CHAPTER 5

RESULTS

5.1 DISTRIBUTION OF DEMOGRAPHIC AND CLINICAL VARIABLES OF STUDY SUBJECTS:

All the demographic and clinical characteristics of study participants were collected through structured prevalidated questionnaire. Clinical data including biochemical parameters were collected from the patient's information sheets.

Demographic parameters such as mean age of study participants was matched among three study groups i.e; HC: 53.35±6.69, DC: 52.18±10.69 and DN: 56.95±12.04 (P_{ANOVA} =0.07). Distribution of male and female ratio across the study groups were found to be insignificant (P=0.6) (Table 5.23).

The mean height of study participants from were matched and was HC (164.2±6.74cm), DC (165.0±6.99 cm) and DN (167.0±6.26 cm), (P_{ANOVA}=0.1). Weight of DC subjects (57.08±8.6 kg) was significantly lower than HC (64.02± 9.5 kg) (P_{Tukeys}<0.001) but not with the DN subjects (62.40±8.87 kg, P_{Tukeys}>0.05) (Table 5.23 Figure 5.43A).

Body mass index (BMI) of the study subjects were within the normal limit, but the level was significantly higher among HC subjects compared to others (DC and DN) (P_{ANOVA}=0.0003). The mean BMI of study participants from HC was 23.68±2.82 kg/m^2. The BMI of DC (21.01±3.17 kg/m^2) and DN (21.78±3.88 kg/m^2) subjects didn't differ significantly (P_{Tukeys}>0.05) from each other (Table 5.23 Figure 5.43B).

The mean systolic blood pressure (SBP) of individuals across the study groups was comparable (130.1±11.34 mmHg, 131.5±14.60 mmHg and 135.2±23.41 mmHg respectively for HC, DC and DN subjects, P_{ANOVA}=0.3). Similarly, there was no significant differences (P_{ANOVA}=0.7) for mean diastolic blood pressure (DBP) in the three study groups (81.49±7.79 mmHg, 82.23±6.89 mmHg and 82.93±8.81 mmHg for HC, DC and DN respectively) (Table 5.23). Duration of Diabetes (DOD) among DC (75.95±54.63 months) subjects was found to be significantly (P_t=0.03) lower than that of DN subjects (104.3±63.33 months) (Table 5.23 and Figure 5.43C). Mean duration of diabetic nephropathy (DODN) among DN subjects was 10.07±14.13 months.

Level of Random Blood Sugar (RBS) was significantly low in the group HC (92.40±15.46 mg/dl) compared to DC (260.4±129.9 mg/dl) and DN (228.3±177.7 mg/dl) (P_{ANOVA}<0.0001) but, between DC and DN the RBS level was not significant (P_{Tukeys}>0.05) (Table 5.23 Figure 5.43D).

Serum creatinine was significantly higher among DN (4.82±3.60 mg/dl) compared to DC (0.63±0.33mg/dl) and HC (0.63±0.19mg/dl, P_{ANOVA}< 0.0001) (Table 5.23 Figure 5.43E).

Values of blood urea and CRP could be accessed for a subset of participants. It was found that blood urea was found to be significantly higher among DN (90.07±55.70 mg/dl) subjects than that of DC (35.67±7.89mg/dl) and HC (16.21±4.12 mg/dl) subjects (P_{ANOVA}<0.0001) (Table 5.23 Figure 5.43F). The CRP level was also significantly higher in DN (8.66±12.21mg/L) subjects followed by DC (4.09±6.09 mg/L) and HC (0.97±0.85mg/L) subjects respectively, (P_{ANOVA}<0.0001) (Table 5.23). Glycemic status (HbA1c%) for the DC (9.46 ±1.96) and DN (8.41± 3.86) subjects were matched in this study (P_t=0.6) (Table 5.23).

Table 5.23: Distribution of Demographic and clinical variables in the study subjects

	Healthy Control	**Diabetic Control**	**Diabetic Nephropathy**	**P_{ANOVA}**	**P_{Tukeys}**	
Age(Years)	53.35±6.69	52.18±10.69	56.95±12.04	0.07	HC vs DC	>0.05
					HC vs DN	>0.05
					DC vs DN	>0.05
Female/ Male	14/46	12/44	12/48	0.63	-	-
Height(cm)	164.2±6.74	165.0±6.99	167.0±6.26	0.1	HC vs DC	>0.05
					HC vs DN	>0.05
					DC vs DN	>0.05
Weight(Kg)	64.02± 9.5	57.08±8.6	62.40±8.87	0.008	HC vs DC	<0.001
					HC vs DN	>0.05
					DC vs DN	>0.05
BMI (kg/m^2)	23.68±2.82	21.01±3.17	21.78±3.88	0.0003	HC vs DC	<0.0001
					HC vs DN	<0.01
					DC vs DN	>0.05
SBP(mmHg)	130.1±11.34	131.5±14.60	135.2±23.41	0.3	HC vs DC	>0.05
					HC vs DN	>0.05
					DC vs DN	>0.05
DBP(mmHg)	81.49±7.79	82.23±6.89	82.93±8.81	0.7	HC vs DC	>0.05
					HC vs DN	>0.05
					DC vs DN	>0.05
RBS(mg/dl)	92.40±15.46	260.4±129.9	228.3±177.7	< 0.0001	HC vs DC	<0.0001
					HC vs DN	<0.0001
					DC vs DN	>0.05
S.Creatinine (mg/dl)	0.63±0.19	0.63±0.33	4.82±3.60	< 0.0001	HC vs DC	>0.05
					HC vs DN	<0.0001
					DC vs DN	<0.0001
Blood Urea(mg/dl)	16.21±4.12	35.67±7.89	90.07±55.70	< 0.0001	HC vs DC	>0.05
					HC vs DN	<0.0001
					DC vs DN	<0.001
S. CRP (mg/L)	0.97±0.85	4.09±6.09	8.66±12.21	< 0.0001	HC vs DC	>0.05
					HC vs DN	<0.0001

					DC vs DN	<0.05
DOD (months)		75.95±54.63	104.3±63.33	0.03		
HbA1c (%)	-	9.46±1.96	8.41± 3.86	0.6	-	
BUN	-	-	106.94±73.59			
Uric Acid	-	-	6.9±3.6			

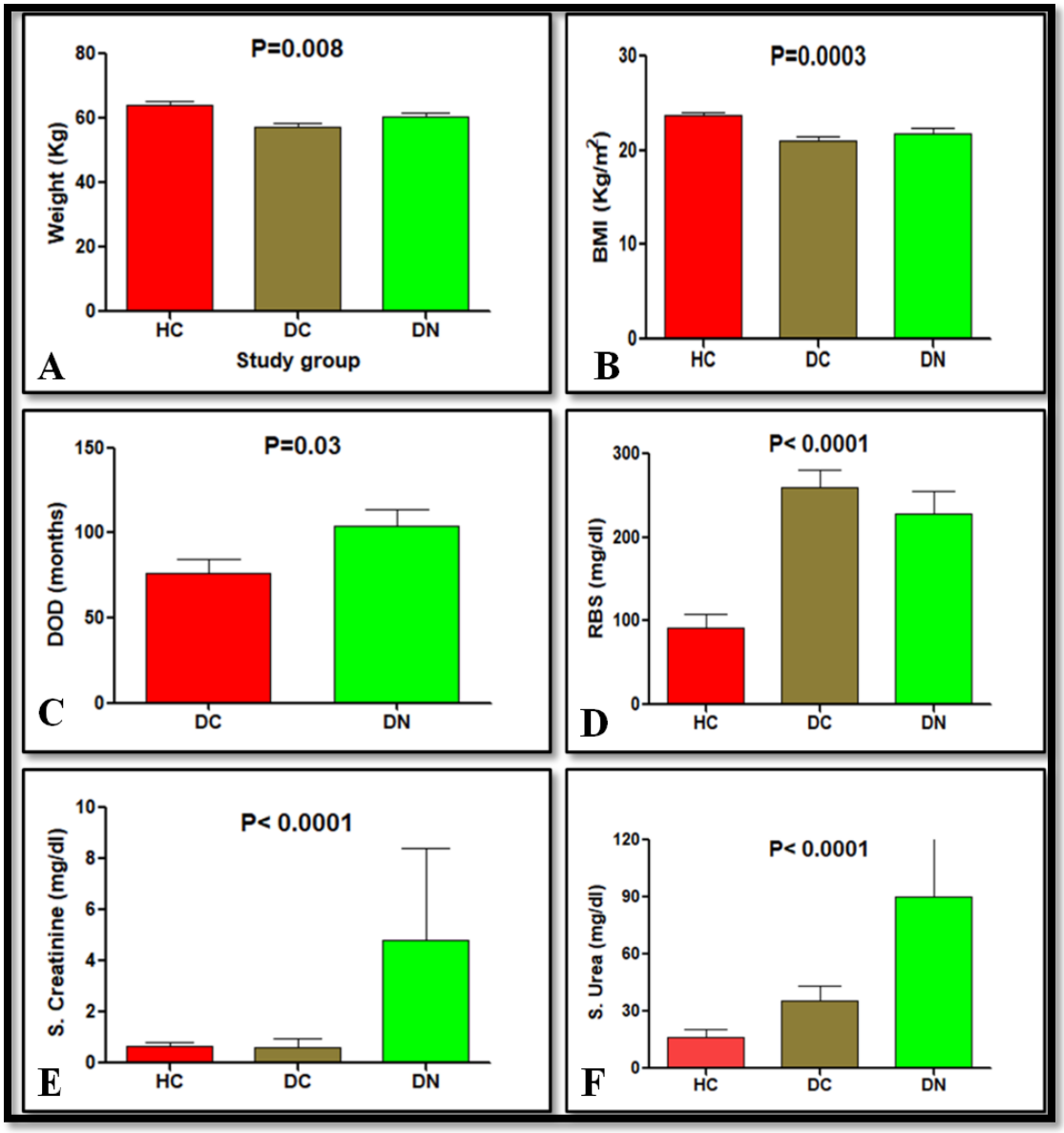

Figure 5.43: Distribution of Demographic and clinical variables among study subjects. [A]Weight of the study population was significantly higher among HC subjects than DC and DN. [B] Distribution of BMI among HC was significantly higher than DC and DN. [C] Duration of Diabetes (DOD) was significantly higher in DN than DC.[D] Random blood sugar (RBS) in mg/dl was higher in DC than DN and HC. [E]Serum creatinine level (mg/dl) was significantly higher in DN subjects compared to DC and HC. [F]Distribution of Serum urea level (mg/dl) of the study population revealed that DN subjects has significantly higher S. creatinine than DC and HC. All data is presented as mean±SD and significant level was determined by $P<0.05$.

5.2 EXPRESSION OF mRNA FOR NLRP3, CASP-1 AND PYCARD IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AMONG CASES AND CONTROLS:

Expressions of mRNAs were quantified in terms of relative quantification through qRT-PCR using SYBR Green chemistry (details are given in methodology section). The GAPDH was used as housekeeping geneand also considered as reference standard to normalize the target signal. The comparativeCt method (ΔΔCt) was used to quantify gene expression, and the relative quantificationwas calculated as $2^{-\Delta\Delta Ct}$ taking HC as reference sample. Amplification specificity was controlled by a melting curveanalysis (Figure 5.44 melting curve). The final gene expression was expressed as fold ($2^{-\Delta\Delta CT}$) change of target gene expression in the target sample (DN and DC) relative to a reference sample (HC).

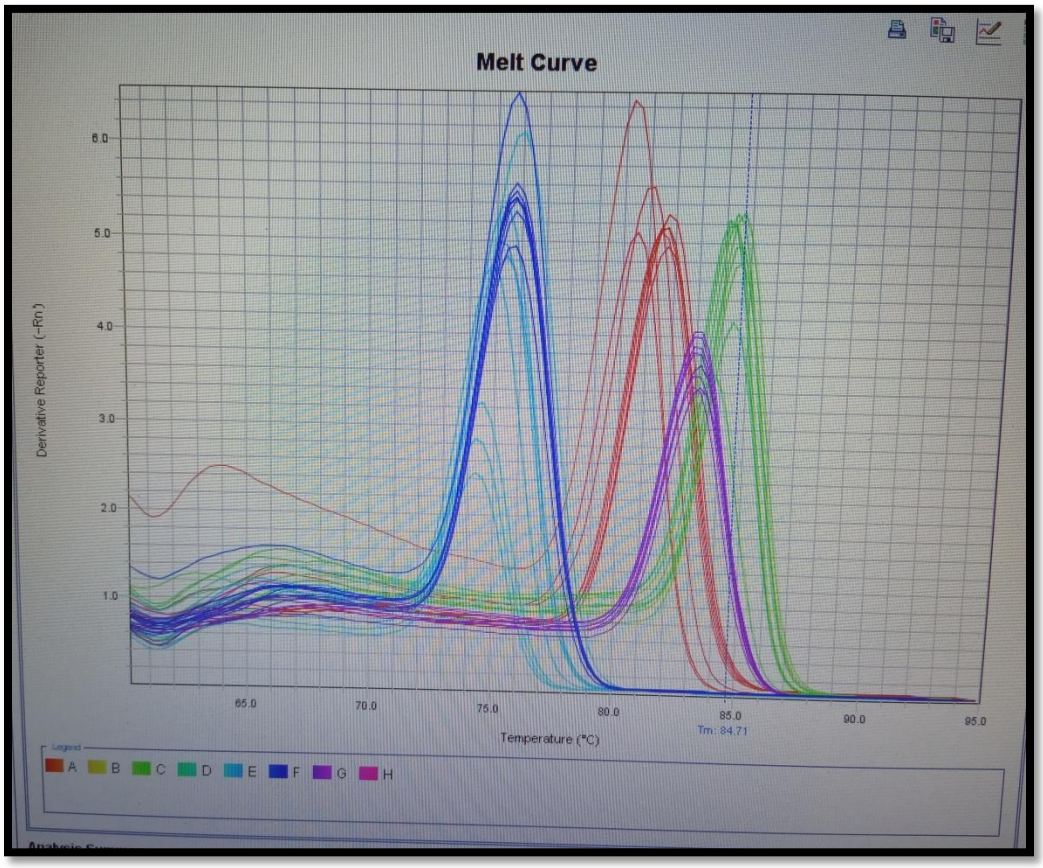

Figure 5.44: Melting curves results of GAPDH, NLPR3, CASP-1 and PYCARD expression in SYBR green based realtime PCR. A single melting peak corresponds to each amplicon generated using specific set of primers for each gene.

All the three genes of Inflammasome complex i.e; NLRP3, CASP1 and PYCARD were expressed in elevated manner among T2DM patients than HC individuals. We have compared the expression of genes as fold change between DC and DN subjects after taking HC as reference subjects. The study reveals that mRNA expression of NLRP3 (fold change) was significantly higher (17.42±22.85) among DN subjects than DC (5.95±12.66) subjects (P_{mw}=0.009) (Figure 5.45 A and Table 5.24A). mRNAs expression of CASP1 was significantly higher fold change (9.04±14.39) in DN subjects compared to DC (2.87±7.89), P_{mw} =0.02 (Figure 5.45 B and Table 5.24A). Fold change of PYCARD was moderately higher in DN (5.01±7.89) than DC (3.21±4.73) but the changes were not significant (P_{mw}=0.2) (Figure 5.45 C and Table 5.24A).

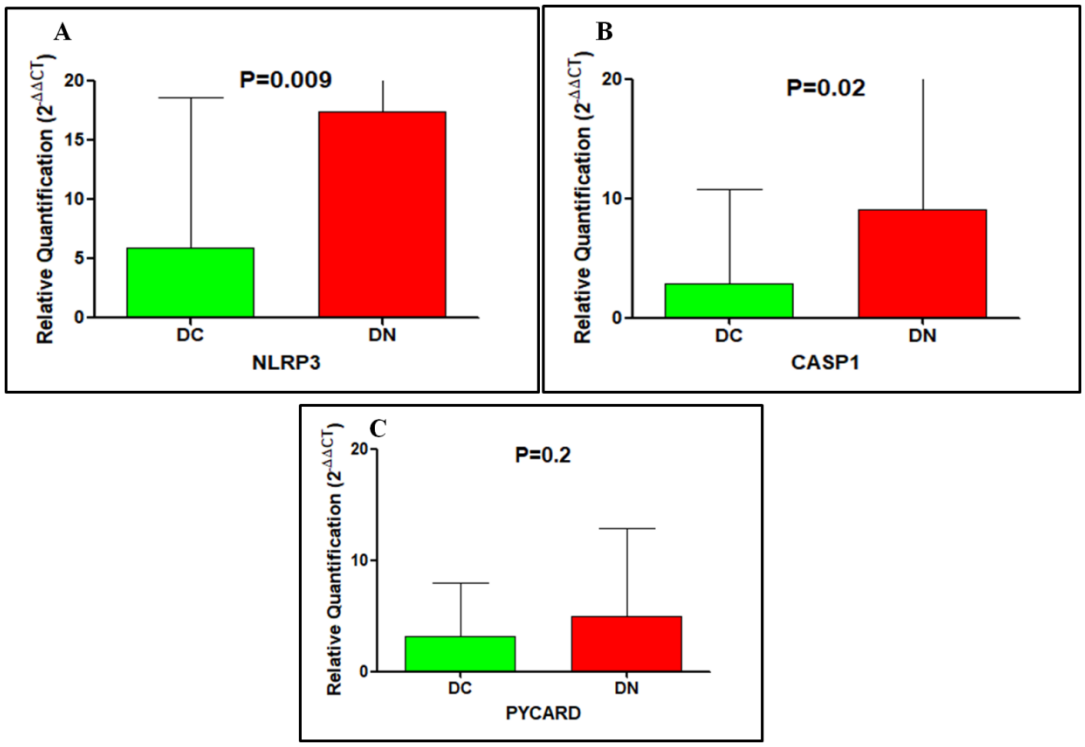

Figure 5.45: Gene expression of NLRP3, CASP-1 and PYCARD from DC and DN patients after normalizing to HC as reference subjects. Bars represent the mean relative expression of gene ± SD of [A] NLRP3 [B] CASP-1 and [C] PYCARD gene expression in PBMC from DC and DN patients (n = 60 per group). m-RNA expression (fold change) of [A] NLRP3, p=0.009 and [B] CASP-1,P_{mw}=0.02 were significantly higher among DN subjects than DC subject. P value considered significant at P< 0.05, P< 0.01 and P< 0.001 between DC and DN.

5.3 EXPRESSION OF miRNA hsa-miR 223, hsa-miR 22-3p, hsa- miR4291 AND hsa-miR 185-3p IN PBMC FROM DN, DC AND HC STUDY POPULATION.

Expression of mi-RNA hsa-miR-223 and hsa-miR-22-3p targeting NLRP3, hsa-miR-4291 targeting CASP-1 and hsa-miR-185-3p targeting PYCARD (Inflammasome components) among DN and DC were assessed individually as described for mRNA expression and fold change was calculated as $2^{-\Delta\Delta ct}$ method. Here we have used Hs-SNORD61-11 as housekeeping miRNA and also considered as reference standard to normalize the target signal. The comparativeCt method (ΔΔCt) was used to quantify miRNA expression, and the relative quantificationwas calculated as $2^{-\Delta\Delta Ct}$ taking HC as reference sample. Amplification specificity was controlled by a melting curveanalysis (Figure 5.46 melting curve).

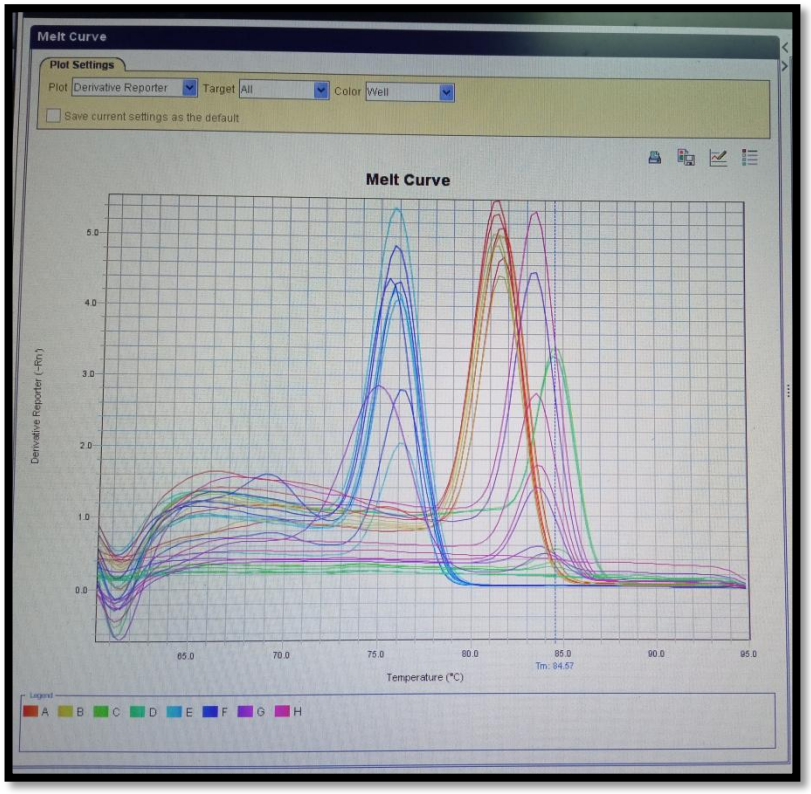

Figure 5.46: Melting curves results of miRNA, hsa-miR-223 and hsa-miR-22-3p targeting NLRP3, hsa-miR-4291 targeting CASP-1 and hsa-miR-185-3p targeting PYCARD expression in SYBR green based realtime PCR. A single melting peak corresponds to each amplicon generated using specific set of primers for each miRNA.

The study reveals that the expression (fold change) of microRNA hsa-miR 223 targeting NLRP3 was found significantly higher among DC (8.67±18.89) subjects compared to DN (1.65±3.03), P_{mw}=0.05 (Figure 5.47 A and Table 5.24B). The another miRNA, hsa-miR-22-3p targeting NLRP3 showed moderately higher level of expression with mean fold change of 1.02±3.46 among DN as compared to DC (0.77±1.81,P_{mw}=0.06). The study reveals that hsa-miR-22-3p is downregulated among DC compared to DN and HC (Figure 5.47 B and Table 5.24B). The study did not reveal any significant difference for miRNA, hsa- miR-4291 targeting CASP1 though higher fold change was observed among DN (27.28 ±58.76) and DC (12.08±25.12) when compared to HC as reference samples (Figure 5.47 C and Table 5.24B). The fold change of hsa-miR 185-3p miRNA targeting PYCARD was significantly higher among DN (31.75 ± 38.18) than among DC (10.24 ± 19.11 folds, P_{mw}=0.04) (Figure 5.47 D and Table 5.24B).

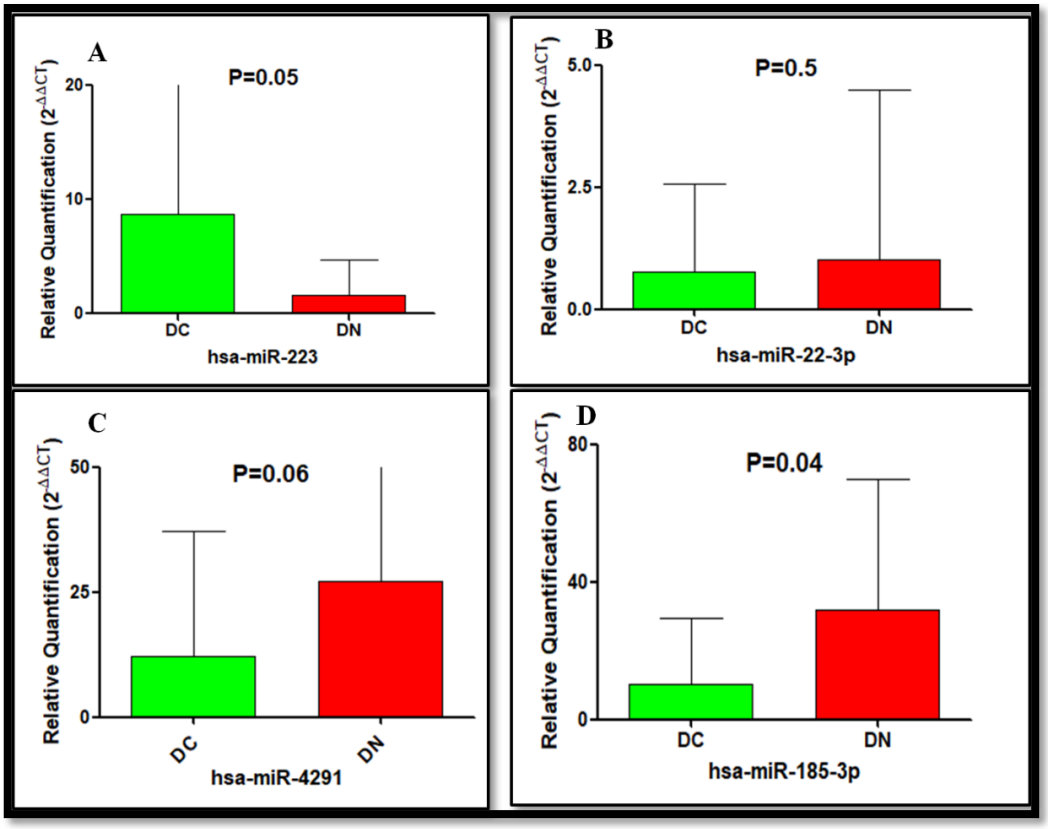

Figure 5.47: mi-RNA expression of hsa-miR-223, hsa-miR-22-3p, hsa-miR-4291and hsa-miR-185-3pfrom DC and DN patients after normalized to HC as reference subjects. Bars represents the mean relative expression of miRNA± SD [A] hsa-miR-223,[B] hsa-miR-22-3p,

[C] hsa-miR-4291and [D] hsa-miR-185-3p expression in PBMC from DC and DN patients (n = 60 per group). Expression (fold change) of [D] hsa-miR-185-3p targeting PYCARD was significantly (P_{mw}=0.04) high among DN subjects compared to DC.

Table 5.24: Relative quantification of Inflammasome complex and their targeting miRNA expression from Diabetic Control and Diabetic Nephropathy patients taking healthy subjects as reference.

	Diabetic Control	**Diabetic Nephropathy**	**P_{mw}**
A] Expression of NLRP3 Inflammasome			
NLRP3	5.95±12.66	17.42±22.85	**0.009**
CASP-1	2.87±7.89	9.04±14.39	**0.02**
PYCARD	3.21±4.73	5.01±7.89	0.2
B] Expression of micro RNAs targeting NLRP3 Inflammasome complex genes			
hsa-miR-223	8.67±18.89	1.65±3.03	0.05
hsa-miR-22-3p	0.77± 1.81	1.02 ± 3.46	0.06
hsa-miR-4291	12.08± 25.12	27.28± 58.76	0.33
hsa-miR-185-3p	10.2± 19.114	31.75± 38.18	**0.04**

5.4 EXPRESSION OF m-RNA FOR TLRS (TLR1-10) IN PBMC AMONG DIABETICS AND CONTROLS:

Keeping GAPDH as housekeeping gene and HC as reference sample, we have calculated $2^{-\Delta\Delta Ct}$ for TLRs that includes TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10. Amplification specificity was controlled by a melting curve analysis (Figure 5.48).

