory damage in AD is mediated by NLRP3 inflammasome and related signaling pathways.

Micro RNA, a ~22 nucleotide long single stranded noncoding endogenous RNA, is abundantly expressed and has regulatory role in central nervous system [20]. In several neurodegenerative diseases, dysregulated expression of microRNAs are frequently observed [21]. In this study, we intended to perform comprehensive analysis of the 3' UTR of NLRP3 gene to find out regulatory microRNAs which can lead to AD pathogenesis through dysregulation of NLRP3 inflammation. Different step by step analysis were taken together and compared to find out the most potential miRNAs in regulation of NLRP3 gene and after that an insilico validation process was also applied to reinforce the outcome.

1. LITERATURE REVIEW:

Neurodegenerative diseases (NDDs) are complex disorders. In most of the cases, the cause of the disease is undetermined which makes these idiopathic. The ultimate fate of the neurons in these diseases is apoptosis. Due to severe complexity of these diseases, therapeutics are very limited. Some significantly prevalent NDDs are Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) etc.

Alzheimer's disease is the most prevalent neurodegenerative disease followed by Parkinson's disease. According to the 2014 statistics, about 5.5 million adults are to be affected by the disease alone in United States of America and nearly 13.8 million people are feared to be affected by 2050 [1]. The disease mostly affects the population who are above 60 in their age. So there is immense financial burden on the countries to deal with the disease. Estimated $818 billion was invested in 2015 to deal with the disease in USA [22]. The main cause is thought to be accumulation of deposited misfolded amyloid beta protein which generates amyloid plaque [23]. Also, tau protein is hyper-phosphorylated which creates neurofibrillary tangles

(NFTs) which leads to misassembled microtubules [24] Ultimately the neuron dies. Aβ and NFT are the major biomarkers of AD. Apart from these, several other reasons can cause the disease. One of the other causes are oxidative stress. Oxidative stress (OS) can induce the production of reactive oxygen species (ROS) and reactive nitrogen species (ROS) in the brain which can create amyloid burden and generate apoptosis signals [25]. Mitochondrial dysfunction and lysosomal dysregulation is also an important cause [26]. Post translational modifications or epigenetic regulation is also a significant cause of the disease as protein clearance process through ubiquitination and chaperone mediated proteasome system get dysfunctional [27]**.** As a consequences of the disease, several symptoms are observed such that dementia, cognitive decline, language problem, difficulty in doing simple things, poor judgement, misplacing object very frequently, disorientation in time and space, behavioral changes etc [28]**.** The most affected region of the brain in AD is hippocampus; although prefrontal cortex is also a significant location of AD affected brain [29]**.**

Microglia act as a surveillance system in the microenvironment of central nervous system. When any undesirable cellular events occurs such as debris accumulation or pathogenic invasion, microglia gets activated and performs phagocytosis and induce neuroinflammation by cytokine secretion. It has also function in brain development. It maintains a healthy neuronal environment inside CNS. The three main functional states of microglia are termed as "M1,""M2," and "M0" are linked to pro-inflammatory, anti-inflammatory, and surveillance activities, respectively. Microglia can be both neuroprotective and neurotoxic. If neuroinflammation caused by reactive microglia cleans the site of damage, it is beneficial but it could be extremely harmful if it starts to damage the peripheral healthy cells too [30]. It is noteworthy that hippocampus is the main affected part of the AD brain which is also a key site for neuroinflammation, which suggests a close correlation between neuroinflammation and AD pathogenesis [31]. Microglia and astrocytes were found to be clustered in AD brain tissue [32].

NLRP3 is a microglia recruited multimeric protein complex termed as "inflammasome", consisting of three main components which are: a sensor like molecule, an adaptor protein containing a CARD (ASC), and procaspase-1 [14]. When pattern recognition receptors (PRRs) recognize an inflammatory harmful stimuli, they makes oligomers with ASC protein via pyrin death domain (PYD) [33]. Most of the inflammasome sensors have NOD-like receptor domain (NLR). These NLRs are consists of three different elements which are following respectively: a "NACHT domain", a "leucine rich repeats (LRRs)" and "PYD" or "CARD" protein which interacts with ASC/CASP1 [34]. The first characterized NLR containing inflammasome complex was NLRP1 (NOD-LRR and pyrin domain containing 1) and subsequently other inflammasomes were identified such as NLRP3, NLRP6, NLRP7 etc. Among them NLRP3 is the most characterized inflammasome complex. Another inflammasome complex is absent in melanoma-2 (AIM2) which has a pyrin and HIN domain-containing protein (PYHIN) domain as its

sensor protein instead of NLR. [35]. Among all the inflammasomes, NLRP3 is the most studies inflammasome complex in context of Alzheimer's disease [36]. As the inflammasome complexes are one of the important mediators of neuroinflammation, so involvement of those in AD pathogenesis is highly likely. NLRP3 induces the formation of active interleukin 1β and interleukin 18 by cleaving their inactive precursors i.e. pro-interleukin 1β and pro-interleukin 18 [37].

Researchers had demonstrated that NLRP3 and caspase-1 knockout mice showing improved cognition and lowered neuroinflammation [15]. NLRP3 or CASP-1 knockout mice also showed reduced amyloid burden due to increased phagocytosis by microglia instead of improved APP processing which suggests that NLRP3 mediated neuroinflammation has direct involvement in amyloid clearance in Alzheimer's disease [36]. ATP released from the dying cells can activate microglial P2X7 receptors in AD [38]. In a microarray study, upregulated expression of both casp1 and interleukin-1β was seen in preclinical stage of AD [29]. Histological analysis of AD brain tissue supported the finding which showed higher level of microglial caspase-1 and ASC specks [39].

For an idiopathic disease, no possibilities should be ruled out, so polymorphism in the NLRP3 gene could also be a potential risk factor for AD progression. 2292 Han Chinese subjects were tested for NLRP3 polymorphism and rs2027432 was found to be significant for LOAD [40]. In a separate study, rs2027432 was found to elevate IL-1β through NLRP3 mRNA overexpression [41]. APP/PS1 mice with NLRP3 knock-out (KO) or NLRP3 inhibitor showed improved memory and reduced amyloid burden [42]. Reduced amyloid deposition was observed in NLRP3 knock-out mice AD model [14]. S100β, a protein localized mainly in astrocytes and a dementia associated biomarker of AD in elderly, is found elevated in the serum samples of AD patients which suggests astrocyte dysfunction in AD [43]**.** In-vitro and in-vivo model showed that interleukin-1β upregulates S100β [23]. In presence of internalized $A\beta_{1-42}$, microglial lysosome gets dysfunctioned which leads to release of cathepsin B triggering NLRP3 inflammasome activation [44]. Upregulation of MAPK p38 is significantly responsible for Tau hyperphosphorylation in AD. The upregulation of MAPK p38 is shown to be caused by increased IL-1β in-vivo. So this will not be wrong to infer in that inflammatory cytokine IL-1β is a responsible factor for MAPK p38 mediated tau hyperphosphorylation in AD. Also inhibiting IL-1β had shown improved cognition and reduced NFT burden [24]. Fibrilar Aβ which is the most significant component for AD pathogenesis, is a particulate sterile molecule which had been shown to activate NLRP3 inflammaome [44].

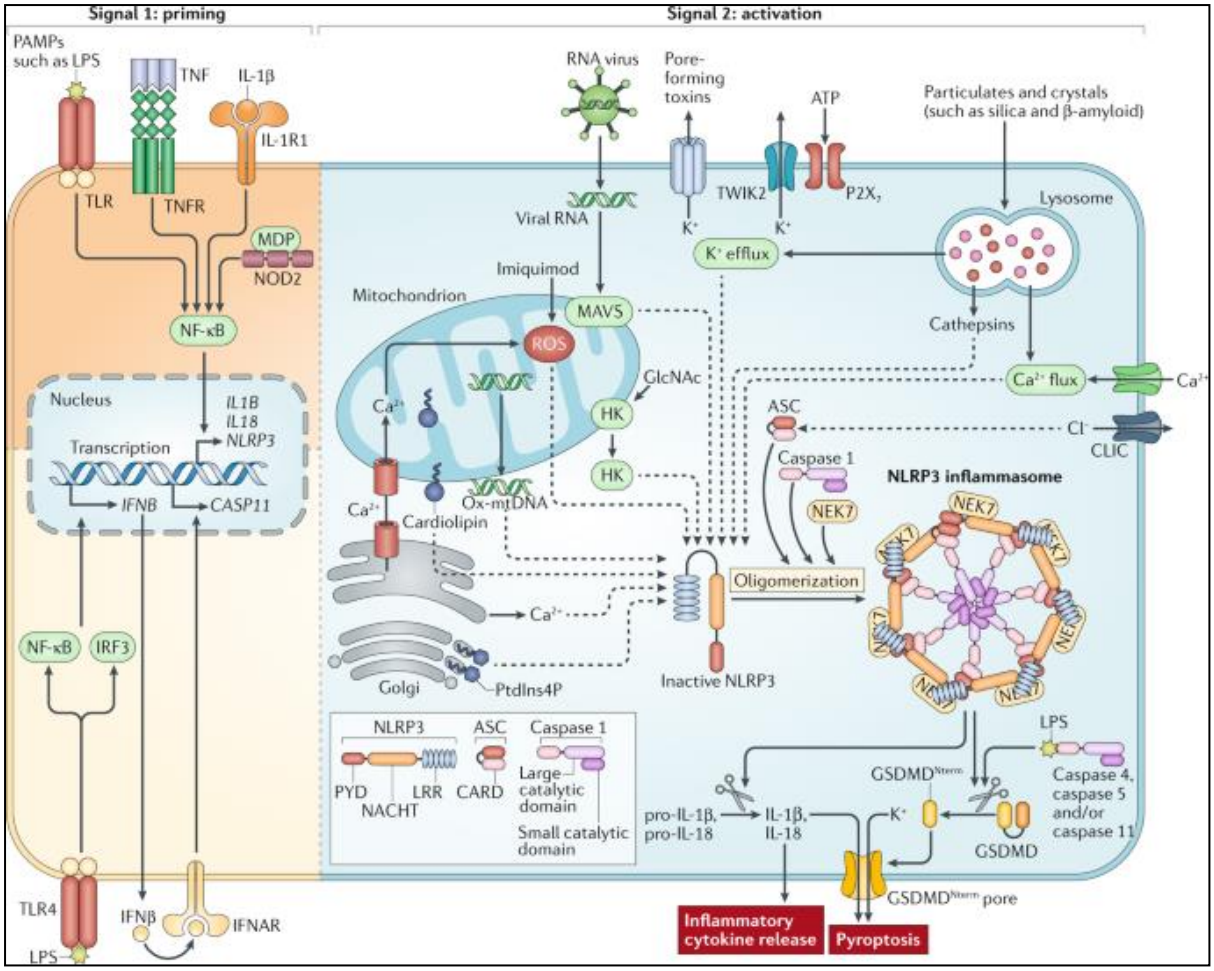


Fig 1: NLRP3 activation pathway

MicroRNA transcribes in the nucleus in form of double stranded hairpin structure named primary microRNA. Drosha and DGCR8 then cleave it first inside the nucleus and double stranded precursor miRNA is formed. After that it is transported to the cytoplasm by the help of EXPORTIN5 protein. The precursor miRNA is then attached to DICER and the hairpin loop is cleaved out. After that ARGOUNATE protein is bound on the double stranded miRNA, forming the RNA induced silencing complex (RISC). The passenger strand is then removed and thus mature miRNA is formed. The mature miRNA then can bind to the target mRNA seed regions according to the Watson-Crick base pairing [20].

