CHAPTER 6

DISCUSSION

Inflammation associated Diabetic complication has been postulated in various animal model studies but the comprehensive data still enigmatic to understand the same in human subjects though the altered lifestyle and gene environment interaction increased the burden of hyperglycemia associated micro and macro vascular complication in exponential manner. In present study we tried to describe the influence of inflammation on Diabetic microvascular complication like Diabetic Nephropathy (DN) in quantitative manner among the human

subjects using a case control study design. Observation of the study indicates the alteration of gene expression related to inflammation like NLRP3 Inflammasome that has been replicated with other population data (Lee, J., *et al.* 2017). Present study has demonstrated that, gene expression of Inflammasome complex like NLRP3 (17.42±22.85 and 5.95±12.66 fold), CASP-1 (9.04±14.39 and 2.87±7.89 fold) and PYCARD (5.01±7.89 and 3.21±4.73 fold) were significantly upregulated in Diabetic subjects including DN in reference to Healthy control (Figure 5.45). Our present data reveals that the variable like NLRP3 of the Inflammasome complex upregulated most efficient manner compare to the other molecules of the complex like CASP-1 and PYCARD (Figure 5.45). Functional studies indicate that apart from the pathogen, hyperglycemia induces the DAMPs significantly to triggers activation of NLRP3 Inflammasome complex. It has been documented that hyperglycemic activates urate, free fatty acids and extracellular ATP release that may ultimately triggers the innate immune mediators through NLRP3-PYCARD-Caspase1 complex or NLRP3 Inflammasome complex (Lee, H. M., *et al.* 2013). The study substantiates the earlier observation that reveals association of NLRP3 and Caspase1 with DN in animal model (Shahzad, K., *et al*. 2015 and 2016, Zhang, X., *et al*. 2017). The replicative phenomenon in animal and human model insists us to postulate that NLRP3-PYCARD-CASPASE1 complex or NLRP3 Inflammasome complex may play an important role in the pathogenesis of microvascular complications including DN where NLRP3 activation is most prominent. Previous study with hemodialyzed chronic kidney disease patients (CKD-HD) documented the higher fold change of NLRP3 (~1.6 fold), CASP1 (~1.4 fold) PYCARD/ASC (~1.2 fold), IL1β (~1.6fold) and IL18 (~1.5 fold) compared to healthy subjects (Granata, S., *et al.* 2015). Present observation along with previous data may establish the pathogenic link for the aberration of nephron microvasculature through the altered homeostasis of Inflammasome complex for diabetic nephropathy and other inflammatory diseases (Shahzad, K., *et al*. 2015). Various animal model studies established the role of NLRP3 in the pathogenesis and development of DN and found increased level of NLRP3, Caspase1 and IL1β in DN cases than control and in diabetic induced rats (Shahzad, K., *et al*. 2016, Fu, Y., *et al.* 2017). In a study with db/db mice found that in-vitro glucose induced NLRP3 expression in glomerular endothelial cells and podocytes (Shahzad, K., *et al*. 2015, Kim, Y. G., *et al.* 2019). Single Cell atlas datasets for Inflammasome complex datasets in different cell type in hyperglycemic condition reveals the altered homeostasis of the

complex highest in monocytes that may associated with the adverse impact of microvasculature of nephron cells for the genesis of the nephropathy associated complication (Figure 5.73, 5.74, 5.75) (Shahzad, K., *et al*. 2015, Segerstolpe, A., *et al.* 2016, Vento-Tormo, R., *et al.* 2018, Schaum, N., *et al.* 2018). Recently pathogenic role of NLRP3 and Caspse1 for aggravate diabetic nephropathy in non myeloid-derived cell has been demonstrated by Zhuang *et al.*, in 2014 (Zhuang, Y., *et al.*, 2014). Mitochondrial Reactive Oxygen Species (ROS) has been identified as potent activator of NLRP3 Inflammasome in diabetic glomeruli and provides a mechanistic link between ROS and sterile inflammation in diabetic nephropathy. Previous studies reported that ROS mediated NLRP3 Inflammasome activation in hyperglycemic condition is a potent contributor for DN occurrence by damaging of renal cells through activating inflammatory cascade via involving of macrophage (Forbes, J. M., *et al.* 2008, Tschopp, J., Schroder, K. 2010, Heid, M. E., *et al.* 2013, Shahzad, K., *et al*. 2015,). ROS dependent activation of NLRP3 mediated inflammation in immune cells of diabetic patients also been documented recently (Mirza, R. E., *et al.* 2014). Mitochondrial ROS activates NLRP3 through free TxNIP, dissociates from thioredoxin and also accumulation of dysfunctional mitochondria which generates ROS also sufficient to activate NLRP3, hence ROS is a common activator of NLRP3 inflammasome in hyperglycemic condition (Tschopp, J., Schroder, K. 2010, Zhou, R., *et al.* 2010, Shah, A., *et al.* 2013). Advance glycation end products (AGE) accumulation due to persistent hyperglycemia and induction of mitochondrial ROS may contribute to ROS-dependent Inflammasome activation, in podocytes for further pathogenesis (Shahzad, K., *et al*. 2015). Several studies indicates that along with ROS, other DAMP such as extracellular ATP, extracellular matrix components etc. are capable to activate NLRP3 Inflammasome in diabetic microvascular complications including DN (Mariathasan, S., *et al*. 2006, Muruve, D. A., *et al*. 2008, Babelova, A., *et al.* 2009, Yamasaki, K., *et al.* 2009, Zhou, R., *et al.* 2010). Poor glycemic status is one of the potent contributors for the further progression of disease severity. UKPDS and Steno 2 ADVANCE clinical trial postulates that intensive glycaemic control leads to a reduction in morbidity and mortality in diabetes related micro and macro vascular complications (Holman, R. R., *et al*. 2008, Gaede, P., *et al*. 2016).

eGFR is coined as crucial marker for kidney function in humans and reduced eGFR is associated with severity of nephropathy including DN (Dabla 2010). eGFR status of 89 to 60