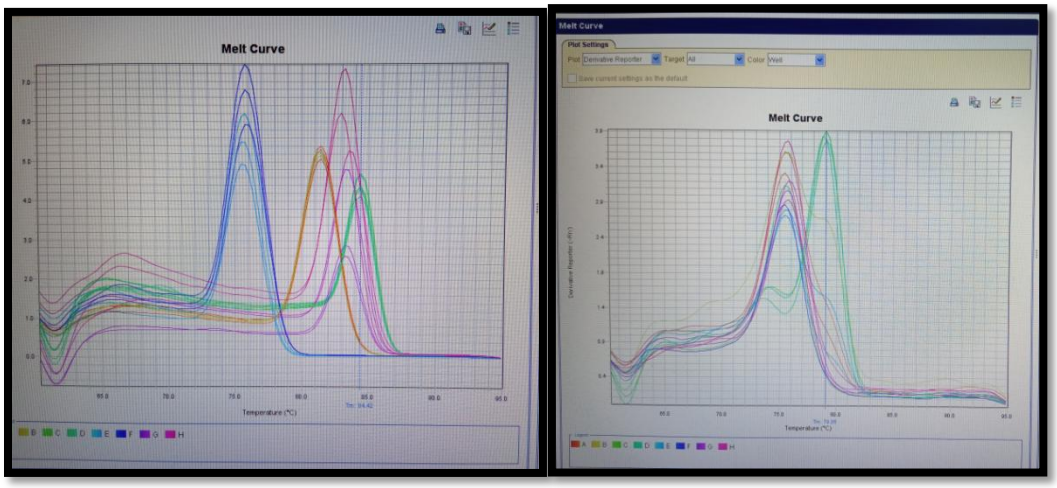

Figure 5.48: Melting curves results of TLR1,TLR2,TLR3,TLR4,TLR5,TLR6,TLR7, TLR8, TLR9 and TLR10 mRNA expression in SYBR green based realtime PCR. A single melting peak corresponds to each amplicon generated using specific set of primers for each mRNA.

The study reveals that mRNA expression (fold change) of TLR1 did not differ significantly between DC (5.52±9.31) and DN subjects (5.14±12.03), P_{mw}=0.9, though higher expression was observed compared to HC (Figure 5.49A and Table 5.25A).The fold change of TLR2 expression was higher in DC (15.06±18.77) than DN (9.98±12.18) but the difference was insignificant (p=0.6) (Figure 5.49B and Table 5.25A).TLR3 expression was 66.68± 114.1 and 57.01±62.87 respectively among DC subjects and DN subjects (P_{mw}=0.3) (Figure5.49C and Table 5.25A). TLR 4 expression was found to be significantly higher among DN patients than DC patients and the fold change was 22.66±31.34 and 9.74±16.27, P_{mw}=0.02 (Figure5.49D and Table 5.25A). TLR5 and TLR 6 expression in DN (10.21±22.64 and 4.44±4.44) patients possess higher compared to DC (9.64±11.02 and 2.17±5.03) but the difference was insignificant, P_{mw} value 0.2 and 0.8 respectively (Figure5.49 E and 5.49F and Table 5.25A). It was observed that the expression of TLR7 was found higher in DC (16.50±25.71) than that of DN subjects (12.65±17.69), P_{mw}=0.6 (Figure5.49G and Table 5.25A). TLR8, TLR9 and TLR10 were found to be up-regulated but did not reveal significant difference among DC and DN subjects (Figure5.49 H-J and Table 5.25A). The expression of TLR8 was 22.97±25.94 and 19.94±28.98 (P_{mw}=0.8) for DC and DN respectively. TLR9 and TLR 10 expression was found to be higher in DC (7.86±22.33 and 16.52±22.93) than DN (4.01±11.00 and 12.09±22.25) but the difference was not significant (Figure 5.49 I and 5.49 J and Table 5.25A).

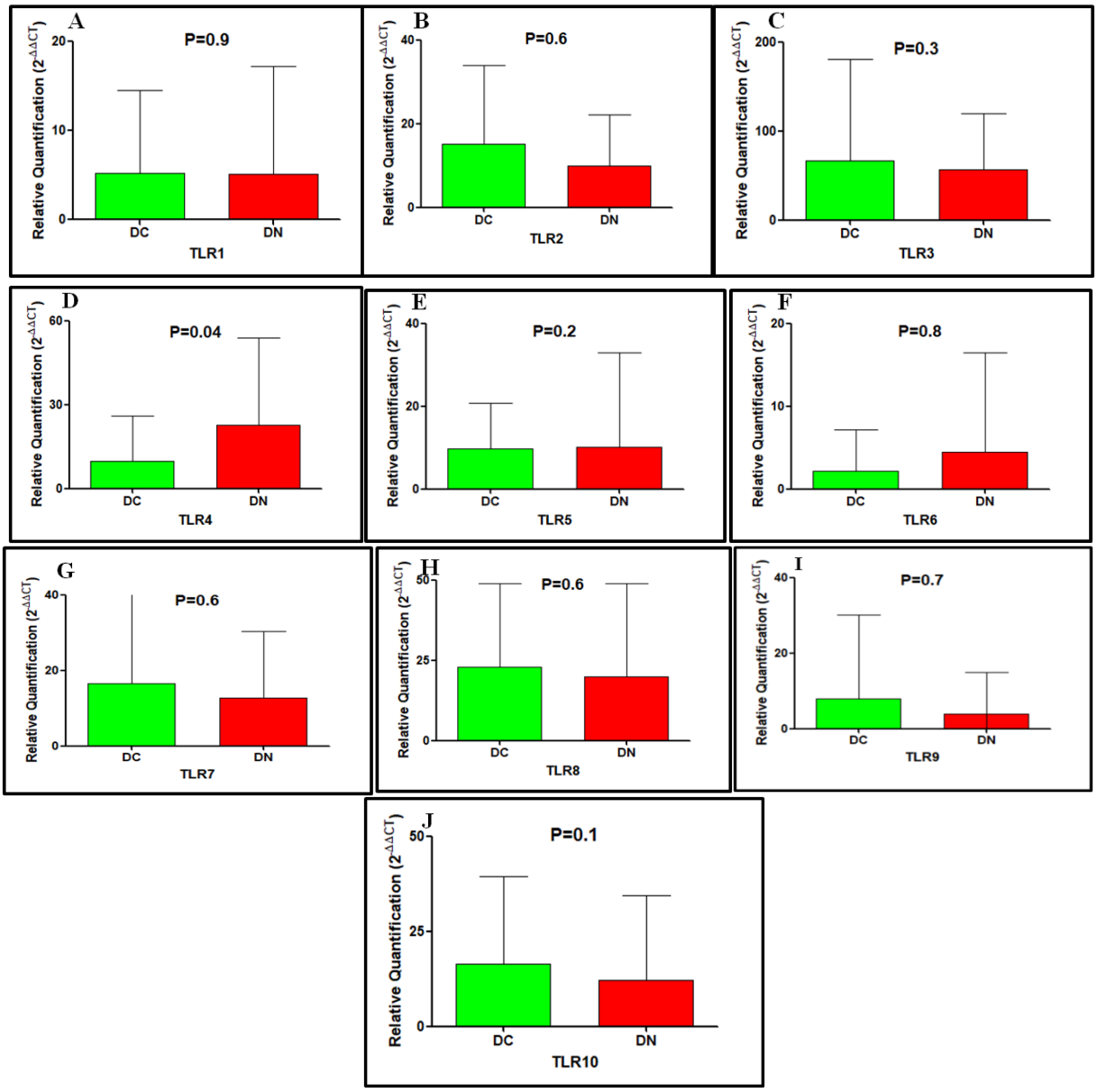

Figure 5.49: Gene expression of TLR1-10 from DC and DN patients after normalizing to HC as reference subjects. Bars represent the mean relative expression of gene ± SD of (A) TLR1 (B) TLR2 (C)TLR3 (D) TLR4 (E)TLR5 (F) TLR6 (G)TLR7 (H)TLR8 (I) TLR9 and (J)TLR10 gene expression in PBMC from DC and DN patients (n = 60 per group). mRNA expression of TLR4 (D) was significantly (p=0.04) elevated among DN subjects than in DC subjects. P value considered significant at P< 0.05, P< 0.01 and P< 0.001 between DC and DN.

5. 5 EXPRESSION OF hsa-miR-561-3p, hsa-miR-4307, hsa-miR-448 AND hsa-miR-4760-3p in PBMC FROM TYPE2 DN PATIENTS, DC AND HC STUDY SUBJECTS.

Here we investigated the relative quantification of microRNA hsa-miR-561-3p, hsa-miR-4307, has-miR-448 and hsa-miR-4760-3p targeting TLR2, TLR3, TLR4 and TLR7 whose mRNA expression was significantly increased among cases (DC and DN) compared to HC. Expression of mi-RNA among DN and DC were assessed individually as described for mRNA expression and fold change was calculated as $2^{-\Delta\Delta ct}$ method. Here we have used Hs-SNORD61-11 as housekeeping miRNA and also considered as reference standard to normalize the target signal. The comparativeCt method (ΔΔCt) was used to quantify miRNA expression, and the relative quantificationwas calculated as $2^{-\Delta\Delta Ct}$ taking HC as reference sample. Amplification specificity was controlled by a melting curveanalysis (Figure 5.50 melting curve).

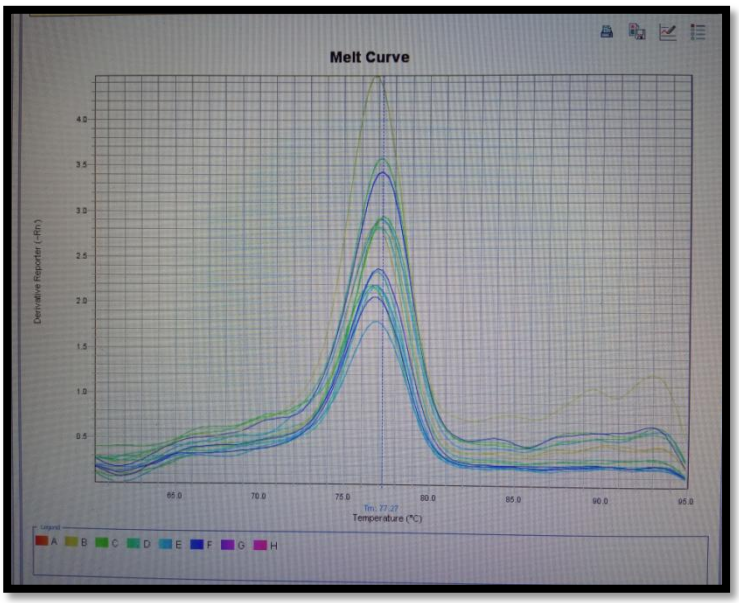

Figure 5.50: Melting curves results of hsa-miR-561-3p, hsa-miR-4307, has-miR-448 and hsa-miR-4760-3p miRNA expression in SYBR green based realtime PCR. A single melting peak corresponds to each amplicon generated using specific set of primers for each miRNA.

The study reveals that the expression (relative fold change) of hsa-miR-448 targeting TLR4 was significantly (p=0.01) high (13.04±16.46) among DN subjects compared to DC

(5.36±7.71folds) subjects (Figure 5.51C and Table 5.25B). The study did not reveal any significant difference for hsa-miR-561-3p which targets TLR2 (DC: 6.99±13.66, DN: 1.54 ± 3.22, P_{mw}=0.3), hsa-miR-4307 that targets TLR3 (DC: 1.48 ± 2.52, DN: 0.12±0.16, P_{mw} =0.09) and hsa-miR-4760-3p that targets TLR7 (DC: 1.21±1.46, DN:1.14±1.65, P_{mw}=0.6) (Figure 5.51A, B and D and Table5.25B).

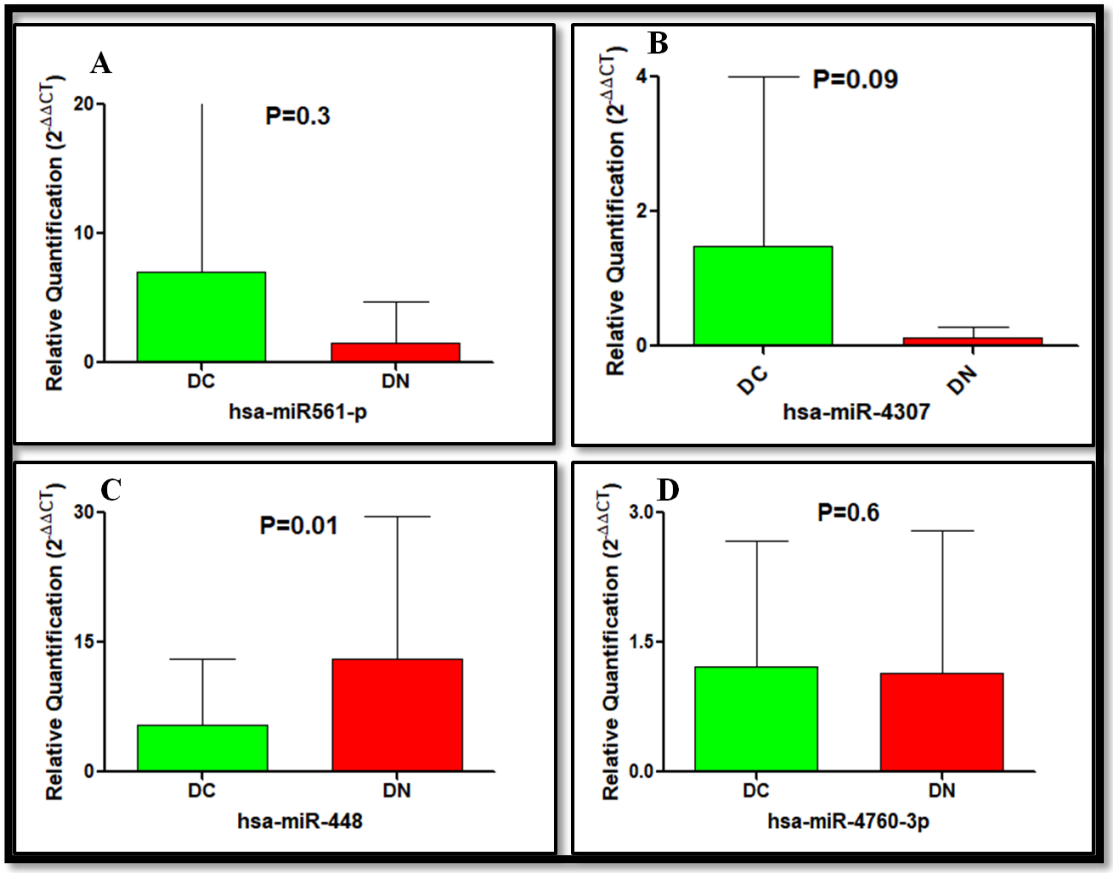

Figure 5.51: miRNA expression of hsa-miR-561-3p, hsa-miR-4307, hsa-miR-448 and hsa-miR-4760-3p from HC, DC and DN patients after normalizing to HC as reference subjects. Bars represents the mean relative expression of miRNA± SD [A] hsa-miR-561-3p [B] hsa-miR-4307 [C] hsa-miR-448 and [D] hsa-miR-4760-3p expression in PBMC from DC and DN patients (n = 60 per group). Expression (fold change) of (C) hsa-miR-448 targeting TLR4 was significantly (P_{mw}=0.01) high among DN subjects compared to among DC. P value considered significant at P< 0.05, P < 0.01 and P< 0.001 between DC and DN of miRNAs.

Table 5.25: Relative quantification of TLRs and their targeting miRNA expression from DC and DN patients taking healthy subjects as reference.

	Diabetic Control	**Diabetic Nephropathy**	**P_{mw}**
[A]Relative expression of TLRs			
TLR1	5.25±9.31	5.14±12.03	0.9
TLR2	15.06±18.77	9.98±12.18	0.6
TLR3	66.68±114.1	57.01±62.87	0.3
TLR4	**9.74±16.27**	**22.66±31.34**	**0.04**
TLR5	9.64±11.02	10.21±22.64	0.2
TLR6	2.17±5.03	4.44±4.44	0.8
TLR7	16.50±25.71	12.65±17.69	0.6
TLR8	55.51±65.01	73.27±103.8	0.8
TLR9	7.86±22.33	4.01±11.00	0.7
TLR10	16.52±22.93	12.09±22.25	0.1
[B] Relative expression of miRNA targets TLRs			
hsa-miR-561-3p	6.99±13.66	1.54±3.22	0.3
hsa-miR-4307	1.48±2.52	0.12±0.16	0.09
hsa-miR-448	**5.36±7.71**	**13.04±16.46**	**0.01**
hsa-miR-4760-3p	1.21±1.46	1.14±1.65	0.6

5.6 EXPRESSION OF m-RNA FOR CYTOKINES IL1β, IL18 AND TNF-α IN PBMC AMONG DIABETICS AND CONTROLS:

We have also evaluated the key cytokines as downstream signaling pathway of Inflammation. We evaluated the relative fold change ($2^{-\Delta\Delta Ct}$) of cytokines IL1β, IL18 and TNF-α using HC as reference sample for DC and DN. Amplification specificity was controlled by a melting curve analysis (Figure 5.52 melting curve).

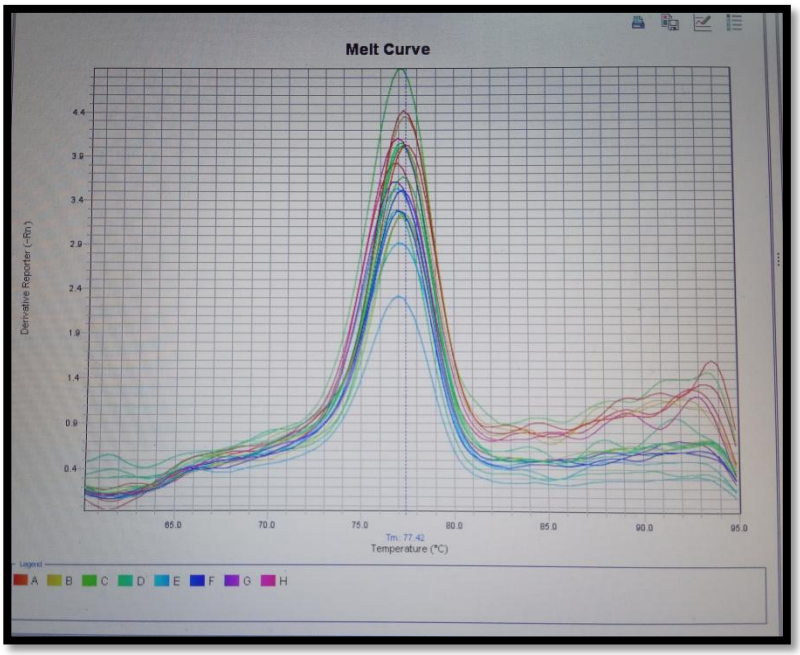

Figure 5.52: Melting curves results of IL1β, IL18 and TNF-α mRNA expression in SYBR green based realtime PCR. A single melting peak corresponds to each amplicon generated using specific set of primers for each mRNA.

The present study observed higher expression of cytokines IL1β, IL18 and TNF-α in PBMC among diabetics compared controls. Statistically significant higher upregulation were observed for IL1β among DC subjects compared to DN and the fold change was 23.14±26.62 and 12.19±19.30, P_{mw}=0.03 (Figure5.53A and Table5.26). IL18 and TNF-α also express in elevated manner among DC subjects than DN subjects but the difference was insignificant. The fold change of IL18 was 16.10±15.57 and 11.78±16.99, P_{mw}=0.3 in DC and DN respectively (Figure5.53B and Table5.26). TNF-α expression was 3.16±6.71 and 2.95±5.48, P_{mw}=0.6 fold in DC and DN subjects respectively (Figure5.53C and Table5.26).

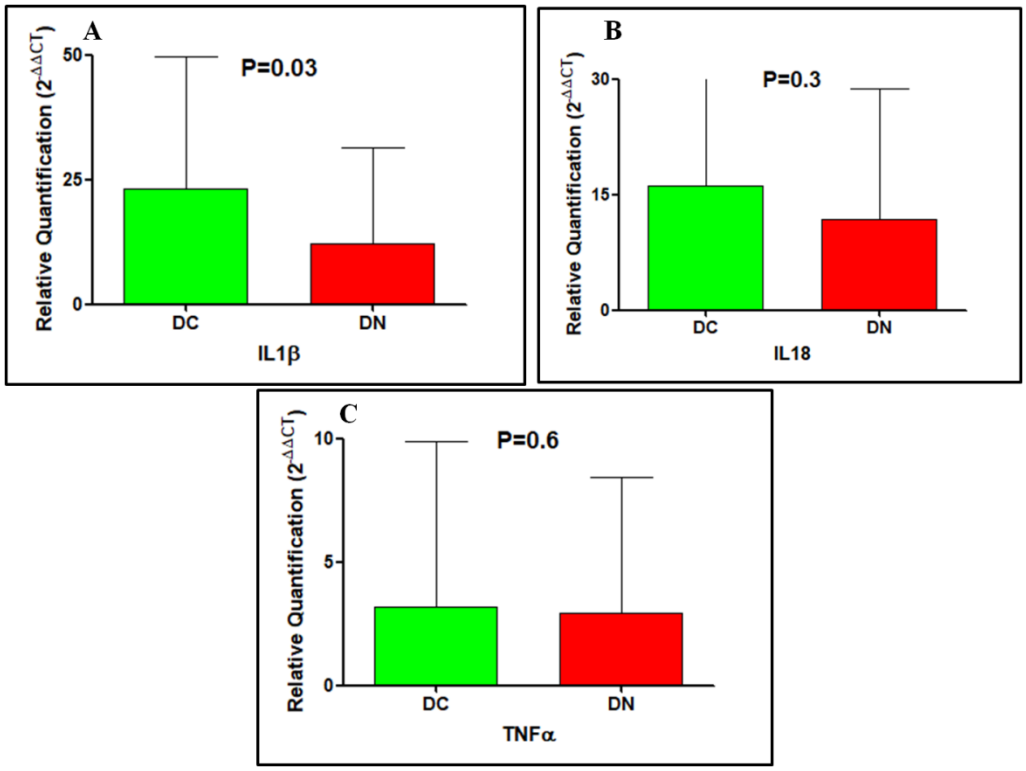

Figure 5.53: mRNA expression of cytokines IL1β, IL18 and TNF-α from DC and DN patients after normalizing to HC as reference subjects. Bars represents the mean relative expression of mRNA± SD [A] IL1β [B] IL18 and [C] TNF-α expression in PBMC from DC and DN patients (n = 60 per group). Expression (fold change) of [A] IL1β was significantly (P_{mw}=0.03) high among DC subjects compared to among DN. P value considered significant at P< 0.05, P< 0.01 and P< 0.001 between DC and DN of mRNAs.

Table 5.26: Relative quantification of cytokines IL1β, IL18 and TNF-α expression from Diabetic Control and Diabetic Nephropathy patients taking healthy subjects as reference subject.

	Diabetic Control	**Diabetic Nephropathy**	**P_{mw}**
IL1β	23.14±26.62	12.19±19.30	**0.03**
IL18	16.10±15.57	11.78±16.99	0.3
TNF-α	3.16±6.71	2.95±5.48	0.3

5.7 LINEAR REGRESSION OF RELATIVE QUANTIFICATION OF INFLAMMASOME COMPLEX mRNA THAT INCLUDES NLRP3, CASP-1 AND PYCARD FOR DN SEVERITY BASED ON ESTIMATED GLOMERULAR FILTRATION RATE (eGFR).

Here reduced eGFR consider as severity of DN. We evaluate the effect of variables like relative quantification of NLRP3, CASP-1 and PYCARD considering (x_i-x_n) on dependent variable eGFR (y). The study revealed that elevation of NLRP3 significantly associated with reduced glomerular filtration rate (P=0.05) (Figure 5.54 and Table 5.27). The estimate of the regression is -0.77 with the standard error (SE):0.39. Residual standard error: 91.92, Multiple R-squared: 0.06, Adjusted R-squared: 0.05, (Figure 5.54 and Table 5.27).The higher expression of CASP-1 among T2DM patients was found to be significantly associated with reduced eGFR (P=0.04). The estimate of the regression is -2.16 with the SE: 1.02. Residual standard error: 87.62, Multiple R-squared: 0.07, Adjusted R-squared: 0.06 (Figure 5.55and Table 5.27). The study did not reveal any significant association of PYCARD gene expression with the severity of DN (P=0.2) (Table 5.27) The estimate of the regression is 0.2 with the SE:0.14. Residual standard error: 89.56, Multiple R-squared: 0.04, Adjusted R-squared: 0.02.

