CHAPTER 4

MATERIAL AND METHODS

4.1 STUDY DESIGN:

The study was a hospital based case control study designed to compare expression of Inflammasome complex, their downstream cytokines, miRNAs targeting Inflammasome complex and TLRs in T2DM patients with nephropathy, T2DM without nephropathy and other micro and macro vascular complications and healthy individuals. The Inflammasome complex studied were NLRP3, CASP1 and PYCARD, TLRs (TLR1-10) and their downstream cytokines (IL1β, IL18 and TNFα). We also estimated the expression of miRNAs targeting Inflammasome complex (hsa-miR-223, hsa-miR-22-3p, hsa-miR-4291 and hsa-miR-185-3p) and TLRs (hsa-miR-561-3p, hsa-miR-4307, hsa-miR-448 and hsa-miR-4760-3p) across the groups. Further, we quantified the shift of GM architecture in T2DM patients with nephropathy in reference to healthy control (HC) and its relation with m-RNA expression of inflammatory genes.

4.2 STUDY SITES:

The present study was carried out at Indian Council of Medical Research-Regional Medical Research Centre, North Eastern Region, (ICMR-RMRC, NER) Dibrugarh, Assam. Study subjects were recruited from out patients department (OPD) and in-patients department of Assam Medical College and Hospitals, Dibrugarh, Assam. All the laboratory works were done at the state of art laboratory of Epidemiology and Nutrition division, ICMR RMRC, NER in support with other divisions.

4.3 ETHICAL APPROVAL TO RECRUIT STUDY SUBJECTS:

Ethical approval was obtained from Institutional-Ethics-Committee of ICMR-Regional Medical Research Centre, NE Region, Dibrugarh vide letter no. RMRC/Dib/IEC (Human)/2019-20/3446 dated: 09/12/2019. Written informed consent was obtained from all participants before recruitment of the study subjects.

4.4 RECRUITMENT OF CASES AND CONTROLS:

The healthy controls (HC) were apparently healthy individuals without any health related complications. HC subjects were recruited from the hospital who came to the hospital as attendant or for minor ailments. They are not related to the cases (T2DM with or without nephropathy).

All the consecutive T2DM (confirmed by the clinician) cases attending Nephropathy, Diabetic and Medicine outpatient department (OPD) and in-patients departments of Assam Medical College and Hospital, Dibrugarh were recruited. For diagnosis of diabetes, American Diabetic Association guidelines (ADA) and Indian Council of Medical Research (ICMR) criteria were adopted. We have recruited total 180 individuals (60 Diabetic Nephropathy cases, 60 Diabetic Control and 60 Healthy Control) for this study.

4.4.1 Diagnosis of Diabetes Case:

Diabetic cases (DC) were diagnosed based on plasma glucose criteria either fasting plasma glucose test (FPG) or 2 hours oral glucose tolerance test (OGTT). Additionally HbA1C (glycated haemoglobin) test was also considered for diagnosis of diabetes. According to American diabetic association diagnostic criteria for diabetes are described in table 4.10.

Table 4.10: ADA/ICMR guidelines for diagnosis of diabetes and intermediate hyperglycaemia

	HbA1C (percent)	**Fasting Plasma Glucose *(mg/dL)**	**Oral Glucose Tolerance Test# (mg/dL)**
Diabetes	≥6.5	≥126	≥200
Prediabetes	5.7-6.4	100-125	140-199
Normal	~5.7	≤99	≤139

* Fasting is defined as no caloric intake for at least 8 h.

OGTT test should be performed as described by the WHO, using a glucose load containing the equivalent of 75-g anhydrous glucose dissolved in water.

4.4.2 Diagnosis of DN:

The DN cases were recruited after confirmation by a nephrologist of AMCH. Selection of DN cases was done as per diagnosis criteria of National Kidney Foundation using standard clinical/biochemical criteria(microalbuminuria i.e. albumin-creatinine ratio >30 to 299 mg/day and macroalbuminuria i.e. >300 mg/day).Different degree of DN was evaluated by estimated glomerular filtration rate (eGFR) from serum creatinine level. Diabetic nephropathy is characterized by progressive kidney damage reflected by increasing albuminuria, impairment in renal function leading to decline in glomerular filtration rate (GFR) and elevated blood pressure. Micro and macroalbuminuria is the main clinical category for the evaluation of DN based on urinary albumin excretion (UAE). According to American Diabetes Association the cut off value for diagnosis of micro and macroalbuminuria is based on timed, 24-h and spot

urine collection for estimation of urinary protein, presented in table 4.11(Persson, F., & Rossing, P. 2018, Care 2004).

Table 4.11: Diabetic nephropathy diagnosis and main clinical characteristics based on urine albumin excretion

Stage	**Albuminuria cutoff values**
Microalbuminuria	20–199 g/min
	30–299 mg/24 h
	30–299 mg/g*
Macroalbuminuria	200 g/min
	300 mg/24 h
	300 mg/g*

* Albumin to Creatinine Ratio (ACR)

Some diabetic patients possessed decreased glomerular filtration rates (GFR) though albuminuria status is normal. **(**Caramori, M. L., *et al.* 2003**,** MacIsaac, R. J., *et al.* 2004) Third National Health and Nutrition Examination Survey (NHANES III) study observed that low GFR (<60 ml min^{-}1 1.73 m^{-}2) was present in 30per cent of patients in the absence of micro or macroalbuminuria and retinopathy (Garg, A. X. *et al.* 2002, Kramer, H. J., *et al.* 2003). Various techniques are used to measure the GFR such as Insulin clearance Cr-EDTA, I-iothalamate and iohexoletc are the major (Gaspari, F., *et al.* 1997). In clinical practice, GFR can be estimated based on serum creatinine, age and weight by Cockroft and Goult formula [CCr={((l40–age) x weight)/(72xSCr)}x 0.85 (if female)] (Cockcroft, D.W. and M.H. Gault. 1976). Estimated GFR (eGFR) from serum creatinine, age and weight based on gender was calculated using an online formula available at http:/www.kidney.org/ klsprofessionals/gfr_calculator.com. The reference range of GFR or eGFR values in young individuals is presented in table 4.12.

Table 4.12: Severity and stages of DN according to GFR (ml/min/1.73m2)

Stage	**GFR (ml/min/1.73m2)**	**Severity**
G1	≥ 90	Normal or High
G2	60-89	Mildly decreased
G3a	45-59	Mildly to moderately decreased
G3b	30-44	Moderately to severely decreased
G4	15-29	Severely decreased
G5	<15	Kidney failure

4.5 INCLUSION CRITERIA:

Confirmed type 2 diabetic patients of either gender, aged 30 years and above and attending diabetic clinic and medicine OPDs of Assam Medical College were eligible for the study. For recruitment of DN cases, individuals with prolonged uncontrolled hyperglycemia along with higher serum creatinine (> 1.2 mg/dl) were considered.

4.6 EXCLUSION CRITERIA:

People with coronary-artery-diseases (CAD), peripheral-vascular-diseases and history of any thrombotic event, acute infection, immune compromised subjects, chronic viral infection,subjectswith chronic debilitating diseases including cancers and subjects unwilling to provide informed consent were excluded from the study.

4.7 COLLECTION OF SOCIO-DEMOGRAPHIC AND BASELINE INFORMATION:

Socio-demographic and clinical information of all participants were obtained. Information on habit of smoking, alcohol consumption, substance uses was recorded. Further,

information on current health status, medical history, medication use, family history of diabetes, hypertension and others diseases were also recorded. Anthropometric measurements including height, weight and blood pressure (BP) were recorded. All information was collected through predesigned and pretested questionnaires. Clinical parameter including fasting blood sugar, serum creatinine, blood urea, uric acid, glycosylated haemoglobin and other biochemistry investigations were recorded from laboratory investigation reports which were prescribed by the clinician as a part of routine management of the cases.