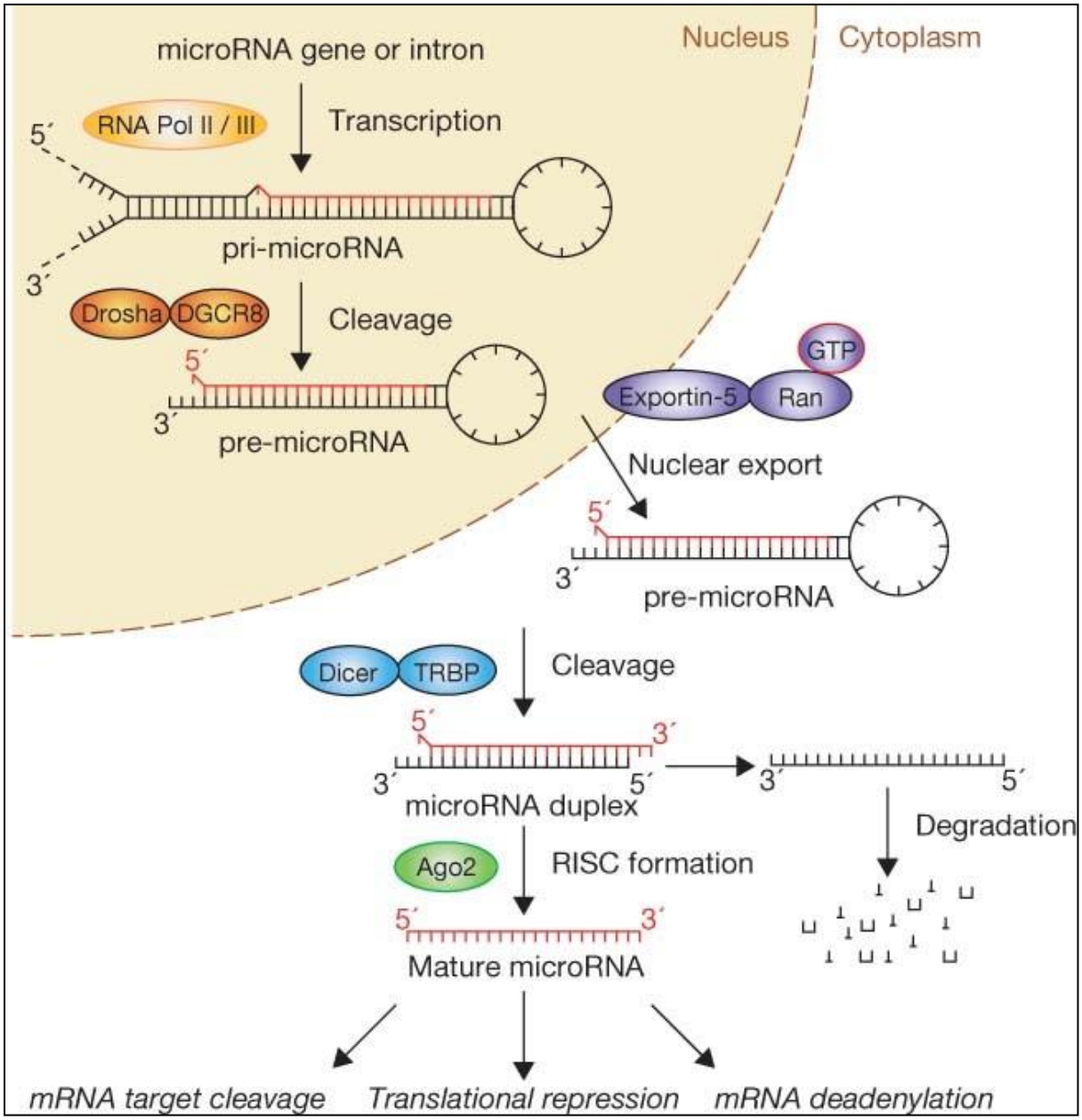


Fig 2: Micro RNA biosynthesis

Noncoding RNAs are highly enriched in brain [21]. Many microRNAs have important regulatory roles in CNS. In neurodegenerative diseases, dysregulated expression of microRNAs were observed [20]. So microRNA has been attracting much attention in the field of neurodegenerative disorders.

2. METHODOLOGY:

3.1 NLRP3 targeting micro RNAs:

Multistep justified analysis was performed to find out the most potential miRNAs which may contribute in Alzheimer's disease pathogenesis through neuro-inflammation by regulating NLRP3 gene. **Figure 3** shows the workflow that was followed in this study.

To pin point the miRNAs which have the potential to target the 3' untranslated region, an integrated miRNA target database "mirDIP" ("http://ophid.utoronto.ca/mirDIP/") [45] was searched. mirDIP is a publicly available database which combines a total of 30 different miRNA target prediction databases.

3.2 Analyzing miRNA expression in brain tissues:

The predicted miRNAs were subjected to tissue expression analysis to find out if they are expressed in healthy brain tissues. For this purpose, "TissueAtlas" ("https://ccb-web.cs.uni-saarland.de/tissueatlas/") [46] database were used. Quantile-normalized expression were obtained for each miRNA.

3.3 Finding presence of SNPs in the miRNA binding site at 3' UTR of NLRP3:

Presence of SNP at the 3' UTR of a particular gene can create or delete a miRNA binding region thus miRNA mediated gene regulation could be affected. So, we have used a SNP predicting database "MirSNP" ("http://bioinfo.bjmu.edu.cn/mirsnp/search/") [47] and "miRdSNP" ("http://mirdsnp.ccr.buffalo.edu/") [48] which predicted the possible presence of SNPs in the miRNA binding site at the 3' UTR of the NLRP3 matured mRNA.

3.4 miRNA mediated NLRP3 transcription factor regulation:

Transcription factor is a very significant regulator of a gene expression. So, if a transcription factors gets somehow dysregulated, it will affect the target gene expression. We are not omitting the possibility that our candidate miRNAs might bind to those transcription factors and as a result the transcription factors would get dysregulated which ultimately could affect the NLRP3 expression. So, keeping the fact in mind we predicted the transcription factors of NLRP3 using TF2DNA [49] database. Then the transcription factor and our candidate miRNAs were subjected to target prediction analysis with the help of mirDIP database.

3.5 Analyzing microarray expression of the candidate miRNAs in Alzheimer's disease:

To find out whether our candidate miRNAs are found to be dysregulated in Alzheimer's disease, we obtained microarray dataset from NCBI GEO [50] and differential expression

analysis was done using R programming language. Two Alzheimer's disease related datasets with accession numbers GSE16759 [51] and GSE48552 [52] were taken for the analysis. Entries with significance value ≤ 0.05 was considered. The fold change of common miRNA entries from both the datasets were averaged. Our candidate miRNAs were checked from the result to find out whether they are found to be dysregulated in AD.

3.6 Analyzing all the results and shortlisting the most potential miRNAs for NLRP3 regulation:

The results obtained from the previous analysis steps were taken together and a comparative evaluation was performed. A final list of miRNAs were made which have the most potential to regulate the expression of NLRP3.

3.7 Involvement of the shortlisted miRNAs in relevant signaling pathways:

In support or justification of the obtained results, the finalized miRNAs were taken for pathway analysis to get an idea whether they could be involved in neurodegeneration through inflammation. The pathways were obtained using miRPathDB (https://mpd.bioinf.uni-sb.de) [53] database. Simply those pathways were obtained which relates to neuroinflammation, apoptosis etc. Only experimentally validated strong evidences were selected.

3.8 Finding miRNA sponges:

A controversial approach of gene expression regulation is ceRNA (competitive endogenous RNA) where it is said that some noncoding RNAs such as lncRNA (long noncoding RNA), circRNA (circular RNA) can bind to miRNA response element (sponging of miRNA) which results in unavailability of the seed region on the miRNA and as a result the miRNA can no longer bind to the target mRNA [54]. So, we identified the lncRNAs which can bind to our shortlisted miRNAs using STARBASE (www.starbase.sysu.edu.cn) [55] database. A lncRNA-miRNA network was built using Cytoscape software [56]. Using cytoHubba plug-in [57] highly connected lncRNAs were identified.

3.9 Predicting miRNA binding site accessibility by analyzing RNA secondary structure:

Sequence complementarity does not necessarily ensures mRNA-miRNA binding, it also relies on binding site accessibility. Keeping that in consideration, we analyzed the secondary structure of the 3' UTR of NLRP3 mature mRNA using "RNAfold web server" ("http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi") [58]. The predicted seed regions were thoroughly checked with base pairing probabilities. Those seed regions were

considered which had high base-pairing probabilities and whose locations were in stem or stem-loop sections of the secondary structure.

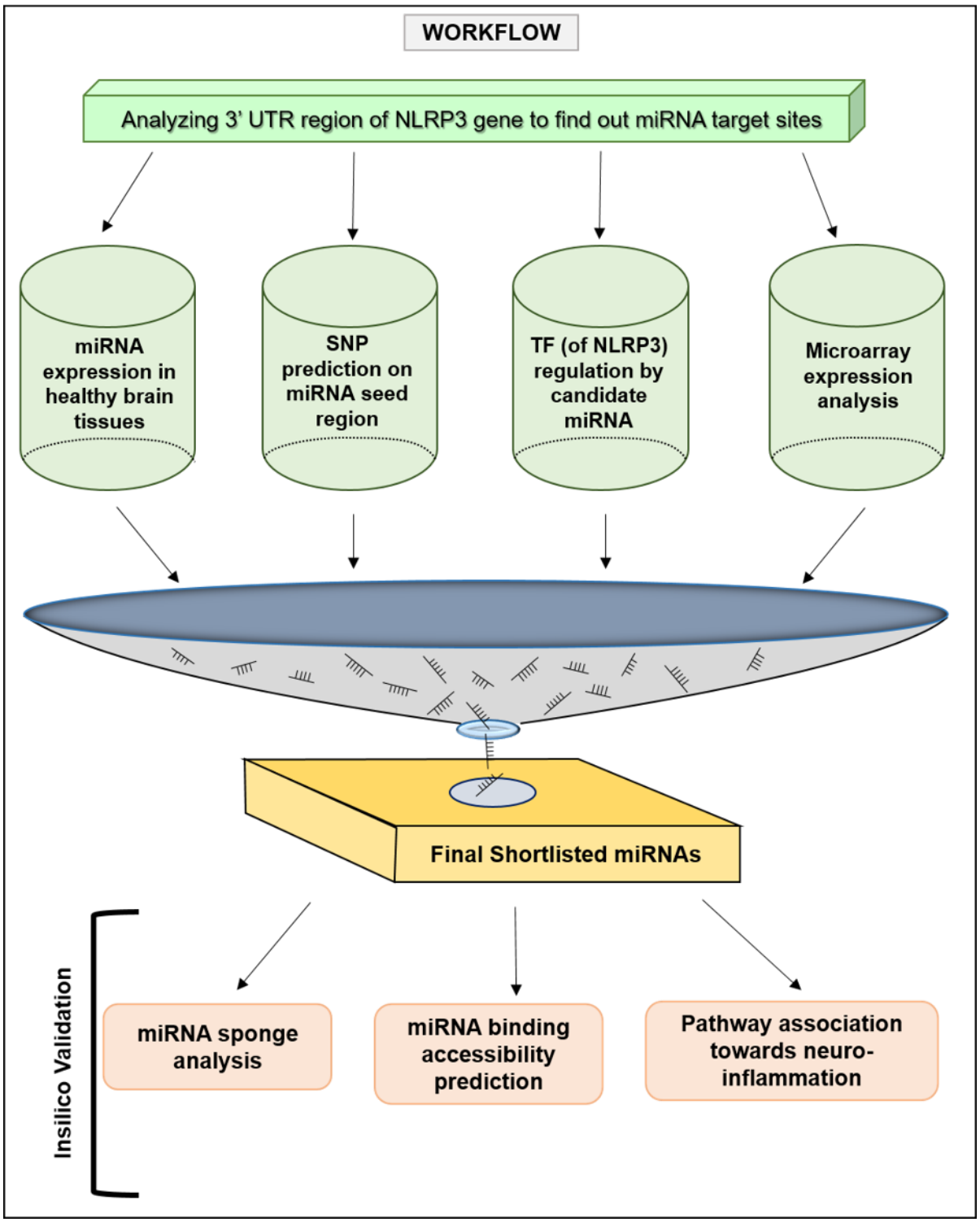


Fig 3: Methodology and flow diagram being used in the current study.