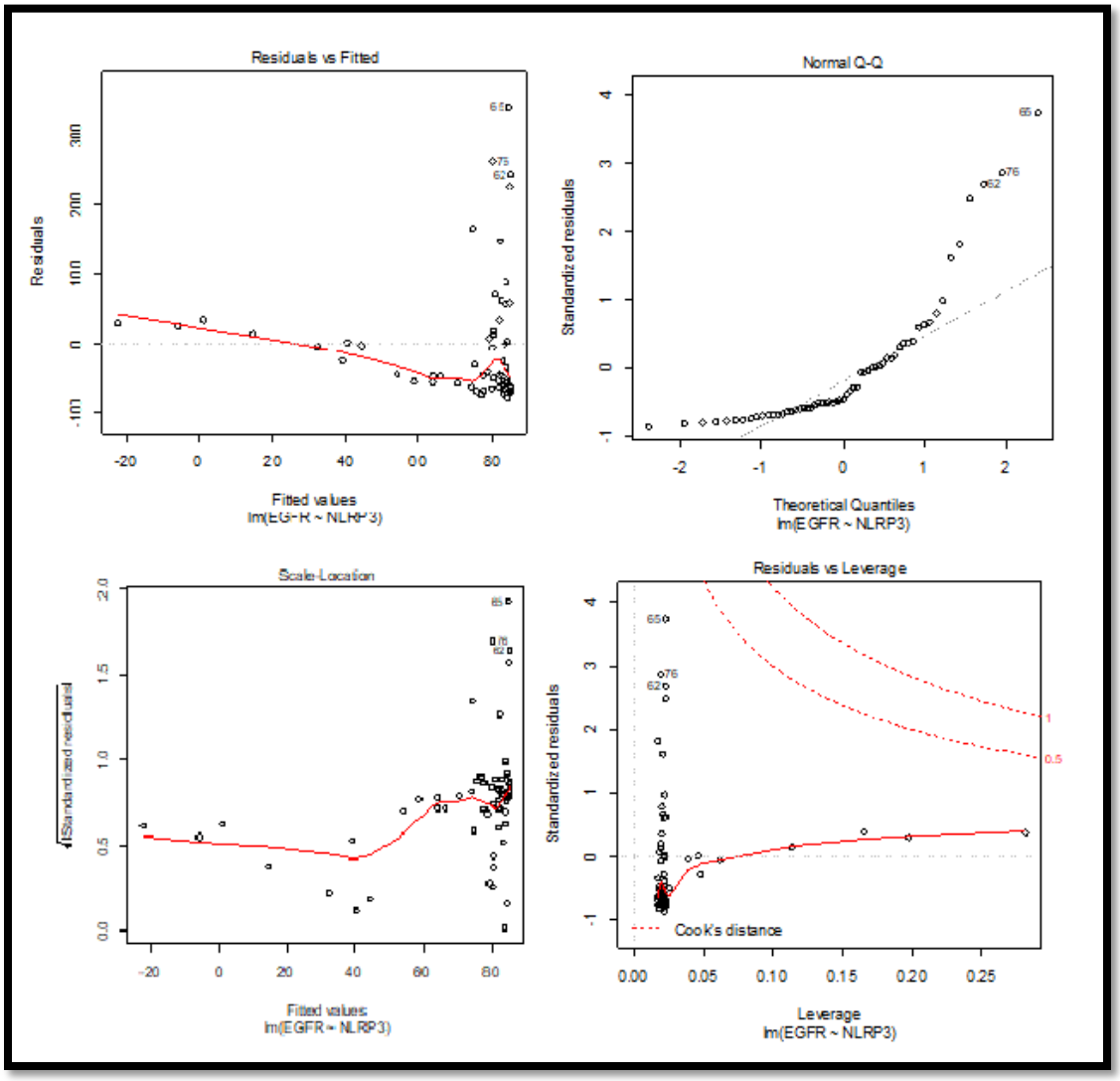

Figure 5.54: Figure represents the linear regression of the interaction of NLRP3 gene expression with eGFR among the Type2 Diabetic subjects. Elevated level of NLRP3 associated with reduced eGFR level (P:0.05). The estimate of the regression is -0.77 with the SE:0.39. Residual standard error: 91.92, Multiple R-squared: 0.06, Adjusted R-squared: 0.048

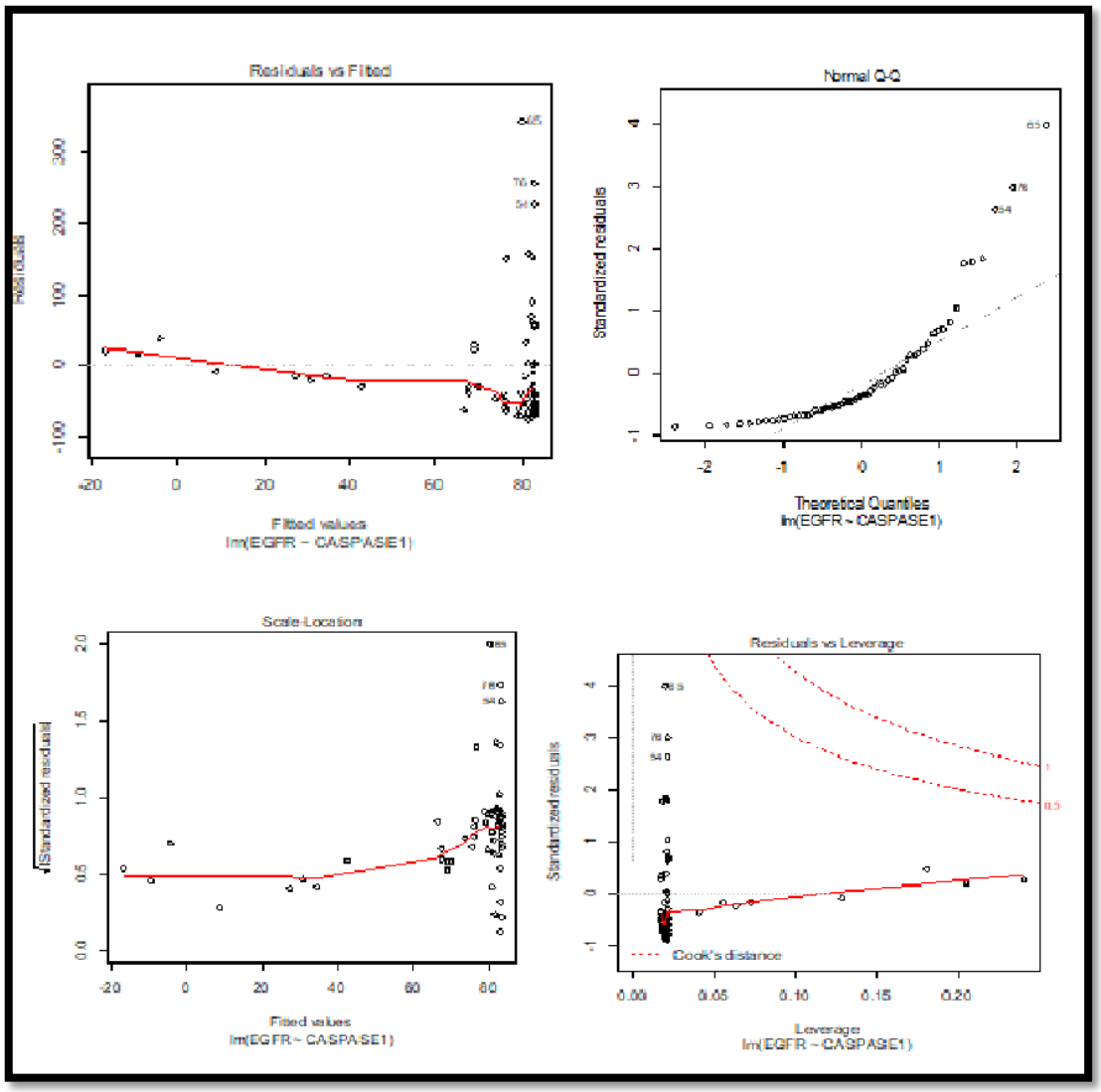

Figure 5.55: Figure represents the linear regression of the interaction of CASP-1 gene expression with eGFR among the Type2 Diabetic subjects. Elevated level of CASP-1 associated with reduced eGFR level (P:0.04). The estimate of the regression is -2.16 with the SE:1.02. Residual standard error: 87.62, Multiple R-squared: 0.07, Adjusted R-squared: 0.06

Table 5.27: Linear regression of relative quantification of Inflammasome complex m-RNA that includes NLRP3, CASP-1 and PYCARD for DN severity based on estimated glomerular filtration rate (eGFR).

	Estimates	**Std. Error**	**T value**	**Pr(> T)**
NLRP3	-0.77	0.39	-1.98	**0.05**
CASP-1	-2.16	1.02	-2.11	**0.04**
PYCARD	0.20	0.14	1.47	0.15

5.8. LINEAR REGRESSION OF RELATIVE QUANTIFICATION OF hsa-miR-223, hsa-miR-22-3p, hsa-miR-4291 AND hsa-miR-185-3p miRNAs TARGETS INFLAMMASOME COMPLEX MRNA WITH DN SEVERITY BASED ON eGFR.

Here reduced eGFR consider as severity of DN. We have evaluate the effect of variables like relative quantification of miRNAs like, hsa-miR-223, hsa-miR-22-3p, hsa- miR-4291 and hsa-miR-185-3p considering (x_i-x_n) on dependent variable eGFR (y). Linear regression analysis revealed the miRNA, hsa-miR-223 and hsa-miR-22-3p targets NLRP3 does not significantly associated with the severity of DN interms of eGFR status though the association is negative (p=0.45 and 0.58) (Table 5.28). The estimate of the regression is -5.24 and -2.05 with the SE:6.86 and 3.73, Residual standard error: 94.87 and 89.09 Multiple R-squared: 0.02 and 0.005, Adjusted R-squared: -0.01 and -0.01 respectively for miRNA, hsa-miR-223 and hsa-miR-22-3p targets NLRP3 (Table 5.28). The expression of miRNA, hsa-miR-4291 targets CASP-1 and hsa-miR-185-3p targets PYCARD was inversely associated with the reduced eGFR status but the association was insignificant (P=0.25 and 0.34 respectively) (Table 5.28). The estimate of the regression is -0.27 and -0.002 with the SE: 0.23 and 0.002, Residual standard error: 89.23 and 90.07 Multiple R-squared: 0.02 and 0.02, Adjusted R-squared:-0.006 and -0.001 respectively for miRNA, hsa-miR-4291 and hsa-miR-185-3p targets CASP-1 and PYCARD respectively (Table 5.28).

Table 5.28: Linear regression of relative quantification of hsa-miR-223, hsa-miR-22-3p, hsa-miR-4291 and hsa-miR-185-3p miRNAs targets Inflammasome complex mRNA with DN severity based on eGFR.

	Estimates	**Std. Error**	**T value**	**Pr(> T)**
hsa-miR-223	-5.24	6.86	-0.76	0.45
hsa-miR-22-3p	-2.05	3.73	-0.55	0.58
hsa-miR-4291	-0.27	0.23	-1.17	0.25
hsa-miR-185-3p	-0.0015	0.0016	-0.96	0.34

5.9 LINEAR REGRESSION OF RELATIVE QUANTIFICATION OF TLR m-RNA THAT INCLUDES TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 AND TLR10 FOR DN SEVERITY BASED ON ESTIMATED GLOMERULAR FILTRATION RATE (eGFR).

Here reduced eGFR consider as severity of DN. We evaluate the effect of variables like relative quantification of TLR m-RNA that includes TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 considering (x_i-x_n) on dependent variable eGFR (y). Linear regression revealed that elevated m-RNA expression of TLR4 was significantly (P=0.04) associated with decreased eGFR status (Figure 5.57 and Table 5.29). The study further reveals that expression of TLR4 is inversely related with reduced eGFR with an estimate of -1.38 with the SE: 0.66. Residual standard error: 90.69, Multiple R-squared: 0.105, Adjusted R-squared: 0.08 (Figure 5.57 and Table 5.29). TLR7 expression was found significantly correlated with the DN severity (P=0.008). TLR7 expression was positively associated with reduced eGFR with an estimate 0.45, SE: 0.15, Residual standard error: 83.19, Multiple R-squared: 0.27, Adjusted R-squared: 0.24 (Figure 5.58 and Table 5.29).

The expression level of TLR1, TLR2, TLR5, TLR6, TLR8, TLR9 and TLR10, inversely related with eGFR status but the association was not significant (Table 5.29). TLR1 upregulation found to be inversely related with the reduced eGFR with an estimate of -0.19, SE: 0.25, Residual standard error: 97.77, Multiple R-squared: 0.01, Adjusted R-squared:-0.009, P:0.45 (Table 5.29). Increased expression of TLR2 was found negatively associated with the reduced eGFR among T2DM patients with an estimate of -0.002, SE: 0.17, Residual standard error: 92.2, Multiple R-squared: 0.00004, Adjusted R-squared:-0.02, P:0.9 (Table5.29). Linear regression revealed the insignificant positive association of TLR3 upregulation with eGFR level among T2DM patients with an estimate of 0.006, SE: 0.01, Residual standard error: 112.3, Multiple R-squared: 0.009, Adjusted R-squared:-0.03, P: 0.6 (Table 5.29). Higher mRNA expression of TLR5 and TLR6 was found negatively associated with reduced eGFR having estimate of -0.42 and -1.20, SE: 0.75 and 1.19. Residual standard error: 99.47 and 92.21, Multiple R-squared: 0.007 and 0.02, Adjusted R-squared: -0.02 and 0.0005, P: 0.6 and 0.3 respectively (Table 5.29). Present study revealed insignificant inverse association of TLR8, TLR9 and TLR10 mRNAs expression with eGFR level among T2DM

patients and the estimates was -0.13, -0.75 and -0.21, SE: 0.18, 1.26 and 0.30. Residual standard error: 116.1, 113.0 and 99.21, Multiple R-squared: 0.02, 0.01 and 0.01, Adjusted R-squared: -0.02,-0.02 and -0.01, P: 0.5, 0.7 and 0.5 respectively (Table 5.29)

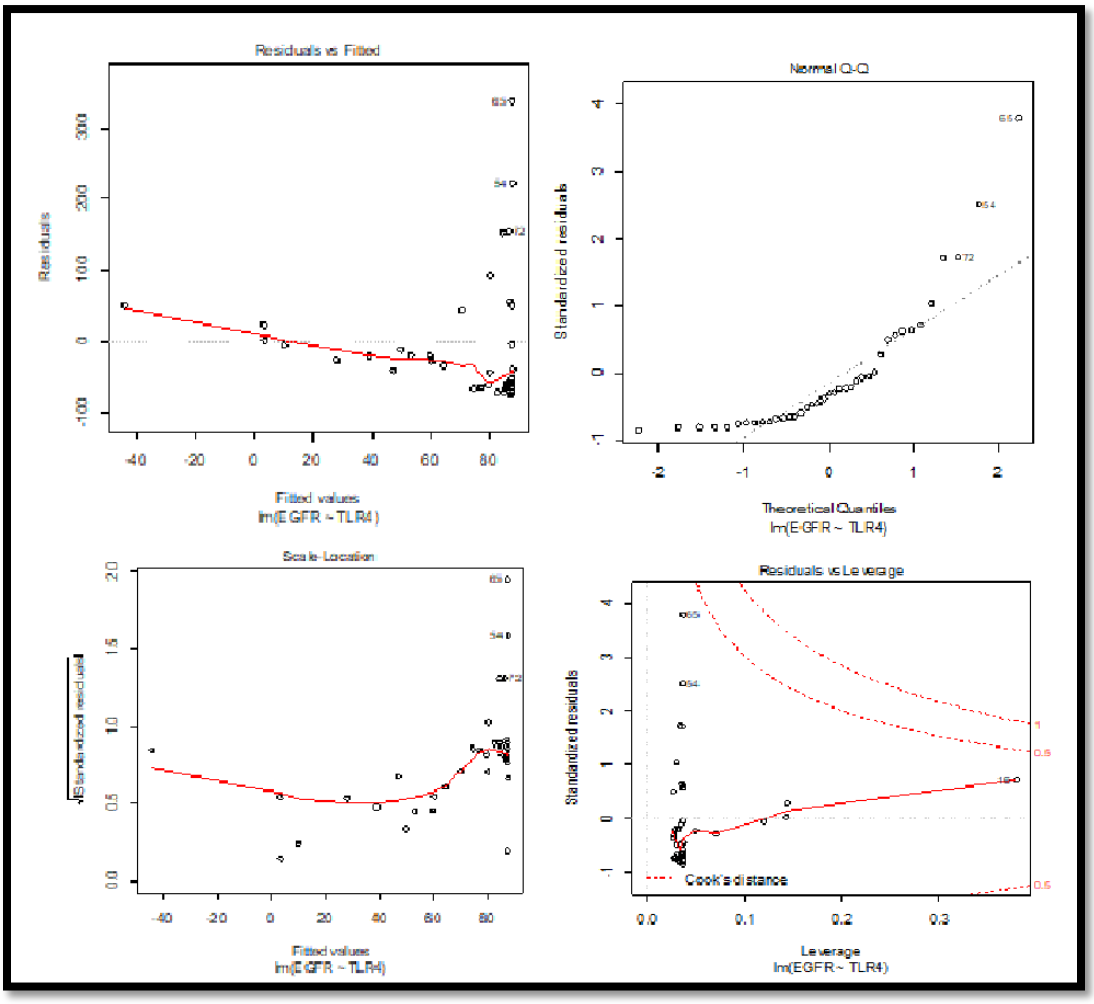

Figure 5.56: Figure represents the linear regression of the interaction of TLR4 gene expression with eGFR among the Type2 Diabetic subjects. Elevated level of TLR4 associated with reduced eGFR level (P:0.04). The estimate of the regression is -1.38 with the SE:0.66. Residual standard error: 90.69, Multiple R-squared: 0.1, Adjusted R-squared: 0.08

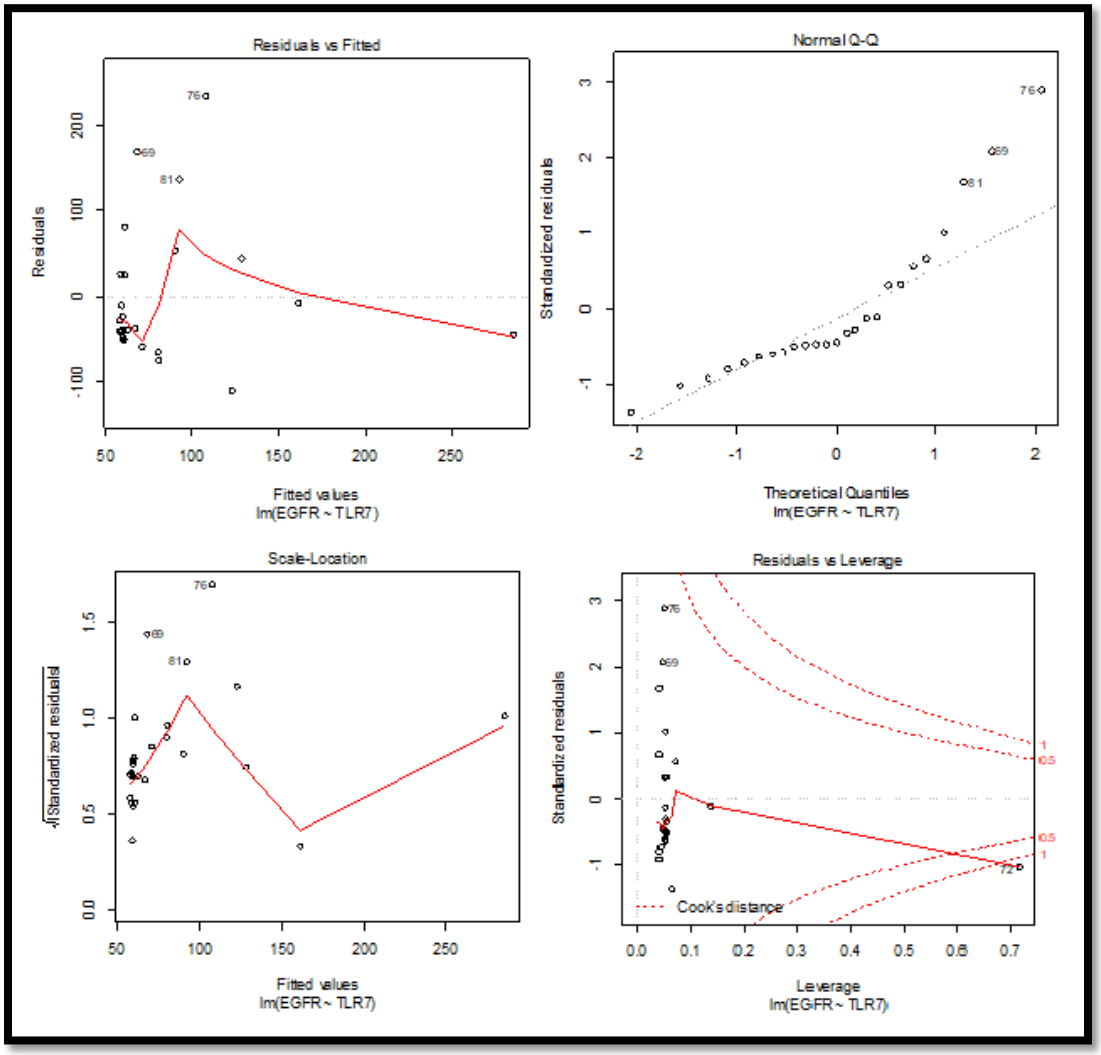

Figure 5.57: Figure represents the linear regression of the interaction of TLR7 gene expression with eGFR among the Type2 Diabetic subjects. Elevated level of TLR7 associated with eGFR level (P:0.008). The estimate of the regression is 0.45 with the SE: 0.15. Residual standard error: 83.19, Multiple R-squared: 0.3, Adjusted R-squared: 0.2

Table 5.29: Linear regression of relative quantification of TLR m-RNA that includes TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 for DN severity based on estimated glomerular filtration rate (eGFR).

	Estimates	**Std. Error**	**T value**	**Pr(> T)**
TLR1	-0.19	0.25	-0.77	0.45
TLR2	-0.002	0.17	-0.01	0.99
TLR3	0.006	0.012	0.48	0.64
TLR4	-1.38	0.66	-2.08	**0.04**
TLR5	-0.42	0.75	-0.55	0.58
TLR6	-1.20	1.19	-1.01	0.32
TLR7	**0.45**	**0.15**	**2.92**	**0.008**
TLR8	-0.13	0.18	-0.71	0.48
TLR9	-0.75	1.26	-0.59	0.56
TLR10	-0.21	0.30	-0.69	0.49

5.10 LINEAR REGRESSION OF RELATIVE QUANTIFICATION OF hsa-miR-561-3p, hsa-miR-4307, hsa-miR-448 AND hsa-miR-4760-3p miRNAs TARGETS TLRS WITH DN SEVERITY BASED ON eGFR.

Here reduced eGFR consider as severity of DN and we have evaluate the effect of variables like relative quantification of miRNAs includinghsa-miR-561-3p, hsa-miR-4307, hsa-miR-448 and hsa-miR-4760-3p considering (x_i-x_n) on dependent variable eGFR (y).We could not find any significant association between relative quantification of miRNAs expression with the severity of DN status interms of eGFR level. Linear regression analysis revealed the miRNA, hsa-miR-561-3p targets TLR2 does not significantly associated with the severity of DN in-terms of eGFR status though the association is negative (P=0.9) (Table 5.30). The estimate of the regression is -0.002 with the SE: 3.89, Residual standard error: 128.6, Multiple R-squared: 0.000003, Adjusted R-squared: -0.07 (Table 5.30). The expression of hsa-miR-4307miRNA, targets TLR3 was found inversely associated with the reduced eGFR status but the association was insignificant (P=0.8) (Table 5.30). The estimate of the

regression is -10.54 with the SE: 45.28, Residual standard error: 147.6, Multiple R-squared: 0.006, Adjusted R-squared:-0.1 for hsa-miR-4307miRNA, targets TLR3 (Table 5.30) Study also found negative association between relative quantification of hsa-miR-448 targets TLR4 but the association was not significant and the estimate of the regression is -1.91 with the SE: 1.21, Residual standard error: 94.61, Multiple R-squared: 0.07, Adjusted R-squared:-0.04 (Table 5.30). Relative quantification of hsa-miR-4760-3p expression which targets TLR7 found positively associated with the eGFR status among T2DM patients and the estimate of the regression is 1.45 with the SE: 12.51, Residual standard error: 105.3, Multiple R-squared: 0.0005, Adjusted R-squared:-0.04 (Table 5.30).

Table 5.30: Linear regression of relative quantification of hsa-miR-561-3p, hsa-miR-4307, hsa-miR-448 and hsa-miR-4760-3p miRNAs targets TLRs with DN severity based on eGFR.

	Estimates	**Std. Error**	**T value**	**Pr(> T)**
hsa_miR561_p	-0.002	3.89	-0.001	0.99
hsa_miR4307	-10.54	45.28	-0.23	0.82
hsa_miR448	-1.91	1.21	-1.58	0.13
hsa_miR4760_3p	1.458	12.51	0.12	0.9

5.11 LINEAR REGRESSION OF RELATIVE QUANTIFICATION OF INFLAMMATORY CYTOKINES IL1β, IL18 AND TNFα WITH DN SEVERITY BASED ON eGFR

Considering reduced eGFR as severity of DN, we evaluate the effect of variables like relative quantification of IL1β, IL18 and TNFα considering (x_i-x_n) on dependent variable eGFR (y). Relative quantification of inflammatory cytokines such as IL1β, IL18 and TNFα expression which is downstream signaling molecules of Inflammasome cascade was not significantly associated with the severity of DN in terms of eGFR status though the association was positive (Table 5.31). Present data revealed that higher upregulation of IL1β mRNA among T2DM patients was positively associated with the reduced eGFR (p=0.1) and estimates of regression was 0.2, with the SE: 0.09, Residual standard error: 92.04, Multiple R-squared: 0.05, Adjusted R-squared: 0.03 (Table 5.31). IL18 and TNFα mRNA expression among T2DM patients was positively associated with the reduced eGFR but the significant level was

not obtained (P=0.5 and 0.3). The estimate of regression was 0.09 and 1.82 with the SE: 0.2 and 1.7, Residual standard error: 97.39 and 81.86, Multiple R-squared: 0.02 and 0.02, Adjusted R-squared: -0.03 and 0.002 (Table 5.31).

Table 5.31: Linear regression of relative quantification of inflammatory cytokines IL1β, IL18 and TNFα with DN severity based on eGFR

	Estimates	**Std. Error**	**T value**	**Pr(> T)**
IL1B	0.16	0.09	1.59	0.12
IL18	0.09	0.15	0.62	0.53
TNF-α	1.82	1.73	1.05	0.29

5.12. CORRELATION OF mRNAs AND ITS TARGETED mi-RNA EXPRESSION AMONG STUDY SUBJECTS.

We have analyzed the correlation of Inflammasome complex (NLRP3, CASP-1, and PYCARD), TLR2, TLR3, TLR4 and TLR7 mRNA expression with the relative quantification of their targeting miRNA like hsa-miR-223, hsa-miR-22-3p, has-miR-4291, hsa-miR-185-3p hsa-miR-561-3p, hsa-miR-4307, hsa-miR-448 and hsa-miR-4760-3p respectively but couldn't found any significant correlation. The study reveal that NLRP3, PYCARD and TLR4 expression inversely related with their respective miRNAs i.e; hsa-miR-223, hsa-miR-185-3p and hsa-miR-448 (P=0.5, 0.7 and 0.8 respectively). The estimate of the correlation for NLRP3 and hsa-miR-223 was -0.21 with the SE: 0.3, Residual standard error: 28.76, Multiple R-squared: 0.01, Adjusted R-squared: -0.01 (Figure 5.59 and Table 5.32). Relative quantification of hsa-miR-22-3p expression was directly correlates with the NLRP3 expression and the estimate of regression was 0.8, SE: 1.3, Residual standard error: 28.64, Multiple R-squared: 0.007, Adjusted R-squared: -0.007 (Figure 5.60 and Table 5.32). Relative expression of CASP-1 and its targeting miRNA, has-miR-4291 showed positive correlation but the significant level was not obtained and the estimate of regression was 0.004, SE: 0.03, Residual standard error: 11.36, Multiple R-squared: 0.00003, Adjusted R-squared: -0.01, P: 0.9 (Figure 5.61 and Table 5.32). No significant correlation was observed between the relative expression

of PYCARD expression and hsa-miR-185-3p expression and the estimate of regression was -0.0005 with the SE: 0.001, Residual standard error: 83.05, Multiple R-squared: 0.002, Adjusted R-squared: -0.01, P: 0.7 (Figure 5.62 and Table 5.32).Positive correlation was observed for the relative quantification of TLR2, TLR3 and TLR7 expression with their respective miRNAs i.e; hsa-miR-561-3p, hsa-miR-4307 and hsa-miR-4760-3p expression but the same was not significant(P=0.12,0.4 and 0.1 respectively) (Figure 5.63, 5.64 and 5.66 and Table 5.32). The estimates of regression were 0.31, 45.72 and 70.06 with the SE: 0.2, 51.54 and 40.70, Residual standard error: 9.09, 169.8 and 121.1, Multiple R-squared: 0.1, 0.07 and 0.2, Adjusted R-squared: 0.09, -0.02 and 0.1 (Figure 5.63, 5.64 and 5.66 and Table 5.32). The inverse correlation of TLR4 expression and its targeted miRNA (hsa-miR-448) expression was estimated by -0.03 and the SE: 0.17, Residual standard error: 23.25, Multiple R-squared: 0.001, Adjusted R-squared: -0.03, P: 0.8 (Figure 5.65 and Table 5.32).