or lower associated with thickening of basement membrane and mesangial proliferation and further Glomerular damage leads to eand stage renal disease (ESRD) (Mogensen 2000, Gheith, O., *et al.* 2015). Kidney biopsy studies postulates that the severity of diabetic nephropathy measured through GFR is associated with the presence of specific inflammatory markers (Krolewski 2015). Linear regression to test predictability of eGFR status based on expressional alteration of NLRP3, CASP-1 and PYCARD revealed the higher expression of NLRP3 (0.05) and CASP1 (0.04) is significantly associated with the severity of DN with the aid of reduced eGFR (Table 5.27 and Figure 5.54 and 5.55). Our findings substantiate the previous in vitro studies suggesting glomerular apoptosis may contribute to diabetic nephropathy (DN), through activation of NLRP3 Inflammasome and Caspase1. Recent study postulates the altered expressional effect of Casapase-1 accelerates the pyroptosis (Shahzad, K., *et al*. 2016). Pyroptosis is a proinflammatory form of programmed cell death mediated by Caspase 1 and studies have confirmed that pyroptosis is involved in bacterial diseases and inflammatory processes (Danelishvili, L., and Bermudez, L. E. 2013). Involvement and mechanism of pyroptosis has been extensively studied in infectious diseases, nervous system-related diseases, atherosclerosis, and other diseases diabetic micro and macro vascular diseases (Chang, W., *et al.* 2013, Li, X., *et al.* 2014, Qiu, Z., *et al.* 2017). Pyroptosis is induced by the action of inflammatory cytokine IL-1β which is processed and matured due to activated Caspase1 (Qiu, Z., *et al.* 2017). In-vitro studies with diabetic rat model revealed that NLRP3 mediated activation of Caspase1 induced pyroptosis play an important role in the development of diabetic cardiomyopathy (Giordano, A., *et al.* 2013, Li, X., *et al.* 2014, Luo, B., *et al.* 2014, Qiu, Z., *et al.* 2017). Cell death due to pyroptosis includes programming of inflammatory caspase1, membrane pore formation and leakage of intracellular contents and NLRP3 Inflammasome may induce Caspase 1 dependent pyroptosis in multi organ disease including DN and other infectious diseases (Fink, S. L., and Cookson, B. T. 2006, Kovarova, M., *et al.* 2012, Doitsh, G., *et al.* 2014, Qiu, Z., *et al.* 2017). Study on Rat model revealed that, hyperglycemia increased the ROS production leads to activate Caspase1 that triggered pyroptotic cell death associated with diabetic related macro and micro vascular complications (Qiu, Z., *et al.* 2017). Data suggested that caspase1 dependent pyroptosis mediated by NLRP3 Inflammasome contribute to myocardium injury in diabetes (Qiu, Z., *et al.* 2017). Results from various in-vitro studies along with present study may postulate that Casapase-1 plays a crucial

role the pathogenesis of DN and further kidney damage in terms of reduced GFR. Activated Inflammasome may contribute to local inflammation in renal parenchymal cells, cell stress or cell lose may promote albuminuria, hypertrophy of the podocytes and later diffuse glomerulosclerosis-related nephron loss. Role of NLRP3, Caspase1 and ASC/PYCARD has also been reported on systemic autoimmunity (Anders 2016). Present study documented the significant association of CASP-1 higher expression with reduced eGFR among T2DM patients (Figure 5.55 and Table 5.27). Reduced eGFR represents the severity of kidney disease as it is directly related to the glomerular function. Observation from present study and previous animal model studies may postulates that Caspase-1 dependent NLRP3 Inflammasome mediated cellular dysfunction potentially leading to pyroptosis further induced diabetic glomerulopathy (Shahzad, K., *et al*. 2016). Linear regression analysis revealed that upregulation of NLRP3 (-0.77) and CASP-1(-2.16) had a negative association with eGFR in T2DM patients indicates that elevation of both gene plays a crucial role in mediating glomerulosclerosis, eventually leading to renal dysfunction and further pathogenesis of DN (Xiong, J., *et al.* 2015).

Various studies and present study shown that, down-stream-signaling cytokine of NLRP3 Inflammasome pathway i.e, IL-1β (21.01±45.00 fold) and IL-18 (40.97±65.34 fold) have significantly elevated during diabetic CKD and able to promote renal tubulointerstitial fibrosis (Vesey, D. A., *et al.*2002**,** Liang, D., *et al.* 2007). NLRP3 mediated proinflamatory cytokines such as IL1β and IL18 activation contributes to pathophysiology of DN by regulating renal necroinflammation. mRNA expression of IL-1β, IL-18 and TNF-α could not significantly associated with reduced eGFR among study T2DM subjects (Table 5.31). Serum level of inflammatory cytokines viz. IL-1β, IL-18 and TNF-α were found higher among DN subjects compared to DC and HC individuals. IL-18 was found significantly ($P<0.0001$) higher in DN (1957±771.0) compared to DC (1247±624.5) and HC (930.6±480.9) which substantiates previous studies (Figure 5.66) (Pickup, J. C., *et al.* 2000, Nakamura, A., *et al.* 2005). Present study also revealed the significant positive correlations between serum IL-18 and eGFR among T2DM subjects. Several studies reported the elevation of C-reactive protein, IL-6, IL-8, IL-18, IL-1β and TNF-α in patient of T2DM and associated microvascular complications diseases. Higher serum TNF-α, IL-18 and IL-1β concentrations have recently been identified as a strong predictor for severeity of coronary artery disease, acute ischemic

stroke and DN (Blankenberg, S., *et al.* 2002, Zaremba, J., and Losy, J. 2003, Anders 2016). IL-18 and IL-1β established as a important factor for progression and development of atherosclerosis, DN and other diabetic related complications as they enables to produced other proinflammatory cytrokines, endothelial apoptosis, upregulation of ICAM-1 (Nakamura, A., *et al.* 2005, Anders 2016). Further we have validated our finding with INT407 cell lines induced with various concentration of glucose to produced hyperglycemic condition to the cells and assessed the expression of NLRP3, CASP1, PYCARD and all TLRs along with their targeting miRNAs. It was observed that expression of mRNAs were higher in cells with highest concentration of glucose for all gene and miRNAs of Inflammasome complex cascade (Figure 5.67 and 5.69). Our finding also substantiates with previous in-vitro studies and NLRP3 Inflammasome established as a potent player of DN pathophisiology (Muruve, D. A., *et al*. 2008, Shahzad, K., *et al*. 2016).

Possible role of NLRP3 Inflammasome complex in pathogenesis of DN with the involvement of proinflammatory cytokines insist researcher to develop effective inhibitors for the complex to prevent further pathogenesis of DN and other inflammatory diseases. Till now there is no NLRP3 antagonists has not been approved for therapeutic use though various clinical trials are going on for various NLRP3 dependent diseases including DN. Interestingly antagonists to inflammasome effectors (eg: IL1 receptor antagonists and IL1β) have been approved as therapeutics for NLRP3-dependent diseases (Mulay 2019). In a previous study, it was reported that IL-22 gene therapy may inhibited NLRP3 activation, caspase-1 cleavage and IL-1β maturation in Streptocotozin (SZT) model (Wang S. *et al.* 2017).

Anti inflammatory effects of angiotensin receptor blocker (ARB) treatment in terms of lowering the proinflammatory cytokines levels were studied but data are substantially lacking and further studies are required (Anders 2016). Metformin is an extensively used oral hypoglycemic agent for treatment of T2DM. Main function of metformin is to improve hyperglycemic condition by increasing glucose uptake by muscle and suppression of hepatic glucose production (Saisho 2015). Randomized controlled trial showed that, metformin also reduced cardiovascular events (Saisho 2015). Metformin suppresses inflammatory response by improving metabolic parameters or directly inhibition of nuclear factor κB (NFκB) via AMP-activated protein kinase (AMPK)-dependent and independent pathways (Saisho 2015). Potential anti-inflammatory effects of anti diabetic agents such as insulin, biguanides,

Sodium-glucose co-transporter-2 (SGLT2) inhibitors, sulfonylureas, thiazolidinediones and dipeptidyl peptidase-4 (DPP-4) inhibitors has been reported that, they may modulates NLRP3 Inflammasome activity in renal tissues and can prevent DN progression but studies are limited (Yaribeygi, H., *et al.* 2019). Upregulation of NLRP3 and CASP1 gene in patients of diabetic kidney disease also open up a new therapeutic targets in diabetic microvascular complications including DN (Dixit 2013, Granata, S., *et al*. 2015, Shahzad, K., *et al*. 2015).