4.8 SAMPLE COLLECTION AND PREPARATION FOR ANALYSIS:

5 ml of whole blood was collected in EDTA vials by venipuncture from antecubital veins of the study participants. Blood samples were collected by trained technician. Plasma was separated by centrifugation in 1000rpm for 5min and stored at -80℃ in deep freeze for further cytokine analysis. Blood cells were further treated for separation of peripheral blood mononuclear cells (PBMCs). PBMCs were separated from the whole blood by a density gradient centrifugation method using Ficoll-Histopaque (Histopaque®1077, Sigma Aldrich, Milan, Italy). PBMCs are chiefly lymphocytes and monocytes. Anticoagulant-treated blood is layered on the Ficoll-histopaque solution and centrifuged for a short period of time. Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layers contain erythrocytes which have been aggregated by the Ficoll and therefore sediment completely through the Ficoll-histopaque. The layer contains mostly granulocytes which at the osmotic pressure of the Ficoll-histopaque solution attain a density which is enough to migrate through the Ficoll-histopaque layer. Because of their lower density, the lymphocytes are found at the interface between the plasma and the Ficoll-histopaque with other slowly sedimenting particles (platelets and monocytes). The lymphocytes are then recovered from the interface and subjected to a short washing step with a balanced salt solution to remove any platelet, ficoll-histopaque and plasma.

4.8.1 Procedure:

2ml of ficoll-histopaque is taken in a 15ml centrifuge tube and 2ml of whole blood cells (plasma separated) is taken from the EDTA vial and gently layered by 45° angle on top

of the ficoll-histopaque using a 1ml pipette. The tubes were centrifuged without any delay for 30min at 2500 RPM in centrifuge. Aspirated the whitish buffy coat (about 1ml) i.e PBMC formed in the interphase between histopaque and medium. The approximate yield of cells from 2ml of blood varies between 10^7 to10^8.

4.9 TOTAL RNA ISOLATION FROM PBMC

Total RNA including micro RNA from isolated PBMCs were separated using miRNeasy Mini kit (Qiagen AG, Basel, Switzerland, Cat No.217004) combines phenol/guanidine-based lysis of samples and silica-membrane-based purification of the total RNA. QIAzol lysis Reagent, included in the kit, is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis of tissues, to inhibit RNases and also to remove most of the cellular DNA and proteins from the lysate by organic extraction. Cells or tissue samples are homogenized in QIAzolLysis Reagent. After addition of chloroform, the homogenate is separated into aqueous and organic phases by centrifugation. RNA partitions to the upper, aqueous phase. The upper, aqueous phase is extracted and ethanol is added to provide appropriate binding conditions for all RNA molecules. The sample is then applied to the RNeasy Mini spin column, where the total RNA binds to the membrane and phenol and other contaminants are efficiently washed away. High quality RNA is then eluted in RNase-free water.

4.9.1 Procedure:

700µl of Trizol (QIAzol) lysis reagent was added to the PBMC sample and disrupted by gentle pipetteting and the homogenate was incubated at room temperature (15-25˚c) for 5mins. 140µl chloroform was added and shacked vigorously for 15 sec. The tube was then incubated at room RT for 2-3min and centrifuged for 15min at 12000Xg at 4˚c. The upper aqueous phase was transferred to a new collection tube and 1.5 volume of 100% ethanol was added and mixed thoroughly. 700µl of the sample was pipetted into an RNeasyMinElute spin column assembled with 2ml collection tube. The column was then centrifuged at ≥ 8000Xg for 15sec at room temperature (RT). The flow through was discarded. The previous step was repeated for the remaining sample. 700µl of wash buffer (RWT) was pipetted into the

RNeasyMinElute spin column. The column was then centrifuged at ≥ 8000Xg for 30 sec at RT. The flow through was discarded. 500µl of wash Buffer (RPE) was pipetted onto the RNeasyMinElute spin column. Centrifugation steps were repeated at ≥ 8000Xg for 15sec at RT and flow through was discarded. A second wash was also done by addition of 500µl of RPE buffer and centrifuged at ≥ 8000Xg for 2minutes at RT. The flow through was discarded. A dry spin was done by placing the spin columns in new 2ml collection tube and centrifuged at at ≥ 8000Xg for 1 minute. The final steps of RNA elution, the RNeasyMinElute spin columns was placed in a new 1.5ml collection tube and 30µl of RNase free water were added directly to the centre of the spin column membrane and centrifuged for 1min at full speed to elute the total RNA. The eluted RNA was stored at -80℃ until further experiments.

4.10 QUANTIFICATION OF TOTAL RNA

The instrument EPOCH (BioTek, USA) used for measuring the quantity and quality of isolated total RNA worked on the principle of Beer-Lambert's Law.Instrument quantities and qualifies nucleic acid on the basis of optical density of the solution *i.e* RNA solution. Purity of the samples could be defined by measuring the ratio of two spectrophotometric absorbance *i.e* at A_{260}/A_{280} (Fleige, S., and Pfaffl, M. W. 2006). Advantages of this instrument was the small volume of sample required (2 µl) and its high sensitivity.

4.10.1 Procedure:

Instrument was set up blank with 2 µl of nuclease free water before quantification. Thereafter, 2 µl of the sample i.e RNA was dispensed on the holder plate and absorbance was measured at 260nm, 280nm and 320nm. RNA purity was assessed by the software, attached with the instrument, on the basis of 260/280nm and 260/320nm ratios. RNA quantity was measured at an optical density of 260nm and the concentration of RNA was expressed as ng/µl. RNA was considered pure when the ratio 260/280nm was approximately 2.0(Table 4.13). After each quantification step, pedestals were wiped using a dry lint- free laboratory wipe to make the instrument ready for measuring next sample. RNA samples obtained the ratio of A_{260}/A_{280} between 1.8 to 2.0 were qualified for further process of cDNA synthesized

and real time PCR. A final cleaning of measurement surfaces was recommended with distilled water.

Table 4.13: Spectrophotometer reading of total RNA

Sl. No.	Sample ID	Conc. ng/ µl	A_{260}	A_{280}	A_{260}/A_{280}	A_{260}/A_{320}
1	HC1	122.5	0.51	0.26	1.96	0.58
2	HC2	123.5	0.50	0.26	1.92	0.43
3	HC3	54.6	1.2	0.59	2.03	1.95
4	HC4	76.77	1.73	0.83	2.08	1.69
5	DC1	88.9	2.13	1.06	2.01	1.67
6	DC2	43.2	0.91	0.45	2.02	1.93
7	DC3	89.6	2.24	1.07	2.09	2.44
8	DC4	212.5	5.31	2.61	2.03	2.18
9	DN1	141.6	3.53	1.72	2.05	1.59
10	DN2	52.6	1.29	0.63	2.05	1.94
11	DN3	142.4	3.48	1.68	2.07	1.59
12	DN4	51.5	1.28	0.63	2.03	1.94

The yield of RNA, expressed as ng/ µl, A_{260}/A_{280} ratio (used to assess protein contamination) and A_{260}/A_{320} values (to assess salt and buffer contamination) as determined for few representative samples in Nanodrop spectrophotometer are shown.

4.11 PREPARATION OF c-DNA FROM ELUTED RNA:

Complementary DNA (cDNA) synthesis was done by QuantiTect® Reverse Transcription Kit (Qiagen AG, Basel, Switzerland, Cat No: 205311). The unique QuantiTect Reverse Transcription kit provides a fast and convenient procedure for cDNA synthesis with integrated genomic DNA removal. Genomic DNA contamination in RNA samples is effectively eliminated by gDNA Wipeout Buffer. All components that are required for fast and efficient reverse transcription are provided with the QuantiTect Reverse transcription kit,

including Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and a unique RT primer mix. The synthesized cDNA is optimized for use in real time PCR, allowing reliable quantification of targets from all regions of mRNA transcript.

4.11.1 Procedure:

The template RNA was thawed on ice. The gDNA wipeout Buffer, Quantiscript Reverse trancriptase, Quantiscript RT Buffer, RT Primer mix and Rnase free water are thawed at RT (15-25˚c). Each solution is mixed by flicking the tubes. The tubes are centrifuged briefly to collect residual liquid from the sides of the tubes and the keep on ice. The genomic DNA elimination reaction is prepared on ice according to table 4.14.