3. **RESULTS:**

4.1 miRNA target prediction:

"mirDIP" were used to find out predicted and experimentally validated miRNAs which can target 3' UTR of NLRP3 transcript. 91 miRNAs were identified (**Table 1**).

Table 1: Obtained target miRNAs for 3' UTR of NLRP3 gene predicted by mirDIP integrated database.

MicroRNA	Integrated Score	MicroRNA	Integrated Score
hsa-223-3p	0.792901633	hsa-141-3p	0.271310445
hsa-106a-5p	0.626399168	hsa-200a-3p	0.260046552
hsa-17-5p	0.603865088	hsa-4662a-3p	0.257574783
hsa-20b-5p	0.597402222	hsa-3613-3p	0.254534932
hsa-22-3p	0.558493408	hsa-5197-3p	0.253663367
hsa-520d-3p	0.557742851	hsa-524-5p	0.249554553
hsa-373-3p	0.554654118	hsa-526b-3p	0.239962071
hsa-302d-3p	0.542980071	hsa-3148	0.239310478
hsa-520e	0.538278932	hsa-548n	0.237948992
hsa-520c-3p	0.517647246	hsa-429	0.236780126
hsa-372-3p	0.510003196	hsa-144-3p	0.235430384
hsa-106b-5p	0.509299903	hsa-548g-3p	0.233781569
hsa-519d-3p	0.503119178	hsa-148b-5p	0.228150022
hsa-610	0.501330684	hsa-548e-3p	0.227287595
hsa-93-5p	0.49942261	hsa-3682-3p	0.225169188
hsa-20a-5p	0.4973222	hsa-876-5p	0.224436203
hsa-520a-3p	0.491282333	hsa-548i	0.223429531
hsa-302a-3p	0.484867624	hsa-412-3p	0.220494956
hsa-520b	0.468418257	hsa-143-3p	0.214431983
hsa-302b-3p	0.463985259	hsa-561-3p	0.213587027
hsa-302c-3p	0.461616283	hsa-548c-5p	0.21222602
hsa-556-5p	0.455525358	hsa-338-3p	0.212124874
hsa-302e	0.425612064	hsa-548ar-3p	0.210562441
hsa-520f-3p	0.416193207	hsa-421	0.209802385
hsa-548a-3p	0.408928539	hsa-1255b-5p	0.206198824
hsa-570-3p	0.374197822	hsa-27a-3p	0.205500849
hsa-4282	0.367458092	hsa-589-3p	0.203613854
hsa-1290	0.361822564	hsa-187-5p	0.202255994
hsa-1305	0.361063989	hsa-200b-3p	0.201934851
hsa-30d-5p	0.332817179	hsa-4803	0.201036643
hsa-186-5p	0.324398242	hsa-200c-3p	0.200680965
hsa-490-5p	0.315564554	hsa-3658	0.2001575
hsa-1253	0.31377036	hsa-559	0.199344487
hsa-548o-3p	0.311342465	hsa-3140-5p	0.198277058
hsa-30a-5p	0.308098976	hsa-4769-3p	0.189865494

hsa-1323	0.302966617	hsa-190a-5p	0.189636157
hsa-30b-5p	0.29872002	hsa-548a-5p	0.188602367
hsa-1272	0.298095882	hsa-1200	0.187062629
hsa-1246	0.298005384	hsa-5692b	0.186113124
hsa-548f-3p	0.29723976	hsa-30c-5p	0.184924996
hsa-30e-5p	0.29673157	hsa-7-5p	0.184862582
hsa-548p	0.295134365	hsa-548at-5p	0.184838876
hsa-513a-3p	0.293229912	hsa-548b-5p	0.184826049
hsa-520d-5p	0.279819074	hsa-5692c	0.183337094

4.2 miRNA expression in healthy brain tissues:

The obtained miRNAs were searched for their natural expression in brain tissues. TissueAtlas database were searched for this purpose. Table 2 shows the miRNAs which had significantly high expression level and moderate level in different brain tissues. 43 miRNAs were found to have very high expression and 9 miRNAs were found to have moderate expression. The rest of the miRNAs did not have that much expression in the brain tissues. **Table 2** and **Table 3** shows the miRNAs having significant expression and moderate expression levels in different healthy brain tissues respectively.

Table 2: list of miRNAs having significantly high expression in healthy brain tissues.

miRNA Name				
hsa-17-5p	hsa-30e-5p	hsa-302a-3p	hsa-4769-3p	hsa-548a-5p
hsa-20a-5p	hsa-93-5p	hsa-338-3p	hsa-520c-3p	hsa-548d-5p
hsa-20b-5p	hsa-106b-5p	hsa-373-3p	hsa-520d-3p	hsa-548f-3p
hsa-30a-5p	hsa-148b-5p	hsa-421	hsa-520e	hsa-548g-3p
hsa-30b-5p	hsa-186-5p	hsa-490-5p	hsa-520f-3p	hsa-548n
hsa-30c-5p	hsa-190a-5p	hsa-513a-3p	hsa-526b-3p	hsa-570-3p
hsa-30d-5p	hsa-302c-3p	hsa-519d-3p	hsa-548a-3p	hsa-610
hsa-876-5p	hsa-1253	hsa-1255b-5p	hsa-1305	hsa-3682-3p

Table 3: List of miRNAs having moderate expression in healthy brain tissues.

miRNA Name		
hsa-7-5p	hsa-187-5p	hsa-520a-3p
hsa-22-3p	hsa-429	hsa-520b
hsa-27a-3p	hsa-223-3p	hsa-1246

4.3 SNP variation in 3' UTR miRNA seed region of NLRP3:

Presence of SNPs can change the binding site of miRNAs on NLRP3 gene which might result into dysregulation of NLRP3 expression. So the existence of polymorphisms were checked for miRNA binding site on NLRP3 with the help of MirSNP database and MiRdSNP database. Only those outputs were retained which overlapped with our candidate miRNAs (**Table 4**). 10

of our candidate miRNAs (hsa-1323, hsa-223-3p, hsa-30d-5p, hsa-4662a-3p, hsa-548a-3p, hsa-548ar-3p, hsa-548at-5p, hsa-548g-3p, hsa-548o-3p, and hsa-570-3p) were found to have SNPs in their target region at the 3' UTR of the matured NLRP3 mRNA. Among them, hsa-223-3p have the highest number of SNPs (8 SNPs) at the 3' UTR target site.

Table 4: List of polymorphisms available in 3' UTR seed section of predicted miRNAs on NLRP3 transcript

miRNA	SNP	Alleles
hsa-1323	rs10802502	C > T
hsa-223-3p	rs10802501	T > A
	rs68080322	-
	rs34691535	dupT
	rs67491721	-
	rs10802502	C > T
	rs10754558	G > C,T
	rs34222187	dupG
	rs60418626	C >-
hsa-30d-5p	rs68080322	-
hsa-4662a-3p	rs10802501	T > A
hsa-548a-3p	rs10802502	C > T
hsa-548ar-3p	rs10802502	C > T
hsa-548at-5p	rs10802501	T > A
hsa-548g-3p	rs10802502	C > T
hsa-548o-3p	rs10802502	C > T
hsa-570-3p	rs10802502	C > T

4.4 Analysis of transcription factors in regulation of NLRP3 transcription:

Transcription factors can regulate the transcription of a gene. miRNA can regulate the expression of those transcription factors thus indirectly regulating the expression of the main target gene. With the help of TF2DNA database, we identified 34 transcription factors responsible for transcription of NLRP3 gene (**Table 5**). To find out potential target site of our candidate miRNAs on the 3' UTR of the transcription factors, we used mirDIP database. The miRNAs which can regulate (predicted) the transcription factors were listed in **Supplementary**

Table S1. The miRNAs which have the potential to bind to atleast 7 transcription factors, were considered as potential NLRP3 transcription regulating miRNAs (**Table 6**).

Table 5: Predicted transcription factors involved in regulation of NLRP3. Using TF2DNA database, the transcription factors responsible for NLRP3 transcription were obtained.

Gene	Transcription Factors
NLRP3	KLF11, MEIS2, JDP2, ZNF140, ZNF813, E2F7, NFATC1, KLF17, ATF7, ZNF438, FOSL1, NFATC3, ZNF708, ZSCAN22, ETV3, MYCL, ZNF560, ZNF121, PRDM13, HLF, ZNF774, ZNF575, ZNF766, ZIC5, RC3H2, NFIL3, FEV, ZNF256, ZNF852, ZNF440, ZXDB, BHLHA15, ZNF827, ZNF101

Table 6: Significant candidate miRNAs having predicted potential to regulate maximum number of transcription factors. miRNAs having binding site in more than equals to 7 transcription factors of NLRP3 were screened.

miRNA	Transcription Factors	Number of TFs Regulated
hsa-93-5p	E2F7, HLF, KLF11, MEIS2, MYCL, NFATC3, RC3H2, ZNF140, ZNF827, ZXDB	10
hsa-338-3p	E2F7,HLF,JDP2,MYCL,NFATC3,PRDM13,RC3H2,ZIC5,ZNF140,ZNF827	
hsa-30b-5p	E2F7, HLF, JDP2, KLF11, MEIS2, MYCL, NFATC3, PRDM13, ZIC5, ZNF827	
hsa-30e-5p	E2F7,HLF,JDP2,KLF11,MEIS2,NFATC1,NFATC3,PRDM13,ZNF827	9
hsa-30a-5p	E2F7, HLF, JDP2, KLF11, MEIS2, MYCL, NFATC3, PRDM13, ZNF827	
hsa-27a-3p	ATF7, E2F7, ETV3, MEIS2, NFIL3, RC3H2, ZIC5, ZNF708, ZNF827	
hsa-106a-5p	E2F7, HLF, KLF11, MEIS2, MYCL, RC3H2, ZNF140, ZNF827, ZXDB	
hsa-372-3p	E2F7, HLF, KLF11, MEIS2, NFATC3, RC3H2, ZNF827, ZXDB	8

hsa-30d-5p	E2F7, HLF, JDP2, KLF11, MEIS2, NFATC3, PRDM13, ZNF827	
hsa-30c-5p	E2F7, HLF, JDP2, KLF11, MEIS2, NFATC3, PRDM13, ZNF827	
hsa-22-3p	E2F7, FOSL1, HLF, MEIS2, MYCL, NFATC1, RC3H2, ZNF827	
hsa-20b-5p	HLF, KLF11, MEIS2, MYCL, NFATC3, ZNF140, ZNF827, ZXDB	
hsa-20a-5p	HLF, KLF11, MEIS2, MYCL, NFATC3, ZNF140, ZNF827, ZXDB	
hsa-186-5p	E2F7, HLF, RC3H2, ZIC5, ZNF440, ZNF708, ZNF813, ZXDB	
hsa-17-5p	HLF, KLF11, MEIS2, MYCL, NFATC3, ZNF140, ZNF827, ZXDB	
hsa-106b-5p	HLF, KLF11, MYCL, NFATC3, PRDM13, ZNF140, ZNF827, ZXDB	
hsa-519d-3p	HLF, KLF11, MEIS2, MYCL, NFATC3, ZNF140, ZNF827	7
hsa-548a-3p	ETV3, HLF, MEIS2, NFATC3, NFIL3, RC3H2, ZXDB	
hsa-513a-3p	HLF, KLF17, MEIS2, ZIC5, ZNF438, ZNF708, ZNF813	
hsa-373-3p	E2F7, HLF, KLF11, MEIS2, RC3H2, ZNF827, ZXDB	
hsa-143-3p	ETV3, MEIS2, MYCL, NFATC1, NFATC3, RC3H2, ZSCAN22	

4.5 Microarray expression analysis of the candidate miRNAs:

Two NCBI GEO microarray datasets GSE16759 and GSE48552 were used to perform differential expression analysis of miRNAs in AD patients. Using R programming language, normalization of the data was carried out and "LIMMA" R-package [59] was utilized to find out differentially expressed micro RNAs. Significance value ≤ 0.05 was considered. Among the resulted miRNAs, 15 such miRNAs were found to be dysregulated which belong to our

candidate miRNA list. Those miRNAs were hsa-148b-5p, hsa-17-5p, hsa-186-5p, hsa-190a-5p, hsa-20b-5p, hsa-223-3p, hsa-27a-3p, hsa-302b-3p, hsa-302c-3p, hsa-30a-5p, hsa-30e-5p, hsa-338-3p, hsa-3613-3p, hsa-421, hsa-589-3p.