Inflammatory cytokines exert an important diversity of actions implicated in diabetic nephropathy, from development to the initial stages of diabetes to progression and to late stages of renal failure. Recent clinical trials documented anti-inflammatory molecule as a potent target to reduce the burden of diabetic microvascular complication but the studies are substantial lacking on PAMP and DAMP mediated inflammation to explain the disease mechanism at least in human model. Previous studies postulates that, DAMPs in response to stress, tissue injury or cell death trigger innate immune molecules by activating Inflammasome (Schroder, K., and Tschopp, J. 2010, Anders, H.J., *et al*. 2011 and 2014, Harijith, A., *et al*. 2014). Receptor for Advance Glycation End product (RAGE) is one of the best-studied DAMP receptors in various metabolic condition including T2DM and AGE-RAGE axis play an important role in the pathogenesis of DN (Yan, S. F., *et al.* 2010, Schaefer 2014). NLRP3 inflammasome activation can occur as a response to a number of distinct DAMPs including Uric acid, Biglycan, Histones release from necrotic cells (Gasse, P., *et al.* 2009, Babelova, A., *et al.* 2009, Allam, R., *et al.* 2013, Schaefer 2014). Importantly, small but seminal human studies have also provided evidences that anti-inflammatory therapy can improve glycemia and βcell function in T2DM and its associated complications including DN (Agrawal, N. K., and Kant, S. 2014). Persistent hyperglycemia was identified as risk factor for the activation of NLRP3 inflammasome (Sakai, N., and Wada, T. 2015, Shahzad, K., *et al*. 2015, Qiu, Y. Y., and Tang, L. Q. 2016).

Present study also assessed the expression of TLR family genes and revealed that TLR family receptors were significantly associated with T2DM patients compared to healthy control (HC) which was evidenced by significant elevation of TLR4 m-RNA expression among DN subjects. Elevation of m-RNA level of TLRs (TLR1-TLR10) among DM patients observed in the present study substantiate the earlier findings (Figure 5.49 and Table 5.25) (Jialal, I., *et al*. 2014, Gupta, S.,*et al*. 2017). Saket Gupta *et al* in 2017 were measured the

expression serum cytokine and mRNA levels of cytokines, TLRs in monocytes (M) and neutrophils (N) in a cohort of 112 diabetic patients and found enhanced gene expression of TLRs (Gupta, S.,*et al*. 2017). Present study revealed significant upregulation of TLR4 among DN (22.66±31.34 fold) patients compared to DC (9.74±16.27 fold, p=0.04) patients (Fig 5.49 and Table 5.25). TLR7 found significantly upregulated among DC (79.23±93.45 fold) compared to DN (21.48±38.20 fold, p=0.04) (Fig 5.49 and Table 5.25). Linear regression analysis revealed the significant association of higher TLR4 (P=0.04) with the reduced eGFR among T2DM patients. Aberrant glucose concentration and inflammatory cytokines secretion through TLR4 activation on mouse mesangial cells and its contribution to develop DN have been postulated (Verzola, D., *et al*. 2014). Potent detrimental effect of inflammation in DN is yet to be understood but previous studies demonstrated the deleterious effect of TLR4/NF-κB inflammatory signaling mediators for nuclear translocation in glomeruli and proximal tubules (Verzola, D., *et al*. 2014, Qiu, S., *et al*. 2016). Annotation of the Single Cell atlas datasets for TLRs particularly expression of TLR4 in different cell type in hyperglycemic condition revealed the altered homeostasis of the same highest in monocytes and endothelial cells and may leads to the genesis of nephropathy associated complication. Close ties between insulin resistance and chronic activation of TLR4 may contribute to the adverse complication of DN. Animal model data and our present observation i.e. significant elevation of TLR4 m-RNA expression and its pathway derived inflammatory cytokine like IL1 β may crucial for pathogenesis of DN. Though the m-RNA expression of TLR3, TLR4 and TLR7 was significantly elevated among T2DM, the level of TLR3 and TLR7 was relatively low among DN patients compared to T2DM subjects not having DN. Genetic susceptibility of TLR3 has been documented among subjects with T2DM and vascular complications for Han Chinese population (Zhou, Z., *et al.* 2017). Elevated m-RNA expression of TLR7 was also documented in acute renal ischemia of STZ-induced diabetic rats but the observation was not replicated in human (Yayi, H., *et al.* 2016).

TLRs are the first well studied PPRs and recognized a wide range of PAMPs and DAMPs to promote inflammation and pathogenesis of DN (Kawai, T., and Akira, S. 2011, Lin, M., and Tang, S. C. 2013). Previous studies evaluated the role of TLR2 and TLR4 in the pathogenesis of DN and other kidney disease through activation of inflammation. TLR2 and TLR4 recognized various endogenous DAMPs generated in hyperglycemic conditions and

activates further inflammation (Mudaliar, H., *et al*. 2014, Panchapakesan, U., and Pollock, C. 2018). Activated TLRs further activates NF-κB (nuclear factor κB), which results in the synthesis and secretion of pro-inflammatory cytokines and chemokines leads to inflammation in renal cells (Mudaliar, H., *et al*. 2014, Panchapakesan, U., and Pollock, C. 2018). Data from present study and recent in-vitro and in-vivo studies have highlighted the role of TLR4 and other TLRs in the pathogenesis of DN (Lin, M., and Tang, S. C. 2013). Overexpression of TLR2 and TLR4 in monocytes of T2DM patients from present study substantiates earlier results showing positive correlation with hemoglobin A1c (HbA1c) levels in T1DM and T2DM patients (Devaraj, S., *et al*. 2008 and 2009, Dasu, M. R., *et al*. 2010). TLRs are expressed in various kidney cell types including lymphocytes, dendritic cells, mesangial cells etc. and involved in the events of renal inflammation in DN and other kidney injury (Lin, M., and Tang, S. C. 2013). Recent study involving $TLR4^{-/-}$ and $TLR2^{-/-}$ mice developed lower level of inflammatory cytokines in serum, kidney and urine compared to wild type mice (Leemans, J. C., *et al*. 2005, Zhang, B.,*et al.* 2008). Several studies postulates the protective role of TLR2 and TLR4 inhibition in DN and other kidney diseases (Lin, M., and Tang, S. C. 2013).