Table 4.14: Genomic DNA elimination reaction components

Components	**Volume/Reaction**
g DNA wipeout buffer 7X	1µl
Template RNA sample	1µl (100 ng)
RNase free water	4µl
Total reaction volume	7µl

This genomic DNA wipe out reaction mixture was incubated for 2min at 42˚C and then placed immediately on ice. The reverse transcription reaction master mix was prepared in 1,5ml tube by combining Reverse transcription master mix, Quantiscript RT buffer and RT primer mix according to the table 4.15. A NO-RT control mix was also prepared simultaneously by combining the entire reaction component except RT enzyme which was supplemented with equal volume of nuclease free water. NO-RT control enabled monitoring of DNA contamination in RNA preparation during amplification in real time PCR. 3 µl of RT mix was dispensed in each PCR tube containing RNA during genomic DNA wipe out reaction. The PCR tube contacting the reaction components were incubated at 42˚c for 15 minutes and 95˚c for 3min in order to inactivate RT enzyme.

Table 4.15: Reverse transcription reaction components

Components	**Volume/reaction**
Reverse transcription master mix	0.5µl in each sample mixture
Quantiscript RT buffer,5X	2µl
RT primer mix	0.5µl
Template RNA	7µl
Total reaction volume	10µl

4. 12 REAL TIME PCR FOR mRNA EXPRESSION ASSAY

4. 12. 1 Introduction:

Real-time PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of at least one specified wavelength and detect the fluorescence emitted by the excited fluorophore. Real time PCR monitors the progress of a PCR reaction in real time and a relatively small amount of PCR product can be quantified. Real time PCR is based on the detection of the fluorescence produced by reporter molecules which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double stranded DNA (SYBR® Green) or sequence specific probes (Molecular Beacons or TaqMan® Probes) (Deepak, S. A., *et al.* 2007, Taylor, S., *et al.* 2010, Wagner 2013). The PCR process generally consists of a series of temperature changes that are repeated 25-50 times. These cycles normally consist of three stages: the 1st round at 95˚c, allows the separation of the nucleic acid double chain; the 2nd, at a temperature of around 50-60˚c, allows the binding of the primers with the DNA template; the 3rd, at between 68-72˚c, facilitates the polymerization carried out by the DNA polymerase. Due to the small size of the fragments the last step is usually omitted in this type of PCR as the enzyme is able to increase their number during the chance between the alignment stage and the denaturing stages.

Real-time PCR amplification reactions for all the studied samples were performed in duplicate in 20 µl of final volume via SYBR green chemistry on ABI-Prism step one plus

(Applied Biosystem, USA). PCR protocol was performed using DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific, USA, Cat No.FNZ415S). The quantitative Real-Time PCR was done to determine mRNA expression of Inflammasome complex (NLRP3, Caspase-1 and PYCARD), TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, IL1β, IL18 and TNFα by pre validated primers (Sigma, Milan, Italy). Details of primers and product lengths are described in table 4.16. In order to normalize the gene expression of targeted genes, GAPDH gene was taken as housekeeping gene for this study (Silver, N., *et al.* 2006). The mean threshold cycle number (C_T), of triplicate reactions, was determined from inbuilt software of Step One Plus real-time PCR.

Table 4.16: Primers used for the detection of expression level for the targeted genes and housekeeping gene

Sl No	**Gene bank Accession No. (Sequence length in bp)**	**Name of Primers**	**Sequence (5'- 3')**	**Length in bp**	**Genome position**	**Product Length (bp)**
1	NM_002046.7	GAPDH_F	CGACCACTTTGTCAAGCTCA	20	988-1007	97
	(1285)	GAPDH_R	TTACTCCTTGGAGGCCATGT	20	1084-1065	
2	NM_004895.4	NLRP3_F	TTCAGGTGTTGGAATTAGAC	20	3574-3593	147
	(4470)	NLRP3_R	CTTCACAGAACATCATGACC	20	3720-3701	
3	NM_001257119.3	CASP1_F	CAACTACAGAAGAGTTTGAGG	21	891-911	92
	(1933)	CASP1_R	AACATTATCTGGTGTGGAAG	20	982-963	
4	NM_013258.	PYCARD_F	AGGCCTGCACT	19	400-418	88

	5		TTATAGAC			
	(757)	PYCARD_R	CTTCCCGTACA GAGCATC	18	487-470	
5	NM_003263. 4	TLR1_F	CCCTACAAAAG GAATCTGTATC	22	196-217	89
	(2851)	TLR1_R	TGCTAGTCATTT TGGAACAC	20	284-265	
6	NM_001318 789.2	TLR2_F	CTTTCAACTGGT AGTTGTGG	20	208-277	176
	(3596)	TLR2_R	GGAATGGAGTT TAAAGATCCTG	22	383-362	
7	NM003265.2 (3057)	TLR3_F	GCTCTCCTTCAC CATTCC	18	1591-1608	143
		TLR3_R	CCGTGCTAAGT TGTTATGC	19	1715-1733	
8	NM_003266. 4	TLR4_F	GAAGTTGAACG AATGGAATGT	21	1997-2017	129
	(12797)	TLR4_R	AGATACTACAA GCACACTGAG	21	2125-2105	
9	NM_003268. 6	TLR5_F	ATCTTTCACATG GGTTTGTC	20	1488-1507	170
	(4235)	TLR5_R	TTCCCCCAGAA GGTTATATG	20	1657-1638	
10	NM_006068. 4	TLR6_F	AGAGATCTTGA ATTTGGACTC	21	48-68	87
	(5891)	TLR6_R	TGTCTTTGGTCA TGATGTTG	20	134-115	
11	NM016562.4 (5003)	TLR7_F	GGTGATGATGA CAGCAAGT	19	2712-2730	113
		TLR7_R	CATAGCAACAG	21	2824-2804	

			TCTGGTGATA			
12	NM_016610.4 (4353)	TLR8_F	ACTGCTGCTGAGTCATAAC	19	2372-2390	118
		TLR8_R	TGCGGATTTGTTGATTGTTT	20	2489-2470	
13	NM017442.3 (3922)	TLR9_F	AATGTCACCAGCCTTTCC	18	824-841	101
		TLR9_R	TTCCACTTGAGGTTGAGATG	20	905-924	
14	NM_001195106.1 (3801)	TLR10_F	CATCTGTAAGGGTTTTGAGC	20	378-397	195
		TLR10_R	CTTTCTTAGAGACATGTTGGAG	22	572-551	
15	NM_000576.3 (1507)	IL1β_F	CTAAACAGATGAAGTGCTCC	20	173-192	183
		IL1β_R	GGTCATTCTCCTGGAAGG	18	355-338	
16	NM_001243211.2 (1103)	IL18_F	CCTTTAAGGAAATGAATCCTCC	22	538-559	95
		IL18_R	CATCTTATTATCATGTCCTGGG	22	632-611	
17	NM_000594.4 (1678)	TNF-α_F	GAAAGCATGATCCGGGACGTG	21	187-207	510
		TNF-α_R	GATGGCAGAGAGGAGGTTGAC	21	696-676	

4.12.2 Procedure for Real Time PCR:

Required amount of cDNA was subjected to Real-Time quantitative RT-PCR using SYBR Green as a fluorescent reporter (SYBR Green PCR Master Mix containing 2X ROX

normalizing dye) in the Appied Biosystem Step One plus Real Time PCR machine. The specific genes and the housekeeping gene were amplified in separate reaction tubes. The PCR mix contained 1X SYBR green, Taq polymerase enzyme, buffer , dNTPs and ions, target and housekeeping gene primers, cDNA template and nuclease free water in the proportion given in table 4.17. The PCR mix preparation also included no template control (i.e, with all the component except cDNA template) and No-RT control (i.e, cDNA prepared without RT enzyme). 9µl of each mastermix was dispensed in a 96 well qPCR plate (Thermo Scientific, USA, Cat No: AB1900) and 1µl of each c-DNA was added to respective wells. The PCR plate was then covered with a sealing foil (Thermo Scientific, USA, cat no.: AB-1170), centrifuged at 3000 rpm for 1 minute in a swing bucket rotor and placed in Step One plus Real Time PCR machine. Thermal cycling condition included a predenaturation step to activate hot start Taq Polymerase followed by 40 cycles of amplification cycle. Annealing and extention was carried out simultaneously at single temperature. A melt curve analysis was also included in the PCR protocol for identification of specific products. The details amplification protocol is given in table 4.18. The comparative Ct method (ΔΔCt) was used to quantify mRNA expression and the relative quantification was calculated as $2^{-\Delta\Delta Ct}$ method (Schmittgen, T. D., and Livak, K. J. 2008, Taylor, S., *et al.* 2010).