4.6 Final selection of candidate miRNAs having potential of regulating NLRP3:

To finalize the potential candidate miRNAs, the results obtained from the previous steps were analyzed. Mainly, potential to target the atleast 7 transcription factors of NLRP3, normal brain tissue expression, microarray dysregulated expression and presence of SNP in 3' UTR of the gene were analyzed for each miRNA and a comparative analysis was implemented to find out the most potential miRNAs having regulating capacity for NLRP3. Those miRNAs were shortlisted which fulfilled atleast 3 criteria out of 4. Ten miRNAs (hsa-17-5p, hsa-20b-5p, hsa-27a-3p, hsa-186-5p, hsa-30d-5p, hsa-30a-5p, hsa-30e-5p, hsa-338-3p, hsa-223-3p, and hsa-548a-3p) were shortlisted. **Table 7** shows the final shortlisted miRNAs along with the criteria which they were tested in.

Table 7: Final shortlisted miRNAs having the potential to regulate the NLRP3 expression in AD. Candidate miRNAs were analyzed with four criteria. miRNAs found common from atleast three of the four criteria were selected and enlisted.

miRNA	Normal Brain Tissue Expression	Microarray Dysregulated Expression in AD	Number of Transcription Factors (of NLRP3) Regulated by miRNAs	Presence of SNP in miRNA Binding Site at 3' UTR of NLRP3
hsa-17-5p	SIGNIFICANT	DYSREGULATED	8	NA
hsa-20b-5p	SIGNIFICANT	DYSREGULATED	8	NA
hsa-27a-3p	MODERATE	DYSREGULATED	9	NA
hsa-186-5p	SIGNIFICANT	DYSREGULATED	8	NA
hsa-30d-5p	SIGNIFICANT	NA	8	rs68080322
hsa-30a-5p	SIGNIFICANT	DYSREGULATED	9	NA
hsa-30e-5p	SIGNIFICANT	DYSREGULATED	9	NA
hsa-338-3p	SIGNIFICANT	DYSREGULATED	10	NA
hsa-223-3p				rs10802501

	MODERATE	DYSREGULATED	NA	rs68080322
				rs34691535
				rs67491721
				rs10802502
				rs10754558
				rs34222187
				rs60418626
hsa-548a-3p	SIGNIFICANT	NA	7	rs10802502

4.7 Signaling pathways of the final miRNAs:

The final listed miRNAs were subjected to pathway analysis through miRPathDB database. Only experimental evidences were considered. As NLRP3 is directly linked to neuro-inflammation in AD so the focus was given on the pathways which are linked to neuro-inflammation, neuronal apoptosis, microglial activation, interleukin activation, innate immunity response etc. **Table 8** shows the significantly relevant pathways in which the shortlisted miRNAs are involved. The resulted miRNAs that were found to be involved in those pathways were hsa-186-5p, hsa-17-5p, hsa-27a-3p, hsa-30a-5p, hsa-30e-5p and hsa-223-3p were. Among these, hsa-223-3p and hsa-17-5p were involved with most of the resulted pathways which signifies critical role of these two miRNAs in NLRP3 mediated neuroinflammation. **Figure 4** represents the validated pathways along with the involved miRNAs.

Table 8: Association of the shortlisted miRNAs in AD through neuro-inflammation related pathways. Shortlisted miRNAs were subjected to pathway analysis to find out if they are involved in neuroinflammation, innate immunity activation, apoptosis and other AD related pathways.

Pathways	miRNAs Involved	P-value	Genes
Regulation of NLRP3 inflammasome	hsa-17-5p	0.014	HSP90AB1, PYCARD, TXNIP
	hsa-186-5p	0.026	HSP90AB1, P2RX7, RELA, SUGT1
Positive regulation of interleukin-1 beta production and secretion	hsa-223-3p	0.027	CCL3, NLRP3

Regulation of oxidative stress-induced neuron intrinsic apoptotic signaling pathway	hsa-223-3p	0.03	FBXW7, PARP1
	hsa-27a-3p	0.03	FBXW7, HIF1A, PINK1, SLC7A11
Structural pathway of Interleukin 1 (IL-1)	hsa-17-5p	0.016	IRAK1, IRAK4, MAP3K14, MAP3K3, MAP3K8, MAPK1, MAPK14, MAPK9, MKNK2, MYC, RPS6KA5
	hsa-223-3p	0.048	CHUK, MKNK2
Alzheimer's disease	hsa-30e-5p	0.037	CASP3, TP53
Regulation of microglial cell activation	hsa-223-3p	0.023	ATM, CCL3, IL6, NAMPT
	hsa-17-5p	0.049	APP, LDLR, TNF
Neuron apoptotic process	hsa-17-5p	0.015	ADARB1, APP, BACE1, BCL2, BCL2L11, BTG2, CASP2, CIT, DRAXIN, EGLN3, F2R, FOXQ1, FZD9, GAPDH, HIF1A, KCNB1, MAP3K12, MCL1, MDK, MECP2, NONO, OXR1, PDPK1, PIK3CA, PRNP, RB1, SOD2, TNF, TNFRSF21, TP53, UBE2V2, CBL, CCL5, CLU, CREB1, EIF2S1, REST, RRAS2, SLC7A11, STAT3
	hsa-223-3p	0.003	ATM, CCL3, FBXW7, FOXO3, MEF2C, NAMPT, PARP1, TP53, RRAS2, STAT3, TP53
	hsa-27a-3p	0.006	ATN1, BNIP3, BTG2, CPEB4, EN2, FBXW7, FZD9, GATA3, GSK3B, HIF1A, HIPK2, KRAS, MAP2K4, NF1, PINK1, THRB, TP53, SLC7A11, VPS35
	hsa-30a-5p	0.038	ABL1, ATM, BAX, CAPN2, CASP3, CTNNB1, JUN, MYBL2, PICALM, TP53, WNT5A
Regulation of neuro-inflammatory response	hsa-223-3p	0.041	ATM, CCL3, IL6, NAMPT
	hsa-27a-3p	0.048	ADORA2B, ALDH9A1, ARL6IP1, CYP1B1, DPYD, GIPC1, GSK3B, IFNG, KHSRP, NF1, PNKD, RAP1B, SLC7A11, SLC7A2, SNCG, EGFR, IGF1, LDLR
	hsa-17-5p	0.041	APP, CLU, LDLR, TLR7, TNF

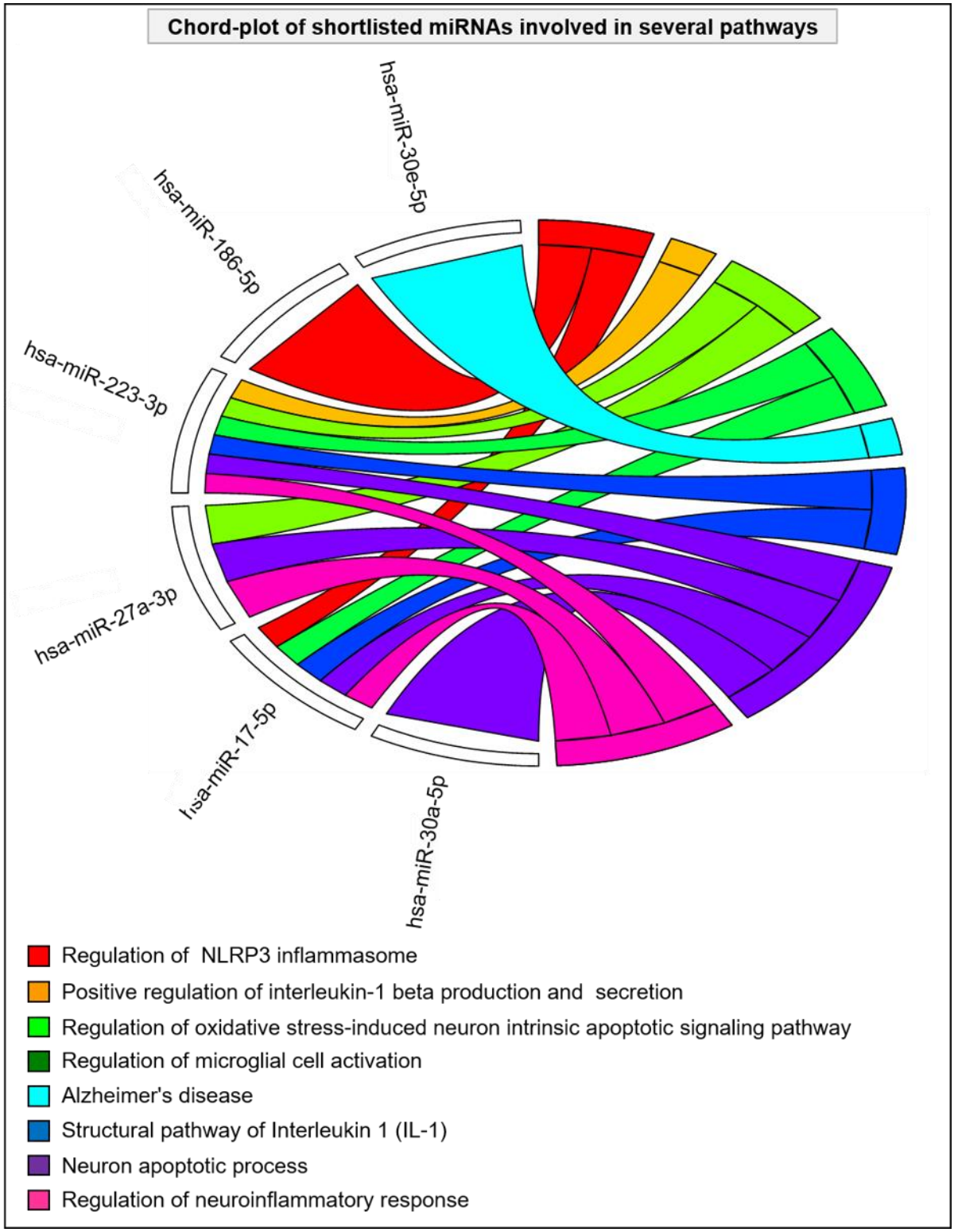


Fig 4: Chord plot of the shortlisted miRNAs involved in neuroinflammation, innate immunity activation, apoptosis and other AD related pathways. hsa-186-5p, hsa-17-5p, hsa-27a-3p, hsa-30a-5p, hsa-30e-5p and hsa-223-3p were found to be involved in those pathways. Among these, hsa-223-3p and hsa-17-5p were found to be involved with most of the resulted pathways which signifies crucial role of these two miRNAs in NLRP3 mediated neuroinflammation.

4.8 Analyzing miRNA sponge activity by different lncRNAs:

ceRNA hypothesis states that noncoding RNAs such as lncRNA. circRNA can bind to miRNA response element on miRNA and inhibit the miRNA to bind to the target mRNA (gene) thus affecting the gene regulation. This activity is referred as miRNA sponging. In this study, we have analyzed the potential lncRNAs which can sponge our shortlisted miRNAs. STARBASE database were used to find out potential lncRNAs having binding site for our candidate miRNAs. RNA-seq and high-throughput data were provided in the database. Only strongly correlated lncRNAs were screened from the database. An lncRNA-miRNA network was constructed using Cytoscape software (**Figure 5**).