Overexpression of TLR4 mRNA in differentially glucose induced INT407 cells from present study also supported from previous study of Glucose Induced TLR4 Expression in Human PTECs (Figure 5.69) (Lin, M., *et al.* 2012). Overexpression of other TLRs also observed in INT407 cells when induced with different concentration of glucose. Various study postulates the tubular TLR4 is one of the main mediator of DN pathogenesis as it was found negatively associated with the reduced eGFR (Lin, M., and Tang, S. C. 2013). Less albuminuria, mesangial expansion, macrophage infiltration was found lower in TLR4 deficient mice (Lin, M., and Tang, S. C. 2013). Another study also revealed the decreased urinary albumin excretion (UAE), glomerulosclerosis and proinflammatory cytokines in db/db mice, treated with a non-specific TLR inhibitor GIT27 (Lin, M., and Tang, S. C. 2013). Various experimental data suggested that, TLR2 and TLR4 along with other TLRs may crucial for the pathogenesis of DN through progressive inflammation established them as therapeutic targets for DN (Lin, M., *et al.* 2012, Lin, M., and Tang, S. C. 2013). In-vitro studies documented the TLR signaling inhibitory effect of current treatment strategies for DN such as ARB, aldosterone receptor antagonist (ARA) statin, vitamin D analog and dipeptidyl peptidase-4

(DPP-4) inhibitor and metformin (Methe, H., *et al.*2005, Niessner, A., *et al.* 2006, Dasu, M. R., *et al.* 2007, Lv, J., *et al.*2009, Sochorova, K., *et al.*2009, Liu, K. H., *et al.*2010, Ta, N. N., *et al.*2010).

Recent development of TLR4 antagonist called "Eritoran" has been developed to treating inflammation in various communicable as well as noncommunicable diseases including insulin resistance in experimental animal models (Lin, M., and Tang, S. C. 2013). Another TLR singnaling antagonists which are undergoing various phases of clinical trials are CRX-526, TAK242, GIT27 (VGX-1027) for both TLR2 and TLR4, OPN-305 a monoclonal antibody that blocks TLR2 and AP177 a TLR2 functional aptamer (Lin, M., *et al.* 2012, Lin, M., and Tang, S. C. 2013). Data from present study and accumulating data from various experimental studies suggested that TLR4 may be a promising therapeutic target in DN. Along with TLRs-NLRs complex may also act as intracellular sensors for development of DN and considered same as inflammatory disease (Lin, M., *et al.* 2012, Lin, M., and Tang, S. C. 2013). Despite promising results from various experimental studies for development of inflammatory antagonist but their efficacy remains elusive for human DN (Kelly, D. J., *et al.* 2005, Ninichuk, V., *et al.* 2007, Kang, Y. S., *et al.* 2010, Sayyed, S. G., *et al.* 2010, Lin, M., *et al.* 2012, Lin, M., and Tang, S. C. 2013). Initiation of clinical trials of TLR and NLR inhibitors may be effective to reduce down the burden of DN though the safety and efficacy to human trial yet to elusive (Lin, M., *et al.* 2012, Lin, M., and Tang, S. C. 2013).

Data from present study substantiate that prolonged hyperglycaemia includes an inflammation for diabetic complications including DN. Apart from the hyperglycemia mediated Chronic Kidney Disease (CKD), activation of inflammation also documented on hyperglycemia independent CKD through polycystic kidney disease, obstractive uropathy, glomerular nephritic and nephritic syndromes including membranous nephropathy, lupus nephritis, amyloidosis and rapidly progressive glomerulonephritist (Karihaloo 2015). The observation of the study among non diabetic CKD (Zammit, A. R., *et al*. 2015) and the present data on diabetic CKD postulate the partial homogenous molecular mechanism from different etiological factor. Peritoneal macrophages in diabetic rats produced elevated level tumour necrosis factor-alpha (TNF-α) (Navarro-Gonzalez, J. F., and Mora-Fernandez, C. 2008). Serum and urinary levels of IL-18 have been reported to be higher in patients with DN

(Mahmoud, R. A., *et al*. 2004). Studies well documented that persistent hyperglycemia induce inflammation and oxidative stress through prolonged biochemical derangement like AGE accumulation, Polyol pathway, hexamine pathway which ultimately develop DN (Singh, V. P., *et al.* 2014). The accumulation of AGEs and its impact on anatomical alteration of glomerular basement membranes (GBMs) may stimulate the expression of TNF-α and IL-1β by macrophages in persistent hyperglycemic condition to develop various inflammatory complications (Singh, V. P., *et al.* 2014, Hamid, S., Gul, A., and Hamid, Q. 2016). Apart from the elevated level of TNF-α in diabetes patients several functional studies postulates that activation of TNF-α induced by TLRs. Further insilico network analysis reveals the strong relation of the molecules like TNF-α and IL-1β with TLRs (Figure 5.75). Though the phenomenon is well established in animal and cellular model but the data with clinical specimen is relatively less and is under power. Recent reports on single cell transcriptomic analysis of early DN revealed strong angiogenic signaling in human diabetic kidney (Wilson, P. C., *et al.* 2019). Another study of transcriptomic analysis of DN identified multiple novel genes and pathways associated with the pathogenesis of DN could serve as biomarkers (Woroniecka, K. I., *et al.* 2011).

Epigenetic modification including DNA methylation have been associated with T2DM but the involvement of other epigenetic regulators including non coding RNA like mi-RNAs for T2DM and its associated vascular complications is still enigmatic. Limited studies evaluated the potent role of mi-RNA for immune modulation and their differential expression in various autoimmune diseases and T2DM (Hamar 2012, Moura, J., *et al.* 2014). miRNA mediated epigenomic alteration play a distinct role in the gene regulation in mammal and thus to be a potential novel class of therapeutic targets as well as biomarker but the data are extremely limited (Wahid, F., *et al.* 2010). Gene expression governs cellular functions that lead to multiple biological processes including development and physiology. Altered gene expression causes disease by altering cell function. It has been hypothesized that interaction between protein-coding and noncoding RNAs seems to be crucial to gene expression, but such relationships are largely unexplored particularly for human cells (Kota *et al.* 2009; van Rooij, E., *et al.* 2012; Xiao and Rajewsky, 2009). miRNAs, the small noncoding RNAs post-transcriptionally regulate mRNA levels and translation (Bartel 2004). There is an increasing awareness of their role in disease pathophysiology as well as potential therapeutics (van Rooij

, E., *et al.,* 2012). Genetic associations along with the systemic relation with miRNA levels and their transmitting role for m-RNA regulation may yield novel insights into molecular mechanisms of disease pathogenesis particularly for the hyperglycaemia mediated hypoxia induced multifactorial disease including DN (Wu, H., *et al.,* 2014). miRNAs are a class of 20–24 nt non-coding RNAs that regulate gene expression during posttranscriptional repression through mRNA degradation in a sequence-specific manner and repressing translation.