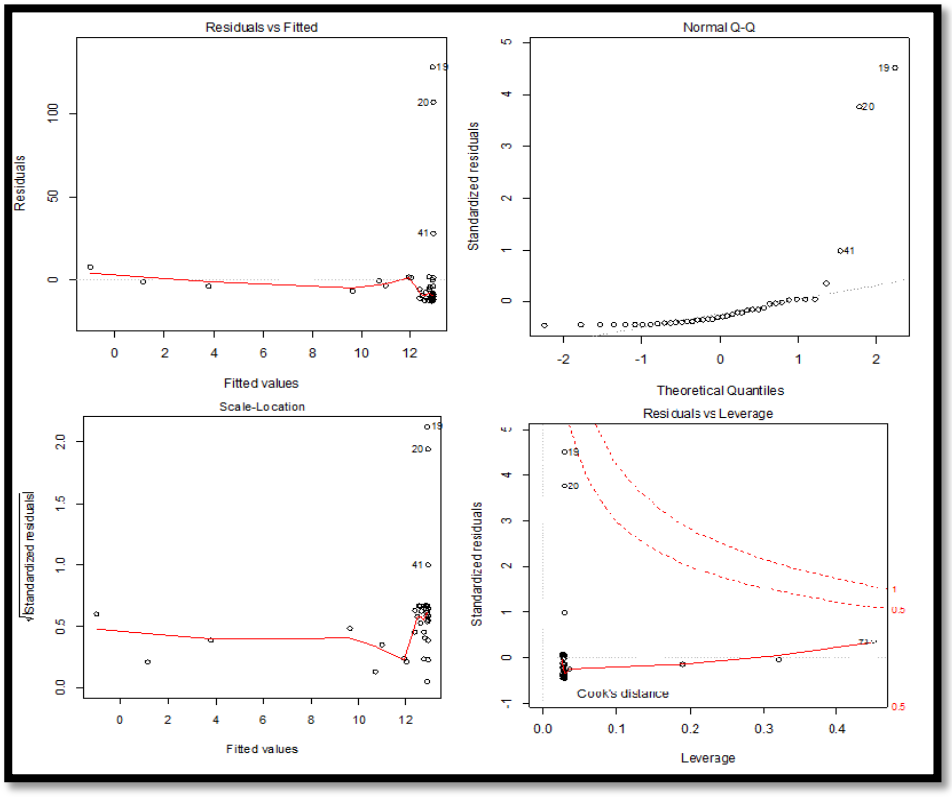

Figure 5.58: Figure represents the correlation of NLRP3 expression with the hsa-miR-223 expression. NLRP3 expression was inversely related with hsa-miR-223 with an estimate -0.21 with the SE: 0.3, Residual standard error: 28.76, Multiple R-squared: 0.01, Adjusted R-squared: -0.01, P: 0.5

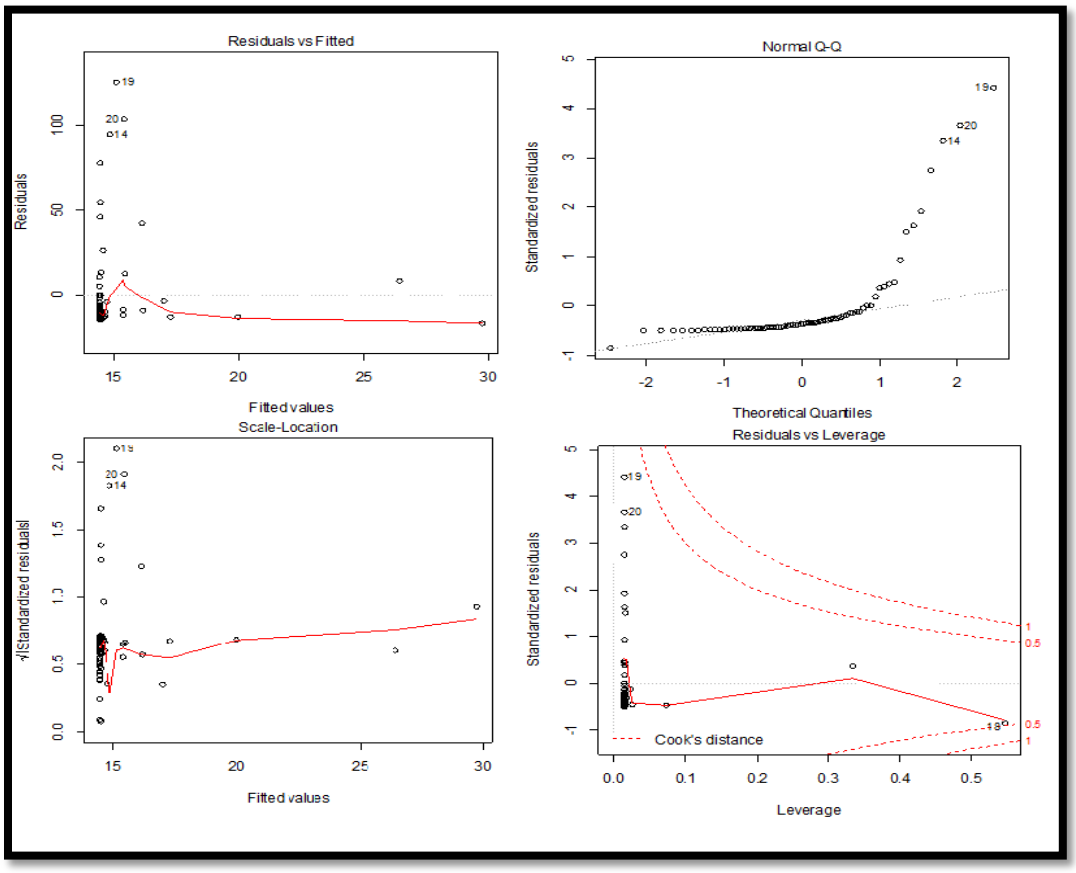

Figure 5.59: Figure represents the correlation of NLRP3 expression with the hsa-miR-22-3p expression. NLRP3 expression was positively related with hsa-miR-223 expression. The estimate of regression was 0.8, SE: 1.3, Residual standard error: 28.64, Multiple R-squared: 0.007, Adjusted R-squared: -0.007, P: 0.5.

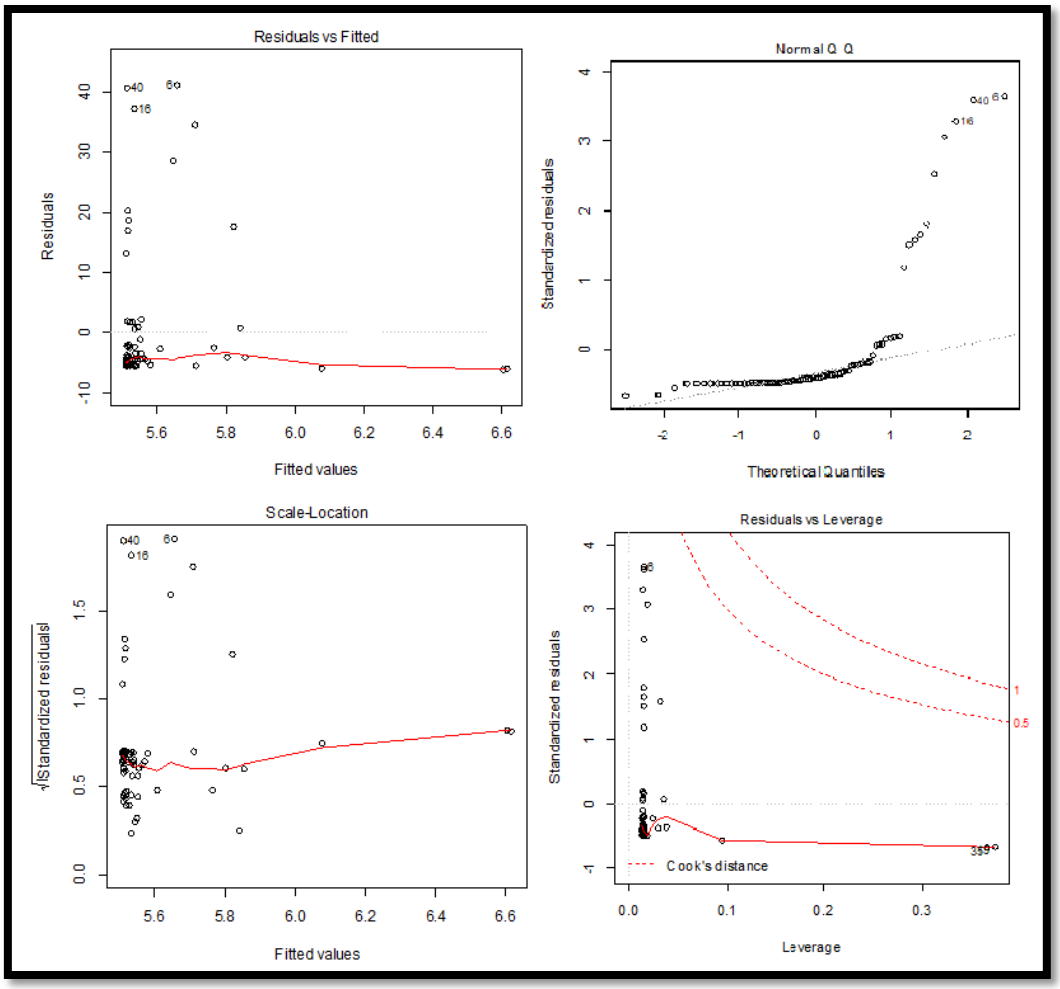

Figure 5.60: Figure represents the correlation of CASP-1 expression with its targeting miRNA, has-miR-4291expression. CASP-1 expression was positively related with has-miR-4291expression. The estimate of regression was 0.004, SE: 0.03, Residual standard error: 11.36, Multiple R-squared: 0.00003, Adjusted R-squared: -0.01, P: 0.9.

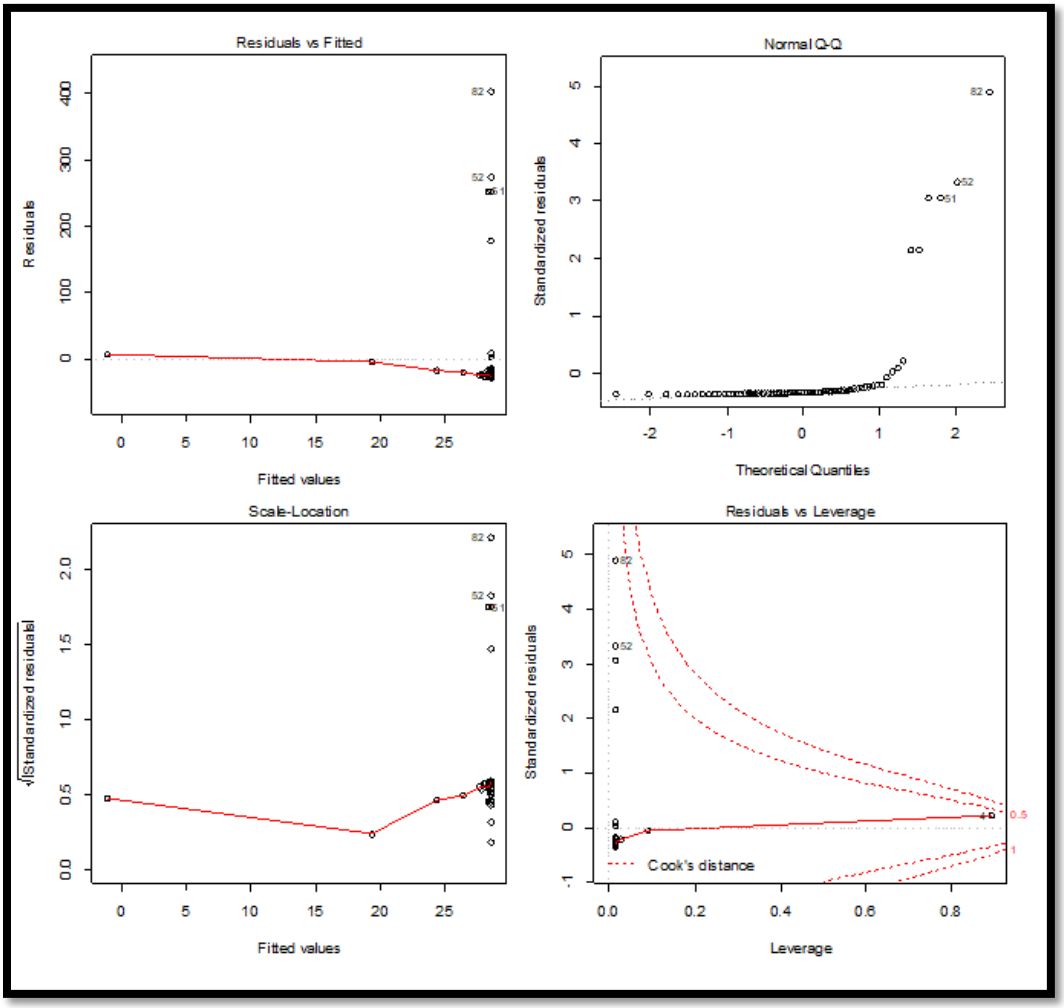

Figure 5.61: Figure represents the correlation of PYCARD expression with its targeting miRNA, has-miR-185-3p expression. PYCARD expression wasinversely related with has-miR-185-3p expression. The estimate of regression was -0.0005 with the SE: 0.001,Residual standard error: 83.05, Multiple R-squared: 0.002, Adjusted R-squared: -0.01, P: 0.7

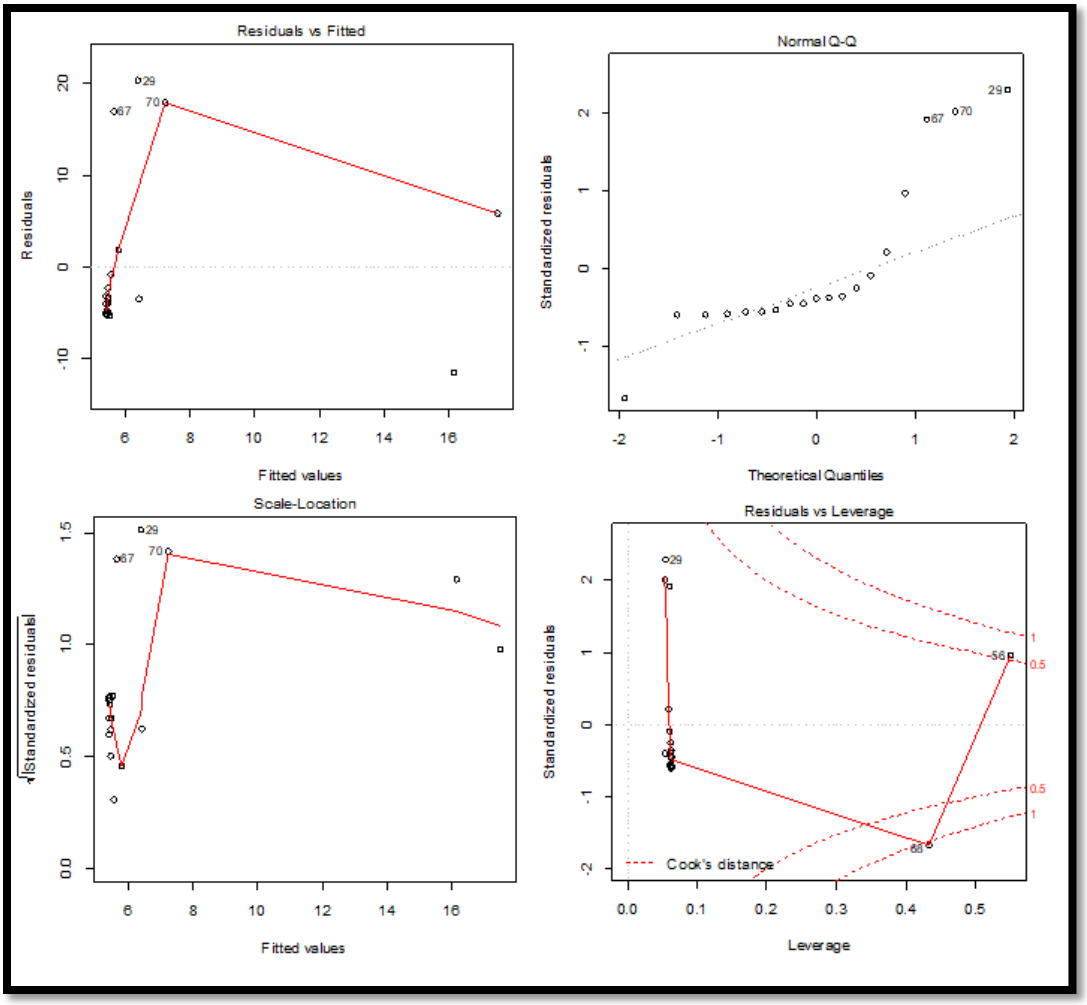

Figure 5.62: Figure represents the correlation of TLR2 expression with its targeting miRNA, hsa-miR-561-3p expression. TLR2 expression was positively related with hsa-miR-561-3p expression. The estimate of regression was0.31 with the SE: 0.2, Residual standard error: 9.09, Multiple R-squared: 0.1, Adjusted R-squared: 0.09, P: 0.1

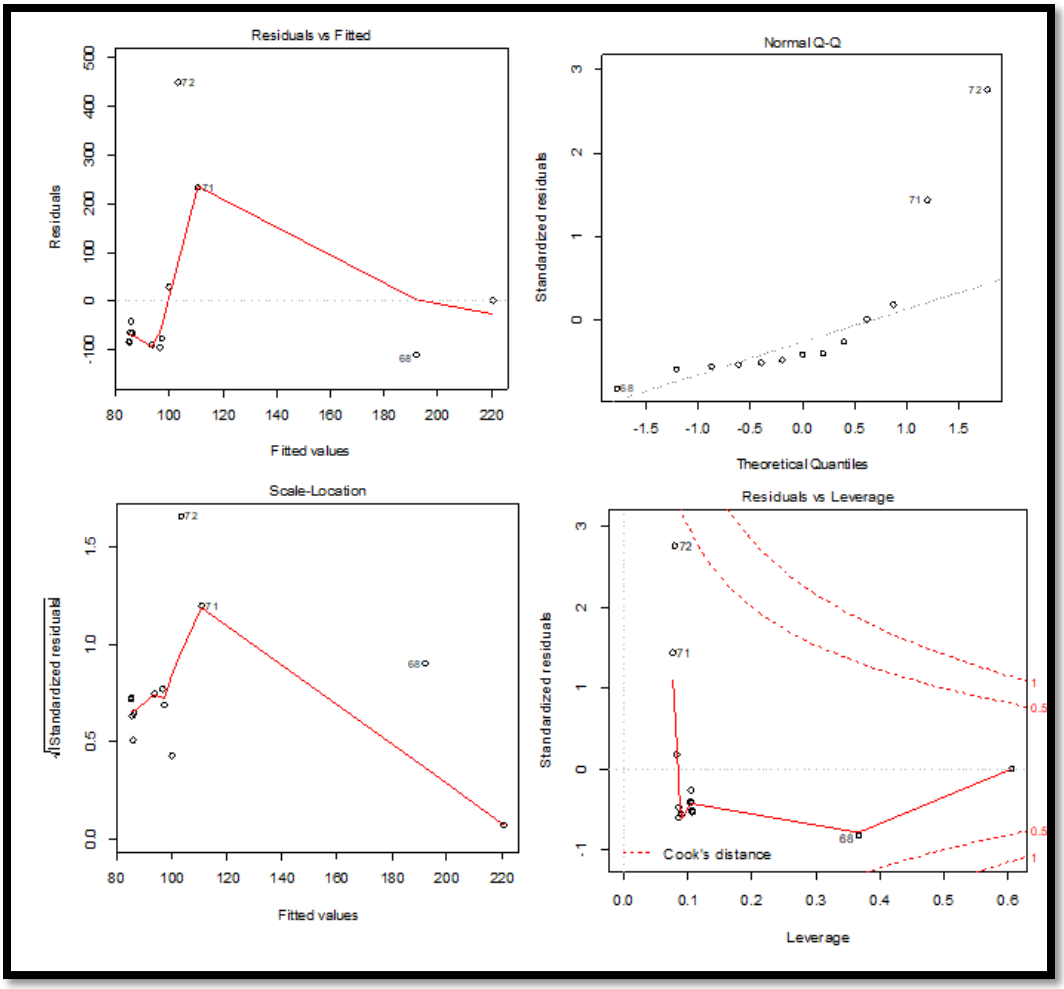

Figure 5.63: Figure represents the correlation of TLR3 expression with its targeting miRNA, hsa-miR-4307 expression. TLR3 expression was positively related with hsa-miR-4307 expression. The estimate of regression was 45.72, with the SE: 51.54, Residual standard error: 169.8, Multiple R-squared: 0.07, Adjusted R-squared:-0.02, P: 0.4.

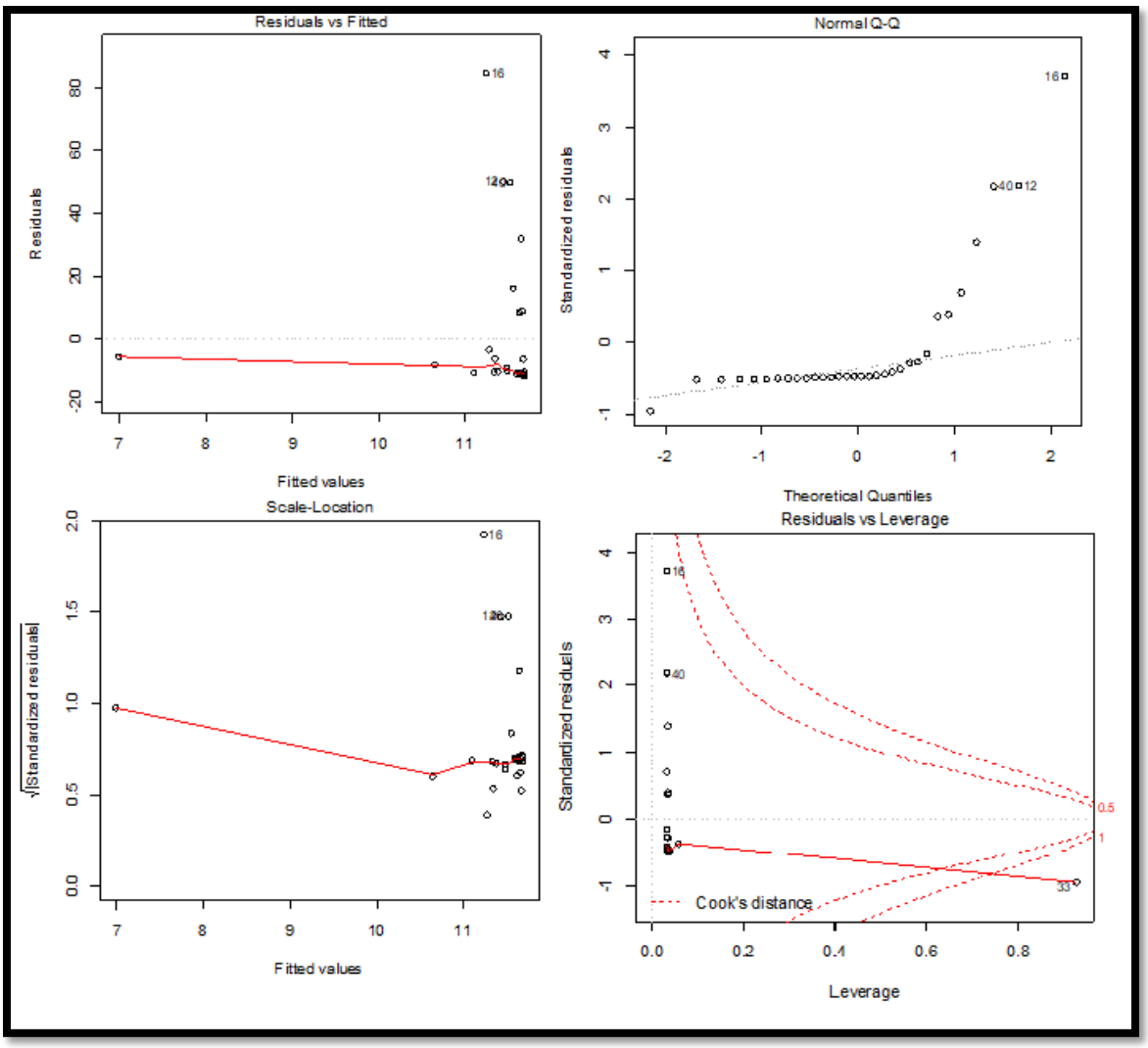

Figure 5.64: Figure represents the correlation of TLR4 expression and the hsa-miR-448 expression. TLR4 expression inversely related with hsa-miR-448 but the association was insignificant (P:0.8). The estimate of regression was -0.03, SE: 0.17, Residual standard error: 23.25, Multiple R-squared: 0.001, Adjusted R-squared: -0.03.

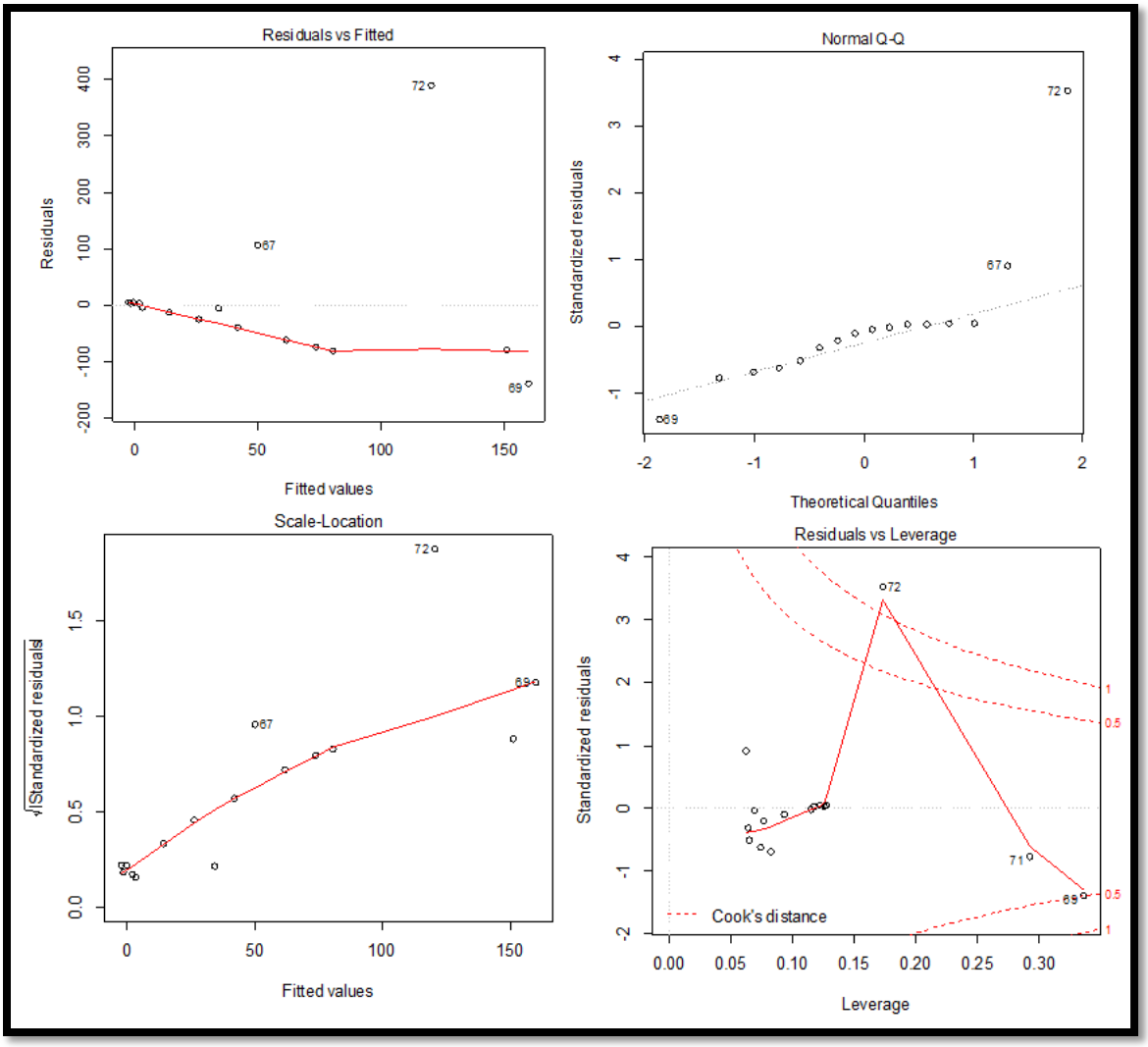

Figure 5.65: Figure represents the correlation of TLR7 expression and the hsa-miR-4760-3p expression. TLR7 expression positively related with hsa-miR-4760-3p but the association was insignificant (P:0.1). The estimate of regression was 70.06, SE: 40.70, Residual standard error: 121.1, Multiple R-squared: 0.2, Adjusted R-squared: 0.1.