Except hsa-548a-3p, all the miRNAs were found in the database to have sponging information. Using cytoHubba network plug-in, highly connected lncRNAs were identified among which NEAT1, XIST, KCNQ1OT1, MALAT1, AC021078.1, EPB41L4A-AS1, OIP5-AS1, and AL035425.3 had significantly high predicted MRE toward our candidate miRNAs.

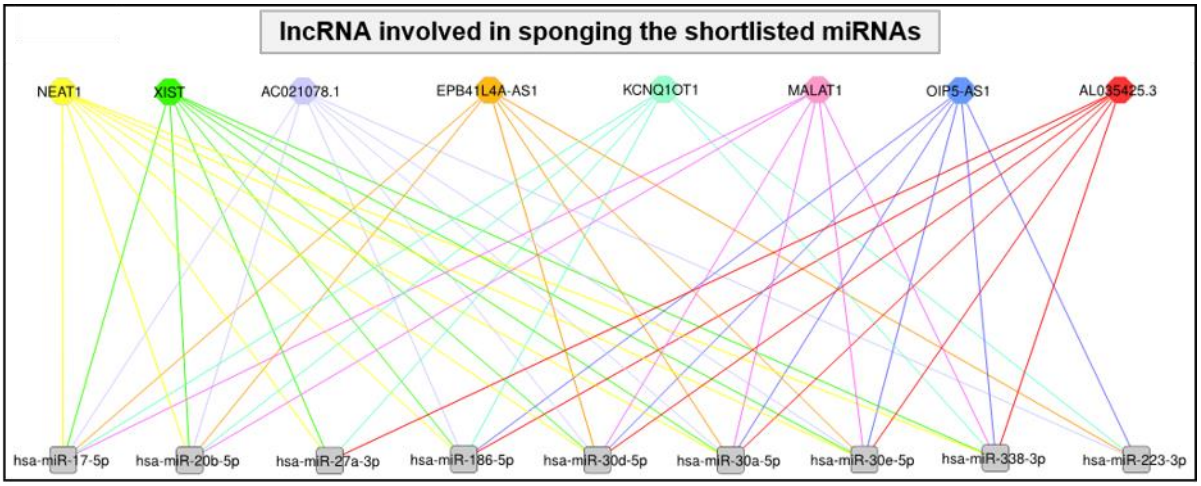


Fig 5: lncRNA mediated ceRNA network for shortlisted miRNAs. lncRNA can act as ceRNA so it can sponge the miRNAs to bind to the target gene. Several lncRNAs were identified having sponging ability for the shortlisted miRNAs and as a result NLRP3 regulation could be hampered. NEAT1, XIST, KCNQ1OT1, MALAT1, AC021078.1, EPB41L4A-AS1, OIP5-AS1, and AL035425.3 were the top 8 lncRNAs which can sponge to atleast 6 of the shortlisted miRNAs. No sponging information was found for hsa-548a-3p. NEAT1 and XIST have the potentiality to bind to the maximum number (eight) of the shortlisted miRNAs.

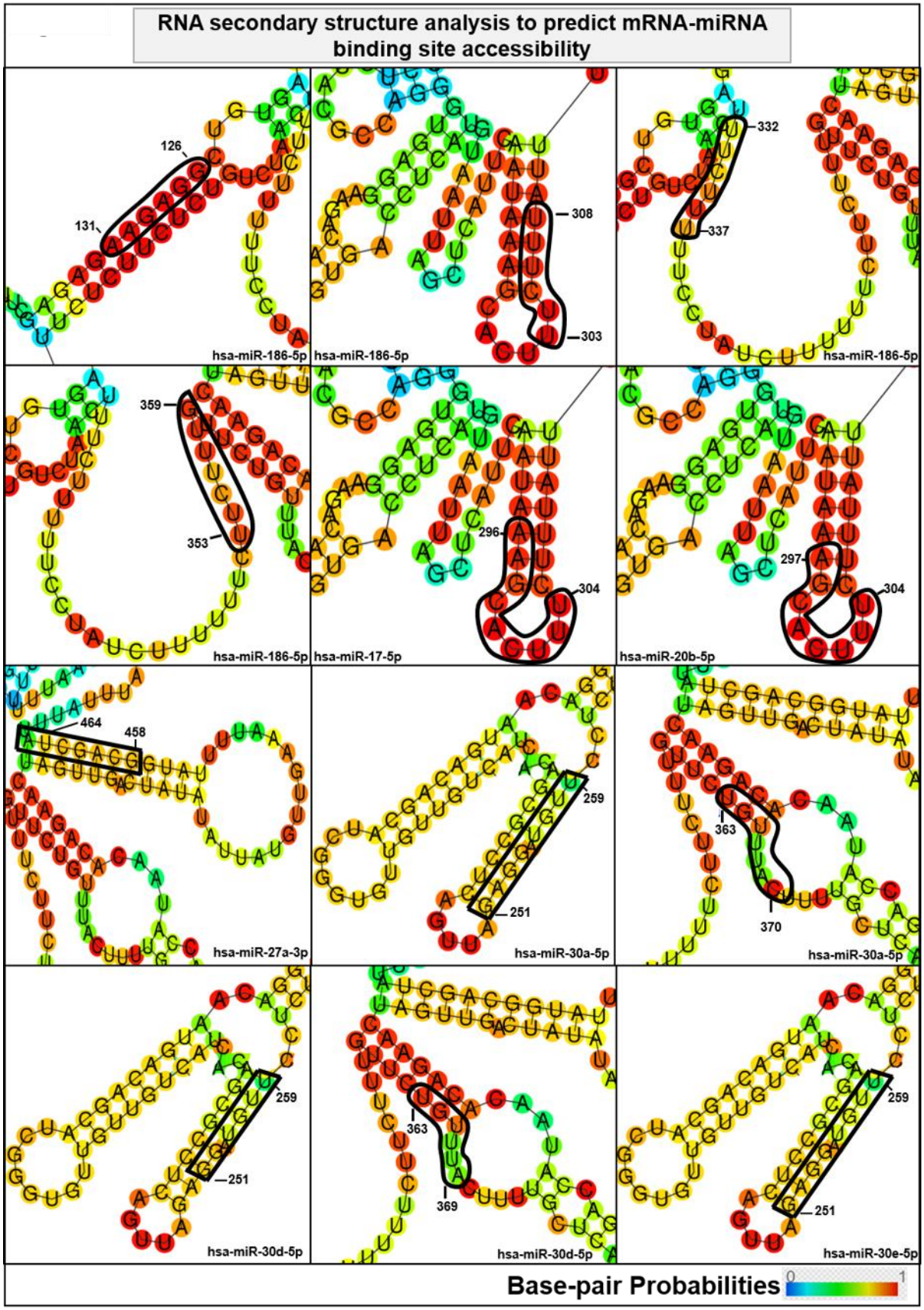
4.9 Secondary structure prediction:

Most of the miRNA binding prediction tools provide sequence based prediction. But one major factor for a miRNA to bind is its target site accessibility. If the target site is complex, steric hindrance is there and as a result the miRNA cannot bind to that region despite having seed match. So, we computed the secondary structure of the 3' UTR of the NLRP3 mature mRNA and the binding site of the shortlisted miRNAs were analyzed. **Supplementary Table S2** shows the 3' UTR sequence of the NLRP3 matured mRNA. **Figure 6** shows the seed regions

of the shortlisted miRNAs at the 3' UTR of the NLRP3 mRNA. The representation depicted on the figures were based on base-pair probabilities. Low base-pair probabilities means the target site accessibility is less so chances of miRNA binding is low and high base-pair probabilities means the miRNAs can access the target regions thus can easily bind. **Table 9** gives a comparative analysis of the shortlisted miRNAs for binding site parameters to access the seed regions.

Table 9: Comparative analysis of the binding site accessibilities of the shortlisted miRNAs by analyzing the secondary structure of NLRP3. The seed sequence of the shortlisted miRNAs were checked by analyzing the secondary structure of the 3' UTR of the NLRP3 mature mRNA sequence to find out binding site accessibility if the shortlisted miRNAs.

miRNA	Seed Sequence on Mature miRNA (5'-3')	Seed Length	Location on 3' UTR of NLRP3	Location on 3' UTR of NLRP3 Secondary Structure
hsa-186-5P	UUCUCC	6 mer	126-131	Stem
	AAAGAA	6 mer	303-308	Stem-hairpin
	AAAGAA	6 mer	332-337	Loop
	CAAAGAA	7 mer	353-359	Loop
hsa-17-5p	AAAGUGCUU	9 mer	296-304	Stem-hairpin
hsa-20b-5p	AAAGUGCU	8 mer	297-304	Stem-hairpin
hsa-27a-3p	UAGCUGC	7 mer	458-464	Stem
hsa-30a-5p	AACAUCCUC	9 mer	251-259	Stem
	GUAAACA	7 mer	363-370	Stem-internal loop
hsa-30d-5p	AACAUCC	7 mer	253-259	Stem
	GUAAACA	7 mer	363-369	Stem-internal loop
hsa-30e-5p	AACAUCCUC	9 mer	251-259	Stem
	GUAAACA	7 mer	363-369	Stem-internal loop
hsa-223-3p	CAAGCU	6 mer	135-140	Stem-multiloop
	CAAGCU	6 mer	163-168	Stem-internal loop
	UGACAA	6 mer	228-233	Stem
hsa-338-3p	UCCUGGUG	8 mer	199-206	Stem
hsa-548a-3p	AAUUAC	6 mer	277-282	Pseudoknot
	CUGGCA	6 mer	16-21	Stem-internal loop
	ACUGGC	6 mer	24-29	Stem
	CAAAACUG	8 mer	489-497	Stem


RNA secondary structure analysis to predict mRNA-miRNA binding site accessibility
126
131
hsa-miR-186-5p
308
303
hsa-miR-186-5p
332
337
hsa-miR-186-5p
359
353
hsa-miR-186-5p
296
304
hsa-miR-17-5p
297
304
hsa-miR-20b-5p
464
458
hsa-miR-27a-3p
259
251
hsa-miR-30a-5p
363
370
hsa-miR-30a-5p
259
251
hsa-miR-30d-5p
363
369
hsa-miR-30d-5p
259
251
hsa-miR-30e-5p
Base-pair Probabilities
0
1