The roles of miRNAs are just beginning to be understood, but the study of miRNA function has been limited by poor understanding of the general principles of gene regulation through miRNAs. mi-RNA directed regulation on inflammasome complex viz. PYCARD, NLRP3 and Caspase1 under hyperglycaemia is substantially lacking (Bauernfeind,F., *et al*., 2012, Chen, H.Y., *et al*., 2014; Qazi, O., *et al.,* 2013, Wu, H., *et al.,* 2014). Through insilico approach we have predicted, miRNAs viz. 'hsa-miR 22-3p and hsa-miR 223' targeting NLRP3, 'hsa- miR4291' targeting CASP-1 and 'hsa-miR 185-3p' targeting PYCARD as putative regulators with a target score having more than 80%. In the present study we have looked for a concrete role of regulatory miRNAs on Inflammasome complex and their interaction on microvascular complication in kidney among persistent hyperglycemic subjects. Present study revealed the upregulation of micro RNA like hsa-miR-223 targeting NLRP3, hsa- miR-4291targeting CASP-1 and hsa-miR-185-3p targeting PYCARD (Inflammasome components) was found significantly higher among T2DM subjects compared to HC. In this present study it is revealed that the miRNA, hsa-miR-22-3p targeting NLRP3 was found downregulated among T2DM patients (Figure 5.47A). The fold change of hsa-miR 185-3p miRNA targeting PYCARD was significantly higher among DN (31.75 ± 38.18) compare to diabetic subjects who did not develop Nephropathy (10.24 ± 19.11 folds) (p=0.04) (Figure 5.47D) where the mRNA of PYCARD showed moderate upregulation compared to other Inflammasome complex mediators among study subjects. Though significant association of miRNA expression with the severity of disease with the aid of reduced estimated glomerular filtration rate (eGFR) was not obtain but the expression was negatively associated with estimates -5.24, -2.05, -0.27 and -0.002 for hsa-miR-223, has-miR-22-3p, has-miR4291 and has-miR-185-3p respectively (Table 5.28). Present study also found the negative correlation of hsa-miR-223 (estimates:-0.21) and has-miR-185-3p (estimates:-0.0005) expression with their targeted mRNAs of Inflammasome complex (Figure 5.58 and 5.61). This findings may serve

as crucial marker for understanding the severity and adverse prognosis of DN. Caspase1 and NLRP3 regulates activation of IL-1 cytokine family through damage associated molecular pattern (DAMPs) and leads to the development of tubulointerstitial inflammation in DN which was evidenced in animal model (Karolina, D. S., *et al*. 2014, Zhang, X., *et al*. 2017). Studies documented that hyperglycemia activate inflammatory cytokines (IL-1β and IL18) through the activation of NLRP3 inflammasome complex (Vilaysane, A., *et al*. 2010, Granata, S., *et al*. 2015). Therefore we may postulate that altered homeosatasis of NLRP3, CASP1, PYCARD and hsa-miR 22-3p, hsa-miR 223, hsa-miR-4291 and hsa-miR 185-3p may actively involved for the adverse prognosis of DN. The involvement of miR-223-3p has been evaluated by M. Ulbing *el al* in 2017 among CKD patients and found the downregulation of miR-223-3p and miR-93-5p in patients with CKD stage 4 and 5 compared to healthy controls and association with the inflammation and kidney function (Ulbing, M., *et al.* 2017). Epigenomic alteration viz. altered mRNA-miRNA homeostasis and interaction may mediate the Inflammasome dependent inflammatory process of DN (Bauernfeind, F., *et al*., 2012, Qazi, O., *et al.,* 2013, Bertoli, G., *et al.* 2015). The present observation supports the previous Caspase dependent pathogenesis for DN in transgenic mice model as well as in human (Bauernfeind, F., *et al*., 2012, Qazi, O., *et al.,* 2013, Bertoli, G., *et al.* 2015, Simpson, K., *et al.* 2016, Shahzad, K., *et al.* 2016, Zeni, L., *et al.* 2017). Further our recent study reveals the potent role of Caspase1 mediated pyroptosis in DN (Bhattacharjee, C. K., *et al.* 2019).

We also observed that significant upregulation of hsa-miR-448 targeting TLR4 among DN (13.04±16.46) compared to DC (5.36±7.71,p=0.01). The study further reveals that expression of TLR4 inversely related with hsa-miR-448 with an estimate -0.03 (Figure 5.64 and Table 5.32). hsa-miR-448 expression also found negatively correlates with the reduced eGFR among T2DM patients and explain its pathogenicity on severity. (Table 5.28) Further insilico prediction revels that, hsa-miR-448 not only target the TLR4 m-RNA, several sets of genes including GAN, KCTD9, VASH2, GRHL3 may be encountered by the same miRNA with higher specificity (miRDB data base). Hence the magnitude of upregulation of hsa-miR-448 to downregulate the TLR4 expression may not enough due to its multigenic specificity with other sets of genes along with the multigenic crosstalk. Wang, Y., *et al.* 2018 revealed that, persistent hyperglycemia and hypoxemia may induce miR-448 and also promoted apoptotic pathway (Wang, Y., *et al.* 2018). The relative expression of hsa-miR-561-3p

targeting TLR2, has-miR-4307 targeting TLR3 and has-miR-4760-3p targeting TLR7 found 6.99±13.66, 1.48±2.52 and 1.21±1.46 fold among DC and 1.54±3.22, 12±0.16 and 1.14±1.65 fold among DN subjects (Figure 5.51). Expression of hsa-miR-561-3p, has-miR-4307 and has-miR-4760-3p found negatively correlates with eGFR but positively with their targeted mRNAs among T2DM patients but the association was not significant (Table 5.30).

Threshold Effects and Endogenous miRNA Competitors has play a crucial role in efficiently repressed of any targeted mRNA by a specific miRNA (Mukherji *et al.* 2011). Available miRNA concentration determines the threshold level and affinity of the target sites also necessary for effective repression (Mukherji *et al.* 2011). A mathematical model for threshold of mRNA for miRNA repression has been predicted by Mukherji *et al.* 2011. Mukherji *et al.* 2011 also revelaed that, threshold transition for regulation by endogenous miR-20 in HeLa (approximately 2,000 miR-20 molecules per cell) was approximately 60 target mRNAs per cell consisting of seven typical sites in the 3′ UTR (Mukherji *et al.* 2011). Different miRNA expression profile exhibits in different tissue or a particular tissue in different conditions as a result of different threshold for a target gene. Various studies reviewed the impact of various miRNA in pathogenesis of DN trough animal and cell culture model. Direct involvement of miR-200a, miR-130a, miR-410 and miR-15a in β cell function, insulin biosynthesis and pathogenesis of DN has been reported (Fiorentino, L., *et al.* 2013, Conserva, F., *et al.* 2013). Discovery of miRNA associated DN disease pathogenesis insist scientists to generate the data on miRNA that target specific m-RNA to restore normal cellular function. miRNA as biomarker has been recognized for the disease including cancer, cardiovascular disease, metabolic and immune disorder (Bauernfeind, F., *et al*., 2012, Qazi, O., *et al.*, 2013, Bertoli, G., *et al.* 2015, Ulbing, M., *et al.* 2017). Urinary miRNA may act as potential noninvasive biomarker for the early detection and progression of kidney diseases including DN (Simpson, K., *et al.* 2016). Wang, L. P., *et al.* 2019 in their meta analysis reported the impact of miRNAs in DN, including urinary albumin excretion rates (UAE), urinary albumin creatinine ratio (UCR), GFR, HbAc1 and creatinine. They have identified fifteen miRNAs (miRNA-21, miRNA-181b, miRNA-194, miRNA-30, miRNA-215, and others) were upregulated and seven miRNAs (miRNA-26a, miRNA-126, miRNA-424, miRNA-574-3p, miR- 223, miR-155, and miR-192) were downregulated in the DN group

compared with control group which suggest miRNAs may participate in the pathogenesis of DN process with affecting various pathways (Wang, L. P., *et al.* 2019).