Table 5.32: Correlation of mRNAs and its targeted mi-RNA expression among study subjects

	Estimates	**Std. Error**	**T value**	**Pr(> T)**
NLRP3 and hsa-miR-223				
Intercept	12.92	4.89	2.64	0.01
hsa-miR-223	-0.21	0.31	-0.68	0.50
NLRP3 and hsa-miR-22-3P				
Intercept	14.43	3.51	4.11	0.0001
hsa-miR-22-3P	0.87	1.25	0.70	0.48
CASP-1 and hsa-miR-4291				
Intercept	5.51	1.39	3.96	0.0002
hsa-miR-4291	0.004	0.03	0.15	0.88
PYCARD and hsa-miR-185-3p				
Intercept	28.52	10.107	2.82	0.006 **
hsa-miR-185-3p	-0.0005	0.001	-0.37	0.71
TLR2 and has-miR-561-3p				
Intercept	5.40	2.27	2.38	0.03 *
hsa_miR561_p	0.31	0.19	1.65	0.12
TLR3 and has-miR-4307				
Intercept	85.06	55.36	1.54	0.15
hsa_miR4307	45.72	51.41	0.89	0.39
TLR4 and has-miR-448				
Intercept	11.68	4.38	2.67	0.01 *
hsa_miR448	-0.03	0.17	-0.19	0.84
TLR7 and has-miR-4760-3p				
Intercept	-7.23	45.32	-0.16	0.88
hsa_miR4760_3p	70.06	40.70	1.72	0.11

5.13 DISTRIBUTION OF IL1β, IL18 AND TNF-α FROM PLASMA OF HC, DC AND DN PATIENTS

Estimation of inflammatory cytokines such as IL1β, IL18 and TNF-α from plasma of HC, DC and DN patients were done by ELISA and found elevated among T2DM patients compared to HC (Figure 5.66 A, B and C). Plasma level of IL18 was significantly higher in DN (1957±771.0 pg/ml) and DC (1247±624.5 pg/ml) compared to HC (930.6±480.9 pg/ml), (P_{ANOVA}< 0.0001) (Figure 5.66A).

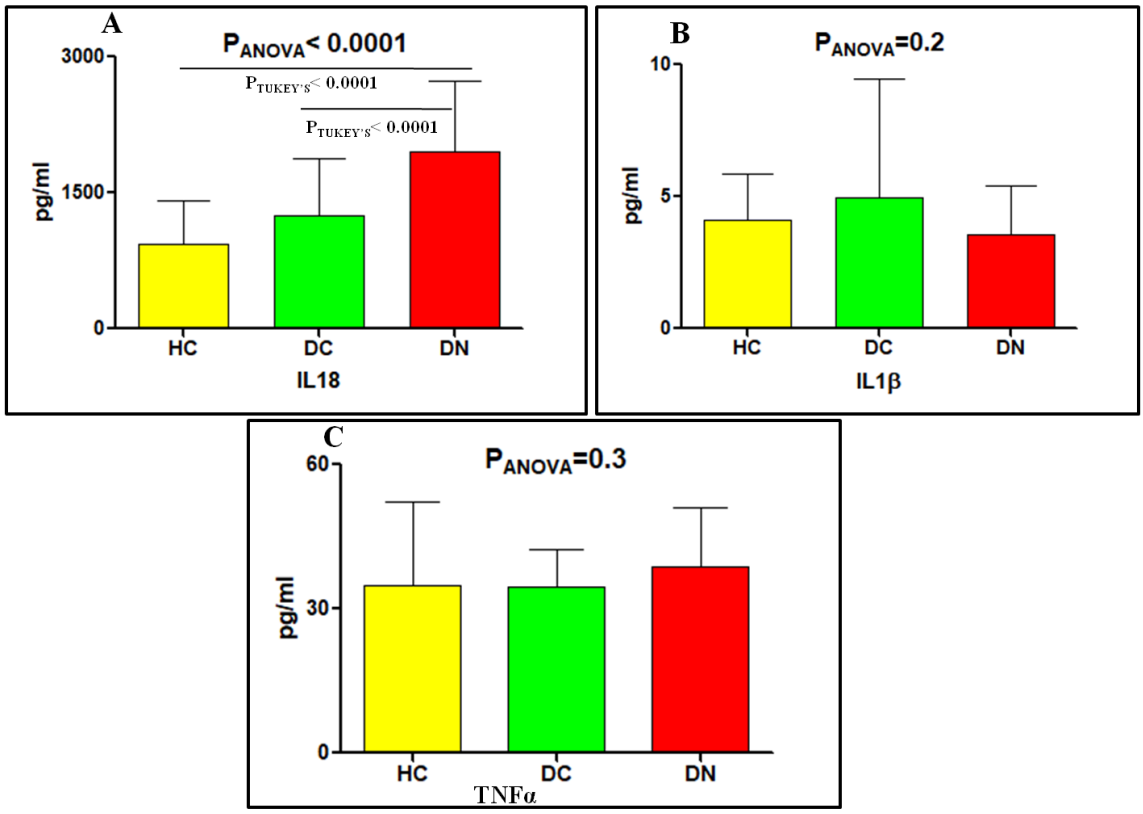

Figure 5.66: Figure represents the distribution of plasma level of cytokines IL1β, IL18 and TNF-α from HC, DC and DN patients. Bars represents the plasma level of [A] IL18 [B] IL1β and [C] TNF-α as mean ± SD (n = 60 per group). Statistically significant difference observed for IL18 level which was higher in DN (1957±771.0 pg/ml) and DC (1247±624.5 pg/ml) compared to HC (930.6±480.9 pg/ml), (P_{ANOVA}< 0.0001). Significantly higher plasma level of IL18 was observed among DN patients than DC ($P_{TUKEY'S}$< 0.0001). P value considered significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Significantly higher plasma level of IL18 was observed among DN patients than DC ($P_{TUKEY'S}$< 0.0001) (Figure 5.66A). Results revealed that, plasma level of IL1β also didn't differ significantly among the study group HC (4.10±1.73pg/ml), DC (4.97±4.49 pg/ml) and DN (3.54±1.89, P=0.2) (Figure 5.66B).TNF-α was found higher among DN (38.74±12.21pg/ml) cases than DC (34.66±7.81 pg/ml) and HC (34.74±17.55 pg/ml) but the level was not significantly differ (P=0.3) (Figure 5.66C).

5.14 LINEAR REGRESSION OF PLASMA IL1β, IL18 AND TNFα CYTOKINE WITH DN SEVERITY BASED ON ESTIMATED GLOMERULAR FILTRATION RATE (eGFR).

Here reduced eGFR consider as severity of DN. We evaluate the effect of variables like plasma level of IL1β, IL18 and TNFα cytokine considering (x_i-x_n) on dependent variable eGFR (y).Study revealed the serum level of IL1β was significantly associated with the eGFR status (p<0.0001). The estimate of this regression was 10.42, SE: 1.84 and the Residual standard error: 44.65, Multiple R-squared: 0.41, Adjusted R-squared: 0.39 (Table 5.33). No significant association were observed from linear regression of plasma TNFα and IL18 with the reduced eGFR status (P=0.6 and 0.5 respectively) (Table 5.33). The estimates of the regression were -0.47 and -0.009, SE: 1.03 and 0.01, Residual standard error: 79.53 and 79.73, Multiple R-squared: 0.009 and 0.005, Adjusted R-squared: -0.01 and -0.02 (Table 5.33).

Table 5.33: Linear regression of plasma TNFα, IL1β and IL18 cytokine with DN severity based on estimated glomerular filtration rate (eGFR).

	Estimates	**Std. Error**	**T value**	**Pr(> T)**
TNFα	-0.47	1.03	-0.46	0.65
IL1β	**10.102**	**1.84**	**5.49**	**1.91e-06*** **
IL18	-0.009	0.014	-0.66	0.51

5.15: VALIDATION OF miRNA-mRNA INTERACTION IN INT407 CELL LINES

Expressions of Inflammasome complex mRNAs (NLRP3, CASP-1 and PYCARD), TLRs (TLR1-TLR10), inflammatory cytokines (IL1β, IL18 and TNFα) and miRNAs targeting Inflammasome and TLRs were quantified in terms of relative quantification through qRT-PCR using SYBR Green chemistry (details are given in methodology section) in INT407 cells. INT407 cells were induced with different concentration (125 mg/dl, 250mg/dl and 500mg/dl) of glucose to maintain hyperglycemic condition along with a controlled experiment i.e cells without glucose treatment (UT). The untreated cells were reffered as reference sample. The GAPDH was used as housekeeping geneand also considered as reference standard to normalize the target signal. The comparativeCt method (ΔΔCt) was used to quantify gene expression, and the relative quantificationwas calculated as $2^{-\Delta\Delta Ct}$ taking untreatted as reference sample.

5.15.1 Expression of Inflammasome Complex Gene Includes NLRP3, CASP-1 and PYCARD in INT407 Cells upon Induced with Different Concentration of Glucose.

Relative quantification of Inflammasome complex mRNAs (NLRP3, CASP-1 and PYCARD) were upregulated in all the cells treated with different concentration of glucose compared to untreated cells. NLRP3 was upregulated by 1.38±0.28, 1.21±0.08 and 1.45±0.19 fold in cells induced with 125mg/dl, 250mg/dl and 500mg/dl glucose respectively (Figure.5.67A). Relative expression of CASP-1 mRNA was found highest in cells treated with 500mg/dl glucose (2.67±0.81) compared 125mg/dl (2.43±0.81) and 250 mg/dl (2.43±1.10) (Figure 5.67B). PYCARD gene was also found upregulated upon treated with glucose compared to untreated cells and the upregulations was 3.12±0.78, 3.09±1.15 and 2.79±0.07 fold in 125mg/dl, 250mg/dl and 500mg/dl cells respectively (Figure 5.67 C).

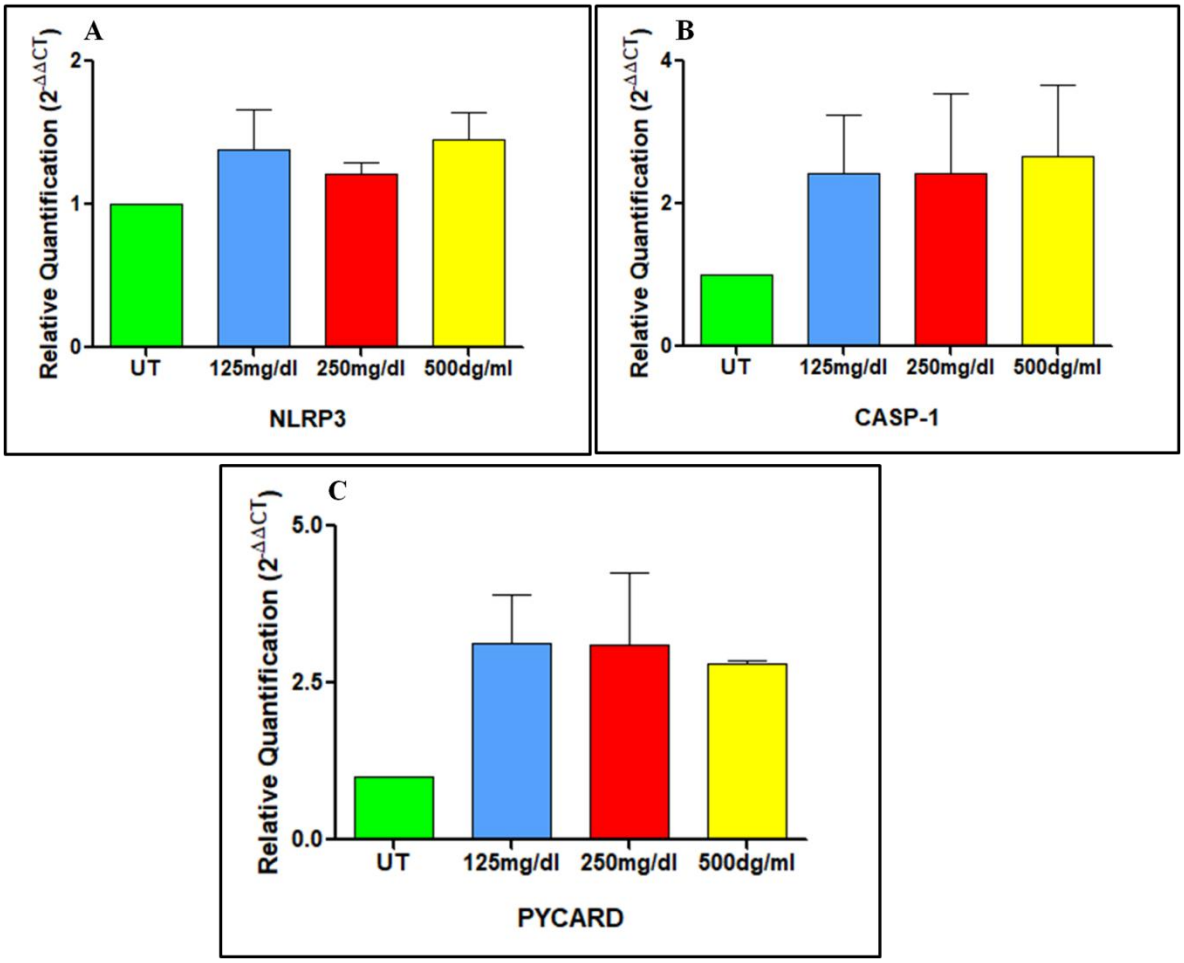

Figure 5.67: Figure represents the relative quantification of Inflammasome complex gene expression in INT407 cell upon glucose induction. [A] NLRP3, [B] CASP-1 and [C] PYCARD gene was upregulated in INT407 cells induced with different concentration of glucose compared to untreated cells.

5.15.2 Expression of miRNAs Includes hsa-miR-223, hsa-miR-22-3p, hsa- miR-4291 and hsa-miR-185-3p Targets NLRP3, CASP-1 and PYCARD Genes in INT407 Cells upon Induced with Different Concentration of Glucose.

Relative quantification of miRNAs targetes Inflammasome complex mRNAs (NLRP3, CASP-1 and PYCARD) in cells treated with different concentration of glucose was found upregulated or downregulated compared to untreated cells. miRNA, hsa-miR-223 targets

NLRP3 gene was found concomitant increase with the increase of glucose concentration (Figure 5.68A) compared to untreated cells. The relative quantification of hsa-miR-223 expression was 1.29±0.48, 1.87±0.78 and 3.39±1.43 fold respectively in cells treated with 125mg/dl, 250mg/dl and 500mg/dl glucose (Figure 5.68A). hsa-miR-22-3p targets NLRP3, hsa- miR-4291 targets CASP-1 and hsa-miR-185-3p targetsPYCARD expression was found downregulated in INT407 cells upon induced with different concentration of glucose compared to untreated cells (Figure5.68 B, 5.68C and 5.68D).

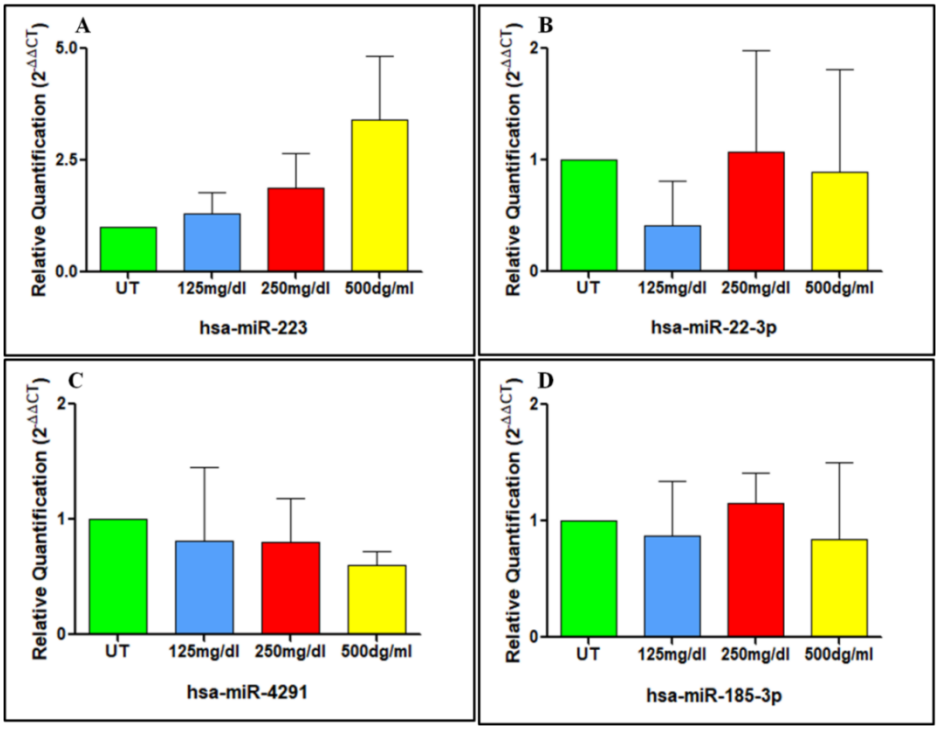

Figure 5.68: Figure represents the relative quantification of miRNAs targets Inflammasome complex gene expression in INT407 cell upon glucose induction. [A] hsa-miR-223 was upregulated gradually with higher glucose concentration. [B] hsa-miR-22-3p, [C] hsa- miR-4291 and [D]hsa-miR-185-3p miRNA was downregulated in INT407 cells induced with different concentration of glucose compared to UT.

5.15.3 Expression of m-RNA for TLRs (TLR1-10) in INT407 Cells upon Induced with Different Concentration of Glucose.

Keeping GAPDH as housekeeping gene and UT cells as reference sample, we calculated $2^{-\Delta\Delta Ct}$ for TLRs that includes TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10 in INT407 cells treated with different concentration of glucose. Relative quantification of TLR gene expression was not in a linear mode with glucose concentration. TLR1 was found upregulation in cells treated with 250mg/dl (1.84±0.27) and 500mg/dl (1.69±0.76) glucose and downregulated in cells treated with 125mg/dl (0.85±0.27) glucose compared to untreated cells (Figure 5.69A). TLR2 expression was found highest in cells treated with 125mg/dl glucose and the fold change was 11.29±4.62 (Figure 5.69B). Relative expression of TLR3, TLR5 and TLR9 were found unregulated in treated cells compared to untreated cells (Figure 5.69 C, 5.69E and 5.69H). TLR4 expression was increased exponentially with the increase of glucose concentration (Figure 5.69D). The fold change was 1.63±1.06, 2.13±0.59 and 2.83±0.82 in cells treated with 125mg/dl, 250mg/dl and 500mg/dl respectively (Figure 5.69D). TLR6, TLR7 and TLR10 had diverse expression in INT407 cells induced with glucose (Figure 5.69F, 5.69G and 5.69I). We couldnot assessed TLR8 expression in INT407 cells.

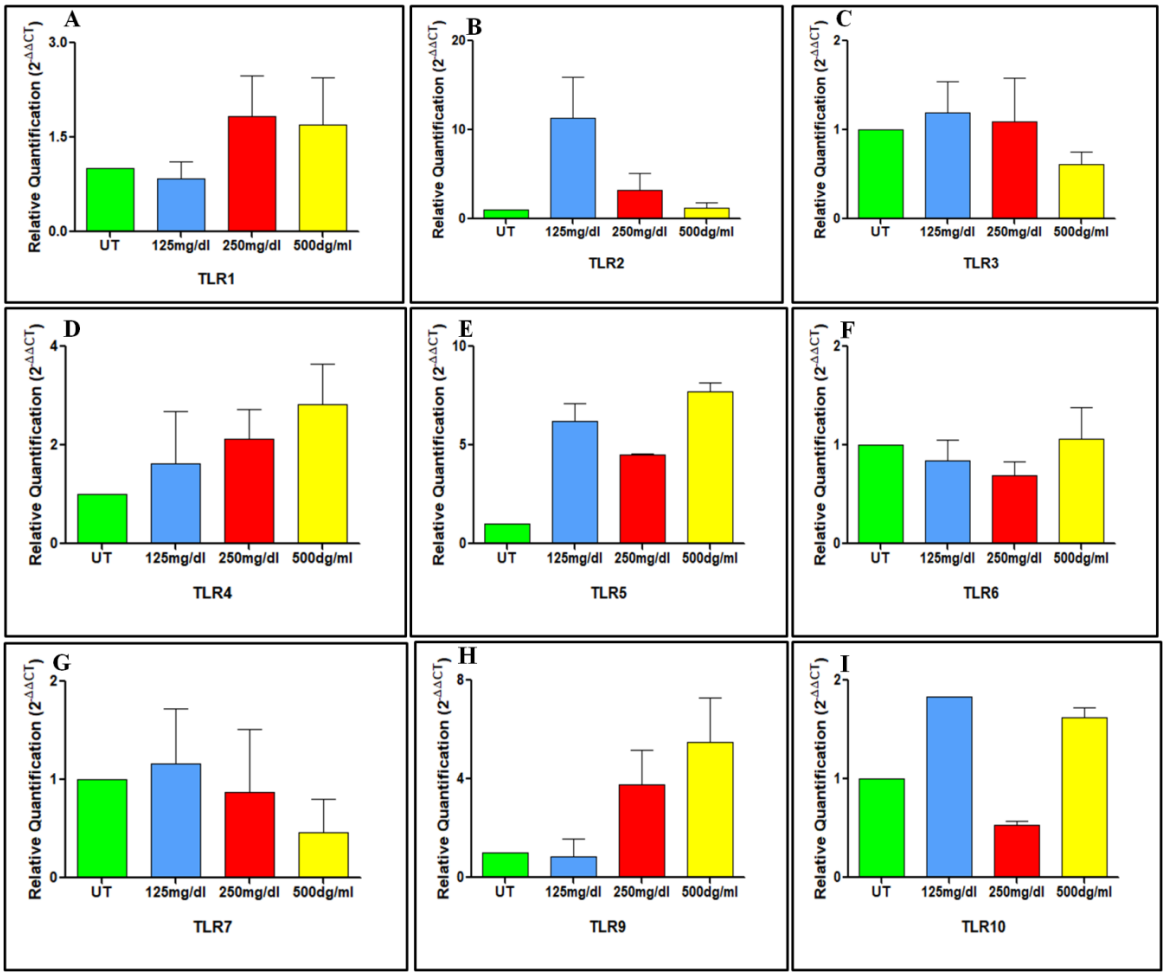

Figure 5.69: Figure represents the relative quantification of TLRs gene expression in INT407 cell upon glucose induction.

5.15.4 Expression of miRNAs Includes hsa-miR-561-3p, hsa-miR-4307, hsa-miR-448 and hsa-miR-4760-3p Targets TLRs Genes in INT407 Cells upon Induced with Different Concentration of Glucose.

Relative quantification of miRNAs targets TLRs in cells treated with different concentration of glucose were found upregulated or downregulated compared to untreated cells. miRNA,hsa-miR-561-3p targets TLR2 gene was found upregulated in cells induced with different glucose concentration (Figure 5.70A) compared to untreated cells. The relative quantification of hsa-miR-561-3p expression was 1.17±0.16, 0.94±0.53 and 1.35±0.47 fold respectively in cells treated with 125mg/dl, 250mg/dl and 500mg/dl glucose (Figure 5.70A). hsa-miR-4307 (1.98±0.97) targets TLR3 and hsa-miR-448 (2.570.79±) targets TLR4 was

found upregulated in INT407 cells induced with 125mg/dl glucose (Figure 5.70B and 5.70C). Expression of hsa-miR-448 was found downregulated in other glucose induced cells (Figure 5.70C). hsa-miR-4760-3p targets TLR7 also dounregulated with the increase of glucose concentration in INT407 cells compared to UT (Figure5.70D).

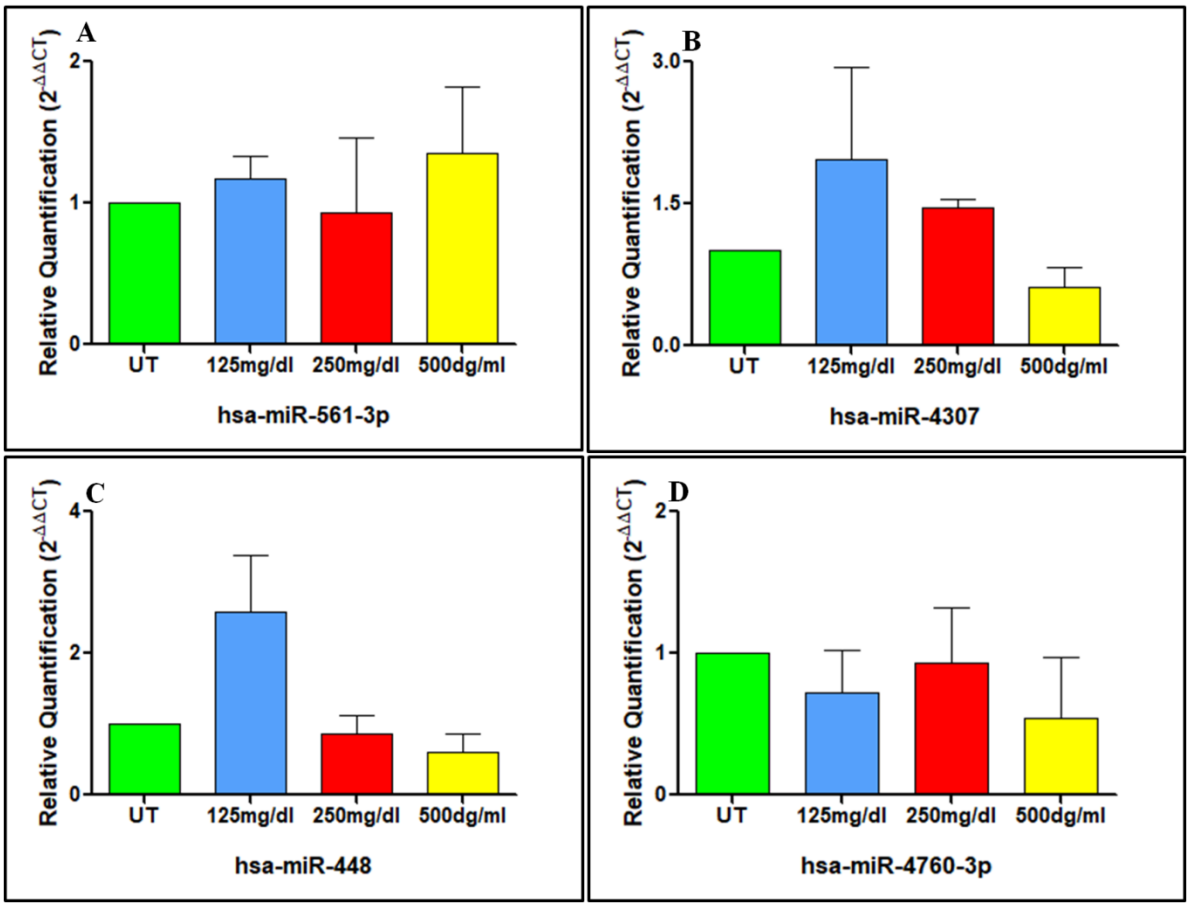

Figure 5.70: Figure represents the relative quantificantion of miRNAs targets TLRs in INT407 cell upon glucose induction. [A] hsa-miR-561-3pwas upregulated with higher glucose concentration. [B] hsa-miR-4307 and [C] hsa-miR-448 was upregulated in cells treated with 125mg/dl glucose. [D] hsa-miR-4760-3p miRNA was downregulated in INT407 cells induced with different concentration of glucose compared to UT.

5.15.5 Expression of IL1β, IL18 and TNFα Genes in INT407 Cells upon Induced with Different Concentration of Glucose.

IL1β, IL18 and TNFα genes expression in INT407 cells upon induced with different concentration of glucose was found upregulated.Relative quantification of IL1β expression was found highest in cells treated 500mg/dl glucose and the fold change was 3.31±0.71 (Figure 5.71A). IL18 (1.74±0.87) and TNFα (2.81±0.78) genes expressionwere higher in cells treated with 125mg/dl and 250 mg/dl concentration of glucose compared to UT and othe glucose concentrations (Figure 5.71B and 5.71C). After reaching the saturation point of higher expression in cells induced with 250mg/dl, TNFα expression shows dounregulation in cells cultured with 500mg/dl glucose (Figure5.71C).