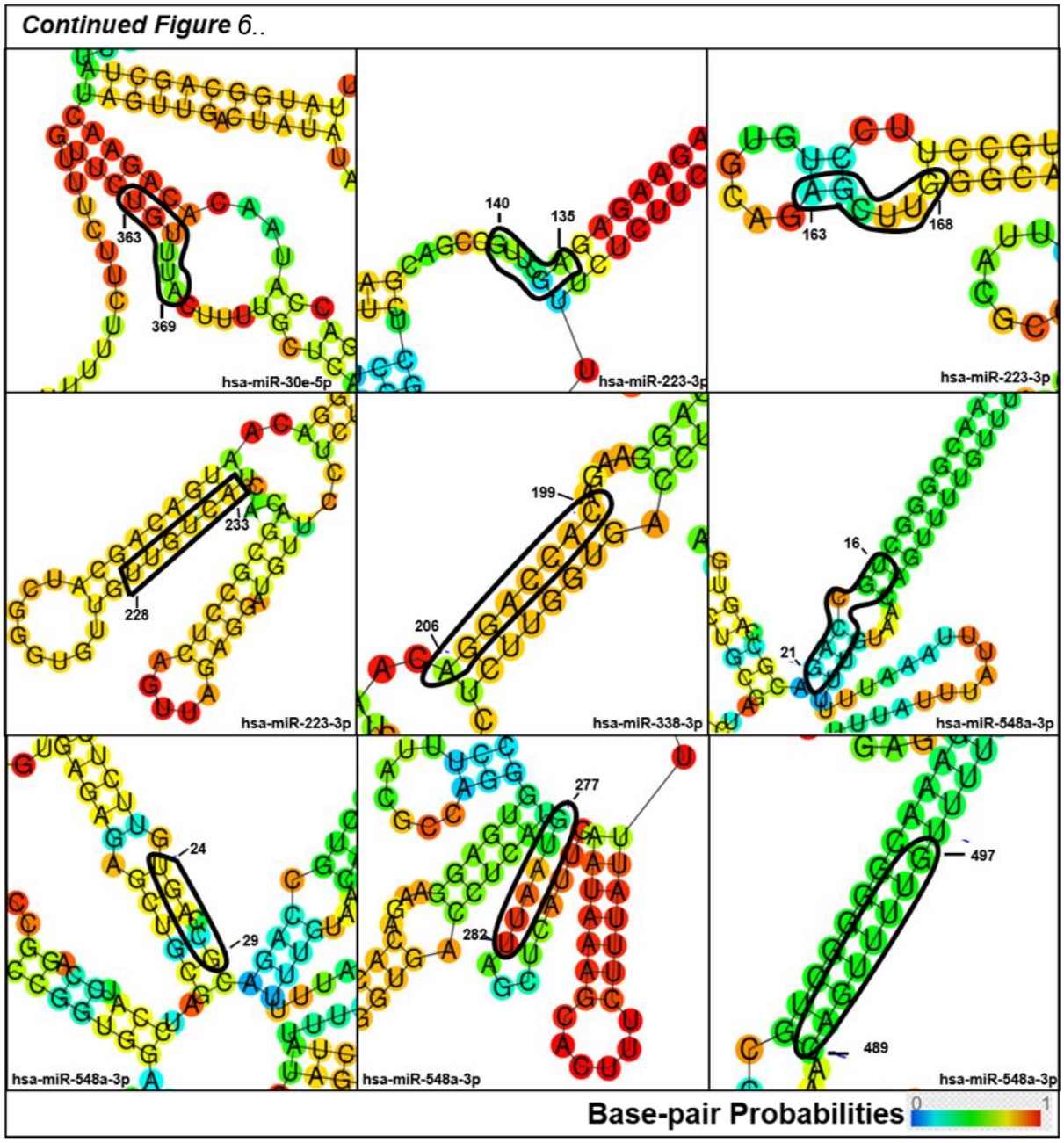


Fig 6: Secondary structure of the 3' UTR of NLRP3 matured mRNA. The miRNA-mRNA binding accessibility prediction was done by analyzing the RNA secondary structure. The seed sites for the corresponding miRNAs on the 3' UTR of the NLRP3 is shown in the black bordered areas. The nucleotides are color coded according to their base-pairing probabilities. Red corresponds to the highest probability of base-pairing and orange and yellow corresponds to moderate base-pairing probabilities but green, sky and blue depicts lower base-pairing probabilities. hsa-186-5p (seed: 126-131, 303-308, 353-359), hsa-17-5p (seed: 296-304), hsa-20b-5p (seed: 297-304) have significantly higher probabilities to bind to the 3' UTR of NLRP3. hsa-223-3p (seed: 135-140, 163-168), hsa-548a-3p (seed: 16-21, 489-497) have very low probability to bind to NLRP3. Rest of the miRNAs have moderate chanced to make base-pairing with the NLRP3.

4. DISCUSSION:

Neuro-inflammation is one of the major suspected causes of neurodegenerative disorders. Severe chronic neuro-inflammation is observed in AD, PD, ALS, HD and other NDDs [60]. Any kind of neuronal shock can trigger neuro-inflammation. It is a natural protective weapon to the CNS. After clearing the infection or aggregation, the inflammation stops but when it starts to damage self-tissues it is very much harmful. In NDDs, neuron undergo apoptosis but the cause is not very clearly understood. AD is the most prevalent NDD and the most common form of dementia in the world. According to the World Alzheimer's Report, 46.8 million people are suffering from dementia. In AD, during post-translational modification, amyloid precursor protein (APP) is cleaved by β-secretase instead of α-secretase and subsequently cleaved by γ-secretase and forms insoluble Aβ oligomers. It accumulates and aggregates as amyloid plaques in extracellular space in different brain regions [61]. On the other hand, microtubule associated protein Tau is hyper-phosphorylated and forms NFT plaques [28]. These aggregations trigger innate immunity inside the brain which is mediated by microglia. Activated microglia induces neuro-inflammation. Inside the microglia, there are inflammoasomes, which are multi domain protein complex, actually creates the inflammation. NLRP3 (NOD-LRR and pyrin domain containing protein 3), or cryopyrin, was found to colocalize with neuritic plaques in Alzheimer's disease [44]. NLRP3 along with adaptor protein ASC and pro-caspase-1 helps producing active IL-1β and IL-18 and triggers neuro-inflammation. Reports showed that Aβ plaque formation and deposition were promoted by injecting ASC specks in APP/PS1 transgenic mice while deletion of ASC prevented the spreading of the plaques [62]. Tau22/Asc−/− and Tau22/Nlrp3−/− transgenic mice showed reduced tau pathology and improved cognitive ability [63]. Deletion of NLRP3 caused reduction in Aβ deposition and brain neuro-inflammation and also relived cognitive impairment in 3 x Tg-AD mice [64].

Micro RNAs are ~22 nt endogenous non-coding, single-stranded RNAs which are not only found in brain aberrantly but also have regulatory mechanisms by controlling several gene expression by mRNA degradation or post translational inhibition. They are involved in neuron differentiation, maturation, synaptic plasticity and many other crucial processes inside the brain [65]. In neurodegenerative disorders, significant dysregulation of miRNA expression level was observed which suggests the involvement of those in the disease pathogenesis. Researchers showed that dysregulated miRNA can cause deregulation of their target genes which in term leads to different abnormalities [66].

In our study, different miRNAs which have the potential to target the NLRP3 inflammasome, were analyzed. Initially the study started with 91 candidate miRNAs. Four step insilico methodology was followed to shortlist the most potential regulatory miRNAs targeting the NLRP3. First the candidate miRNAs were checked whether they have mentionable expression level in different brain tissues or not. 43 miRNAs were found to have significantly high expression level and 9 miRNAs were found to have moderate expression. After that, presence of polymorphism were checked in the miRNA binding region at the 3' UTR of NLRP3 mRNA. A total of SNPs were found in the binding region of 10 miRNAs (hsa-1323, hsa-223-3p, hsa-30d-5p, hsa-4662a-3p, hsa-548a-3p, hsa-548ar-3p, hsa-548at-5p, hsa-548g-3p, hsa-548o-3p, and hsa-570-3p) at the 3' UTR of NLRP3. Among those, hsa-223-3p has 8 SNPs alone. Third step was to find out whether the candidate miRNAs can target the transcription factors of NLRP3. The rationale behind performing the step was that the transcription factors of a gene is responsible for its normal transcription so deregulated TFs can lead to dysregulated then the gene transcription get deregulated. So, if our candidate miRNA targets and inhibits the TFs of NLRP3 then there is a possibility that the NLRP3 normal transcription might get hampered. In this analysis we have selected only those miRNAs which can target atleast 7 TFs of NLRP3. 21 such miRNAs (hsa-93-5p, hsa-338-3p, hsa-30b-5p, hsa-30e-5p, hsa-30a-5p, hsa-27a-3p, hsa-106a-5p, hsa-372-3, hsa-30d-5p, hsa-30c-5p, hsa-22-3p, hsa-20b-5p, hsa-20a-5p, hsa-186-5p, hsa-17-5p, hsa-106b-5p, hsa-519d-3p, hsa-548a-3p, hsa-513a-3p, hsa-373-3p, and hsa-143-3p) were identified. Lastly we carried out microarray expression study to identify whether our candidate miRNAs were found to be dysregulated in AD patient's postmortem brain tissues. 16 miRNAs were identified which belong to our candidate miRNA list and were found to be dysregulated in the microarray expression analysis. The miRNAs are hsa-148b-5p, hsa-17-5p, hsa-186-5p, hsa-190a-5p, hsa-20b-5p, hsa-223-3p, hsa-27a-3p, hsa-302b-3p, hsa-302c-3p, hsa-30a-5p, hsa-30e-5p, hsa-338-3p, hsa-3613-3p, hsa-421, hsa-589-3p. After that results from all these 4 criteria were compared and those miRNAs were shortlisted which could satisfy 3 out of the 4 criteria. After a thorough comparison, 10 such miRNAs were considered as the final shortlisted miRNAs having the most potential to regulate the expression of NLRP3 gene expression. Those 10 miRNAs are hsa-17-5p, hsa-20b-5p, hsa-27a-3p, hsa-186-5p, hsa-30d-5p, hsa-30a-5p, hsa-30e-5p, hsa-338-3p, hsa-223-3p, and hsa-548a-3p. To validate our result, three additional insilico validation steps were followed. Firstly, the shortlisted miRNAs were checked for pathway involvement. Experimentally validated pathway analysis were performed. Only those pathways were considered which involved NLRP3 inflammasome, neuro-inflammation, microglial activation, neuronal apoptosis, Alzheimer's disease, innate immunity

etc. 6 out the 10 miRNAs (hsa-186-5p, hsa-223-3p, hsa-27a-3p, hsa-17-5p, hsa-30e-5p, hsa-30a-5p) were found to be related to those mentioned pathways. Though no experimentally validated pathways were found for hsa-548a-3p, hsa-338-3p, hsa-30d-5p, and hsa-20b-5p but predicted pathways significantly included all the 10 miRNAs. The next insilico validation step was to find for miRNA sponges for those 10 miRNAs. Long non-coding RNAs can sponge the miRNAs which in turn disables the miRNAs to bind to their target genes thus inducing dysregulation of the genes. So, using network biology, 8 crucial miRNA sponging lncRNAs (NEAT1, XIST, KCNQ1OT1, MALAT1, AC021078.1, EPB41L4A-AS1, OIP5-AS1, and AL035425.3) were identified which can sponge atleast 6 out of the 10 shortlisted miRNAs. The final step of insilico validation was to predict the miRNA binding site accessibility on 3' UTR of the NLRP3 matured mRNA. Usually miRNA target prediction tools uses sequence complementarity but they usually disregard the steric property of the binding site. So a predicted binding site may not be available on an mRNA due to steric hindrance. So we analyzed the secondary structure of the 3' UTR of the mature NLRP3 mRNA. All the seed sites on the 3' UTR were checked whether miRNA can get binding accessibility there. Considering the base-pairing probabilities, hsa-186-5p (seed: 126-131, 303-308, 353-359), hsa-17-5p (seed: 296-304), and hsa-20b-5p (seed: 297-304) had very high probabilities of binding to the 3' UTR of NLRP3. hsa-223-3p (seed: 135-140, 163-168), hsa-548a-3p (seed: 16-21, 489-497) had very low probability of binding to the NLRP3 and rest of the seed sites of the shortlisted miRNAs had moderate chanced to make base-pairing with the NLRP3 matured mRNA. But as there is limitation of research in RNA secondary and tertiary structure, so, the shortlisted miRNAs should be taken for further in-vitro and in-vivo validation.