Recent animal model study predicts miRNA-29b inhibits DN as its overexpression reverse pathological changes of DN and may have therapeutic potential for diabetic kidney complication (Chen, H.-Y., *et al*. 2014). Another study by Bauernfeind, F., *et al*. in 2012 promises the inhibitory role of miR-223 against NLRP3 inflammasome through various experiment, this finding shed light on inflammatory cascade involved in numerous disease (Bauernfeind, F., *et al*. in 2012). miRNA-mediated RNA interference regulates many immune processes and miR-223 is a potent regulator of NLRP3 inflammasome atleast in inflammatory bowel disease (IBD) (Kanneganti 2017). Shen, S. C., *et al.* in 2016 revealed the role of miR22 in regulation of inflammation among coronary artery disease (CAD) patients by modulating MCP-1 expression (Shen, S. C., *et al.* 2016). Another study suggested that higher miR-185 levels might be associated with prognosis of dilated cardiomyopathy (DCM) as its involvement was observed in repression of B cell function (Yu, M., *et al.* 2016). TLR4 mediated inflammation is associated with the pathogenesis of diabetes. Type 2 diabetes models "C57BL/6J" mice were studied for the evaluation of TLR4 suppression by miR-448 and the inhibitory role of miR-448 against TLR4 was confirmed (Zhao, Q., *et al.* 2019).

Inhibition of inflammation is crucial to prevent tissue damage and miRNAs has the potential to regulate inflammation as it is speculated as fine-tune signaling regulators to prevent uncontrolled progress of inflammatory reactions. Various miRNA has been discovered to regulate inflammatory response and their expression levels may offer promising diagnostic as well as prognostic. miRNA microarrays and qRT-PCR arrays are the most sensitive and specific method to detection and measurement of miRNAs expression level from human blood which may leads to develop new therapeutic methods in the future (Tahamtan, A., et al. 2018)

Increasing evidence based on animal and cell culture model along with case control studies suggested naturally occurring endogenous miRNA are important antisense therapeutic molecules (Tahamtan, A., et al. 2018). Preclinical and clinical trial for treatment hepatitis C, liver cancer, and other diseases are going on by correcting the expression of miRNAs by their mimics or inhibitors to develop the same as potential therapeutic approaches (Walayat, A., *et al.* 2018). In cancer research the utility of miRNA based therapeutics emphasized on their stability and optimized delivery system as targeted therapy (Walayat, A., *et al.* 2018).

Regulation of miRNA expression for the therapeutic purpose includes oligonucleotides or virus-based constructs, which are used either to directly block the expression or to directly substitute for the loss of expression of a disease associated signature miRNAs (Walayat, A., *et al.* 2018). miRNA based therapeutics also adopted indirect employing of drugs to alter miRNA expression by targeting their transcription and processing (Walayat, A., *et al.* 2018). Antisense oligonucleotides, miRNA sponges, miRNAmask and small RNA inhibitors are the approaches to achieve the blocking of miRNA expression (Walayat, A., *et al.* 2018). Restoring downregulated miRNA expression can be achieved by using synthetic miRNA (miRNA mimic) or by inserting genes coding for miRNA into viral constructs (Walayat, A., *et al.* 2018).

Chakraborty, C., *et al.* in 2017 reviewed the patents of miRNA and siRNA-based new drugs, and their therapeutic stability as well as the delivery system (Chakraborty, C., *et al*. 2017). Approximately 20 clinical trials have been initiated using miRNA and siRNA-based therapeutics but only one miRNA therapeutic, (SPC3649 (miravirsen)), an inhibitor of miR-122 developed by Santaris Pharma from Denmark, is entered in a clinical trial. Several others are in preclinical stage (Gebert, L. F., *et al.* 2013).

Functional studies propose that damage due to accumulation of extracellular matrix (ECM) in kidney cells is potentially regulated by a complex interaction between NLRs and TLRs with reactive oxygen species (ROS) (Anders, H. J., and Schaefer, L., 2014). With the aid of previous observation, present study postulates that elevated level of TLR4 may potentially activate inflammatory cytokines production and the hypoxia induced has-miR-448 targeting TLR4, to activate apoptosis via activation of Caspase-1 of NLRP3 inflamasome complex among T2DM which ultimately accelerates the adverse microvascular complication like DN.

Our observation may postulate that NLRP3, CASP1, TLR4 and TLR7 may serve as better explanatory marker to understand the adverse prognosis of DN as there expression was significantly associated with the severity of DN (Tabel 5.27 and 5.29). This study may shed light on the role of the inflammatory cascade in the pathogenesis and severity of DN. Further validation of our findings in a different cohort is required.

Recent clinical trials documented anti-inflammatory molecule as a potent target to reduce the burden of diabetic microvascular complication but the studies are substantial

lacking on PAMP and DAMP mediated inflammation to explain the disease mechanism at least in human model. TLRs and Nod Like Receptors (NLRs) family containing 3 (NLRP3) inflammasome has been postulated for the pathogenesis of DN. Significantly, small but seminal human studies have also provided evidences that anti-inflammatory therapy can improve glycemia and β cell function in T2DM and its associated complications including DN. Present study also reveal the strong association of NLRP3 and TLRs pathways in network analysis. (Figure 5.75) As anti-inflammatory agent recently seems a promising therapeutic candidate hence we believe that our observation may be crucial to understand the role of inflammasome during the pathogenesis and severity of DN which remains still enigmatic till today. Again very recently scientific community have an access to capture the novel class of non-coding RNAs particularly micro-RNA (miRNAs) which found their way into the clinic due to their fundamental roles in cellular processes such as differentiation, proliferation and apoptosis. In this context we also focused to assess the impact of miRNAs targeting inflammasome which may deliver as diagnostic and therapeutic biomarkers for DN in future.

Previous reports and present findings may postulate that persistent hyperglycemia may alter the homeostasis of mRNA and mi-RNA of inflammasome complex seems crucial for deeper understanding the pathogenesis of DN. Present study may open a new horizon for deeper understanding of DN patho mechanism with the aid of interaction between micro RNA and Inflammasome complex to find out better disease explainable marker. Studies postulated that inflammatory molecules are the key players for disturbance of endothelial cell–podocyte dysfunction leading to DN (Bauernfeind, F., *et al.* 2012, Chen, H.Y., *et al.* 2014, Shahzad, K., *et al.* 2015 and 2016). Progression of microalbuminuria to overt nephropathy is accompanied by predictable structural changes in the glomerulus, including podocyte damage and loss (Zeni, L., *et al*. 2017). Present study is limited with its sample size. Another replicative study in larger cohort is extremely required.

Gut microbiome (GM) seems a crucial regulator for host metabolism through intestinal glucose absorption as well as energy balance (Parekh, P. J., *et al*. 2014). Apart from temporal and spatial alteration of GM architecture, several metabolic and lifestyle disorder like diabetes-mellitus (DM) and hypertension associated with the same (Qin, J., *et al*. 2012). Host-GM interactions play a central role in bile acid (BA) metabolism which is essential for metabolic (Parekh, P. J., *et al*. 2014).