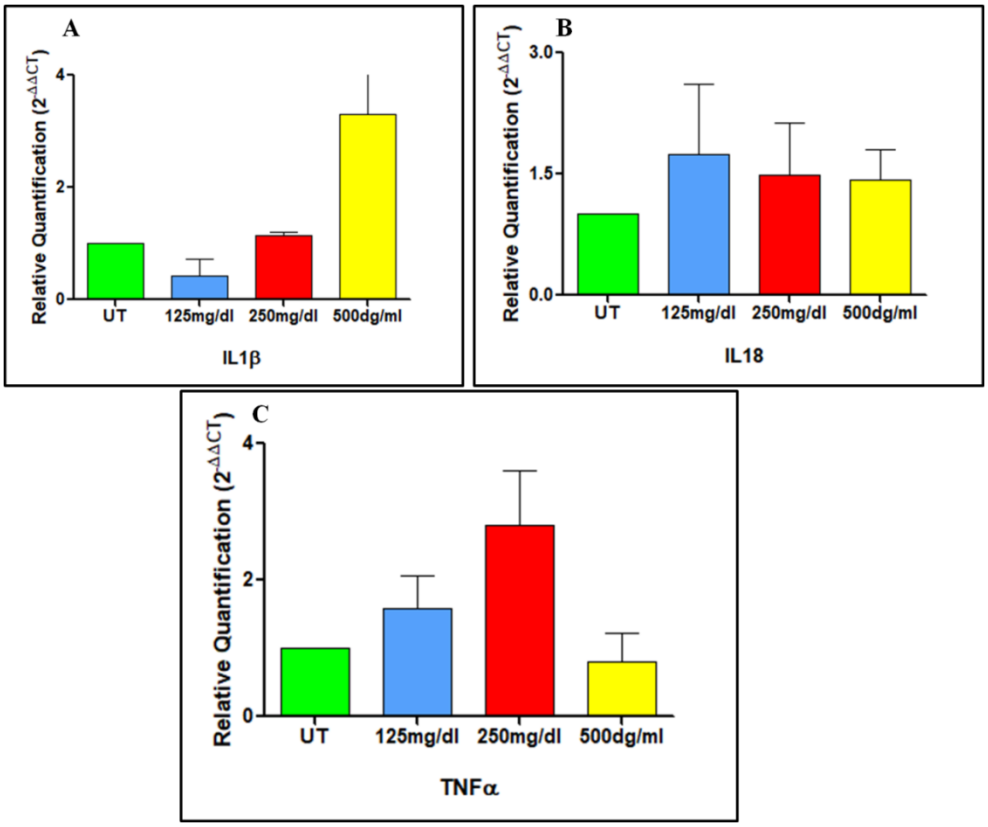

Figure 5.71: Figure represents the relative quantificantion of IL1β, IL18 and TNFα genes gene expression in INT407 cell upon glucose induction. [A] IL1β was upregulated gradually with higher glucose concentration. [B] IL18 expressed highly in cells treated with 125mg/dl glucose, [C] TNFα expression shows dounregulation in cells cultured with 500mg/dl glucose.

5.16: EXPRESSION OF NLRP3, CASP-1, TLR4 AND TLR7 FORM SINGLE CELL EXPRESSION EXPERIMENT AND NETWORK ANALYSIS OF STUDIED GENES OF INFLAMMATION CASCADE

We have analysed data of NLRP3, CASP-1, TLR4 and TLR7 form single cell transcriptomic experiments of human pancreas from healthy individuals and type 2 diabetes patients, Immune cell and 20 organs and tissues from individual mice. Cluster of single cell from single cell RNA sequencing are represented as Figure 5.72A, 5.73A and 5. 74A. Single cell RNA sequencing analysis of 3200 Human Pancreatic Islets cells from healthy and T2DM patients revealed the higher expression of NLRP3 in Pancreatic ductal cell (Figure 5.72B). Higher level of CASP-1 gene were expressed in Pancreatic ductal cell and Endothelial cells (Figure 5.72C). TLR4 expression was found upregulated in few cells of Pancreatic ductal cell and Endothelial cells (Figure 5.72D). Expressed TLR7 was found distributed in various cells like Pancreatic ductal cell, Endothelial cells and Pancreatic stellate cell of T2DM patients (Figure 5.72E) (Segerstolpe, A., *et al.* 2016). Another single cell RNA sequencing experiment revealed the NLRP3 expression was highly distinctive among Monocytes (Figure 5.73B). CASP-1 expression also elevated among Monocytes and Tcells (Figure 5.73C). TLR4 and TLR7 expression was alos found prominent in Monocytes and T Cells (Figure 5.73D and 5.73E) (Vento-Tormo, R., *et al.* 2018) Single-cell RNA-seq analysis of 20 organs and tissues from individual mice predicted the upregulation of NLRP3 expression in Granulocytes, CASP-1 in Blood cells, TLR4 and TLR7 in Granulocytes (Figure 5.74B, 5.74C, 5.74D and 5.74E) (Schaum, N., *et al.* 2018). Insilico net work analysis were done by GeneMANIA (https://genemania.org/) to understand the interaction like physical interactions, co-expression, prediction, co-localization, pathways, genetic interactions and shared protein domains between our studied molecules including NLRP3 Inflammasome, TLRs and inflammatory cytokines. Strong network of NLRP3 Inflammasome, TLRs, TNFα, IL1β, IL18 and other inflammatory molecules were observed (Figure 5.75A). Stong network could be observed betweenNLRP3 and other inflammatory molecules and TLR4 and other inflammatory molecules (Figure 5.75B and 5.75C).

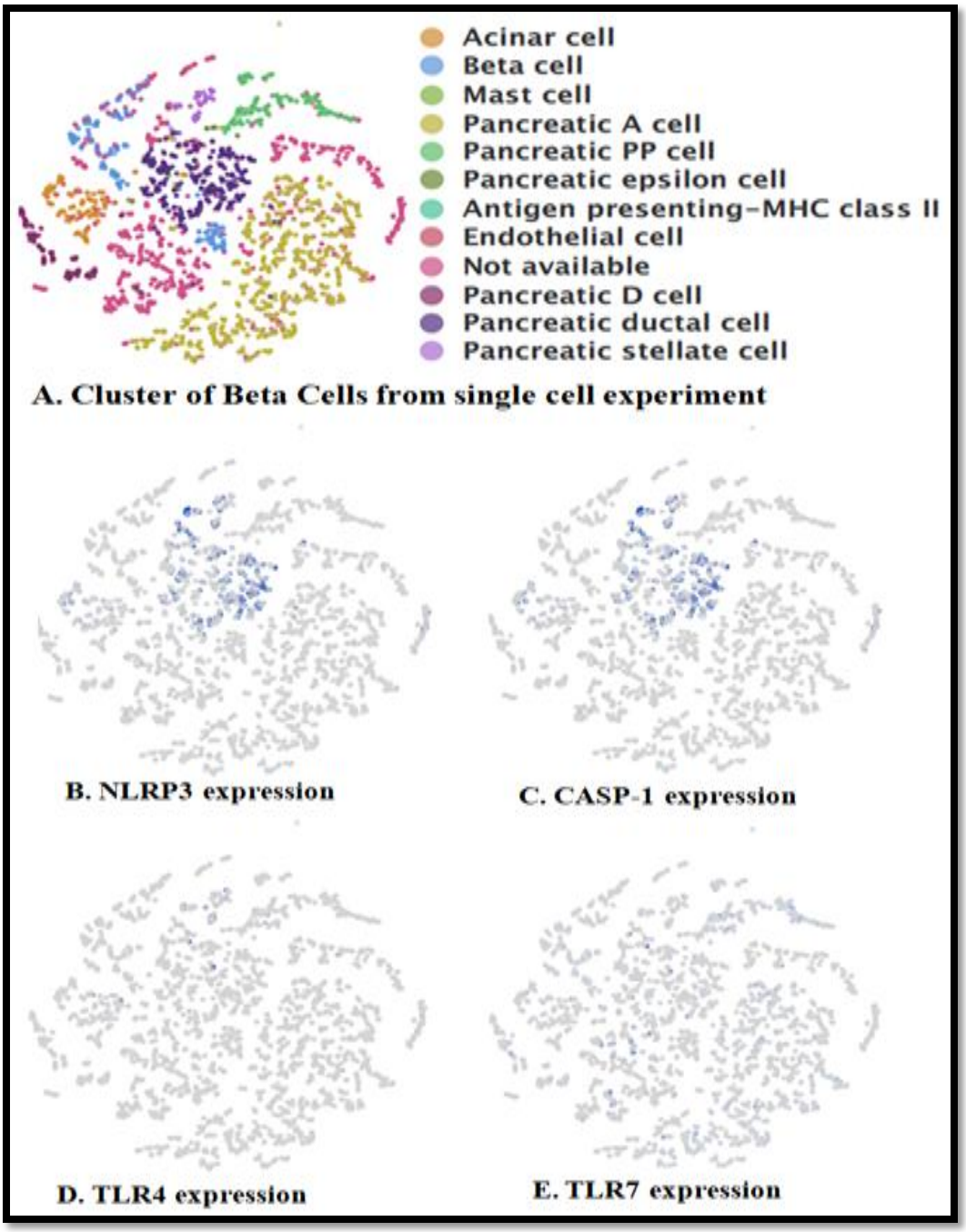

Figure 5.72: Expression of NLRP3, CASP-1, TLR4 and TLR7 form single cell expression experiment of Pancreatic Islets cells from healthy and T2DM patients

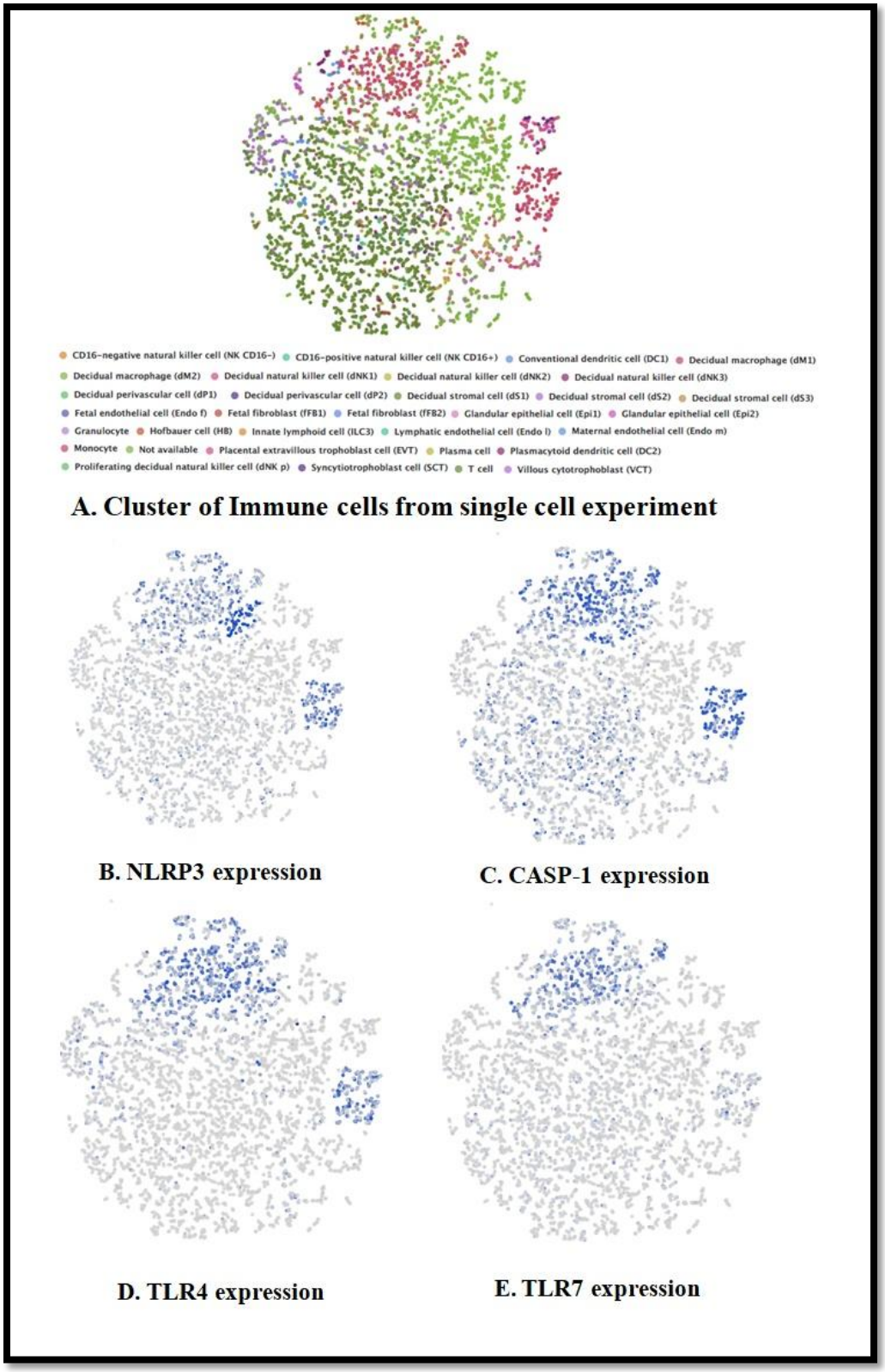

Figure 5.73: Expression of NLRP3, CASP-1, TLR4 and TLR7 form single cell expression experiment of Immune cells

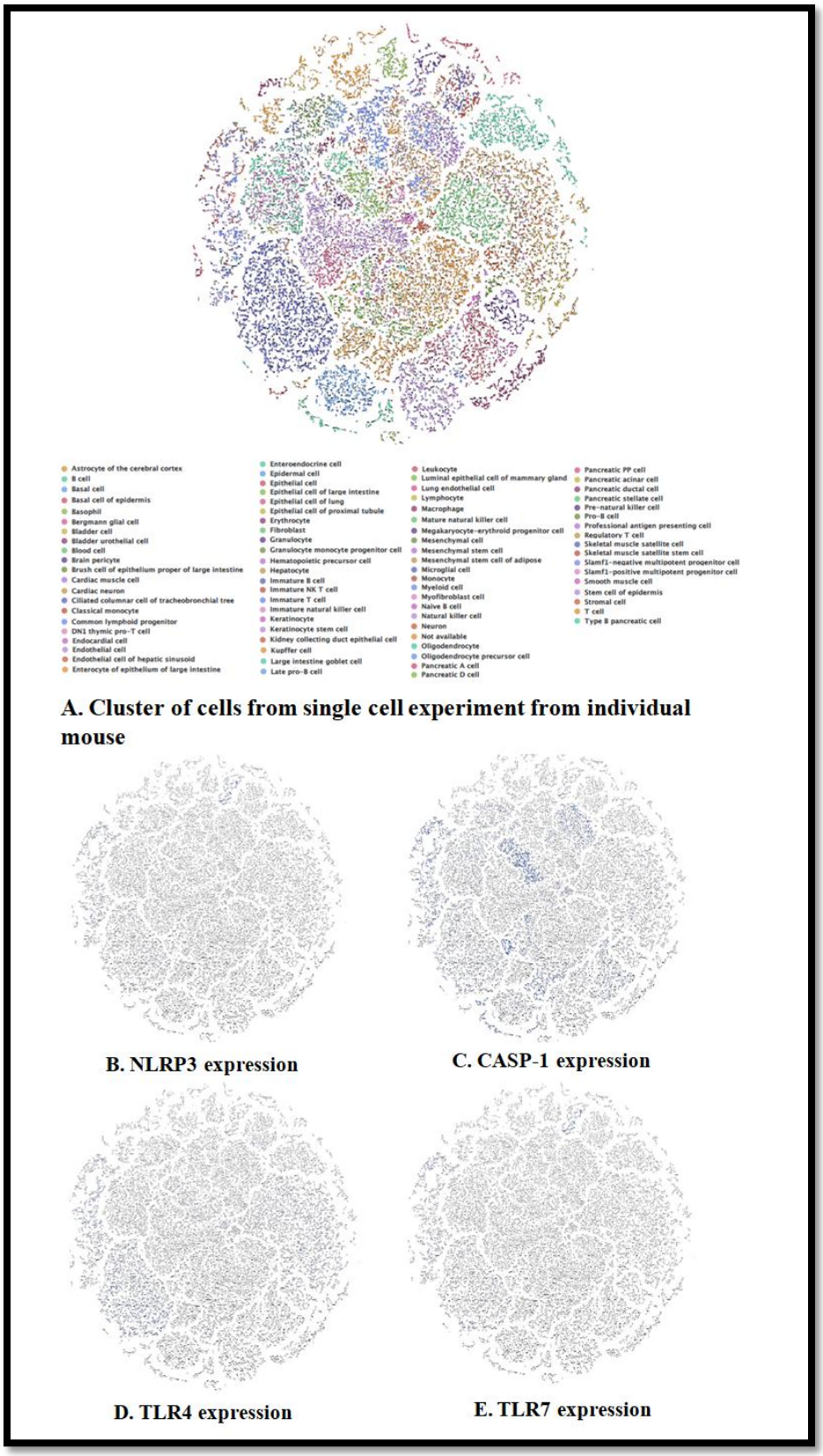

Figure 5.74: Expression of NLRP3, CASP-1, TLR4 and TLR7 form single cell expression experiment from organs and tissues from individual mice

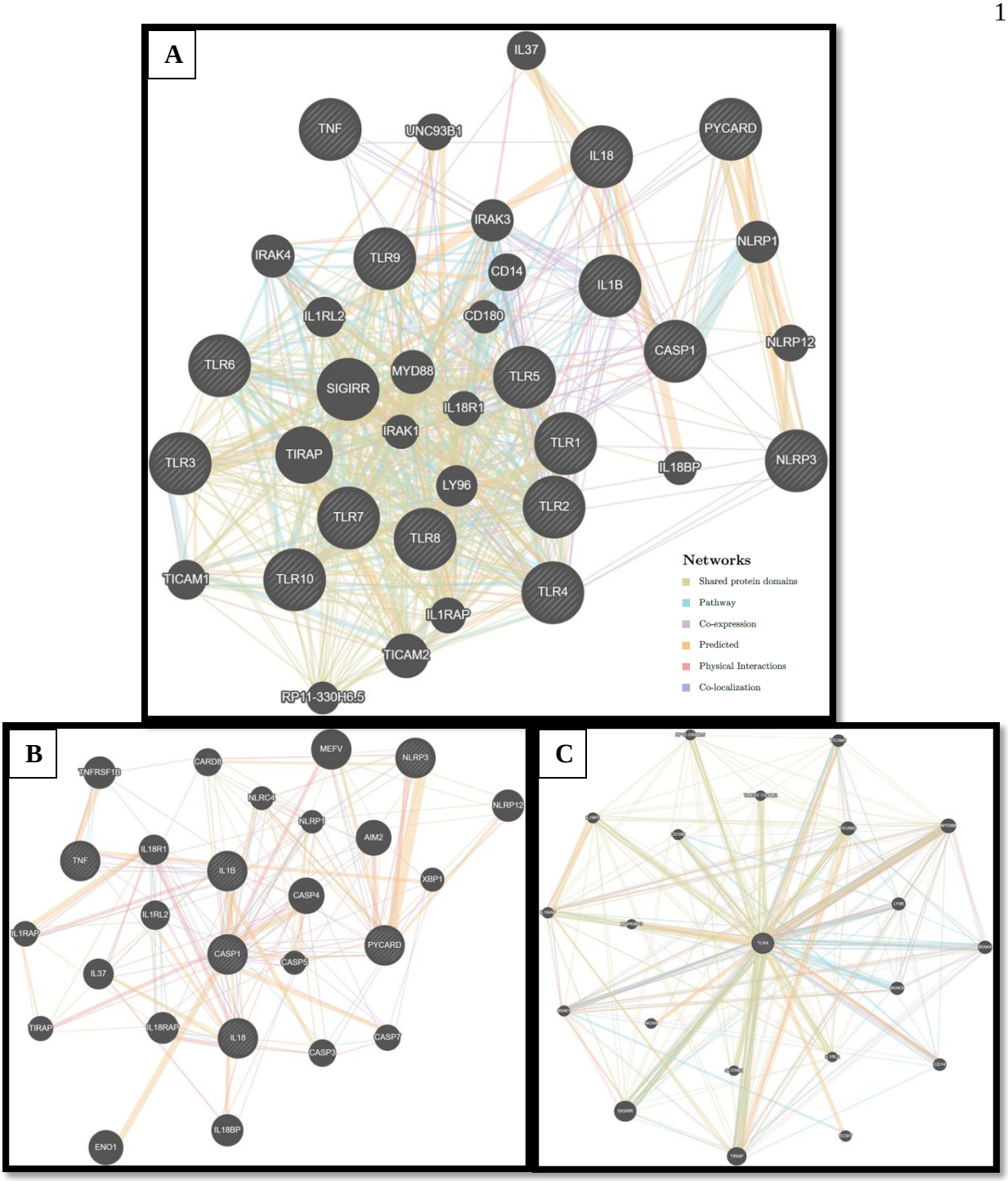

Figure 5.75: Insiliconet work analysis were done by Gene mania to understand the interaction like physical interactions, co-expression, prediction, co-localization, pathways, genetic interactions and shared protein domains between [A] NLRP3 Inflammasome, TLRs ,TNFα, IL1β, IL18 and other inflammatory molecules [B] NLRP3 and other inflammatory molecules [C] TLR4 and other inflammatory molecules

5.17 MICROBIAL ARCHITECTURE OF STUDY SUBJECTS:

We have estimated the gut microbiota from 28 study individuals (12DN, 10DC and 6HC) through 16s metagenome sequencing. The study documented about 10 numbers of phyla among the study subjects (Figure 5.76). The study reveal that the phylum *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria* are dominated the GM architecture among controls as well as T2DM (DC+DN) for the population. Abundance of *Actinobacteria* was found to significantly higher in HC (3.07±3.37) compared to DN (0.19±0.31, P_{Tukeys}<0.05) and DC (0.85±2.38, P_{ANOVA}=0.05) subject (Figure 5.77A). Significant shift in *Proteobacteria* abundance has been documented among the groups as it was found higher among T2DM (26.16±26.94 and 41.36±14.66 in DC and DN respectively) subjects compared to HC (9.84±7.67), P_{ANOVA}=0.02 (Figure 5.77D). No significant differences were observed for the abundance of phylum *Bacteroidetes* (P_{ANOVA}=0.5) and *Firmicutes* (P_{ANOVA}=0.08) among HC (33.37±6.23 and 50.87±3.79), DC (35.40±16.65 and 37.50±17.07) and DN (28.38 ±10.44 and 29.78 ±21.51) respectively (Figure 5.77 B and 5.77 C).

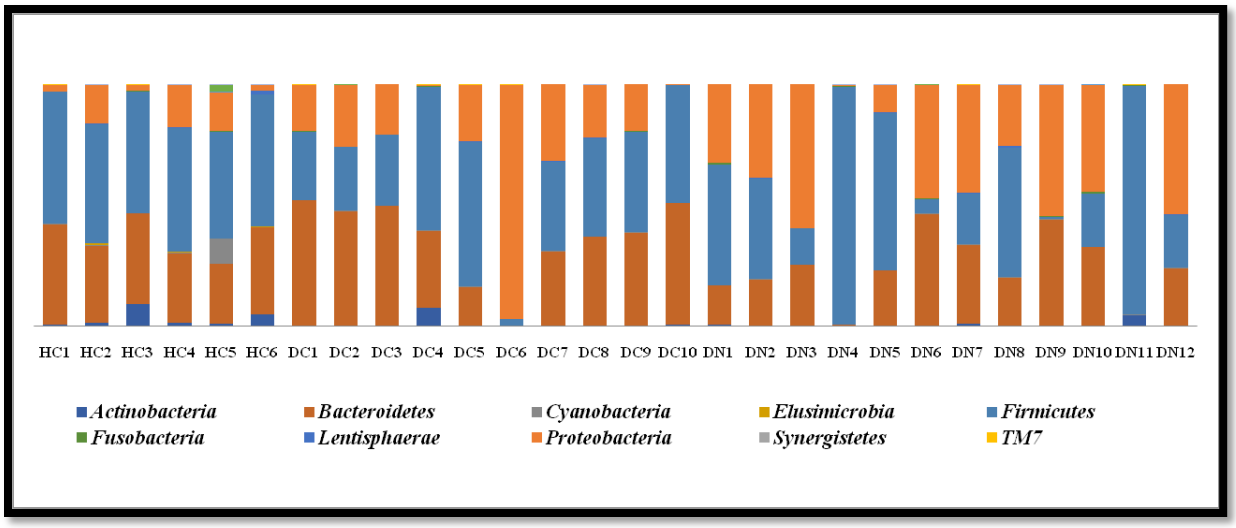

Figure 5.76: Bacterial abundance of Phylum among study individuals

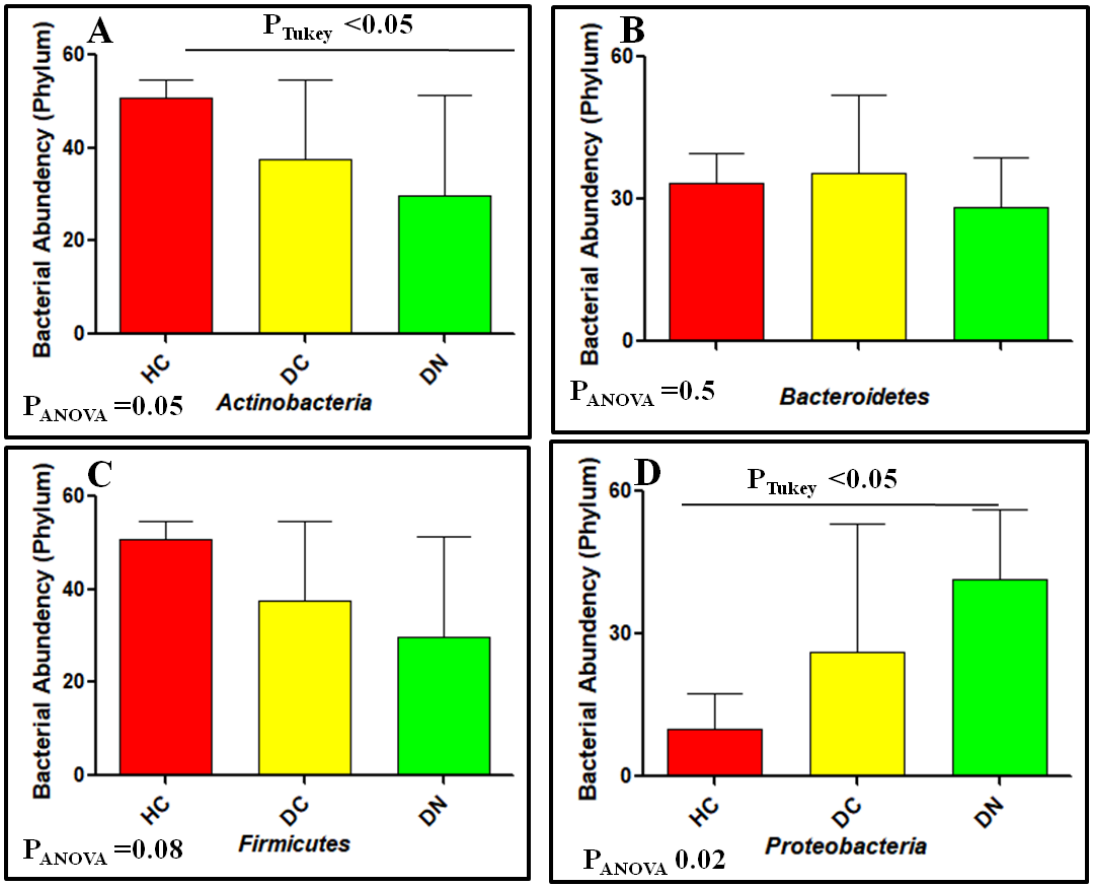

Figure 5.77: Represents the abundance of *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria* in HC, DC and DN subjects. [A] Abundance of *Actinobacteria is* significantly lower in DN (0.19±0.31) than HC (3.07±3.37) subjects. B] Abundance of *Proteobacteria* issignificantly increased in DN (41.36±14.66) than HC (9.84±7.67), p<0.05. Statistically significant was considered P<0.05, P<0.001 and P<0.0001

Further present study could documents 26 different types of *Class* of bacteria and dominated by *Gammaproteria* in T2DM and *Flavobacteria* in HC subjects (Figure 5.78). Study also revealed 49 different types of *Order* of bacterial specis and dominated by *Bacteridales, Clostridiales* and *Lactobacillalis* in some samples (Figure 5.79). GM of present study was comprises of 95 different types of bacterial *Family* and some high abundance familes of were *Bacteroidaceae, Enterobacteriaceae, Enterococcaceae, Fusobacteriaceae, Prevotellaceae* and *Ruminococcaceae* (Figure 5.80).