Table 4.17: Preparation of reaction mix for real-time PCR for mRNA expression

Components	**Stock conc.**	**Final volume**	**Final conc.**
2X DyNAmo Flash SYBR Green qPCR Master Mix	2X	5µl	1X
Primers (sense and antisense)	5µM	1 µl (0.5µl each primer)	0.5µM
cDNA	5ng/µl	1µl	10ng
Nuclease free water		3µl	
Total		10µl	

Table 4.18: The amplification protocol of real-time PCR for mRNA expression

Amplification Stage			
Temperature	**Time**	**Purpose**	**Cycle**
95 ˚C	15 minutes	Initial Incubation	1 cycle
95 ˚C	5 sec	Cycle denaturation	40 cycle
60 ˚C	15 sec	Annealing and Extension, (fluorescence data collection)	
Melt Curve Stage			
A] 95 ˚C for 5 min			
B] 60 ˚C to 95 ˚C with increasing + 3 ˚C			

4.13 REVERSE TRANSCRIPTION AND REAL TIME PCR FOR miRNA EXPRESSION ASSAY

4.13.1 miRNA selection:

miRNAs are the best-studied of the small noncoding RNAs, which are capable of post-transcriptional regulation of mRNA expression. mi-RNAs for this study were selected with a target score having more than 80% from miRDB data base. miRDB is an online database for miRNA target prediction and functional annotations (Wong, N., and Wang, X. 2014). miRDB uses bioinformatics tool MirTarget to predicts all the targets by analysing thousands of miRNA-target interaction from high-throghput sequencing experiments (Wong, N., and Wang, X. 2014). miRDB hosts predicted miRNA targets in five species: human, mouse, rat, dog and chicken(Wong, N., and Wang, X. 2014). We have selected below listed miRNA (table 4.19) for this study.

Table 4.19: List of miRNA and their target genes with target score from miRDB

Target Gene	Name of mi-RNA	mRNA Targeting Score	mature miRNA sequence (length in base)	miRBase ID	Precursor miRNA Genomic Location
NLRP3	hsa-miR-22-3p	94	5'-aguucuucaguggcaagcuuua - 3' (22)	MIMAT0004495	chr17:1713903-1713987
	hsa-miR-223-3p	80	5'-ugucaguuugucaaauaccca - 3' (22)	MIMAT0000280	chrX:66018870-66018979 (+)
CASP-1	hsa-miR-4291	92	5' - uucagcaggaacagcu - 3' (16)	MIMAT0016922	chr9:93819357-93819421 (+)
PYCARD	hsa-miR 185-3p	78	5' – aggggcuggcuuuccucugguc - 3' (22)	MIMAT0004611	chr22:20033139-20033220 (+)
TLR2	hsa-miR-561-3p	91	5' – caaaguuuaagauccuugaagu - 3' (22)	MIMAT0003225	chr2:188297492-188297588 (+)
TLR3	hsa-miR-4307	87	5' – aauguuuuuuccuguuucc - 3' (19)	MIMAT0016860	chr14:26908642-26908725 (+)
TLR4	hsa-miR-448	93	5' – uugcauauguaggaugucccau - 3' (22)	MIMAT0001532	chrX:114823454-114823564 (+)
TLR7	hsa-miR-4760-3p	92	5' – aaauucauguucaaucuaaacc - 3' (22)	MIMAT0019907	chr21:40212352-40212431 (-)

4.13.2 Reverse Transcription for miRNA Expression Assay:

Reverse transcription of isolated miRNA to synthesize cDNA was performed using the miScript reverse transcription kit (QIAGEN AG, Basel, Switzerland, cat no: 218161), following the manufacturer's instructions. The miScript II RT Kit includes miScript Reverse

Transcriptase Mix, 10xmiScriptNucleics Mix, 5x miScriptHiSpec Buffer and 5x miScriptHiFlex Buffer. miScript Reverse Transcriptase Mix is an optimized blend of poly(A) polymerase and reverse transcriptase. 10x miScriptNucleics Mix contains dNTPs, rATP, oligo-dT primers. miScriptHiSpec Buffer are used to reverse transcribe mature miRNAs into cDNA. Mature miRNAs are polyadenylated by poly (A) polymerase and reverse transcribed into cDNA using oligo-dT primers. The oligo-dT primers have a 3' degenerate anchor and a universal tag sequence on the 5' end, allowing amplification of mature miRNA in the real-time PCR step. The reverse transcription reaction master mix was prepared in 1,5ml tube by combining according to the table 4.20.

Table 4.20: Preparation of reverse-transcription master mix for cDNA synthesised

Component	**Volume**	**Final Concentration**
5x miScriptHiSpec Buffer	4 µl	1X
10x miScriptNucleics Mix	2 µl	1X
RNase-free water Variable	11 µl	-
miScript Reverse	2 µl	1X
Transcriptase Mix		
Template RNA Variable (up to 1 µg)	1 µl	100 ng
Total Volume	20 µl	-

Incubate the mixture for 60 min at 37ºC, another incubation was done 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix and place on ice.

4.13. 3 Real Time PCR for mi-RNA Expression Assay:

Real-time PCR amplification reactions were performed in duplicate in 20 µl of final volume via SYBR Green chemistry on ABI-Prism step one plus (Applied Biosystem). PCR protocol was performed using miScript SYBR Green PCR Kit (QIAGEN AG, Basel, Switzerland, cat no: 218073) for eight miRNA viz.hsa-miR 22-3p and hsa-miR 223’ targeting NLRP3, hsa- miR4291 targeting CASP1, hsa-miR 185-3p targeting PYCARD,hsa-miR-561-3p targeting TLR2, hsa-miR-4307 targeting TLR3, hsa-miR-448 targeting TLR4 and hsa-miR-

4760-3p targeting TLR7 according to manufacturer's instruction. The primer assay for miRNA real time PCR was procured from QIAGEN AG, Basel, Switzerland, (cat no 218300).

Real time protocol initiated with initial activation step for 15 min at 95°C then 3step cycling condition for 40 cycle was 15 second for 94°C, 30 second at 55°C and 30 second at 70°C. The Hs_SNORD61_11miScript primer assay (QIAGEN AG, Basel, Switzerland) was used as a reference standard to normalize the target signal. cDNA prepared using the miScript II RT Kit with miScriptHiSpec Buffer are used for measuring the expression of targeted miRNAs through real time PCR. This real time PCR protocol enables quantification of mature miRNA using target-specific miScript Primer Assays (forward primers) and the miScript SYBR Green PCR Kit (QIAGEN AG, Basel, Switzerland, cat no: 218073), which contains the miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix. The comparative Ct method (ΔΔCt) was used to quantify micro RNA expression and the relative quantification was calculated as $2^{-\Delta\Delta Ct}$ (Schmittgen, T. D., and Livak, K. J. 2008, Taylor, S., *et al.* 2010). Amplification specificity was controlled by a melting curve analysis and the amount of miRNA target was evaluated using the comparative Ct method.

4.13.4 Procedure of real-time PCR: All the kit components were thawed and PCR master mix was prepared as per describe in table 4.21.

Table 4.21: Reaction setup for real-time PCR

Component	**Volume/reaction**	**Final Concentration**
2x QuantiTect SYBR Green PCR Master Mix	10 µl	1X
10x miScript Universal Primer	2 µl	1X
10x miScript Primer Assay	2 µl	1X
RNase-free water Variable Variable	7 µl	
Template cDNA	1 µl (1–3 ng)	-
Total volume	20 µl	-

Mixed the reaction mix thoroughly and dispense 19 µl volumes into the 96 well plates. All the reactions were consisting of no template controls (all component except cDNA) The PCR plate was then covered with a sealing foil (Thermo Scientific, USA, cat no.: AB-1170),

centrifuged at 3000 rpm for 1 minute in a swing bucket rotor and placed in Step One plus Real Time PCR machine. Thermal cycling condition included a predenaturation step followed by 40 cycles of amplification cycle. A melt curve analysis was also included in the PCR protocol for identification of specific products. The details amplification protocol is described as in table 4.22.