Microbial dysbiosis in Type2-Diabetes Mellitus (T2DM) patients possibly contributes to the adverse prognosis of the same through its several micro and macrovascular complications (Qin, J., *et al*. 2012). Studies postulates that prolonged hyperglycemia associated biochemical aberration and chronic inflammation associated with severity of T2DM where inflammation also coined as mediator of GM dysbiosis (Qin, J., *et al*. 2012, Parekh, P. J., *et al*. 2014, Demirel, I., *et al*. 2018, Bhattacharjee, C. K., *et al*. 2019). Hyperglycemia, retarded carbohydrate hydrolysis, inflammation and GM dysbiosis may impair glucose absorption to alter microbial fermentation in gut which ultimately altered intestinal environment (Qin, J., *et al*. 2012, Parekh, P. J., *et al*. 2014). Shift of GM architecture may potentially contribute to metabolic endotoxemia which may damage the prolonged glomerular cells but yet to explore.

As GM reflect the metabolic cooperation between different phylotypes and may alter due to hyperglycemia, hence present study quantities the shift of GM architecture among T2DM with the aid of DN in reference with healthy control (HC) and its relation with the expression of host inflammatory machinery that includes NLRP3, CASP1, TLR4 and IL1β. Significant shift of GM have been documented among DN (32.53±16.84 and 19.95± 15.67) subjects compared to DC (13.87±25.95 and 1.27± 3.17) as well as HC (0.18±0.25 and 9.55± 15.68) atleast for the abundance of the genus *Escherichia* (P=0.01) and *Bacteroides* (P=0.02) (Figure 5.81 and Table 5.34). Abundance of genus *Prevotella* found significantly highest among DC (24.55±21.00) compared to HC (22.70±18.04) and DN (4.76±9.94, P=0.03) (Table 5.34). Present study also revealed the higher abundance of significant genus of gut microbiome community such as *Faecalibacterium*, *Dialister* etc along with lower abundance of *Lactobacillus* in DN compared to DC and HC (Table 5.34). Concomitant increased abundance for the phyla *Proteobacteria* (P=0.02) and its genus *Escherichia* among DC and DN compare to healthy control may suggests the impair metabolism and dysbiosis of GM (Table 5.35). *Bacteroidetes* and *Firmicutes* are the other major phylum found in this present study. North East India population along with other population around the globe possesses lesser abundance *Proteobacteria* in healthy individual compared diabetic population (Larsen, N., *et al.*2010, Qin, J., *et al.* 2012, Lambeth, S. M., *et al.* 2015, Barrios, C., *et al.* 2015, Li, C., *et al.* 2016, Pushpanathan, P., *et al.* 2016, Mrozinska, S., *et al.* 2016, Sanchez-Alcoholado, L., *et al.* 2017, Lazar, V., *et al.* 2019). It is well documented from mice model study that elevated

level of *Proteobacteria* was found gastrointestinal track (GIT) of rats under high fat and sugar diet (Lazar, V., *et al.* 2019). Previous study substantiate our findings that, the higher abundance of gram negative bacteria including phylum *Proteobacteria* in T2DM patients and a decrease gut *Bifidobacterium* species are associated with the inflammation (Table 5.34) (Li, C., *et al.* 2016). True to the findings of our observation, we observed that the phenomenon like increment of the proportion of phylum *Proteobacteria* among T2DM subjects documented for the population like Poland (2.82%), Denmark (3.54%), Germany (1.2-1.3%), Spain (3.67%), China (5.5%) and USA (3.78%) along with another Indian population (30.75%) but differed with the species (Larsen, N., *et al.*2010, Qin, J., *et al.* 2012, Lambeth, S. M., *et al.* 2015, Barrios, C., *et al.* 2015, Li, C., *et al.* 2016, Pushpanathan, P., *et al.* 2016, Mrozinska, S., *et al.* 2016, Sanchez-Alcoholado, L., *et al.* 2017, Lazar, V., *et al.* 2019).

The study did not reveal any significant difference in terms of numbers of OTUs for HC (117.8±17.68), DC (103.0±33.69) and DN (101.3±20.82) (Table 5.37). The α-diversity (Shannon-diversity-index (SDI) and Simpsion) and β-diversity (Bray-Curtis dissimilarity index (BCDI)) also computed and found SDI were 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 significantly highest in DC (0.45±0.12) compared to DN (0.43±0.11) and minimum in HC (0.27±0.03) (P <0.0001) (Figure 5.89 and Table 5.38). The inter group variation for BCDI is significantly higher in DC (P_{Tukeys}<0.0001) and DN (P<0.0001) compared to HC but the variation is not significant for T2DM subjects i.e, for DC and DN (Figure 5.89). Intra group variation of BCDI revealed that diversity is significantly increased in 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 5.89 and Table 5.38).

Extent of the severity of nephropathy including DN, eGFR identified as a potent marker for kidney function in humans and reduced eGFR is associated with severity of DN. Linear regression to test predictability of eGFR status based on GM architecture showed that the abundance of bacterial genus *Escherichia* could be a better explanatory marker for retarded eGFR (Figure 5.88 and Table 5.36) to explain the diseases severity.

Recent study reported the LPS derived from gram negative bacteria induced signaling cascade via Toll-like receptors (TLRs) for production of proinflammatory molecules in T2DM (Lambeth, S. M., *et al.*2015). Our recent reports also documented the activation of inflammasome complex for DN pathogenesis through pyroptosis (Bhattacharjee, C. K., *et al.* 2019). Functional studies hypothesize that the genus *Escherichia* activate of host NLRP3 and CASP-1 for Inflammation and cell death to the epithelial cells (Demirel, I., *et al*. 2018). Activation of the NLRP3 Inflammasome is dependent on various extracellular priming including the virulence factors like α-hemolysin and type-1 fimbriae, which derived from *Escherichia* which regulate intracellular Inflammasome signaling cascades (Demirel, I., *et al*. 2018, Bhattacharjee, C. K., *et al.* 2019). The present study documented that the increment of the proportion of the genus *Escherichia* significantly associated with host inflammatory gene expression atleast for NLRP3 (p=0.0004, Estimates: 1.64) and CASP1 (P=0.0009, Estimates: 1.04) The phenomenon may postulate Gut dysbiosis through increase abundance of Escherichia may leads to glomerular damage due to metabolic endotoxemia and inflammation for the severity of DN (Upadhyaya, S., and Banerjee, G. 2015, Demirel, I., *et al*. 2018, Bhattacharjee, C. K., *et al.* 2019). Several studies reported the altered gut microbiota in T2DM patients is different from non-diabetic persons (Larsen, N., *et al*. 2010). Previous study also found the higher abundance of *Bacteroidetes* and *Proteobacteria* and relatively lower abundance of *Firmicutes* which substantiate our findings with individuals from NE India (Larsen, N., *et al*. 2010, Schwiertz, A., *et al*. 2010). Gut microbiota of T2DM subjects from various study was found enriched with Gram-negative bacteria belongs to the phyla *Bacteroidetes* and *Proteobacteria* along with genus *Escherichia.* The phenomenon may postulate Gut dysbiosis through increase abundance of *Escherichia* may leads to glomerular damage due to metabolic endotoxemia and inflammation for the severity of DN as outer membranes of these bacteria are lipopolysaccharides (LPS) (Allcock, G. H., *et al*. 2001, Larsen, N., *et al*. 2010, Schwiertz, A., *et al*. 2010). Another study by Diaz-Rizzolo, D. A., *et al*. in 2019 stated dysbiosis of GM among obese and non obese individuals from Spain and found decrease abundance *Prevotella* (0.026 per cent) an increase of *Faecalibacteriumprausnitzii* (93.6 per cent) and an increase in lactic acid bacteria of healthier dietary clusters (Diaz-Rizzolo, D. A., *et al*. 2019). They have also found higher abundance of *Escherichia coli* (0.872 vs 0.188 per cent) in obese than non obese group (Diaz-Rizzolo, D.