5. CONCLUSION:

NLRP3 is the most characterized inflammasome complex which is a key mediator of neuro-inflammation in CNS and was reported to be involved in several neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. Upregulation of NLRP3 expression reported to cause neuroinflammation in Alzheimer's disease. In this study, multistep computational analysis was performed to investigate the role of miRNAs to regulate the expression of NLRP3 gene. Several miRNAs were identified by analyzing the 3' UTR of NLRP3 mRNA. Different insilico approaches was applied such as healthy brain tissue expression, presence of SNPs in miRNA binding site on NLRP3, ability of miRNAs to regulate the transcription factors of NLRP3 and microarray expression analysis of AD patients. Combining the results, 10 novel miRNAs were identified (hsa-17-5p, hsa-20b-5p, hsa-27a-3p, hsa-186-5p, hsa-30d-5p, hsa-30a-5p, hsa-30e-5p, hsa-338-3p, hsa-223-3p, and hsa-548a-3p) which may have the most potentiality to regulate the NLRP3 expression. Lastly, a three steps insilico validation was performed comprising i) involvement of the shortlisted miRNAs in neuroinflammation related pathways, ii) miRNA sponge analysis and iii) Secondary RNA structure prediction of 3' UTR of NLRP3 mRNA to find out miRNA-mRNA binding site accessibility. Experimental validation is need to find out the detailed regulatory role of the miRNAs in maintaining the NLRP3 expression level in Alzheimer's disease.

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Supplementary material:

Supplementary Table S1: Predicted Interaction between predicted miRNAs and obtained transcription factors to know how transcription factors get regulated by miRNA binding.

miRNA	Transcription Factors
hsa-miR-106a-5p	E2F7, HLF, KLF11, MEIS2, MYCL, RC3H2, ZNF140, ZNF827, ZXDB
hsa-miR-106b-5p	HLF, KLF11, MYCL, NFATC3, PRDM13, ZNF140, ZNF827, ZXDB
hsa-miR-1253	MEIS2
hsa-miR-1272	MEIS2
hsa-miR-1305	MEIS2
hsa-miR-1323	KLF11, ZXDB
hsa-miR-141-3p	HLF, KLF11, MEIS2, RC3H2
hsa-miR-143-3p	ETV3, MEIS2, MYCL, NFATC1, NFATC3, RC3H2, ZSCAN22
hsa-miR-144-3p	JDP2, MEIS2, PRDM13, RC3H2, ZIC5, ZNF827
hsa-miR-17-5p	HLF, KLF11, MEIS2, MYCL, NFATC3, ZNF140, ZNF827, ZXDB
hsa-miR-186-5p	E2F7, HLF, RC3H2, ZIC5, ZNF440, ZNF708, ZNF813, ZXDB
hsa-miR-187-5p	ETV3, MEIS2, RC3H2
hsa-miR-190a-5p	RC3H2, ZIC5

hsa-miR-200a-3p	HLF, KLF11, MEIS2, RC3H2
hsa-miR-200b-3p	HLF
hsa-miR-200c-3p	HLF
hsa-miR-20a-5p	HLF, KLF11, MEIS2, MYCL, NFATC3, ZNF140, ZNF827, ZXDB
hsa-miR-20b-5p	HLF, KLF11, MEIS2, MYCL, NFATC3, ZNF140, ZNF827, ZXDB
hsa-miR-223-3p	HLF, MEIS2, ZXDB
hsa-miR-22-3p	E2F7, FOSL1, HLF, MEIS2, MYCL, NFATC1, RC3H2, ZNF827
hsa-miR-27a-3p	ATF7, E2F7, ETV3, MEIS2, NFIL3, RC3H2, ZIC5, ZNF708, ZNF827
hsa-miR-302a-3p	E2F7, HLF, KLF11, RC3H2, ZNF827, ZXDB
hsa-miR-302b-3p	E2F7, HLF, KLF11, RC3H2, ZNF827, ZXDB
hsa-miR-302c-3p	E2F7, HLF, KLF11, RC3H2, ZXDB
hsa-miR-302d-3p	E2F7, HLF, KLF11, RC3H2, ZNF827, ZXDB
hsa-miR-302e	E2F7, HLF, RC3H2, ZNF827
hsa-miR-30a-5p	E2F7, HLF, JDP2, KLF11, MEIS2, MYCL, NFATC3, PRDM13, ZNF827
hsa-miR-30b-5p	E2F7, HLF, JDP2, KLF11, MEIS2, MYCL, NFATC3, PRDM13, ZIC5, ZNF827
hsa-miR-30c-5p	E2F7, HLF, JDP2, KLF11, MEIS2, NFATC3, PRDM13, ZNF827

hsa-miR-30d-5p	E2F7, HLF, JDP2, KLF11, MEIS2, NFATC3, PRDM13, ZNF827
hsa-miR-30e-5p	E2F7,HLF,JDP2,KLF11,MEIS2,NFATC1,NFATC3,PRDM13,ZNF827
hsa-miR-3148	ETV3, MEIS2, MYCL
hsa-miR-338-3p	E2F7,HLF,JDP2,MYCL,NFATC3,PRDM13,RC3H2,ZIC5,ZNF140,ZNF827
hsa-miR-3613-3p	MEIS2, NFATC3
hsa-miR-3658	ZXDB
hsa-miR-3682-3p	ETV3
hsa-miR-372-3p	E2F7, HLF, KLF11, MEIS2, NFATC3, RC3H2, ZNF827, ZXDB
hsa-miR-373-3p	E2F7, HLF, KLF11, MEIS2, RC3H2, ZNF827, ZXDB
hsa-miR-412-3p	MYCL, ZNF813
hsa-miR-421	HLF, KLF11, MEIS2, RC3H2, ZNF813
hsa-miR-4282	HLF, NFATC3, ZNF121
hsa-miR-429	HLF, NFATC3, RC3H2
hsa-miR-490-5p	MEIS2, ZXDB
hsa-miR-513a-3p	HLF, KLF17, MEIS2, ZIC5, ZNF438, ZNF708, ZNF813
hsa-miR-519d-3p	HLF, KLF11, MEIS2, MYCL, NFATC3, ZNF140, ZNF827
hsa-miR-520a-3p	E2F7, HLF, KLF11, NFATC3, ZNF827, ZXDB

hsa-miR-520b	E2F7, HLF, KLF11, NFATC3, ZNF827, ZXDB
hsa-miR-520c-3p	E2F7, HLF, KLF11, NFATC3, ZNF827, ZXDB
hsa-miR-520d-3p	E2F7, HLF, KLF11, RC3H2, ZNF827, ZXDB
hsa-miR-520d-5p	E2F7, FOSL1, KLF11, NFATC3, ZIC5, ZNF708
hsa-miR-520e	E2F7, HLF, KLF11, ZNF827, ZXDB
hsa-miR-520f-3p	E2F7, HLF, ZIC5
hsa-miR-524-5p	E2F7, FOSL1, KLF11, NFATC3, ZIC5, ZNF708
hsa-miR-526b-3p	HLF, KLF11, ZNF827
hsa-miR-548a-3p	ETV3, HLF, MEIS2, NFATC3, NFIL3, RC3H2, ZXDB
hsa-miR-548a-5p	E2F7, MEIS2, MYCL, ZNF813, ZXDB
hsa-miR-548b-5p	E2F7, MEIS2, MYCL, ZNF813, ZXDB
hsa-miR-548c-5p	E2F7, MEIS2, MYCL, ZNF813, ZXDB
hsa-miR-548d-5p	E2F7, MEIS2, MYCL, ZNF813, ZXDB
hsa-miR-548e-3p	MEIS2, NFATC3, ZXDB
hsa-miR-548f-3p	HLF, MEIS2, NFATC3, ZXDB
hsa-miR-548g-3p	HLF, MEIS2
hsa-miR-548i	MEIS2, ZXDB

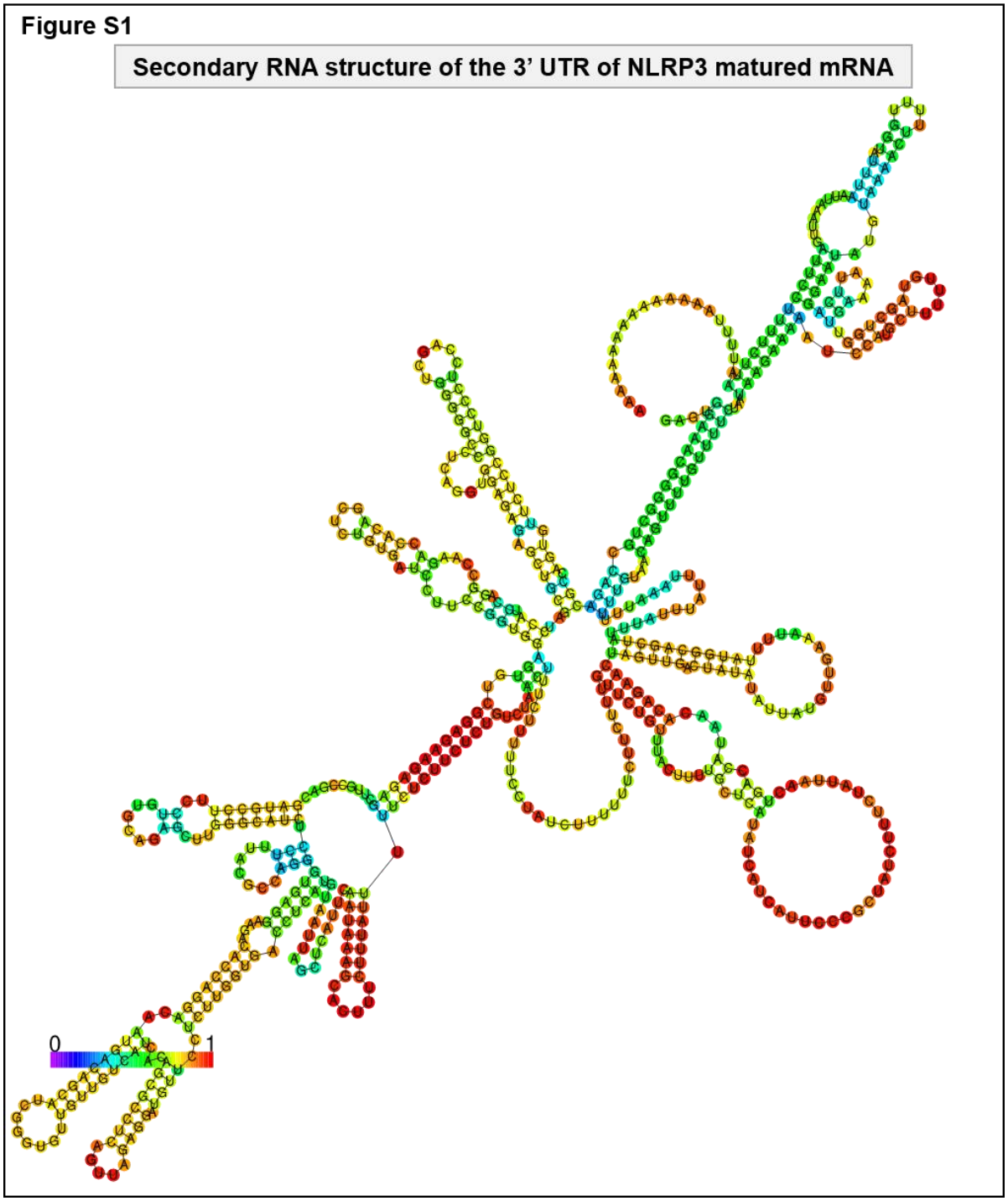
hsa-miR-548j-5p	MEIS2, NFIL3, ZXDB
hsa-miR-548n	E2F7, MEIS2, NFATC3, NFIL3, ZNF708, ZNF813
hsa-miR-548o-3p	NFATC3, ZXDB
hsa-miR-548p	E2F7, MEIS2, RC3H2, ZXDB
hsa-miR-556-5p	NFATC3, ZNF827
hsa-miR-559	E2F7, MEIS2, MYCL, ZNF813
hsa-miR-561-3p	E2F7, HLF, MEIS2, ZNF708
hsa-miR-5692b	ZIC5
hsa-miR-5692c	ZIC5
hsa-miR-570-3p	E2F7, HLF, MEIS2, ZNF813
hsa-miR-589-3p	E2F7
hsa-miR-610	E2F7
hsa-miR-7-5p	MEIS2, NFATC3, RC3H2, ZIC5
hsa-miR-876-5p	ETV3
hsa-miR-93-5p	E2F7, HLF, KLF11, MEIS2, MYCL, NFATC3, RC3H2, ZNF140, ZNF827, ZXDB