Table 4.22: Thermal cycling conditions for real-time PCR

Amplification Stage			
95 ˚C	15 minutes	Initial Incubation	1 cycle
94 ˚C	15 sec	Cycle denaturation	40 cycle
55˚C	30 Sec	Annealing	
70 ˚C	30 sec	Extention, Perform fluorescence data Collection.	
Melt Curve Stage			
A] 95 ˚C for 5 min			
B] 60 ˚C to 95 ˚C with increasing + 3 ˚C			

After completion of run in real time PCR system data were analyzed by calculating ct value. The comparative Ct method (ΔΔCt) was used to quantify gene expression, and the relative quantification was calculated as $2^{-\Delta\Delta Ct}$ method (Schmittgen, T. D., and Livak, K. J. 2008, Taylor, S., *et al.* 2010).

4.14 CALCULATION OF RELATIVE QUANTIFICATION:

The comparative Ct method (ΔΔCt) was used to quantify gene expression, and the relative quantification was calculated as $2^{-\Delta\Delta C}$ method (Schmittgen, T. D., and Livak, K. J. 2008).

The steps to calculate the relative quantification (fold change) of gene expressions are as follows:

1. ΔCT = Ct of target gene - Ct of housekeeping gene (GAPDH).

2. ΔΔCT = ΔCT of target sample (DN and DC) - ΔCT of reference sample (HC).

[A] ΔΔCT for target sample = ΔCT of target sample (DN and DC) - ΔCT of reference sample (HC) = +/- value

[B] ΔΔCT of reference sample = ΔCT of reference sample- ΔCT of reference sample= 0

3. The final gene expression was expressed as fold i.e; $2^{-\Delta\Delta CT}$

[A] Fold change for target samples = $2^{-(+/-value)}$

[B] Fold change for reference samples = $2^{-(0)} = 1$

Therefore the fold change of healthy control subjects which was taken as reference samples was '1'. Based on fold change of healthy control i.e; 1, we have compared other two groups viz. DC and DN fold change wheather it has upregulated or downregulated.

Same procedure was followed for calculation of miRNA expression, here we have taken mi-RNA: Hs_SNORD61 (Qiagen, Germany) as reference miRNA instead of GAPDH for mRNA.

4.15 CELL CULTURE TECHNIQUE FOR VALIDATION OF MIRNA-MRNA INTERACTION FOR THE PROPOSED STUDY:

Animal cell culture is a technique of invitro growth of cells. Culture conditions vary for each cell type, but artificial environments consist of a suitable desired substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (CO2, O2), and regulates the physio-chemical environment (pH buffer, osmotic pressure, temperature). Most cells require a surface or an artificial substrate (adherent or monolayer culture) whereas others can be grown free floating in culture medium. Cell line (colony of cells) INT 407 was purchased from NCCS, pune. This epithelial line was originally thought to be derived from normal embryonic intestinal tissue and are adherent in nature (Henle, G., and Deinhardt, F. 1957). Cell culture technique is highly prone to contaminations and therefore all the procedures were performed in sterile environment (Freshney 2015). It was required to wear sterile aprons, gloves, mask, head covers and shoe covers before performing the experiment. The media used for cell culture was Minimum Essential Medium (MEM) with 1000mg/L L-glutamine and sodium biocarbonate, (Sigma, USA, Cat No: AL-178A) generally stored at 4^{o}C. The Trypsin Phosphate Versene Glucose

(TPVG) (Himedia, India, Cat No: TCL031-500ML) and Fetal Bovine Serum (FBS) Heat inactivated, (Gibco, USA, Cat No: 10082147, 500ML) were stored at -20^{0}C and used for trypnisation and growth of cells. The bacterial and fungal contamination could be distinguished by naked eye but the mycoplasma contamination was required to be detected by molecular method. Antibiotic Antimycotic Solution (100×) (A5955-20ML: A5955 SIGMA), Stabilized with 10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL was used to control the contamination in cell culture.

4.15.1 Principle:

The cell culture is a process of growing the cells in artificially controlled environment which are derived directly from animal. The isolation of cells from these tissues could be done through a range of processes like enzymatic or mechanical disruption and then the cells are grown in a suitable media. There are two types of cell cultures i.e primary cell culture and secondary cell culture. Primary cell culture is the growth of the cells which are directly derived from the animal or plant tissue but has limited life span due to various reasons. Exhaustion of media and available space make these cells in the primary culture to die. Therefore to keep the continuity of cell growth and proliferation, the cells in the primary culture are sub cultured and this process of subculturing is known as secondary culture. So, the secondary cell cultures are derived from the already established primary cell culture and have a finite life span (Freshney 2015).

4.15.2 Subculture of INT407 Cell line:

A T-25 Flask containing monolayer of INT407 cells procured from NCCS, Pune were used for subculturing. INT407 cell line was maintained with 10% FBS (Gibco) in MEM(Sigma). First the previous medium present in the flask was discarded and cells were washed with sterile 1XPBS. Then the cells were detached with 1ml TPVG and incubated for 1-2 minutes in 5% CO_2 incubator for 1 min at 37℃. Thereafter the flask was removed from the incubator and with gentle tapping the cells were detached from the surface. After adding 2ml MEM the cells were flushed extensively to prevent clumping of cells. Thereafter, cells

were distributed as per to the requirement in MEM with10% FBS. Finally after wiping the outer surface of flask with 70% alcohol, the flask were kept at 37^0C in a 5% CO_2 incubator. The cells were regularly observed for contamination and growth in inverted microscope and were regularly sub cultured using the above procedure (Freshney 2015).

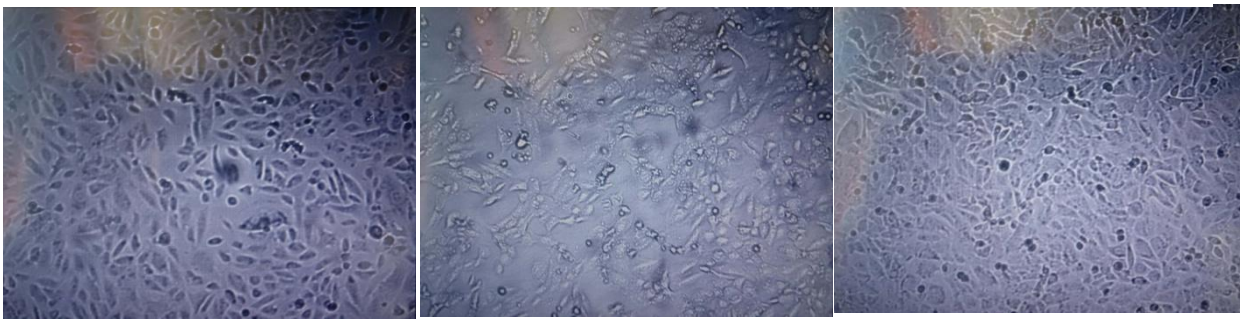

Figure 4.41: Image of INT407 cells

4.15.3 Cell Counting:

Cell counting was traditionally done by using haemocytometer though the method is still widely adapted. In order to count the live or viable cells, a dye is used to differentiate live and dead cells. It was based on the principle that live cells have intact cell membrane, so they excludes the dye when added, however, the dead cells could not do the same and therefore stain blue if trypan blue is used to stain them due to uptake of the dye without the hindrance of cell membrane (Strober 1997). Present study utilized an automated cell counter TC20^{TM} (BIORAD) for cell counting purpose which gives the direct cell counting for percentage of live and dead cells present in a given sample.

4.15.3.1 Procedure:

The T-25 flask with the INT407 cell line was washed with 1X PBS and then trypsinised using TPVG as described in section earlier. Cells were resuspended in 2ml MEM and flushed extensively to get single cell suspension. To count the cells, 10ul of the cell suspension was taken out with a sterile pipette and mixed with 10ul of 0.4% trypan blue dye (Biorad, USA). Cells are counted and numbers of cells per ml are calculated.

4.15.4 Cryopreservation of cell lines:

4.15.4.1 Principle:

Cryopreservation is a process which allows the long term preservation of cells at very low temperature usually at -196^{o}C. The method utilizes the use of cryoprotectants like dimethyl sulphoxide (DMSO), glycerol, propanediol etc. If cells are preserved directly at low temperature then due to fast cooling, the formation of ice crystals can damage the cells however cryoprotectants preserves the fine cellular structure by reducing the amount of ice formed at any given temperature by increasing the amount of solutes in the system, The cryoprotectants should be less toxic to the cells and capable of penetrating inside the cells. The use of DMSO is most common due to its low toxicity and cost effectiveness. It was discovered by the Russian scientist Alexander Zaytsev in 1866.It is known to act by reducing the concentration of electrolyte at any given temperature in the unfrozen residue inside and outside the cell (Pegg 2015, Jang, T. H., *et al.* 2017).