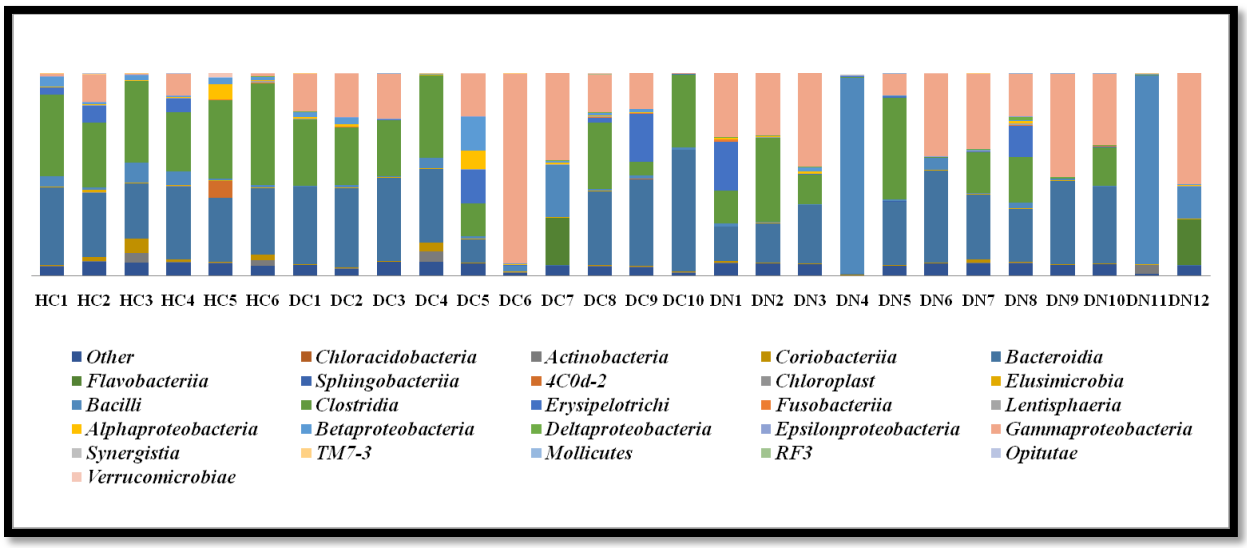

Figure 5.78: Bacterial abundance of Class among study individuals

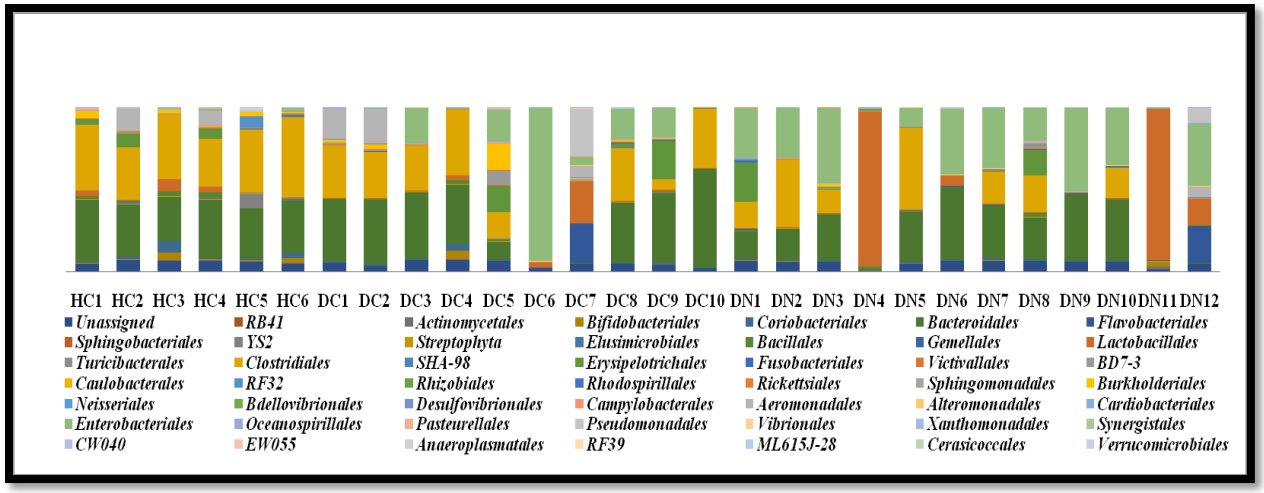

Figure 5.79: Bacterial abundance of Order among study individuals

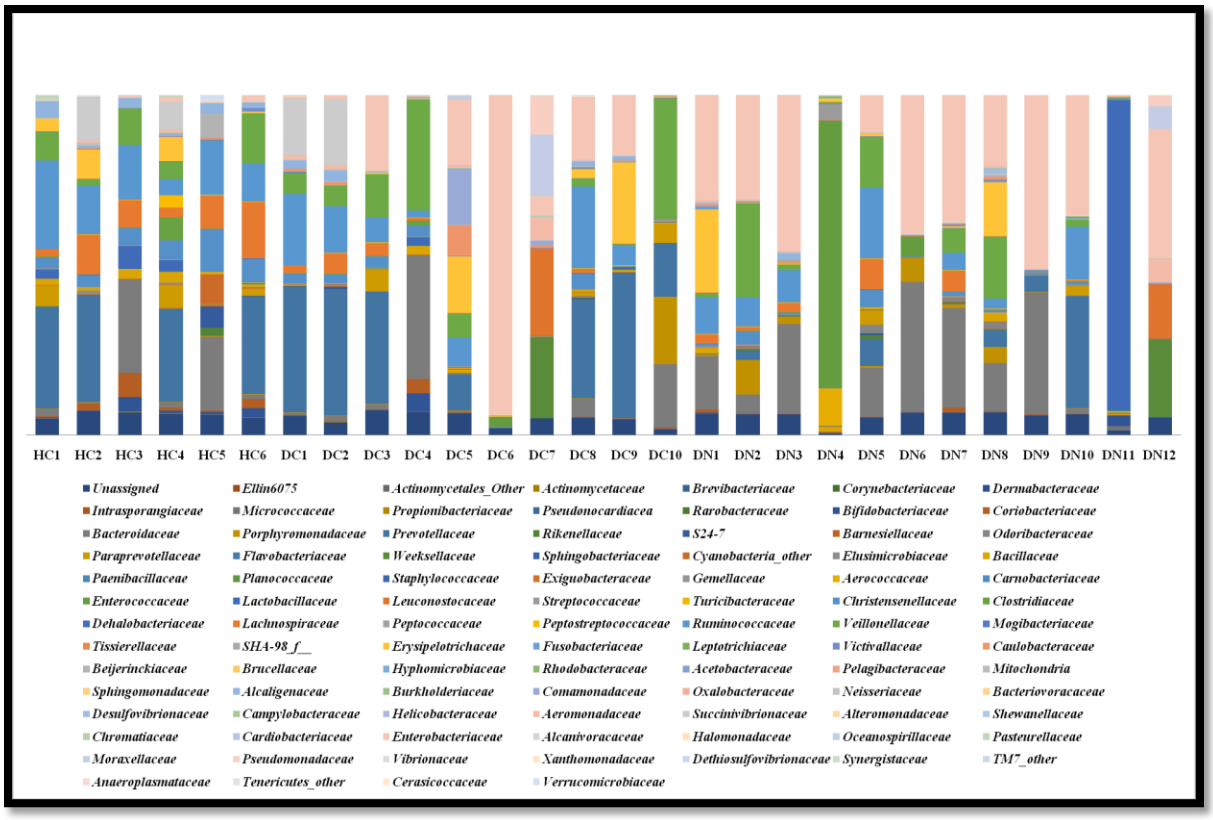

Figure 5.80: Bacterial abundance of Family among study individuals

Present study could estimate about 142 different Genus of bacteria. Genus *Acinetobacter*, *Bacteroides*, *Bifidobacterium*, *Dialister*, *Escherichia*, *Faecalibacterium*, *Lactobacillus*, *Prevotella* and *Wautersiella* were found dominating among HC, DC and DN study subjects. The abundance of *Escherichia* found significantly higher in DN (32.53±16.84, P_{ANOVA}=0.01) and lowest in HC (0.18±0.25) (Figure 5.81 and Table 5.34). The abundance of *Escherichia* was 13.87±25.95 among DC subjects. Bacterial abundance of Genus *Bacteroides* found highest in DN subjects (19.95±15.67) compared to DC (1.27±3.17) and HC (9.55±15.68), P_{ANOVA} =0.02, on the other hand abundance of *Prevotella* was significantly highest in DC (24.55±21.00) compared to DN (4.76±9.94), DC (22.70±18.04) and P_{ANOVA} =0.03 (Table 5.34). *Bifidobacterium*, *Lactobacillus* and *Faecalibacterium* proportion was found highest in HC (1.13±1.24, 0.09±0.12 and 12.65±9.46) subjects compared to DC (0.40±1.26, 0.03±0.04 and 6.25±5.78) and lowest in DN (0.006±0.014, 6.25±5.78 and 6.94±7.35)subjects but difference was not significant (P=0.11, 0.22 and 0.12 respectively)

(Table 5.34). The proportion of *Acinetobacter*, *Dialister* and *Wautersiella* also found dominating among DC (1.34± 3.9, 7.18 ± 14.53 and 3.06± 9.68) compared to DN (0.33± 1.04, 0.06± 0.16 and 2.40± 7.59) and HC (0.0005± 0.001, 2.74 ± 4.77 and 0.0007± 0.001) study subjects (P=0.53, 0.25 and 0.74 respectively) (Table 5.34).

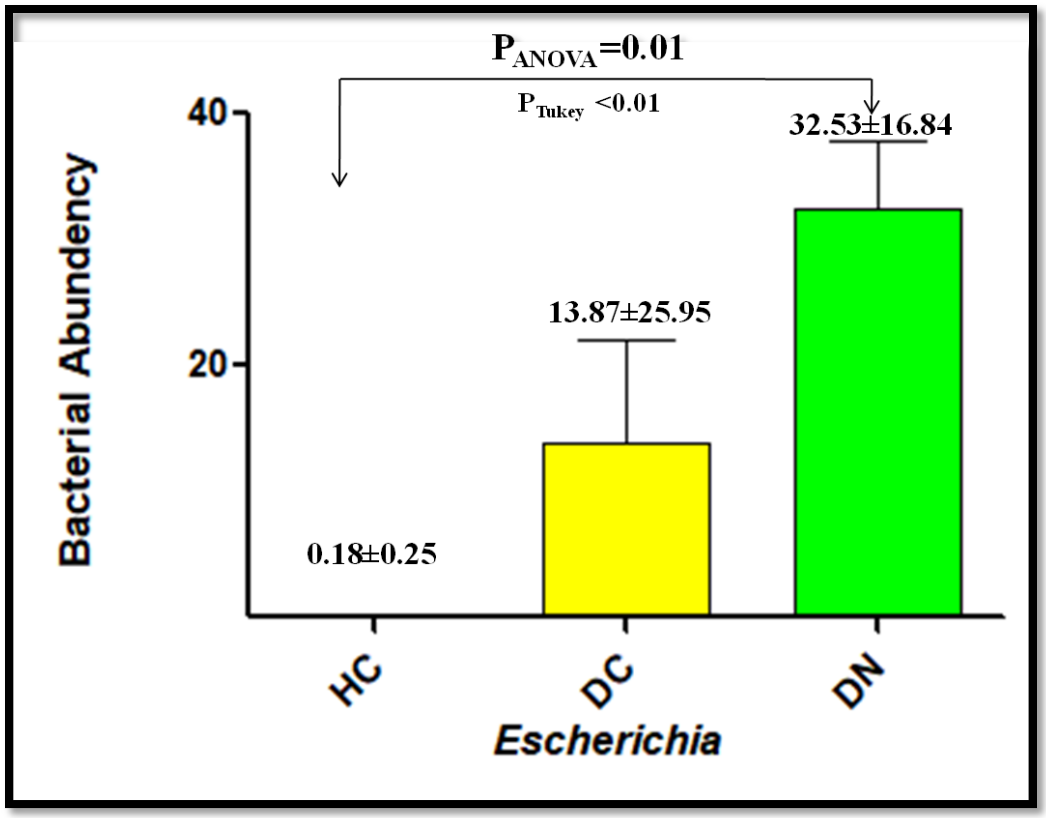

Figure 5.81: Represents the abundance of *Escherichia* among HC, DC and DN study participants. Abundance of *Escherichia* significantly increased in DN subjects than HC and DC. Statistically significant difference for abundance of *Escherichia* observed between HC (0.18±0.25) and DN (32.53±16.84), P<0.01.

Table 5.34: Bacterial abundance of Genus *Acinetobacter*, *Bacteroides*, *Bifidobacterium, Dialister, Escherichia, Faecalibacterium, Lactobacillus, Prevotella*and *Wautersiella* among HC, DC and DN study subjects

	HC	**DC**	**DN**	**P ANOVA**	**P_{TUKEYS}**	
Acinetobacter	0.0005± 0.001	1.34± 3.9	0.33± 1.04	0.53	HC Vs DC	>0.05
					HC Vs DN	>0.05
					DC Vs DN	>0.05
Bacteroides	9.55± 15.68	1.27± 3.17	19.95± 15.67	**0.02**	HC Vs DC	>0.05
					HC Vs DN	>0.05
					DC Vs DN	**<0.01**
Bifidobacterium	1.13±1.24	0.40±1.26	0.006±0.014	0.11	HC Vs DC	>0.05
					HC Vs DN	>0.05
					DC Vs DN	>0.05
Dialister	2.74 ± 4.77	7.18 ± 14.53	0.06± 0.16	0.25	HC Vs DC	>0.05
					HC Vs DN	>0.05
					DC Vs DN	>0.05
Escherichia	0.18±0.25	13.87±25.95	32.53±16.84	**0.01**	HC Vs DC	>0.05
					HC Vs DN	<0.001
					DC Vs DN	>0.05
Faecalibacterium	12.65±9.46	6.25±5.78	6.94±7.35	0.22	HC Vs DC	>0.05
					HC Vs DN	>0.05
					DC Vs DN	>0.05
Lactobacillus	0.09±0.12	0.03±0.04	0.02±0.05	0.12	HC Vs DC	>0.05
					HC Vs DN	>0.05
					DC Vs DN	>0.05
Prevotella	22.70±18.04	24.55±21.00	4.76±9.94	**0.03**	HC Vs DC	>0.05
					HC Vs DN	>0.05
					DC Vs DN	**<0.01**
Wautersiella	0.0007± 0.001	3.06± 9.68	2.40± 7.59	0.74	HC Vs DC	>0.05
					HC Vs DN	>0.05
					DC Vs DN	>0.05

The significant shift in abundance of *Gammaproteobacteria* (Class), *Enterobacteriales* (Order) and *Enterobacteriaceae* (Family) represents the genus *Escherichia* was found significant among HC, DC and DN study subjects.Significant increase of bacterial abundency was observed in DN (0.36±0.13, 0.34±0.12 and 0.34±0.12) compared to HC (0.045±0.06, 0.006±0.007 and 0.006±0.007) for *Gammaproteobacteria* (P_{Tukeys}<0.05), *Enterobacteriales* (P_{Tukeys} <0.001) and *Enterobacteriaceae* (P_{Tukeys} <0.001) respectively (Table 5. 35).

Table 5.35: Bacterial abundance of *Gammaproteobacteria* (Class), *Enterobacteriales* (Order), *Enterobacteriaceae* (Family) and *Escherichia* (Genus) among HC, DC and DN study subjects

	HC	DC	DN	P_{ANOVA}	P_{TUKEYS}	
Gammaproteobacteria	0.045±0.06	0.26±0.27	0.36±0.13	0.01	HC Vs DC	>0.05
					HC Vs DN	<0.05
					DC Vs DN	>0.05
Enterobacteriales	0.006±0.007	0.18±0.28	0.34±0.12	0.008	HC Vs DC	>0.05
					HC Vs DN	<0.001
					DC Vs DN	>0.05
Enterobacteriaceae	0.006±0.007	0.18±0.28	0.34±0.12	0.008	HC Vs DC	>0.05
					HC Vs DN	<0.001
					DC Vs DN	>0.05

Linear regression was performed with the independent variables (X) including the level of relative quantification of mRNA (NLRP3, CASP1, TLR4 and IL1β), estimated glomerular filtration rate (eGFR), BMI, abundance of dominated phylum in this study (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria) and other dominating genus (*Acinetobacter, Bacteroides, Bifidobacterium, Dialister, Faecalibacterium, Lactobacillus, Prevotella* and *Wautersiella*) with the proportion of genera *Escherichia* (Y) as an dependent variable to predict the association of dependent variables. We used the proportion of Escherichia as dependent variable as the shift of the proportion of the same most significant on disease status (Table 5.36).

The study documented that m-RNA expression of NLRP3 and CASP-1 are elevated in DN compared to DC in reference of healthy control upon the estimation of relative expression through qPCR. Linear regression also showed the positive association with the elevated expression of NLRP3 (P=0.0004, Estimates: 1.64) and CASP-1 (P=0.0009, Estimates: 1.04) gene expression with the concomitant increased proportion of *Escherichia* on the severity of disease state among the guts of study subjects (Table 5.36 and Figure 5.82, 5.83). Further the study documented the negative correlation with the proportion of relative abundance of *Bacteroidetes* (P=0.03, Estimates: -0.75) with the proportion of *Escherichia*. (Table 5.36 and Figure 5.84). The abundence of *Escherichia* could predict the relative abundance of other genus- *Actinobacter* (P = 0.001, Estimates: 5.56) and *Wautersiella* (P=0.0009, Estimates: 2.29)

and also predicts negative correlation with the abundance of geneus *Prevotella* (p=0.04, Estimates: -0.48) (Table 5.36 and Figure 5.85, 5.86, 5.87).

The proportion of *Escherichia* also predicts the clinical variable like eGFR that explain the disease severity. Increased proportion of Escherichia associated with decreased rate of eGFR (P=0.02, Estimates: -0.2) or the adverse prognosis of DN. (Table 5.36 and Figure 5.88).

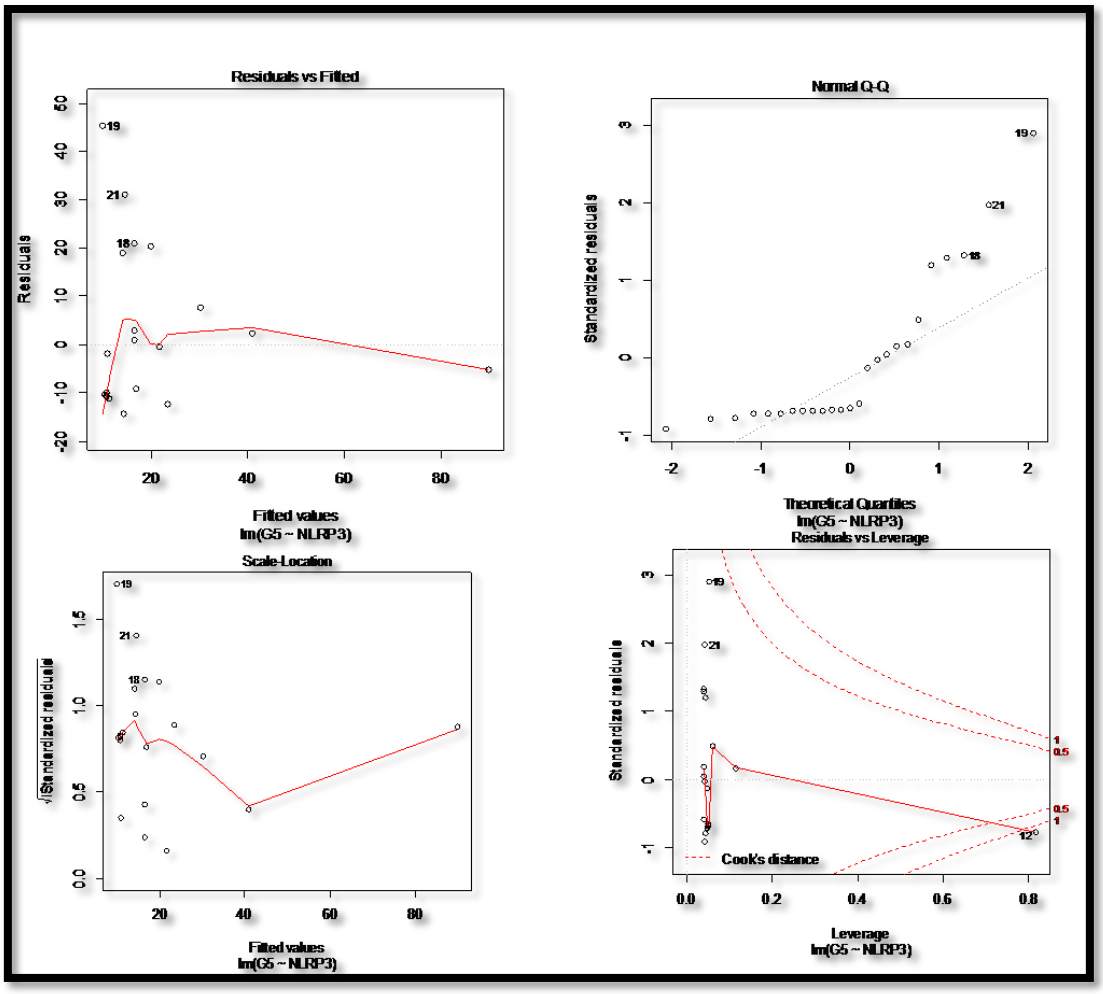

Figure 5.82: Figure represents the linear regression of the interaction of *Escherichia* with the NLRP3 gene expression among the Type2 Diabetic subjects. Elevated level of *Escherichia* associated with increased NLRP3 expression (P:0.0004). The estimate of the regression is 1.64 with the SE:0.33. Residual standard error: 16.04, Multiple R-squared: 0.53, Adjusted R-squared: 0.50, F-statistic: 25.47

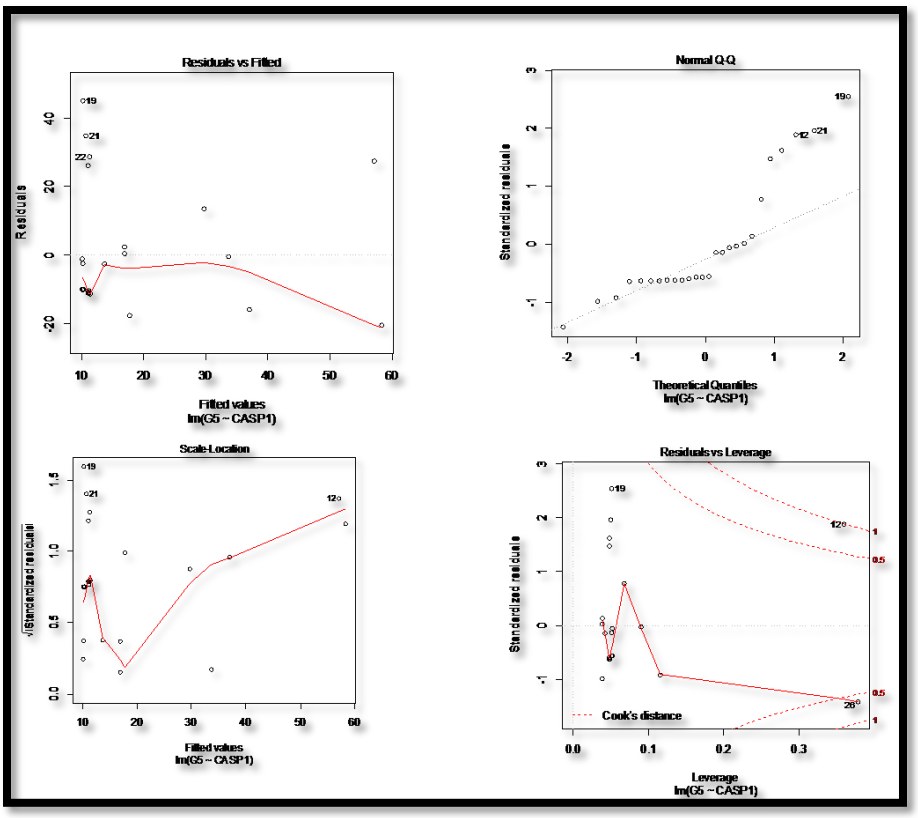

Figure 5.83: Figure represents the linear regression of the interaction of *Escherichia* with the CASP1 gene expression among the Type2 Diabetic subjects. Elevated level of *Escherichia* associated with increased CASP1 expression (P:0.0009). The estimate of the regression is 1.04 with the SE:0.27. Residual standard error: 18.26, Multiple R-squared: 0.37, Adjusted R-squared: 0.35, F-statistic: 14.38

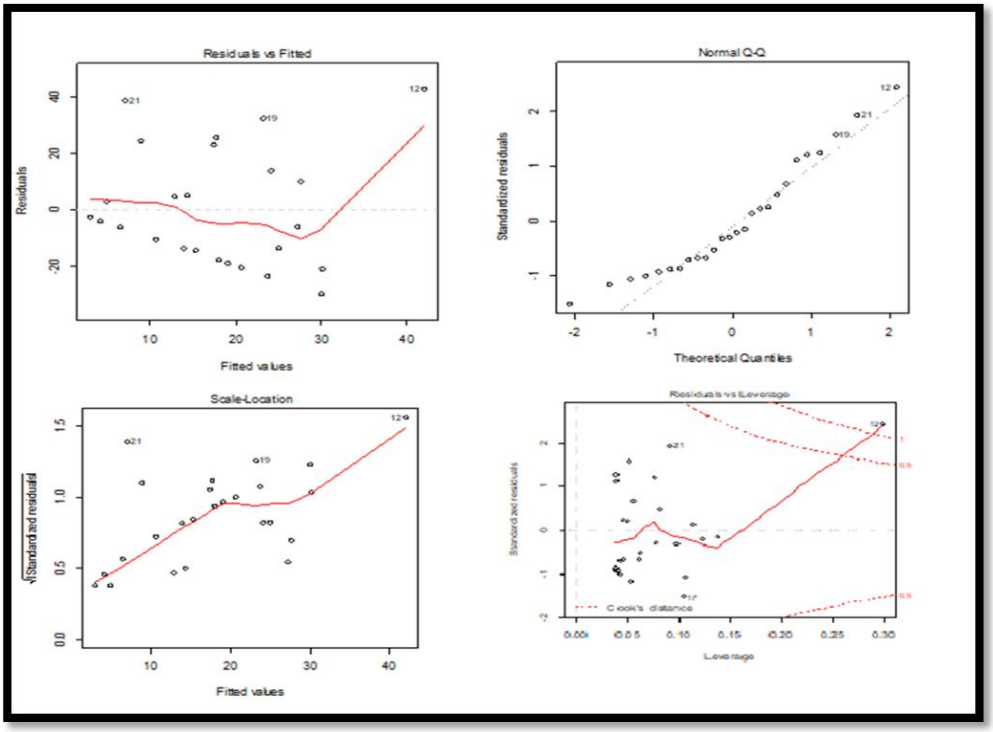

Figure 5.84: Figure represents the linear regression of the interaction of *Escherichia* with the phylum *Bacteroidetes* among the Type2 Diabetic subjects. Elevated level of *Escherichia* associated with decreased *Bacteroidetes* (P:0.03). The estimate of the regression is -0.75 with the SE:0.33. Residual standard error: 20.98, Multiple R-squared: 0.17, Adjusted R-squared: 0.1, F-statistic: 5.08.