A., *et al*. 2019). Beneficial effects of gut microbiota for development of protection against promoting T2DM in obese and prediabetes depends on healthy dietary pattern and increase population of growth promoting microbiota (Diaz-Rizzolo, D. A., *et al*. 2019). Abundance of Gram-negative bacteria in T2DM patients or animal models may influence absorption of macromolecules from the intestine resulting in low grade inflammation and altered signalling pathways influencing lipid and glucose metabolism leads to further pathophysiology and pathogenesis of type 2 diabetes (Allin, K. H., *et al.* 2015). Human gut microbiota plays a crucial role in maintaining homeostasis of intestine and metabolizing the xenobiotics including drugs. Advance in research provides the use of probiotics species like *Lactobacillus* and *Bifidobacterium* into a new height and extensively in use for a long time in various diseases including diabetes (Hehemann, J. H., *et al.* 2010, Arumugam, M., *et al.* 2011, Siezen, R. J., and Kleerebezem, M. 2011, Wu, G. D., *et al.* 2011). Various experimental data suggested the beneficial role of probiotics in human health and modulation of blood glucose level (Sanz, Y., *et al.* 2013). Shift of gut microbiota may cause chronic inflammation in T2DM associated micro vascular complications including DN severity. Some probiotic like *Lactobacillus* and *Bifidobacterium*can provide immune-modulatory effects to counter chronic inflammation by inducing IL10 production, which is an anti-inflammatory cytokine reported downregulates IFN- IFN-γ and IL-2/IL-1β in mice (Cani, P. D., *et al.* 2012, Sanz, Y., *et al.* 2013, Cano, P. G., *et al.* 2013). Recent study on rat model found that, use of *Lactobacillus reuteri* (GMNL-263) associated with reduce T2DM markers like serum glucose, glycated hemoglobin and c-peptide and also reduction in inflammatory cytokines Il-6 and TNF-α in adipose tissue (Hsieh, F. C., *et al.* 2013). Various clinical trials reported that, probiotic strains such as *Lactobacillus casei* Shirota, *Bifidobacterium animalis* subsp. lactis 420 and *L. casei Zhang* shows promises to reduce the metabolic bacteremia in early phases of T2DM hence may involve in further reduction of the of DN severity (Ooi, L. G., and Liong, M. T. 2010, Naito, E., *et al*. 2011, Amar, J., *et al*. 2011, Asemi, Z., *et al*. 2011 and 2013, Ejtahed, H. S., *et al.* 2012, Zhang, Y., *et al.* 2014, Sharma, S., and Tripathi, P. 2019).

Multidisciplinary approaches such as metagenomics, transcriptomic and metabolomics required to explore the molecular basis of metabolic interactions between specific microbes in healthy or patients with diabetes related disorders including DN. Though studies bridging the link between GM with the diseases including inflammatory bowel disease, cancer, obesity and

T2DM, asthma and multiple sclerosis but enthusiasm leads to GM with diagnostics, prognostics and therapeutics of the same (Allin, K. H., *et al.* 2015, Sharma, S., and Tripathi, P. 2019). The vast majority of microbiome research in humans represents only the association. Recent transplantation studies with germ-free mice support causal role of gut microbiota in metabolic diseases including T2DM but it is also not direct translation as human biology quite far from well controlled laboratory mice. Therefore to establish causality, statistically well-powered prospective studies, intervention studies and randomized clinical trials are required (Allin, K. H., *et al.* 2015, Sharma, S., and Tripathi, P. 2019). As the present study from north east India may postulate the potent role of Inflamosome complex for the severity of DN where low-grade inflammation associated with GM dysbiosis. Association of NLRP3 Inflammasome and TLRs with the pathogenesis of DN found in this study strengthens the postulation of various in-vitro and in-vivo studies where activation of NLRP3 and TLRs established as a key mechanism to induce inflammation and insulin resistance in diabetic complications including DN. Present study has also measured the expression of miRNAs targeting NLRP3 Inflammasome and TLR pathways genes associated with DN severity. Recent experimental data advocates the use of miRNAs as new therapeutics for repression of inflammation and further reduction of disease severity. miRNA inhibitors or mimic to change expression of target genes is the main clinical importance of miRNA therapy. Therapeutic miRNAs may have several advantages over alternative gene/protein targeting strategies, because these sequences can be synthesized, one miRNA can have multiple target genes, which may be more beneficial (Tahamtan, A., *et al.* 2018, Hanna, J., *et al.* 2019). Expression levels of studied miRNAs targeting NLRs and TLRs are from human blood as miRNAs are stable in blood and measured via qRT PCR (Tahamtan, A., *et al.* 2018, Hanna, J., *et al.* 2019). Further study is required to investigated the molecular mechanisms of miRNAs in DN pathogenesis and possibly to develop new therapeutics in future. As present study also assessed the significant association of NLRP3, CASP1 and TLR4 expression with the reduced eGFR for the severity of DN insist for further research to inhibit inflammation to reduce further pathogenesis of DN. Prolonged hyperglycemia associated differential carbohydrate hydrolysis, inflammation and GM dysbiosis impair glucose absorption in the intestine to alter microbial fermentation in the gut which ultimately altered intestinal environment (Qin, J., *et al.* 2012, Parekh, P.J., *et al.* 2014). Shift of GM architecture may potentially contribute to metabolic endotoxemia which

may damage the prolonged hyperglycaemic inflamed glomerular cells but yet to explore. Various interventional randomised control trails reported that, probiotics administrations are associated with the reduced bacterial translocation and altered the gut microbiota among T2DM patients (Allin, K. H., *et al.* 2015, Sharma, S., and Tripathi, P. 2019). Hence further study may aim to quantities the shift of GM architecture among T2DM upon the probiotic supplement and its subsequent impact on inflammation and metabolome.

Increment of gut derived *Escherichia* abundance matched with the clinical outcome like retarded eGFR among T2DM may postulate that probiotic supplement may be useful to reduce down the *Escherichia* associated inflammation. A considerable research data suggested that GM may play an important role in common disease including T2DM and associated micro and macro vascular complications through inducing inflammation. Further comprehensive research is required to address underlying mechanisms of causes, inhibitors and confounders for the severity of T2DM microvascular and macrovascular complications including DN with the aid of GM architect, Inflammasome, TLRs and their targeting miRNAs for better management of the disease pathogenesis. Technological advancement can be utilize for the better prognosis of type 2 diabetic related micro and macro vascular complication especially by modulating innate immune regulatory gene expression especially for NLRP3 inflammasome and TLR4 through their targeting miRNAs.