4.15.4.2 Procedure:

Cells in their exponential phase are usually preserved to obtain good viability upon cell retrieval. The T-25 or T-75 flask with 70% confluency was trypsinised. Then the cells were suspended into the plain medium without FBS and flushed extensively to detach all the cells from the flask and to create single cell suspension. The whole cell suspension was then taken into the 50 ml or 15ml falcon tube and centrifuged at 1500 rpm for 10 minutes. In the mean time the cryomix was prepared which is a mixture of 20% DMSO (Himedia), 30% FBS and 50% MEM per 2ml vial. Cryomix was kept in ice until utilized. After centrifugation, tubes were removed and the supernatant was discarded slowly with the help of sterile pipette without disturbing the pellet. The cell pellet was resuspended in 1ml medium and the cell count was taken. The cells are preserved with 1million cells per vial and accordingly the numbers of vials were made. The cells were suspended slowly by adding drop by drop the cryo medium and then dispensed in pre chilled 1ml cryovials. The vials were kept in -1^{o}C

cooler containing acetone and stored at -80^{o}C for 24hrs. Finally vials were removed from -80^{o}C and preserved in liquid nitrogen.

4.15.5 Induction of Glucose in Cultured cells:

The maintained cell line was subjected to induction by D-glucose to attain the hyperglycaemic condition (Nishikawa, T., *et al*. 2000, Mohamadpour, Z., *et al.* 2016, Ito, M., *et al.* 2017). Alteration in gene expression along with mi-RNA expression was measured in real time PCR. A 70-80-% confluence T-25 Flask containing monolayer of INT407 was used for glucose induction. INT407 cells were detached with 1ml TPVG and incubated for 1-2 minutes in 5% CO_2 incubator for 1 min at 37℃. Thereafter the flask was removed from the incubator and with gentle tapping the cells were detached from the surface. Cells were counted in automated cell counter and approximately 1 million cells were dispensed in well of 6 well cell culture plate. 2ml of 10% FBS containing MEM media were dispensed to the every wells. The culture plates were incubated in 5% CO_2incubator for 12-16 hours at 37℃. After 100% adherence, cells were washed with 1X PBS and again cultured with 10% FBS containing MEM media along with glucose. Experiment was design to induce cells with various concentration of D-glucose (Himedia). 50 µl of glucose stock solution were added to the cells to attain the different concentration of glucose such as 125 mg/dl/ 250 mg/dl and 500mg/dl in triplicate. Control experiments were also done by culturing cells without glucose. Then the culture plates were incubated in 5% CO_2 incubator for 4 hours at 37℃. Supernatant were separated and cells were lysed with Trizol for further total RNA isolation and real time PCR.

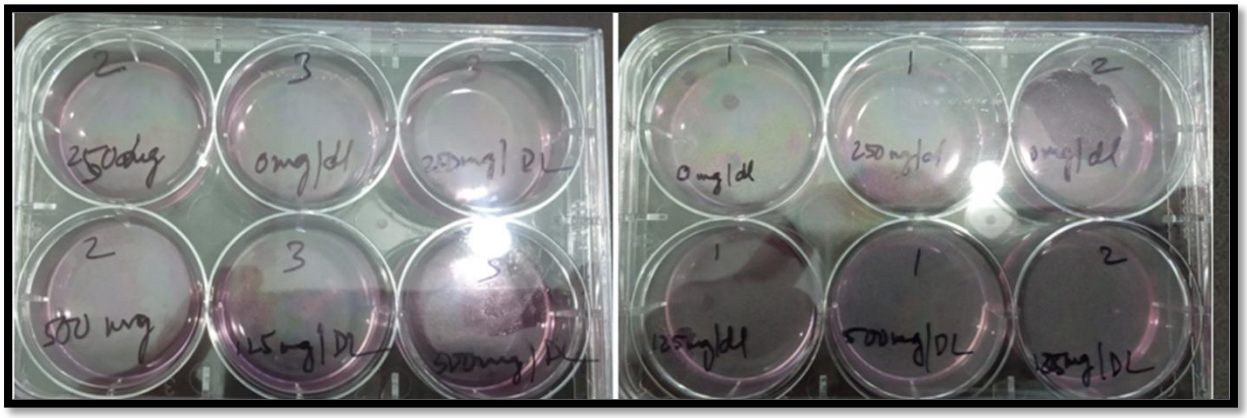

Figure 4.42: Induction of glucose on INT407 cells

4.16 ESTIMATION OF IL1β, IL18 AND TNFα BY COMMERCIALLY AVAILABLE ELISA KIT

4.16.1 Estimation of IL1β:

Serum IL1βwere estimated from all the collected samples using RayBio$^{@}$ Human IL-1beta ELISA kit (RayBiotech, GA, cat no: ELH-IL1b).This kit is an in vitro enzyme-linked immunosorbent assay for the quantitative estimation of IL1β from human serum, plasma and cell culture supernatant. Antibody specific to human IL1β coated on a 96 well plate. Quantitative estimation of IL1β is based on antibody antigen reaction and then enzyme substrate reaction. Optical density of coloured product is measured at 450nm and quantification is measured by plotting standard curve. This kit provided an IL1β standard in lyophilized form that was to be reconstituted to 20 ng/ml with 880 µl of assay diluents. Seven standards were prepared from stock standard vial by serial dilution and the concentration were such as 20 ng/ml, 100 pg/ml, 40 pg/ml, 16 pg/ml, 6.4 pg/ml, 2.56 pg/ml, 1.02 pg/ml and 0.48 pg/ml.

4.16.1.1 Procedure:

All the reagent and samples were kept in room temperature and the antibody coated wells were arranged according to samples and standards in duplicates. 100 µl of standards and plasma samples (samples were diluted 2 folds by assay diluents containing 0.09% sodium azide provided with the kit) were dispensed to the well and the plate were incubated for 2.5 hours at room temperature with gentle shaking. A blank was also set by adding nuclease free water instead of samples. Discarded the solution and washed the plate 4 times with 1X wash solution. After washing 100 µl 1X biotinylated anti-Human IL1β were added to the wells and incubated for 1 hour at room temperature. After incubation the plate was washed 4 times with 1X wash buffer and 100 µl of 1X horse reddish peroxidase (HRP)-conjugated with streptavidin to all wells. Then the plate was incubated for 45 minutes at room temperature with gentle shaking. Washing procedure was repeated to wash away unbound antibodies and 100 µl of 1X 3,3,5,5’-tetramethylbenzidine (TMB) substrate were added to all wells and incubated for 30 minutes at room temperature in dark with gentle shaking. 100 µl of stop solution

containing 0.2M sulphuric acid were added to inactivate the enzymatic reaction and measured the optical density (OD) of solution in wells at 450nm immediately on Thermo Scientific Multiskan FC.

4.16.1.2 Calculation of Results:

Calculated the mean absorbance for each set of duplicate standards, controls and samples and subtract the average zero standard optical density. Quantitative estimation of serum IL1β level was obtained by plotting the standard curve on Graph Pad prism software by extrapolating OD of unknown samples. Standard curve was prepared by plotting standard concentration on the x-axis and OD on y-axis.

4.16.1.3 Sensitivity and Reproducibility and Specificity of the IL1β ELISA kit:

The minimum detectable dose of Human IL-1b was determined to be 0.3 pg/ml. The Intra-Assay coefficient of varience (CV%) is <10% and the Inter-Assay CV% is <12% of this kit. This ELISA kit shows no cross-reactivity with any of the other cytokines tested.