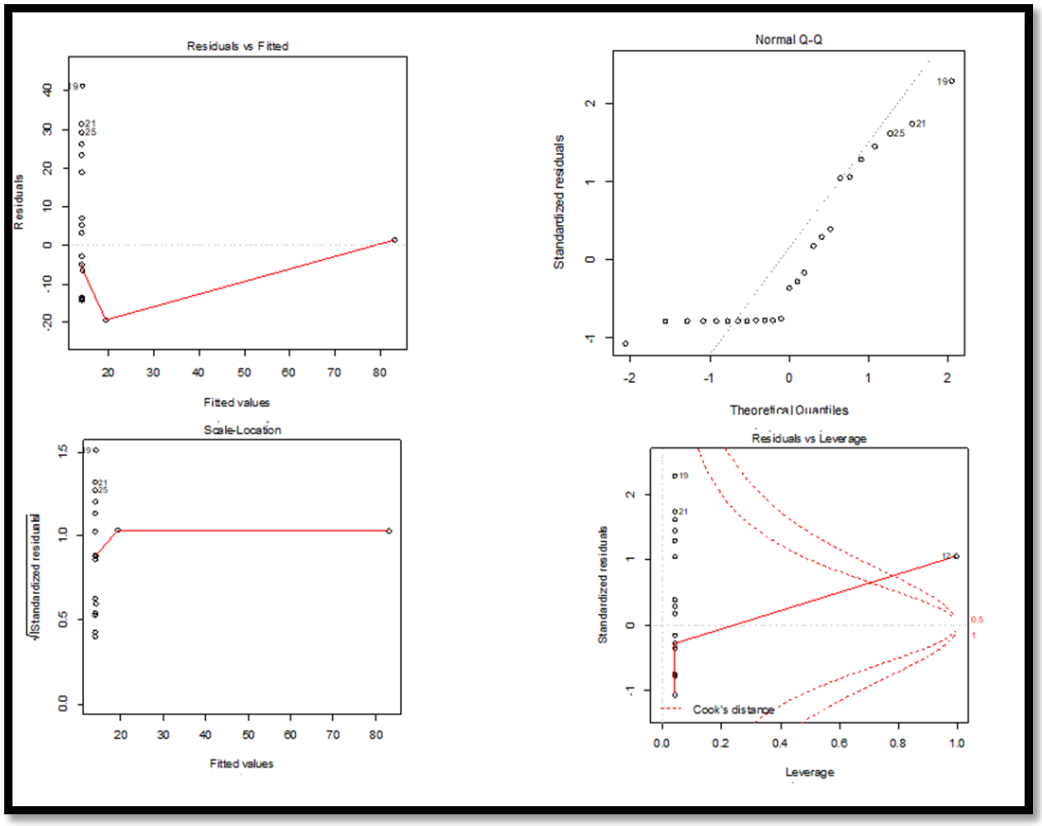

Figure 5.85: Figure represents the linear regression of the interaction of *Escherichia* with the genus *Acinetobacter* among the Type2 Diabetic subjects. Elevated level of *Escherichia* associated with increased *Acinetobacter* (P:0.001). The estimate of the regression is 5.56 with the SE:1.52. Residual standard error: 18.43, Multiple R-squared: 0.37, Adjusted R-squared: 0.34, F-statistic: 13.47.

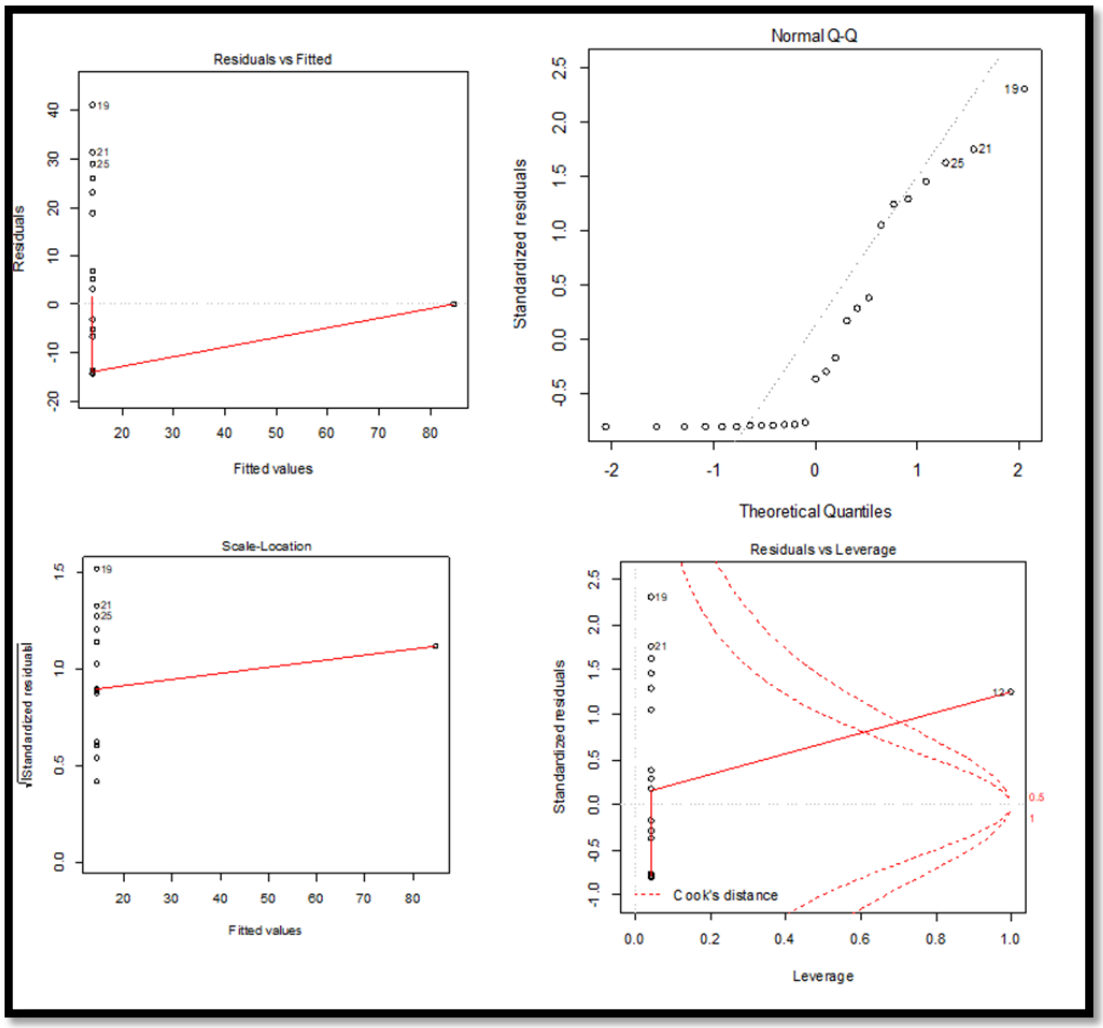

Figure 5.86: Figure represents the linear regression of the interaction of *Escherichia* with the genus *Wautersiella* among the Type2 Diabetic subjects. Elevated level of *Escherichia* associated with increased *Wautersiella* (P:0.0009). The estimate of the regression is 2.29 with the SE:0.61. Residual standard error: 18.22, Multiple R-squared: 0.38, Adjusted R-squared: 0.36, F-statistic: 14.31.

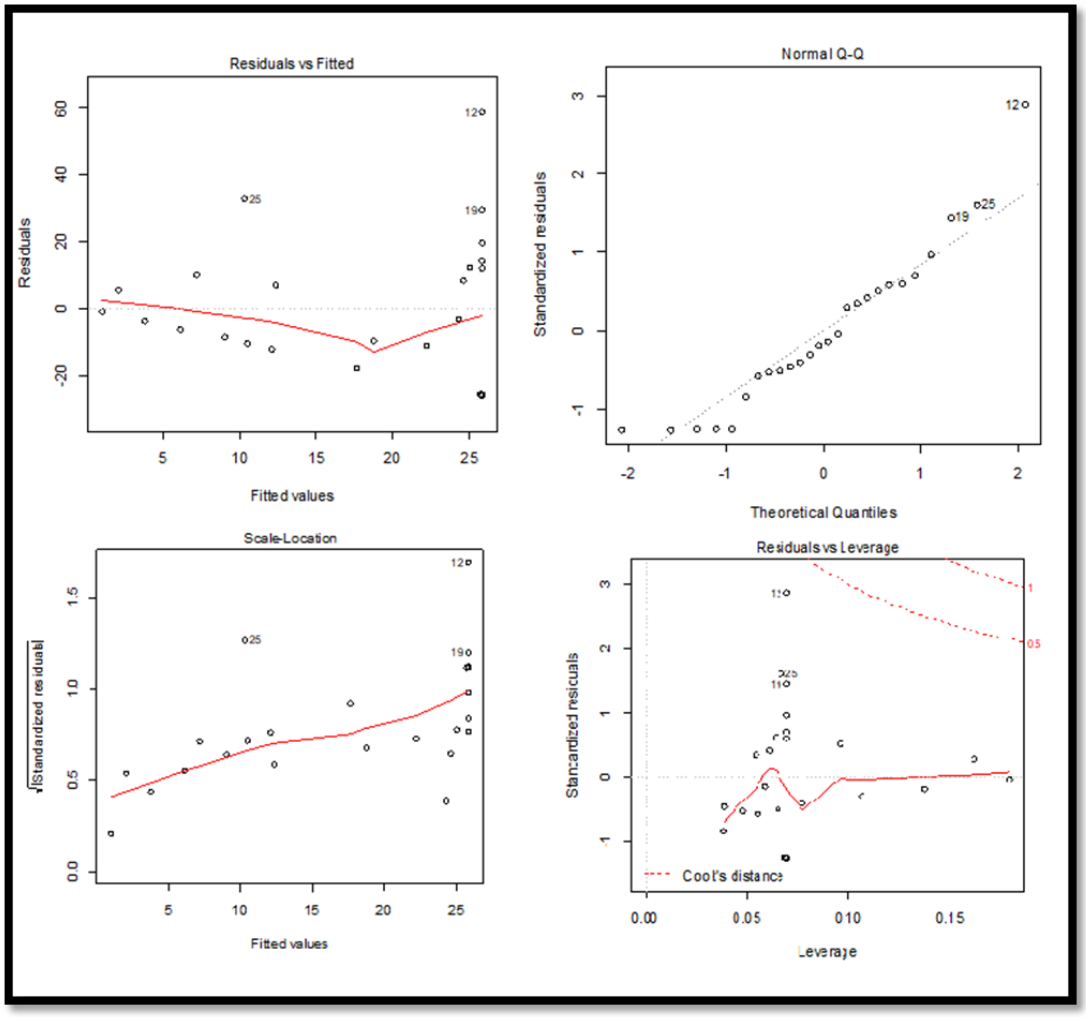

Figure 5.87: Figure represents the linear regression of the interaction of *Escherichia* with the genus *Prevotella* among the Type2 Diabetic subjects. Elevated level of *Escherichia* associated with decreased *Prevotella*(P:0.04). The estimate of the regression is -0.48 with the SE:0.23. Residual standard error: 21.2, Multiple R-squared: 0.16, Adjusted R-squared: 0.12 , F-statistic: 4.49.

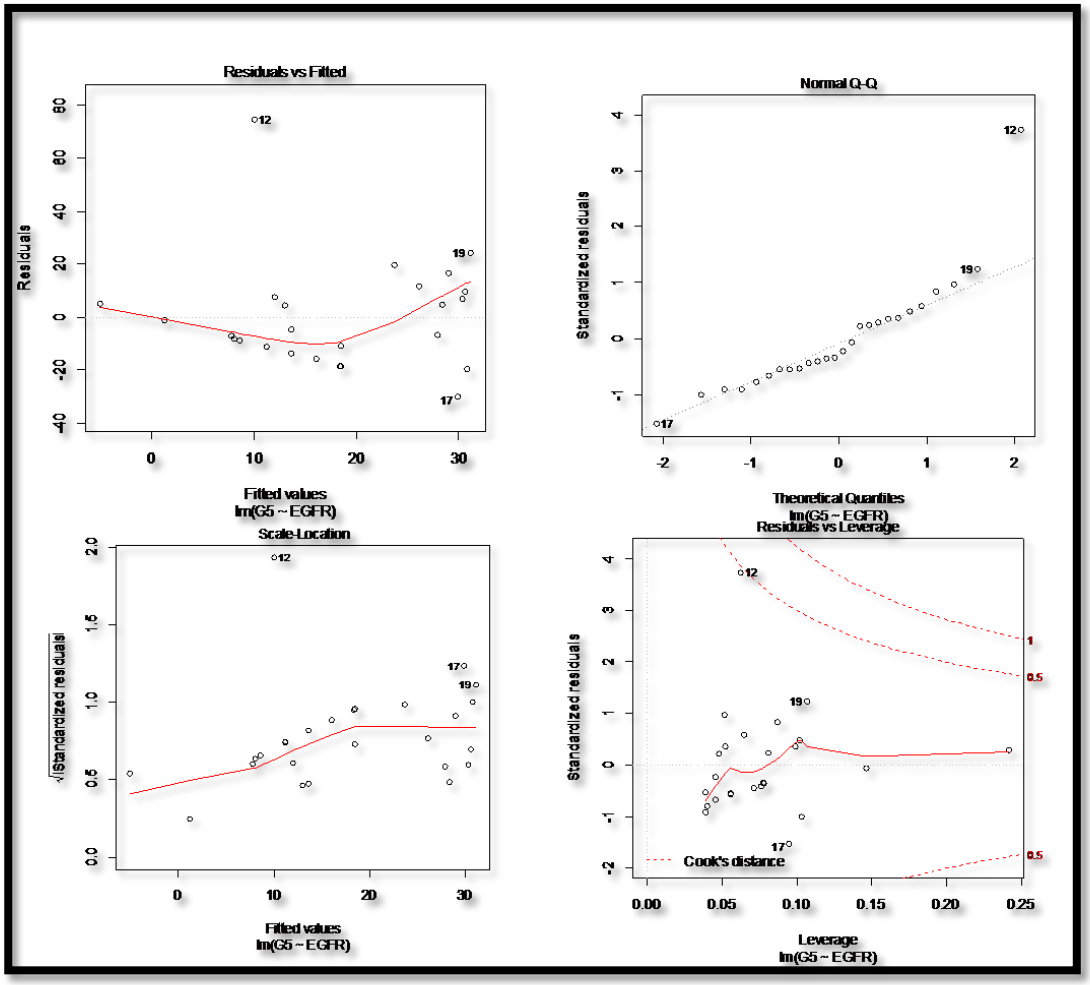

Figure 5.88: Figure represents the linear regression of the interaction of *Escherichia* with the eGFR status among the Type2 Diabetic subjects. Elevated level of *Escherichia* associated with decreased eGFR status (P:0.02). The estimate of the regression is -0.2 with the SE:0.08. Residual standard error: 20.63, Multiple R-squared: 0.20, Adjusted R-squared: 0.17, F-statistic: 6.08

Table 5.36: Linear regression to predict the relative abundance of phylum, gene expression and clinical parameters with the genus *Escherichia*

Genus	Estimate	Std. Error	t value	Pr(>|t|
Linear regression to predict the relative abundance of phylum with the genus *Escherichia*				
Actinobacteria	-3.21	1.83	-1.75	0.09
Bacteroidetes	**-0.75**	**0.33**	**-2.25**	**0.03**
Firmicutes	-0.11	0.24	-0.47	0.6
Proteobacteria	0.21	0.19	1.06	0.3
Linear regression to predict the relative abundance of other major genus of bacteria with the genus *Escherichia*				
Acinetobacter	**5.56**	**1.52**	**3.67**	**0.001**
Bacteroides	0.31	0.29	1.07	0.3
Bifidobacterium	-7.15	4.24	-1.69	0.1
Dialister	-0.23	0.47	-0.49	0.6
Faecalibacterium	-0.62	0.56	-1.10	0.3
Lactobacillus	-0.18	0.24	-0.74	0.5
Prevotella	**-0.48**	**0.23**	**-2.12**	**0.04**
Wautersiella	**2.29**	**0.61**	**3.78**	**0.0009**
Linear regression to predict the relative abundance of **gene expression with the genus *Escherichia***				
NLRP3	**1.64**	**0.33**	**5.04**	**0.0004**
CASP1	**1.04**	**0.27**	**3.79**	**0.0009**
TLR4	0.43	0.32	1.35	0.2
IL1B	0.06	0.05	1.2	0.24
Linear regression to predict the relative abundance clinical parameters with the genus *Escherichia*				
eGFR	**-0.2**	**0.08**	**-2.47**	**0.02**
BMI	-2.71	1.73	-1.56	0.13

 # It may be noted we include the genus who had abundancy atleast ≥1 %.

The study did not reveal any significant difference in terms of numbers of Operational Taxonomic Units (OTUs). The mean OTUs for HC, DC and DN were 117.8±17.68, 103.0±33.69 and 101.3±20.82, respectively P_{ANOVA}=0.42 (Table 5.37). To compare the GM diversity among HC, DC and DN, we computed the α (Shannon-diversity-index (SDI) and Simpson) (Table 5.37) and β-diversity (Bray-Curtis dissimilarity index (BCDI) (Figure 5.89 and Table 5.38). The SDI decreased in DN (2.24±0.41) and DC (2.17±0.61) compare to HC (2.75±0.24) but the level is insignificant (P=0.06), (Table 5.37). Inter individual BCDI is highest in DC (0.45±0.12) compared to DN (0.43±0.11) and minimum in HC (0.27±0.03) (P_{ANOVA}<0.0001) (Figure 5.89A and Table 5.38). The inter group variation for BCDI is significantly higher in DC (P_{Tukeys}<0.0001) and DN (P_{Tukeys}<0.0001) compared to HC but the variation is not significant for T2DM subjects i.e, for DC and DN (P_{Tukeys} >0.05) (Figure 5.89A and Table 5.38). Further, we have computed the intra group variation of BCDI and revealed that distance of diversity is significantly increased between DN and DC (0.45±0.10) compare to HC and DN (0.41±0.10) and minimum in HC and DC (0.34±0.12), P<0.001(Figure 5.89B and Table 5.38).

Table 5.37: Represents the Operational Taxonomic Units (OUT) and Alpha diversity (Shannon and Simpson diversity-index) among HC, DC and DN study subjects

	HC	**DC**	**DN**	**P ANOVA**
OTU	117.8±17.68	103.0±33.69	101.3±20.82	0.42
Shannon-diversity-index	2.75±0.24	2.17±0.61	2.24±0.41	0.06
Simpson-diversity-index	0.88±0.03	0.78±0.18	0.83±0.06	0.22

Table 5.38: Bray-Curtis dissimilarity index among intra-group and intergroup of study plot

HC	**DC**	**DN**	**P ANOVA**	**PTUKEYS**	
0.27±0.03	0.45±0.12	0.43±0.11	**< 0.0001**	**HC vs DC**	**< 0.0001**
				HC vs DN	**< 0.0001**
				DC vs DN	>0.05
HC /DC	**HC/DN**	**DC/ DN**	**P ANOVA**	**PTUKEYS**	
0.34±0.12	0.41±0.10	0.45±0.10	**0.001**	HC/ DC vs HC/DN	>0.05
				HC/DC vs DC/DN	**< 0.001**
				HC/ DN vs DC/DN	>0.05

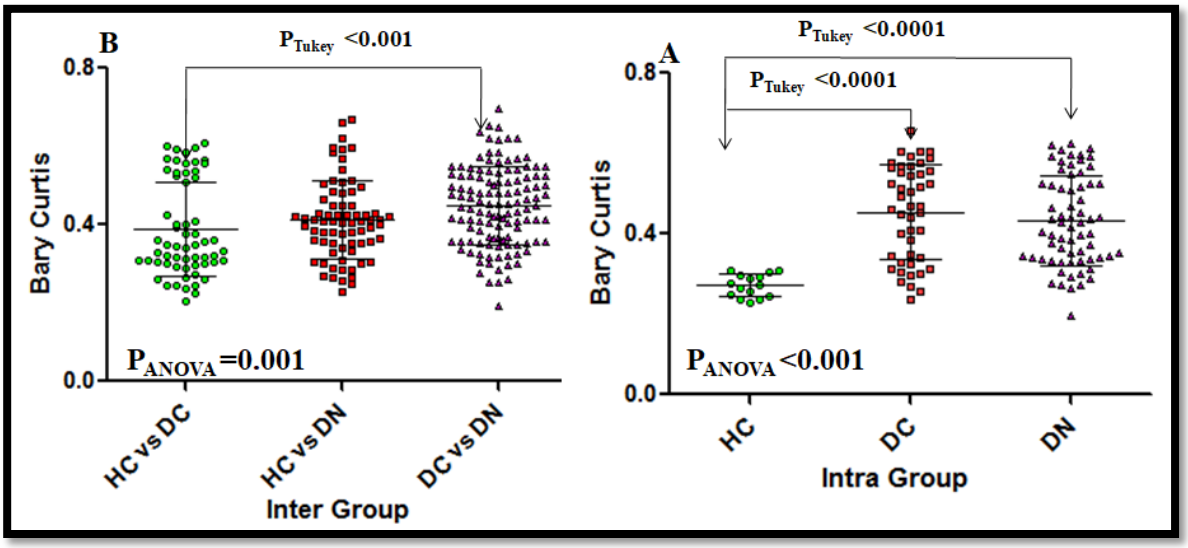

Figure 5.89: Bray-Curtis diversity index (BCDI) of inter and intra group of study subjects. BCID reveals larger inter-individual distance among the DN whereas BCDI significantly reduced for HC and DC. (A) Intergroup BCDI significantly significantly higher among diabetic subjects (DC and DN) compared to HC (P<0.0001). (B) Intra individual BCDI was higher in HC/DN and DC/DN than HC/ DC.

Further we have computed the frequency of Genus representing gut microbiome of study subjects from present syudy (Figure 5.90) and Principal component analysis (PCA) of Diabetic and non diabetic subjects (Figure 5.91).

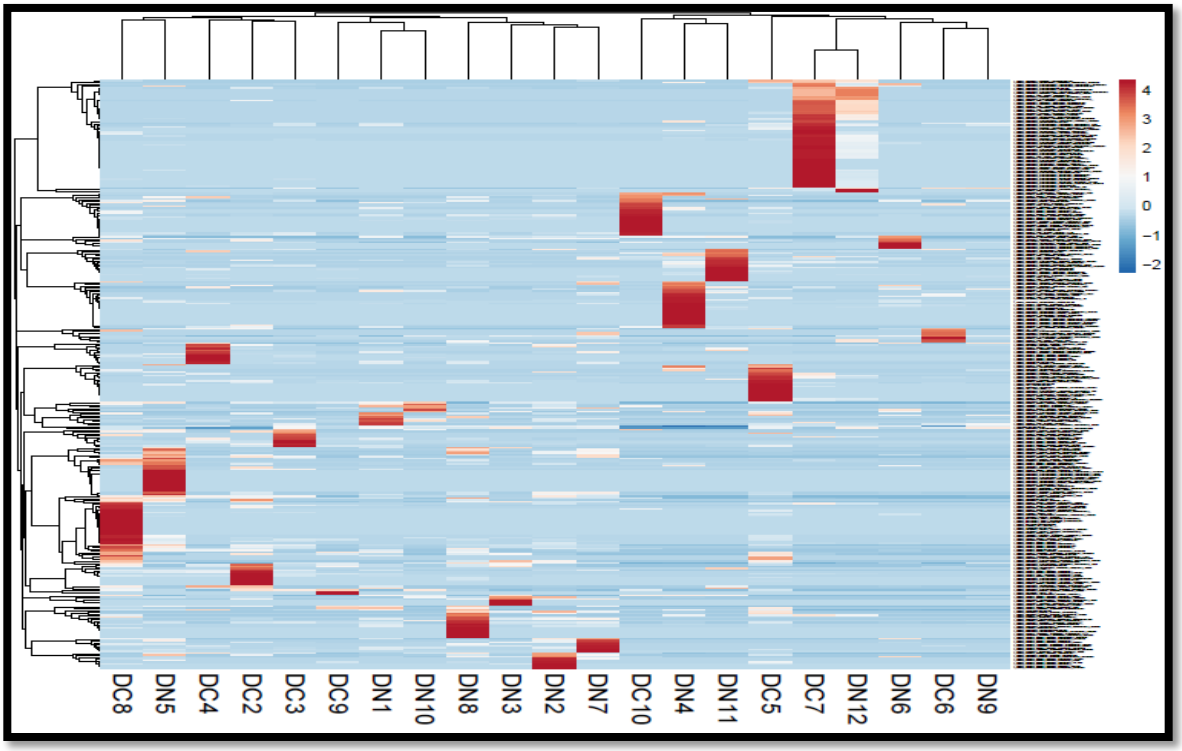

Figure 5.90: Frequency of genus in Gut microbiota among study subjects

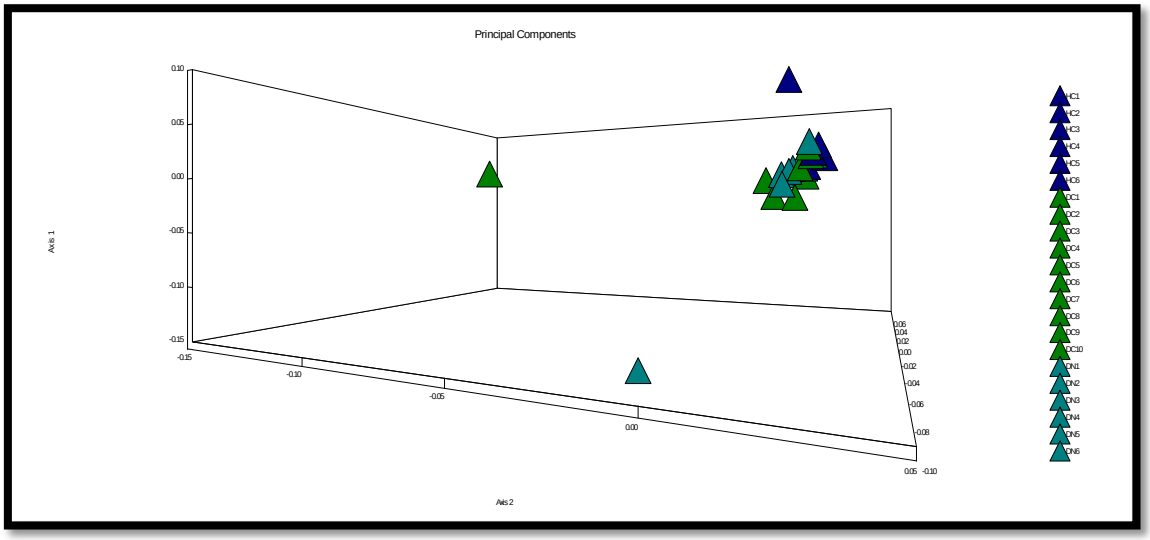

Figure5.91: Principal component analysis of Diabetic and non diabetic subjects