Supplementary Table S2: The 3' UTR sequence of NLRP3 (Transcript ID ENST00000336119.8)

Gene	Transcript ID	3' UTR Sequence

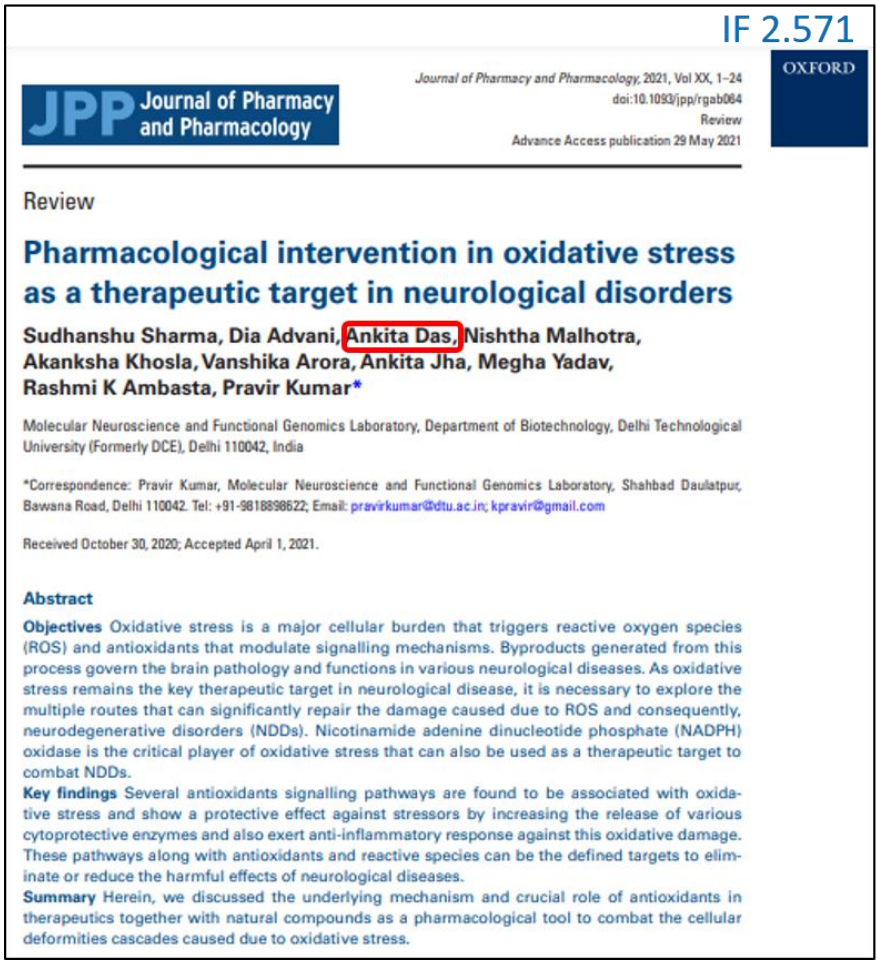
NLRP3	ENST00000336119.8	GAGUGGAAACGGGGCUGCCAGACGCCAGUGUUCUCC GGUCCCUCCAGCUGGGGGCCCUCAGGUGGAGAGAGC UGCGAUCCAUCCAGGCCAAGACCACAGCUCUGUGAUC CUUCCGGUGGAGUGUCGGAGAAGAGAGCUUGCCGAC GAUGCCUUCCUGUGCAGAGCUUGGGCAUCUCCUUUA CGCCAGGGUGAGGAAGACACCAGGACAAUGACAGCA UCGGGUGUUGUUGUCAUCACAGCGCCUCAGUUAGAG GAUGUUCCUCUUGGUGACCUCAUGUAAUUAGCUCAU UCAAUAAAGCACUUUCUUUAUUUUUCUCUUCUCUGU CUAACUUUCUUUUUCCUAUCUUUUUUCUUCUUUGUU CUGUUUACUUUUGCUCAUAUCAUCAUUCCCGCUAUC UUUCUAUUAACUGACCAUAACACAGAACUAGUUGAC UAUAUAUUAUGUUGAAAUUUUAUGGCAGCUAUUUAU UUAUUUAAAUUUUUUGUAACAGUUUUGUUUUCUAAU AAGAAAAAUCCAUGCUUUUUGUAGCUGGUUGAAAAU UCAGGAAUAUGUAAAACUUUUUGGUAUUUAAUUAAA UUGAUUCCUUUUCUUAAUUUUAAAAAAAAAAAAAAA

Figure S1



Supplementary Figure S1: Secondary structure of the 3' UTR of NLRP3 matured mRNA. Base-pairing probabilities are color coded.

I.



IF 2.571

JPP Journal of Pharmacy and Pharmacology

Journal of Pharmacy and Pharmacology, 2021, Vol XX, 1–24
doi:10.1093/jpp/rgab064
Review
Advance Access publication 29 May 2021

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Review

Pharmacological intervention in oxidative stress as a therapeutic target in neurological disorders

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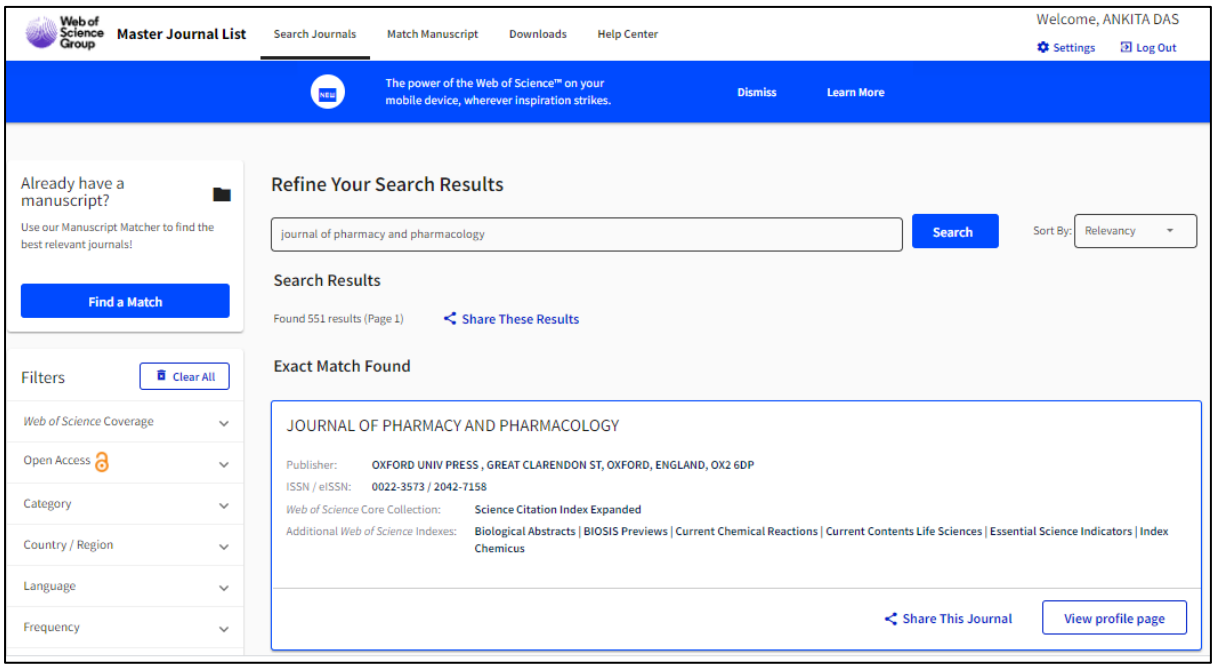
Received October 30, 2020; Accepted April 1, 2021.

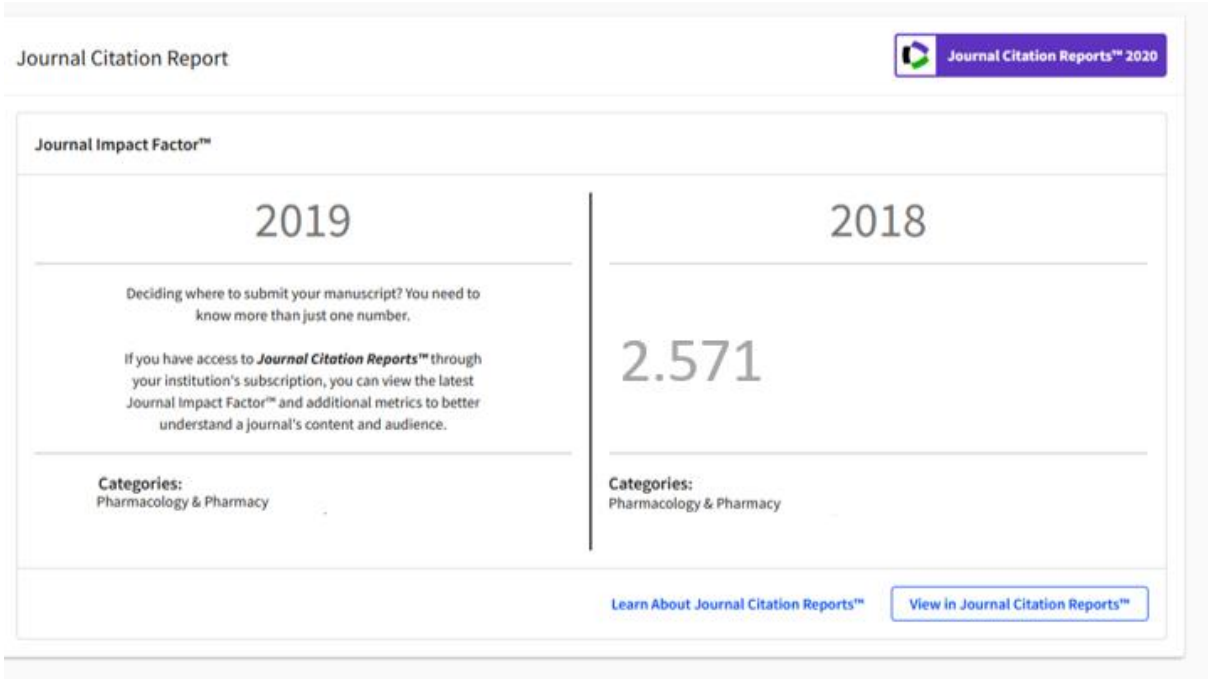
Abstract

Objectives Oxidative stress is a major cellular burden that triggers reactive oxygen species (ROS) and antioxidants that modulate signalling mechanisms. Byproducts generated from this process govern the brain pathology and functions in various neurological diseases. As oxidative stress remains the key therapeutic target in neurological disease, it is necessary to explore the multiple routes that can significantly repair the damage caused due to ROS and consequently, neurodegenerative disorders (NDDs). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the critical player of oxidative stress that can also be used as a therapeutic target to combat NDDs.

Key findings Several antioxidants signalling pathways are found to be associated with oxidative stress and show a protective effect against stressors by increasing the release of various cytoprotective enzymes and also exert anti-inflammatory response against this oxidative damage. These pathways along with antioxidants and reactive species can be the defined targets to eliminate or reduce the harmful effects of neurological diseases.

Summary Herein, we discussed the underlying mechanism and crucial role of antioxidants in therapeutics together with natural compounds as a pharmacological tool to combat the cellular deformities cascades caused due to oxidative stress.

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Letter Of Acceptance

Abstract id: ICETSEM_2506124798

Abstract Title: Missing link between NLRP3 mediated neuroinflammation and micro RNA in Alzheimer's disease

Author: Ankita Das

Co-Authors: Pravir Kumar

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