4.16.2 Estimation of IL18:

Serum IL18 were estimated from all the collected samples using RayBio$^{@}$ Human IL-18 ELISA kit (RayBiotech, GA, cat no: ELH-IL18).This kit is an in vitro enzyme-linked immunosorbent assay for the quantitative estimation of IL18 from human serum, plasma and cell culture supernatant. Antibody specific to human IL1β were coated on a 96 well plate. Quantitative estimation of IL18 is based on antibody antigen reaction and then enzyme substrate reaction. Optical density of coloured product is measured at 450 nm and quantification is measured by plotting standard curve. This kit provided IL18 standard as lyophilised and was to be reconstituted to 5000pg/ml with 400µl of assay diluents. Seven standards were prepared from stock standard vial by serial dilution and the concentration are such as 5000 pg/ml, 75 pg/ml, 30 pg/ml, 12 pg/ml, 4.8 pg/ml, 1.92 pg/ml, 0.77 pg/ml and 0.31 pg/ml.

4.16.2.1 Procedure:

All the reagent and samples were kept in room temperature and antibody coated wells were arranged according to samples and standards in duplicates. 100 µl of standards and plasma samples (samples were diluted 5 folds by assay diluents containing 0.09% sodium azide provided with the kit) were dispensed to the well and plate is incubated for 2.5 hours at room temperature with gentle shaking. A blank was also set by adding nuclease free water instead of samples. Discarded the solution and washed the plate 4 times with 1X wash solution. After washing 100 µl 1X biotinylated anti-Human IL1β were added to the wells and incubated for 1 hour at room temperature. After incubation the plates was washed 4 times with 1X wash buffer and 100 µl of 1X horse reddish peroxidase (HRP)-conjugated streptavidin to all wells. Then the plate was incubated for 45 minutes at room temperature with gentle shaking. Washing procedure was repeated to wash away unbound antibodies and 100 µl of 1X 3, 3, 5, 5'-tetramethylbenzidine (TMB) substrate were added to all wells and incubated for 30 minutes at room temperature in dark with gentle shaking. 100 µl of stop solution containing 0.2M sulphuric acid were added to inactivate the enzymatic reaction and measured the optical density (OD) of solution in wells at 450nm immediately on Thermo Scientific Multiskan FC.

4.16.2.2 Calculation of Results:

Calculated the mean absorbance for each set of duplicate standards, controls and samples and subtract the average zero standard optical density. Quantitative estimation of serum IL18 level was obtained by plotting the standard curve on Graph Pad prism software by extrapolating OD of unknown samples. Standard curve was prepared by plotting standard concentration on the x-axis and OD on y-axis.

4.16.2.3 Sensitivity and Reproducibility and Specificity of the IL18 ELISA kit:

The minimum detectable dose of Human IL-18 was determined to be 0.5 pg/ml. The Intra-Assay coefficient of varience (CV%) is <10% and the Inter-Assay CV% is <12% of this kit. This ELISA kit shows no cross-reactivity with any of the other cytokines tested.

4.16.3 Estimation of TNFα:

Serum TNFαwere estimated from all the collected samples using Human TNF-α ELISA kit (Invitrogen, Thermo Fisher Scientific, USA, cat no: KHC3011).This kit is an solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for the quantitative estimation of TNFα from human serum, plasma and cell culture supernatant. Antibody specific to human TNFα are coated on a 96 well plate. Quantitative estimation of TNFα is based on antibody antigen reaction and then enzyme substrate reaction. Optical density of coloured product is measured at 450 nm and quantification is measured by plotting standard curve. This kit provided a standard as lyophilised and reconstitute Hu TNFα standard to 2000pg/ml with standard diluents buffer. Seven standards were prepared from stock standard vial by serial dilution and the concentration are such as 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.2 pg/ml and 15.6 pg/ml.

4.16.3.1 Procedure:

All the reagent and samples were kept in room temperature and antibody coated wells were arranged according to samples and standards in duplicates. 50 µl of Incubation buffer contains 8 mM sodium azide to wells for plasma and standard and 50 µl of standard diluents buffer contains 8 mM sodium azide for cell culture supernatant. And a blank was set for chomogen blanks left empty. 100 µl standards and plasma samples, cell culture supernatant (samples were diluted 2 folds by standard diluents buffer containing 0.09% sodium azide provided with the kit) were dispensed to the well, cover the plate and incubated the plate for 2 hours at room temperature with gentle shaking. A blank was also set by adding nuclease free water instead of samples. Discarded the solution and washed the plate 4 times with 1X wash solution. After washing 100 µl 1X Hu TNF-α Biotin Conjugate (contains 0.09% sodium azide) solution were added to all wells except the chromogen blank and incubated for 1 hour at room temperature. After incubation the plates was washed 4 times with 1X wash buffer and 100 µl of 1X horse reddish peroxidise (HRP)-conjugated streptavidin (contains 1.3mM thymol) to all wells except the chromogen blank. Then the plate was incubated for 30 minutes at room temperature with gentle shaking. Washing procedure was repeated to wash away unbound antibodies and 100 µl of Stabilized Chromogen (3,3,5,5’-tetramethylbenzidine, TMB)

substrate were added to all wells and incubated for 30 minutes at room temperature in dark with gentle shaking. The substrate solution turned blue. 100 µl of stop solution were added to inactivate the enzymatic reaction and measured the optical density (OD) of solution in wells at 450nm immediately on Thermo Scientific Multiskan FC.

4.16.3. 2 Calculation of Results:

Calculated the mean absorbance for each set of duplicate standards, controls and samples and subtract the average zero standard optical density. Quantitative estimation of serum TNFα level was obtained by plotting the standard curve on Graph Pad prism software by extrapolating OD of unknown samples. Standard curve were prepared by plotting standard concentration on the x-axis and OD on y-axis.

4.16.3.3 Sensitivity and Reproducibility and Specificity of the TNFα ELISA kit: The minimum detectable dose of Human TNFα is 1.7pg/ml. This ELISA kit shows no cross-reactivity with any of the other cytokines tested.

4.17 ESTIMATION OF GUT MICROBIOTA FROM STUDIED PARTICIPANTS

4.17.1 Methodology for DNA Isolation from Stool Samples for Gut Microbiota Estimation from Studied Participants

Human fresh stool were collected from 28 individuals (DN=12, T2Diabetic Control, (DC=10) and (HC=6)] at Assam Medical College, India. Bacterial DNA was extracted using the Qiagen stool DNA Kit (Qiagen, Germany, Cat No: 51604) and amplicons were generated through 16s universal primer for variable region 3 and 4 and sequenced on Illumina-HiSeq2500 and analyzed through QIIME (Version 1.9.0).

QIAamp DNA Stool Mini Kits contain QIAGEN Protease and provide fast and easy purification of DNA from fresh or frozen stool samples. Stool samples typically contain various compounds that can degrade DNA and inhibit down stream procedures. Removal of such compound can be ensured by the use of InhibitEX tablet or buffer contained in the

QIAamp DNA stool mini kit. InhibitEX Tablets or buffer efficiently adsorbs these substances early in the purification process so that they can easily be removed by a quick centrifugation step. This kit also contains Buffer ASL, which is specially developed to remove inhibitory substances from stool samples. DNA of up to 20 kb can be purified by the QIAamp DNA Stool Mini Kit. This kit contains proteinase K, which is the enzyme of choice for SDS-containing lysis buffer used in the tissue protocol, but which performs equally well in the blood and body fluid protocol. The activity of the proteinase K solution is 600 mAU/ml solution (or 40 mAU/mg protein). This activity provides optimal results in QIAamp protocols. The QIAamp DNA purification procedure comprises 4 steps and is carried out using QIAamp Mini spin columns in a standard microcentrifuge, on a vacuum manifold, or fully automated on the QIAcube. Adsorption to the QIAamp membrane, the lysate buffering conditions are adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample is loaded onto the QIAamp Mini spin column. DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation or vacuum step. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane. Removal of residual contaminants DNA bound to the QIAamp membrane is washed in 2 centrifugation or vacuum steps. The use of 2 different wash buffers, Buffer AW1 and Buffer AW2, significantly improves the purity of the eluted DNA. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding. Elution of pure nucleic acids Purified DNA is eluted from the QIAamp Mini spin column in a concentrated form in either Buffer AE or water. Yields will be increased if the QIAamp Mini spin column is incubated with the elution buffer at room temperature for 5 minutes before centrifugation. The eluted genomic DNA is up to 50 kb in length (predominantly 20–30 kb) and is suitable for direct use in PCR or Southern-blotting applications.

4.17.1.1 Procedure:

180-200 mg stool were measured and placed into 2 ml centrifuge tube in maintaining cold chain. 1.4 ml of ASL buffer was added to all samples and vortexes continuously for 1 minute or until complete homogenized. The tubes were then incubated for 5 minutes in 70℃ and another 5 minutes in 95℃ at dry bath with gentle shaking. Tubes were centrifuged at

13000 rpm for 1 minute to pellet stool particles. Pipetted 1 ml of the supernatant into new 2 ml centrifuge tube and discarded the pellet. 1ml of InhibitEX buffer was added to each tube and vortex immediately. Centrifuged the tubes at 13000 rpm for 3 minutes. 200 µl of supernatant were collected in new microcentrifuge tube containing 20 µl QIAGEN Protease (or proteinase K). 200 µl of lysis buffer (AL) were pipetted to the sample and mixed by vortexing. Incubated for 10 min at 70 ºC and added 200 µl of 96-100% ethanol to the samples and mixed by vortexing. Briefly centrifuged the 1.5 ml tube to remove drops from the inside of the lid. Carefully applied the mixture from step 6 to the QIAamp Mini spin column attached with 2 ml collection tube without wetting the rim. Centrifuged the tube at 13000 rpm for 1 min. Placed the QIAamp Mini spin column in a clean 2 ml collection tube and discarded the tube containing the filtrate. 500 µl AW1 (wash) buffer were added to the QIAamp Mini spin column without wetting the rim. Centrifuged the tube at 13000 rpm for 1 min. Placed thaeQIAamp Mini spin column in a clean 2 ml collection tube and discarded the tube containing the filtrate. 500 µl AW2 (wash) buffer were added to the QIAamp Mini spin column without wetting the rim. Centrifuged the tube at 13000 rpm for 3 min. A dry spin was done by placing the QIAamp Mini spin column in a clean 2 ml collection tube and discarded the tube containing the filtrate and centrifuged at full speed for 1 min. QIAamp Mini spin column were placed in a clean 1.5 ml microcentrifuge tube and discarded the tube containing the filtrate. Carefully opened the QIAamp Mini spin column and added 60 µl buffer AE (Elution). Incubated at room temperature (15-25 ºC) for 1 min then centrifuged at 13000 rpm for 1 min. Stored the eluted DNA in -20 ºC freezer for further use.

4.17.2 16S Metagenome Sequencing

16S Metagenome Sequencing was done using next generation sequencing (NGS) technology targets to aplifly 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions. 16S rRNA variable regions are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations (Illumina, I. 2013, Jovel, J., *et al*. 2016)

4.17.2.1 Gene-specific sequences

The gene specific sequences used in this protocol targets the 16S V3 and V4 region. The full length primer sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are:

Primer sequences:

16S Forward: 5' AGAGTTTGATCCTGGCTCAG 3'

16S Reverse: 5' GGTTACCTTGTTACGACTT 3'

V3V4F 5' CCTACGGGNGGCWGCAG 3'

V3V4R 5' GACTACHVGGGTATCTAATCC 3'

Each sequenced sample is prepared according to the Illumina16S Metagenomic Sequencing Library protocols. The quantification of DNA and the DNA quality was measured by PicoGreen and Nanodrop. 2ng Genomic DNA was used in the first amplification step using the following conditions: 5pmol forward tailed target specific primer; 5pmol reverse tailed target specific primer; and Herculase II polymerase (Agilent). The PCR for each variable region was carried out in triplicate in a 25ul reaction in a thermal cycler PCR system with the following parameters: initial denaturation at 95°C for 3mins, followed by 25cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s with a final extension at 72°C for 5min and then stored at 4°C hold. The amplicon libraries were cleaned to remove excess nucleotides, salts and enzymes using 20ul of the AgencourtAMPure XP system (Beckman Coulter Genomics) and eluted in 25ul of TE buffer. The 10ul of the first step reaction was submitted to a second amplification step using the following conditions: Nextera XT Index Primer (N7xx); Nextera XT Index Primer (S5xx); Herculase II polymerase (Agilent). The PCR for each variable region was carried out in triplicate in a 25ul reaction in the above-mentioned thermal cycler with the following parameters: initial denaturation at 95°C for 3min, followed by 8cycles of 95°C for 30s, 55°C for 30s, and 72°C for 30s with a final extension at 72°C for 5min and then stored at 4°C hold. The amplicon libraries were cleaned to remove excess nucleotides, salts and enzymes using 20ul of the AgencourtAMPure XP system (Beckman Coulter Genomics) and eluted in 25ul of TE buffer. The final purified product is then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantificatoin kits for IlluminaSequecing platforms) and qualified using the Tape Station DNA screentape D1000

(Agilent). In preparation for cluster generation and sequencing, pooled libraries are denatured with NaOH, diluted with hybridization buffer, and then heat denatured before sequencing. Each run includes a minimum of 5% PhiX to serve as an internal control for these low-diversity libraries. These libraries were pooled based on the data required and sequenced on HiSeq 2500 sequencer using Rapid SBS kit V2 (500 cycles) to generate 250 paired-end reads.

4.17.2.2 Metagenominc Sequence Analysis

The initial quality control was performed based on the FASTQC reports (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) for each sequence file. The files were then demultiplexed for the MID sequences and trimmed using the following filter criteria: Number of Ambiguous bases = 0–2, QV per base = 20–25, Minimum sequence length = 150–200bp, Maximum Sequence Length = 250–300bp and Number of Homopolymers = 4–6 based on the nature of the data. The 16's sequence read files were analysed for the 28 samples on QIIME (Version 1.9.0) (Caporaso, J. G., *et al.* 2010). Chimera filtering was performed using UCHIME. Maximum and minimum read count among the samples were (pair end read) 31.6 million and 27.2 million, respectively with an average Q30 (phred score) of 79.8%. The Green genes database was used for Taxonomy assignment. Operational Taxonomic Units (OTUs) picking was performed using the *pick_otus.py* command with the default UCLUST algorithm. The UCLUST algorithm uses the USEARCH algorithm to assign sequences to a cluster. The USEARCH algorithm works by searching a query sequence against target sequences and recording the k-mers in common between the two sequences. Rather than inferring sequence similarity as the number of matching k-mers between a query and target sequence, USEARCH arranges the target sequences in decreasing order of the number of unique k-mers shared between the two sequences. The query sequences are arranged into clusters. Each cluster centroid shares a level of similarity below a set identity threshold level with each other centroid. The remaining query sequences are then assigned to a centroid (target sequence) based on identity threshold using the USEARCH algorithm. If the query sequence does not share similarity with a centroid above the threshold a new cluster is created. The most abundant read in each OTU was selected as the representative sequence; this

step was performed using *pick_rep_set.py. Assign_taxonomy.py* was used for the classification of each of the representative sequences.

Software Name	Version
FLASH	v1.2.11
vsearch	v2.9.1
QIIME	v1.9
Perl	v5.16.3
Python	v3.7
R	v3.5.0

4.18 Statistical Analysis:

Variable with multiple covariates (BMI, age, HbA1c, DOD) and a set of explanatory variables (mRNA, miRNA expression levels and protein level) were analyzed. The significance of difference between corresponding groups of observations was evaluated by the Mann-Whitney U test among the variables that are not in normal distribution though we performed two tailed t test for the variables which are in normal distribution and ANOVA and post hoc Tukey's test performed for more than two groups such as Age, height, weight, BMI, blood glucose, serum creatinine and blood urea whenever required. All values were expressed as mean ± standard deviation (SD) and range. Pearson and Spearman's rank correlation coefficient on the logged data was used to determine the relation between variables having continuous data. Apart from the group we used linear regression model to define the severity of DN using eGFR data as a dependent variable (y) whose value consider as continuous data where the expression value of different genes and protein used as independent variable (Xi-Xn) to obtain the estimate to quantities the risk prediction. eGFR from serum creatinine, age and weight based on gender was calculated using an online formula available at http:/www.kidney.org/ klsprofessionals/gfr_calculator.com. All statistical analysis was done using graph pad and R3.1 (The R Foundation for Statistical Computing R version 3.2.3). Graphs were developed by Graph pad prism v.7 (GraphPad Prism version 7.00 for Windows). For all statistical analysis, p value <0.05 was taken as significant.